The effect of rumen adaptation to oxalic acid on selection of oxalic-acid-rich plants by goats

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Rumen microbial degradation is an important route for detoxification of secondary plant compounds encountered in the diets of free-grazing ruminants. Exposure to diets containing particular secondary plant compounds can lead to increased rates of secondary compound degradation in the rumen. An experiment was conducted to determine whether rumen adaptation to oxalic acid would influence the diet selection of goats offered choices between plant species differing in their oxalic acid content. Twelve adult female goats were divided into two groups of six animals each. One group received a daily oral dose, in gelatin capsules, of 0·6 mmol oxalic acid/kg live weight per d throughout the experiment while the other group received placebos consisting of empty gelatin capsules. After an adaptation period of 8 d, the animals were allowed to graze a mixture of spinach (rich in oxalic acid) and cabbage (low in oxalic acid) for 7 h/d on two consecutive days per week during four consecutive 1-week periods. Intervening days were spent on grass pasture. Diet composition and intake were measured using cuticular wax *n*-alkanes as internal markers. Results showed that adapted goats included a higher proportion of spinach in their diet ($P < 0.05$) although absolute intakes of spinach were the same for the two groups. Goats in the oxalic-acid-adapted group consumed less cabbage than control animals ($P < 0.05$) suggesting that adaptation to oxalic acid at the rumen level may have interfered with detoxification of cabbage-derived secondary plant compounds. Voluntary intake increased progressively through the four experimental periods ($P < 0.001$) with a tendency for higher intakes among control than among adapted animals ($P < 0.1$). The experiment demonstrates how differences in the rate of degradation of secondary plant compounds may influence diet selection in ruminants.

Diet selection: Oxalic acid: Rumen detoxification

Certain plant secondary compounds have toxic properties and have been found to deter herbivore feeding on particular plant species in a large number of contexts (Palo & Robbins, 1991). This phenomenon has an important influence on the diet selected by herbivores and therefore affects the impact of foraging animals on vegetation composition over time (Jefferies *et al.* 1994). One of the mechanisms whereby herbivores avoid potentially toxic food items may be through conditioned food aversions (Provenza & Balph, 1987). Thus herbivores may learn to associate plant cues with toxic post-ingestive consequences over a series of encounters and show partial aversion to toxic plants during subsequent encounters. The strength of aversion to toxic sources is known to depend on the strength of post-ingestive physiological effects (du Toit *et al.* 1991). This behavioural adaptation to the presence of secondary plant compounds in the diet has been shown to operate in certain

simple situations using artificial feeding deterrents and pen-fed domestic ruminants (Provenza *et al.* 1994). The significance of conditioned food aversions as mediators of diet selection by free-ranging herbivores is still in question.

As well as behavioural mechanisms for limiting the toxicity of plant secondary compounds, large herbivores are equipped with important physiological detoxification mechanisms (Smith, 1992). Detoxification of ingested plant secondary compounds has been shown to operate both in the digestive tract and at the systemic level. The flexibility of these detoxification mechanisms gives large herbivores the potential to adapt readily to inclusion of new toxic items in their diet, provided sufficient time is available for induction of detoxification pathways. For example, oxalic acid is present in a range of food plants commonly consumed by herbivores and renal toxicity and hypocalcaemia may occur after abrupt exposure to oxalic-acid-containing plants

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(James, 1972). Gradual exposure to increasing levels of oxalic acid leads to a change in the composition of the rumen microbial population and the resulting breakdown of oxalic acid prevents these symptoms (Allison *et al.* 1977; Duncan *et al.* 1997).

Different rates of secondary plant compound detoxification in the digestive tract, arising as a result of adaptation, are likely to yield different rates of absorption of secondary compounds and therefore differences in post-absorptive consequences. An experiment is reported which tested the impact of rumen adaptation to a secondary plant compound on learned aversion to a potentially toxic plant. Goats were adapted to oxalic acid by regular oral administration; they were then offered choices between plants containing high and low concentrations of oxalic acid and their diet selection was compared with that of non-adapted animals.

