1744?81 ...

Effects of γ-terpinene, terpinolene, α-copaene, and α-terpinene on consumption of alfalfa pellets by sheep[1,2]

R. E. Estell*[3], E. L. Fredrickson*, D. M. Anderson*, and M. D. Remmenga†

*ARS, USDA, Jornada Experimental Range; and †University Statistics Center, New Mexico State University, Las Cruces, NM 88003-8003

ABSTRACT: Although plant secondary chemistry influences shrub consumption by free-ranging ruminants, the effects of many specific compounds on herbivores have not been examined. We conducted four experiments to examine effects of individual terpenes on alfalfa pellet intake by lambs. Forty-five lambs were individually fed alfalfa pellets sprayed with γ-terpinene, terpinolene, α-copaene, or α-terpinene at one of five concentrations in an ethanol carrier. Treatments (0, 0.5, 1, 2, and 10×) were multiples of the concentration (×) of a specific terpene on the leaf surface of *Flourensia cernua* (a low-preference shrub for domestic ruminants). Terpenes were applied to alfalfa pellets (0.64 kg·lamb^{-1}·d^{-1}, DM basis), and consumption was measured during a 20-min interval for 5 d. A day effect was detected for γ-terpinene on intake ($P < 0.001$ for both linear and quadratic contrasts). No effect of terpinolene, α-copaene, or α-terpinene on intake was detected in this study. None of the terpenes tested was strongly related to intake of alfalfa pellets by lambs under the conditions of this study.

Key Words: Diet Selection, Herbivory, Intake, Sheep, Terpenes

 J. Anim. Sci. 2005. 83:1967–1971

Introduction

Shrub encroachment and subsequent loss of grasslands is a serious problem for livestock producers and land managers in the Chihuahuan Desert. Shrubs often contain secondary chemicals that render them unpalatable and/or toxic, even though they may contain substantial nutrients. Tarbush (*Flourensia cernua* DC) is an unpalatable shrub that contains 16 to 25% CP, but it has high concentrations of terpenes and phenolics (Estell et al., 1996b). Sheep consuming tarbush in mixed diets (up to 30% of diet) for several weeks did not have abnormal digesta kinetics or fermentation patterns or exhibit symptoms of toxicity (Fredrickson et al., 1994; King et al., 1996). When forced to consume tarbush in small paddocks, ruminants exhibited differential selectivity among plants that was related to the quantity of epicuticular wax on the leaf surface (Estell et al., 1994b). Removal of surface compounds from tarbush by rinsing with organic solvents increased preference by sheep (Estell et al., 1994a), and specific mono- and sesquiterpenes on the leaf surface were related to tarbush consumption using multivariate analysis (Estell et al., 1998a); however, specific compounds must be tested individually to prove cause and effect (Clausen et al., 1992). We have previously examined effects of 15 volatile compounds (camphor, borneol, limonene, *cis*-jasmone, α-pinene, β-caryophyllene, *p*-cymene, α-humulene, 1,8-cineole, 3-carene, sabinene, camphene, myrcene, β-pinene, and caryophyllene oxide) on intake by lambs (Estell et al., 1998b; 2000; 2002). Only camphor, α-pinene, camphene, and caryophyllene oxide were related to intake when tested individually.

The objective of the present experiments was to individually examine effects of four additional terpenes present on the leaf surface of tarbush (γ-terpinene, terpinolene, α-copaene, and α-terpinene) on consumption of alfalfa pellets by lambs. Our hypothesis was that alfalfa pellet intake would decrease as the concentration of a specific terpene increased.

Materials and Methods

Treatments, Animal Management, and Experimental Protocol

Four experiments were conducted to examine effects of three hydrocarbon monoterpenes (γ-terpinene, terpi-

[1]Authors are grateful for the assistance of A. Gonzalez, W. Hooper, and Y.-F. Wang.

[2]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

[3]Correspondence—phone: 505-646-4842; fax: 505-646-5889; e-mail: restell@nmsu.edu.

Received December 14, 2004.
Accepted May 13, 2005.

nolene, and α-terpinene) and a hydrocarbon sesquiterpene (α-copaene) on alfalfa pellet intake by lambs. Mean concentrations of these terpenes on the leaf surface of tarbush in previous studies (Tellez et al., 1997; Estell et al., 1998a) were approximately 15, 5, 5, and 10 μg/g of DM for γ-terpinene, terpinolene, α-copaene, and α-terpinene, respectively. These concentrations represent the concentration of each compound to which animals are exposed when browsing tarbush. One compound was examined in each experiment at five concentrations, and treatments were multiples (0, 0.5, 1, 2, or 10×) of the exposure concentration of that terpene.

