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The effect of excesses and deficiencies in amino acids on the feeding behaviour of the common brushtail possum (*Trichosurus vulpecula*)

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Abstract In this study of the amino acid nutrition of a marsupial we tested three hypotheses: (a) that brushtail possums eat less when diets contain excesses or deficiencies in essential amino acids, (b) that brushtail possums choose diets that do not contain amino acid excesses, and (c) that amino acid consumption is mediated partly by the 5HT₃ receptor. Possums ate less when 0.2–1.0% methionine (wet matter) was added to the diet, but similar concentrations of lysine and threonine had little effect. However, when given a choice, possums always selected the basal ration over one with added lysine, methionine or threonine at concentrations between 0.05% and 0.9%. In contrast to the experiments with excess amino acids, possums did not eat less of a diet almost devoid of an essential amino acid. Instead, the possums ate less when their diets contained synthetic amino acids rather than similar amounts and proportions of amino acids as casein. Contrary to the third hypothesis, the 5HT₃ receptor antagonist, ondansetron, did not affect feeding by possums given a diet containing 0.8% methionine, suggesting that post-ingestive feedback, via the 5HT₃ receptor, does not regulate amino acid intake when diets contain amino acid excesses.

Keywords Lysine · Methionine · Threonine · Feeding · Marsupial · Ondansetron

Abbreviations AA Amino acid · DM Dry matter · DMI Dry matter intake · 5HT Serotonin · WM Wet matter

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Introduction

It is commonly believed that the foraging strategies of folivorous marsupials are limited by nutrient availability. In order to remain in nutritional balance animals must match their requirements for specific nutrients with the temporal and spatial availability of foods (Simpson and Raubenheimer 1999). Australian environments are widely perceived as deficient in available nutrients, and this is reflected in both the growth forms of plants and in the life histories of many mammals. Indeed, limited availability of nitrogen has been advocated as the essential factor limiting the productivity of animals and constraining their food choices (White 1992). Regarding arboreal folivores, this has led to a variety of studies and speculation. For example, Degabriele (1981), Braithwaite et al. (1984) and Cork and Catling (1996) examined the correlation between the distribution patterns of folivores in Australian forests and the concentration of foliar nitrogen, while various researchers measured the nitrogen requirements of the arboreal marsupial species that eat eucalypt foliage (see Hume 1999).

Despite the vast number of eucalypts only four arboreal species eat eucalypt foliage. These marsupial folivores span the range from extreme dietary specialists, such as the koala (*Phascolarctos cinereus*; Cork and Sanson 1990) and the greater glider (*Petauroides volans*; Marples 1973), through the intermediate common ringtail possum (*Pseudocheirus peregrinus*; Pahl 1987) to the generalist herbivore, the common brushtail possum (*Trichosurus vulpecula*; Kerle 1984) that relies less on eucalypt foliage than do the other species. Differences in digestive physiology separate the species. The gut of the brushtail appears not to have an extensive particle separation mechanism and, unlike the ringtail possum, the species does not practice caecotrophy to retrieve the microbial protein synthesised in the hindgut. This means that the brushtail possum has little use for the non-amino acid nitrogen in plants and instead has a dietary requirement for amino acids (AAs).

Extensive literature focuses on all aspects of essential AAs in the nutrition and diet selection of domesticated and laboratory species such as chickens and rats, but there has been little explicit recognition of the importance of essential AAs for wild species. Marsupials are a prime example. Although many species must obtain their AA requirements from dietary sources, there are only scattered reports of the AA content of potential foods (Hume 1999). Amino acid nutrition may be particularly relevant for folivorous marsupials that must acquire AAs from *Eucalyptus* foliage containing low concentrations of proteins (Cork and Catling 1996) but relatively high concentrations of secondary chemicals, some of which, including tannins, bind to dietary proteins and make them unavailable to the animal (Karasov et al. 1992).

Animals probably detect excesses and deficiencies in AAs via alterations in the pattern of plasma AA concentrations (Krauss and Mayer 1965; Leung et al. 1968) and competition for transport across the blood-brain barrier (Hrupka et al. 1997). The neurotransmitter serotonin (5HT) has been implicated in this signalling process by inducing sensations of satiety in rats fed AA-deficient diets (Erecius et al. 1996; Gietzen et al. 1991). The 5HT₃ receptor subtype, in particular, has been implicated as part of the pathway in nutrient regulation (Gietzen et al. 1991). Lawler et al. (1998) proposed this same signalling pathway for regulating the ingestion of plant toxins by brushtail possums, suggesting integration of nutrient and toxin pathways.

Alternatively, animals may regulate their AA nutrition through gustatory mechanisms using taste and odour cues as markers to identify foods that exert negative post-ingestive effects (Arnold et al. 1980). Provenza et al. (1996) and Day et al. (1998) argue that herbivores do not simply avoid foods that they recognise as being nutritionally deficient, but continually sample a range of novel and familiar foods to glean information about potential food sources.

This study considers the interactions between post-ingestive and pre-ingestive cues in the diet selection decisions of wild-caught brushtail possums offered diets differing in their AA patterns. We first compared the effects of dietary excesses and deficiencies of three essential AAs – lysine, methionine and threonine – on feeding by brushtail possums. We anticipated that a dietary excess of a single AA would elicit a reduction in feeding by possums due to elevated plasma concentrations of that AA, but that responses would vary between the AA sub-groups due to differences in rates of uptake (Hrupka et al. 1999). We were interested also in determining whether possums would respond differently to dietary AA deficiencies and excesses. Finally, we sought to determine the physiological mechanisms by which possums recognise AA excesses and regulate their feeding behaviour. Specifically, we attempted to determine a role for the 5HT₃ receptor in the regulation of AA intake by wild marsupials. We anticipated that if possums rejected diets containing AA excesses, due to

serotonergic feedback, antagonism of this pathway would restore food intake.

Materials and methods

Animals

Twelve male common brushtail possums were captured in wire cage traps in woodland around Canberra, in southeastern Australia. The animals were housed in individual metabolism cages as previously described (Foley and Hume 1987) in an air-conditioned room maintained at approximately 21°C, on a 12 h:12 h light:dark cycle. A dimmer switch allowed a gradual change in brightness over a 1-h period, simulating dusk and dawn. In the 2 weeks after capture possums were fed *Eucalyptus* foliage and fruits before being gradually introduced to basal diet 1 (see below) until this was the only food on offer.