Materials and methods

Experimental design

Twelve female Scottish Cashmere goats (mean live weight 38·3 (SD 3·47) kg) were assigned to two treatment groups of six animals each, balanced for live weight. One group was adapted to oxalic acid by once daily oral dosing (0·6 mmol/kg live weight per d) throughout the experiment (treatment group) while the other group received a placebo (control group). The experiment consisted of a preliminary 8 d adaptation period followed by four consecutive experimental periods lasting 7 d each. In total, there were eight 5 × 10 m spinach–cabbage plots and during each spinach–cabbage grazing day, goats were given access to a fresh plot of spinach (*Spinacea oleracea*; high concentrations of oxalic acid) and cabbage (*Brassica oleracea*; low concentrations of oxalic acid) plants on the first 2 d of each experimental period. DM intake and diet composition were measured during this time. During the remaining 5 d of each period, goats were allowed to graze a perennial ryegrass pasture.

Procedures

Oxalic acid (Aldrich, Poole, Dorset, UK) contained in gelatin capsules (Davcaps, Monmouth, Gwent, UK) was administered to animals in the treatment group by once daily oral dosing at 09.00 hours. Placebos in the form of empty gelatin capsules were given at the same time to the control group of goats. During the preliminary 8 d adaptation period, oxalic acid dose rates were increased from zero to the full dose in equal increments over the first 5 d and the full dose was given for the remaining 3 d. During each of the subsequent experimental periods oxalic acid was administered daily at the full dose rate except on days during which animals had access to the spinach–cabbage plots. On these days, no oxalic acid was administered to avoid potential toxicity from the combined effects of naturally ingested and artificially administered oxalic acid. Rumen samples were collected under anaerobic conditions by stomach tube at 10.00 hours on the day before, and the day after, the spinach–cabbage feeding days. Animals were taken indoors

for this purpose. During each experimental period, spot samples of faeces were collected by rectal grab sampling immediately before releasing animals onto a spinach–cabbage plot (09.00 hours) and at 24, 33, 48, 55 and 72 h thereafter. Faecal samples were stored at −20° pending *n*-alkane analysis for estimation of herbage intake and diet composition.

Diet options

From 09.00 hours to 16.00 hours on each of the days when spinach and cabbage were offered, goats had access to a fresh experimental plot measuring 5 × 10 m containing cabbage (variety 'First Early Market 218'; Breeders Seeds, Ormskirk, Lancs., UK) and spinach (variety 'Carambole'; Breeders Seeds) plants sown 10 weeks before the start of the first experimental period. Each plot contained alternate spinach and cabbage plants at 0·3 m intervals. A fresh 50 m^2 plot was used on each spinach–cabbage feeding day. Animals grazed a perennial ryegrass pasture overnight between the two spinach–cabbage feeding days and during the remaining 5 d of each experimental period.

Measurements

Rates of oxalic acid degradation were measured by a modification of the method of Allison *et al.* (1985) as described by Duncan *et al.* (1997). Samples of ruminal contents obtained by stomach tube were collected, kept at 37° and processed within 60 min of collection.

Standing biomass of DM was measured by cutting, to ground level, six randomly chosen plants of each species per feeding period, pooling herbage by species and drying at 85°. The mean dry weight per plant was multiplied by the number of plants counted per plot to determine total biomass of DM per hectare. A further three randomly chosen plants of each species per period were cut to ground level, pooled by species and subsequently sub-sampled for oxalic acid analysis. The remaining material was then freeze-dried for further chemical analysis. Samples were then milled through a 1 mm screen and analysed for total N (Perkin Elmer elemental analyser; Perkin Elmer Ltd, Beaconsfield, Bucks., UK), neutral-detergent fibre (Van Soest & Wine, 1967), acid-detergent fibre (Van Soest, 1963) and lignin (Van Soest, 1963). Samples were analysed for oxalic acid by GC. Fresh, frozen samples were prepared by a modification of the method of Libert (1981) as follows. Fresh herbage (20 g) was homogenized in 250 ml water for 3 min. To 25 ml homogenate, 20 ml 5 M-HCl was added along with 0·5 ml internal standard (2 mg succinic acid/ml absolute alcohol) and distilled water to make up to 50 ml. The homogenate was heated in a water-bath at 95° for 30 min and then filtered. Oxalic acid was measured using GC following preparation of derivatives by silylation with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA; Aldrich). For this purpose homogenates were acidified and extracted with diethyl ether. Ether was removed by evaporation and MTBSTFA reagent (100 ml/l solution in acetonitrile) was added before heating at 80° for 30 min. Samples were analysed by capillary GC using a

Supelco SBP-20 bonded phase capillary column ($0.53\,\mu\text{m}\times 30\,\text{m}$) with oven temperature programmed from $60°$ to $200°$ at $7°/\text{min}$.

