

Mechanisms for Eliminating Monoterpenes of Sagebrush by Specialist and Generalist Rabbits

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Abstract Pygmy rabbits (*Brachylagus idahoensis*) are one of only three vertebrates that subsist virtually exclusively on sagebrush (*Artemisia* spp.), which contains high levels of monoterpenes that can be toxic. We examined the mechanisms used by specialist pygmy rabbits to eliminate 1,8-cineole, a monoterpene of sagebrush, and compared them with those of cottontail rabbits (*Sylvilagus nuttalli*), a generalist herbivore. Rabbits were offered food pellets with increasing concentrations of cineole, and we measured voluntary intake and excretion of cineole metabolites in feces and urine. We expected pygmy rabbits to consume more, but excrete cineole more rapidly by using less-energetically expensive methods of detoxification than cottontails. Pygmy rabbits consumed 3–5 times more cineole than cottontails relative to their metabolic body mass, and excreted up to 2 times more cineole metabolites in their urine than did cottontails. Urinary metabolites excreted by pygmy rabbits were 20 % more highly-oxidized and 6 times less-conjugated than those of cottontails. Twenty percent of all cineole metabolites recovered from pygmy rabbits were in feces, whereas cottontails did not excrete fecal metabolites. When compared to other mammals that consume

cineole, pygmy rabbits voluntarily consumed more, and excreted more cineole metabolites in feces, but they excreted less oxidized and more conjugated cineole metabolites in urine. Pygmy rabbits seem to have a greater capacity to minimize systemic exposure to cineole than do cottontails, and other cineole-consumers, by minimizing absorption and maximizing detoxification of ingested cineole. However, mechanisms that lower systemic exposure to cineole may come with a higher energetic cost in pygmy rabbits than in other mammalian herbivores.

Keywords *Artemisia tridentata* · *Brachylagus idahoensis* · Cineole · Detoxification · Pygmy rabbits · *Sylvilagus nuttalli*

Introduction

Big sagebrush (*Artemisia tridentata*) rangelands dominate 1.1 million km² of the western United States and Canada (Meyer and Karasov, 1991), and provide important habitat for a wide range of endemic wildlife species. However, reduction, fragmentation and degradation of sagebrush rangelands has threatened many sagebrush-steppe obligates such as sage grouse (*Centrocercus* spp., Connelly et al., 2000), Brewer's sparrows (*Spizella breweri*), sage sparrows (*Amphispiza belli*), sage thrashers (*Oreoscoptes montanus*, Knick and Rotenberry, 2000), Washington, Idaho, and Columbian ground squirrels (*Spermophilus washingtoni*, *S. brunneus*, *S. columbianus*, Betts, 1990; Weddell, 1991; Sherman and Runge, 2002), and pygmy rabbits (*Brachylagus idahoensis*, Federal Register, 2003). Despite its importance as wildlife habitat, however, few vertebrate species consume substantial amounts of sagebrush. Only three species, pronghorn (Ngugi et al., 1992), sage grouse (Connelly et al., 2000), and pygmy rabbits

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(Thines et al., 2004) consume sagebrush as more than a minor component of their seasonal diets, and thus are considered dietary specialists (Shipley et al., 2009). For example, pygmy rabbits consume up to 99 % of their diet from big sagebrush (*Artemisia tridentata*) in winter and up to 50 % in summer (Green and Flinders, 1980; Thines et al., 2004). Sagebrush contains high concentrations of plant secondary metabolites (PSMs) such as terpenes, sesquiterpene lactones, and phenolics, which can influence intake (Kelsey et al., 1973; Bray et al., 1991; Wilt et al., 1992). Monoterpenes, which comprise 1–4 % of dry mass (DM) of sagebrush (White et al., 1982), generally are toxic to mammals (Nagy et al., 1964; Cork and Foley, 1991), compromise energy budgets (Sorensen et al., 2005), and are avoided by most herbivores (Sinclair et al., 1988; Meyer and Karasov, 1991). Consuming moderate amounts of sagebrush (15–50 % of the diet) have caused digestive upset, reduced rumen motility, rumen lesions, rumenitis, and even death in mice and wild and domestic ruminants (Nagy et al., 1964; Johnson et al., 1976).

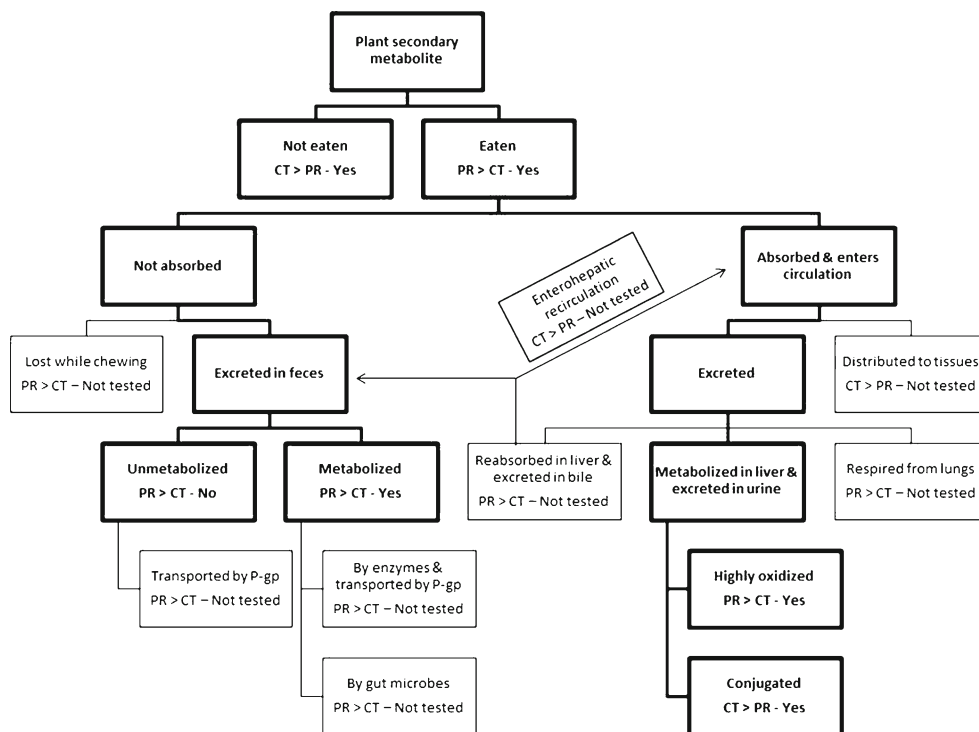
Herbivores that regularly consume plants with PSMs like those in sagebrush have developed the ability to avoid or tolerate PSMs through detoxification and elimination. Herbivores have a variety of options when confronted with PSMs in their diet (Fig. 1). First, animals can avoid plants or plant parts containing high amounts of PSMs through conditioned flavor aversion (Provenza, 1995). Second, animals may consume the plant, but avoid absorbing PSMs by releasing volatiles while chewing (White et al., 1982) or by excreting unmetabolized parent PSMs or metabolites in

feces. PSMs that are lipid-soluble, nonpolar, and nonionic may be actively effluxed from enterocytes back into the lumen via ATP-dependent transporters and, therefore, excreted unmetabolized in the feces (Sorensen and Dearing, 2006; Stanley et al., 2009). In addition, PSMs can be metabolized by detoxification enzymes in enterocytes (Sorensen and Dearing, 2006; Sorensen et al., 2007) or by microflora (Malecky and Broudiscou, 2009) before absorption and excreted in feces as metabolites.

