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Do herbivores associate flavours with specific consequences in flavour aversion learning?

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Abstract

Goats were first offered a novel, maple-flavoured diet paired with either lithium chloride (LiCl) or oxalic acid (OA) delivered intraruminally. Aversion to maple diet persisted for 6 days, regardless of toxin. Following a 10-day intermission, aversion to maple diet was reacquired (spontaneous recovery) among subjects previously dosed with LiCl but not for subjects receiving OA. All subjects were then offered two diets: the maple diet previously paired with LiCl or OA and a novel grape-flavoured diet. Immediately following consumption of both diets, all subjects were dosed with LiCl. Preference for maple and grape diets was assessed by two-choice test. Goats avoided maple diet for only 1 day, regardless of their previous experience with maple diet (paired with LiCl or OA). These results indicate that goats generalized the postingestive consequences of the two toxins. Furthermore, presence of a novel flavour (grape) during toxin exposure contributed to rapid extinction of the aversion to maple flavour.

Keywords: Flavour aversion learning; Foraging; Prior illness; Spontaneous recovery; Wildlife damage management

1. Introduction

Simple experiments of flavour aversion learning (FAL) are numerous (see for example Riley and Freeman, 2004). In these controlled experiments, a food is offered to test subjects and an aversion is produced by administration of a toxin. In subsequent feeding bouts, subjects limit

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intake of flavours associated with toxins (Launchbaugh and Provenza, 1993; Lawler et al., 1999; Provenza et al., 2000). Avoidance of the flavour occurs even when the toxin is no longer present.

Wildlife damage management professionals continue to look at FAL with great interest to minimize damage to agriculture by pest herbivores (Mason, 1998; Sayre and Clark, 2001; Gentle et al., 2004). Numerous toxicants that promote conditioned aversions have been identified (Riley and Tuck, 1985). However, applied FAL is hampered by the limited number of these toxicants that are registered for pesticide use. Regulation may preclude use in certain areas, at certain times of the year, and/or on specific agricultural products. Thus, it would be desirable to condition an aversion to a specific non-toxic flavour and exploit this aversion by using only the flavour when the toxin is unavailable. The fundamental relationship between the toxin and associated cue must be better understood if there is any hope to successfully exploit cue avoidance in applied FAL.

For this study, we asked the question, ‘‘once a flavour has been associated with a toxin, what happens when that same flavour is presented with a different toxin and a novel flavour?’’ This was achieved by using two toxins with different toxicological effects. Lithium chloride (LiCl) produces gastrointestinal malaise without dangerous side effects (Ralphs, 1992). Oxalic acid (OA) promotes aversions through hypocalcemia which results in central nervous system irritability and reduced motor activity (Frutos et al., 1998).

Two possible outcomes were considered: generalization of illness or toxin–cue specificity. If generalization occurs, the original flavour would be associated with any subsequent toxicosis. If flavour cues are specifically associated with the physiological consequences of the toxin, the original flavour would be associated only with the original toxin. Meanwhile, the novel flavour would be linked to the novel consequences of the second toxin. As in previous studies, model ruminant herbivores were used as experimental subjects (Kimball et al., 2002; Kimball and Nolte, 2005).

2. Methods

2.1. Subjects

Twenty-four pigmy goats (*Capra aegagrus hircus*) were group housed in a 2.0 ha pen when not being tested. Shelter, trace mineral salt block, and water were provided ad libitum in this pen. For 30-min test periods, subjects were moved into individual, 3 m × 6 m pens constructed of rigid wire fencing (1.2 m high). Goats were deprived of basal rations (alfalfa hay) for 5 h prior to 30-min testing bouts. Test foods were offered individually or side-by-side in 2 L plastic buckets attached to the inside of the pen (45 cm off the ground). When two diets were offered, the positions of the test diets were alternated daily. Water was not present in the individual pens during testing. The experiments described here were conducted during the period of 19 July to 2 September 2005 and approved by the Institutional Animal Care and Use Committee of the USDA National Wildlife Research Center.