Diets

Two basal diets were used throughout the experiments. We maintained animals on Basal diet 1 and fed it in experiments that did not require us to restrict AA intake. This basal diet consisted of (% wet matter, WM) grated apple (50.0), grated carrot (15.0), mashed banana (13.0), ground rice hulls (8.0), sugar (5.7), ground lucerne (5.0), ground Weetbix (a wheat-based breakfast cereal; 3.0) and acid casein (0.3). This diet contained on average 28% dry matter (DM) of which 0.94% was N, enough for possums to easily satisfy their N requirements. Although it is conventional to report dietary composition as DM, these diets are easier to reproduce if the information is in WM.

To determine the short-term effects of feeding possums diets with AA deficiencies we formulated a low protein basal diet that we supplemented with synthetic L-forms of AAs obtained from Ajinomoto (Japan). This diet (Basal diet 2) consisted of (%WM): grated apple (87.0), sugar (9.2), Solka-Floc (wood cellulose, 2.9), vitamin and mineral supplement (0.35), NaCl (0.25), monounsaturated vegetable oil (0.15) and polyunsaturated vegetable oil (0.15) and contained on average 25% dry matter (DM) of which 0.18% was N. Apart from requirements for special functions (e.g. moulting birds require more cystine) the AA requirements of animals across taxa, expressed as ratios, are remarkably similar, and what varies most is the requirement for protein. We assumed that brushtail possums follow this trend and calculated their proportional AA requirements as the mean of those for the pig, laying chicken, rat and trout. On this basis we prepared six separate AA mixtures (Table 1): (a) all essential AAs present in the ‘‘correct ratios’’, (b) all non-essential AAs present in the correct ratios, (d) all essential AAs except Lys, (d) all essential AAs except Met, (e) all essential AAs except Thr, (f) all essential AAs except Lys and Thr.

When pure AAs were added to the basal diet, a batch of the dry ingredients, sufficient for the experiment, were lightly sprayed with honey diluted in warm water, and then AAs were sprinkled over and mixed thoroughly. The control diet was sprayed with diluted honey only.

Unless stated otherwise diets were prepared each day, and about 400 g was presented to each possum as a wet mash at 17:00 h with drinking water available ad libitum. The uneaten food was removed at 08:30 h the next morning. The food refusals along with a sample of the food offered were oven-dried to constant mass at 80°C to determine dry matter intake (DMI). Food intake was measured in the days before and after each experiment to ensure that animals were not adversely affected by experimentation. The possums were weighed at regular intervals throughout the study to provide an indication of their welfare, both during and between experiments. This also provided information on the long-term effects of a low protein diet.

The experiments described below are summarised in Table 2.

Table 1. The composition of the essential and non-essential AA mixtures used for the deficiency experiments and the determined nitrogen and amino acid composition of Basal diet 1 fed in part A (excess AAs), the complete diet fed in part B (AA deficiencies) and the casein diet fed in parts B and C. Possums fed AA excesses in part A received the basal diet supplemented with various concentrations of Lys, Met or Thr. Likewise, the possums fed depleted diets in part B were fed the “complete diet” lacking the AA of interest (*ND* not determined)

Amino acid	AA mixture (mg/g)	Basal diet 1 (mg/g DM)	Complete diet (mg/g DM)	Casein diet (mg/g DM)
Nitrogen	–	9.95	11.5	11.6
Non-essential				
Alanine	36	2.8	1.3	2.2
Arginine	101	2.4	2.3	2.3
Asparagine	101	ND	ND	ND
Aspartic acid	50	5.8	4.8	6.0
Glutamine	157	ND	ND	ND
Glutamic acid	316	12.3	12.1	9.0
Glycine	101	2.4	2.2	1.4
Proline	101	5.2	3.0	7.5
Serine	36	2.7	2.9	3.7
Essential				
Cystine	90	ND	ND	ND
Histidine	98	1.3	1.3	1.8
Isoleucine	133	2.6	3.0	3.6
Leucine	222	4.3	4.2	4.2
Lysine	0	2.4	2.6	4.4
Methionine	0	0.5	1.3	1.2
Phenylalanine	111	2.7	2.4	2.4
Threonine	0	2.5	1.8	2.9
Tryptophan	35	ND	ND	ND
Tyrosine	111	1.6	1.9	2.5
Valine	200	3.1	4.5	5.7

Table 2. A summary of the experiments

Experiment	Subject	AA studied	Notes
1	AA excess	Lys, Met, Thr	AAs added to basal diet at 0.2%, 0.4%, 0.6%, 0.8% and 1.0% WM
2, part A	AA excess	Lys, Met, Thr	Choice experiment: basal or 0.3%, 0.6%, 0.9% WM of study AA
2, part B	AA excess	Lys, Met, Thr	Choice experiment: basal or 0.05%, 0.1%, 0.15%, 0.2%, 0.3%, 0.5% WM of study AA
3	5HT ₃ receptor and AA excess	Met	Use of ondansetron as an antagonist of the 5HT ₃ receptor. Basal diet containing 0.8% WM Met
4	AA deficiency	Lys, Met, Thr	Basal diet containing a mixture of synthetic AAs devoid of the study AA
5	Free AAs	General	Basal diet containing a complete complement of synthetic AAs to test whether possums adapt to free AAs if given enough time.
6	Protein bound versus free AA	General	Possums fed a basal diet containing casein or hydrolysed casein

Experiment 1: the short-term effect of lysine, methionine and threonine excesses on the food intake of common brushtail possums

This experiment was divided into three parts, each testing the response to a single AA, starting with Thr. We used a six treatment Latin square design to measure the feeding response of six brushtail possums to graded concentrations of excess Thr (0.0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% WM). Possums were offered each diet for two consecutive days. A rest day, in which possums received the basal diet without additional AAs, was included between treatments to reduce carry-over effects and to ensure that all animals were well fed before exposure to the next treatment. This experiment, using the same AA concentrations and possums, was repeated twice to measure how much possums ate of diets containing an excess of Met or Lys. The results obtained using excess Thr showed no significant difference between food intake on the first and second experimental days so in the Met and Lys studies each treatment diet was offered for only 1 day, separated by a rest day.

