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ORIGINAL PAPER

# Acid loads induced by the detoxification of plant secondary metabolites do not limit feeding by common brushtail possums (*Trichosurus vulpecula*)

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**Abstract** We fed common brushtail possums artificial diets containing a buffer and the plant secondary metabolite (PSM), orcinol, to test the hypothesis that organic acids, common products of PSM metabolism, limit feeding by common brushtail possums (*Trichosurus vulpecula*). We introduced several diets containing orcinol and a buffer (urinary alkalising agent) over a course of three experiments. A diet containing 2% orcinol (wet matter) caused possums to reduce their food intake immediately, but feeding returned to normal 1–2 days later. Even though possums excreted strongly acidic urine (pH 5.1) and had perturbed nitrogen metabolism, they maintained their food intake and body mass until the experiment terminated 9 days after the introduction of orcinol. Possums ate 52% less when the basal diet contained 4% orcinol. As expected, the acid loads caused a change in the composition of urinary nitrogen with possums excreting more ammonium than urea and a large amount of unidentified nitrogenous material. Supplementing the diet containing orcinol with buffer neutralised the metabolic acid load and partly restored normal nitrogen metabolism, but did not restore feeding. Also, animals eating orcinol excreted normal amounts of 3-methylhistidine, indicating no increase in muscle protein catabolism. This suggests that a limitation to the rate of detoxification or toxicosis, rather than acid loads, limits the ingestion of acid-inducing PSMs.

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**Abbreviations**

| | |
|---|---|
| DM | Dry matter |
| DMI | Dry matter intake |
| 3-MeH | 3-Methylhistidine |
| N | Nitrogen |
| PSM | Plant secondary metabolite |
| REML | Residual maximum likelihood |
| WM | Wet matter |

## Introduction

Plants contain a multitude of secondary metabolites (PSMs), some of which defend them against herbivores (see Harborne 1988). Whilst animals may be able to avoid specific PSMs, these substances are so widespread that complete avoidance is impossible (Iason 2005). Most compounds that are absorbed are detoxified in the liver to a compound that is readily excreted (Williams 1959). Detoxification typically biotransforms a non-polar lipophilic PSM into a polar hydrophilic compound that the animal can excrete in urine or bile (Scheline 1978). Although most herbivores consume and detoxify PSMs, there is a limit to the amount they can ingest. Freeland and Janzen (1974) suggested that PSM intake might be regulated by the limitations and costs involved in detoxifying and excreting them. This has become known as the detoxification limitation hypothesis (Marsh et al. 2006; Sorensen and Dearing 2003).

Most studies of animal–PSM interactions examine factors that limit detoxification, for example, specific nutrients or enzymes that may be in short supply. Amongst these, a

convincing study was that of Marsh et al. (2005), who showed that the amount of glycine available for conjugation determined the amount of benzoic acid common brushtail possums (*Trichosurus vulpecula*) were willing to eat. Alternatively, researchers studied the costs involved in detoxification, such as the effect on overall energy budgets (Sorensen et al. 2005). Another measure of the cost of detoxifying PSMs comes from monitoring the strong organic acids that are a product of detoxification (Foley et al. 1995). Mammals use several buffering systems to counter excess hydrogen ions [$H^+$]. The main [$H^+$] buffer is bicarbonate, a component of the $CO_2/HCO_3^-$ buffer system, followed by the secondary buffers, phosphates and proteins (Heisler 1986; Henderson 1909). Because herbivores must eliminate excess [$H^+$] to maintain their blood pH within narrow limits, studying these acid loads provides a general approach to understanding the costs and consequences of detoxification (Foley 1992; Foley and Hume 1987).

Few studies have examined this aspect of the detoxification limitation hypothesis. Foley (1992) found that the acid load induced by the detoxification of terpenes and phenolic compounds ingested by common ringtail possums (*Pseudocheirus peregrinus*) was correlated with a reduced amount of urea that was recycled back to the gut, thus depressing their nitrogen (N) balance. Elsewhere, Campbell and Hewitt (2000) showed that acidotic white-tailed deer (*Odocoileus virginianus*) excreted large amounts of calcium. Dearing and Cork (1999) found that common brushtail possums ate more when offered two species of eucalypts simultaneously rather than separately. When fed separately, one eucalypt resulted in possums producing acidified urine and the other did not.

If an animal cannot buffer [$H^+$] with plasma bicarbonate then it must obtain additional supplies from the diet or from body reserves. Plants provide a rich source of potential bicarbonate for animals through the metabolism of citrate and malate salts contained in many leaves, fruits and vegetables (Atkinson and Bourke 1987; Atkinson and Camien 1982; Sherman and Gettler 1912). Alternatively, an animal can obtain more bicarbonate from stores of $CO_3^{2-}$ and $HCO_3^-$ in its skeletal system, which drives excess [$H^+$] into the bone matrix (Heisler 1986; Krieger et al. 2004), or through ammoniagenesis via the metabolism of the amino acids, glutamine, alanine and asparagine (Atkinson and Bourke 1984). For every mole of bicarbonate produced, an equimolar amount of ammonium is excreted in the urine. Therefore, urinary ammonium is a direct index of bicarbonate production (Halperin et al. 1989).

