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DETOXIFICATION RATES CONSTRAIN FEEDING IN COMMON BRUSHTAIL POSSUMS (*TRICHOSURUS VULPECULA*)

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Abstract. A central theory in mammalian herbivore–plant interactions is that feeding decisions depend on an animal's capacity to detoxify plant secondary metabolites (PSMs). Thus, if a herbivore could detoxify a PSM faster, it might eat more. This theory has been difficult to test because the mechanisms by which herbivores detoxify most PSMs are largely unknown. We investigated whether common brushtail possums alter aspects of their feeding in response to an increased capacity to detoxify benzoic acid, a common PSM. Animals detoxify benzoic acid primarily by conjugating it with glycine to form benzoyl glycine (hippuric acid). Therefore, adding glycine to a diet that contains benzoic acid provides a simple way to study how detoxification constrains feeding in mammals. Brushtail possums offered supplementary glycine metabolized benzoate faster and, in response, ate more. Furthermore, when given a choice, brushtail possums selected a diet containing both benzoate and glycine over diets with a high concentration of just one of these supplements. The possums' aversion to glycine may explain why their ability to select a favorable diet combination from feeders containing diets with each of the supplements seemed less acute: they preferred diets containing benzoate alone over those containing glycine and did not mix their diet even though it would have allowed them to eat more benzoate and thus more food. Administering the anti-emetic drug, ondansetron (a specific 5-HT_3 receptor antagonist), did not alter the amount eaten of diets containing benzoate, glycine, or both, suggesting that 5-HT_3 receptors do not regulate benzoate or glycine intake. The results show that common brushtail possums recognize changes in their detoxification capacity and alter their feeding response accordingly.

Key words: *benzoic acid; detoxification; herbivore; plant secondary metabolite; serotonin.*

INTRODUCTION

Feeding decisions of mammalian herbivores are seen as a balance between nutrient acquisition and regulation of toxin intake (e.g., Wang and Provenza 1997, Villalba et al. 2002, Duncan et al. 2003, Provenza et al. 2003). The relative contributions of each are almost certainly dynamic, depending on the herbivore, the time it has available for feeding, the plants being eaten, and temporal factors like season. Several studies have shown that, given the resources, animals can correct nutrient imbalances (e.g., Richter et al. 1937, Leung and Rogers 1986, Markison et al. 1999) and mix their diet to obtain favorable nutrient combinations (e.g., Sanchez-Vazquez et al. 1998, Atwood et al. 2001, Raubenheimer and Simpson 2003). In contrast, we know relatively little about how toxins influence feeding decisions. Outright avoidance is not possible, as most plants contain toxins (Foley et al. 1999). Instead, cattle (Pfister et al. 1997), common brushtail possums (Stapley et al. 2000), common ringtail possums (Lawler et al. 2000), desert woodrats (Mangione et al. 2000), and ruffed grouse (Jakubas and Gullion 1990) are some of the herbivores known to closely regulate their intake of toxins.

Freeland and Janzen (1974) proposed that limitations in detoxification processes regulate toxin intake. In other words, when a detoxification pathway becomes saturated, a herbivore must either stop feeding until toxin concentrations in its body fall, or find something else to eat. This theory is widely accepted (Foley et al. 1999) but difficult to test. It requires understanding the sequence of enzymatic reactions that constitutes detoxification, something that is lacking for most herbivore–toxin combinations. Consequently, although detoxification limitations are central to theories of animal–plant interactions, there is no direct evidence that they influence feeding.

In circumstances where detoxification rates determine feeding rates, we might expect herbivores to modify their feeding behavior to match their detoxification capacity. Therefore, if we could manipulate the rate of the limiting reaction, it should be possible to simultaneously alter the rate of food intake and thus test Freeland and Janzen's detoxification limitation hypothesis. This type of manipulative experiment is difficult and has several prerequisites. First, the toxin of interest should be one encountered by the study animal. Second, the toxin must be a single molecule that is readily quantifiable, rather than a complex plant extract

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that may contain many potentially deleterious compounds. Third, it is necessary to know the detoxification pathways of the chosen toxin. Fourth, it must be easy to manipulate the metabolism of the compound. Finally, the study assumes greater ecological relevance if the detoxification pathway is one that is common to a variety of molecules. With these factors in mind, we chose to study benzoic acid and a generalist herbivore, the common brushtail possum (*Trichosurus vulpecula*; see Plate 1).

