Comparison of leaf terpenoids and tannins in *Juniperus monosperma* from woodrat (*Neotoma stephensi*) browsed and non-browsed trees

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ABSTRACT

Neotoma stephensi is the only vertebrate herbivore to specialize on *Juniperus* that is heavily defended by terpenes and tannins. A comparison between heavily, woodrat (*Neotoma stephensi*) browsed and non-browsed *Juniperus monosperma* trees revealed that the percentage of total volatile leaf oil yields was not significantly different between browsed trees (0.53%, 2hr dist., DM-basis) and non-browsed trees (0.57%, 2hr dist., DM basis). Only one terpene, p-cymene, was significantly different between browsed and non-browsed trees. Condensed tannins did not differ significantly between browsed (3.36%) and non-browsed trees (3.02%). Woodrats, in this population, may be selecting browsed trees on convenience, proximity to their midden, and safety from predators. Published on-line www.phytologia.org *Phytologia 96(2): 63-70 (April 1, 2014).*

KEY WORDS: *Juniperus monosperma, Neotoma stephensi,* woodrats, browsing, terpenes, tannins, diet selection.

Seminal papers in the 1970s (Rhoades and Cates, 1976; Cates and Rhoades, 1977; Feeny, 1976) enlightened biologists that plants produce defensive compounds against herbivores. Terpenes and tannins are two types of compounds produced by juniper that are known to deter herbivores (Bernays et al. 1989; Gershenzon and Dudareva 2007). Terpenes can act as feeding deterrents (Gershenzon and Dudareva 2007) and have numerous toxic actions such as central nervous system depression, contact dermatitis, lung function impairment, liver and kidney cysts and even death (Sperling et al., 1967; Savolainen, 1978; Falk et al., 1990) as well as alter microbial fermentation (Schwartz et al. 1980 a,b, Nagy et al. 1964). Tannins readily form complexes with protein that decrease the palatability of forages (McArthur et al. 1995), reduce digestive enzyme function (Pridham, 1963), and alter microbial fermentation (Lowry et al. 1996), which can lead to decreased growth rates (Mole et al. 1993). In order to consume chemically defended plants, vertebrate herbivores' may choose plants with lower levels of defensive compounds, eat a variety of plants to avoid toxic levels of any one compound, or have efficient mechanisms to deal with defensive compounds (Freeland and Janzen 1974).

Neotoma stephensi is a small mammalian herbivore (~250g) and is the only vertebrate to specialize on juniper. Vaughan (1982) investigated the feeding behavior of *Neotoma stephensi* from several sites dominated by *Juniperus monosperma* and *J. californica*. He reported that juniper leaves accounted for over 90% of the diet in woodrats. Vaughan (1982) suggested that woodrats have fine-tuned foraging abilities to select trees with low levels of defensive chemicals as seen in other pine specialists like *Sciurus abert* (Abert's squirrel, Snyder 1992). More recent evidence suggests that *Neotoma*

stephensi has highly efficient physiological mechanisms to effectively deal with the terpenes present in *Juniperous monosperma,* especially when compared to a sympatric generalist like *Neotoma albigula* that can only consume ~25% of its diet as juniper (Boyle and Dearing 2003; Sorenson et al. 2004; Skopec et al. 2007; Skopec and Dearing 2011; Torregrossa et al. 2011)

Considering the amount of research on the specialist woodrat (*N. stephensi*), it is surprising that we could find no publication concerning the composition of *J. monosperma* leaves from browsed trees vs. non-browsed trees. The purpose of this paper is to present new data on leaf volatile oils and condensed tannins from *J. monosperma* leaves from *N. stephensi* browsed and non-browsed trees.