Foley et al. (1995) suggested that animals can obtain additional amino acids for ammoniagenesis from the catabolism of skeletal muscle, in a manner similar to that used by animals producing acid loads by other means (Caso and Garlick 2005; DelGiudice et al. 1998; Pickering et al. 2003; Williams et al. 1991). Protein is often in short supply in the diets of herbivores, especially marsupial folivores (DeGabriel et al. 2009; Hume 1999), whilst protein turnover consumes large amounts of energy (Reeds et al. 1985). Presumably, animals cannot depend on the catabolism of skeletal muscle protein for long and so we might expect animals to limit feeding to limit the production of acid loads. Alternatively, diet selection, which provides access to extra means of buffering [$H^+$] or protein, should alleviate the need to catabolise skeletal muscle protein. Researchers generally accept the concept of PSM-induced acid loads, prompting several attempts to study it with natural plant diets (Dearing et al. 2000; Foley 1992; Guglielmo et al. 1996; Mangione et al. 2001). The problem with this approach is that plants often contain a diversity of PSMs, which complicate the picture. This prompted us to tackle the problem in a simpler system by adding a PSM and buffer to an artificial diet fed to the common brushtail possum, a marsupial folivore. We chose the phenolic compound, orcinol (1,3-dihydroxy-5-methylbenzene), which is metabolised to a strong acid via a known detoxification pathway (Boyle et al. 1999; Marsh et al. 2006). In this way, we could study: the time course of urine acidification; determine whether the addition of dietary buffers helps common brushtail possums eliminate acid loads and whether the production of organic acids limits feeding; and determine how the excretion of PSMs as organic acids influences the N balance of the animal.

## Materials and methods

### Animals

We caught eight male common brushtail possums in cage traps in the peri-urban area of Canberra, adjacent to large areas of natural forest. We maintained them individually in large outdoor pens and fed them a variety of leaves and fruits whilst gradually introducing them to an artificial diet over a period of 1 month. Once acclimated to captivity, the possums were housed in individual metabolism cages as previously described by Foley and Hume (1987). The metabolism cages were kept in a temperature-controlled room (18 ± 2°C) conditioned on a 12:12 h, light:dark cycle, with the transitions occurring gradually over 1 h at 0600 and 1800 hours.

### Diets

An artificial diet (Table 1) was the sole food source during the experimental periods. We substituted Solka-Floc® (a purified wood cellulose powder) when increasing the dietary concentration of orcinol monohydrate (1,3-dihydroxy-5-methylbenzene) or buffer. We increased the

**Table 1** The composition of the basal diet fed in experiment 1 expressed as both dry matter and wet matter

| Ingredient | Inclusion (wet matter) | Inclusion (dry matter) |
|---|---|---|
| Apples | 39 | 16.05 |
| Bananas | 28 | 16.46 |
| Carrot | 10 | 3.23 |
| Oats | 8 | 22.33 |
| Rice hulls | 5.2 | 14.52 |
| Lucerne | 4.2 | 11.73 |
| HCl-casein | 0.5 | 1.40 |
| Solka-Floc®[a] | 3.5 | 9.77 |
| Oil | 1.25 | 3.49 |
| NaCl | 0.03 | 0.09 |
| $CaHPO_4 \cdot 2H_2O$ | 0.254 | 0.75 |
| Premix[b] | 0.066 | 0.19 |
| | 100 | 100 |

The diet contained about 34% dry matter

[a] Solka-Floc® is powdered wood cellulose that was used to allow the addition of buffer and orcinol to the diet. We increased the concentration of Solka-Floc® to 5% in experiment 2 and 6.5% in experiment 3 and reduced the concentrations of the apples through to lucerne

[b] Mineral and vitamin premix, Souters Min-A-Vit® for horses

concentration of Solka-Floc® to 5 and 6.5%, respectively, in experiments 2 and 3, and proportionately reduced the concentration of the apples to lucerne to accommodate higher concentrations of orcinol and buffer.

Although some researchers have delivered buffers via a gastro-oesophageal tube or intravenous infusion (Phy and Provenza 1998a, b), we wanted to mix the alkalising agent with the diet so that the animal had exogenous buffer whilst metabolising the orcinol. A preliminary experiment to determine a suitable alkalising agent showed that $KHCO_3$ reduced food intake by common brushtail possums, whilst a formulation based on a proprietary buffer to alkalinise the urine of humans experiencing urinary tract infections did not. This product contains sodium bicarbonate (44% with 5.2 mmol of bicarbonate generated per g), tartaric acid (22% and 0 mmol), citric acid (18 and 2.8 mmol) and sodium citrate (16% and 1.6 mmol). We made a buffer using this formulation and added it to diets to supply possums with bicarbonate at 1.25 times the molar concentration of orcinol.

## Measurement of food intake and collection and storage of urine and faeces

We fed the possums at 1700 hours each day and retained a sample of the food offered for immediate determination of dry matter (DM) content by oven drying at 60°C. Food refusals and faeces were collected at 0830 hours the next morning and dried to a constant mass. A collection tray beneath the metabolism cages allowed the separation of urine and faeces. A plug of glass wool in a funnel prevented any faeces or food from contaminating the urine, which accumulated in polythene bottles immersed in pellets of solid $CO_2$. We thawed the urine at room temperature and immediately collected two samples in 20-ml glass vials, which we stored at −20°C pending analysis.

## Experiment 1: the effect of orcinol on the time course of urine acidification and ammoniagenesis

Eight common brushtail possums (mean body mass ± SE 2.8 ± 0.1 kg), naïve to orcinol, were offered two diets, the basal diet and the basal diet supplemented with 2% orcinol wet matter (WM) in place of some of the Solka-Floc®. The amount of orcinol added was determined previously by Marsh et al. (2006), who described the amount as 'non-limiting' because it induces an acid load, but does not reduce feeding. The experiment was a one-group time series design with all possums receiving the same diet on the same day. The first 2 days the possums ate the basal diet, followed by 9 days of the orcinol diet and then 4 days of the basal diet.

