Effect of individual terpenes on consumption of alfalfa pellets by sheep[1,2]

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ABSTRACT: We examined effects of individual terpenes on alfalfa pellet intake of lambs in five experiments. Forty-five lambs (nine lambs/treatment) were individually fed alfalfa pellets sprayed with either *p*-cymene, α-humulene, 1,8-cineole, 3-carene, or sabinene at one of five concentrations (one terpene per experiment). Treatments (0, .5, 1, 2, and 10×) were multiples of the concentration (×) of a specific terpene in tarbush that was related to differential herbivory by livestock in previous studies. Terpenes were applied to alfalfa pellets (.64 kg·lamb^{-1}·d^{-1}, DM basis), and consumption was measured during a 20-min interval for 5 d. Lambs were adapted to handling and pen feeding for 10 d and were maintained and fed alfalfa pellets in one group (except during 20-min tests) at a mean total daily intake of 4.7% of BW (DM basis). None of the five compounds decreased alfalfa pellet consumption during the 20-min interval. These five mono- and sesquiterpenes do not seem to be responsible for differential herbivory of individual tarbush plants by livestock.

Key Words: Food Preferences, Intake, Sheep, Terpenoids

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Introduction

Arid southwestern rangelands are typically dominated by shrubs. Antiherbivory compounds in desert shrubs are usually terpenoids and phenolics (Meyer and Karasov, 1991). On the Jornada Experimental Range, *Flourensia cernua* DC (tarbush) has increased at the expense of desert grasslands on the heavier, more productive soils (Buffington and Herbel, 1965). Though use as a forage by livestock is generally low, tarbush has composed 12% of cattle diets during early summer (Anderson and Holechek, 1983). When forced to browse tarbush, ruminants exhibited differential use of individual plants, which was related to epicuticular wax concentration (Estell et al., 1994b). Removing surface compounds with organic solvents resulted in increased tarbush consumption by sheep (Estell et al., 1994a). Specific mono- and sesquiterpenes on the leaf surface of tarbush were associated with tarbush use (Estell et al., 1998a).

Although relationships of dietary components with plant use can be identified using multivariate analyses, cause and effect cannot be verified (McArthur et al., 1993); consequently, effects of specific compounds must be tested individually (Clausen et al., 1992). Six compounds related to tarbush intake using multivariate analysis (camphor, borneol, limonene, *cis*-jasmone, α-pinene, and β-caryophyllene) were initially tested (Estell et al., 1998b). Only two of these compounds (camphor and α-pinene) were related to intake by lambs when tested individually. The objective of the following experiments was to individually examine five additional terpenes that were related to differential tarbush use (Estell et al., 1998a). Our hypothesis was that consumption of alfalfa pellets by lambs would decrease as the concentration of a specific terpene increased.

Materials and Methods

Treatments, Animal Management, and Experimental Protocol

Effects of three hydrocarbon monoterpenes (3-carene, *p*-cymene, and sabinene), one oxygenated monoterpene (1,8-cineole), and a hydrocarbon sesquiterpene (α-humulene) on intake of alfalfa pellets by sheep were examined individually. These compounds were related to tarbush (*Flourensia cernua*) herbivory in previous studies (Estell et al., 1994b, 1998a), except 1,8-cineole, which was related to diet selection in other research (Lawler et al., 1998; Pass et al., 1998). Mean concentrations

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of these compounds on the leaf surface of tarbush in previous studies at this location (approximately 5, 25, 50, 100, and 10 μg/g DM for *p*-cymene, α-humulene, 1,8-cineole, 3-carene, and sabinene, respectively) were considered the level (×) of each chemical to which livestock browsing tarbush were exposed.

Five experiments were conducted in accordance with USDA guidelines and reviewed by the New Mexico State University Institutional Animal Care and Use Committee. The experimental protocol was a modified version of Estell et al. (1998b). One terpene was examined at five concentrations (treatments) in each experiment. Treatments were multiples (0, .5, 1, 2, or 10) of the exposure concentration for that specific compound. Forty-five ewe lambs (Polypay, approximately 5 mo of age, mean initial BW of 43.0 ± .4 kg, without previous browsing experience) were randomly assigned to one of 15 pens and three groups. Pen and group assignments for each lamb remained the same across experiments. Lambs were randomly assigned to one of the five terpene concentrations (nine lambs/treatment) in a randomized complete block design before each experiment, with the restriction that each group (block) contained three lambs from each 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). Each experiment was 5 d in length. Groups were fed in succession at 0800, 0830, and 0900 each morning. Two 5-d periods were conducted initially to familiarize lambs with handling procedures and 20-min feeding (wk 1) and establish baseline intake during the 20-min interval without treatments (wk 2). *p*-Cymene, α-humulene, 1,8-cineole, 3-carene, and sabinene treatments were examined during wk 3 through 7, respectively, with a 2-d interval between experiments. Order of testing the five compounds in wk 3 to 7 was selected randomly.