Experiment 2: do possums prefer a diet with the correct proportions of amino acids to one containing an excess of one or more amino acids?

The possums in experiment 1 reacted in varying ways to AA excesses. They reduced their intake of a basal diet containing

increasing concentrations of Met but still ate diets containing high concentrations of Thr and Lys. We investigated the hypothesis that when offered a choice between a basal diet containing the correct proportions of all AAs and one containing AA excesses, brushtail possums would prefer the basal diet and show an aversion to the excessive diets, regardless of the AA in question.

Experiment 2a: high concentrations of amino acids

Six common brushtail possums, housed in separate metabolism cages, were paired according to similarity in average meal size. In a Latin square design each pair of animals was offered two separate containers of food at 17:00 h. One contained Basal diet 1 only, whilst the other contained Basal diet 1 supplemented with either 0.3%, 0.6% or 0.9% WM of Thr, Met or Lys. Both food containers were removed at 21:00 h to allow measurement of food intake over the main feeding period. Additional basal diet was offered to all possums at 21:00 h and removed at 0830 h. The 9-day experiment was divided into three periods of 3 days each, with each period testing the animals' response to a single AA.

Experiment 2b: low concentrations of excess amino acids

We repeated these experiments to determine the range of concentrations over which animals are able to respond to free AAs. Using

the same experimental protocol as in experiment 2a, we observed the feeding preferences of six possums exposed to Met, Thr and Lys during three periods of 6 days each at concentrations of 0.05%, 0.10%, 0.15%, 0.20%, 0.30% and 0.50% (WM) of Basal diet 1.

Experiment 3: the effect of the selective 5HT₃ receptor antagonist ondansetron on the food intake of brushtail possums fed a methionine-rich diet

We chose Met for this experiment because it invoked the strongest depression in feeding in experiments 1 and 2. Eight common brushtail possums were randomly allocated to four groups with each possum pair receiving one of four treatments per period in a digram balanced Latin square design (Ratkowski et al. 1993). The dietary treatments were: Basal diet 1 only; Basal diet 1 plus 0.035 mg/g (WM) ondansetron; Basal diet 1 plus 0.8% (WM) methionine; Basal diet 1 plus 0.8% methionine plus 0.035 mg/g (WM) ondansetron. To ensure an even distribution in the diet, the ondansetron was first diluted with glucose using the method of doubling. Equal amounts of the drug and glucose were combined, and this mixture was then combined sequentially with equal amounts of glucose to yield the desired amount (36 g for the experiment). The final compound was mixed with the dry ingredients needed for the ondansetron treatments for the entire experiment. The ondansetron-free diets were supplemented with glucose.

Each experimental period lasted for 4 days, the first two of which served to introduce Met to those animals in the Met and Met + ondansetron groups. These possums received 0.8% (WM) Met in their diets, while the other possums received Basal diet 1 only on these days. On day 3 the calculated dose of ondansetron was added to Basal diet 1 fed to the groups receiving Met plus ondansetron and basal diet 1 plus ondansetron groups. Day four was a rest day when all possums received Basal diet 1 to ensure they were well fed before the next treatment and to reduce possible carry-over effects.

Experiment 4: the effect of a dietary deficiency in threonine, methionine or lysine on feeding by brushtail possums

We compared the DMI of six common brushtail possums fed Basal diet 2 to which we added one of three treatments: (a) the mixture containing all the essential and non-essential AAs (here termed the "complete diet"), (b) the non-essential AA mixture and the essential mixture with a single AA omitted ("AA-deficient diet"), and (c) an equivalent amount of amino acids offered as the milk protein, casein, which is considered a high-quality protein ("casein diet"). The experiment was designed as a combination of two 3×3 Latin squares and each animal was offered each of the diets for two consecutive days to counter any naivety effects. This experimental design was used in four separate experiments to compare the DMI of possums fed diets devoid of Thr, Met or Lys, as well as a diet that was simultaneously devoid of both Thr and Lys. One animal stopped eating early in the Thr and Lys deficiency experiment. In the animal's interest we fed a recovery diet, and therefore the results for this experiment are based on measurements for five animals only.

Experiment 5: acclimation of common brushtail possums to a diet containing a balanced complement of free amino acids

In experiment 4 possums ate less of the complete diet relative to the casein diet, although both contained a balanced mixture of AAs. Possums were exposed to each experimental diet for two consecutive days only, and thus it is possible that this was insufficient time to elicit an adaptive phase response to a diet containing a balanced mix of free AAs. Therefore in this experiment we tested over a longer period. Six common brushtail possums were offered the complete diet, described in experiment 4, for 6 consecutive days, during which time we measured the average daily DMI. This diet was available to possums ad libitum day and night. Refusals were

collected each day at 08:30 h at which time fresh food was provided.

Experiment 6: protein-bound versus free amino acids

The results of experiments 4 and 5 examined whether possums eat less of a diet formulated from synthetic AAs than they do of one containing mainly protein-bound AAs, as casein. Therefore in this experiment we tested whether possums eat different amounts when offered diets containing either casein or hydrolysed casein. In three 2×2 Latin square designs six possums were offered either Basal diet 1 with 1.8% WM casein or Basal diet 1 supplemented with the same amount of nitrogen as hydrolysed casein (Sigma). Food intake was measured for two nights.

Statistical analysis

We know from experience that there is marked between-animal variation in feeding by brushtail possums. Furthermore, this variation is usually not explained by differences in body mass. In order to account for this variation we performed all experiments as Latin square designs and analysed them using the analysis of variance procedure in Genstat. The full model always included interaction terms and body mass as a covariate. Non-significant terms were dropped from the full model to give the final reduced model. All models were tested to ensure that residual values were normally distributed and randomly distributed against fitted values.

