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The Role of Sensory Cues and Feeding Context in the Mediation of Pine-Needle Oil's Repellency in Prairie Voles

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ABSTRACT

Pine-needle oil inhibits feeding in vertebrate species through sensory cues. Prairie voles *(Microtus ochrogaster)* significantly decreased their ingestion of raw apple pieces when pine-needle oil (10% vol/vol) was applied as a repellent coating. During single-choice tests, voles selected similar amounts of sunflower seeds from pine-needle oil-scented jars and vegetable oil-treated jars. However, when jars containing both stimuli were presented simultaneously, voles retrieved significantly more food from the vegetable oil-treated jars than the pine-needle oil-treated jars. Neonatal administration of capsaicin chronically depletes neurotransmitters in C– and A–∂ fibers greatly diminishing or abolishing pain transmission in the affected neurons (i.e., trigeminal nerve). Capsaicin-desensitized prairie voles decreased their ingestion of pine-needle oil-treated apples to the same extent as the sham-injected control group of animals, suggesting that pine-needle oil's repellency was not mediated by activation of pain fibers. In settings where alternative foods are available, and where minimal sampling of the product does not represent a hazard, Siberian pine-needle oil may be useful as a rodent repellent.

KEY WORDS

ingestive behavior, Microtus ochrogaster, prairie voles, repellent, taste, trigeminal

INTRODUCTION

There is an increasing demand for environmentally compatible, nontoxic repellents for wildlife damage management. Naturally-occurring essential oils, containing plant chemical defense compounds, inhibit feeding in a number of mammalian species and may represent useful

sources of repellents (Radwan et al. 1982, Bell and Harestad 1987, Roy and Bergeron 1990, Harbone 1991, Langenheim 1994).

The monoterpenes, α– and β–pinene and myrcene, are present in the essential oils extracted from many conifers (Radwan et al. 1982, Lindroth 1988). Plant chemical defense compounds vary greatly between and within plant species and the rate of their synthesis can be regulated by plant damage due to herbivory (Harbone 1991). It is unknown which of the many types of plant secondary metabolites present in essential oils contribute to their repellency.

Rapid evaluation of potentially useful compounds can be done using laboratory tests prior to performing more extensive field trials. Investigating the mechanisms mediating repellency in a controlled setting will add to our understanding of the basis of avoidance behavior. We have established that voles do not avoid apple pieces associated with the pine-needle oil's volatile cues if they are forced to consume them in the immediate vicinity of the pine-needle oil stimulus (Wager–Pagé et al., unpubl. data). In the absence of an alternative food, the voles may have been consuming apple to maintain their nutrient intake or for its hedonic value or both.

Chemosensations following exposure to irritant compounds commonly found in human cuisines such as capsaicin, gingerol, and piperine, are conducted by unmyelinated primary sensory neurons (Szolcsàyi 1990). Neonatal treatment of mammals with capsaicin depletes neurotransmitters present in unmyelinated C– and A–∂ fibers peripheral sensory neurons resulting in an interruption of pain transmission (Gamse et al. 1981, Nagy 1982). Because of their inability to detect substance P-mediated chemosensation, capsaicin-desensitized animals will not avoid irritant compounds (Nagy et al. 1982, Mason et al. 1987). In the present studies, we examined the contribution of primary sensory afferents in mediating the avoidance of pine-needle oil in prairie voles using the capsaicin-desensitized technique. To determine whether chemosensory irritation is the mechanism by which this essential oil elicits avoidance behavior, we evaluated the role of C– and A–δ pain fibers in mediating pine-needle oil's repellency in prairie voles.

We also evaluated the ingestive responses of prairie voles following exposure to a monoterpene mixture containing α– and β–pinenes and myrcene to determine whether it was an equally effective inhibitor of feeding behavior as pine-needle oil. The role of food availability on modulation of pine-needle oil's effect on feeding behavior in prairie voles was examined.