During the 20-min feeding, .64 kg of pellets (DM basis) was offered daily to lambs (a level established in previous study to exceed the intake of lambs of similar weight and intake within 20 min). Alfalfa pellets (≥ 15% CP; .95 cm diameter) from sun-cured alfalfa hay were fed in all experiments. Orts were weighed daily and lambs were weighed on d 5 of each experiment before the 0800 feeding. Alfalfa pellets were sampled randomly throughout the study, composited, ground to pass a 2-mm screen in a Wiley Mill, and analyzed for dry matter (93.8%; AOAC, 1990).

Lambs were adapted to alfalfa pellets for 2 wk and the drylot pen for 1 wk before individual feeding began. 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 fed 4.7% of BW (DM basis) daily throughout the study. In addition to the .64 kg of treated pellets fed during the 20-min interval, lambs were group-fed untreated pellets twice daily (at 1000 and 1300). Lambs received .95 kg DM·lamb^{-1}·d^{-1} (pen average) at 1300. The amount of feed offered at 1000 was adjusted weekly to maintain a mean daily intake of 4.7% of BW in an attempt to maintain a more uniform growth rate and a more consistent level of appetite across all studies (mean ADG = .20 ± .006 kg/d). The total amount of feed refused during 20-min tests was calculated daily and an equivalent amount of untreated feed was also fed at 1000. All 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 1000.

Compounds were obtained from Aldrich Chemical (Milwaukee, WI) except sabinene, which was obtained from Pfaltz and Bauer (Waterbury, CT). Manufacturer-specified purities were 99, 98, 99, 95, and 99% for *p*-cymene, α-humulene, 1,8-cineole, 3-carene, and sabinene, respectively. Stock solutions of *p*-cymene, α-humulene, 1,8-cineole, 3-carene, and sabinene containing 1, 5, 10, 20, and 2 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 10×, 2×, 1×, and .5× treatment levels, respectively. The control (0×) consisted of alfalfa pellets sprayed with ethanol.

Solutions were mixed in amber glass containers and transferred to graduated (34-mL increments) high-density polyethylene spray bottles each morning. Treatments were applied in a stream pattern to minimize volatilization. Feed pans (one stainless steel pan per lamb, ethanol-rinsed between studies) were tilted repeatedly during application in an effort to apply treatments evenly. Order of application was rotated within and across days. Approximately 10 min lapsed between spraying and feeding. Treatments were applied in an adjacent room, and an exhaust fan was operated in the metabolism unit. A detailed description of the treatment application appears elsewhere (Estell et al., 1998b).

The amount of chemical loss due to volatilization between application and feeding was examined for each compound as described previously (Estell et al., 1998b), with minor modifications. Briefly, 34 mL of stock solution was sprayed on .64 kg of alfalfa pellets (DM basis), and quadruplicate samples (35 to 40 g) were collected at 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**), using instrumentation parameters and column conditions as described by Tellez et al. (1997) and peak area ratios relative to known standards for quantitation. Extraction efficiency for each compound was determined by adding 34 mL of each stock solution to .64 kg of pellets. After flasks were sealed and allowed to stand for 30 min, ethanol (700 mL) was added, and sealed flasks were extracted for 6 h with continual shaking. Extracts were filtered and subjected to GCMS analysis, and re-

covery at various times after spraying was corrected for extraction efficiency. The corrected mean recovery at 10, 20, and 30 min, respectively, was 91.3, 71.9, and 76.7% for *p*-cymene; 73.4, 69.5, and 82.9% for α-humulene; 96.5, 85.1, and 84.9% for 1,8-cineole; 98.6, 65.7, and 81.7% for 3-carene; and 75.3, 71.8, and 72.2% for sabinene. These three sampling times approximate the beginning, midpoint, and end of the 20-min feeding period (assuming a 10-min lag between spraying and feeding). The CV for recovery estimates at 10, 20, and 30 min, respectively, was 1.5, 19.2, and 5.0% for p-cymene; 13.0, 14.8, and 6.9% for α-humulene; 12.4, 17.2, and 18.2% for 1,8-cineole; 5.8, 10.1, and 6.4% for 3-carene; and 11.7, 12.7, and 11.8% for sabinene. The variability among replicates indicates some difficulty achieving uniform treatment application. Feed pans used to measure recovery were rinsed with ethanol (34 mL) the following day to test for residual chemicals. Minor traces of chemical residues (.0003, .003, .003, .0002, and .0008% for *p*-cymene, α-humulene, 1,8-cineole, 3-carene, and sabinene, respectively) were detected the following day. None of the compounds examined was present above our detection limits in untreated alfalfa pellets extracted in a similar manner.