Benzoic acid is widespread in the plant kingdom, occurring in species as diverse as tobacco (Chong et al. 2001), cranberries (Zuo et al. 2002), and eucalypts (S. McLean, *personal communication*). It is a precursor of other plant secondary metabolites, such as salicylic acid (Coquoz et al. 1998, Yalpani et al. 1998), and ecologists have used its hydroxy derivatives (e.g., 3,4,5-trihydroxybenzoic acid [gallic acid]) as model phenolics (Wiggins et al. 2003). More importantly, its metabolism is well documented (Bridges et al. 1970, Awaluddin and McLean 1985). Animals, including the brushtail possum (Awaluddin and McLean 1985), usually excrete most of the benzoic acid they ingest as its glycine conjugate, benzoyl glycine (hippuric acid), although conjugates with glucuronic acid and ornithine have also been recorded (Bridges et al. 1970, Awaluddin and McLean 1985). The rate at which hippuric acid is synthesized depends on the quantity of glycine available for conjugation (Griffith and Lewis 1923). Thus, humans and rats supplemented with glycine excrete hippurate faster (Amsel and Levy 1969, Gregus et al. 1993).

The ability to manipulate the concentrations of benzoate and glycine in a purified basal diet allowed us to test three hypotheses: (1) that supplementary glycine increases the rate of excretion of benzoate as hippuric acid, (2) that supplementary glycine allows possums to ingest more benzoic acid, and (3) whether or not the release of serotonin explains how possums detect enhanced excretory capacity and translate this into a change in feeding behavior. Earlier studies in our laboratory have shown that serotonin receptor antagonists can enable animals to ingest more PSMs, presumably by attenuating nauseous feelings resulting from the release of serotonin from damaged cells (Lawler et al. 1998). Therefore, we tested whether the serotonin receptor (5-HT_3) antagonist, ondansetron, would have the same effect in brushtail possums consuming benzoate and glycine.

Methods

Eight male common brushtail possums (body mass = 2.86 ± 0.13 kg, mean ± SE) were captured on the Australian National University (Canberra, Australia) campus. They were housed in individual metabolism cages as described in Wallis et al. (2002). All animals were initially offered *Eucalyptus melliodora* foliage as a familiar food they eat in the wild and a basal diet

Plate 1. A common brushtail possum feeding on *Eucalyptus melliodora* foliage. Photo credit: K. Marsh.

(55.5% apple, 28% banana, 5.5% ground rice hulls, 4.7% ground lucerne, 4.7% ground Weet-Bix [a whole wheat breakfast cereal; Sanitarium, Berkeley Vale, New South Wales, Australia] and 1.6% acid casein on a wet matter basis) in the form of a wet mash. The average dry matter (DM) content of the diet was 31% and 1.9% DM was nitrogen. The amount of foliage offered was reduced over several weeks until possums were eating the artificial diet only. Water was offered ad libitum. All possums were weighed weekly and maintained body mass throughout experiments.

Sodium benzoate was slowly introduced to the diet to reduce naivety effects and to determine the range of experimental concentrations. When compounds, such as benzoate or glycine, were added to the diet, they were mixed thoroughly with the dry ingredients before the addition of wet ingredients. Diets were prepared immediately before they were offered at 1700 hours and were removed the following morning at 0900 hours. Dry matter intake (DMI) was calculated by drying a subsample of the food offered and all of the uneaten food, at 60°C.

Response to variation in concentrations of benzoate and glycine

Dry matter intake was measured for six brushtail possums (a subset of the eight acclimated to captivity) fed the basal diet containing six concentrations of so-

dium benzoate (0, 0.13, 0.28, 0.41, 0.56, and 0.69 mmol/g DM; Aldrich, Milwaukee, Wisconsin, USA). Each treatment was offered to each possum for one night in a Latin square design. Treatment nights were separated by a night when possums were provided with the basal diet to ensure the welfare of the animals and to reduce carryover effects.

A Latin square design was used to measure the DMI of the same six possums fed the basal diet containing six concentrations of glycine (0, 0.13, 0.28, 0.41, 0.56, and 0.69 mmol/g DM; Merck, Kilsyth, Victoria, Australia). These concentrations were deliberately made equimolar to the concentrations of benzoate fed in the previous experiment because, in the formation of benzoyl glycine, one mole of glycine binds to one mole of benzoate.