If absorbed, PSMs are distributed to organs via the circulation where they can accumulate in tissues or be detoxified by metabolizing enzymes (primarily in the liver) and excreted via several routes (Fig. 1). Detoxification of PSMs generally increases water solubility for excretion in urine (McLean and Duncan, 2006; Sorensen et al., 2006), bile (Grigoleit and Grigoleit, 2005), or respired air (Boyle et al., 2002). In Phase I detoxification, microsomal enzymes such as Cytochrome P450 catalyze reactions such as oxidation, reduction, or hydrolysis. The more oxidized the PSM metabolite, the more polar it becomes. In Phase II, PSMs or Phase I metabolites are conjugated to polar molecules, such as glycine and glucuronic acid.

Herbivores, like pygmy rabbits, that eat specialized diets consisting of large quantities of a few taxa of plants containing PSMs must limit the absorption of PSMs and rapidly and efficiently detoxify and eliminate them. Although specialists voluntarily consume more PSMs in their diets than do generalist herbivores, they often employ more effective mechanisms to limit absorption than do their generalist

Fig. 1 Possible fates of plant secondary metabolites (PSMs) encountered by herbivores (adapted from Marsh et al., 2006), expected differences in strategies used by specialist pygmy rabbits (PR, *Brachylagus idahoensis*) and generalist cottontails (CT, *Sylvilagus nuttalli*) consuming monoterpenes in sagebrush, and outcome of tests of our hypotheses (in bold) and those not yet tested



counterparts (Sorensen and Dearing, 2003; Sorensen et al., 2004). Furthermore, specialists may use detoxification pathways that are less energetically expensive than do generalists. For example, specialists may oxidize PSMs to a greater degree, thus reducing the need for subsequent conjugation with glucuronic acid, a derivative of endogenous glucose, or with glycine, an amino acid (Boyle et al., 1999, 2000). Finally, specialists (e.g., ringtail possums, *Pseudocheirus peregrinus*, and koalas, *Phascolarctos cinereus*) produce fewer metabolites conjugated with glucuronic acid than do generalists (e.g., lab rats and brushtail possums, *Trichosurus vulpecula*, Boyle et al., 1999, 2000) and excrete less glucuronic acid per unit of energy intake (e.g., Stephens woodrat, *Neotoma stephensi*, Sorensen et al., 2005).

Our goal in this study was to examine mechanisms that allow pygmy rabbits to tolerate nearly monospecific diets of sagebrush by comparing their intake and fecal and urinary excretion of 1,8-cineole, a common monoterpene of sagebrush (Fig. 2, Shipley et al., 2006) and its metabolites, with that of mountain cottontails (*Sylvilagus nuttalli*). Cottontails are generalist herbivores that often co-inhabit sagebrush rangelands with pygmy rabbits in the northwestern United States, but consume diets of <4 % sagebrush year

round (e.g., Johnson and Hansen, 1979; MacCracken and Hansen, 1984). We predicted that pygmy rabbits would (Fig. 1): 1) voluntarily consume more cineole than cottontails; 2) excrete more unmetabolized cineole and cineole metabolites in their feces than cottontails; 3) excrete more highly-oxidized cineole metabolites in their urine and feces than cottontails; 4) excrete a lower proportion of conjugated metabolites, thus their urine would have lower amounts of glucuronic acid and gross energy, and a higher pH than cottontails.

Methods and Materials

Measuring Intake of Cineole To compare the tolerance for 1,8-cineole between pygmy rabbits and cottontails, we conducted a series of intake trials with rabbits captured in wire box traps from southern Idaho (pygmy rabbits) and eastern Washington (cottontails). Four pygmy rabbits (3 female, 1 male) weighing a mean of 432 g (SD=83 g) and 6 cottontail rabbits (4 female, 2 male) weighing a mean of 1,174 g (SD=215 g) were acclimated to a completely-balanced grain-alfalfa rabbit pellet in 0.4-m² stainless steel metabolic crates, with fresh water *ad libitum*. All protocols were approved by WSU's Institutional Animal Care and Use Committee (protocol #3097). During trials, rabbits received increasing amounts of 1,8-cineole (99 %, Alfa Aesar, Ward Hill, MA, USA) mixed with pellets in experimental blocks of 3 d per concentration, including 0, 1, 3, 5, and 7 % cineole by dry weight. Concentrations of cineole were achieved by mixing pellets with cineole in an amber-colored glass jar each morning, and storing them in the freezer when not in use. Pellets were weighed before offering them to the rabbits in ceramic bowls, and refreshed 3 times per day to avoid excessive evaporation of cineole from the pellets. Samples of food offered and refused were collected at each feeding for later chemical analysis. We measured the dry matter of food offered by weighing a fresh subsample, drying it at 100 °C for 24 hr, then re-weighing it. We collected all refusals and dried them at 100 °C for 24 hr. On the third day at each cineole concentration, we collected urine from the rabbits on dry ice for 24 hr and froze it at -20 °C. Although ice was routinely used for urine collection, it was not considered essential because cineole metabolites are stable at room temperature (S. McLean, pers. comm.), and ice was omitted for the feces collection because of practical difficulties. We determined dry matter from a subset of feces. Volume and pH (using a calibrated, glass-probe pH meter) of thawed urine was subsequently measured for each rabbit at each level of cineole concentration. Animals were removed from the experiment if they lost >20 % of initial body weight. We removed 1 cottontail on day 10 (day 2 of

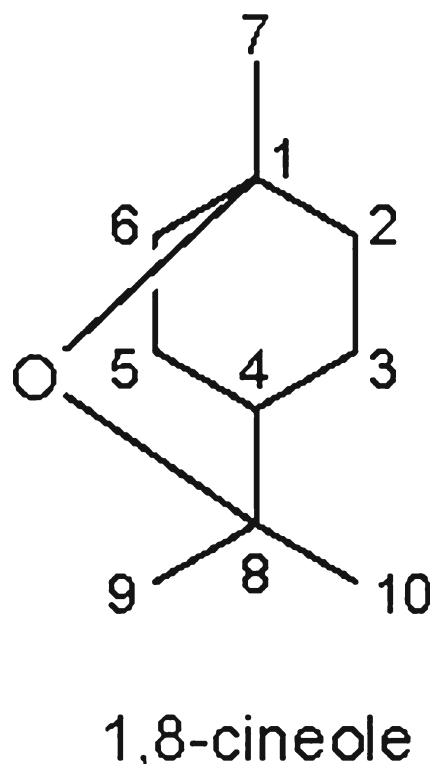


Fig. 2 The common naming and numbering system used throughout this paper for 1,8-cineole and its oxygenated metabolites was based on the *p*-menthane scaffold as shown. Thus, the oxygenated derivatives discussed in the text occur at C2, C3, C7 or C9 and can occur as simple hydroxylations, carboxylations or combinations thereof

5 % cineole trial) and 1 pygmy rabbit on day 13 (day 2 of 7 % cineole trial).