2.2. Test materials

Individual diet constituents were ground prior to formulating test diets. The control test diet was prepared with 15 kg Formula 135 deer feed (X-Cel Feeds, Tacoma, WA, USA), 7 kg alfalfa (Concentrates Inc., Portland, OR, USA), 2 kg soybean meal (Land O’Lakes Farmland Feed, Seattle, WA, USA), and 1 L vegetable oil (Wesson brand). To produce flavoured test diets, 500 g of maple livestock feed additive (Lucta USA Inc., Northbrook, IL, USA) or grape pomace (Wilber-Ellis, Portland, OR, USA) were added directly to 25 kg of control diet, yielding a flavour concentration of 2.0%.

LiCl, sodium chloride (NaCl), and OA were purchased from Sigma–Aldrich Co. (Milwaukee, WI, USA). A 150 mg/mL solution of LiCl and a 250 mg/mL OA solution (also containing 150 mg/mL NaCl) were prepared in tap water for delivery to the test subjects by intraruminal intubation. NaCl was added to the OA solution to equate the salinity and molality of the OA and LiCl solutions. Salinity, the concentration of dissolved ions measured on a mass basis, was equal in the two solutions (150 ppt). The molal concentration of the LiCl solution was 4.68 mol/kg and the combined molality of the NaCl and OA solution was 4.54 mol/kg. Because osmolality is proportional to molal concentration, it was assumed that the osmotic load of the two solutions was equivalent. Because OA solubility decreased with addition of NaCl (''salting out'' effect), the OA solution was continuously stirred by hand to ensure a homogenous OA dosage.

2.3. Test schedule

On test days, goats were subjected to a 5-h food deprivation period commencing at 08:00 h daily. Thirty-min feeding trials were begun at 13:00 h. Alfalfa hay was made available upon completion of each feeding trial until 08:00 h the next morning. Alfalfa hay was available ad libitum on those days when feeding trials were not conducted. Subjects were offered flavoured foods and dosed with toxins according to treatment group (Table 1).

2.4. Pre-treatment feeding trial

All subjects were offered 500 g of control diet for 30 min daily for 5 consecutive days. Intake was determined by measuring refusals. Subjects were assigned to one of three treatment groups (Flavour, LiCl, or OA; eight subjects per group) such that mean intake and standard deviation were similar among treatments.

2.5. Flavour preference

Responses to the two flavours were assessed by offering subjects in the Flavour treatment the choice of maple diet and grape diet in a two-choice test over 5 consecutive days (Table 1). Five hundred grams of each diet were offered for 30 min. Daily intake was determined by measuring mass of refusals on the 5 days. During this phase of the experiment, subjects in the LiCl and OA treatments were not tested.

2.6. Initial toxin exposure

On day 0, subjects from LiCl and OA treatments were offered 500 g of maple diet for 30 min (Table 1). Intake was recorded and a 95% confidence interval was constructed from this intake data. Immediately after feeding, subjects were administered a toxin, according to treatment group, by intraruminal intubation.

Table 1
Experimental design: duration and identity of diets and toxin exposures offered to goats according to treatment group (LiCl = lithium chloride; OA = oxalic acid)

Procedure	Duration/test day	Flavour treatment	LiCl treatment	OA treatment
Pre-treatment	5 days	Control	Control	Control
Flavour preference	5 days	Maple, grape		
Initial toxin exposure	Day 0		Maple (LiCl)	Maple (OA)
Extinction	Days 1–9		Maple	Maple
Delay period	Days 10–20		Control	Control
Spontaneous recovery	Day 21		Maple	Maple
New toxin exposure	Day 22		Maple, grape (LiCl)	Maple, grape (LiCl)
Two-choice preference	Days 23–30		Maple, grape	Maple, grape

Subjects in the LiCl treatment were administered the LiCl solution at a rate of 1.0 mL solution/kg body mass, resulting in a dose of 150 mg/kg. Subjects in the OA treatment were similarly dosed with the OA solution at a rate of 1.0 mL/kg, resulting in an OA dose of 250 mg/kg. Basal rations (alfalfa hay) were withheld for 2 h following toxin administration to ensure that toxicosis was not associated with the basal diet.