Herbage intake and diet composition during spinach–cabbage feeding periods were estimated using a modification of the *n*-alkane technique (Dove & Mayes, 1991) modified for estimation of short-term intake and diet composition (Duncan *et al*. 1999). Briefly, the method relies on the use of *n*-alkanes which are components of the cuticular wax of most plants. *n*-Alkanes are relatively indigestible and can thus be used as internal markers for intake estimation following collection and analysis of faecal samples. The accuracy of estimation is markedly improved by oral administration of synthetic *n*-alkanes with similar chain lengths, which allows correction for incomplete faecal recovery of herbage *n*-alkanes. Diet composition can be estimated from faecal *n*-alkane patterns by virtue of the differing *n*-alkane profiles of different plant species. In the current experiment, for example, the fact that cabbage contains high levels of C_{29} *n*-alkane, while spinach is rich in C_{31} *n*-alkane, was exploited. Measurement of faecal concentrations of these two *n*-alkanes allowed accurate estimation of the proportions of the two source plant components in the diet. In the present experiment, animals were dosed with two *n*-alkane pellets on each dosing occasion at 0, 7, 24 and 31 h after the start of each 2 d spinach–cabbage feeding period. Each *n*-alkane pellet consisted of a paper bung impregnated with 200 mg C_{28} *n*-alkane and 200 mg of C_{32} *n*-alkane. Excretion curves of dosed and herbage *n*-alkanes were plotted using *n*-alkane concentrations measured in faecal grab samples. The areas under the excretion curves for each *n*-alkane were used in place of true *n*-alkane concentrations to estimate intake and diet composition according to conventional *n*-alkane calculation methods (Dove & Mayes, 1991). Intake of grass during the rest period between each cabbage–spinach grazing day was not calculated. The contribution of *n*-alkanes derived from grass consumed during this time to the excretion curves was disregarded since *n*-alkane concentrations in *Lolium perenne* (100–200 mg/kg DM; Dove & Mayes, 1991) are an order of magnitude lower than those in cabbage and spinach (1000–2000 mg/kg DM; current experiment). Herbage and faecal samples were analysed for *n*-alkanes using the method of Mayes *et al*. (1986).

Statistical analysis

The experiment was analysed using ANOVA with a factorial treatment structure (two treatments × four periods). Variance associated with the adaptation treatment was estimated between animals. Variance associated with period and interactions with period was estimated within animals. Rates of degradation of oxalic acid were expressed as means of values measured before and after the spinach–cabbage feeding periods and were log transformed to reduce systematic variation of residuals. Data relating to the proportion of spinach in the diet were subjected to an arcsine transformation before statistical analysis, again, to reduce systematic variation of the residuals. Data were analysed using GENSTAT 5 (Lawes Agricultural Trust, 1989).

Results

Daily oral administration of oxalic acid had a marked influence on rates of degradation of oxalic acid in the rumen. Mean untransformed rates of degradation were 0.592 (range 0.236–1.685) $\mu\text{mol/ml}$ rumen fluid per h for adapted animals and 0.269 (range 0.165–0.360) $\mu\text{mol/ml}$ rumen fluid per h for control animals ($P < 0.05$). Rates of degradation were not significantly affected by period and there was no significant interaction between period and treatment.

Concentrations of oxalic acid in spinach (5.85 (SE 0.76) g/kg DM) were considerably greater than in cabbage which contained negligible amounts (0.17 (SE 0.05) g/kg DM). Cabbage had a higher nutritive value than spinach with lower levels of neutral-detergent fibre (213.5 (SE 12.80) *v.* 371.5 (SE 12.49) g/kg DM) and acid-detergent fibre (199.2 (SE 17.60) *v.* 272.0 (SE 10.84) g/kg DM) and higher crude protein concentrations (134.7 (SE 12.07) *v.* 103.1 (SE 6.13) g/kg DM). Average standing biomass was similar for the two plant species (1.91 (SE 0.62) *v.* 1.77 (SE 0.51) tonnes DM/ha for cabbage and spinach respectively).