METHODS

Subjects

Seventy prairie voles (male and female, 60–120 days of age) were obtained from the Monell breeding colony and individually caged (17.8 × 29.2 × 12.7 cm) under a 12:12 hr light-dark cycle at 23 °C. All voles within each test group were born within a 30-day period. All voles had free access to feed (Purina Lab Rabbit Chow RP5321) and tapwater. Cages and bedding were changed weekly.

Capsaicin-Desensitization Procedure

Litters of prairie vole pups (age 1–3 days) were removed from their dams, weighed, wrapped in surgical gauze, and lightly packed in crushed ice for 10 min to achieve an anesthetic state (Phifer and Terry 1986). Pups were removed from the ice and rapidly injected subcutaneously with either capsaicin (50 mg/kg BW) (n = 11) or an equal volume (~15 μl) of the vehicle, 10:10:80 tween:ethanol:saline solution, (n = 10) (Silver et al. 1991). Pups were allowed to recover for 5 min and then were returned to their home cages. All pups were weaned at 21 days of age and placed into individual cages. Two vehicle-injected pups died prior to weaning. The sex ratio was not determined in this study because the sexes can not be differentiated prior to maturation at 21+ days of age.

Chemicals

The Siberian pine-needle oil used throughout testing was purchased from the Penn Herb Company (Philadelphia, PA) (hereafter, referred to as pine-needle oil). Myrcene (CAS No. 123-35-3), α-pinene (CAS No. 7785-26-4), and β-pinene (CAS No. 18172-67-3) were purchased from Aldrich Chemical Company, Milwaukee, WI. Capsaicin (8-methyl-N-Vanillyl-6-Nonamamide, CAS No. 404-86-4) was purchased from Sigma Chemical Co., St. Louis, MO. Crisco® vegetable oil was purchased locally.

