Effects of Volatile Compounds on Consumption of Alfalfa Pellets by Sheep[1,2]

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ABSTRACT: We examined the effects of six volatile compounds on alfalfa pellet consumption by lambs. In each experiment, 45 lambs were individually fed alfalfa pellets sprayed with a selected compound (camphor, limonene, *cis*-jasmone, β-caryophyllene, borneol, or α-pinene) at one of five concentrations. Treatment concentrations were multiples (0, .5, 1, 2, and 10) of the concentration of a specific compound (X) that was related to differential herbivory of tarbush by livestock in previous studies. Treatments were applied to alfalfa pellets (.64 kg·lamb^{-1}·d^{-1}, DM basis), and consumption was measured during a 20-min interval each morning for 5 d. Lambs were adapted to handling procedures and the pelleted diet (without treatments) for 10 d. Lambs were maintained and fed (approximately 4.5 to 5% of BW) as one group except during 20-min tests. A negative linear effect of treatment concentration on intake was observed for camphor ($P < .02$) and α-pinene ($P < .01$), and a quadratic response was detected for borneol ($P < .02$). The other three compounds had no discernible effect on consumption. Although volatile compounds generally had only minor influences on consumption, the negative influences of α-pinene and camphor concentrations on pellet consumption suggest that these monoterpenes may partially explain differential herbivory of individual tarbush plants by livestock.

Key Words: Diets, Intake, Sheep, Terpenoids

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Introduction

Northern Chihuahuan Desert rangelands are typically shrub-dominated. Secondary compounds are particularly prevalent in woody plants (Bryant et al., 1991). Defense compounds in desert shrubs are usually terpenoids and phenolic compounds (Meyer and Karasov, 1991). We have examined interactions between desert shrubs and mammalian herbivory using *Flourensia cernua DC* (tarbush). Ruminants exhibited differential use of individual plants when forced to browse tarbush (Estell et al., 1994b). Removal of surface compounds with organic solvents increased tarbush consumption by sheep (Estell et al., 1994a), and terpenes on the leaf surface of tarbush were related to diet selection (Estell et al., 1996).

Specific substances rather than groups of compounds deter vertebrate herbivores from woody plants (Sinclair et al., 1988), and classes of molecules are not uniform in their deterrent properties (Reichardt et al., 1985; Bryant et al., 1991). Duffey and Stout (1996) suggested that plant allellochemical-herbivore interactions must be examined with multivariate rather than univariate approaches because a chemical action is not independent of surrounding chemicals. Although relationships of dietary components and selection can be demonstrated using multivariate analyses, cause and effect cannot be verified (McArthur et al., 1993), and compounds must ultimately be tested individually (Clausen et al., 1992). The objective of these experiments was to determine effects of volatile compounds that were related to differential tarbush use in previous studies on intake by lambs when applied individually to alfalfa pellets. Our hypothesis was that pellet consumption would decrease as treatment concentration of a given chemical increased.

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[2]Mention of a trade name, proprietary product, or vendor does not constitute a warranty of the product by the USDA or imply its approval to the exclusion of other products or vendors that may also be suitable.

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Materials and Methods

Treatments, Animal Management, and Experimental Protocol

Effects of six compounds on intake of alfalfa pellets by sheep were examined individually. These compounds included two hydrocarbon monoterpenes (α-pinene and limonene), two oxygenated monoterpenes (camphor and borneol), a green-leaf volatile (*cis*-jasmone), and a hydrocarbon sesquiterpene (β-caryophyllene). Criteria for selection of compounds were that they had been identified in tarbush and exhibited relationships with herbivory in previous studies (Estell et al., 1994b; 1996). Other factors considered were importance of compounds in other plant-animal interaction literature and cost and availability of pure compounds. Mean concentrations of these six compounds on the leaf surface of tarbush in those previous studies were approximately 100, 100, 5, 300, 25, and 100 μg/g (DM basis) for α-pinene, limonene, camphor, borneol, *cis*-jasmone, and β-caryophyllene, respectively. These concentrations were assumed to represent the level of exposure (X) to each chemical for livestock browsing tarbush.