Results

Body mass

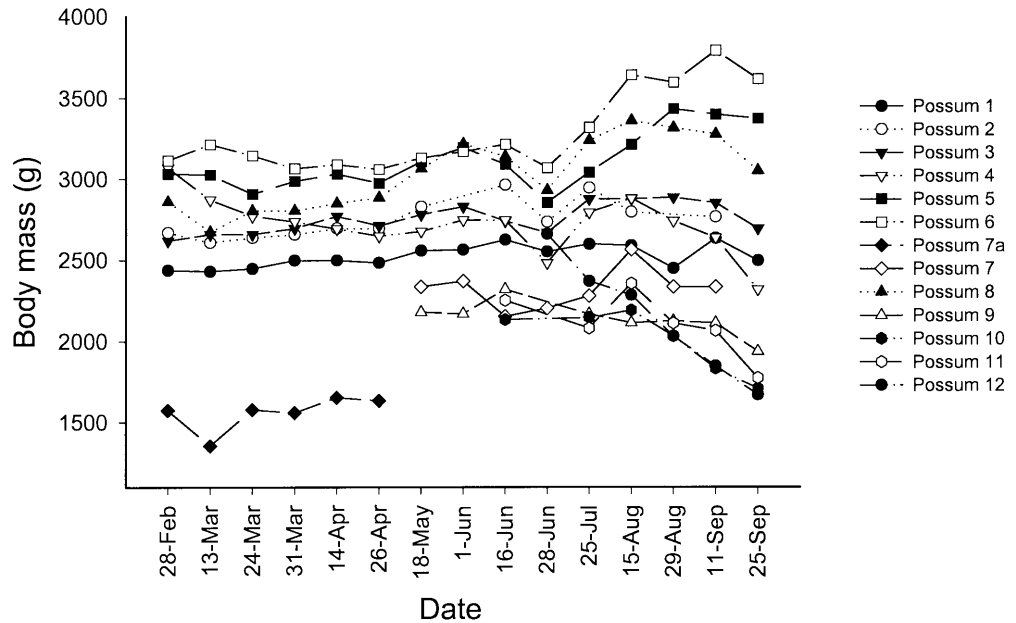
Feeding brushtail possums low protein diets for a long period (March–July) interspersed with various other treatments including diets containing excesses and deficiencies in Lys, Met and Thr had remarkably little effect on their body masses (Fig. 1). In the latter part of the study body masses drifted apart when animals were split into two groups and were not always being used for experiments. For example, three possums (nos. 5, 6 and 8) gained mass during July when they were not used for experimentation and were fed Basal diet 1 ad libitum.

Experiment 1: the short-term effect of lysine, methionine and threonine excesses on the food intake of common brushtail possums

Brushtail possums fed diets with high concentrations of either Thr, Met or Lys maintained body masses and showed no obvious signs of adverse reactions to the diets (Fig. 2). Brushtail possums did not eat less in response to dietary excesses of between 0.2 and 1.0% Thr ($F_{5,20} = 0.27$, $P = 0.927$). Interestingly, even when they were offered the diet with no excess Thr, possums tended to eat less on the first (68.4 ± 4.99 g DM/day) experimental day than they did of the identical diet on the rest days (78.4 ± 1.89 g Dm/day, $P = 0.052$).

In comparison to the results for Thr, possums ate significantly less of diets containing increasing concentrations of free Met ($F_{5,20} = 6.12$, $P = 0.001$). This curve

Fig. 1. Changes over time in the body masses of common brushtail possums maintained on low protein diets



is best described by a linear fit. Possums ate about 34% less of the diet with the highest concentration of Met (1.0%) than they did of the basal diet containing no synthetic Met.

DMI was greater at all concentrations during the Lys feeding experiment than for both the Thr and Met experiments. Adding excess Lys to the diet had a significant effect on DMI ($F_{5,20} = 3.05$, $P = 0.033$), but in a seemingly random way, including an obvious fluctuation around 0.6% Lys. At a concentration of 1.0% added Lys, possums ate 15% less DM than they did of the basal diet.

Experiment 2: do possums prefer a diet with the correct proportions of amino acids to one containing an excess of one or more amino acids?

Experiment 2a: high concentrations of amino acids

Regardless of the treatment possums ate between 38 and 45 g DM over the 4-h feeding period (Fig. 3). When offered a choice between Basal diet 1 and the same diet containing an excess of AAs, possums always preferred the basal diet. Methionine elicited the strongest response ($F_{1,14} = 228.06$, $P < 0.001$), with possums obtaining at least 75% of their DMI from the basal diet. The reaction of the possums depended on the concentration of excess Met ($F_{2,14} = 10.25$, $P = 0.002$), with possums eating more of the basal diet as the concentration of Met increased. Possums also ate significantly more of the basal diet than one containing excess Thr ($F_{1,15} = 59.24$, $P < 0.001$). A similar, but weaker, effect was observed with possums given a choice between the basal and excess Lys diets ($F_{1,15} = 12.44$, $P = 0.003$). In contrast to the result for Met, there was no dose-dependent response either for Thr ($F_{2,15} = 1.07$, $P = 0.369$) or for Lys ($F_{2,15} = 0.51$, $P = 0.613$).

Experiment 2b: low concentrations of excess amino acids

At all six concentrations of excess AA tested (0.05–0.5% WM) brushtail possums preferred the basal diet (Fig. 4). We observed a significant difference between the amount of the treatment diet eaten, relative to the basal diet for Thr ($F_{1,29} = 122.8$, $P < 0.001$), Met ($F_{1,30} = 205.27$, $P < 0.001$) and Lys ($F_{1,30} = 47.82$, $P < 0.001$). In keeping with the first part of experiment 2, the greatest and smallest differences in food intake between the basal and treatment diets occurred with added Met and Lys, respectively. There was no significant dose-dependent effect for Thr ($F_{5,29} = 0.63$, $P = 0.675$), Met ($F_{5,30} = 0.18$, $P = 0.969$) or for Lys ($F_{5,30} = 0.65$, $P = 0.667$).