Measuring Cineole in Food and Refusals The amount of cineole in the food offered and refused was quantified by grinding composite samples in liquid nitrogen and extracting 5 times with 2 ml pentane. We used capillary gas chromatography (GC, flame ionization detection, FID) to identify and quantify cineole with a Hewlett-Packard model 5890 Series II gas chromatograph (Palo Alto, CA, USA) using a 30-m \times 0.25-mm id fused-silica capillary column with a 0.25- μ m film of 5 % phenyl-95 % dimethylpolysiloxane (Zebron ZB-5, Phenomenex, Torrance, CA, USA). Cool on-column injection was used with oven programming from 40 °C to 200 °C at 10 °C/min, 200 °C to 300 °C at 40 °C/min. Helium was used as carrier gas and the FID was kept at 350 °C. We used a Hewlett-Packard 6890 Series GC-Mass Spectrometer (GC-MS, similar separation conditions, spectra collected at 70 eV and analyzed using Hewlett-Packard Chemstation software) for product identification by comparing retention times and mass spectra to authentic 1,8-cineole and for further quantitation by using standard curves of the authentic standard.

Measuring Cineole and Cineole Metabolites in Urine and Feces We determined the total amount of free and conjugated cineole metabolites in the urine of pygmy rabbits and cottontails consuming 0, 1, 3, 5, and 7 % cineole diets by using a 2-step procedure following that of Boyle et al. (2000). Samples were run in triplicate. Each sample was enzymatically hydrolyzed, acidified, and extracted twice with 1.5 ml ethyl acetate, with terpinen-4-ol added as an internal standard. Following reduction of the organic phases, methyl ester- and trimethylsilyl derivitization was performed using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and chlorotrimethyl silane (TMCS) as the catalyst according to manufacturer's instructions (Alltech Associates, Deerfield, IL, USA). Cineole metabolites were analysed with GC and GC-MS using authentic standards of 2-, 3-, 9-, and 7-hydroxycineole, 7- and 9-cineolic acid, and 7-hydroxy, 9-cineolic acid that were similarly treated (Table 1, Fig. 2). Tentative identification of cineole metabolites for which no standards were available was based on polarity (retention times) and mass spectra (parent ions and characteristic mass peaks).

To determine the relative proportions of free and conjugated metabolites in both urine and feces, we conducted a two-step procedure using urine (0.5 ml) and feces (0.5 g suspended in 2.5 ml acetate buffer, pH 7) from 4 rabbits of each species that consumed 5 % cineole. Free metabolites were removed by ethyl acetate extraction whereas the remaining conjugated metabolites (in the aqueous extract) were released by enzymatic hydrolyzation; both samples were reduced and derivitized, as described in the previous paragraph, before analysis by GC and GC-MS.

Measuring Unmetabolized Cineole in Feces To measure the amount of unmetabolized cineole in the rabbits' feces, we used headspace analysis on feces from both cineole-free and 5 % cineole diets, because it offered a more sensitive approach for detecting low concentrations of a highly volatile compound. Wet feces were ground, and 100 mg were placed into a 20-ml glass headspace autosampler vial and sealed. A cineole standard was prepared by adding 5 μ l pure cineole to 100 mg (wet weight) of control feces obtained from rabbits on control diets and sealing the vial. Samples were incubated at 100 °C for 30 min in an Agilent 7694 headspace sampler (Agilent Technology, Santa Clara, CA). One ml of headspace gas was injected into a J&W DB-5 capillary column (30 m \times 250 μ m \times 0.25 μ m) installed in an Agilent 6890N GC. Splitless injection mode was used and injector temperature was 250 °C. The flow rate of helium as carrier gas was 1 ml/min. The flame ionization detector was maintained at 300 °C. Chromatographic separation was achieved with a temperature program initially held at 40 °C for 2 min and increased to 150 °C at 15 °C/min and held for 10 min.

Glucuronic Acid and Energy Content of Urine We measured the production of glucuronic acid (μ mol/ml) in the urine of 4 pygmy rabbits and 4 cottontails on cineole-free diets and diets containing 5 % cineole by using the methods of Blumenkranz and Asboe-Hansen (1973). We measured the gross energy content (j/g) of 1–3 ml urine soaked in cotton from the cineole-free diets and diets containing 5 % cineole by using a bomb-calorimeter (IKA C-5000, IKA Works, Inc., Wilmington, NC, USA).

Statistical Analyses All data were reported as mean \pm SE unless otherwise indicated. We examined the effects of cineole concentration and rabbit species on dry matter (g/d/kg^{0.75}) and cineole (mmol/d/kg^{0.75}) intake, production, and composition of urinary metabolites (μ mol/g DM intake/day, μ mol/ μ mol cineole ingested/day), and urine pH using a two-way repeated measures mixed linear model with interactions (PROC MIXED, SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). For significant effects of cineole concentration and interactions, we compared least squares means by using *post hoc* pairwise analyses with $\alpha=0.05$. We compared the proportion of free metabolites (%) in the urine among metabolite types and rabbit species using a two-way analysis of variance with interactions (PROC GLM). We compared the proportion of fecal metabolites (% of total) within pygmy rabbits using a one-way ANOVA, and composition of cineole metabolites between urine and feces in pygmy rabbits using a paired *t*-test, after checking for equal variances (PROC TTEST). We compared the increase in glucuronic acid (μ mol/ μ mol cineole/d) and gross energy (j/ μ mol cineole/d) in the urine between the cineole-free and 5 % cineole diets between the rabbit species by using a two-sample *t*-test.

Table 1 Retention time on capillary gas chromatography (GC) and GC-mass spectrophotometer (GC-MS) and percent detected (mean \pm SE) of the internal standard, cineole and cineole metabolites recovered inurine and feces in pygmy rabbits (PR, *Brachylagus idahoensis*) and cottontail rabbits (CT, *Sylvilagus nuttalli*)

Group	Metabolite	Retention time		Standard available	Percent in urine		Percent in feces	
		(GC-MS)	(capillary GC)		PR	CT	PR	CT
Internal standard	Terpinen-4-ol	9.51	8.71	Yes	–	–	–	–
Cineole C ₁₀ H ₁₈ O	1,8-cineole	6.81	6.23	Yes	0	0	0	0
Hydroxycineoles C ₁₀ H ₁₈ O ₂	3-hydroxycineole	10.28	9.96	Yes	3.0 \pm 1.3	0.7 \pm 0.2	21.8 \pm 3.8	0
	2-hydroxycineole	10.91	10.37	No	20.5 \pm 15.3	9.1 \pm 1.8	24.3 \pm 0.5	0
	9-hydroxycineole	11.30	11.00	Yes	39.2 \pm 8.4	60.6 \pm 1.7	32.5 \pm 2.8	0
	7-hydroxycineole	11.54	11.38	Yes	5.8 \pm 1.4	7.1 \pm 0.4	5.6 \pm 0.5	0
Cineolic acids C ₁₀ H ₁₆ O ₃	9-cineolic acid	12.12	12.03	Yes	9.1 \pm 3.3	10.4 \pm 1.0	3.5 \pm 0.1	0
	7-cineolic acid	12.68	12.73	Yes	6.1 \pm 2.0	1.5 \pm 0.4	2.9 \pm 0.6	0
Major dihydroxycineoles C ₁₀ H ₁₈ O ₃	Unknown A	13.87	13.54	No	3.2 \pm 1.0	1.6 \pm 0.2	0	0
	Unknown B	14.26	13.82	No	4.8 \pm 0.9	3.8 \pm 2.4	3.8 \pm 0.3	0
	Unknown C	14.52	14.14	No	7.9 \pm 1.3	5.4 \pm 0.6	5.6 \pm 0.3	0
Hydroxycineolic acids C ₁₀ H ₁₆ O ₄	7-hydroxy-9-cineolic acid	15.43	15.18	Yes	0	0	0	0

Finally, we examined the effect of cineole intake on urine pH between rabbit species by using analysis of covariance (PROC GLM).