2.7. Extinction

For 9 days following initial toxin exposure, subjects in the LiCl and OA treatments were offered 500 g of maple diet for 30 min (Table 1). Intake was recorded. Maple diet was offered daily in this manner until extinction was observed. Extinction, or the ending of the aversive response, was defined as 3 consecutive days of mean intake falling within (or above) the 95% confidence interval derived from day 0 maple intake. At this stage of the experiment, one subject from the LiCl treatment group was removed from the study because of health concerns (broken tooth).

2.8. Delay period

Once extinction of the aversion was indicated, subjects in both treatments were offered 500 g of control diet (no flavour) for 30 min on 10 consecutive days.

2.9. Spontaneous recovery

All subjects were then (day 21) offered 500 g of maple diet for 30 min. Maple diet intake was recorded for this single day to assess spontaneous recovery of the aversion.

2.10. Second toxin exposure

On day 22, all subjects of both LiCl ($n = 7$) and OA ($n = 8$) treatments were offered 50 g each of maple and grape diet in side-by-side containers. The mass of each diet was limited to 50 g to ensure that all subjects would eat both diets in the 30-min test period. Immediately following consumption of both diets, LiCl was administered to all goats by intraruminal intubation (150 mg/kg). Basal rations were withheld for 2 h following delivery of LiCl.

2.11. Two-choice preference test

On days 23–30, all subjects were offered 500 g of maple diet and 500 g of grape diet as described for the two-choice flavour preference test (Section 2.5). Intake was recorded.

2.12. Statistical analyses

Mean intakes of the flavour treatment (grape and maple diet) were calculated for the 5-day flavour preference test (maple versus grape flavour in the absence of an aversion). Intake data were analyzed by analysis of variance (ANOVA; SAS, 2002). Flavour (grape, maple), days (1–5), and flavour × day interaction were fixed effects. Subject and subject × day interaction were random effects. Residuals were evaluated by constructing a normal probability plot as well as plotting Studentized residuals versus predicted values to test ANOVA assumptions (SAS, 2002).

Intake data (g) from the initial toxin exposure and extinction experiments were analyzed by ANOVA (SAS, 2002). Treatment (LiCl, OA), day, and treatment × day interaction were fixed effects. Subject (nested in treatment) and day × subject (treatment) interaction were random effects. Residuals were evaluated as described for analysis of flavour preference data.

Four a priori hypotheses related to formation of an aversion and spontaneous recovery of it were tested by linear contrast—(1) within the LiCl treatment: day 1 intake was less than day 0 intake; (2) within the OA

treatment: day 1 intake was less than day 0 intake; (3) within the LiCl treatment: day 21 intake was less than day 0 intake; (4) within the OA treatment: day 21 intake was less than day 0 intake. To monitor persistence of the aversion, nine post hoc multiple comparisons of intake by day (day 0 intake versus each of days 1–9) were made by controlling false discovery rate according to the method of Benjamini and Hochberg (1995).

Intake data obtained from the two-choice preference test were similarly analyzed by ANOVA except that flavour (grape, maple) and all two- and three-way interactions were also considered in the model. Two a priori hypotheses were tested by linear contrast: (1) on day 23: maple diet intake of the LiCl treatment was less than the OA treatment; (2) on day 23: grape diet intake of the OA treatment was less than the LiCl treatment. Residuals were inspected graphically to test ANOVA assumptions.

3. Results

3.1. Pre-treatment and flavour preference

Among the LiCl, OA, and Flavour treatments, mean control diet intakes (S.D.) were 345 (69) g, 335 (66) g, and 323 (48) g, respectively. In the absence of an aversion, goats in the Flavour treatment did not prefer one of the flavoured diets to the other ($P = 0.507$; Fig. 1). Furthermore, there were no day ($P = 0.506$) or day × flavour interaction ($P = 0.359$) effects.

3.2. Initial toxin exposure and extinction

Both LiCl and OA administration impacted maple diet consumption, as indicated by a significant day effect (Fig. 2; $P < 0.0001$). Linear contrasts indicated day 1 test diet intake was significantly less than day 0 intake for both the LiCl treatment ($P < 0.0001$) and OA treatment ($P < 0.0001$), further indicating an aversion to the maple diet. Absence of a significant treatment effect ($P = 0.899$) indicated that both toxins similarly conditioned an aversion. The extinction component of the experiment was terminated after day 9 because the condition of 3 consecutive days of intake equal to day 0 was met. Post hoc comparisons of daily intake indicated that aversion to the maple diet persisted for 6 days and extinction occurred on day 7.