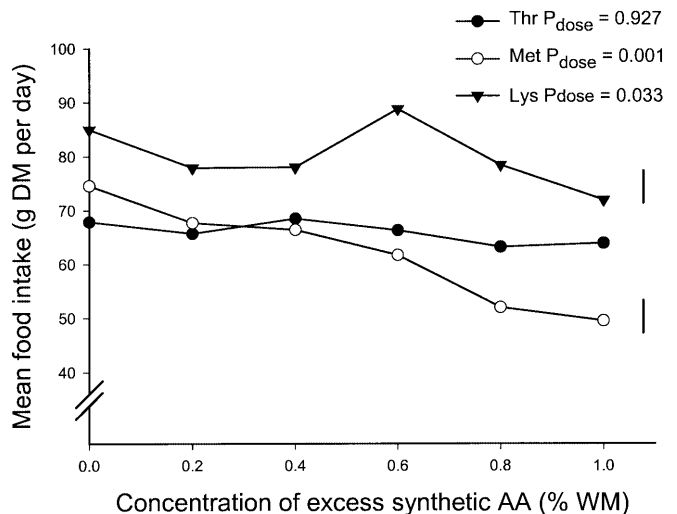


Fig. 2. The mean DMI of brushtail possums fed an artificial diet containing graded amounts of excess synthetic Lys, Met or Thr. Bars Least significant differences ($P = 0.05$)

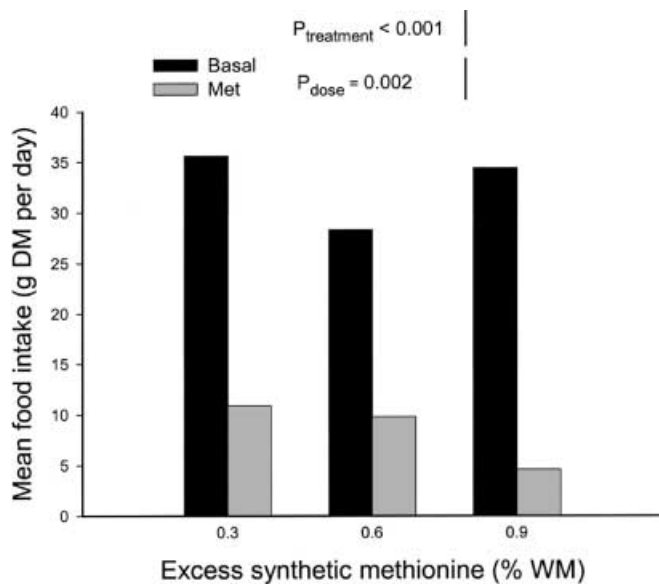


Fig. 3. The mean DMI of brushtail possums given a choice between a basal diet and the same diet supplemented with 0.3%, 0.6%, or 0.9% Met (WM). Bar Least significant difference ($P=0.05$)

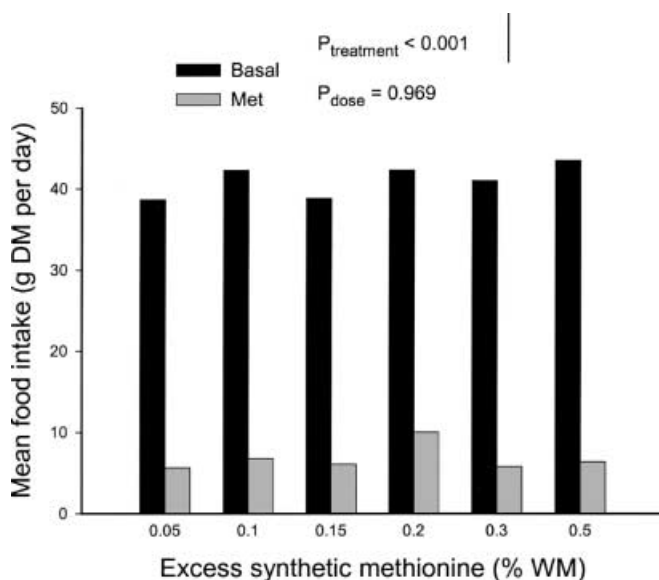


Fig. 4. The mean DMI of brushtail possums given a choice between a basal diet and the same diet supplemented with 0.05%, 0.10%, 0.15%, 0.20%, 0.3% or 0.5% Met (WM). Bar Least significant difference ($P=0.05$)

Experiment 3: the effect of the selective 5HT₃ receptor antagonist ondansetron on the food intake of brushtail possums fed a methionine-rich diet

As expected, adding 0.8% Met to Basal diet 1 significantly reduced (ca. 47%) the amount that possums ate relative to their intake on the basal diet ($F_{1,18}=173.3$, $P<0.001$; Fig. 5). Adding ondansetron to the diet had no effect on feeding by brushtail possums and did not result in them eating more of the Met-rich diet

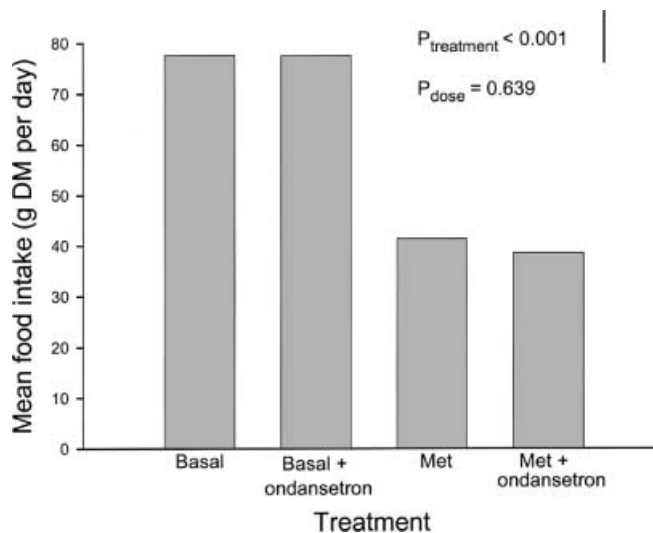


Fig. 5. The mean DMI of brushtail possums fed a basal diet or the same diet containing 0.8% Met. Animals in both groups were then offered the diets with an oral dose of the anti-nausea drug, ondansetron. Bar Least significant difference ($P=0.05$)

($F_{1,18}=0.24$, $P=0.627$). Similarly, there was no interaction between the ondansetron treatment and diet ($F_{1,18}=0.23$, $P=0.639$).

An analysis of covariance revealed that the Met ingested by possums on days 1 and 2 of each period (acclimation days) had a significant effect on the amount of the Met-rich diet eaten on the following experimental day ($F_{1,17}=10.63$, $P=0.005$). By using the amount of the Met diet eaten in the acclimation period as a covariate we showed that DMI decreases the longer possums are fed a Met-rich diet (mean DMI day 1 = 43.7 ± 17.9 g compared with DMI day 3 = 40.0 ± 14.2 g). Therefore the effect on DMI of adding excess Met was stronger on day 3 than would have been observed if possums had not received preliminary treatment with Met ($F_{1,17}=4.38$, $P=0.052$).