Total daily DM intakes from spinach and cabbage increased progressively over the four experimental periods (Fig. 1; $P < 0.001$) averaging 0.20 (SE 0.034) kg DM/d during period 1 and reaching 1.20 (SE 0.115) kg DM/d during period 4. The adaptation treatment had a small influence on DM intake with control animals tending to consume more than adapted animals (Fig. 1; 0.846 (SE 0.0996) *v.* 0.627 (SE 0.0878) kg DM/d; $P < 0.1$).

The proportion of the spinach in the diet was significantly higher among oxalic-acid-adapted animals than among control animals (Fig. 2; 0.445 (SE 0.0293) *v.* 0.283 (SE 0.0177); $P < 0.01$) over the whole experiment. The overall proportion of spinach in the diet decreased as the experiment progressed (0.462 (SE 0.0397), 0.357 (SE 0.0393), 0.314 (SE 0.0385), 0.292 (SE 0.0236) for periods 1–4; $P < 0.001$) but the interaction between adaptation treatment and period was not significant.

The intake of DM from spinach was not affected by adaptation treatment (Fig. 3) and intake of spinach increased over the course of the experiment (0.086 (SE 0.0109), 0.204 (SE 0.0209), 0.298 (SE 0.0412), 0.347 (SE 0.0431) kg DM/d for periods 1–4; $P < 0.001$). By contrast the intake of DM from cabbage was significantly lower among oxalic-acid-adapted animals than among control animals (Fig. 4; 0.372 (SE 0.0264) *v.* 0.631 (SE 0.0783) kg DM/d; $P < 0.05$). DM intakes from cabbage also steadily increased over the course of the experiment (0.116 (SE 0.0248), 0.399 (SE 0.0577), 0.685 (SE 0.0766), 0.852 (SE 0.0885) kg DM/d; $P < 0.001$).

Discussion

The experiment reported here was set up to test a hypothesis originally proposed by Provenza *et al*. (1992), that increased rates of rumen degradation of particular secondary plant compounds may result in greater selection by ruminant herbivores of plants containing those compounds. The hypothesis has not previously been tested in a free-grazing situation with choices of naturally occurring toxic plants. In
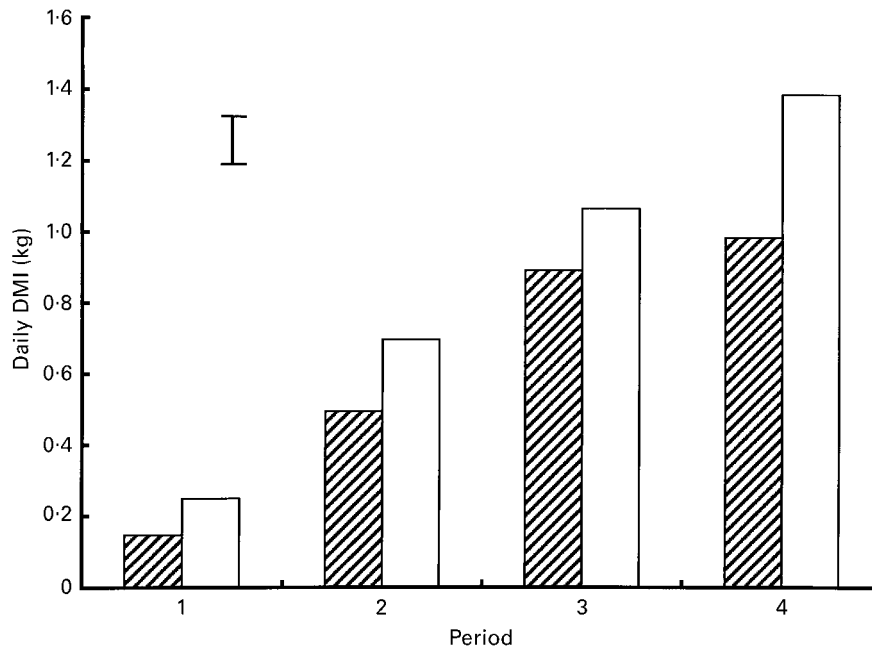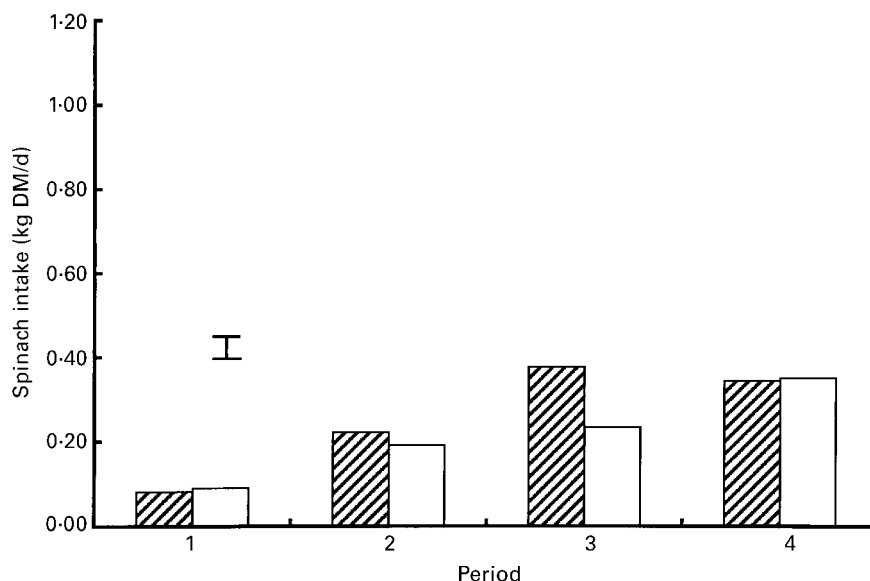
Fig. 1. Total daily dry matter intake (DMI, kg) of control goats (□; daily oral dosing with empty gelatin capsules) and goats adapted to oxalic acid (▨; daily oral dosing with 0·6 mmol/kg live weight) offered a matrix of cabbage and spinach plants for 7 h/d on two consecutive days per week during four consecutive 1-week periods. Values are means for six goats per treatment. The vertical bar represents the standard error of difference for the interaction between treatment and period.