Six experiments were conducted in accordance with guidelines established by the New Mexico State University Institutional Animal Care and Use Committee. Each 5-d experiment measured intake by growing lambs of alfalfa pellets treated with one chemical. Treatment concentrations were multiples (0, .5, 1, 2, or 10) of the exposure concentration of each compound. Before each experiment, 45 ewe lambs (Polypay, 6 mo of age, mean initial BW = 35.0 ± .8 kg) without previous experience browsing tarbush were randomly assigned to one of five treatments using a completely randomized design with nine lambs per treatment. Lambs were individually fed treated pellets each morning during a 20-min interval in an enclosed metabolism unit (1.22- × 2.44-m pens). Lambs were randomly assigned to one of three groups and 15 pens daily, and groups were fed in succession each morning at 0800, 0830, and 0900. Sequential 5-d periods were conducted to familiarize lambs with pelleted feed and handling procedures (wk 1) and establish baseline intake without treatments (wk 2). Camphor, limonene, *cis*-jasmone, β-caryophyllene, borneol, and α-pinene treatments were examined during wk 3 through 8, respectively, with a 2-d interval between experiments.

During a preliminary study, mean consumption by three lambs of similar BW during a 20-min interval was .43 kg of DM·lamb^{-1}·d^{-1}. Consequently, .64 kg of pellets (DM basis) were offered daily to lambs during experiments (a level exceeding the upper intake limit of any lamb during the preliminary period) to ensure that feed availability did not limit intake. Alfalfa pellets (≥ 15% CP; .95 cm diameter) from the same batch of sun-cured alfalfa hay were fed in all experiments. Orts were weighed daily and mean pellet consumption during the 20-min period was calculated by lamb across days. Lambs were weighed on d 5 of each experiment immediately preceding the 0800 feeding. Alfalfa pellets were sampled randomly from each bag and composited. Samples were ground to pass a 2-mm screen in a Wiley Mill and analyzed for DM (94%; AOAC, 1990).

Except for the morning feeding period, lambs were maintained as one group in a drylot with free access to water and trace-mineralized salt (93 to 97% NaCl, 3 g/kg Mn, 2.5 g/kg Zn, 1.5 g/kg Fe, .15 g/kg Cu, .09 g/kg I, .025 g/kg Co, and .01 g/kg Se). Lambs were group-fed untreated pellets twice daily (.64 kg DM/lamb at 1000 and .43 kg DM/lamb at 1300), equating to approximately 4.9% of initial BW (including morning feeding). Also, the total amount of feed refused was calculated each morning, and an equivalent amount of untreated feed was fed at 1000. Feed was typically consumed by 1500. During the 2-d intervals between experiments, lambs were fed in a similar manner, except that they were group-fed an additional .64 kg DM·lamb^{-1}·d^{-1} of untreated feed at 0800. Because of the moderate growth rate of lambs (mean ADG = .24 ± .005 kg/d), feed offered in drylot was adjusted to regulate appetite and ensure some orts for all lambs following the 20-min feeding. Total feed offered was 1.71 kg DM·lamb^{-1}·d^{-1} initially and was increased three times in increments of .11 kg DM·lamb^{-1}·d^{-1}; during wk 4 because three lambs had no orts and at the end of wk 4 and 7 because several lambs had less than .1 kg of orts.