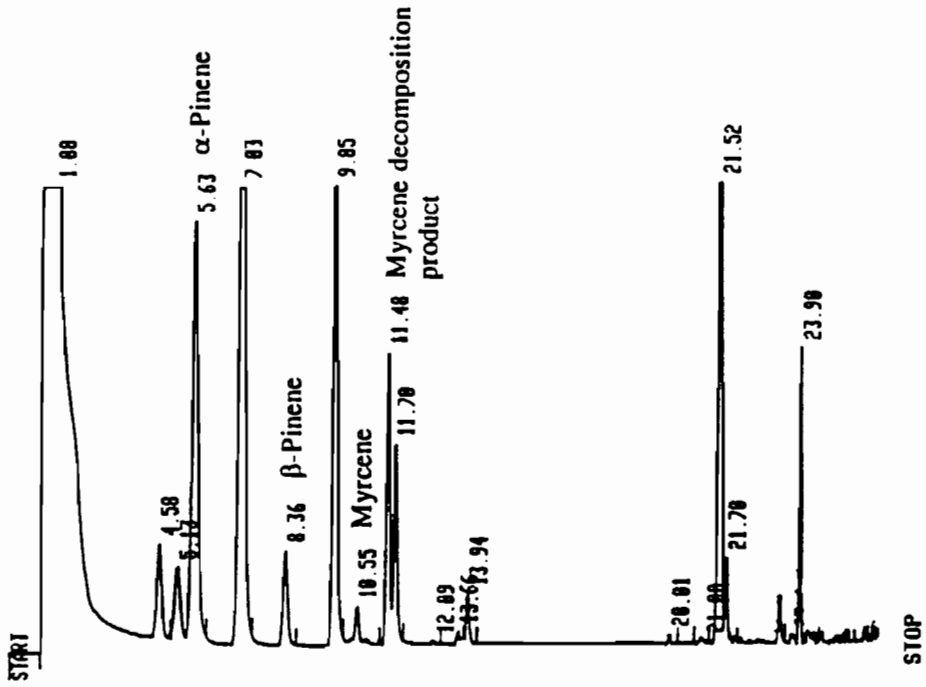
Chemical Analysis

Pine-needle oil was analyzed for the presence of α-pinene, β-pinene, and myrcene using capillary gas chromatography. Analyses were performed using a Varian 3300 instrument equipped with a Stabilwax DA (15 m) column, a flame ionization detector, and a Hewlett-Packard 3390A recording integrator. The analysis conditions were as follows: carrier gas—helium at 30 cm/sec linear velocity; detector temperature—240 °C; injector temperature—240° C; initial column temperature—35 °C; temperature gradient—5 °C/min to 135° C, then 25° C to 240° C; sample size—1 μl; sample concentration—0.1 mg/ml in pentane. Components were identified by comparison of their retention times to those of commercial standards and their abundance was calculated by integration (Figure 1).

Behavioral Testing

Experiment 1

In experiment 1, we compared the ingestive responses of prairie voles following exposure to 10% Siberian pine-needle oil and 10%-terpene-mixture repellent coatings on raw apple. Twenty male prairie voles were randomly selected from the laboratory colony. Ten voles were assigned to the pine-needle oil group and the remaining 10 voles to the terpene mixture group.



RT	COMPOUND	% PRESENT
5.63	α-Pinene	12.0
8.36	β-Pinene	2.32
10.55	Myrcene	0.52
11.48	Myrcene decomposition product	5.14

FIGURE 1. Capillary gas chromatogram of pine-needle oil. Peaks of components identified are labeled as α–pinene, β–pinene, myrcene and myrcene decomposition product. The retention times (RT) are listed on the top of each peak, and the percentage of α–pinene, β–pinene and myrcene present in Siberian pine-needle oil solution are listed below the chromatogram.

Stimuli were prepared by diluting pine-needle oil or the stock monoterpene solution in vegetable oil (10.0% vol/vol) and mixing it thoroughly on a vortex genie. The stock terpene mixture was prepared by adding myrcene (5.7 ml [equivalent to myrcene + decomposition product]), α–pinene (12.0 ml), and β–pinene (2.3 ml) to 80.0 ml vegetable oil to formulate a solution approximating the concentrations of these compounds present in Siberian pine-needle oil (Figure 1). Raw apples were cut into uniform pieces (10–15 g) and evenly coated with the stimuli solutions (pine-needle oil and terpene mixture) or the control solution (vegetable oil) by vigorous shaking in a closed container. All preference tests were two-choice of 2-hr duration. Baseline apple consumption was determined on 2 pretest days during which two pieces of oil coated apple were placed in opposite corners of the voles' home cages. After 2 hr, apple pieces were removed

and weighed to determine intake. The stimulus presentations were counterbalanced to compensate for side preferences. All tests were performed twice and 5–7 days elapsed between test periods. On test day 1, one piece of apple coated with pine-needle oil or terpene mixture (10.0% vol/vol) was presented in one front corner, and a second piece of apple coated with the vegetable-oil vehicle solution only was placed in the opposite corner. On test day 2, one apple piece coated with the test stimuli and one vegetable-oil coated apple piece were presented in opposite corners than those used on test day 1. The data from the two test day 1 and 2 were combined and averaged.

Experiment 2

In experiment 2, the effect of the volatile cues of pine-needle oil on food retrieval behavior of prairie voles was evaluated in single- and two-choice tests. Female prairie voles (n = 10) were selected from the laboratory breeding colony by birth date. Testing was performed in cages with the following dimensions: single-choice tests: (17.8 × 29.2 × 12.7 cm) and two-choice tests (23 × 44 × 20 cm). Glass jars (ht: 5 cm, diam: 4.8 cm, opening diam: 3.9 cm) were glued to the walls of the test cages at a 90° angle with the top opening of the jar 1 cm from the cage floor. Filter paper circles (diam: 5.5 cm) were securely fitted into the bottom of each jar and saturated with 500 μl of vegetable oil for the control condition or 500 μl of 100% pine-needle oil for the treatment condition. Twenty sunflower seeds were placed into the jars directly in front of the filter paper. Care was taken to prevent contact between the saturated filter paper and the sunflower seeds.