Experiment 4: the effect of a dietary deficiency in threonine, methionine or lysine on feeding by brushtail possums

Brushtail possums ate significantly different amounts of the casein, complete and Thr-deficient diets ($F_{2,8}=7.94$, $P=0.013$; Fig. 6). The DMI of the Thr-deficient diets was significantly lower than for both the AA balanced diets, while possums ate less of the complete diet than they did of the casein diet.

Brushtail possums also significantly reduced their DMI when fed the Met-deficient diet compared to the casein diet ($F_{2,8}=13.78$, $P=0.003$). However, we observed no significant difference in feeding between possums offered the complete diet or the Met-deficient diet. In this experiment we found that body mass had a significant effect on DMI ($F_{1,4}=17.98$, $P=0.013$), and therefore the means were adjusted accordingly.

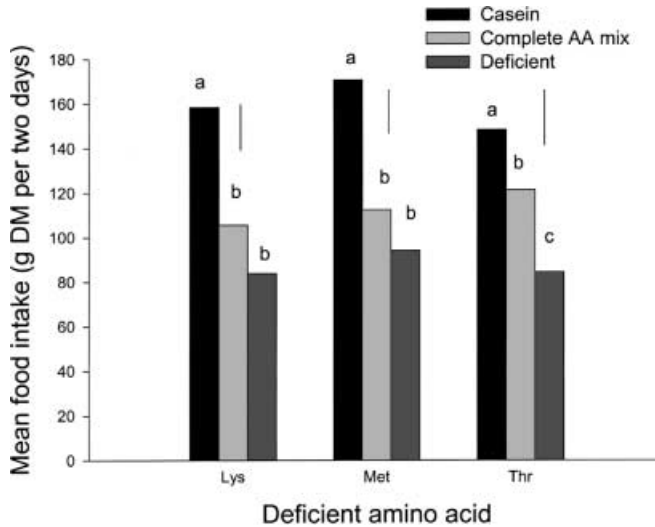


Fig. 6. The mean food intake over 2 days of brushtail possums fed a basal diet containing either casein, a complete mixture of AAs or a mixture devoid of one AA. Different letters Means within each AA group that are significantly different ($P < 0.05$); bars least significant differences ($P = 0.05$)

The dietary treatment significantly affected DMI in both the Lys deficiency and combined Lys and Thr deficiency experiments ($F_{2,8} = 14.89$, $P = 0.002$ and $F_{2,8} = 19.20$, $P = 0.002$, respectively). In both experiments possums ate similar amounts of the complete and deficient diets, but these amounts were less than they ate of the casein diet.

Experiment 5: acclimation of common brushtail possums to a diet containing a balanced complement of free amino acids

Brushtail possums did not show any signs during 6 days of acclimating to a diet containing a balanced complement of AAs by eating more. We found no significant difference in food intake for any day within this period ($F_{5,30} = 0.28$, $P = 0.922$)

Experiment 6: protein bound-versus free amino acids

After correcting for the significant effect of body mass ($F_{1,6} = 14.62$, $P = 0.009$), brushtail possums ate less of the diet containing hydrolysed casein than they did of the diet formulated with casein ($F_{1,6} = 10.48$, $P = 0.018$; Fig. 7).

Discussion

To our knowledge, this is the first study of the AA nutrition of a marsupial and among few studies of the responses of wild animals to deficiencies and excesses in AAs. There are various analyses of the AA composition of the diets of wildlife (e.g. Boren et al. 1995; Izhaki

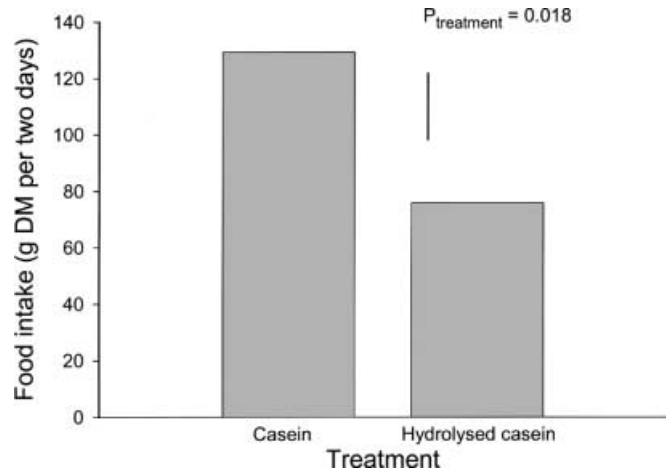


Fig. 7. The mean food intake over 2 days of brushtail possums fed a basal diet with either casein or hydrolysed casein. Bar Least significant difference ($P = 0.05$)

1998), measures of the AA requirements of a passerine bird (Murphy 1993) and various assessments of protein quality that make inference to AA nutrition (e.g. Van Tets and Hulbert 1999; Van Tets et al. 2000). Consequently the study is preliminary, wide-ranging, and based on short experiments. We chose the common brushtail possum because it is a generalist herbivore, it is the most widely distributed marsupial in Australia, it readily adapts to captivity, and it can be maintained for many weeks on simple, low protein fruit and cereal diets. Importantly, it is easy to manipulate the nutrient composition of such diets. Moreover, it is a species with hindgut fermentation and presumably no way of obtaining AAs other than from its diet: we might therefore expect it to be susceptible to changes in dietary AA profiles.

The effect of dietary excesses of amino acids on food intake

The feeding responses of our brushtail possums to dietary AA excesses differed between the individual AAs tested. They did not eat less of diets containing excess Thr despite reports of depressions in food intake and growth for chickens fed excess Thr (Barkley and Wallis 2001). This is remarkable because the possums failed to respond even when the diet contained 1% WM of added Thr, corresponding to roughly 3.5% of DM. In contrast, the chickens studied by Barkley and Wallis (2001) ate less, grew slower and deposited less carcass protein when fed a diet containing a 0.05% excess of Thr on a DM basis. Contrasting their response to Thr, the possums ate less of diets containing excess Met. The linear negative dose-response curve shown in Fig. 2 differs to observations on rats and chickens, which often ate more of diets containing relatively low concentrations of free Met but, with increasing Met increments, eventually reached a threshold of tolerance after which food intake was

depressed (Harper et al. 1970). In this situation the rats and chickens were responding to a deficiency in Met in the basal diet, which turned to a sufficiency and finally an excess with serial additions of the AA. The negative response of the possums suggests either that the lowest increment of Met was excessive, or that synthetic Met stimulated sensory cues eliciting an adverse effect. The intermediate response of the possums to synthetic Lys is difficult to interpret due to the prolific feeding on the diet containing 0.6% added Lys. Nevertheless, the possums responded more to excess Lys than they did to excess Thr but less to Lys than they did to excess Met. The possums, as with rats and chickens, seemed exceedingly tolerant of excess Lys (Harper et al. 1970).

