J Comp Physiol B (2000) 170: 185–192

ORIGINAL PAPER

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Plant secondary metabolites as mammalian feeding deterrents: separating the effects of the taste of salicin from its post-ingestive consequences in the common brushtail possum (*Trichosurus vulpecula*)

Accepted: 7 December 1999

Abstract The effect of the phenolic glycoside, salicin, on food intake of the common brushtail possum (*Trichosurus vulpecula*) was studied in a series of feeding experiments. Increasing the concentration of salicin in a diet of fruits and cereals led to significant reductions of food intake in the short term (6 days). After prolonged (20 days) exposure to salicin, food intake (19 g $kg^{-0.75}$ day^{-1}) was still reduced relative to controls (31 g $kg^{-0.75}$ day^{-1}) but not reduced to the same extent as in the short-term experiments. Nonetheless, over these 20 days, common brushtail possums regulated their intake of salicin so as not to exceed a threshold limit of 1.9 ± 0.1 g $kg^{-0.75}$ day^{-1}. Manipulative experiments sought to determine whether this threshold intake was in response to pre-ingestive factors (taste) or the post-ingestive consequences of ingesting salicin. Dietary salicin (0.17–5.0% DM) had no significant effect on nitrogen balance or urea metabolism and injection of a specific serotonin receptor antagonist, ondansetron, did not lead to increases in salicin intake as has been found for some other plant secondary metabolites. Similarly, administration of 1.3 g salicin by gavage had no significant effect on the subsequent intake of salicin compared to controls that were gavaged with water. We concluded that pre-ingestive factors were responsible for common brushtail possums limiting their intake of salicin-rich diets rather than any measurable post-ingestive consequence of feeding.

Communicated by I. D. Hume

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Key words Phenolic glycoside · Jensenone · Antifeedant · Marsupial · Detoxification

Abbreviations *5-HT* 5-hydroxytryptamine · *DM* dry matter · *DMI* dry matter intake · *PSM* plant secondary metabolite

Introduction

It is now widely accepted that plant secondary metabolites (PSMs) influence food choices of herbivorous mammals (Palo and Robbins 1991). However, the reasons why animals eat plants containing some PSMs yet avoid others are still poorly understood. Correlative studies can tell us something about the preferences of animals for different plants but 25 years after Freeland and Janzen's (1974) seminal hypotheses on the role of PSMs on herbivore foraging, there is still no clear framework for understanding how PSMs influence diet choice of herbivores (Foley and McArthur 1994). Generalisations across different plant/herbivore systems are rare.

Animals that feed on plants containing secondary metabolites must make continuous and rapid decisions about the quality of their food. Only rarely do animals totally reject particular plants. More commonly, intake is carefully regulated so that the amount of food consumed does not exceed some threshold. This threshold is most often set by the presence of PSMs but knowing the chemical principle is not always enough to predict feeding intensity (Pfister et al. 1990). Understanding how animals arrive at decisions in foraging is the key step in explaining food choices and the level of food intake (Provenza 1995).

Early studies of PSM-herbivore interactions focused on tannins because they were ubiquitous and because it was believed that their actions as agents to bind protein in the gastrointestinal tract were relatively straightforward. However, it is becoming increasingly evident that the role of tannins as reducers of protein digestibility has

been over-emphasised. This may be because in at least some animals, physiological defences against tannins (e.g. tannin-binding salivary proteins: Foley et al. 1999) themselves result in elevated faecal nitrogen losses that are difficult to distinguish from other sources of faecal nitrogen. In many places (e.g. Mole and Waterman 1987; Bernays et al. 1989; Foley and McArthur 1994) the importance of tannins as agents which reduce vertebrate feeding is being questioned.

Plants contain an array of other small phenolic metabolites that may deter herbivores because of their taste (Bate-Smith 1972), or post-ingestive consequences. These include potential toxic effects at the tissue level, the cost of biotransformation and excretion (Jakubas et al. 1995) or their effects on whole body acid-base balance (Foley et al. 1995). Determining whether taste or post-ingestive consequences play a role in diet selection is thus a first step towards understanding foraging decisions.

We studied the effects of the phenolic glycoside salicin on feeding in marsupial common brushtail possums (*Trichosurus vulpecula*). Correlative studies suggested that feeding intensity of free-living common brushtail possums was inversely proportional to the concentration of salicin in foliage of different willow cultivars (Markham 1970; Edwards 1978). Common brushtail possums are generalist browsers that are believed to be highly susceptible to the effects of PSMs (Freeland and Winter 1975; McArthur and Sanson 1993) but there are few physiological or ecological data to explain the basis of this susceptibility.

We anticipated that salicin would reduce the food intake of common brushtail possums in a dose-dependent or threshold manner. We wanted to investigate how the animals regulated their intake of salicin, in particular, by separating pre-ingestive (i.e. taste) factors from post-ingestive (nutritional or toxic) effects.