After determining the responses of possums to varying concentrations of benzoate and glycine, we repeated the study but combined equimolar concentrations (0, 0.13, 0.28, 0.41, 0.56, and 0.69 mmol/g DM) of the two compounds in the same diet. The previous experiments suggested that the rest night between treatment nights was unnecessary so in this experiment the treatment nights were consecutive.

Effect of benzoate and glycine on hippuric acid excretion

We decided that it would be much easier to interpret the results of this experiment if possums ingested the same amount of sodium benzoate with and without supplementary glycine. Thus, we offered six brushtail possums 20 mmol of sodium benzoate, glycine, or both compounds in only 150 g wet matter of food. This is about half the amount brushtail possums voluntarily eat in a night. All possums received every treatment for a single night in a Latin square design. Each treatment night was followed by two nights where the basal diet was offered ad libitum.

Urine fell through the mesh floor of cages onto a plastic collection tray and was funneled into preweighed plastic bottles surrounded by solid pellets of carbon dioxide in a vacuum flask. The bottles were changed every three hours for 27 hours from the time that the possums were first offered the treatment. DMI was measured at the same times so that rates of benzoate intake could be calculated. Bottles were weighed to determine the volume of urine collected, after which they were thawed and a subsample was kept for analysis. Subsamples were stored in the dark in glass vials at -20°C.

Do brushtail possums choose diets that enhance detoxification rates?

Six brushtail possums were housed in covered pens ($1.8 \times 2.0 \times 3.2$ m) containing branches for climbing, a nest box, and a water bowl. In the week before experiments we placed feeders in different positions to ensure that possums were used to looking for more than one food source.

There were two parts to this experiment. The first was to determine whether possums, given the choice, would select a diet that contains equimolar concentrations of benzoate and glycine rather than just one of these compounds. We formulated six choice combinations: three concentrations each of benzoate and glycine (0.13, 0.41, and 0.69 mmol/g DM) paired with the same concentrations of both compounds mixed in a single diet. For example, possums were offered one diet containing 0.13 mmol/g DM benzoate and another containing 0.13 mmol/g DM benzoate and 0.13 mmol/g DM glycine. The combinations were offered in a Latin square design so that every possum received each of the six combinations for one night. The concentrations were chosen because in dose response experiments they covered a range of responses by the possums, i.e., the lower concentration did not limit intake, the intermediate one was at the point where intake started to decline, and the highest concentration significantly reduced intake.

The aim of the second part of the experiment was to determine whether possums were able to choose a diet combination to enhance the rate of detoxification of benzoate. A diet containing 0.89 mmol/g DM glycine was offered with a separate diet containing either 0, 0.37, or 0.74 mmol/g DM benzoate. Each possum received each combination for two consecutive nights in a Latin square design. The first night was to introduce them to the combinations so they could learn the consequences of the choices, while DMI was measured on the second night. To ensure possums were sufficiently well fed they were offered the basal diet each day between 1000 and 1400 hours.

Do possums recognize toxin excesses through serotonin-mediated feedback?

Four diets were formulated: basal diet, and diets containing sodium benzoate, glycine, and benzoate and glycine, respectively. The concentration of benzoate and glycine used in each case was 0.46 mmol/g DM. Possums were fed each diet for four consecutive nights, and on the fourth night were given an intraperitoneal (IP) injection of either 0.55 mL of isotonic sodium chloride or 0.55 mL of the serotonin receptor antagonist, ondansetron (2 mg/mL; Glaxo, Stevenage, Hertfordshire, UK). This made eight possible treatments, but because of the cost of ondansetron we measured DMI for eight possums on only four of the treatments each in a truncated Latin square. To reduce carryover effects, possums received the basal diet for two nights following each treatment.