As generalist herbivores that eat an enormous array of foods (Owen and Norton 1995), brushtail possums presumably integrate a wide range of sensory and environmental cues to choose foods that allow them to cope with nutritional limitations (Provenza et al. 1996). Thus it is appealing to suggest that possums respond to disproportionate amounts of dietary AAs in more complex ways than do domesticated species, such as poultry, pigs and rats, that invariably eat uniform diets for long periods. However, our results provide no evidence for this suggestion. It is important to note that studies with domesticated species focus on juvenile animals selected for high growth rates and on reproductive animals, both of which show marked responses to subtle dietary changes (Harper et al. 1970). As pointed out by Harper et al. (1970), mature animals are not as susceptible to the adverse effects of disproportionate amounts of AAs as are young ones. The possums used in the present study were all mature males. As with other marsupials, their resting metabolic rate is roughly 70% that of eutherian mammals (Dawson and Hulbert 1970). Furthermore, they are extremely calm in captivity, and their daily metabolic rate is therefore probably a small multiple of their resting metabolic rate.

As with many other animals, brushtail possums can catabolise sub-toxic quantities of essential AAs. The differences shown by the possums in response to excesses of individual AAs, in which they showed a strong aversion to Met, may be partially explained by variation in the rates of catabolism and thus the toxicity of the three AAs. Thr is catabolised faster than Lys, whilst excess Met is removed relatively slowly (Hrupka et al. 1999; Leung et al. 1968). Likewise, Thr and Lys are considered to be among the least toxic and Met the most toxic of the essential AAs (Harper et al. 1970). If the responses we observed in our possums reflect the rates of catabolism of the AA then this implies that possums and rats catabolise AAs in similar ways.

Possoms select nutritionally balanced foods when provided with a choice

When provided with a choice, the brushtail possums consistently rejected diets containing AA excesses in

favour of the basal diet, despite not exhibiting strong aversions to Thr and Lys when no other foods were available. It seems that possums can detect dietary excesses of these AAs, but in the absence of choice their hunger overrides any aversion. It is likely that foraging possums select diets that do not cause them to ingest disproportionate amounts of AAs, although catabolising AAs provides an energy source. Thus, why do animals avoid ingesting foods with disproportionate amounts of AAs? One possible answer is that in doing so the animal would have trouble meeting AA requirements. Another possible answer is that urea, the end-product of AA catabolism, requires water for excretion and this may be scarce.

The degree of aversion to treated diets was specific to the individual AAs, with excess Met eliciting the greatest response. However, the choice experiments gave no evidence of a dose-dependent response, suggesting that any concentrations of free AAs beyond requirements exert some unfavourable effect. Even at the lowest concentration (0.05% WM) of excess Thr, Met or Lys the possums preferred the basal diet. We have maintained possums on Basal diet 1 for long periods, and it therefore presumably it supplies them with the nutrients required for maintenance. Thus any additional nutrients are surplus to requirements. The animals' decision to choose against diets containing supplemental AAs suggests both that they can select, and that they favour a diet supplying them with AAs in the correct proportions. Alternatively, brushtail possums may detect and then reject even slight amounts of synthetic or free AAs in their foods.

Is there a role for the 5HT₃ receptor in the regulation of AA nutrition?

In order to explain the response of brushtail possums to dietary AA excesses we attempted to determine the physiological mechanisms by which possums recognise these diets. It is thought that the neurotransmitter 5HT is associated with food intake regulation by mediating appetite and satiety (Blundell 1984; Fernstrom 1985). Thus we postulated that excesses of free AAs would increase the activity of 5HT acting at the 5HT₃ receptor and cause brushtail possums to eat less of diets containing excess Met. Consequently, blocking the 5HT pathway, using the 5HT₃ receptor antagonist ondansetron, would allow animals to restore their food intake to basal levels. Such responses have been shown in other animals, but always to AA deficient diets. For example, ondansetron ameliorated the peripherally mediated anorectic response to an AA deficient diet in rats (Jiang and Gietzen 1994).

Administration of ondansetron in the food (0.035 mg/g WM) did not reverse the food intake depression produced by adding excess Met (0.8%) to the basal diet. The complete lack of a response ($P=0.63$) makes it unlikely that an inadequacy of ondansetron

explains the possums' continued aversion to Met. A more likely explanation is that 5HT₃ receptors are not involved in mediating excess AA consumption by brushtail possums.

Recently Gietzen et al. (1998) suggested that animals initially recognise AA imbalances using other neural signalling systems, such as norepinephrine, and that 5HT is involved in the later phases. Another possibility is that the 5HT₃ receptor partially governs the responses of possums to AA deficiencies, but that the metabolic effects associated with ingestion of free AAs may exert stronger signals (Gietzen et al. 1998).

The effects of dietary AA deficiencies on diet selection

The varied feeding responses and the relatively high tolerance of our brushtail possums to dietary excesses of specific AAs prompted us to determine the effects of dietary deficiencies, again using Lys, Met and Thr. We reasoned that AA-deficient diets must exert negative metabolic effects because they prevent animals from synthesising the proteins required for tissue maintenance (Markison et al. 2000). One problem with carrying out this type of experiment with brushtail possums is that they require little protein. Wellard and Hume (1981) reported a digestible nitrogen requirement of 189 mg/kg mass^{0.75} per day, or 315 mg per day for a 2 kg possum fed a diet of predominantly honey and bran. If possums are eating about 70 g DM per day, the dietary DM must contain 0.45% digestible N, or about 3% digestible crude protein for the possum to meet its nitrogen requirement. We attempted to force possums into negative nitrogen balance by formulating a basal diet in which the only source of N was the 87% apple (0.2% N on a DM basis). The determined nitrogen content of this diet was 0.18% or roughly 40% of that required by the possum to attain nitrogen balance if all the N is digested. To this diet we added various AA mixtures that were devoid of either Lys, Met, Thr or both Lys and Thr so that the analysed nitrogen content of the diet fed was 0.9%. Of course, the four deficient diets were almost devoid of the AA of interest.