Firstly, we examined the effect of acclimation on the intake of salicin because many studies have shown that animals prefer familiar foods. The brushtail possums used in the experiments were captured in mangrove forests where there were no plants containing salicin and therefore may have avoided foods containing this compound due to a lack of experience with the taste and/or its post-ingestive consequences. Therefore, feeding animals salicin over an extended period would familiarise them with the effects of the compound and eliminate the possibility of the possums avoiding the food due to inexperience alone.

We then examined the effect of salicin on nitrogen and urea metabolism to evaluate the possibility that excretion of absorbed salicin could act to reduce food intake by exerting either nitrogen costs through conjugation with glycine or energy costs through conjugation with glucuronic acid. The varied effects of PSM ingestion reported in other studies (Foley et al. 1999) suggested that a broad study of nitrogen and urea metabolism and glucuronic acid excretion could be useful in elucidating the effect of salicin on the animal's metabolism.

We then tested the possibility that salicin induced food aversions through stimulation of the emetic system via serotonin (5-hydroxytryptamine; 5-HT) receptors. Previous studies in common brushtail possums (Lawler et al. 1998) have shown that the antifeedant action of *Eucalyptus* constituents is mediated through 5-HT_3 and linked to conditioned aversions. These experiments sought to identify whether the same was true of salicin in brushtail possums. Alternatively, if salicin does not cause any post-ingestive toxicity and was avoided due to pre-ingestive effects (i.e. taste), administering salicin by gavage and bypassing the sense of taste should have no effect on the subsequent intake of food containing salicin.

Materials and methods

Animals and basal diets

Twelve common brushtail possums (*T. vulpecula*) (mean body mass 2.28 kg) were captured in wire cage traps in mangrove forest on Magnetic Island (latitude 19° 08′, longitude 146° 50′). Only eight different plant species are present in this forest and none contain salicin. The animals were housed in individual metabolism cages in an air-conditioned room (22 ± 2 °C and 65% relative humidity) under a 12:12 light:dark routine. The lights were connected to a dimmer which allowed a gradual change in intensity of the light to simulate dawn and dusk.

After initial capture the animals were offered foliage from a range of mangrove and *Eucalyptus* species as well as apples and bananas. An artificial diet based on fruit and cereals was then gradually introduced until it was the only food on offer. This diet consisted of (% wet weight): grated apple (55%), grated banana (15%), grated carrot (15%), sucrose (5.7%), ground lucerne hay (3%), ground Weet-Bix (Sanitarium whole-wheat breakfast cereal) (3%), ground rice hulls (3%) and casein (0.3%). This diet contained 68% water and (as a percentage of DM) 1.0% N, 23% neutral detergent fibre and 12% acid lignin. This diet served as the basal diet for all experiments and was prepared fresh each day and was presented as a wet mash. There was no evidence of selective feeding on this diet. Salicin was purchased from Sigma as a white, water-soluble powder (> 99% purity) and added to the dry ingredients of the diet as required to produce diets of varying salicin concentration.

The same brushtail possums were used in all experiments described and food intake was monitored before and after experimentation to ensure all animals were eating well. There was at least 3 weeks between each feeding trial to minimise possible carry-over effects and our statistical design allowed any carry-over effects to be detected.

Dose-response experiments

Two types of feeding experiments were carried out to measure the effects of salicin on food intake in common brushtail possums. The first was a short-term experiment in which naïve animals were given different concentrations of salicin over 6 days. The second experiment involved slowly introducing salicin into the diet of the common brushtail possums and gradually increasing the concentration over a 20-day period to determine if the animals acclimated to salicin over an extended period of time.

Experiment 1: effect of short-term dietary salicin on food intake in common brushtail possums

The experiment was designed as a digram-balanced 6 × 6 Latin square (Ratkowsky et al. 1994) where each treatment was preceded

and followed equally often by each other treatment to reduce the possibility of carry-over effects. Six male common brushtail possums were used with six concentrations of salicin being added to the food over 6 days. The concentrations were 0%, 2.8%, 5.5%, 6.9%, 8.3% and 11.1% salicin on a DM basis. These concentrations were chosen based on the range of salicin concentrations found in *Populus* sp. plantations in New Zealand (Edwards 1978). DM intake (DMI) was measured after oven-drying (60°C) subsamples of the food offered and food refused to constant mass. To correct for comparisons between animals of different body mass, all parameters of intake were expressed in terms of metabolic body mass ($kg^{-0.75}$) (Demment and Van Soest 1985).