MATERIALS AND METHODS

Plant material: Juniperus monosperma -

2008: Skopec 1-10, not- browsed, 11-20, browsed (= lab acc. *Adams 13867-13876*, heavily browsed; *Adams 13877-13886*, not browsed), common lava cinders, 35° 26.708' N; 111° 21.572' W, elev. 5290 ft, March, 2007, Coconino Co., AZ

2013: Adams (with A. Allgood and D. Thornburg) re-collected from the same site as Skopec, *Adams 13920-13929*, heavily browsed; *Adams 13930-13939*, not browsed, common lava cinders, 35° 26.708' N; 111° 21.572' W, elev. 5290 ft, 27 June 2013, Coconino Co., AZ

Herbarium vouchers are deposited in the herbarium, Baylor University (BAYLU).

Essential oils analysis - A portion (200 g FW) of the fresh foliage was kept cool (20ºC) and in the dark, then, exhaustively steam-distilled for 24 h using a modified circulatory Clevenger-type apparatus (Adams 1991). Oil samples were concentrated (diethyl ether trap-removed) with nitrogen and stored at -20ºC until analyzed. Steam distilled leaves were oven dried to a constant weight (48 hr, 100ºC) for the determination of oil yield as [oil wt./(oil wt. + oven dried extracted foliage wt.)]. The extracted oils were analyzed on a HP5971 MSD mass spectrometer: 0.2 ul of a 10% solution (in diethyl ether) oil injected, split, 1:10, temperature programmed, linear, 60º - 246ºC at 3ºC/min. (62 mins.), carrier gas He, flow 34.96 cm/sec or 1.02 ml/min, injector 220ºC, detector 240ºC, scan time 1/sec, directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25-micron coating thickness, fused silica capillary column (see Adams 2007, p. 4, for detailed operating conditions). Identifications were made by searches of our volatile oil library (Adams 2007) using HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantification was by flame ionization detector on an HP 5890 gas chromatograph operated under the same conditions as the GCMS (above) using the HP Chemstation software.

Condensed tannin analysis - Condensed tannins in air dried (48 hr, 42ºC) leaves were assayed for ECT, PCT, and FCT fractions by methods described by Terrill et al. (1992). Samples were oven-dried and standards prepared from Ashe juniper as recommended by Wolfe et al. (2008).

Statistical analyses - Terpenoids (as percentage of total oil and as mg per g dry foliage weight) were compared among the samples by ANOVA and SNK (Student-Newman-Keuls) analyses as described by Steele and Torrie (1960). Differences were considered significant at $P \leq 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

Facing the uncertainty of chemical changes in leaf samples collected in 2008 and stored (-20°C) for 5 years, the population was re-sampled in 2013. The yields of volatile leaf oils (terpenoids) in browsed (0.53%) and non-browsed (0.57%) was not significantly different for 2008 samples, nor for 2013 samples (0.56, 0.57% ns, Table 1). Likewise, for the condensed tannins, browsed vs. non-browsed, 2007:

3.36, 3.02%, ns; 2013: 3.41, 2.91%, ns, Table 1). No differences in oils or tannins were found between the fresh (2013) and 5 yr. old samples (2008).

A detailed compositional analysis of *J. monosperma* volatile leaf oils from browsed and non-browsed trees is shown in Table 1. ANOVA of the leaf volatile oils components (% total oil basis) for browsed and non-browsed trees revealed only p-cymene was significantly different (Table 1). Analysis on a mg/g basis found no significant differences in any terpenoid between browsed and non-browsed samples (Table 1).

From an examination of *J. monosperma* trees in the field, there did not appear to be any mature trees that were complete avoided (not browsed). Although, the 10 trees collected as 'non-browsed' were clearly scarcely touched (Fig. 1) and the 10 'browsed' trees were very heavily browsed (Fig. 2). In every case, a midden was present in the browsed trees sampled in this study.

Figure 1. Non-browsed *J. monosperma* tree. The ground cover is lava cinders.

Figure 2. Heavily browsed *J. monosperma* tree. A woodrat midden is under the tree at ground level.