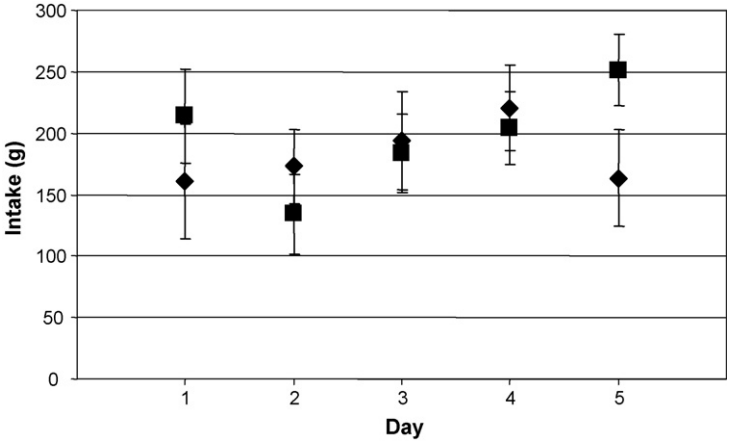

Fig. 1. Intake (g) of test diets offered to goats ($n = 8$) in a 30-min two-choice test in the absence of an aversion (maple diet, ◆; grape diet, ■). Standard errors are represented by the vertical lines.

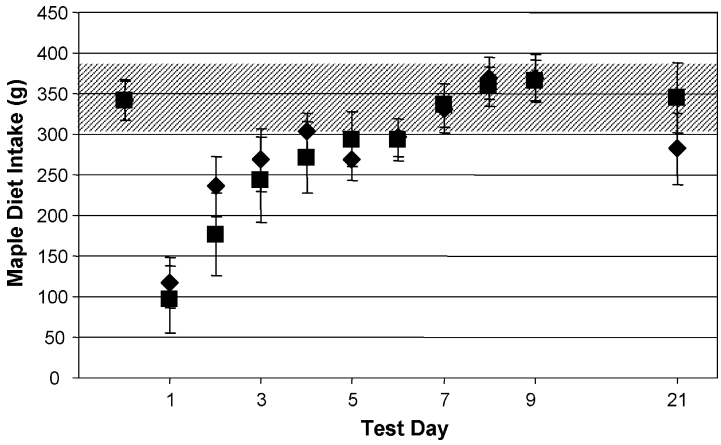

Fig. 2. Maple diet intake (g) during a 30-min test of goats intraruminally dosed with either oxalic acid (OA, ■; $n = 8$) or lithium chloride (LiCl, ◆; $n = 7$). The cross-hatched area represents a 95% confidence interval about the day 0 mean. Standard errors are represented by the vertical lines.

3.3. Spontaneous recovery

As tested by linear contrasts, spontaneous recovery of the aversion after 10 days without access to the maple diet (indicated by less intake on day 21 versus day 0) was observed for the LiCl treatment ($P = 0.026$) but not the OA treatment ($P = 0.873$).

3.4. Second toxin exposure and two-choice preference

Following the second toxin exposure, significant day ($P < 0.0001$) and flavour × day interaction ($P < 0.0001$) effects were observed for the two-choice test intake data (Fig. 3). Furthermore, no treatment effects were significant, including no significant three-way interactions. Evaluation of linear contrasts similarly indicated that maple diet intake did not differ between treatments on day 1 ($P = 0.601$); nor did grape diet intake differ between treatments on day 1 ($P = 0.622$). Inspection of residuals indicated the assumptions of ANOVA were met for all statistical models.

4. Discussion

Because goats did not prefer either flavour in the absence of an aversion (Fig. 1), it was anticipated that toxin-flavour pairing, not properties of the flavours themselves, would determine test diet preferences of the goats. Subjects in the Flavour treatment were not used in further experiments because of their experience with the flavours. Prior experience with a non-toxic food diminishes the likelihood of associated aversions to the flavour of that non-toxic food (Provenza et al., 1996).