During single-choice tests, only one jar containing either the vegetable-oil or pine-needle oil on filter paper was present in the animals' test cages. Five voles were placed into the cages containing the vegetable oil-scented jars, and the remaining animals were exposed to the pine-needle oil-scented jars for 30 min during single-choice trials. At the end of each test period, the animals were removed from the test cages and the number of sunflower seeds retrieved were counted. The tests were repeated until all of the voles had been exposed to both control and treatment conditions.

During two-choice tests, one jar containing vegetable oil saturated filter paper and a second jar lined with pine-needle oil-saturated filter paper were attached to the front walls at the opposite ends of the cages. Voles were placed into the cages containing both the vegetable oil-scented jars and pine-needle oil-scented jars for 30 min. At the end of each test period, the animals were removed from the test cages and the number of sunflower seeds retrieved from the vegetable oil- and the pine-needle oil-scented jars were counted. The tests were performed in duplicate, and the position of the vegetable oil and pine-needle oil stimuli presentations were counterbalanced, as described in experiment 1 to reduce side preference bias.

Experiment 3

In experiment 3, we evaluated the contribution of primary sensory neurons in the mediation of the repellent effects of pine-needle oil. The ingestive responses of sham-injected and capsaicin-

desensitized voles were compared following exposure to either capsaicin or pine-needle oil-treated apples. Repellency testing began when the vole pups were 30 days of age. The voles were adapted to the preference testing regime for 1 week prior to the start of the experiment. On test days in the first study, voles were given apples soaked in either the treatment solution, capsaicin (0.1% wt/vol) or the vehicle solution (3.0% ethanol-distilled water) in 2-hr, two-choice tests. Pine-needle oil-coated (10.0% vol/vol) apples were paired with vegetable oil-treated apples in the second part of this experiment using the procedures previously described in experiment 1. All tests were performed twice, and the stimulus presentations were counterbalanced, as described in experiments 1 to reduce bias due to side preferences. Two weeks elapsed between the tests using capsaicin and pine-needle oil as stimuli. The voles' neurological status was assessed following completion of each study. This was assessed in unrestrained voles using no behavioral responses (i.e., escape, head shaking, eye/face wiping) following a brief exposure to acetic acid vapors as an indicator that the animals were desensitized.

Analysis

Significant differences between the means of apple (grams) consumed were identified using two-way analysis of variance in experiments 1 and 3. The factors in these experiments were as follows: experiment 1 (factors = solution and concentration) and experiment 3 (factors = neurological status and stimulus concentration). To compare ingestive responses between stimuli, preference ratios were calculated by dividing the amount ingested from the treated apple by the total amount of apple consumed. Preference ratios (PR) $\sim$ 0.5 denote indifference, PR $<<$ 0.5 indicate a repellency effect, and PR $>>$ 0.5 are associated with stimulus attraction. Means of the preference ratios from two test periods were subjected to the same analysis as the raw data. Differences between individual means were determined by Tukey's post-hoc tests. Analysis of the means of the percentages of sunflower seeds removed from the treatment and control jars in experiment 2 was performed by one-way analysis of variance.

RESULTS

Pine-needle oil decreased apple ingestion in prairie voles during two-choice feeding trials ($F(1,42) = 12.2$, $P<0.001$) (Figure 2). Preliminary inspection of the data indicated that pine-needle oil's inhibition of apple intake was present during the initial exposure. Although, the terpene coating mixture containing α–pinene, β–pinene, and myrcene did not consistently decrease apple consumption in voles, evaluation of the preference ratios indicated that its repellent effects did not significantly differ from the pine-needle oil coating (Figure 2).