an indoor experiment reported by Frutos *et al.* (1998), diet selection between two artificial feeds, differing only in their flavour and oxalic acid content, was not influenced by adaptation of the rumen to oxalic acid. In that experiment, however, oxalic acid concentrations in the diet did not present a serious toxic challenge to the animal; furthermore such effects may be more apparent in free-grazing situations where herbivores are making choices in circumstances more
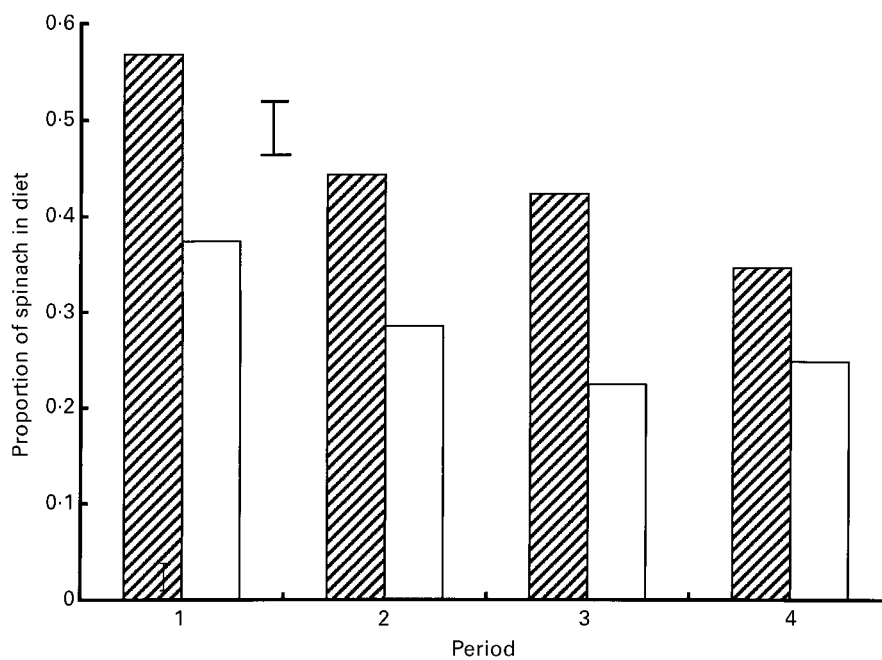
Fig. 2. Proportion of spinach in the diet selected by control goats (□; daily oral dosing with empty gelatin capsules) and goats adapted to oxalic acid (▨; daily oral dosing with 0·6 mmol/kg live weight) offered a matrix of cabbage and spinach plants for 7 h/d on two consecutive days per week during four consecutive 1-week periods. Values are means for six goats per treatment. The vertical bar represents the standard error of difference for the interaction between treatment and period (untransformed data).
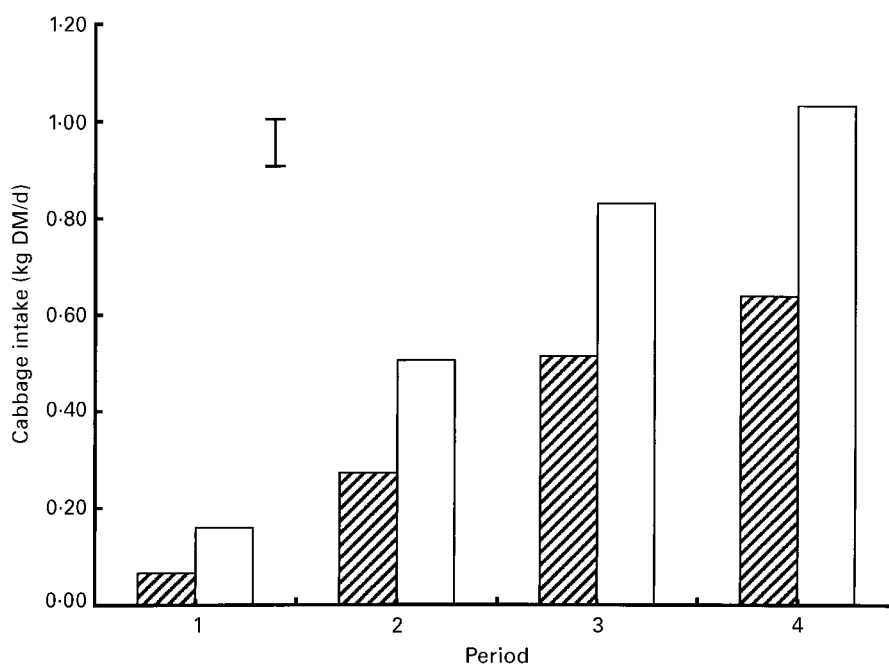
Fig. 3. Intake of dry matter from spinach (kg/d) by control goats (□; daily oral dosing with empty gelatin capsules) and goats adapted to oxalic acid (▨; daily oral dosing with 0·6 mmol/kg live weight) offered a matrix of cabbage and spinach plants for 7 h/d on two consecutive days per week during four consecutive 1-week periods. Values are means for six goats per treatment. The vertical bar represents the standard error of difference for the interaction between treatment and period.