Goats formed an aversion to maple diet regardless of the toxin (LiCl or OA) after a single exposure (Fig. 2). Extinction of the aversion was observed at day 7 for both treatment groups. This is typical of one-choice tests following a single toxin exposure. If an alternative food had been available, aversion to the maple diet would likely have been more persistent (Kimball et al., 2002).

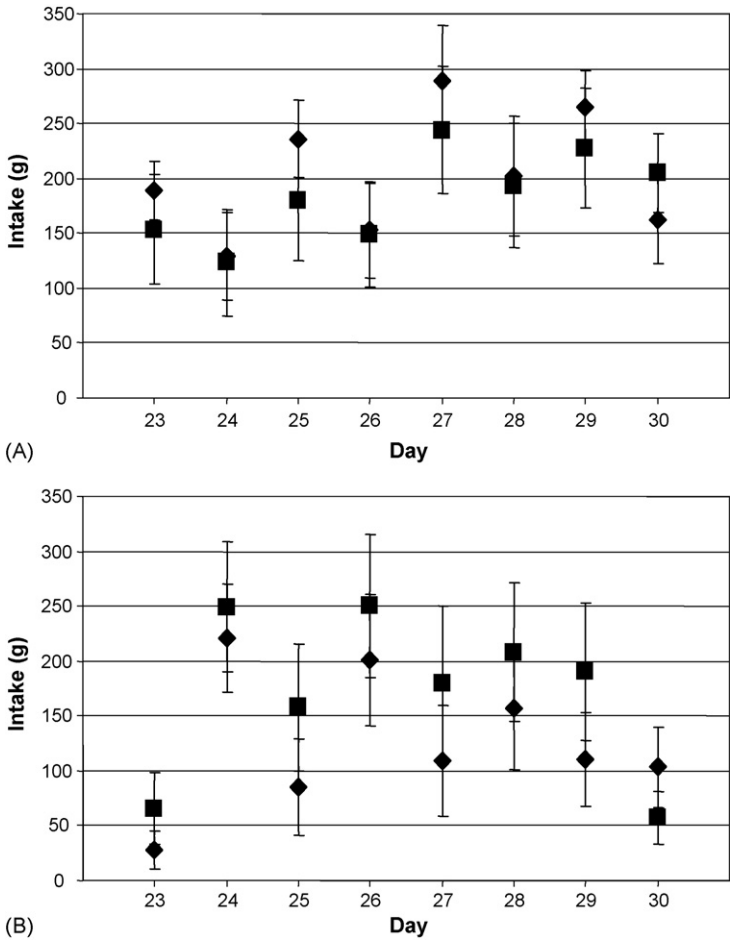

Fig. 3. (A) Grape diet intake (g) and (B) maple diet intake (g) of goats dosed with lithium chloride (LiCl) during a 30-min two-choice test. Subjects ($n = 7$) in the LiCl treatment (◆) were previously dosed with LiCl in association with the maple diet. Goats ($n = 8$) in the oxalic acid treatment (OA, ■) were previously dosed with OA in association with the maple diet.

Toxin dose has a significant impact on the intensity of flavour aversions as measured by persistence of the aversion (Shumake et al., 1982). Because this study was designed to compare goat responses to toxins differing in toxicological effects, not intensity, it was imperative that each dose resulted in similar avoidance behaviour of the maple diet. The LiCl dose chosen for this study (150 mg/kg) was based on previous LiCl experiments and anticipated behavioural results (see for example Kimball et al., 2002). The OA dose (250 mg/kg) was extrapolated from a free-feeding study in which lambs were offered an OA-adulterated diet (Burritt and Provenza, 2000). The doses used here produced aversions of similar intensity, as evidenced by similar persistence and the absence of a significant treatment effect.