We found that the brushtail possums ate at least 40% less when their diets were deficient in Thr, Met or Lys, relative to their intake of the casein diet. This is consistent with observations of depressed food intake, reduced growth and organ damage in rats fed diets that were lacking in any of the essential AAs (Gietzen et al. 1998; Hrupka et al. 1999; Nasset et al. 1967).

In contrast to the situation for AA surpluses, the possums fed deficient diets did not respond to individual AAs. This is emphasised by the observation that the possums ate similar amounts of the diets that were deficient in Thr and Lys and the diet that was deficient in both of these AAs.

A surprising result was the size of the reduction in feeding in the brushtail possums offered the complete

diet relative to the casein diet, even though both were formulated to contain similar amounts of essential AAs. Leung and Rogers (1973) also found that rats ate less when all AAs were present in the free form. However, they reported that food intake was further reduced when one of these AAs was omitted from the diet. In comparison, the possums in this study did not reduce their intake of either a Met- or a Lys-deficient diet relative to the complete diet, although they did eat significantly less of the Thr-deficient diet. We considered that possums may have rejected both the AA deficient and complete diets based on aversions to the taste of free AAs. This theory assumes that eating diets containing free AAs relies solely on feedback from pre-ingestive cues. However, this assumption is contradicted by the findings of Leung et al. (1972), who showed that repleting a deficient diet with the limiting AA resulted in immediate resumption in feeding by rats when all AAs were present in the free form.

Previous studies have shown that rats are cautious in eating diets corrected with the limiting AA after associating them with the deleterious effects of an AA deficiency. However, given time to learn about the nutritional benefits of a complete diet, they adjust their food intake and restore feeding (Leung et al. 1986). We tested this idea by offering possums the complete diet ad libitum for 6 days so that they could learn about the longer term metabolic effects of eating the complete mix of AAs. We found that possums did not eat more of this diet as the experiment progressed but instead showed a classic AA deficiencies type response, whereby animals do not adapt but instead exhibit reduced feeding after prolonged exposure (Hrupka et al. 1999).

The similar responses of the brushtail possums to the complete and deficient diets suggest a role for post-ingestive feedback in response to free AAs. We asserted previously that hunger masked any negative effects of eating diets with AA surpluses. Therefore the failure of brushtail possums to eat the complete diet after several days suggests that they experienced negative metabolic effects. It is likely that the metabolic effects of the AA deficiency were compounded by the rapid absorption of free AAs that signalled for sensations of satiety and simulated the situation of an AA deficiency or excess (Canolty and Nasset 1975; Hrupka et al. 1999). Supporting this possibility is the finding that our animals ate significantly less of a basal diet supplemented with hydrolysed casein than they did of one containing acid casein. This finding has ecological implications because eucalypt foliage may contain high concentrations of free AAs. In the only published study that we know of, Journet and Cochrane (1978) showed that free AAs represent 33–50% of the total leaf amino-N in *Eucalyptus blakelyi* and that young leaves contain more free AAs than do mature leaves. We might speculate that free AAs deter possums from feeding on eucalypt foliage, but this aspect clearly requires more research.

Learning behaviour in diet selection

A common finding throughout these experiments was that the brushtail possums learn to recognise diets containing excesses of AAs and alter their feeding behaviour accordingly. For instance, despite their preference for a basal diet, the brushtail possums did not avoid diets containing excess AAs when given a choice. Instead, they continued to eat some of the treatment diet, even after several days of this experimental regime. Likewise, the possums did not completely reject AA deficient diets but regulated their DMI between 40 and 50 g per day. Similar foraging behaviour has been documented by both Provenza et al. (1996) and Day et al. (1998) who proposed that animals do not completely avoid nutritionally inadequate diets but instead sample them in subsequent encounters.

Physiological mechanisms governing diet selection: an integration of pre-ingestive and post-ingestive stimuli?

An important aim of this study was to determine the physiological mechanisms that allow common brushtail possums to recognise and discriminately select foods that provide them with an adequate supply of essential AAs. We established that the possums chose against dietary excesses of AAs, while deficiencies in AAs must also have exerted negative metabolic effects. However, the physiological pathways mediating this process remain unclear, and the evidence provided remains tenuous. Rather, we have alluded to the possibility that pre-ingestive cues and post-ingestive feedback work together to dictate diet selection. The relative importance of taste and of the rapid absorption of free AAs are difficult to separate. Threonine is perceived to taste sweet, in comparison to Met and Lys, which have both been reported to be bitter (Mori et al. 1991; Tabuchi et al. 1991). This may explain why the possums, in the absence of choice, did not eat less of a diet with excess Thr but became averse to diets containing free Met and Lys, especially at high concentrations. Leung et al. (1986) concluded that rats only use the taste of free AAs as markers to signal for AA deficiencies, as flavour cues were not strong enough to overcome cues from metabolic change. It is likely that the brushtail possums integrated post-ingestive and pre-ingestive signals to regulate their intake of AA deficient diets. By associating the taste of free AAs with the metabolic consequences of eating the possums could select diets containing a balanced proportion of essential AAs.

Conclusions

This study shows that the brushtail possums regulate their feeding behaviour in response to the abundance of essential AAs, and therefore studies of AA nutrition are more appropriate, than a focus on total nitrogen, to

understanding the limitations faced by foraging animals. The ability of brushtail possums to effectively regulate their intake of essential AAs appears to require a complex process of learning, through which they continually sample an array of novel and familiar foods. This allows them to assess the metabolic costs and benefits of eating diets of varying AA composition.

Results suggest that further research should focus on the link between the high proportion of free AAs in eucalypt foliage and the seeming reluctance of brushtail possums to eat diets composed of free AAs. Further research on the responses of possums to diets composed of large amounts of free AAs would benefit by slowing their absorption, perhaps by incorporating free AAs in an agar gel. We did not attempt to develop a series of purified diets for possums, but this would allow detailed studies of learned taste aversions without the risk of carryover effects.

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