Prairie voles retrieved a lower percentage of sunflower seeds from jars scented with pine-needle oil volatiles (18.3% $\pm$ 6.4) than seeds retrieved from vegetable oil-treated jars (42.0% $\pm$ 9.2), (F (1,18) = 4.50, $P<0.05$, Figure 3). During single-choice tests, removal of sunflower seeds from pine-needle oil-treated jars did not differ from the amount selected from vegetable oil-scented jars (Figure 3).

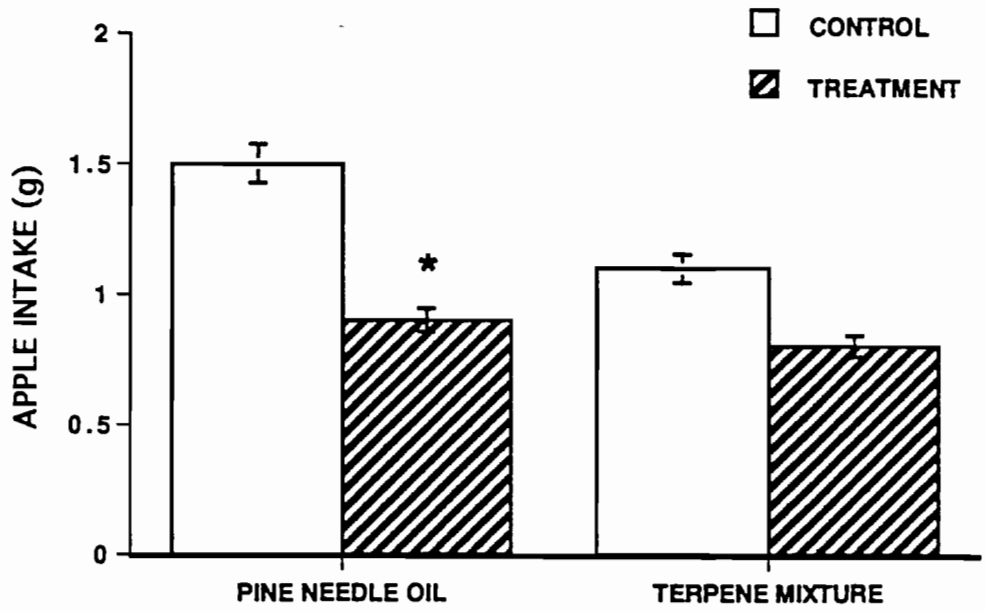


FIGURE 2. Effects of pine-needle oil and terpene mixture (10.0% vol/vol in oil) on 2-hr apple consumption by prairie voles in two-choice tests. Data are expressed as mean amounts of apple ingested in grams (g). *($P<0.05$) indicates a significant difference between means. Capped vertical bars represent standard errors of the means.