We collected urine for later analysis of pH, titratable acidity, total N, ammonium and urea. As the urea and ammonium did not account for all of the urinary N excreted, we also analysed urine for several other nitrogenous compounds: hippurate, allantoin, uric acid, xanthine and creatinine (Wright 1995) and protein. Confirmation of protein prompted us to use specific clinical urinary protein assays to confirm the excretion of protein and to determine its origin. These included the benzethonium chloride urine/cerebrospinal fluid method (Iwata and Nishikaze 1979), biuret total protein method (Foster et al. 1952) and albumin bromocresol green method (Rasanaya et al. 1973). We excluded days 3, 4, 5 and 12 from the statistical analyses to avoid diet change effects.

## Experiment 2: the effect of acid loads on feeding

We measured food intake by the same eight common brushtail possums fed four diets: (1) the basal diet, but with additional water (17% total WM to increase urine volume) and Solka-Floc® (5% total WM); (2) a 4% orcinol diet; (3) a diet with buffer only at 1.25 the molar concentration of orcinol; and (4) an orcinol and buffer diet that included orcinol at 4% and buffer at 1.25 the molar concentration of orcinol. We allocated the eight possums to two groups and fed the diets over four 3-day periods in a modified cross-over design (Table 2).

We analysed urine for pH, titratable acidity, total urinary N, ammonium N and urea N, and used data from days 2 and 3 of each measurement period for statistical analyses.

**Table 2** The design of experiments 2 and 3

| Experiment 2 | Period 1 | Period 2 | Period 3 | Period 4 | |
|---|---|---|---|---|---|
| Group 1 | B | O | O + U | U | |
| Group 2 | B | U | O + U | O | |
| Experiment 3 | Period 1 | Period 2 | Period 3 | Period 4 | Period 5 |
| Group 1 | B | O | B | B@O | O + U |
| Group 2 | O | O + U | B | B | B@O |
| Group 3 | B@O | B | B | O + U | O |
| Group 4 | O + U | B@O | B | O | B |

In experiment 2 a period was 3 days and a group contained four possums and in experiment 3 each period lasted 7 days and a group was two possums

*B* Basal, *O* orcinol, *U* buffer, *O* + *U* orcinol and buffer, *B@O* basal at orcinol intake

### Experiment 3: the effect of acid loads on nitrogen balance

We measured N balance in the same eight possums offered three diets in four treatments: (1) the basal diet with additional water (28.5% total WM to increase urine volume) and Solka-Floc® (6.5% total WM); (2) orcinol; and (3) orcinol and buffer, as described in "Experiment 2". The fourth treatment was a pair-fed control to match the intake of possums offered the basal diet with that measured in animals fed orcinol (Diet 2) to determine the effect of reduced intake alone on N metabolism. The amount offered in the pair-fed control was determined by offering the basal diet supplemented with orcinol to each possum for 3 days before the experiment.

We designed the experiment with pair-fed controls for food intake, because variation in feeding has a large influence on N retention. The experiment was conducted using a 4 × 4 digram-balanced Latin square modified from Cochran and Cox (1950) with the pairs randomly allocated to four groups (Table 2). Each period lasted 7 days, with a 3-day pre-collection period followed by a 4-day collection period. After period two, we fed the basal diet to the possums for 5 days to enable them to regain their condition and to minimise any carryover effects.

We combined the four samples of urine and the four samples of faeces for each possum for each collection period, and analysed the samples of the diets, faeces and urine to calculate N excretion rates, digestibility of N and N balance. Two urine samples from possums eating each diet were also analysed for creatinine and 3-methylhistidine (3-MeH), which is a quantitative index of muscle protein catabolism (Watson et al. 1996). The experiment was analysed using the Latin square algorithm in the analysis of variance command in Genstat, with body mass included as a covariate. One possum ate very little of the orcinol diet in period two, so we removed the data for this animal from all analyses.

### Analytical

Dried food and faeces were ground using a Tecator Cyclotec 1093 sample mill (Tecator: Höganäs, Switzerland) to pass a 1-mm screen and stored in plastic vials at room temperature in the dark.

Urine pH was measured using a PHM62 standard pH meter (Radiometer A/S: Copenhagen, Denmark). Titratable acidity was determined by titrating 10 ± 0.1 ml of urine to pH 7.40 using a 716 DMS Titrino-6 with a 727 Ti stand and pH glass electrode (Metrohm: Herisau, Switzerland). Urine that was more acidic than pH 7.40 was titrated using 0.2 N sodium hydroxide, whilst urine that was more alkaline than pH 7.40 was titrated using 0.2 N hydrochloric acid.

The total N of food, faeces and urine was determined on duplicate 250-mg or 1-ml samples using a semi-micro Kjeldahl technique with a Tecator 2012 digester, selenium catalyst and a Gerhardt Vapodest-5 distillation and titration apparatus.

Ammonium N was determined on the Gerhardt Vapodest-5 distillation and titration apparatus by adding an excess of 40% (w/v) NaOH to 2 ± 0.1 or 1 ± 0.05 ml of urine, depending on expected concentration, to convert the ammonium to ammonia, which was then distilled and measured by titration. Urea N was determined directly using the reaction of urea and diacetyl monoxime in the presence of acidic ferric chloride (Crocker 1967).