akin to the environment in which their selective behaviour evolved.

In the current experiment, goats whose rumen microbial population was pre-adapted to oxalic acid showed higher rates of oxalic acid degradation in the rumen; absorption of dietary oxalic acid following consumption of spinach is therefore likely to have been less than in naïve animals.

Adapted animals selected a higher proportion, although not a higher amount, of spinach in their diets than did control animals. Control animals consumed greater proportions and greater amounts of cabbage than oxalic-acid-adapted animals. Thus, diet selection was modified by the oxalic acid treatment, but not in a manner consistent with the original hypothesis.

Fig. 4. Intake of dry matter from cabbage (kg/d) by control goats (□; daily oral dosing with empty gelatin capsules) and goats adapted to oxalic acid (▨; daily oral dosing with 0·6 mmol/kg live weight) offered a matrix of cabbage and spinach plants for 7 h/d on two consecutive days per week during four consecutive 1-week periods. Values are means for six goats per treatment. The vertical bar represents the standard error of difference for the interaction between treatment and period.

Differences in diet selection between adapted and control animals are unlikely to have been affected by food availability since the same relative proportions of the two diet options would have been available to all animals at any given point in time. Intakes of both diet options increased as the experiment progressed. This was probably due, in part, to the increasing familiarity of the experimental animals with the foods on offer. Initial caution with novel foods has been demonstrated before and probably represents an adaptive mechanism for avoiding over-consumption of potentially toxic plants (Provenza *et al.* 1994). Food neophobia also facilitates physiological adaptation to secondary compounds allowing time for induction of detoxification mechanisms at both rumen and systemic levels.