Experiments were conducted according to USDA guidelines and approved by the New Mexico State University Institutional Animal Care and Use Committee. Forty-five Rambouillet ewe lambs (approximately 7 mo of age, 40.3 ± 1.5 kg initial BW, without previous browsing experience) were assigned randomly to one of 15 pens and three groups (constant across experiments). Lambs were assigned randomly to one of five treatments (terpene concentrations) before each experiment (nine lambs per treatment, restricted to three lambs·treatment^{-1}·group^{-1}) using a randomized complete block design.

Each morning, lambs were individually fed (1.22 × 2.44 m pens) treated pellets in an enclosed metabolism unit for 20 min. Three groups (blocks) were fed at 0800, 0830, and 0900, respectively (15 lambs per group). All experiments lasted 5 d, with two preliminary 5-d periods to familiarize lambs with handling and pen feeding (first adaptation period) and to determine baseline intake of untreated alfalfa pellets during the 20-min interval (second adaptation period). γ-Terpinene, terpinolene, α-copaene, and α-terpinene were tested in Exp. 1 through 4, respectively (order selected randomly; 2-d interval between experiments). Alfalfa pellets (0.64 kg, DM basis; ≥15% CP; 0.95 cm diameter) were offered to lambs during the 20-min feeding, and orts were recorded each day. Alfalfa pellets were sampled throughout the study, composited, ground in a Wiley Mill (2-mm screen), and analyzed for DM by drying at 100°C for 24 h (DM = 92.3%). Lambs were weighed weekly before the 0800 feeding on d 5.

Lambs were adapted to alfalfa pellets for 5 d in a drylot pen before individual feeding began. Lambs were maintained as one group with free access to water and trace mineral salt (93 to 97% NaCl, 3 g/kg of Mn, 2.5 g/kg of Zn, 1.5 g/kg of Fe, 0.15 g/kg of Cu, 0.09 g/kg of I, 0.025 g/kg of Co, and 0.01 g/kg of Se), except during the 20-min feeding period. Lambs were group-fed untreated alfalfa pellets at 3.9% of BW (DM basis, adjusted weekly) at 1000. On test days, the quantity of untreated pellets offered was decreased by the total amount of treated pellets consumed that day.

All compounds were purchased from Fluka (Buchs, Switzerland) except γ-terpinene (Acros Organics, Geel, Belgium). Manufacturer-specified purities were 98, 90, 95, and 97% for γ-terpinene, terpinolene, α-copaene, and α-terpinene, respectively. Stock solutions containing 3, 1, 1, or 2 mg/mL of the respective terpene in 100% ethanol were diluted 5-, 10-, and 20-fold in ethanol. When applied at 0.05 mL/g of DM, the stock and 5-, 10-, and 20-fold dilutions correspond to 10×, 2×, 1×, and 0.5× treatments, respectively. Control (0×) pellets were sprayed only with ethanol. Treatments were applied with polyethylene spray bottles in an adjacent room. The protocol for application of treatments to alfalfa pellets has been described in detail previously (Estell et al., 1998b, 2000, 2002).

Chemical loss due to volatilization between application and feeding was measured using gas chromatography/mass spectrometry as described in Estell et al. (2002). Mean recovery (corrected for extraction efficiency) at 10, 20, and 30 min, respectively, was 91.0, 89.7, and 83.9% for γ-terpinene; 84.4, 73.6, and 70.1% for terpinolene; 99.3, 99.1, and 97.3% for α-copaene; and 74.3, 70.5, and 67.7% for α-terpinene. With a lag time of approximately 10 min between application and feeding, the 10-, 20-, and 30-min estimates equate to the beginning, midpoint, and end of the 20-min period. The sesquiterpene (α-copaene) exhibited low volatility even after 30 min, and as expected, the three monoterpenes were more volatile. Untreated alfalfa pellets also were extracted and analyzed as described by Estell et al. (2002) to determine background terpene concentrations. None of the four terpenes was present above the detection limits of gas chromatography/mass spectrometry in untreated alfalfa pellets.