Each morning of the experimental period, all animals were offered 100 g of basal diet to ensure that each animal had sufficient food to maintain itself and to reduce the possibility of carry-over effects of the previous treatment. Urine volume was measured daily and a subsample collected and frozen for later analysis of total glucuronic acid content using the colorimetric method described by Blumenkrantz and Asboe-Hansen (1973).

Experiment 2: effect of prolonged dietary salicin on food intake

Twelve common brushtail possums were randomly allocated to two diets: (1) a test diet that contained 1.87% (DM) salicin, and (2) a control diet that comprised only the basal ration. Those animals on the test diet were maintained on this regime for 2 days and the concentration of salicin in the food increased by 0.94% (DM) on this and every 2nd day subsequent. The maximum concentration offered was 9.36% (DM) salicin on day 20. The experiments were terminated at this time because further salicin could not be obtained. DMI was measured each day as described above.

Post-ingestive effects and taste of salicin

Three experiments were carried out to separate possible post-ingestive effects of salicin from the taste of the compound as limits to intake. The first experiment measured the effect of dietary salicin on nitrogen balance and urea metabolism. The second experiment tested whether salicin stimulated the emetic system of the animals, whereas the third experiment tested whether rejection of salicin-rich diets was due to pre-ingestive effects such as taste.

Experiment 3: effect of salicin on nitrogen and urea metabolism

The experiment was designed as a combination of two digram-balanced Latin squares with each period lasting 8 days. Three diets, varying in their concentration of salicin were used and two possums were randomly allocated to each diet. Six common brushtail possums were used in total. The diets were prepared so as to have low (0.17%), medium (1.67%) and high (5.0%) concentrations of salicin. These concentrations were chosen based on the effect salicin had on food intake in common brushtail possums as determined in experiments 1 and 2. Each diet was fed for 8 days with food intake and faeces excretion being measured on days 3–7 and urine output on days 3–6. These times were chosen based on previous studies of the passage of food through the gut of common brushtail possums (Wellard and Hume 1981) so as to ensure that the faeces and urine collected were derived from the diet being fed. Subsamples of the urine were used to identify urinary metabolites (see below). On day 7 we measured the rate of formation of urea in the body by injecting each animal with 0.37 MBq of [^{14}C]-labelled urea (Amersham, Australia). Discrete samples of urine were collected hourly for the next 24 h.

Food refusals and faeces were oven dried at 60 °C and samples of the diet offered was freeze dried prior to chemical analysis. All samples were ground in a cyclone mill to pass a 1-mm screen and assayed for total nitrogen by a semi-micro-Kjeldahl procedure using selenium as a catalyst. Urea concentrations in urine were assayed by the diacetyl monoxime method of Crocker (1967) and the activity of [^{14}C]urea in urine was measured by liquid scintillation spectrometry after the urine was acidified with glacial acetic acid to remove bicarbonate (Foley and Hume 1987). We calculated parameters of urea metabolism as described by Cocimano and Leng (1967).

A subsample of the urine excreted by each animal on day 6 of the experiment was retained for quantitative analysis of salicin metabolites which will be reported elsewhere (S. McLean, G. Pass and W. Foley, unpublished data).

Experiment 4: effect of a selective 5-HT_3 receptor antagonist (ondansetron) on salicin intake

The experiment was designed as a digram-balanced 4×4 Latin square with eight animals being allocated randomly amongst four treatments. These were (S), dietary salicin at a concentration of 5.5% of basal diet plus an injection of saline; (S + O) dietary salicin plus an injection of 0.5 mg $kg^{-0.75}$ of ondansetron; (B + O) basal diet plus ondansetron and (B) basal diet plus saline.

Ondansetron is a potent and highly selective antagonist of 5-HT_3 receptors used to control acute nausea and vomiting during cancer therapy (Veyrat-Follet et al. 1997). We purchased ondansetron (''Zofran'', Glaxo) as an aqueous solution (2 mg ml^{-1}) and used the contents of a single 2-ml vial within 30 min.

Each period lasted 4 days. The first 2 days served to introduce salicin to those animals receiving treatments S and S + O. Injections of ondansetron or saline were then made on day 3 by intraperitoneal injection at 1600 hours and food was then offered ad libitum. On average each animal (mean mass = 2.28 kg) received 1 mg of ondansetron per injection. On day 4, animals were offered only the control diet to ensure that all were eating well before the start of the next period.