Protein in the urine was detected using the Lowry method (Lowry et al. 1951). Allantoin, uric acid, xanthine and creatinine in urine were determined simultaneously using reversed-phase high-performance liquid chromatography (HPLC) (Shingfield and Offer 1999). We also analysed urinary hippurate by HPLC (Marsh et al. 2005).

We measured 3-MeH using the Waters AccQ.Tag analysis method (Millipore Corporation; Massachusetts, USA). Briefly, urine was made alkaline (pH 11) with NaOH, sonicated and heated at 55°C for 10 min to drive off ammonium. After returning the samples to neutral pH with HCl, we mixed the sample with 60 ml of borate buffer and 40 ml of Waters AccQ.Fluor reagent, let it stand for 1 min, heated it to 55°C for 10 min and then injected 10 ml onto a Nova-Pak $C_{18}$, 4 μm column at 37°C. The HPLC system was a Waters 600 system fitted with a 717 autosampler and a 474 fluorescence detector set to an excitation wavelength of 250 nm, emission wavelength of 250 nm, filter of 0.5 nm and gain of 100.

### Statistical analyses

We used the residual maximum likelihood (REML) and general analysis of variance (ANOVA) algorithms in Genstat 6.2 (6th edn) for all analyses. We started with complete models to identify all effects and progressively dropped non-significant effects. The animal's body mass was included as a covariate in all analyses. Body mass had a significant effect on some, but not all, analyses and was omitted from the model when it was not significant, and left in the model if it was marginally significant (i.e. $0.05 > P < 0.10$). Because proportional data are potentially misleading, in experiment 2, we compared the excretion of N as ammonium, urea and unknown sources by correcting for the total excretion of N. To avoid part–whole correlations (Christians 1999), we used "total N − ammonium N", "total N − urea N" and "total N − unknown N" as covariates for the three analyses, respectively. For all of the analyses, the residual plots satisfied the requirements for parametric statistics. The means were separated using a least significant test (l.s.d) (two-sided $t$ set to a probability of 0.05) when a significant difference was detected.

## Results

### Experiment 1

Overall, the common brushtail possums maintained their body mass throughout the experiment starting at 2.8 kg (SE = 0.1 kg) and ending at 2.9 kg (SE = 0.06 kg). Two possums lost 1 and 4% of their mass during the experiment. Animals immediately reduced their feeding from about 70 to 40 g DM day$^{-1}$ with the introduction of orcinol, but feeding returned to basal amounts 2 days later and remained there for the duration of the orcinol treatment (Fig. 1a). Mirroring these changes were those in urine pH, which declined from 7.8 to 5.1 (Wald 1 $df = 3{,}850.5$, $\chi^2$ $P < 0.001$; Fig. 1b), and in titratable acidity excretion, which increased from −0.6 to 5.9 mmol day$^{-1}$ (Wald 1 $df = 634.1$, $\chi^2$ $P < 0.001$; data not shown).