Analytical

Urine samples were analyzed for hippuric acid by the following method. In duplicate, a known mass ($\sim$1 mL) of urine was placed in a glass vial and a known

mass (~4 mL) of HPLC grade acetonitrile was added. Samples were mixed on a vortex every 15 min for 2 h, after which 6 mL of Milli-Q water containing 0.1% trifluoroacetic acid (TFA) was added. They were filtered (0.45 μm) and then 15 μL was injected onto a YMC-Pack ODS-A column (150 × 4.6 mm ID C18 column with a particle size of 5 μm and pore size 1.2 × 10^{-8} m; YMC, Wilmington, North Carolina, USA) maintained at 37°C with a flow rate of 0.75 mL/min on a Waters Alliance Model HPLC (Millipore Corporation, Milford, Massachusetts, USA). Hippuric acid was eluted under gradient conditions with 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B) as follows: 90% A/10% B increasing linearly to 70% A/30% B at 15 min, before returning to the starting conditions at 20 min. Samples that gave very large peaks were reinjected with a smaller volume. Four independent standards were prepared from hippuric acid (Aldrich, Milwaukee, Wisconsin, USA) and another from benzoic acid (Sigma, St. Louis, Missouri, USA). Absorbance was measured at 254 nm.

Because hippuric acid did not account for all benzoate ingested, we selected one urine sample from each of six possums fed a diet supplemented with benzoate and used the following method to identify the peak corresponding to benzoyl glucuronide, the likely minor metabolite. We added 50 μL β-glucuronidase (134 800 glucuronidase units/mL and 4000 sulfatase units/mL, Sigma, St. Louis, Missouri, USA), 250 μL Milli-Q water, and 200 μL acetate buffer (1.1 M, pH 5.2) to 0.5 mL urine and incubated overnight at 37°C. β-glucuronidase cleaves benzoyl glucuronide into glucuronic acid and benzoic acid. In the morning 2 mL of HPLC grade acetonitrile were added. Methods then followed those for the determination of hippuric acid except that 3 mL rather than 6 mL of Milli-Q water were added. After identifying which peak corresponded to benzoyl glucuronide, we used the peak area in chromatograms from which we had previously determined hippuric acid as a crude measure of relative concentration (in other words, a larger peak area presumably equals a greater amount of benzoyl glucuronide in the urine).

Statistical analysis

Data were analyzed using the residual maximum likelihood (REML) algorithm in Genstat 7.1 (Numerical Algorithms Group, Oxford, UK). For the no-choice experiments, fixed effects were the identifiable sources of variation, such as treatment, while random effects were the individual possums and the day on which the treatment was offered. When a fixed effect was significant ($P < 0.05$), means were compared with a least significant difference test ($LSD_{0.05}$). All data were checked to ensure that they satisfied the assumptions for parametric statistics and did not require transformation. Data from choice experiments were analyzed both as the total amount of food eaten, and the amount eaten of each of the diets. Once again, fixed effects included treatment and combination (i.e., which other diet was offered), while random effects were possum and day.

The final experiment with ondansetron was analyzed using the ANOVA function in Genstat. The treatment structure was the individual diet offered and whether or not ondansetron was administered. Body mass was included as a covariate.

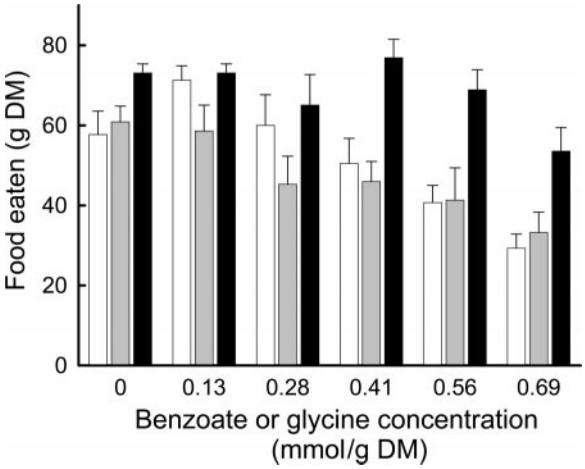

FIG. 1. The effect of increasing concentrations of benzoate (open bars) and glycine (gray bars) and of supplementing diets containing benzoate with equimolar glycine (black bars) on the amount of food eaten by six brushtail possums (mean + SE). DM = dry matter.

RESULTS

Response to variation in concentrations of benzoate and glycine

Brushtail possums offered the lowest concentration of benzoate ate more than they did of the basal diet. This was followed by a linear decrease in food intake with increasing concentrations of benzoate ($P < 0.001$), so that intake at the highest concentration of benzoate (0.69 mmol/g DM) was less than half that of the basal diet (Fig. 1). The amount of benzoate ingested reached a plateau at ~20 mmol/night (Fig. 2).