Experiment 5: effect of salicin given by gavage on food intake of common brushtail possums fed a diet containing salicin

Ten common brushtail possums were randomly allocated between two groups: test and control. All were fed a diet including salicin at 1.7% (DM). They were maintained on this diet for 2 days and the concentration of salicin in the diet was increased by 0.83% (DM) on this and every 2nd day subsequent until all animals were receiving a diet containing 3.3% salicin. Animals in the test group were then given 1.3 g salicin administered by gavage as an aqueous solution at approximately 1600 hours. Animals in the control group were dosed in the same way with an equivalent volume of distilled water. We measured food intake of the salicin-rich diet in both groups over the next 24 h. If salicin acted via a post-ingestive effect, then we expected that animals in the test group would eat only 16.0 g $kg^{-0.75}$ day^{-1} of DM. In experiment 2, at 3.75% salicin the average food intake for the test possums was 28.4 g $kg^{-0.75}$ day^{-1}. At 8.43% salicin, the point at which salicin intake plateaued in experiment 2, food intake was 22.5 g $kg^{-0.75}$ day^{-1}. If the animals ate 28.4 g $kg^{-0.75}$ day^{-1} at 8.43% salicin, salicin intake would be 2.4 g $kg^{-0.75}$ day^{-1}. Therefore, the dose given to the animals was calculated as the difference between the amount of salicin they were willing to ingest at 3.75% salicin content (1.1 g $kg^{-0.75}$ day^{-1}, see Fig. 2B) and the amount of salicin they would ingest if the animals consumed 28.4 g $kg^{-0.75}$ day^{-1} at 8.43% (see above). This difference was calculated to be 1.3 g $kg^{-0.75}$. The difference between this oral dose and expected salicin intake at 8.43% was 0.6 g $kg^{-0.75}$. Therefore, the expected DMI at 3.75% salicin was calculated to be 16 g $kg^{-0.75}$ day^{-1}. We expected the intake of the control group to remain unchanged.

Statistics and calculations

For experiments 1, 3 and 4, possible differences between means of DMI, salicin intake, DM digestibility and parameters of nitrogen metabolism between treatments were compared by analysis of variance (Ratkowsky et al. 1994). Terms accounting for the main

effects of treatment, individual animal, day and possible carry-over effects of treatments were included in all analyses. In experiment 2, the mean DMI of the 2 days at each concentration was calculated. Possible differences between DMI and salicin intake at different concentrations for both test and control groups were then analysed by a repeated measures analysis of variance. In experiment 5, the mean differences in DMIs of test and control groups, before and after dosing the animals with salicin, were compared using the paired sample *t*-test option in SPSS 8.0 (SPSS). The mean difference in intake in the test group alone was then compared to a predicted change in intake by the same method of analysis.

Results

Dose-response experiments

Experiment 1: effect of short-term dietary salicin on food intake

Increasing the concentration of salicin in the food led to significant ($P < 0.001$) reductions in DMI of common brushtail possums (*T. vulpecula*) (Fig. 1a). DMI decreased with increasing dietary salicin up to 8.3% (DM) dietary salicin but there was no significant difference between DMI at this concentration and a concentration of 11.1% (DM). At the highest concentration of dietary salicin, DMI was about 75% lower than that measured on diets containing no salicin. There was no significant difference between the same treatments on the six days of the experiments ($P = 0.550$) and no carry-over effect of treatments ($P = 0.629$). There was, however, significant variation between individual animals ($P = 0.006$).

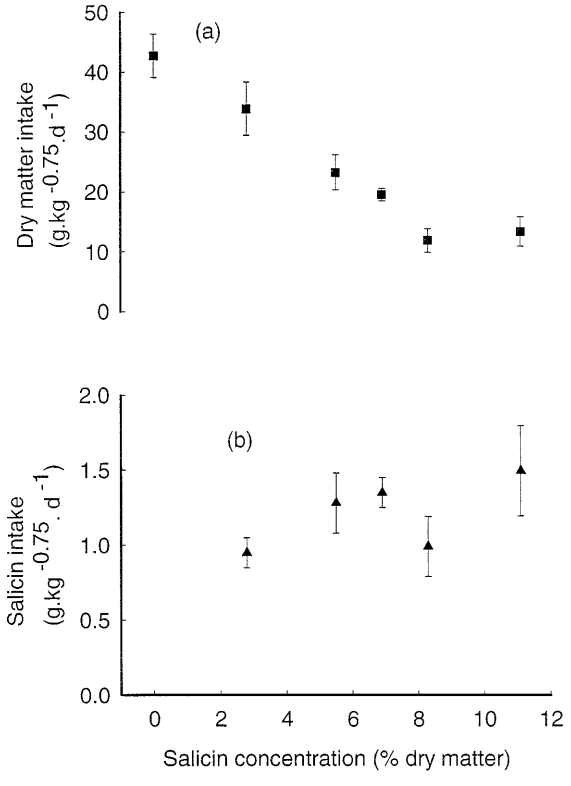

Fig. 1 Intake of **a** dry matter (DM) and **b** salicin in common brushtail possums fed diets with variable concentrations of salicin. Mean ± SE; $n = 6$

Although the mean salicin intake was highest when animals were fed 11.1% salicin, this difference was not statistically significant and there was no significant difference in the salicin intake over all concentrations ($P = 0.181$, excluding 0%). Salicin intake plateaued at 1.3 ± 0.1 g $kg^{-0.75}$ day^{-1} (Fig. 1b). Urinary glucuronic acid output did not differ significantly between treatments ($P = 0.545$) and the mean value was 1.24 ± 0.15 mmol $kg^{-0.75}$ day^{-1}.