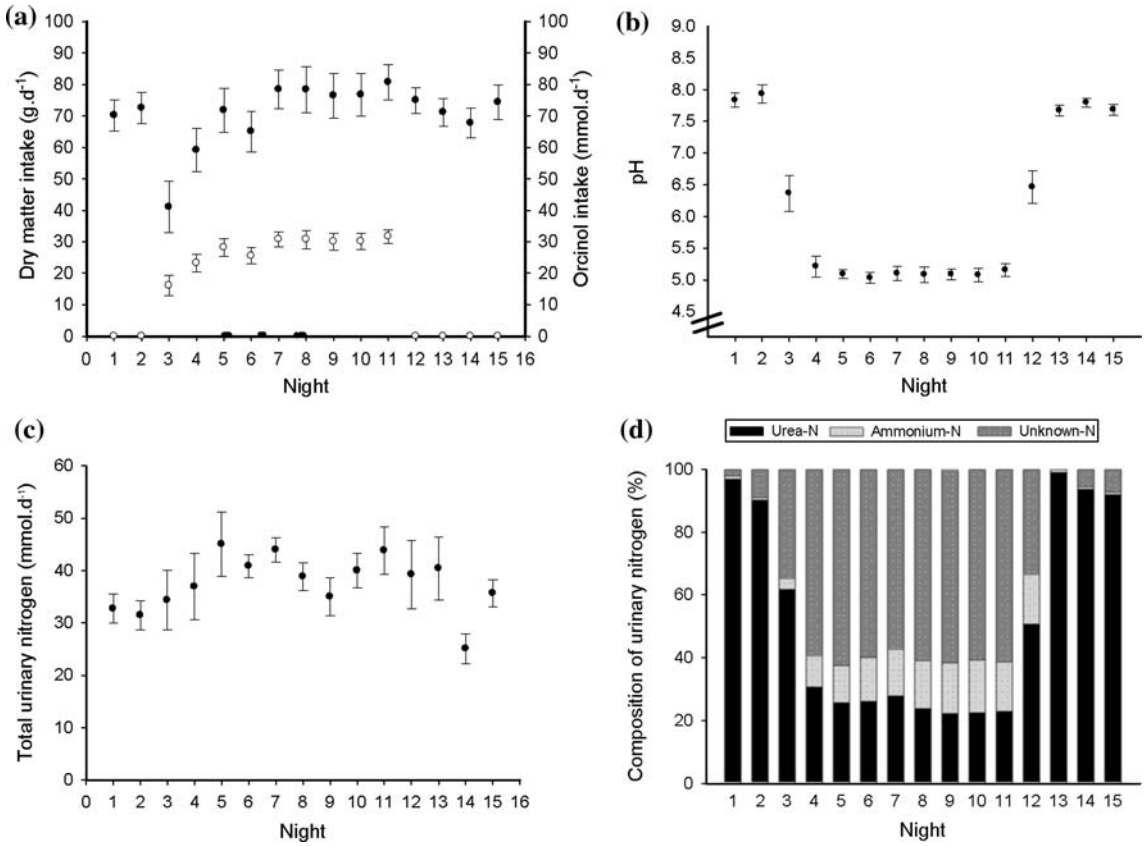


**Fig. 1** Food and orcinol intake and the composition of the urine for 15 days for common brushtail possums in experiment 1. The *arrows* indicate the period orcinol was incorporated in the diet. Values are mean ± SE and $N = 8$ for each day. **a** The daily intake of dry matter (*black*) and orcinol (*white*), **b** the time course of urine acidification, **c** total urinary nitrogen excretion, and **d** the proportional composition of urinary nitrogen

The introduction of orcinol also caused a change in the possums' N metabolism. The total N excreted in the urine increased from 33.1 to 40.4 mmol day$^{-1}$ (Wald 1 $df = 13.62$, $P < 0.001$; Fig. 1c). Ammonium N excretion increased from 0.3 to 6.0 mmol day$^{-1}$ and urea N decreased from 31.2 to 10.1 mmol day$^{-1}$ (Wald 1 $df = 808.7$, $P < 0.001$; Wald 1 $df = 175.2$, $P < 0.001$, respectively).

For the first two nights on the basal diet, the possums excreted urinary N primarily as urea (92.8%) and ammonium (1.5%), but on the last eight nights eating orcinol, urea N was about 26% and ammonium N about 23% of the total urinary N. Over half of the N excreted in the urine comprised unknown nitrogenous compounds (Fig. 1d). On reintroducing the basal diet, there was an immediate increase in the composition of urea N to 48% of the total urinary N, and a decrease in the unknown nitrogenous compound to 30%.

We analysed the urine for compounds that might explain the unknown source of N. HPLC traces revealed that there was insufficient allantoin, uric acid, xanthine, creatinine or hippurate to explain the additional N. A protein analysis (Lowry method) suggested that possums were excreting 1,000% more protein in their urine when they ingested orcinol than when they ate basal diet. Because some compounds interfere with the Lowry method, especially plant phenolic compounds (Smith 1982), we reanalysed the samples using various other assays. The biuret assay showed that possums excreted protein in their urine, but there was no difference between the protein content of the urine when possums were eating the basal diet compared to when they were ingesting orcinol. Likewise, the benzethonium chloride assay showed that possums eating both the basal diet and the basal diet supplemented with orcinol excreted less than 68 mg protein per L of urine (the minimum detectable concentration), whilst the albumin bromocresol green assay also suggested negligible losses.

## Experiment 2

Possums ate about 65 g DM of the basal diet each day, whilst adding 4% orcinol to the diet depressed this by 52%. Adding buffer to the basal diet also suppressed intake (16%), whilst supplementation with both buffer and orcinol caused possums to eat roughly what they did on orcinol alone (Fig. 2a).

The dietary treatments had a highly significant effect on the pH of urine (Wald 3 $df = 95.0$, $P < 0.001$). Urine pH dropped to about 5.4 when the possums ate the orcinol diet and increased to 8.2 on feeding buffer, whilst possums eating the diet containing both buffer and orcinol, produced urine with a pH intermediate of that when they ingested orcinol or buffer separately. The pattern of titratable acid excretion resembled that of urinary pH, with a highly significant treatment effect (Wald 3 $df = 68.5$, $P < 0.001$). Both the basal diet and that containing buffer caused the excretion of negative amounts of titratable acidity (−0.3 and −3.7 mmol day$^{-1}$, respectively), whilst possums ingesting orcinol alone excreted 4.3 mmol day$^{-1}$. Feeding buffer with orcinol alleviated this effect, with possums excreting 0.9 mmol day$^{-1}$ of titratable acidity.

Unlike experiment 1, the treatments did not affect the excretion of total urinary N (Wald 3 $df = 1.87$, $P = 0.132$). There was, however, a trend towards increased urinary N excretion, with possums excreting less when eating the basal diet (24 mmol day$^{-1}$) and much more when ingesting orcinol and buffer simultaneously (35 mmol day$^{-1}$). Even after correcting for differences in the excretion of urinary N, the treatment still affected the rates of excretion of ammonium, urea and the unknown urinary N (Fig. 2b), which resembled the pattern in