Compounds were obtained from Aldrich Chemical (Milwaukee, WI), except camphor (Fluka Chemical, Ronkonkoma, NY). Purities of α-pinene, limonene, camphor, borneol, *cis*-jasmone, and β-caryophyllene specified by manufacturers were 98, 97, 95, 98, 85, and 90%, respectively. Stock solutions of α-pinene, limonene, camphor, borneol, *cis*-jasmone, and β-caryophyllene containing 20, 20, 1, 60, 5, and 20 mg/mL in ethanol, respectively, were diluted 5-, 10-, and 20-fold in 100% ethanol. Application of .05 mL of stock and 5-, 10-, and 20-fold dilutions to 1 g of DM corresponded to 10X, 2X, 1X, and .5X treatment levels, respectively. The 0X (control) treatment consisted of alfalfa pellets sprayed with ethanol only. Solutions were mixed before each experiment in amber glass containers and transferred to graduated (34-mL increments) high-density polyethylene spray bottles (rinsed thoroughly with ethanol) each morning. New spray bottles (one per treatment) were used for each experiment, and nozzles were adjusted to apply treatments in a stream rather than a mist pattern in an attempt to minimize volatilization. Pellets were placed in one end of feed pans, tilted, and gently shaken while sprayed in an effort to apply treatments completely and evenly. Pelleted alfalfa was used to provide a uniform surface for treatment application. Approximately 10 min lapsed between

spraying and feeding, and the order of application was rotated systematically to minimize bias due to time between spraying and feeding. Treatments were applied in an adjacent separately ventilated room, and an exhaust fan in the metabolism unit was used to remove aromas and minimize potential drift among pens. One metal pan was used for each lamb in each experiment to minimize cross-contamination, and pans were steam-cleaned between experiments.

The appropriate combination of stock concentration and spray volume to uniformly spray .64 kg of pellets with a minimum of carrier was determined before experiments began. The amount of chemical loss due to volatilization between application and feeding was examined for all six compounds by spraying 34 mL of stock solutions (i.e., 10X treatment) on .64 kg of alfalfa pellets (DM basis). Each chemical was applied using the same protocol and pans as during experiments. Pellet samples (approximately 30 to 40 g) were collected in quadruplicate at 2, 10, 20, and 30 min after spraying, extracted with 50 mL of ethanol for 6 h with constant shaking, and filtered through a glass fiber filter. Extracts were subjected to gas chromatography-mass spectrometry (**GCMS**) to determine recovery, using instrumentation parameters and column conditions as described by Tellez et al. (1997) and external standard curves for quantification. Extraction efficiency for each compound was determined by adding 34 mL of each stock solution to .64 kg of pellets. Flasks were sealed and allowed to stand for .5 h. Ethanol (700 mL) was then added, and sealed flasks were extracted for 6 h with continual shaking. Extracts were filtered and subjected to GCMS as described previously, and recovery at various times after spraying was corrected for extraction efficiency. The corrected mean recovery at 2, 10, 20, and 30 min, respectively, was 67.4, 62.9, 49.5, and 54.8% for α-pinene; 63.1, 64.9, 60.1, and 64.6% for limonene; 80.1, 81.1, 77.6, and 67.5% for camphor; 57.6, 67.3, 66.0, and 71.0% for borneol; 58.2, 68.1, 73.0, and 75.9% for *cis*-jasmone; and 84.8, 79.8, 85.8, and 70.3% for β-caryophyllene. The 20-min sample (approximate midpoint of 20-min feeding period, assuming a 10-min lag between spraying and feeding) should approximate the actual concentration on pellets in these experiments. No adjustment to spray volume was made to account for volatilization loss. The coefficient of variation for recovery estimates at 2, 10, 20, and 30 min, respectively, was 3.4, 20.2, 6.9, and 11.9% for α-pinene; 6.6, 6.1, 4.7, and 8.4% for limonene; 20.0, 23.2, 7.9, and 11.3% for camphor; 13.4, 6.4, 7.3, and 12.9% for borneol; 2.6, 12.3, 13.3, and 23.1% for *cis*-jasmone; and 6.8, 10.2, 5.3, and 12.1% for β-caryophyllene. Variability among replicates was fairly large, suggesting some difficulty achieving uniform treatment application. However, most of the volatilization loss occurred within 2 min after application, suggesting that most of the loss occurred before the pans were moved to the feeding room. Some deviation in percentage of volatilization loss among chemicals occurred, as expected, due to varying chemical properties (e.g., molecular weight and vapor pressure). Feed pans were also rinsed with ethanol (34 mL) the following day to test for residual chemicals. Minor traces of chemical residues (.1, .9, .4, .5, .4, and .5% for α-pinene, limonene, camphor, borneol, *cis*-jasmone, and β-caryophyllene, respectively) were detected the following day. A similar extraction procedure (ethanol only) was used to determine baseline concentrations of compounds in alfalfa pellets. None of the compounds examined were present above our detection limits in alfalfa pellets, probably because sun curing and pelleting procedures resulted in terpene volatilization.