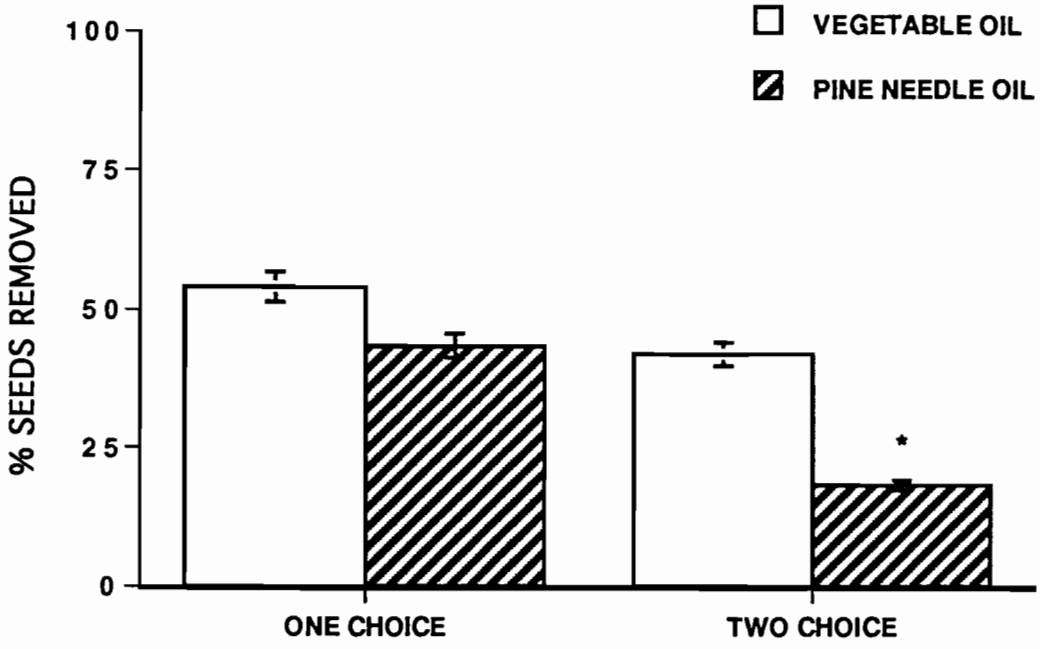


FIGURE 3. The effect of pine-needle oil on sunflower seed retrieval during single- and two-choice tests. Data are expressed as mean percent of seeds removed (%). *($P<0.05$) indicates a significant difference between means. Capped vertical bars represent standard errors of the means.

Prairie voles that were capsaicin-desensitized showed a greater preference for apples treated with 0.1% capsaicin (PR = 0.6 ± 0.06) than their sham-injected littermates (PR = 0.3 ± 0.08), (F (1,17) = 12.37, $P<0.003$, Figure 4*a*). The preference ratios evaluating consumption of 10% pine-needle oil-treated apple by voles (PR = 0.2 ± 0.06) were similar to those of the sham-injected group (PR = 0.3 ± 0.09), $F(1,17) = 1.025$, $P = 0.32$, (Figure 4*b*).

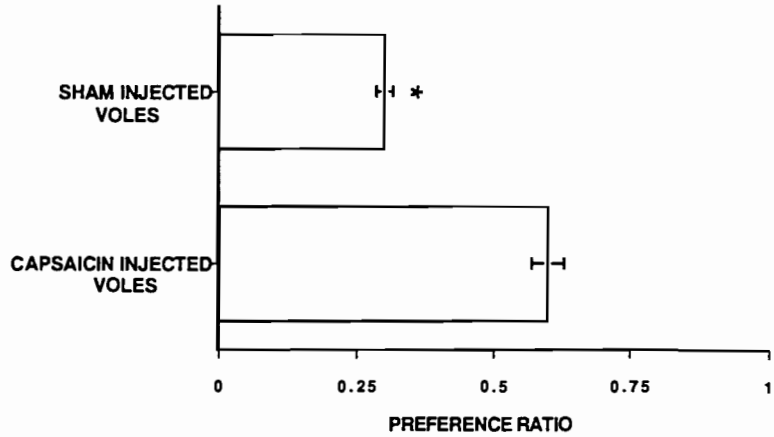


FIGURE 4a. Effect of capsaicin (0.1% wt/vol) on 2-hr apple consumption by prairie voles in two-choice tests.

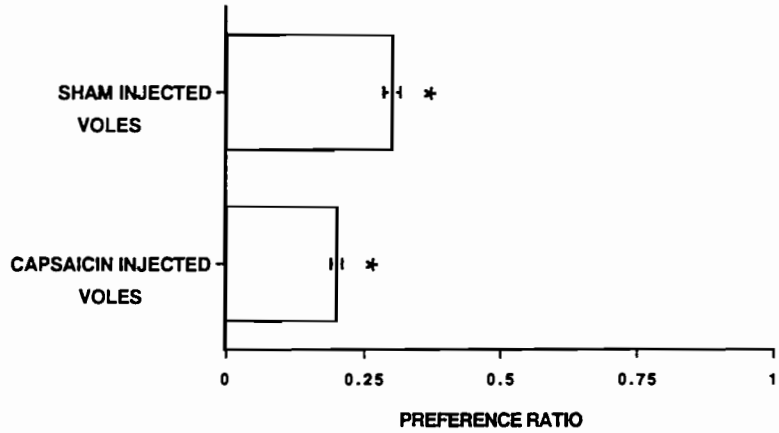


FIGURE 4*b*. Effect of pine-needle oil (10.0% vol/vol) on 2-hr apple consumption by prairie voles in two-choice tests. Data are expressed as mean preference ratios. *($P<0.05$) indicates a significant difference between means. Capped vertical bars represent standard errors of the means.2