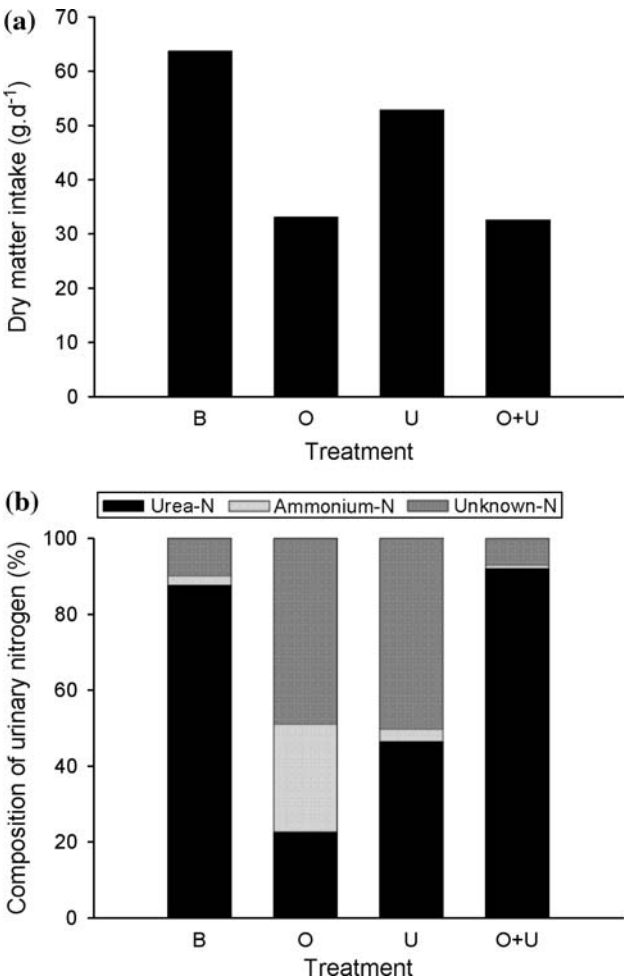


**Fig. 2** **a** Food intake (dry matter g day$^{-1}$) and **b** the composition of urinary nitrogen excreted by common brushtail possums offered various diets in experiment 2 where *B* basal, *O* orcinol and *U* buffer. $N = 16$ for each treatment

experiment 1. The urinary N excreted by possums eating both the basal diet and the basal diet supplemented with buffer contained mainly urea N and a little ammonium N. Ingestion of orcinol caused possums to excrete urine with more ammonium and less urea, with these components again explaining only 50–60% of the total urinary N excreted. Similarly, when possums ingested orcinol and buffer simultaneously, they excreted much more urea and much less ammonium N than they did when ingesting orcinol alone, but these compounds still accounted for only 50–60% of the total N excreted.

Experiment 3

Possums offered the basal diet ate about twice as much as they did when the diet contained orcinol or both orcinol and buffer (64 vs ~32 g DM day$^{-1}$; $P = 0.005$). Possums maintained body mass when fed the basal diet but lost mass when eating diets containing orcinol, both orcinol and buffer and a reduced amount of the basal diet ($P = 0.016$). Possums digested more DM when ingesting orcinol or orcinol and buffer simultaneously, than they did on either of the basal treatments ($P = 0.002$) (Table 3).

All diets contained similar concentrations of N, so the wide differences in dry matter intake (DMI) caused similar differences in N intake. In contrast, possums excreted similar amounts of N on all diets (38.4–48.5 mmol day$^{-1}$), but the pattern of excretion differed markedly between treatments. Of note was the reduced excretion of faecal N by possums ingesting orcinol or both orcinol and buffer and the relatively high excretion of faecal N by possums consuming the basal diet at a reduced intake. When the data were analysed with DMI as a covariate, the possums excreted similar amounts of faecal N when they were fed the basal diets, and this was greater than when they were fed either diet containing orcinol ($F_{3,6} = 18.1$, $P = 0.004$). In contrast to faecal N, possums excreted less urinary N when they were fed the basal diets (20.3 and 20.7 mmol day$^{-1}$) compared to when they were fed the diets containing orcinol (33.7 and 33.8 mmol day$^{-1}$; Table 3). The vastly different ingestion of N, but similar excretion of N, by possums on the four treatments explains why the treatments strongly affected N balance. Possums were in positive N balance (6.9 mmol day$^{-1}$) when eating the basal diet ad libitum, but were in negative N balance on the other treatments (−10.9, −14.6, −14.7 mmol day$^{-1}$, respectively for the basal at reduced intake, the orcinol and the combined orcinol and buffer diets).

## Discussion

It is difficult to measure the costs of ingesting and metabolising PSMs. One of the consequences of conjugation reactions to detoxify PSMs, such as orcinol, is the production of acid loads (e.g. Dearing and Cork 1999; Foley 1992; Mangione et al. 2001; Marsh et al. 2006). Foley proposed that this represents a major metabolic cost to an animal, whilst the ability of the animal to maintain acid–base homeostasis in the face of an acid load could determine the amount of the given compound an animal will eat. Marsh et al. (2006) support this thesis. When increasing the concentration of orcinol in the diet, they showed that common brushtail possums reach a plateau of orcinol intake at roughly 30 mmol per day. At this asymptote, food intake is at basal levels, so any increase in the concentration of orcinol in the diet causes possums to eat less. In keeping with Marsh et al. (2006), we found that possums capped their intake of orcinol at a similar level (~34 mmol day$^{-1}$ in experiments 1–3). We also showed