Results

Intake of Cineole Dry matter intake (g/d/kg^{0.75}) of pellets differed by cineole concentration ($F_{4,30}=35.44$, $P<0.001$) and by rabbit species ($F_{1,8}=60.95$, $P<0.001$), and the cineole concentration \times species interaction also was significant ($F_{4,30}=16.97$, $P<0.001$). Dry matter intake of pygmy rabbits remained constant until cineole concentration reached 7 %, whereas dry matter intake of cottontails declined at the lowest level of cineole offered (1 %), and continued to decline as cineole concentration increased (Fig. 3a). Pygmy rabbits consumed more pellets in relation to metabolic body mass at all cineole concentrations than did cottontails (Fig. 3a). Voluntary intake of cineole (mmol/d) through pellet consumption varied with cineole concentration ($F_{4,30}=147.32$, $P<0.001$) and rabbit species ($F_{1,8}=55.84$, $P<0.001$), and the cineole concentration \times species interaction also was significant ($F_{4,30}=40.37$, $P<0.001$). Cineole intake increased with cineole concentration in the pellets for pygmy rabbits, peaking at 5 % cineole (Fig. 3b). Cineole intake of cottontails also increased with cineole concentration, but at a lower rate than pygmy rabbits (Fig. 3b). Pygmy rabbits voluntarily consumed 2–4 times more cineole in relation to metabolic body mass than cottontails at all concentrations of cineole (Fig. 3b). The average loss

of cineole from pellets from the time fed to the time refusals were collected was 11.1 % (SD=1.9 %).

Cineole Metabolites in Urine and Feces No unmetabolized 1,8-cineole was detected in the urine or feces of either pygmy rabbits or cottontails when consuming diets of any cineole concentration. We classified cineole metabolites detected in the urine and feces according to the extent of oxidation they had undergone and the functional groups they acquired during oxidation (i.e., hydroxycineoles = 1 oxygen, cineolic acids = 2 oxygens, dihydroxycineoles = 2 oxygens, and hydroxycineolic acids = 3 oxygens, Table 1, Fig. 2). We confirmed the presence of 2-, 3-, 7-, and 9-hydroxy cineole, and 7- and 9-cineolic acid, which had been previously identified in brushtail possums consuming cineole (Boyle et al., 2000), in the urine from both species of rabbits and in the feces of pygmy rabbits (Table 1). However, we did not find 7- hydroxy-9 cineolic acid, or many other unidentified dihydroxycineoles and hydroxycineolic acids also found in possums (Boyle et al., 2000), in the urine or feces of either rabbit species. We also detected 3 major unidentified cineole metabolites (dihydroxycineoles A, B, and C) in the urine of both pygmy rabbits and cottontails, which were most likely dihydroxycineoles based on their mass spectra and parent ions (Boyle et al., 2000). However, only dihydroxycineoles B and C were detected in the feces of pygmy rabbits (Table 1). Using the GC-MS, we detected up to 5 additional minor peaks that may have been dihydroxycineole or hydroxycineolic acids, but quantitation and absolute identification were not possible

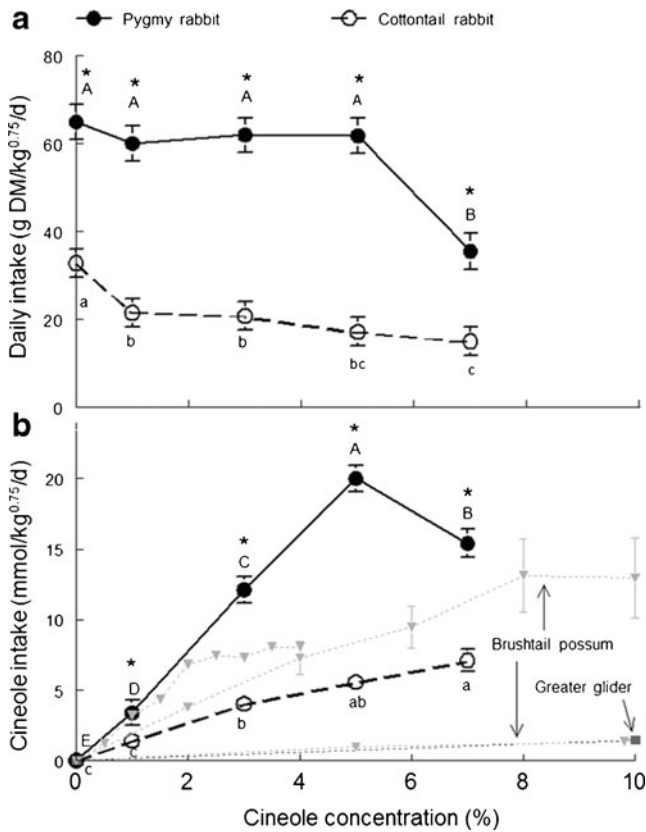


Fig. 3 Mean daily dry matter (DM) intake **a** and cineole intake **b** in relation to metabolic body mass in pygmy rabbits (*Brachylagus idahoensis*, solid circles and line, capital letters) and cottontails (*Sylvilagus nuttalli*, open circles and dashed line, lower case letters) as a response to cineole concentration of a pelleted diet. Intake in relation to metabolic body mass for brushtail possums (*Trichosurus vulpecula*, gray diamonds, Lawler et al., 1999; Boyle and McLean, 2004; DeGabriel et al., 2009) and greater gliders (dark gray square, *Petauroides volans*, Lawler et al., 1999) are provided for comparison. Each line represents a distinct study. Different letters denote significant differences among cineole concentrations within rabbit species and asterisks denote a significant difference between pygmy rabbits and cottontails for each cineole concentration ($\alpha=0.05$)

because of low quantities. An average of 28.1 ± 4.3 % of ingested cineole was recovered as cineole metabolites in the urine and 3.8 ± 0.3 % as metabolites in feces of pygmy rabbits, whereas only 15.7 ± 4.2 % was recovered in the urine, and no metabolites were recovered in the feces of cottontail rabbits. Therefore, we recovered twice as much of the ingested cineole in pygmy rabbits than in cottontails ($t=2.79$, $df=6$, $P=0.02$).