Statistical Analysis

Analysis of variance was conducted separately for each experiment using the GLM procedures of SAS (1989) with pellet consumption (5-d means) during the 20-min interval as the dependent variable and treatment as the independent variable in the model. Because lambs were rerandomized daily to pen and group, these variables were not included in the model. Orthogonal contrasts for unequally spaced treatment levels (0X, .5X, 1X, 2X, and 10X) were constructed to determine linear and quadratic effects of treatment levels on intake. Also, intake of control lambs ($n = 9$) was subjected to analysis of variance with week as the independent variable to evaluate the consistency of intake of control lambs across experiments. Means were separated ($P < .05$) by LSD (SAS, 1989) in the case of a significant F-value ($P < .05$). Repeated measures analysis of variance was conducted using the GLM procedures of SAS (1989) for each experiment with day in the model to evaluate the consistency of intake among days within experiment. Orthogonal polynomial contrasts were tested among days in this repeated measures analysis.

Results and Discussion

Lambs adapted quickly to the feeding protocol during wk 1, although intakes were less than for other weeks except wk 5 and 6 ($P < .05$; data not shown). Baseline intake during the 20-min interval for control lambs during wk 2 did not differ from intakes during wk 3, 4, 5, 7, or 8 (Table 1). During wk 1 and 2, all 45 lambs were fed alfalfa pellets without ethanol, and control lambs in wk 3 to 8 received diets sprayed with ethanol; however, ethanol application to milo or alfalfa hay did not deter feeding by sheep (our unpublished observations). Control lambs consumed less feed during wk 6 than other weeks, except wk 5 ($P < .05$). Differences in intake during the 20-min interval among weeks for controls (Table 1) are likely attributed to increased feed in drylot. Results are not

Table 1. Mean consumption by lambs during a 20-min interval of alfalfa pellets treated with volatile compounds[a,b]

Concentration[c]	Adaptation[d]	Camphor[e]	Limonene	*cis*-Jasmone	β-Caryophyllene	Borneol[f]	α-Pinene[e]
	kg·lamb^{-1}·d^{-1}, DM basis						
0X	.43^{ghi}	.47^{gh}	.49^{g}	.38^{ij}	.33^{j}	.40^{hij}	.41^{hij}
.5X	.47	.41	.48	.35	.33	.44	.45
1X	.38	.45	.43	.33	.37	.39	.45
2X	.40	.41	.51	.33	.33	.50	.39
10X	.44	.37	.49	.36	.38	.41	.34
SEM	.027	.027	.028	.027	.028	.027	.026

[a]n = 45 except for wk 6 (n = 40, 41, 44, 42, and 41 for 0X, .5X, 1X, 2X, and 10X, respectively) and wk 8 (n = 44 for .5X and 2X treatments).
[b]Adaptation, camphor, limonene, *cis*-jasmone, β-caryophyllene, borneol, and α-pinene correspond to wk 2 to 8, respectively.
[c]Concentrations of compounds applied to alfalfa pellets were multiples (0, .5, 1, 2, or 10) of the concentration of that compound in tarbush (X).
[d]No ethanol was applied during the adaptation period (wk 2).
[e]Linear concentration response ($P < .05$).
[f]Quadratic concentration response ($P < .05$).
[g,h,i,j]Within the 0X row, means lacking a common superscript letter differ ($P < .05$).

directly comparable among experiments because of confounding effects of time and drylot feeding level. By design, the exposure level (X) on which treatment concentrations were based varied among compounds; our objective was to examine chemicals at concentrations commonly encountered in tarbush rather than to make direct comparisons among chemicals at uniform concentrations.