Statistical Analyses

For each experiment, daily intake during the 20-min interval was analyzed as repeated measures using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The model included group as a fixed block effect, treatment (terpene concentration), day, and day × treatment interaction. A first-order autoregressive, AR(1), covariance structure was used for the repeated measurements on individual animals across days. Linear and quadratic models were fit to intake across concentrations averaged over days and across days averaged over concentrations when no day × treatment interaction was detected. Intake of control lambs across experiments (including second adaptation period) also was analyzed with the MIXED procedure of SAS, with fixed effects of group, experiment, day, and day × experiment interaction. In the case of an experiment effect ($P < 0.05$), means were separated by pairwise approximate *t*-tests within the MIXED procedure of SAS. Two lambs were removed from the study, one at the beginning of Exp. 2 and one at the beginning of Exp. 3, for reasons unrelated to the study.

Results and Discussion

The control analysis revealed a day × experiment interaction ($P < 0.001$) due to the extremely low intake on d 1 in Exp. 1 (Figure 1). Averaged over days, intake

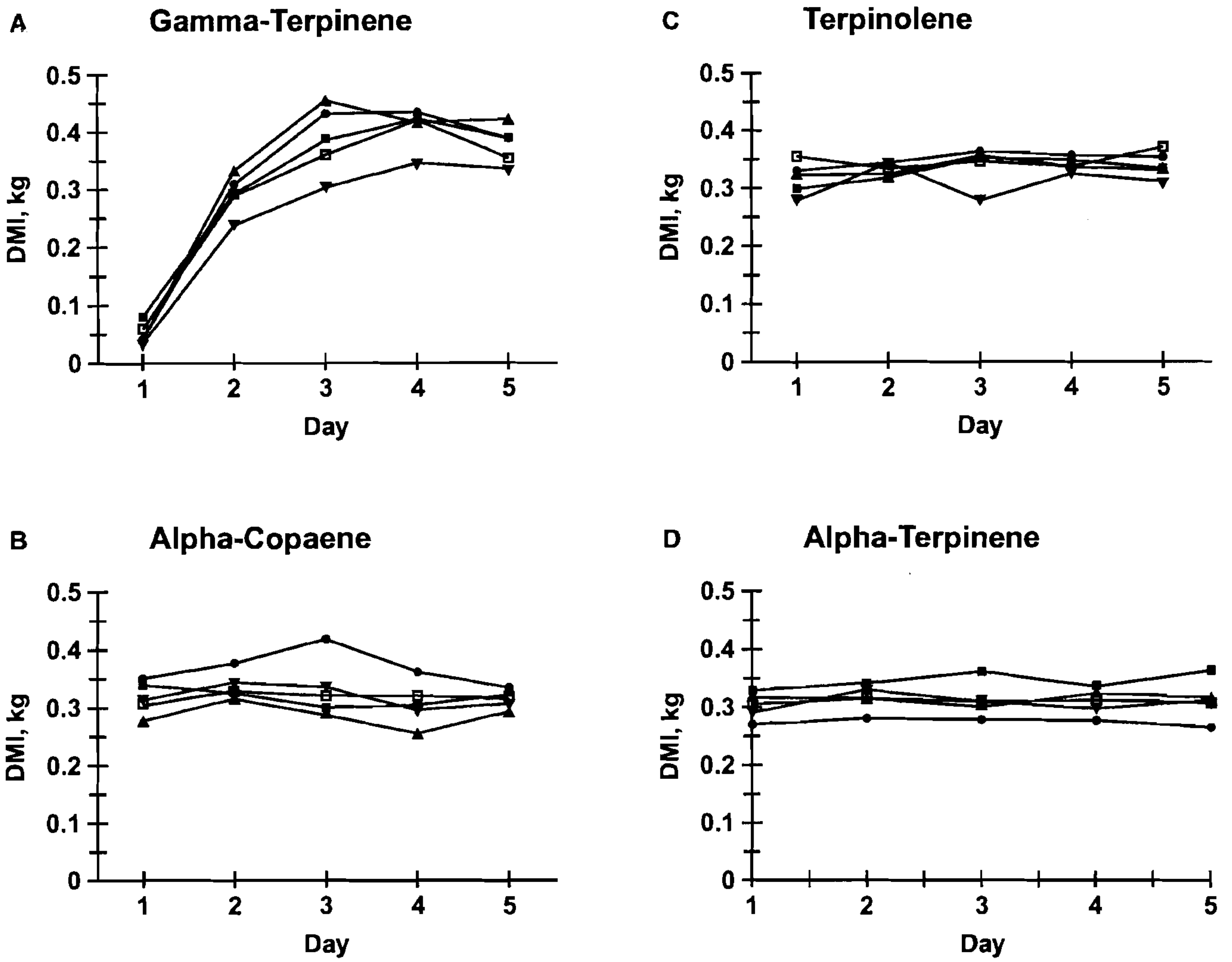

Figure 1. Mean intake of treated alfalfa pellets by lambs during a 20-min interval for five consecutive days; n = 9 lambs/d for all terpene/concentration combinations except 0.5× terpinolene (n = 8), 2× α-copaene (n = 7), 0× α-terpinene (n = 8), and 0.5× α-terpinene (n = 8); SEM = 0.032, 0.035, 0.029, and 0.030 for γ-terpinene, terpinolene, α-copaene, and α-terpinene, respectively, except 0.5× terpinolene (0.037), 2× α-copaene (0.033), 0× α-terpinene (0.032), and 0.5× α-terpinene (0.032). Treatments (multiples of the concentration of specific compounds in tarbush) are designated □, ●, ■, ▲, and ▼ for 0, 0.5, 1, 2, and 10×, respectively. Linear and quadratic day effects ($P < 0.001$) were observed for γ-terpinene pooled across treatments.