The total amount (μmol) of cineole metabolites produced in the urine in relation to food consumed increased with increasing cineole in the diet for both rabbit species ($F_{3,17}=10.05$, $P<0.001$, Fig. 4a). Each individual cineole metabolite ($P<0.05$), and the amount of all hydroxycineoles ($F_{3,17}=10.24$, $P=0.02$), cineolic acids ($F_{3,17}=56.45$, $P<0.001$), and dihydroxycineoles ($F_{3,17}=28.16$, $P<0.001$),

increased in the urine as the cineole concentration in the diet increased (Fig. 4a). However, the total amount of cineole metabolites, groups of metabolites, and individual cineole metabolites produced per μmol of cineole consumed remained constant with increasing concentration of cineole in the diet ($P>0.05$). Although pygmy rabbits tended to produce more total metabolites ($F_{1,6}=4.24$, $P=0.08$) and hydroxycineoles ($F_{1,6}=4.66$, $P=0.07$) in relation to ingested cineole than did cottontails (Fig. 4b), differences were not statistically significant. However, pygmy rabbits produced 4–5 times more cineolic acids ($F_{1,6}=5.75$, $P=0.05$), 3-hydroxy- ($F_{1,6}=11.02$, $P=0.02$), 2-hydroxycineole ($F_{1,6}=15.54$, $P=0.008$), and 7-cineolic acid ($F_{1,6}=27.99$, $P=0.002$) per μmol cineole consumed than did cottontails, and there was a cineole concentration \times species interaction for dihydroxycineole A ($F_{3,17}=8.02$, $P=0.002$) and C ($F_{3,17}=3.73$, $P=0.03$).

Extent of Oxidation of Cineole Metabolites The proportion of each individual metabolite, and groups of metabolites, in the urine remained constant across all cineole concentrations (all $P>0.05$, Fig. 4b), and there were no species \times cineole concentration interactions (all $P>0.05$). In their urine, pygmy rabbits ($\bar{X} = 69.8 \pm 3.1\%$) and cottontails ($\bar{X} = 77.3 \pm 0.7\%$) produced the highest proportion of hydroxycineoles, the least oxidized cineole metabolites with one added oxygen, followed by cineolic acids (pygmy rabbits: $\bar{X} = 14.9 \pm 2.6\%$; cottontails: $\bar{X} = 12.0 \pm 0.7\%$), and dihydroxycineoles, both with 2 added oxygens (pygmy rabbits: $\bar{X} = 15.4 \pm 0.6\%$, cottontails: $\bar{X} = 10.7 \pm 1.0\%$, Table 1, Fig. 5). As in their urine, pygmy rabbits produced the greatest proportion of their fecal metabolites as hydroxycineoles ($\bar{X} = 84.3 \pm 0.4\%$, ($F_{1,7}=52.57$, $P<0.001$), with much lower proportions of cineolic acids ($\bar{X} = 6.4 \pm 0.6\%$) and dihydroxycineoles ($\bar{X} = 9.3 \pm 0.5\%$, Table 1, Fig. 5). Fecal metabolites comprised 19.8 ± 3.8 % of the total metabolites, 23.6 ± 5.4 % of the total hydroxycineoles, 8.9 ± 1.4 % of the total cineolic acids, and 12.9 ± 2.8 % of the total dihydroxycineoles produced by pygmy rabbits in the urine and feces combined (Table 1, Fig. 6).

Pygmy rabbits produced a 30 % greater proportion (30 ± 3.1 %) of more highly-oxidized cineole metabolites (cineolic acids and dihydroxycineoles) relative to less-oxidized metabolites (hydroxycineoles) in their urine than did cottontails (22.7 ± 0.7 %, $t=2.43$, $df=6$, $P=0.04$). Specifically, 3-hydroxycineole ($t=3.50$, $df=6$, $P=0.04$), 2-hydroxycineole ($t=7.13$, $df=6$, $P<0.001$), and 7-cineolic acid ($t=4.57$, $df=6$, $P=0.02$) composed a greater proportion of total metabolites in pygmy rabbits than in cottontails, whereas 9-hydroxycineole ($t=5.07$, $df=6$, $P=0.01$), and dihydroxycineole C ($t=4.20$, $df=6$, $P=0.006$) composed a greater proportion of total

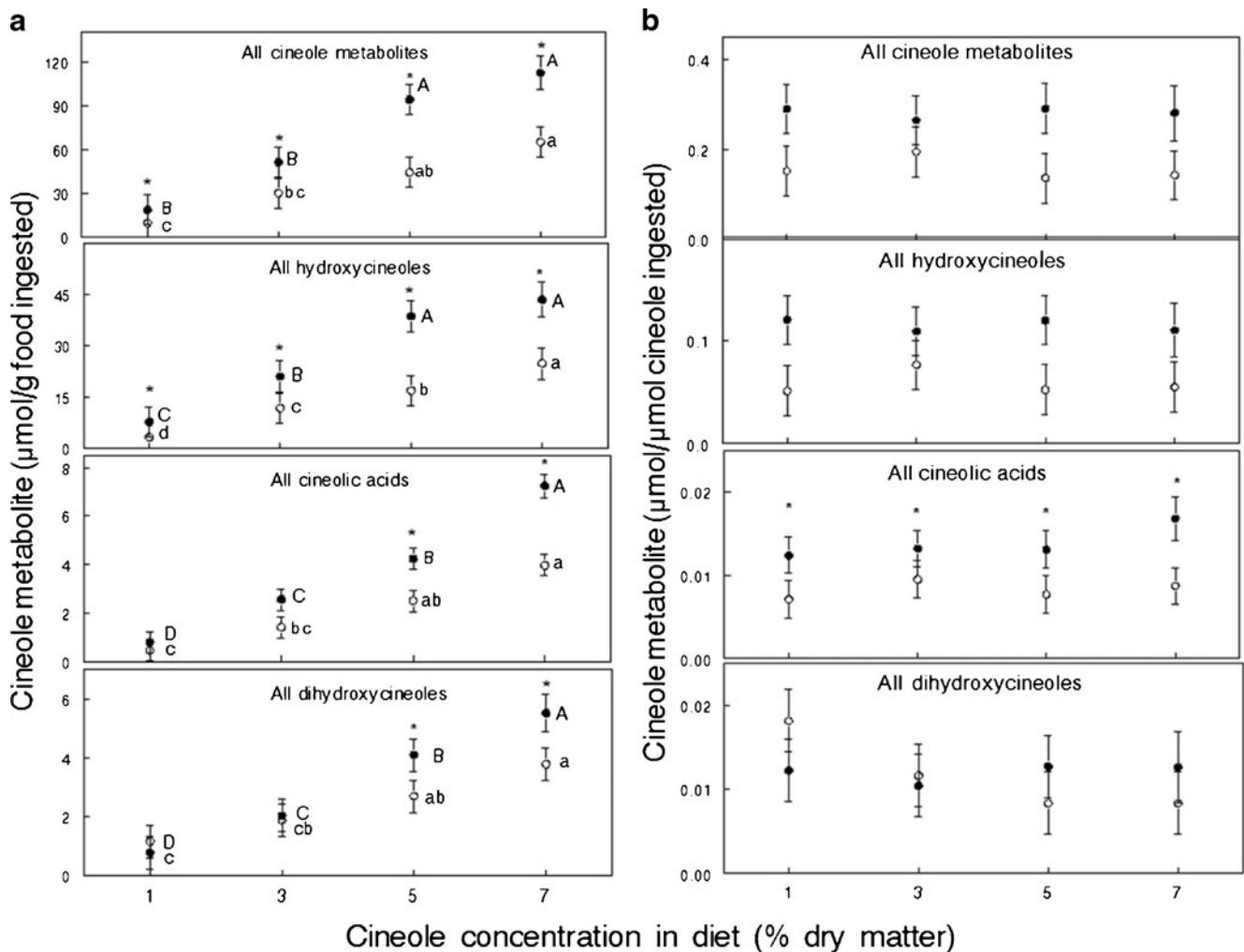
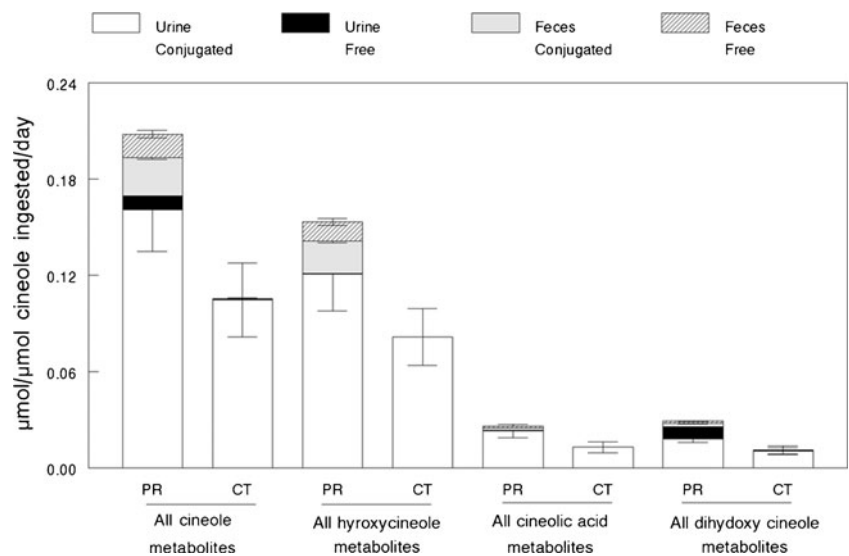