**Table 3** Mean nitrogen intake and excretion in common brushtail possums fed various diets

| | B | O | O + U | B@O | l.s.d (5%) | *F* | *P* |
|---|---|---|---|---|---|---|---|
| Nitrogen (% dry matter) | 1.1$^{a}$ | 1.1$^{a}$ | 1.2$^{a}$ | 1.1$^{a}$ | – | 0.25 | ns |
| Dry matter intake (g day$^{-1}$) | 64.0$^{a}$ | 34.9$^{b}$ | 31.6$^{b}$ | 31.6$^{b}$ | 14.9 | 13.4 | 0.005 |
| Body mass change (kg week$^{-1}$) | 0.07$^{a}$ | −0.23$^{c}$ | −0.14$^{bc}$ | −0.11$^{b}$ | 0.12 | 21.5 | 0.016 |
| Dry matter digestibility (%) | 72$^{a}$ | 82.3$^{b}$ | 86.5$^{b}$ | 70.8$^{a}$ | 6.2 | 18.7 | 0.002 |
| Nitrogen intake (mmol day$^{-1}$) | 55.4$^{a}$ | 31.0$^{b}$ | 27.9$^{b}$ | 27.5$^{b}$ | 13.69 | 11.4 | 0.007 |
| Faecal nitrogen (mmol day$^{-1}$) | 28.1$^{a}$ | 11.9$^{c}$ | 8.9$^{c}$ | 17.7$^{b}$ | 5.8 | 25.4 | <0.001 |
| Apparent nitrogen digestibility (%) | 52.0$^{a}$ | 62.0$^{ac}$ | 71.7$^{c}$ | 37.7$^{b}$ | 10.3 | 23.8 | <0.001 |
| Urinary nitrogen (mmol day$^{-1}$) | 20.3$^{a}$ | 33.7$^{b}$ | 33.8$^{b}$ | 20.7$^{a}$ | 6.9 | 14.6 | 0.004 |
| Total nitrogen excreted (mmol day$^{-1}$) | 48.5$^{a}$ | 45.5$^{a}$ | 42.6$^{a}$ | 38.4$^{a}$ | – | 2.4 | ns |
| Nitrogen balance (mmol day$^{-1}$) | 6.9$^{a}$ | −14.6$^{b}$ | −14.7$^{b}$ | −10.9$^{b}$ | 12.3 | 8.4 | 0.014 |

Mean with a common superscript are not significantly different ($P < 0.05$)

*B* Basal, *O* orcinol, *O + U* orcinol and buffer, *B@O* basal at orcinol intake

that the acid load experienced by possums ingesting orcinol did not determine the amount of orcinol they willingly ingested. Instead, when we neutralised the acid load, possums still did not eat more and continued losing large amounts of unidentified nitrogenous compounds in their urine, likely indicating an unknown toxic effect of orcinol.

In the first experiment, we manipulated the concentration of orcinol in the diet so that possums could still eat their basal amount of food whilst remaining slightly below the ceiling of orcinol intake determined by Marsh et al. (2006). The introduction of orcinol caused an immediate drop in food intake, but intake quickly returned to normal after 2 days. Simultaneously, possums started excreting large acid loads and N metabolism shifted towards ammoniagenesis. The possums maintained this state for 9 days and there seemed no reason, judging from their stable body mass, why they could not maintain it much longer. Nonetheless, closer inspection showed the expected increased concentration of urinary N, of which half remained unidentified. Furthermore, it was not a typical urinary metabolite such as allantoin, creatinine, hippurate or xanthine, nor was it protein. As orcinol is detoxified through conjugation with glucuronic acid (Marsh et al. 2006), the excess N in the urine was not a result of conjugation with nitrogenous conjugates. Clearly, there was severe perturbation of N metabolism that would presumably be reflected in measures of whole body protein synthesis and metabolic rate.

The main mechanism for excreting acid loads is to combine protons with bicarbonate ions and to excrete water and carbon dioxide. Animals produce bicarbonate (without simultaneously producing protons) only by catabolising amino acids that also produce ammonium ions (Atkinson and Camien 1982). Under normal metabolic conditions, ureogenesis uses the bicarbonate and ammonium ions produced from amino acid catabolism and the urea is recycled or excreted in the urine. In contrast, where there is perturbation of acid–base homeostasis, use of the bicarbonate ions for acid–base balance leaves excess ammonium that the animal excretes in the urine (Frassetto et al. 1997; James and Kyriazikis 2002; Sabboh et al. 2005; Walker et al. 1978).

Thus, one cost of detoxification is an increased catabolism of amino acids and perhaps reduced protein synthesis. In more extreme circumstances, the animal may need to catabolise body protein to supply the amino acids for bicarbonate production. This did not appear to be a major factor in these experiments, because there was no difference in urinary 3-MeH excretion. In common ringtail possums (Watson et al. 1996), as well as many other mammals (Haverberg et al. 1975; Long et al. 1977; Wassner et al. 1977), urinary 3-MeH is a quantitative index of muscle protein catabolism. Another cost, which we did not measure, is the drain on glucose reserves in supplying glucuronic acid. The glucose may come from circulating supplies, glycogen or the animal may synthesise it from ascorbate (Dash 1988) or glycogenic amino acids.

In experiment 2, we doubled the concentration of orcinol in the diet to ensure that possums reached the plateau of their potential orcinol intake whilst eating less than normal. Then, based on the acid–base limitations hypothesis, we predicted that alleviating the effects of acid loads, by adding an alkalising agent to a diet containing orcinol, would enable the possums to eat as much as they did of the basal diet. The large increase in urinary pH and significant decline in titratable acidity indicated that the exogenous buffer successfully neutralised the metabolically produced acid load. Likewise, the buffer partly reversed the change in N metabolism, with a decline in the excretion of ammonium and increase in the excretion of urea from that observed when the animals ingested orcinol in the absence of buffer. In contrast, the buffer did not reduce the loss of unidentified N in the urine of animals ingesting orcinol. More importantly, the buffer did not facilitate increased feeding. This suggests that factors other than the acid loads, resulting from the ingestion of orcinol, limits feeding.