by lambs during the second adaptation period differed from controls in Exp. 1, 3, and 4 ($P < 0.05$; Table 1). It is unclear why control intake varied across experiments, given that lambs were weighed weekly and feed was adjusted accordingly to a constant percentage of BW. Control lambs received the ethanol carrier in Exp. 1 to 4, but not in the adaptation period; however, treatment of alfalfa pellets with the same amount of ethanol did not affect intake by lambs previously (Estell et al., 2001, 2002).

A day effect on intake was detected for γ-terpinene ($P < 0.001$ for both linear and quadratic contrasts), which was due to the low intake on d 1 for lambs at all concentrations (Figure 1). Although consumption of alfalfa pellets by lambs was not affected by the ethanol carrier in previous studies (Estell et al., 2001; 2002), a neophobic reaction to the carrier on d 1 of Exp. 1 may have occurred given the low intake of alfalfa pellets for all lambs (including controls). No day effect on intake was observed for terpinolene, α-copaene, or α-terpinene, and no treatment effect was detected for any of the four terpenes examined (Table 1). Results for different terpenes are not directly comparable because treatment levels varied based on the concentration in tarbush leaves. In addition, volatility (based on recovery values) of α-copaene was lower than for the three monoterpenes; thus, actual exposure level was less affected by time after application.

Individual terpenes have been shown to be related to plant use for a number of mammalian species. Specific

Table 1. Mean (±SEM) consumption of alfalfa pellets treated with terpenes by lambs during a 20-min interval

Concentration[a]	Adaptation[b]	γ-Terpinene	Terpinolene	α-Copaene	α-Terpinene
	kg·lamb^{-1}·d^{-1}, DM basis				
0×	0.39 (0.012)[c]	0.30 (0.023)[d]	0.35 (0.028)[cd]	0.32 (0.023)[d]	0.31 (0.028)[d]
0.5×		0.32 (0.023)	0.35 (0.030)	0.37 (0.023)	0.28 (0.028)
1×		0.31 (0.023)	0.33 (0.028)	0.32 (0.023)	0.35 (0.026)
2×		0.33 (0.023)	0.33 (0.028)	0.29 (0.027)	0.32 (0.026)
10×		0.25 (0.023)	0.31 (0.028)	0.32 (0.023)	0.31 (0.026)

[a]Concentrations of compounds applied to alfalfa pellets were multiples (0, 0.5, 1, 2, or 10) of the concentration of that compound in tarbush (×); n = 9 lambs per concentration for all terpene/treatment combinations except 0.5× terpinolene (n = 8), 2× α-copaene (n = 7), 0× α-terpinene (n = 8), and 0.5× α-terpinene (n = 8).

[b]No ethanol was applied during the adaptation period (second adaptation period to establish baseline intake of alfalfa pellets without terpenes).