Experiment 2: effect of prolonged dietary salicin on food intake

DMI in the control group remained constant throughout the experiment (mean of 30.7 ± 0.8 g $kg^{-0.75}$ day^{-1}); however, there was large variation between individuals due to irregular intakes of the female and one male who stopped eating completely for several days early in the experiment. Omission of these two animals reduced variation but meant that the control group contained four animals and the test group six.

Results from the repeated measures ANOVA dictated the use of multivariate analysis to test whether there was a significant effect of time or if there was an interaction between treatment and time. This was indicated by a significant Mauchly sphericity test ($P = 0.001$), which is a test that checks if the correlations between the observed values over time are constant. A significant result indicates that the more powerful univariate model, which assumes constant correlations over time, should not be used. Because multivariate analysis can only be used if the number of observations (times) is less than or equal to the between degrees of freedom [i.e. (group-1) (possums-1)], one time period needed to be removed. Therefore, the final time period was chosen randomly as the variable to be removed. Multivariate analysis (Pillai's test) showed a significant time effect and a marginal difference in treatment by time effect ($P = 0.041$ and $P = 0.055$). DMI in the test group at 9.36% dietary salicin was reduced by about 36% compared with the control group (19.5 ± 2.6 versus 30.7 ± 0.8 g $kg^{-0.75}$ day^{-1}) (Fig. 2a).

Salicin intake in the test group increased until the concentration of salicin in the food reached 6.55% (DM) ($P < 0.001$) and then plateaued at 1.9 ± 0.1 g $kg^{-0.75}$ day^{-1} ($P = 0.543$) between 6.55% and 9.36% (Fig. 2b). A repeated-measures ANOVA showed that there were no significant differences in salicin intake when animals were fed dietary concentrations between and including 6.55% and 9.36% salicin ($P = 0.543$).

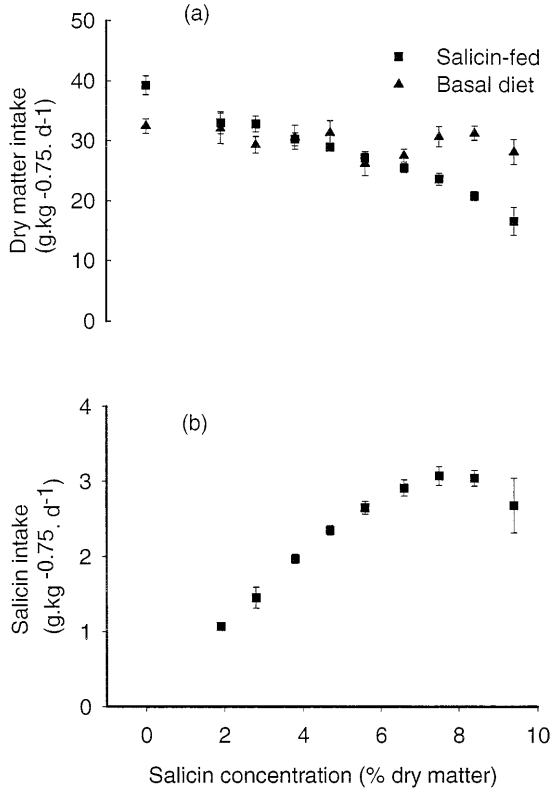

Fig. 2 Intake of **a** DM and **b** salicin in common brushtail possums fed a basal diet or one containing increasing concentrations of salicin. Mean ± SE; $n = 6$ for each treatment

Post-ingestive effects and taste of salicin

Experiment 3: effect of salicin on nitrogen and urea metabolism

Nitrogen intake was significantly higher at low and medium dietary salicin concentrations largely as a result of the higher DMI ($P = 0.004$) (Table 1). Accompanying this was a lower excretion of faecal nitrogen ($P = 0.042$) when animals were fed the highest concentration of salicin but there was no significant difference in the apparent digestibility of nitrogen or in urinary nitrogen loss or nitrogen balance between treatments ($P = 0.436$, 0.642, and 0.397, respectively). Similarly, there were no significant difference in any parameter of urea metabolism (Fig. 3) across the three treatments nor did the proportion of urea synthesised that was degraded in the gut differ between treatments ($P = 0.807$).