Brushtail possums responded in a similar manner to increasing concentrations of glycine ($P = 0.001$). At the highest concentration of glycine (0.69 mmol/g DM), DMI was slightly more than half that of the basal diet (Fig. 1). Glycine intake reached a plateau at ~22 mmol/night (Fig. 2).

Simultaneous ingestion of equimolar benzoate and glycine abolished the dose-dependent effects of each compound fed separately. Brushtail possums maintained food intake at the level of the basal diet at all but the highest concentration of benzoate and glycine ($P = 0.002$; Fig. 1). Of greater consequence was the ability of possums to almost double their intake of each compound (38 mmol) compared to the compounds offered singly (20–22 mmol; Fig. 2).

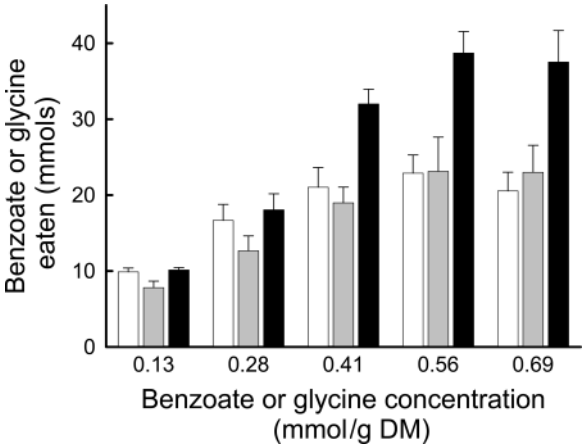

FIG. 2. The effect of increasing concentrations of benzoate (open bars) and glycine (gray bars) and of supplementing diets containing benzoate with equimolar glycine (black bars) on the amount of benzoate or glycine ingested by six brushtail possums (mean + SE). The black bars can be interchangeably viewed as benzoate or glycine intake because the compounds were equimolar in the diet.

Effect of benzoate and glycine on hippuric acid excretion

Possums fed a diet containing glycine but not benzoate excreted only trace amounts of hippuric acid. When possums were offered benzoate, on most occasions (8 out of 12) they ate all of their food and thus ingested 20 mmol of benzoate. However, on the other four occasions, possums left some food and thus consumed only 18 to 19 mmol of benzoate. The amount of benzoate ingested did not depend on which diet they were offered ($P = 0.34$) and did not affect how much hippuric acid they excreted ($P = 0.62$). Possums on all diets ate at the same rate ($P = 0.67$).

Possums excreted more hippuric acid when they were offered sodium benzoate with supplemental glycine (18.2 ± 2.2 mmol, mean ± SE) than when offered benzoate alone (13.5 ± 1.3 mmol; $P = 0.01$). Feeding benzoate and glycine together allowed faster excretion of hippuric acid than when benzoate was fed alone ($P < 0.001$; Fig. 3).

Benzoic acid was found in trace amounts in the urine samples before the addition of β-glucuronidase, but was found in greater concentrations after, confirming the presence of benzoyl glucuronide. Possums offered benzoate excreted approximately twice as much benzoyl glucuronide as those offered benzoate with supplementary glycine ($P < 0.001$). Benzoyl glucuronide was never found in the urine of possums offered diets supplemented only with glycine.

Do brushtail possums recognize diets that enhance detoxification rates?

Possums offered a choice between a diet containing either benzoate or glycine and one containing both of the compounds ate less when the concentration of the compounds was high ($P = 0.004$). This did not depend on which compound was offered singly ($P = 0.101$). However, both the concentration and identity of the single compound offered influenced which diet possums chose. Possums ate a high proportion of the diet containing both benzoate and glycine when glycine was the alternative diet. In contrast, the possums preference for the diet with both compounds increased as the concentration of benzoate in the alternative diet increased ($P = 0.003$; Fig. 4).

Brushtail possums offered diets supplemented with benzoate and glycine in separate feeders ate the same amount of the diet containing glycine no matter the

FIG. 3. The cumulative excretion of hippuric acid over 27 hours (measured every three hours) for six brushtail possums offered glycine (short-dashed line), sodium benzoate (solid line), or sodium benzoate and glycine (long-dashed line). Dotted lines represent the 95% confidence intervals.