Statistical Analysis

Analysis of variance was conducted separately for each experiment (specific terpene) using GLM procedures of SAS (1989) with pellet consumption during the 20-min interval as the dependent variable and treatment (terpene concentration) and group (block) as the independent variables in the model. Although pen and animal are confounded and pen was not in the model, pen effects were not observed under similar conditions in a previous study (Estell et al., 1998b). Orthogonal contrasts for unequally spaced treatment levels (0×, .5×, 1×, 2×, and 10×) were constructed to determine linear and quadratic effects of treatment levels on intake for wk 3 to 7. Also, intake of lambs on the control treatment (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 for each experiment using GLM procedures of SAS (1989) to examine intake by day with treatment and group as independent variables 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

Repeated measures analyses revealed a mean linear effect for wk 2, 3, 5, and 7; a mean quadratic effect for wk 4 and 5; a linear treatment effect for wk 5; a quadratic treatment effect for wk 5 and 7; and a quadratic group effect for wk 2 and 5 ($P < .05$). These linear and quadratic effects within week were generally due to increased intake during the week. Quadratic treatment effects for wk 5 (1,8-cineole) seemed to be due to lower intake of lambs on the 10× treatment on d 1 but were difficult to explain for wk 7 (sabinene). In general, repeated measures analyses identified a tendency for intake to increase within week, but the increase was not treatment-specific.

Intake of control lambs in wk 2 did not differ from that of controls in any other week (Table 1). Intake of control lambs during the 20-min interval in wk 3 differed from other weeks, except wk 2 ($P < .05$; Table 1). Control lambs were fed pellets with the ethanol carrier in wk 3 to 7, but not in wk 1 or 2; however, treatment of alfalfa pellets with ethanol had no effect on intake by lambs previously (our unpublished observations). Intake of control lambs was generally consistent across experiments, except during wk 3 (*p*-cymene). Lambs were fed at a constant percentage of body weight (adjusted weekly); thus, intake for controls among weeks was not expected to differ. However, low intake for wk 3 was consistent across treatments. Although feeding level was constant, results among weeks are not directly comparable because of the confounding effects of time and because treatment levels were based on their concentration in tarbush leaves rather than being applied as a constant percentage of the diet. The reason for lower intake in wk 3 is unclear.

No linear or quadratic responses in intake to treatment concentration were detected for any compound (Table 1). Although small visual differences (approximately 50 g maximum) in intake for higher treatment concentrations were observed in many cases, inconsistent responses and within-treatment variability overshadowed any numerical differences. Exposure level and treatment concentration varied among chemicals because of the specific objectives of the study, but a wide range of exposure occurred. In particular, *p*-cymene (5 μg/g of DM for 1× treatment) was applied at a substantially lower concentration than other chemicals. Nevertheless, the compounds examined had no obvious effect on intake, which does not support our hypothesis. Moreover, because effects were not observed at the lower treatment concentrations, these compounds are not likely important determinants of diet selection.

Lack of effect of these compounds on consumption contrasts with our previous findings and(or) general expectations based on the literature. *p*-Cymene was positively related to degree of use by livestock when examined using univariate analysis, whereas sabinene, 3-carene, α-humulene, and *p*-cymene were in the subset of important variables for distinguishing between high- and low-use plants when subjected to multivariate analysis (Estell et al., 1998a). However, multivariate analysis using a subset of variables that successfully distinguished between high- and low-use plants considers all variables and their interrelationships simultaneously. In the present experiments, compounds were ap-

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

Concentration	Adaptation	*p*-Cymene	α-Humulene	1,8-Cineole	3-Carene	Sabinene
	kg·lamb^{-1}·d^{-1}, DM basis					
0×	.36[de]	.27[e]	.42[d]	.43[d]	.42[d]	.38[d]
.5×	.34	.26	.37	.46	.42	.44
1×	.40	.26	.42	.44	.38	.39
2×	.40	.22	.38	.44	.41	.35
10×	.39	.23	.36	.41	.37	.39
SEM	.036	.031	.034	.035	.034	.033

[a]Concentrations of compounds applied to alfalfa pellets were multiples (0, .5, 1, 2, or 10) of the concentration of that compound in tarbush (×); n = 9 lambs/treatment.
[b]No ethanol was applied during the adaptation period.
[c]No linear or quadratic treatment responses were detected for any terpene. Linear (adaptation, *p*-cymene, and sabinene) and quadratic (α-humulene and 1,8-cineole) day effects were detected ($P < .05$). Day × treatment interactions were observed ($P < .05$) for 1,8-cineole and sabinene (quadratic).
[d,e]For the control treatment (0×), means within a row lacking a common superscript differ ($P < .05$); SEM = .034 for control lambs across experiments.

plied singularly; furthermore, lambs had no opportunity to exhibit preference, whereas in previous studies animals could choose among plants. Because none of the individual compounds had an effect on pellet consumption, previous relationships with plant use may have been coincidental (i.e., correlated with a compound related to intake) or part of a cumulative or synergistic effect.