Fig. 4 Mean amount of cineole metabolites in urine produced per day **a** relative to food pellet intake, and **b** relative to cineole ingested by pygmy rabbits (*Brachylagus idahoensis*, solid circles, capital letters) and cottontails (*Sylvilagus nuttalli*, open circles, lower case letters) as

the concentration of cineole increased in their pelleted diet. Different letters denote significant differences among cineole concentration within rabbit species, and asterisks denote significant differences between rabbit species ($\alpha=0.05$)

Fig. 5 The amount of conjugated and free cineole metabolites produced in the urine and feces of pygmy rabbits (PR, *Brachylagus idahoensis*) and cottontails (CT, *Sylvilagus nuttalli*) per day when rabbits were consuming pellets containing 5 % cineole



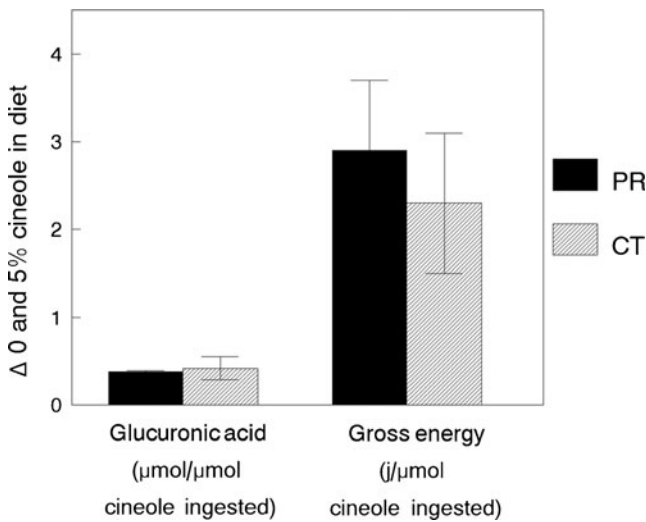


Fig. 6 Change in glucuronic acid and gross energy in the urine of pygmy rabbits (PR, *Brachylagus idahoensis*, solid bars) and cottontail rabbits (CT, *Sylvilagus nuttalli*, hatched bars) between diets of cineole-free (0 %) food pellets and pellets with 5 % cineole concentration in relation to cineole ingested. Glucuronic acid and gross energy in urine did not differ between rabbit species ($\alpha=0.05$)

metabolites in cottontails than in pygmy rabbits. Fecal metabolites of pygmy rabbits were less oxidized than urinary metabolites, with a significantly lower proportion of cineolic acids and dihydroxycineoles ($\bar{X} = 15.6 \pm 0.3\%$) than in the urine ($\bar{X} = 22.5 \pm 0.6\%$, $t=4.85$, $df=6$, $P=0.003$).

Extent of Conjugation of Cineole Metabolites The proportion of cineole metabolites in the urine that were free (unconjugated) rather than conjugated varied with specific metabolite ($F_{2, 24}=242.74$, $P<0.001$) and rabbit species ($F_{1, 24}=156.52$, $P<0.001$), and the species \times metabolite interaction was significant ($F_{1, 24}=120.76$, $P<0.001$). A greater proportion of dihydroxycineoles in the urine was free (pygmy rabbits: $\bar{X} = 30.0 \pm 0.7\%$; cottontails: $\bar{X} = 5.5 \pm 1.6\%$) than of cineolic acids (pygmy rabbits: $\bar{X} = 1.5 \pm 0.1\%$; cottontails: $\bar{X} = 1.5 \pm 0.1\%$) and hydroxycineoles (pygmy rabbits: $\bar{X} = 2.0 \pm 0.1\%$; cottontails: $\bar{X} = 0 \pm 0\%$) in both pygmy rabbits ($F_{2, 11}=375.4$, $P<0.001$) and cottontails ($F_{2, 11}=10.19$, $P=0.005$, Fig. 5). Pygmy rabbits produced an average of $5.2 \pm 0.3\%$ free metabolites in urine overall, a proportion 6 times greater than that of cottontails, which averaged only $0.7 \pm 0.1\%$ free metabolites ($t=14.58$, $df=6$, $P<0.001$). More specifically, pygmy rabbits produced a greater proportion of free hydroxycineoles ($F_{1, 7}=42.42$, $P<0.001$) and free dihydroxycineoles ($F_{1, 7}=193.1$, $P<0.001$) in their urine than did cottontails (Fig. 5). The proportion of cineole metabolites that were free in the feces of pygmy rabbits averaged $37.3 \pm 3.6\%$; but did not differ among metabolite types ($F_{2, 6}=2.73$, $P=0.14$,

Fig. 5). However, a greater proportion of fecal metabolites vs. urinary metabolites was free ($t=10.65$, $df=5$, $P<0.001$, Fig. 5).