A negative linear response in intake to treatment concentration was detected for camphor ($P < .02$) and α-pinene ($P < .01$), and a quadratic response was observed for borneol ($P < .02$; Table 1). Ruminants forced to use tarbush in a previous study at this location preferred for certain plants to the exclusion of others, and this differential use was negatively related to epicuticular wax concentration (Estell et al., 1994b). Removal of surface compounds with organic solvents increased tarbush consumption (Estell et al., 1994a). Although not statistically different, mean concentration of *cis*-jasmone was approximately fivefold greater in plants receiving low use than in those receiving high use (Estell et al., 1994b), but limonene, borneol, and β-caryophyllene were not related to plant use in that study (α-pinene and camphor were not analyzed).

Mono- and sesquiterpenes on the leaf surface of tarbush were identified that were related to diet selection in a subsequent study, but inconsistencies were noted between univariate and multivariate analyses (Estell et al., 1996). When data were subjected to univariate analysis of variance, α-pinene concentration was approximately twofold greater and *cis*-jasmone concentration was less (approximately half) in low use than high use plants, but limonene, camphor, borneol, and β-caryophyllene were not related to use category. However, limonene, camphor, borneol, β-caryophyllene, α-pinene, and *cis*-jasmone were all important variables for distinguishing between high and low use plants using a procedure that selected chemical subsets for multivariate analysis. Nevertheless, *cis*-jasmone did not seem to be related to consumption in the present study. Neither limonene nor β-caryophyllene was related to consumption, in agreement with univariate analyses from previous studies. Although borneol was not related to use in previous studies with univariate analysis, a treatment effect was observed in the present study. In agreement with Estell et al. (1996), α-pinene was negatively related to use, but camphor was not related to use in that study. Although all six compounds tested in the present study were in the variable subset that successfully distinguished between high and low use plants using multivariate analysis (Estell et al., 1996), that analysis considers all variables and their interrelationships. In the present experiments, compounds were applied singularly, and lambs had no opportunity to exhibit preference; whereas, in previous studies animals could choose among plants.

Camphor and *cis*-jasmone were applied at much lower concentrations than other chemicals. However, the 10X concentration of *cis*-jasmone had no effect on pellet consumption, suggesting previous relationships with plant use may have been coincidental (i.e., correlated with a compound related to intake) or part of a cumulative (synergistic) effect. Loughrin et al. (1995) reported *cis*-jasmone to be released in response to insect herbivory, possibly explaining discrepancies with previous studies if induced in response to tarbush herbivory. The fact that exposure level and treatment concentration varied among chemicals may be germane to the search for chemicals affecting diet selection and intake. In particular, the level of exposure for camphor (5 μg/g of DM) was substantially less than for other chemicals, yet a negative linear effect of concentration on intake was observed. Conversely, the level of exposure for borneol (300 μg/g of DM) was greater than for other compounds examined. Yet, a quadratic response to borneol was noted.