Individual terpenes have been associated with degree of herbivory in several mammalian species. Personius et al. (1987) indicated that *p*-cymene was negatively related to sagebrush preference by mule deer. Reichardt et al. (1985) reported cineole was associated with reduced intake by hares. Zhang and States (1991) found the concentration of three monoterpenes (including sabinene) was inversely related to feeding of Abert squirrels on ponderosa pine. Riddle et al. (1996) reported specific monoterpenes were either positively (including cymene) or negatively (including sabinene + β-pinene) correlated to intake of juniper by goats. 1,8-Cineole has been negatively associated with intake in various marsupials consuming eucalyptus (Lawler et al., 1998; Pass et al., 1998). However, recent evidence suggests 1,8-cineole is simply correlated with other compounds that cause formation of conditioned flavor aversions and serves as a cue to detect these toxins (e.g., jensenone; Lawler et al., 1998, 1999). We are not aware of published data concerning relationships of α-humulene or 3-carene to mammalian herbivory. Many of the examples in the literature that suggest a relationship of terpenes to dietary preferences were identified using animals on a low plane of nutrition, whereas the lambs in our study were fed a high-quality diet at nearly 5% of BW. Plane of nutrition may affect preference and animals may consume more aversive phytochemicals with a higher nutrient status (Illius and Jessop, 1996; Wang and Provenza, 1996).

Design of experiments with large animals results in a paradoxical situation in which short length is necessary to avoid adaptation effects, but length is needed to achieve replication for small numbers of animals to minimize variability among individuals (Narjisse et al., 1996). 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, and minimizes the confounding of palatability and postingestive effects (Baumont, 1996). Five-day trials were a compromise to minimize day-to-day intake variation as well as opportunity for learning and adaptation. Although effects of each chemical were assumed to be independent of effects of compounds tested subsequently, previous experience can affect diet selection through the integration of cognitive and affective processes (Provenza et al., 1992). In fact, Provenza (1995) stated that "it is impossible to conduct an experiment on food selection that is not affected by experience, or lack of it." The effect of a given chemical could change even within a 5-d period if an aversion is formed (Provenza et al., 1990). However, no evidence of formation of conditioned aversion to terpenes was observed in our studies. In fact, repeated measures analysis indicated that daily intake within a week typically was constant or gradually increased and then stabilized after 1 or 2 d. Because these responses to a given chemical were generally similar among controls and other treatments, it does not seem that this pattern was due to adaptation to a novel taste. The possibility of acclimation of lambs to volatile aromas as a cause of increased intake during some weeks cannot be ruled out. Odor and taste both affect preference of sheep, and effects are often difficult to separate (Arnold et al., 1980; Narjisse et al., 1996). 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, using exhaust fans and application of chemical solutions in a separate room should have minimized feeding area contamination (based on timing of volatilization losses discussed previously). Nevertheless, it is difficult to ascertain effects

of aroma and taste with volatile compounds. For example, low intake during wk 3 may have been due to an effect of *p*-cymene odor diffusing throughout the room, even though precautions were taken to remove odors and even though this compound was applied at the lowest concentration. Narjisse et al. (1996) examined effects of a monoterpene mixture applied to alfalfa pellets on preference, and the effect of the aroma on feeding. Anosmic sheep did not discriminate against either the terpene aroma or terpene-treated pellets, but intact sheep discriminated against pellets when exposed to terpene aroma and preferred pellets without terpene mixture applied, supporting the involvement of odor in rejection.

In summary, the five compounds tested in these experiments were not related to consumption of alfalfa pellets by sheep. Thus, these particular leaf-surface compounds are probably not responsible for differential use of tarbush by ruminants. Possibly, these compounds may have been correlated with intake rather than causal in previous studies, or they may exert an effect only in concert with other plant chemicals.

Implications

The five terpenes examined in this study did not affect consumption of alfalfa pellets by lambs when fed individually for a short time without the potential for synergism among terpenes or a choice of alternative feed. Determining which compounds influence feed intake is critical information that will ultimately lead to mechanisms to alter 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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