The higher voluntary intake of cabbage observed among control animals and the tendency of control animals to have higher total voluntary food intakes were unexpected. It may be that the adaptation dose of oxalic acid caused a general depression of rumen microbial activity or that it was partially absorbed and caused low-level systemic toxicity. This could explain the tendency for oxalic-acid-adapted animals to consume slightly less than control animals. The effect of the oxalic acid adaptation treatment on diet selection may have been mediated through effects on ruminal breakdown of other secondary compounds in the diet. The chosen 'non-toxic' food option was cabbage which itself contains secondary plant compounds such as glucosinolates and *S*-methyl cysteine sulfoxide. *S*-methyl cysteine sulfoxide is known to break down in the rumen to yield the haemolytic agent dimethyl disulfide which is responsible for the well-known haemolytic anaemia which results from chronic brassica consumption (Smith *et al.* 1974). In the current experiment, the short period of exposure (2 d at a time) makes it highly unlikely that this route of toxicity could have influenced diet selection patterns. Glucosinolates are thought to break down in the rumen under the action of a plant thioglucosidase enzyme to yield a range of potentially toxic metabolites including isothiocyanates and nitriles (Duncan, 1991). Although these metabolites are potentially highly toxic, previous experiments have demonstrated that adaptation of rumen microbes can protect the host against toxicity (Duncan & Milne, 1992). Chronic administration of oxalic acid, while increasing the rumen population of oxalic-acid-degrading micro-organisms, may have had a negative impact on microbial breakdown of other secondary compounds such as the glucosinolate breakdown products through its effects on the rumen microbial population. The effect of this would be to increase the negative impact of glucosinolates among oxalic-acid-adapted animals. This is a possible explanation for the higher intake of cabbage among control animals.

These results raise interesting questions concerning the interaction between different groups of secondary compounds in the diet with respect to their toxic mechanisms and detoxification pathways. Such effects were unforeseen in the current experiment and in the absence of data collected to address these questions we cannot draw definitive conclusions about the role of glucosinolates and their metabolites in the observed patterns of diet selection. Interactions between secondary compounds have been demonstrated in other contexts (Freeland *et al.* 1985) and the effect of such interactions on diet choice has recently been examined in a series of preliminary indoor experiments (F.D. Provenza, personal communication). These experiments suggest that in some circumstances herbivores may select mixed diets to dilute the effects of individual secondary compounds but that in other situations toxins may interact to produce unexpected effects as found here. There is a need for closer examination of the role of interactions between the toxic mechanisms of different plant secondary compounds and the potential repercussions for diet selection behaviour.

Different diet selection patterns were observed in control and adapted animals despite the fact that animals grazed as one group. Social facilitation between animals has been found to influence diet selection patterns (Ralphs *et al.* 1994). This effect would have tended to dilute any treatment-related differences. Allowing treatment and control groups to graze separately might have led to greater differences in food selection patterns between control and adapted animals but would have introduced plot differences as a confounding factor. Although social facilitation can cause rapid extinction of conditioned food aversions in the absence of toxicosis, it is important to note that toxicosis can be as powerful as social facilitation in its effects on diet selection. For example, lambs do not continue to eat the food their mothers consume when the food continues to cause toxicosis (Provenza *et al.* 1993).

The long-term importance of rumen adaptation to plant secondary compounds as a factor influencing diet selection is likely to depend on the speed of induction of detoxification pathways. The rumen microbial population can respond rapidly to dietary changes and adaptation to oxalic acid can occur in a matter of a few days (Duncan *et al.* 1997). In the case of oxalic acid and other compounds to which the rumen adapts rapidly, long-term inter-animal differences in diet selection patterns are therefore unlikely to be related to differing rumen microbial states. For plant secondary compounds requiring longer intervals for adaptation to occur, the state of rumen adaptation could have a significant influence on diet selection patterns. Species differences in detoxification pathways (Duncan *et al.* 1997) may be partially responsible for the diet selection patterns found in different herbivore species.

In conclusion, this experiment demonstrates that adaptation to secondary compounds can modify diet selection patterns. The unexpected pattern of diet selection observed highlights the complexity of secondary compound biotransformation in the rumen but indicates that such chemical processes can have important consequences for free-grazing animals.

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