Table 1 Intake and excretion of dry matter (DM) and nitrogen in common brushtail possums fed a diet containing low (0.17%), medium (1.67%) and high (5.0%) concentrations of salicin (± SE). Values are expressed as g $kg^{-0.75}$ day^{-1} (mean ± SE; $n = 6$) unless otherwise indicated

Diet	Low salicin (0.17% DM)	Medium salicin (1.67% DM)	High salicin (5.0% DM)
DM			
Intake	25.5 ± 2.0	24.7 ± 1.9	17.7 ± 1.3
Digestibility (%)	81.1 ± 0.8	84.9 ± 1.8	85.6 ± 2.0
Nitrogen			
Intake	0.28 ± 0.02	0.29 ± 0.01	0.21 ± 0.01
Faecal	0.14 ± 0.02	0.11 ± 0.01	0.08 ± 0.02
Urinary	0.15 ± 0.02	0.16 ± 0.01	0.14 ± 0.01
Balance	−0.01 ± 0.02	0.02 ± 0.02	−0.02 ± 0.01
Apparent digestibility (%)	50.6 ± 4.0	61.3 ± 3.6	60.4 ± 7.7

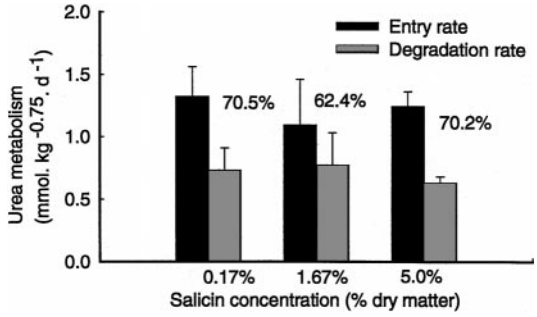

Fig. 3 Mean urea entry rate and degradation rate in common brushtail possums fed a diet containing 0.17%, 1.67% or 5.0% (DM) of salicin. Amount of urea recycled given as a percentage of urea entry rate for each treatment diet. Mean ± SE; $n = 6$

Experiment 4: effect of a selective 5-HT_3 receptor antagonist (ondansetron) on salicin intake

There was no effect of ondansetron on DMI in common brushtail possums fed salicin (S versus S + O; $P = 0.830$). Animals that received ondansetron ate no more basal diet than animals that received injections of saline (B + O versus B; $P = 0.888$) (Fig. 4). Animals that received salicin in their diets (S and S + O) still ate, on average, 63% less than animals that received no salicin (B and B + O) (10.4 ± 1.3 g $kg^{-0.75}$ day^{-1} compared with 28.6 ± 0.9 g $kg^{-0.75}$ day^{-1}). There was no significant difference between individual animals ($P = 0.184$), or between periods ($P = 0.347$) and no carry-over effect of treatments ($P = 0.616$).

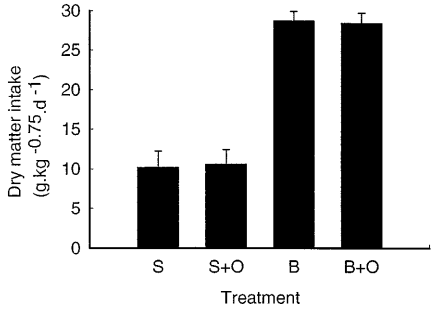

Fig. 4 The effect of an intraperitoneal injection of ondansetron (0.5 mg $kg^{-0.75}$) (*O*) or saline on DM intake (DMI) of common brushtail possums fed 5.5% (DM) dietary salicin (*S*) or a basal diet (*B*). Mean ± SE; $n = 8$

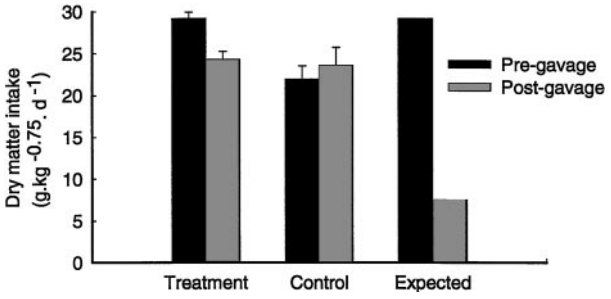

Fig. 5 The effect of 1.3 g salicin given by gastric gavage to common brushtail possums fed 3.3% (DM) dietary salicin. The expected DMI is calculated assuming that salicin causes a dose-dependent decrease in food intake. Mean ± SE; $n = 5$ for each treatment

Experiment 5: effect of salicin given by gavage on food intake of common brushtail possums fed a diet containing salicin

The expected DMI in the test group, (if 1.3 g of salicin caused post-ingestive effects proportional to its intake) was 16 g $kg^{-0.75}$ day^{-1}. There was no significant difference in DMI in the test group ($P = 0.126$) or the control group ($P = 0.572$) after oral doses of either 1.3 g salicin or water and DMI was not reduced to the predicted amount of 16 g $kg^{-0.75}$ day^{-1} (Fig. 5). Although common brushtail possums are able to vomit (Lawler et al. 1998), none did so after gavage of salicin nor was there any sign of excessive salivation or facial grooming. Food intake was also monitored several days after gavage and was found to be high and consistent (results not shown), suggesting that there were no delayed post-ingestive symptoms of an overdose from the bolus dose of salicin.