Linear reductions in consumption with increased concentration of α-pinene and camphor support our hypothesis and agree with general expectations based on the literature. Camphor has been negatively related to feeding in snowshoe hares (Sinclair et al., 1988) and deer (Personius et al., 1987). Elliott and Loudon (1987) noted that red deer rejected a pelleted diet when exposed to monoterpene odors, including α-pinene, limonene, and borneol. Epple et al. (1996) found that oil containing α-pinene reduced intake by pocket gophers, although Reichardt et al. (1985) reported that α-pinene had little effect on feeding preferences of hares. Riddle et al. (1996) reported positive (camphor) and negative (α-pinene, limonene) correlations of specific monoterpenes with juniper intake by goats. Limonene concentration was inversely related to meadow vole damage on conifers (Bucyanayandi et al., 1990), but it was not related to feed preference in guinea pigs (Nolte et al., 1994). The quadratic response for borneol was counter to our expectations and is difficult to explain biologically. Possibly, the 10X treatment may have exceeded a threshold stimulation level. We are not aware of published data that identify relationships between β-caryophyllene and herbivory.

Chemical effects were assumed to be independent in that previous exposure to a compound would not alter the response to the compound tested subsequently. However, previous experience can affect diet selection through the integration of cognitive and affective processes (Provenza et al., 1992). No methods presently used to assess palatability can avoid prior learning effects; however, for measuring palatability of one feed (i.e., no opportunity to exhibit preference), initial eating rate during a short interval at the beginning of the feeding period is a good criterion (Baumont, 1996). Because studies were conducted sequentially, previous experience differed from week to week and could affect comparisons among chemicals. Also, the effect of a given chemical could change even within a 5-d period if an aversion is formed (Provenza et al., 1990). Because intake is affected by sensory and postingestive effects, monitoring intake during the first few minutes following feeding minimizes the confounding of palatability and postingestion factors (Baumont, 1996). However, no evidence of aversion to terpenes was observed in our studies. Repeated measures analyses revealed linear (wk 2, 5, and 6) and quadratic (wk 3, 4, 7, and 8) intake responses by day within week ($P < .05$) for the average response to treatments. The same analyses were not significant when examined by treatment, indicating that no day × treatment interactions were present for intake. The intake response for all treatments within the 5-d periods was positive and generally increased after 1 or 2 d and then stabilized. Because these responses for controls were not different from the other treatments within a week, it does not seem that adaptation to taste of compounds was responsible for this pattern. The possibility of acclimation of lambs to volatile aromas cannot be ruled out. Odor and taste affect preference of sheep and effects are often difficult to separate (Arnold et al., 1980). Adaptation to certain odors has been observed in sheep (Arnold et al., 1980), but lambs in the present study were maintained outdoors and fed untreated feed except during the 20-min feeding, minimizing exposure to odors. Also, application of chemical solutions in a separate room should have minimized feeding area contamination, based on timing of volatilization losses discussed previously.

Information on the metabolic consequences of these compounds and potential feedback on mammalian physiology and behavior is limited. Hiroi et al. (1995) administered four monoterpenes, including α-pinene and borneol, to rat hepatic microsomes and measured P450 induction; all compounds increased some forms of P450. Because mammals have sophisticated detoxification systems based on cytochrome P450 oxidases, general effects of terpenes on mammals are probably limited to reduced intake, and then only when consuming terpene-containing forages in quantity and alternative forages are not available (Harborne, 1991). Monoterpenes are typically toxic to insects but safe for consumption by mammals (Rice and Coats, 1994). Because many monoterpenes are classified as "Generally Recognized as Safe" and are natural plant products that are abundant and easily synthesized (Rice and Coats, 1994), they are potential candidates for use in manipulating feeding patterns of browsing herbivores.

Overall, these data suggest that some leaf surface compounds (e.g., camphor and α-pinene) may be responsible for differential use of tarbush by ruminants, but others seem either to be correlated rather than causal or to have an effect only in concert with other plant chemicals. Other phytochemicals in the terpene family and other classes of chemicals should be examined independently and in combination to determine their influence on feed intake.

Implications

Leaf surface terpenoids may be involved in diet selection. Knowledge of specific chemical interactions with feed intake may ultimately lead to mechanisms for altering feeding behavior and diet selection. Potential benefits of increased shrub consumption would depend on the balance of nutritional benefits and metabolic impacts of phytotoxins.

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