While the intensity of the aversions was similar between the two toxins, spontaneous recovery data indicated that LiCl and OA produced different consequences in goats. After a 10-day intermission when maple diet was not available, subjects in the LiCl treatment demonstrated reacquisition of the aversion, while the OA treatment did not (Fig. 2). Reacquisition of a

lithium-induced aversion has been demonstrated in rats after an 18-day intermission (Rosas and Bouton, 1996) and after a 68-day intermission with lambs (Kimball et al., 2002).

If flavour cues are associated with specific toxic effects, the OA treatment should have associated the novel consequences of LiCl in the second exposure with the novel grape flavour. Cue–toxin specificity is an indication of flavour cue salience. Salience describes the tendency of a novel flavour to be associated with a toxin (Kalat and Rozin, 1970). Novel flavours are thought be more salient because they are readily associated with postingestive consequences.

Alternatively, if goats generalized OA and LiCl-induced toxicosis, maple diet would be avoided following the second toxin exposure, regardless of the nature of the toxin. Generalization results from prior illness and a lack of discrimination between the toxins. Prior illness is an effect of FAL observed following extinction (Burritt and Provenza, 1996). Subjects having experience with a toxin-flavour pair (e.g. LiCl and maple) avoid that flavour on subsequent pairings with the toxin. In other words, prior illness is a memory of past flavour association.

Following the second toxin dose, goat response to the flavours was independent of treatment. Thus, generalization was indicated. On day 23, both treatments readily consumed grape diet (Fig. 3A) and avoided maple diet following lithium-induced toxicosis (Fig. 3B). While subjects familiar with the maple–LiCl pairing avoided maple diet because of prior illness, the OA treatment also associated the negative consequences produced by LiCl with maple diet. Generalization occurred despite the fact that maple diet was previously paired with a toxin (OA) having different consequences. Generalization is a beneficial behaviour that allows herbivores to forage efficiently and avoid potential toxins (Villalba and Provenza, 2000).

Instead of a persistent aversion to maple diet following the second toxin exposure, goats only avoided maple diet for 1 day (day 23; Fig. 3). Mean maple diet intake of the LiCl treatment was less than the OA treatment on days 24–29, but the differences were not statistically significant. Although the novel flavour (grape) was not associated with toxicosis by subjects in either treatment group, its availability may have reduced persistence of aversion to maple diet in the LiCl treatment. In a study with lambs, Burritt and Provenza (1996) examined prior illness using two familiar foods. In their study, aversion to the food causing prior illness persisted for at least 4 days. The current study differs from this experiment because the alternative food available at the second exposure was completely novel. As goats sampled both diets in an attempt to determine the source of the negative consequences (prior illness or salient flavour), they learned both were safe because the toxin was not present in either diet.

Flavour aversions arise from the mechanical, osmotic, and chemical consequences of ingestion (Provenza et al., 1998). To ensure that the contrasting postingestive consequences of the LiCl and OA solutions were limited to their toxicological effects, NaCl was added to the OA solution to equate salinity and osmotic load of the two solutions. Although the ionic concentrations (salinity) and molality (osmotic pressure) of the two solutions were similar, it remains possible that presence of NaCl in the OA solution may have produced unintended circumstances. For example, nutrient excesses may lead to aversions. Lambs provided access to mineral block avoided foods associated with NaCl because sodium was in excess in their diets (Villalba and Provenza, 1996). However, NaCl-induced aversions are rare. Thus, it is unlikely that the postingestive consequences of the OA solution were affected by the addition of NaCl. In fact, NaCl is often used as a control solution in flavour aversion studies. For example, equimolar concentrations of LiCl and NaCl were administered to rats, resulting in strong avoidance of solutions associated only with LiCl (Cross-Mellor et al., 2004). Similarly, NaCl was administered to cattle at the same dosage rate as LiCl, resulting in no aversion (Kronberg et al., 1993).

5. Conclusion

In practice, protection of agricultural resources from damage caused by foraging herbivores will come from frequent exposures to aversion-causing toxins. The results of this study not only demonstrate that aversions produced by a single toxicant exposure are not persistent, but also that persistence is further limited by the presence of novel foods. However, this study does suggest that using a single flavour cue with multiple toxins would be beneficial in applied FAL. The use of a single flavour cue amid many repellent formulations containing different toxins would promote broader avoidance of the flavour cue via generalization.

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