[c,d]For the control treatment (0×), within a row, means without a common superscript differ, $P < 0.05$; n = 45 for adaptation; n = 9 for γ-terpinene, terpinolene, and α-copaene; and n = 8 for α-terpinene; SEM = 0.012 for adaptation; 0.028 for γ-terpinene, terpinolene and α-copaene; and 0.029 for α-terpinene.

monoterpenes have been related to sagebrush intake by mule deer (Personius et al., 1987), juniper intake by goats (Riddle et al., 1996), tarbush intake by sheep (Estell et al., 1998b; 2002), and western red cedar intake by deer (Vourc'h et al., 2002); however, little information exists about the specific compounds examined in our study. Zhang and States (1991) reported a negative relationship between inner bark concentration of three monoterpenes (including terpinolene) and feeding on ponderosa pines by Abert squirrels. Elliott and Loudon (1987) reported that red deer rejected a pelleted diet when exposed to the odor of five monoterpenes (including terpinolene). When cattle, sheep, and goats were forced to use tarbush, they exhibited differential use of tarbush plants, and α-copaene was in the subset of important variables for distinguishing between high, moderate, and low use plants when subjected to multivariate analysis (Estell et al., 1996a). In contrast, α-copaene and terpinolene were not related to intake in the present study. We are not aware of data regarding the relationship of γ- or α-terpinene with herbivory in mammals.

In contrast to our hypothesis, none of the chemicals tested in these four experiments was strongly related to intake of alfalfa pellets by lambs under the conditions of this study. Discrepancies between this study and previous research may be due in part to the fact that studies reporting relationships of volatiles with herbivory were correlative and did not examine the application of specific compounds. Moreover, compounds could be synergistic or have a cumulative effect because total terpene concentration has been related to intake by ruminants in other studies (Schwartz et al., 1980; Riddle et al., 1996). The total essential oil yield from tarbush (0.84 to 1.01% of DM; Tellez et al., 1997) is approximately two orders of magnitude greater than the 10× treatment concentrations used in this study. Furthermore, volatile compounds may serve as cues for detection of other compounds. For example, 1,8-cineole was reported to be negatively related to intake for certain marsupials consuming eucalyptus, but it was subsequently found to be correlated with the concentration of other aversive compounds and to serve as a cue for detecting these toxins (Lawler et al., 1998; 1999). In addition, many studies suggesting terpenes are related to dietary preferences involved animals on a low plane of nutrition, whereas the lambs in this study were fed a high-quality diet at 3.9% of BW. Animals on a high plane of nutrition may be capable of consuming more aversive phytochemicals without negative consequences (Illius and Jessop, 1996; Wang and Provenza, 1996), which might explain the lack of effects of terpenes in this study.

Lambs in our study had no opportunity to exhibit preference. Initial eating rate during a short interval at the beginning of the feeding period is a good criterion to measure palatability and minimizes the confounding of palatability and postingestive effects (Baumont, 1996). Nonetheless, there was no evidence of postingestive aversion to terpenes in this study. The only significant day effect on intake was due to low intake at all concentrations of γ-terpinene on d 1 (Figure 1).

In summary, none of the four terpenes examined in this study greatly affected alfalfa pellet consumption by lambs when fed individually. Perhaps other terpenes or compounds from other chemical classes may be responsible for the low preference of livestock for tarbush. Alternatively, the lack of response of individual compounds may be due to a cumulative or synergistic effect of several compounds. Understanding how plant chemicals interact with herbivores is critical for development of mechanisms to alter foraging behavior and selectivity of livestock.

Literature Cited

Baumont, R. 1996. Palatability and feeding behaviour in ruminants. A review. Ann. Zootech. 45:385–400.

Clausen, T. P., P. B. Reichardt, J. P. Bryant, and F. D. Provenza. 1992. Condensed tannins in plant defense: A perspective on classical theories. Page 639 in Plant Polyphenols. R. W. Hemingway and P. E. Laks, ed. Plenum Press, New York.

Elliott, S., and A. Loudon. 1987. Effects of monoterpene odors on food selection by red deer calves (*Cervus elaphus*). J. Chem. Ecol. 13:1343–1349.

Estell, R. E., D. M. Anderson, and K. M. Havstad. 1994a. Effects of organic solvents on use of tarbush by sheep. J. Chem. Ecol. 20:1137–1142.