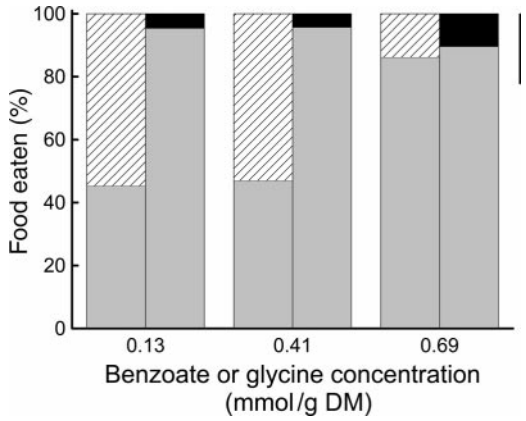

FIG. 4. The percentage of each diet eaten by six brushtail possums given a choice between two diets: one containing both benzoate and glycine (gray bars) and the second either benzoate (cross-hatched bars) or glycine (black bars). Each stacked bar represents one of the choices offered. The values listed on the *x*-axis describe the concentrations at which benzoate and glycine were added to the diets. The vertical bar to the right of the histograms represents the $LSD_{0.05}$ between treatments for the diet containing both benzoate and glycine. Therefore, there is a significant difference at the two lowest concentrations, but not at 0.69 mmol/g DM.

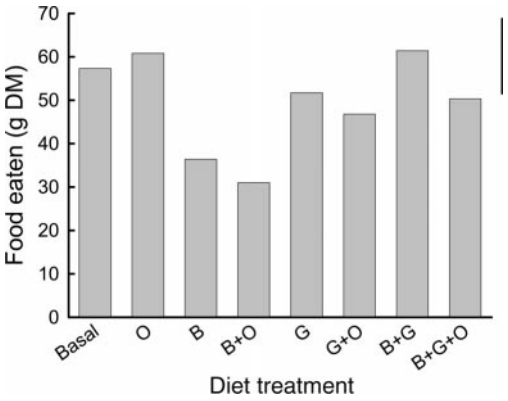

FIG. 5. The effect of an intraperitoneal injection of ondansetron (O) on food intake when diets were supplemented with benzoate (B), glycine (G), or both compounds (B+G). Four of the eight brushtail possums received each treatment; because body mass significantly affected intake ($P = 0.05$), means are adjusted. The vertical bar to the right of the histograms represents the $LSD_{0.05}$ between treatments.

concentration of benzoate in the other feeder (~5 g DM; $P = 0.397$). Furthermore, the actual amount of benzoate they ingested did not influence their intake of glycine ($P = 0.279$). Possums also ate less food in total at the highest benzoate concentration than at the two lower concentrations, even though glycine was available ($P < 0.001$).

Do possums recognize toxin excesses through serotonin-mediated feedback?

Heavier possums ate more food than lighter ones ($P = 0.05$). As expected, adding 0.46 mmol/g DM of benzoate significantly depressed intake ($P = 0.01$). Intake was restored after adding similar amounts of both benzoate and glycine. Surprisingly, adding 0.46 mmol of glycine did not affect food intake. In animals fed the basal diet alone, an IP injection of the serotonin receptor (5-HT_3) antagonist, ondansetron, did not affect food intake. But, ondansetron did not restore food intake to basal levels in possums fed 0.46 mmol/g DM of benzoate ($P = 0.595$; Fig 5).

DISCUSSION

By manipulating dietary concentrations of benzoate and glycine, we have shown that the amount of food that brushtail possums eat depends on the rate at which they detoxify a secondary plant chemical. It has long been known that the formation of hippuric acid is limited by the availability of glycine, rather than the rate of conjugation of glycine to benzoate via benzoyl CoA (Griffith and Lewis 1923, Gregus et al. 1993). We found that supplementary glycine increased the rate of hippuric acid excretion, which coincided with possums eating more of the food containing benzoate. Furthermore, when given the choice of eating the deterrent diets containing supplements of either glycine or benzoate, or the diet containing both supplements, whereby the glycine aids detoxification of the benzoate, the possums chose the latter. Surprisingly, when given the choice of the basal diet supplemented with benzoate in one feeder, or with glycine in another, they failed to maintain their food intake. Clearly, this is a more complex choice than the previous one because both benzoate and glycine, offered as single supplements, deter feeding. Perhaps this requires longer experimentation so that the possums can learn to eat each diet at a rate that enhances the detoxification of the benzoate.