Glucuronic Acid, Gross Energy, and Urine pH Pygmy rabbits and cottontails had a similar increase in the amount of glucuronic acid ($\mu\text{mol}/\mu\text{mol}$ cineole/d, $t=0.28$, $df=6$, $P=0.80$) and gross energy ($\text{J}/\mu\text{mol}$ cineole/d, $t=0.55$, $df=5$, $P=0.61$) in the urine when consuming 5 % cineole diet compared to the cineole-free diet (Fig. 6). Likewise, urine pH differed by cineole concentration ($F_{4, 27}=14.10$, $P<0.001$), but not by rabbit species ($F_{1, 8}=0.40$, $P=0.51$) nor was there a cineole \times species interaction ($F_{4, 27}=0.14$, $P=0.97$). pH remained constant as cineole increased from 0 % to 3 %, but was significantly lower for both species at cineole concentration of 5 and 7 % than at other concentrations (Fig. 7a). Similarly, pH declined with increasing cineole intake (mmol/d), ($F_{1, 44}=12.87$, $P<0.001$) but this relationship did not differ between rabbit species ($F_{1, 44}=0.69$, $P=0.41$), nor was there a cineole \times species interaction ($F_{1, 44}=1.47$, $P=0.23$, Fig. 7b).

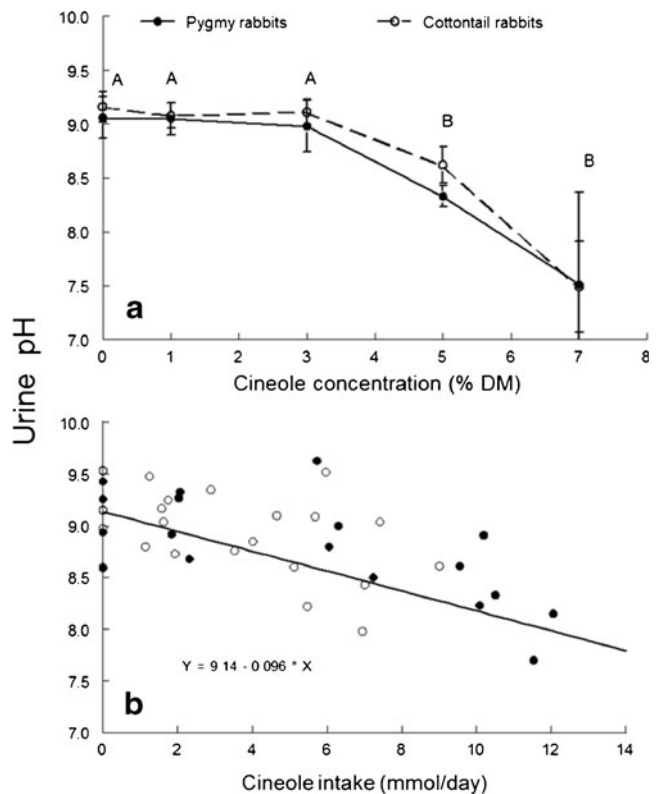


Fig. 7 The relationship between urine pH and **a** cineole concentration of the pelleted diet, and **b** amount of cineole ingested by pygmy rabbits (*Brachylagus idahoensis*, closed circles) and cottontail rabbits (*Sylvilagus nuttalli*, open circles). Different letters denote significant differences in pH among cineole concentrations. pH did not differ between rabbit species ($\alpha=0.05$)

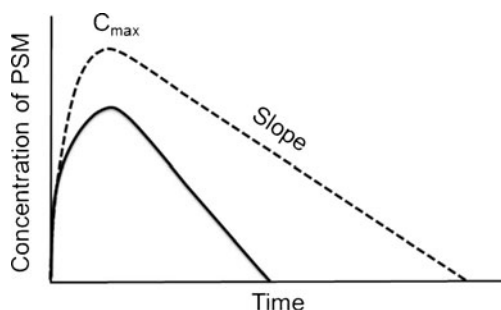


Fig. 8 A theoretical schematic of the systemic concentration-time course of cineole for pygmy rabbits (*Brachylagus idahoensis*, solid line) and cottontails (*Sylvilagus nuttalli*, dashed line) following ingestion of the same dose of cineole. The proposed lower absorption of ingested cineole in the intestine by pygmy rabbits would result in a lower maximum concentration (C_{\max}). The proposed faster or more complete excretion of cineole metabolites by pygmy rabbits would increase the elimination rate (slope) of cineole. Both of these proposed mechanisms would lower the overall systemic exposure (area under the concentration-time curve) to cineole for pygmy rabbits compared to cottontails

Discussion

Compared to generalist cottontails, pygmy rabbits, which specialize on diets of sagebrush that contain high levels of monoterpenes, were able to consume and detoxify high levels of cineole, by using many, but not all, of the hypothesized strategies (Fig. 1). Pygmy rabbits were able to maintain their intake of pellets until cineole content exceeded 5 %, nearly twice the total monoterpene content of sagebrush (White et al., 1982; Shipley et al., 2006), whereas cottontails immediately reduced intake at only 1 % cineole (Fig. 1). As a result, pygmy rabbits consumed a maximum of about 20 mmol of cineole/d/kg^{0.75} for 3 days. In marsupial folivores, both a generalist (brushtail possum, Lawler et al., 1999; Boyle and McLean, 2004; DeGabriel et al., 2009) and a specialist (greater glider, *Petauroides volans*, Lawler et al., 1999) of *Eucalyptus*, which also contains high levels of cineole, began reducing their intake when fed diets of 2–3 % cineole (Fig. 3a).

Pygmy rabbits, like cottontails, brushtail possums, and koalas (specialists on *Eucalyptus*, Boyle et al., 2001), did not avoid absorption and detoxification of cineole by excreting it unchanged in their feces as we had hypothesized (Fig. 1). In contrast, Stephens woodrats (*Neotoma stephensi*) that specialize on one-seeded juniper (*Juniperus monosperma*) had a concentration of the monoterpene α -pinene 5 times lower in their bloodstream (Sorensen and Dearing, 2003) and excreted 3 times more α -pinene in their feces when consuming juniper and α -pinene than did the generalist woodrat (*N. albigula*, Sorensen et al., 2004). However, pygmy rabbits did have an unexpectedly high concentration of metabolized cineole in their feces, whereas none was detected in the feces of our cottontails or in

koalas (Boyle et al., 2001). Although cineole metabolites also have been found in brushtail possum feces, they constituted only 6.5 % of all cineole metabolites (Boyle et al., 2000) compared to nearly 20 % in pygmy rabbits (Fig. 5). Fecal metabolites represented a major avenue of elimination of cineole in pygmy rabbits, which has been a hypothesized (Sorensen and Dearing, 2006, Fig. 1), but previously undocumented, difference between specialist and generalist herbivores.

Cineole metabolites in the feces of pygmy rabbits may have originated from microbial, intestinal, or hepatic detoxification (Fig. 1). For example, monoterpenes are readily metabolized by fiber-adapted microflora in the rumens of Mediterranean dairy goats (Malecky and Broudiscou, 2009). Microbes also inhabit the cecum and the mucosal lining of the intestine before the cecum in many animals (Yamamoto et al., 2009; Angert et al., 2010) and, therefore, may contribute to detoxification of PSMs before absorption in hind-gut fermenting-herbivores like pygmy rabbits. The location and function of intestinal microbes deserves the recent attention by ecologists as a mechanism of tolerance to PSMs in herbivores (Kohl et al., 2011).