Estell, R. E., E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. D. Remmenga. 1996a. Tarbush leaf surface terpene profile in relation to mammalian herbivory. Page 237 in Shrubland Ecosystem Dynamics in a Changing Climate. J. R. Barrow, E. D. McArthur, R. E. Sosebee, and R. J. Tausch, ed. Gen. Tech. Rep. INT-GTR-338. USDA Forest Service, Intermountain Research Station, Ogden, UT.

Estell, R. E., E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. D. Remmenga. 1998a. Relationship of leaf surface terpene profile of tarbush with livestock herbivory. J. Chem. Ecol. 24:1–12.

Estell, R. E., E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. D. Remmenga. 2000. Effects of individual terpenes on consumption of alfalfa pellets by sheep. J. Anim. Sci. 78:1636–1640.

Estell, R. E., E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. D. Remmenga. 2002. Effects of four mono- and sesquiterpenes on the consumption of alfalfa pellets by sheep. J. Anim. Sci. 80:3301–3306.

Estell, R. E., E. L. Fredrickson, D. M. Anderson, W. F. Mueller, and M. D. Remmenga. 1994b. Relationship of tarbush leaf surface secondary chemistry to livestock herbivory. J. Range Manage. 47:424–428.

Estell, R. E., E. L. Fredrickson, and K. M. Havstad. 1996b. Chemical composition of *Flourensia cernua* at four growth stages. Grass Forage Sci. 51:434–441.

Estell, R. E., E. L. Fredrickson, M. R. Tellez, K. M. Havstad, W. L. Shupe, D. M. Anderson, and M. D. Remmenga. 1998b. Effect of volatile compounds on consumption of alfalfa pellets by sheep. J. Anim. Sci. 76:228–233.

Estell, R. E., M. R. Tellez, E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. R. Remmenga. 2001. Extracts of *Flourensia cernua* reduce consumption of alfalfa pellets by sheep. J. Chem. Ecol. 27:2275–2285.

Fredrickson, E., J. Thilsted, R. Estell, and K. Havstad. 1994. Effects of chronic ingestion of tarbush (*Flourensia cernua*) on ewe lambs. Vet. Human Toxicol. 36:409–415.

Illius, A. W., and N. S. Jessop. 1996. Metabolic constraints on voluntary intake in ruminants. J. Anim. Sci. 74:3052–3062.

King, D. W., R. E. Estell, E. L. Fredrickson, K. M. Havstad, J. D. Wallace, and L. W. Murray. 1996. Effects of *Flourensia cernua* ingestion on intake, digestion, and ruminal fermentation of sheep consuming tobosa. J. Range Manage. 49:325–330.

Lawler, I. R., W. J. Foley, B. M. Eschler, D. M. Pass, and K. Handasyde. 1998. Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. Oecologia 116:160–169.

Lawler, I. R., J. Stapley, W. J. Foley, and B. M. Eschler. 1999. Ecological example of conditioned flavor aversion in plant-herbivore interactions: Effects of terpenes of *Eucalyptus* leaves on feeding by common ringtail and brushtail possums. J. Chem. Ecol. 25:401–415.

Personius, T. L., C. L. Wambolt, J. R. Stephens, and R. G. Kelsey. 1987. Crude terpenoid influence on mule deer preference for sagebrush. J. Range Manage. 40:84–88.

Riddle, R. R., C. A. Taylor, Jr., M. M. Kothmann, and J. E. Huston. 1996. Volatile oil contents of ashe and redberry juniper and its relationship to preference by Angora and Spanish goats. J. Range Manage. 49:35–41.

Schwartz, C. C., W. L. Regelin, and J. G. Nagy. 1980. Deer preference for juniper forage and volatile oil treated foods. J. Wildl. Manage. 44:114–120.

Tellez, M. R., R. E. Estell, E. L. Fredrickson, and K. M. Havstad. 1997. Essential oil of *Flourensia cernua* DC. J. Essent. Oil Res. 9:619–624.

Vourc'h, G., de Garine-Wichatitsky, A. Labbe, D. Rosolowski, and J. L. Martin. 2002. Monoterpene effect on feeding choice by deer. J. Chem. Ecol. 28:2411–2427.

Wang, J., and F. D. Provenza. 1996. Food preference and acceptance of novel foods by lambs depend on the composition of the basal diet. J. Anim. Sci. 74:2349–2354.

Zhang, X., and J. S. States. 1991. Selective herbivory of ponderosa pine by Abert squirrels: A re-examination of the role of terpenes. Biochem. Syst. Ecol. 19:111–115.