Hippuric acid is not the only metabolite of benzoic acid—some is conjugated with glucuronic acid and is excreted as benzoyl glucuronide. There was about twice as much benzoyl glucuronide in the urine of possums that had eaten benzoate without supplemental glycine. This suggests that, in the absence of glycine, possums metabolized benzoate via an alternative pathway. A third and even more minor product of benzoate metabolism in brushtail possums, which we did not attempt to measure, is β-hydroxyphenylpropionic acid (Awaluddin and McLean 1985). The existence of at least two pathways other than glycine conjugation to detoxify benzoate poses the question of why brushtail possums do not continue eating at normal levels when benzoate is added to their food? The addition of benzoate to the basal diet reduced feeding by up to 50%. An animal's rate of ingestion of a compound should depend on how quickly it can metabolize it (Freeland and Janzen 1974). It seems that even by recruiting other pathways and increasing production of benzoyl glucuronide, brushtail possums are unable to detoxify benzoate at a sufficient rate to allow them to maintain food intake. The production of hippuric acid by conjugation of benzoate and glycine was the most efficient way for brushtail possums to eliminate benzoate. In most cases, the increase in detoxification rate that came with supplementary glycine allowed possums to eat as much of a diet containing benzoate as they did of the basal diet.

Glycine is a nonessential amino acid that can be formed from precursors such as serine, threonine, and glutamate (Sato et al. 1969, Rowsell et al. 1975, Bird et al. 1984). The basal diet was formulated so that a possum eating 60 g DM of the diet would consume ~1100 mg of nitrogen per day. This is ample to meet the truly digestible maintenance nitrogen requirement (210 $mg \cdot kg^{-0.75} \cdot d^{-1}$ or 480 mg/d for a 2.9-kg possum), determined with a similar diet by Wellard and Hume (1981). However, it will not supply adequate amounts of glycine, or the precursors of glycine, to allow possums to detoxify large amounts of benzoate. It would be interesting to investigate whether supplementing the diet of possums ingesting benzoate with glycine precursors or an excess of protein would result in a change in feeding similar to that observed when glycine itself was supplemented.

Even when provided with supplementary glycine, possums ate less at the highest concentration of benzoate offered than they did of the basal diet. Benzoate

and glycine were always added in equimolar proportions so if the rate of uptake of both compounds remained constant between the diets, it is unlikely that glycine availability restricted feeding. It is more likely that when a diet contains 0.69 mmol/g DM of both benzoate and glycine, other factors limit the rate of formation of hippuric acid. Hippuric acid is formed in two stages. Benzoic acid is first activated to benzoyl-CoA, and then the CoA is replaced with glycine (Hutt and Caldwell 1990). Depending on the amount of benzoate consumed, the availability of CoA or the activity of medium-chain acyl CoA-synthetase may restrict the rate of glycine conjugation (Gregus et al. 1992, 1993, Polonen et al. 2000).

Wild animals have access to plants that vary in their concentrations of plant secondary metabolites (PSMs) and nutrients. The ability to recognize plants with favorable combinations of toxins and detoxification substrates can simplify detoxification. To test whether possums can distinguish between diets that differ in detoxification potential, we offered diets supplemented with benzoate that either contained or did not contain supplementary glycine. The feeding choices made by the possums depended on the benzoate concentration. If the benzoate concentration was low (0.13 and 0.41 mmol/g DM), the possums fed equally from both diets. However, once the concentration of benzoate increased to 0.69 mmol/g DM, the possums preferred the diet that contained both benzoate and glycine. This concurs with the results from the dose response experiments, in which diets supplemented with 0.13, 0.41, or 0.69 mmol of benzoate did not restrict feeding, had an intermediate effect on feeding, and drastically reduced feeding, respectively.

At the lowest concentration of benzoate offered in the dose response experiments, possums ate ~10 g DM more than they had of the basal diet. We have no explanation for this finding other than suggesting that a small amount of benzoate has a favorable effect on nutrient balance and by doing so stimulates feeding. The amount possums ate of the basal diet also varied across the three experiments. We often notice that individual possums alter the amount they eat of the basal diet over time, yet remain consistent in their intake of food containing PSMs. Perhaps physiological constraints limit feeding in the presence of high concentrations of PSMs, while intake of the basal diet, as well as any diet that contains nonlimiting PSM concentrations, is governed by other unknown factors.