DISCUSSION

Pine-needle oil (10% vol/vol) coating on apples inhibited ingestion by prairie voles. However, a composite terpene mixture with levels of pinenes and myrcene, similar to those present in pine-needle oil solution, was less effective, indicating that additional compounds are necessary for full repellency.

Foraging theory implies that animals will maximize their rate of energy intake within the constraints of risk (Krebs 1978). In keeping with this theory, Bell and Harestad (1987) suggested that the use of plant chemical defense compounds as repellents masks the nutritive value of the food by signaling its detrimental effects (1987). When a food is associated with terpenoids, voles are able to exercise two foraging strategies: they can seek an alternative energy source or consume only a small quantity of the test food, thereby minimizing its detrimental effects.

Microtine species employ runway systems and depots for protection and for caching harvested plants (Wolf 1985). We observed that ingestion by prairie voles was not altered when they were prevented from removing apples from the area of the pine-needle oil stimulus (Wager–Pagé, unpubl. data). The test food (apples) may have been highly valued by voles compared to their normal chow diet with the benefit of ingestion outweighing the associated risk of feeding in that experimental context. Data from our present study utilizing two food availability conditions indicate that avoidance of food associated with pine-needle oil occurs only when the alternative vehicle-treated food was available.

Prior to ingestion, mule deer differentiated between several concentrations of volatile oils present in feeds using gustation and olfaction (Schwartz et al. 1980). Bitter taste, volatile olfactory cues, and malaise have all been suggested as mediators of avoidance behavior in herbivores (Harbone 1991, Langenheim 1994). Chemosensory irritants are also avoided by animals and many terpenoid compounds are included in this classification (The Merck Index 1976). The data in experiment 2 indicate that the pine-needle oil's volatile cues are repellent to prairie voles. However, the experimental design did not allow us to determine whether the sensory signals were detected through olfaction or nasal trigeminal chemoreception.

Repeated exposures to pine-needle oil increased its aversiveness to prairie voles during feeding trials, suggesting that it caused malaise (Wager–Pagé et al., unpubl. data). However, the formation of a conditioned taste aversion in response to malaise requires a period of associative learning (Kalat and Rozin 1973). In experiment 1, feeding was inhibited in naïve animals during their initial brief exposure to pine-needle oil. Although the mediators of pine-needle oil's repellency are unknown, sensory cues such as painful sensations, olfaction, or taste signals may elicit an innate avoidance response by indicating potential toxicity. Since desensitized voles were responsive to pine-needle oil, the avoidance response was probably not mediated through pain transmission via primary sensory neurons in the trigeminal or vagus nerves.

MANAGEMENT IMPLICATIONS

In an environment where alternative food sources of equal nutritive or hedonic value are available, pine-needle oil can effectively deter ingestion of a target commodity by prairie voles. Pine-needle oil is an excellent candidate for use in settings where a volatile repellent is desirable. Pine-needle oil's inhibitory effect on feeding occurs in naive voles suggesting that it is not dependent on the development of malaise. Nor does it appear that activation of C- and A-δ fibers mediates the repellency of pine-needle oil. It is unknown whether pine-needle oil's repellency can be maintained for extended periods of time in the absence of additional reinforcing cues. Future studies will examine the physiological mechanisms governing avoidance behavior in vertebrate species to maximize the effectiveness of pine-needle oil or its constituents when applied in economically feasible and ecologically safe concentrations.

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