Alternatively, metabolites of cineole in the feces of pygmy rabbits may be formed by detoxification enzymes in the liver and excreted back into the intestine via the bile or formed by detoxification enzymes in the enterocytes of the gut. Several PSMs are excreted primarily from the liver into the bile and excreted in the feces (Grigoleit and Grigoleit, 2005; Barnes et al., 2011). In addition, relatively high activity of the enzyme Cytochrome P450 3A (CYP3A), which metabolizes monoterpenes like 1,8-cineole (Miyazawa et al., 2001), has been found in the duodenal tissue of brushtail possums (Sorensen et al., 2007). The role of these detoxification enzymes in the intestine in facilitating tolerance to PSMs deserves further attention.

Despite a relatively large amount of cineole metabolites in the feces, the majority of cineole ingested by pygmy rabbits was presumably detoxified in the liver and excreted in the urine. Although only 3- and 2-hydroxycineoles have been identified previously in domestic rabbit urine (Miyazawa et al., 1989), pygmy rabbits and cottontails produced all of the hydroxycineoles and cineolic acids identified in brushtail possum urine, but few of the dihydroxycineoles and no hydroxycineolic acids. We did not detect an induction of detoxification pathways in the rabbits during our trial, because excretion rate relative to cineole ingested and composition of metabolites remained constant over 15 days and 4 levels of cineole in the diet (Fig. 4b).

To a limited degree, pygmy rabbits excreted cineole metabolites that were more oxidized and less conjugated than did cottontails, and thus they used less energetically expensive methods to detoxify cineole, as we had hypothesized (Fig. 1). Both rabbit species produced mono- and

dioxygenated metabolites, but pygmy rabbits excreted over 30 % more metabolites as dioxygenated compounds than did cottontails. However, both rabbit species excreted much less of the highly-oxidized cineole metabolites in their urine than did the generalist brushtail possum and the specialist koala (Boyle et al., 2000, 2001). Brushtail possums and koalas excreted between 60 % and 85 % of the trioxygenated cineole metabolites (i.e., hydroxycineolic acids), and 9 % of the koala's urinary metabolites were tetraoxygenated (i.e., dicineolic acids), neither of which were detected in the urine or feces of either rabbit species. Also, brushtail possums excreted less of the least oxidized metabolites (hydroxycineoles) in the feces than urine, whereas pygmy rabbits excreted more of the least oxidized metabolites in the feces than urine.

Similarly, pygmy rabbits excreted a greater proportion (5 %) of their urinary metabolites in the unconjugated, free form, than did cottontails (0.7 %), and over a third of their fecal metabolites were free. In comparison, brushtail possums have been shown to excrete over 3/4 of their urinary metabolites, and all of their fecal metabolites, in the free form (Boyle et al., 2000). Thus, both rabbit species used the relatively more energetically expensive methods of detoxification (conjugation vs. oxidation) compared to the *Eucalyptus*-eating marsupials. Although conjugation is thought to be more energetically expensive than oxidation (Boyle et al., 1999, 2000), it generally proceeds faster and results in metabolites that are more readily excreted than oxidation reactions (Casarett et al., 2008). Moreover, conjugation may not pose a large energetic cost if substrates for these pathways are readily available in the diet (Dash, 1988). Because they were able to consume and metabolize greater quantities of 1,8-cineole than cottontails, and even marsupial folivores, yet produced similar amounts of urinary energy and glucuronic acid relative to cineole consumption, pygmy rabbits seem to rely on capacity rather than efficiency for coping with monoterpenes in their diet.

We recognize that we measured the fate of only one of the many PSMs in sagebrush. Cineole is not necessarily the most toxic PSMs in sagebrush nor the most difficult to eliminate. Other monoterpenes such as artemiseole and methacrolein recently have been identified as influencing diet selection by pygmy rabbits more than cineole (Ulappa, 2011). Identification of the detoxification pathways of other PSMs in sagebrush deserves additional attention.

Our relatively low (~46 %) recovery of cineole in urine and feces suggests that we may have missed some important fates of ingested cineole. First, we may have overestimated intake of cineole in our rabbits. Approximately 11 % of the cineole added to the food pellets evaporated over the 8-h periods between refreshing the pellets offered, and this loss could account for a small portion of the cineole not recovered. If present, a small amount of unmetabolized

cineole also may have volatilized from fecal pellets during the day and further lowered recovery. In addition, some of the cineole likely volatilized during chewing and was absorbed and released in the breath of rabbits. White et al. (1982) found that more than 90 % of the expected cineole in sagebrush consumed by wild pygmy rabbits was lost, presumably from the breath, by the time the ingesta reached the stomach. Similar losses of volatile monoterpenes from the contents of the upper intestine compared to the diet have been observed in sage grouse (Welch et al., 1989) and mule deer (Cluff et al., 1982). However, these studies did not quantify the amount of monoterpenes consumed, nor did they account for possible absorption or detoxification of monoterpenes before the small intestine.

An additional explanation for our low recovery of cineole may be caused by recycling of metabolized cineole through enterohepatic recirculation (Fig. 1, Grigoleit and Grigoleit, 2005) or ingestion of cecal droppings. Both of these processes could slow the elimination of ingested cineole and reduce its recovery. Enterohepatic recirculation may explain why concentrations of metabolites of cineole consumed by brushtail possums were highest in the feces two days after feeding of cineole stopped (Boyle et al., 2000). Unlike brushtail possums, rabbits are cecotrophic, and this behavior could further lengthen the time for completely eliminating cineole. Future studies should measure urinary and fecal excretion of cineole until metabolites are no longer detected, and under conditions where cecal droppings can be investigated.

Finally, we may not have detected some cineole metabolites because they may have been conjugated to another polar molecule. Although glucuronidation is the major pathway of detoxification in mammals (Casarett et al., 2008), it may receive biased attention from ecologists because glucuronidated metabolites are relatively easy to measure (Blumenkranz and Asboe-Hansen, 1973). Many substrates that undergo glucuronidation also undergo sulfonation, and several monoterpenes can be sulfonated (Welniak, 2003). Monoterpenes also may be conjugated to amino acids such as glycine, glutamine, taurine, serine, and proline and the tripeptide glutathione (Casarett et al., 2008). Several of the enzymes responsible for these alternative conjugation pathways can be induced in herbivores following ingestion of PSMs and can differ among dietary specialists and generalists (Haley et al., 2007; Skopec et al., 2007). Results from these *in vitro* assays indicate a need to use advanced bioanalytical techniques (e.g., Liquid Chromatography and Mass Spectrometry) to investigate a wider range of conjugation metabolites excreted by herbivores consuming PSMs.

In summary, this study suggests that pygmy rabbits have a greater capacity to minimize systemic exposure to cineole than cottontails by minimizing absorption and maximizing detoxification of ingested cineole. Although cineole was not excreted unchanged in the feces, excretion of its metabolites

in the feces formed by either microbial or rabbit enzymes in the gastrointestinal tract represents lower absorption, and thus lower systemic exposure to cineole (per dose consumed, Fig. 8). Second, greater excretion of cineole metabolites in urine relative to cineole dose by pygmy rabbits compared to cottontails would further lower systemic concentration of unchanged cineole and its metabolites. However, mechanisms that lower systemic exposure to cineole may come with a higher cost in pygmy rabbits than in cottontails in the form of excretion of glucuronic acid and energy. We propose that the benefits associated with energetically expensive mechanisms that minimize absorption and maximize detoxification and excretion allow pygmy rabbits to specialize on chemically defended sagebrush.

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