Discussion

These experiments showed that common brushtail possums could regulate their feeding so as not to exceed a threshold intake of salicin. This result accords with observations made in common brushtail possums with other PSMs (Lawler et al. 1998). In the experiments described here, the amount of compound required to affect feeding was one to two orders of magnitude greater than observed by Lawler et al. (1998). Additionally, the difference between our short-term and long-term experiments showed that the animals had a substantial ability to acclimate to dietary salicin. When the common brushtail possums were offered increasing doses of salicin they regulated their intake of salicin equally as well but at a much higher dose than naive animals.

Nonetheless, acclimation did not continue indefinitely and the intake of salicin plateaued at a dietary concentration of 6.55% (DM). In other words, salicin was a deterrent to feeding in common brushtail possums when dietary concentrations exceeded 6.55%. These concentrations are similar to those that Edwards (1978) found to be associated with low palatability of *Salix* spp. for common brushtail possums in New Zealand.

In contrast, similar short-term experiments by Tahvanainen et al. (1985) showed that addition of salicin to an artificial diet had no impact on feeding by hares (*Lepus timidus*) even in the absence of any acclimation. They concluded that the negative correlation between food choice and phenolic glycoside concentrations of a range of plant material was due to compounds other than salicin.

These results suggest that salicin can act as a feeding deterrent in one species of mammalian browser but not in another. Why this should be so, depends on establishing the nature of the signals that common brushtail possums use to recognise and regulate their intake of salicin. Recall that animals did not refuse absolutely to eat diets with high concentrations of salicin; rather they ate less of these diets. This result suggested to us that the signals that common brushtail possums were using to regulate intake were most likely related to the effects of salicin or its metabolites on some aspect of the their metabolism.

Provenza has suggested several times (Provenza et al. 1992, 1994; Provenza 1995) that animals may be able to detect impending toxicosis from PSMs by feedback from the emetic system of the brain. Elsewhere Lawler et al. (1998) discussed the nature of this process and emphasised the need to describe "emetic stimulation" more precisely in terms of the specific receptors involved in signalling to the brain. For example, the intake of another phenolic PSM, jensenone, in common brushtail possums is partly mediated by 5-HT_3 receptors. Additionally it is important to recognise that the animal need not vomit or become overtly ill as the emetic system could be stimulated and lead to a food aversion in the course of any normal feeding bout.

We reasoned that if salicin acted similarly to jensenone in common brushtail possums then dosing the animals with a selective 5-HT_3 antagonist should show whether post-ingestive feedback was acting as the feedback to signal animals to moderate their intake. Few experiments of this nature have been attempted and those have chosen either a high dose of non-specific antiemetic drugs (Aldrich et al. 1993) or else mixtures of three different drugs (Provenza et al. 1994). In contrast our protocol is based on injection of low doses of the drug ondansetron because it is a highly selective serotonin antagonist (at the 5-HT_3 receptor) that is widely used to control emetogenic chemotherapy.

The results showed that ondansetron had no effect on the common brushtail possum's intake of salicin. Although this could be attributable to factors such as insufficient dose, we had shown in a previous study (Lawler et al. 1998) that exactly the same protocol led to an increased intake of another small phenolic metabolite, jensenone. Therefore, we are confident that factors such as the size and timing of the dose in relation to feeding were appropriate and sufficient to detect a real effect. We conclude that in contrast to the situation with jensenone,

common brushtail possums do not regulate their intake of salicin by feedback from the emetic system.

It is feasible that other post-ingestive effects could provide a signal for the animals to regulate their feeding on salicin-rich diets. We focused on nitrogen metabolism since nitrogen is such an important element for herbivores and because we believed that much of the salicin would be eliminated in the urine conjugated with glycine. If this was so, we could have expected to see elevated levels of non-urea nitrogen in the urine. This did not occur (most of the salicin was excreted as a glucuronic acid conjugate (see below)) and the lack of significant difference in total urinary nitrogen excretion or nitrogen balance or in urea metabolism was evidence that salicin intake was not regulated because of a recognisable cost in this essential element.