We know of no other studies in which researchers have attempted to alleviate the effects of PSM-induced acid loads by providing alkalising agents. Perhaps the closest analogy is of the sheep that either selects or eats more of diets containing $NaHCO_3$, because it alleviates intraruminal acid loads resulting from the rapid fermentation of diets rich in soluble carbohydrates (Cooper et al. 1996; Phy and Provenza 1998b). In this case, however, feeding delivers the alkali directly to the site of acid production in the animal's rumen. This raises the question of how acid loads in the absence of toxicosis affect feeding (i.e. a positive control), a subject L'Estrange and McNamara (1975) studied by feeding sheep grass diets supplemented with HCl and by infusing the acid directly into the rumen. They concluded that the feeding depression was due to both reduced palatability and to acidosis. There seem, however, several anomalies to their research. For example, urinary pH remained unexpectedly high (pH 7.2–8.7) and did not differ between treatments, whilst the bicarbonate treatment was administered only with the intra-ruminal acid treatment and not fed with the grass plus acid or alone. Unfortunately, they did not study the urinary metabolites.

Although our preliminary experiment suggested otherwise, adding buffers to the diet of possums was another factor that caused them to eat less. This is not surprising given the mixed reports in literature about the physiological responses of animals to potassium salts and to alkalising agents and the expectation that such responses would affect feeding. For example, Sabboh et al. (2005) found that supplementing the diet of rats with potassium in

several forms, KCl, $KHCO_3$ and potassium malate, caused increases in urinary N excretion. In contrast, Walker et al. (1978) found no change in the excretion of urinary N in lambs fed an acid-producing diet supplemented with $NaHCO_3$. Complicating the picture further, Frassetto et al. (1997) found that $KHCO_3$ decreased urinary N excretion in postmenopausal women. The amount of N excreted in the urine usually depends on the amount of protein ingested or the amount the body catabolises (Meakins et al. 1998; Meijer et al. 1990; Steenbock et al. 1914). Thus, we might expect that an increase in the ionic content of the basal diet might cause possums to eat less, but we expected that an acid load would increase the demand for buffer and that the combined effects of orcinol and buffer would stimulate feeding. Clearly, detoxification and acid–base homeostasis are not so simple.

The complexity of detoxification and acid–base homeostasis is clear from the research of Mangione et al. (2001), who rejected the hypothesis that the regulation of PSMs may result from a change in pH homeostasis produced from the detoxification of PSMs. The reason they gave was that neither the maximum value for glucuronide excretion nor the minimum urinary pH corresponded with tolerable intake of phenolic resin from creosote bush (*Larrea tridentata*). Although they recorded a decline in the pH of urine with increasing ingestion of *Larrea* phenolic resin, the relationship was poor and the lowest pH they recorded was between 7.5 and 8, many times more alkaline than we recorded in possum urine here. It seems that some factor independent of acid loads limited the amount of resin woodrats willingly ate, just as something independent of acid loads limited feeding by possums.

Although PSMs, such as orcinol, generate acid loads, so do other mechanisms. For instance, a reduction in urinary pH and changes in urinary N composition also occur when an animal fasts or ingests a diet containing mineral acids (Hannaford et al. 1981; Hannaford et al. 1982). We foresaw this possibility in experiment 3 and included a pair-fed treatment matching the possums' intake of the basal diet to the intake recorded when the diet contained orcinol. Even when eating half their normal amount of food, their urine resembled that of fully fed possums rather than those ingesting orcinol. Thus, the acid load and the pattern of N excretion generated in this study were not caused by a reduction in food intake, although the negative effects of an acid load may be exacerbated by the plane of nutrition.

Orcinol may affect the N metabolism of the common brushtail possum in at least two ways: through generating acid loads and by reducing food intake. Experiment 3 aimed to test these possibilities. The possums excreted similar amounts of N on all four diets, but the proportion of N excreted in the faeces or urine differed. Possums eating the basal diet, even at a reduced intake, lost about 60% of their N in the faeces, whilst those ingesting orcinol, with or without buffer, lost about 70% in their urine.

Common brushtail possums usually excrete more faecal N than urinary N, because they do not have the physiological ability to retain small particles, which has evolved in other marsupials, such as ringtail possums, greater gliders (*Petauroides volans*) and koalas (*Phascolarctos cinereus*) (Foley and Hume 1987). The indication that the increased urinary N excretion is unrelated to the production of acid loads or reduced intake suggests that orcinol has some other effect.

Although the results suggest that possums excrete less faecal N when eating diets containing orcinol, differences in dietary composition confound this interpretation. Possums eating basal diets ingested more Solka-Floc®. Many researchers argue that additives should replace inert constituents in the diet, so that the nutritional composition of the food remains unchanged. Inclusion of Solka-Floc® probably explains the lower digestibility of the basal diet, but it may also explain the higher rates of faecal N excretion compared to diets containing orcinol or buffer. Perhaps, Solka-Floc® is not inert in the sense that it increases faecal N output and disadvantaged possums eating reduced amounts of the basal diet.

Two studies suggest a link between detoxification, acid–base status and N balance. In the first, Guglielmo et al. (1996) showed that PSMs affected N metabolism. Ruffed grouse (*Bonasa umbellus*) eating aspen buds, containing coniferyl benzoate, required more ornithine for conjugation and excretion, indicating a substantial increase in protein requirements for maintenance. Foley (1992) also found an effect, but, as in the present study, the reason remained unclear. Ringtail possums fed *E. radiata* produced an acid load and were in negative N balance. In contrast, ringtail possums that ate *E. ovata* foliage did not produce an acid load and remained in positive N balance, despite ingesting less N than those feeding on *E. radiata*.

Researchers have studied the interactions between PSMs and herbivores in several ways, probably because there are many thousands of PSMs and herbivores and a great many detoxification pathways. In this study, we used an animal, the diet of which could be manipulated and matched with a PSM with a known detoxification pathway in the animal. By doing so, we demonstrated that there is a limit to the amount of PSM the animal can ingest, independent of the acid load it generates.

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