Despite our original focus on benzoate as a plant-derived feeding deterrent, we soon found that at the same molar concentrations, glycine was equally deterrent. From earlier work in our laboratory we know that brushtail possums tend to avoid diets containing free amino acids (DeGabriel et al. 2002). Therefore, an alternative interpretation of our results is that free amino acids also have detrimental effects on brushtail possums and so adding benzoate to a diet containing glycine ''mops up'' the excess glycine and allows possums to eat more. Polonen et al. (2000) found that plasma glycine concentrations were elevated in mink fed supplementary glycine in the absence, but not the presence of benzoic acid. Although we have not measured plasma glycine in possums, we would anticipate a similar effect.

Further studies of glycine showed that possums consistently preferred diets supplemented with both benzoate and glycine to those containing only glycine. This contrasts with the situation discussed earlier in which possums, given the choice, preferred a diet supplemented with both benzoate and glycine to one containing benzoate alone, only when the latter was at high concentrations. The two lowest glycine concentrations did not significantly reduce feeding in the dose response experiments, but in the choice experiments were rejected in favor of the diet containing both benzoate and glycine. These different responses suggest two things. First, that possums can distinguish between diets containing benzoate that differ in detoxification potential and, second, that possums can eat a certain amount of free glycine when they have no choice, but they would rather not.

The possums' aversion to glycine probably explains why, when given benzoate and glycine in separate feeders, they did not select a mixed diet to increase their detoxification capacity. We expected that brushtail possums would regulate their intake of glycine based on the concentration of benzoate offered. Several other animals are able to ''self-medicate'' when offered feeding deterrents along with items that counteract the detrimental effects of the deterrents. For example, lambs eating diets with high concentrations of tannins will ingest more polyethylene glycol (PEG), a compound that binds to tannins, than will lambs ingesting little tannin (Provenza et al. 2000). Although one of our possums ate some of the glycine diet at each benzoate concentration and two sampled it at the highest benzoate concentration, the others refused to eat any. There are at least two possible reasons why brushtail possums did not learn to self-medicate. The first and most obvious is that we did not give them enough time to detect the beneficial consequences of eating from both feeders. The possums were well aware of the negative consequences of eating the basal diet supplemented with either benzoate or glycine. Presumably, it is a complex series of learning steps for a possum to realize that feeding from both dishes is beneficial. Thus, two days may not have been long enough. Second, the concentration of glycine we offered was very high, even higher than in previous experiments. Offering a lower concentration of glycine may encourage initial sampling and thus speed learning.

The ability of brushtail possums to regulate their intake of benzoate and glycine when the compounds are offered separately or mixed together suggests that they can recognize amino acid or benzoate excesses

and modify their feeding accordingly. The signals that animals use to detect these excesses are key to understanding the selection of diets containing toxins (Foley et al. 1999). Researchers have suggested involvement of the neurotransmitter serotonin (5-HT) in detecting amino acid imbalances (Gietzen et al. 1991, Erecius et al. 1996), although it is unknown whether this initiates the inhibition of feeding or is a consequence of other events (Gietzen et al. 1998). Following Lawler et al. (1998), who found that 5-HT_3 receptors helped brushtail possums regulate their intake of a PSM, jensenone, we tested whether the same pathway was involved in regulating the intake of benzoate and glycine. Possums injected with ondansetron ate no more of a benzoate, glycine, or benzoate + glycine diet than did possums injected with saline. Thus, the possums in this experiment were not relying on signals from 5-HT_3 receptors to inform them of how much glycine or benzoate to eat. Despite this, our results suggest that a systemic signal regulates the ingestion of these compounds even though the nature of the feedback signal remains unknown.

Thirty years ago Freeland and Janzen (1974) proposed that detoxification limitations could drive the feeding decisions of mammalian herbivores. Although widely accepted and central to theories of plant defense, our poor understanding of detoxification pathways has made the theory difficult to test. As far as we know this is the first mammalian study to conclusively link detoxification capacity and feeding behavior. We have shown that saturation of detoxification pathways can constrain feeding. Furthermore, saturation of the primary detoxification pathway is critical and recruitment of secondary pathways cannot overcome the bottleneck to detoxification and restore feeding. Elucidation of detoxification pathways for more PSMs would allow us to determine how widespread this method of regulation is and whether detoxification limitations contribute to diet mixing as envisaged by Freeland and Janzen.

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