Studies of the urinary metabolites excreted by the animals in this study (S. McLean, G. Pass and W. Foley, unpublished data) and our measurements of total glucuronic acid excretion suggested that there was no significant cost to brushtail possums of the detoxification of salicin. If salicin was absorbed across the intestinal surface it could act to reduce food intake by exerting significant energy costs through conjugation with glucuronic acid. The predominant urinary metabolite of salicin in the brushtail possum was the glucuronide of salicyl alcohol. That is, after an oral dose the glucoside of salicyl alcohol (i.e. salicin) is excreted largely as its glucuronide. This result suggests that although the animals are using glucuronic acid, they are also gaining glucose from salicin itself and therefore this is an unusual case in which the net loss of endogenous substrate is minimal. A simple oxidation reaction could convert salicin directly to the corresponding glucuronide, but there is no evidence that this reaction can occur in common brushtail possums (S. McLean, personal communication). A small amount of unchanged salicin was also detected, along with salicyluric acid, free salicyl alcohol, free salicylic acid and conjugated salicylic acid. This is surprising and contrary to what would be expected as it indicates salicin can be absorbed and excreted in its natural form. The low molecular weight and water solubility of salicin may explain this observed excretion pathway. This again suggests that very little energy cost is required for the detoxification of salicin. Although a small amount of energy would be expended in conjugation with glucuronic acid, because salicin is a simple phenolic glucoside, the net loss of endogenous substrates in the metabolism of salicin is zero.

To confirm the absence of post-ingestive effects, we dosed some animals with salicin by gavage. Our reasoning was that a bolus dose given just prior to feeding would reduce the amount of salicin that the animals would voluntarily ingest only if the animals were sensitive to the total body load of salicin. Our rationale for choosing a 24-h period for these observations was based on the pattern of excretion of urinary metabolites of salicin (S. McLean, G. Pass and W. Foley, unpublished observations). In animals fed a diet containing 5.0%, 1.17% and 0.17% salicin (dry weight), the metabolites identified accounted for 64%, 68% and 100% of the salicin dose, respectively, indicating that most of the dose was excreted from the animals within 24 h. Therefore, if salicin exerted post-ingestive effects, we should have observed these effects over the time in which salicin was being excreted from the body, i.e. within 24 h of dosing. We observed no change in food intake over the 24-h period. The results of this experiment clearly supported our earlier conclusion that salicin had no measurable post-ingestive effects on common brushtail possums. That animals would eat just as much salicin after receiving a large dose by gavage suggested that salicin intake is regulated largely by pre-ingestive effects or the taste of the compound.

Edwards (1978) had raised the possibility that the intensely bitter taste of salicin may have been the factor that limited feeding by common brushtail possums on willow clones. Taste is a very subjective measure and human perceptions of bitterness are a poor indicator of rejection thresholds in other animals. Many herbivores have been shown to be indifferent to foods that contain compounds at concentrations three orders of magnitude greater than those described as intensely bitter to humans (e.g. Nolte et al. 1994). Although this study has provided strong evidence that salicin is not avoided due to post-ingestive effects but rather, is avoided due to pre-ingestive effects, it is difficult to conclude that salicin is avoided by common brushtail possums due to its taste alone without further investigation into the trigeminal sensory system of these animals.

The buccal cavity is richly endowed with fibres of the trigeminal sensory system. Many plant compounds stimulate the trigeminal system – most noticeably in humans by those such as capsaicin that are sensed as a burning taste. This effect can lead to a reduction in the size of individual meals or the frequency of meals containing the compound. A similar reduction in the size and frequency of salicin-rich meals is thus a possible explanation of the patterns that we have observed. No studies have examined the potential of salicin as a trigeminal stimulant in mammals but Liu and Simon (1998) showed that many bitter compounds stimulated cultured rat trigeminal cells. Other studies in birds have clearly shown the importance of the trigeminal senses in detecting PSMs. For example, Jakubas and Mason (1991) showed that coniferyl benzoate which is an effective antifeedant in ruffed grouse (*Bonasa umbellus*) was avoided by starlings largely because of its effect on the trigeminal receptors. Further studies of the interactions between bitter PSMs and mammalian trigeminal receptors would be most useful in extending our understanding of the regulated intake of these kinds of diets.

Conclusions

We have shown that pre-ingestive factors are responsible for common brushtail possums limiting their intake of salicin-rich diets rather than emetic stimulation or any

other measurable post-ingestive cost or mechanism. This result raises the possibility that browsing mammals recognise and regulate their intake of PSMs through a variety of different mechanisms rather than their being a central control as envisaged by Provenza (1995). Better tests of this idea would result from studies of compounds that have known post-ingestive consequences that could be reversed or countered independent of anti-emetic effects.

Acknowledgements This research was approved by the Animal Experimentation Ethics Committee of James Cook University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. We thank Dr I. Lawler, Mr R. Gegg and Ms D. Mendez for assistance throughout the experiments, and Dr D. Ratkowsky and Dr M. Blows for statistical advice. This work was funded by grants from the Australian Research Council (to WJF) and James Cook University.

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