The lack of differences in the volatile leaf oils and yield of condensed tannins between browsed and non-browsed trees was surprising in view of the report of Vaughn (1982) about the selection of trees. However, it is instructive to compare browsing (mostly goats) on two juniper species growing in the same population. For *J. ashei*, Adams et al. (2013a) found the browsers selected for lower leaf oil yield. But, in a companion study of browsed *J. pinchotii* (in the same population with *J. ashei* in the 2013a study), Adams et al. (2013b) found no significant difference in % oil yield, nor in condensed tannins or any measure of *in vitro* digestion except for neutral detergent fiber (NDF) and acid detergent fiber (ADF). Adams et al. (2013b) hypothesized that the goats (principal browser) did not select trees on any chemical character but rather used the trees for shade and congregated at trees, then took a few bites, and over time, these 'community meeting' trees became progressively more browsed.

The severe browsing by woodrats at the Arizona *J. monosperma* site (Fig. 2) suggests that proximity to the midden may be a major factor in selecting browse trees. A few young junipers about 1 to 1.5 m tall were seen in the population and none of these appeared to have been browsed. Analysis of the oil yields and condensed tannins showed no difference from older trees, either browsed or not. It may be that the small trees do not offer the woodrats sufficient cover from predators that larger tree affords.

In the present population studied, there is very little variation in yields of leaf oils composition or the concentration of condensed tannins among the *J. monosperma* trees and, thence, scant opportunity for woodrats to select for or against leaf oils or tannins. Of course, there are thousands of chemicals in leaves, so some other chemical may be selected for (or against) such as nitrogen or fiber. Or the juniper specialists unique physiological mechanisms for metabolizing terpenes (Boyle and Dearing 2003; Sorenson et al. 2004; Skopec et al. 2007; Skopec and Dearing 2011; Torregrossa et al. 2011) may be so efficient that they don't select trees to browse on for a biochemical reasons but, instead are selecting trees based on convenience, proximity to their midden and safety from predators.

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Table 1. Comparison of leaf oils obtained from woodrat browsed and non-browsed trees of *J. monsperma*. F, signif. = F ratio and significance, P= 0.05 = *; ns = non significant, nt = not tested. p-cymene, the only compound with a significant difference, is in boldface.

	Factor tested	browsed %	non-browsed %	F, signif.	browsed mg/g DW	non-browsed mg/g DW	F, signif.
	oil yields, 2h dist. (% DW & mg/g DW)						
	March, 2008	0.53%	0.57%	3.65 ns	5.30 mg	5.74 mg	3.65 ns
	June, 2013	0.56%	0.57%	0.08 ns	5.55 mg	5.74 mg	0.08 ns
	total % condensed tannins(TCT):						
	March, 2008	3.36%	3.02%	t=1.4	P= 0.26 ns		
	June, 2013	3.41%	2.91%	t=1.5	P= 0.15 ns		
	Leaf volatile terpenoids						
KI	Compound	browsed %	non-browsed %	F sig	browsed mg/g	non-browsed mg/g DW	F sig
921	tricyclene	0.1	0.2	nt	t	t	nt
924	α-thujene	t	t	nt	t	t	nt
932	α-pinene	51.3	60.0	4.228 ns	2.80	3.51	2.091 ns
945	α-fenchene	0.2	0.2	nt	t	t	nt
946	camphene	0.3	0.3	nt	t	t	nt
961	verbenene	0.2	0.2	nt	t	t	nt
969	sabinene	0.1	0.2	nt	t	t	nt
974	β-pinene	1.1	1.2	0.109 ns	0.06	0.06	1.071 ns
988	myrcene	1.7	1.8	0.669 ns	0.09	0.10	2.096 ns
1001	δ-2-carene	0.1	0.1	nt	t	t	nt
1002	α-phellandrene	0.7	0.8	0.155 ns	0.04	0.04	0.317 ns
1008	δ-3-carene	3.0	1.9	0.214 ns	0.20	0.12	0.396 ns
1014	α-terpinene	0.1	0.1	nt	t	t	nt
1020	**p-cymene**	**1.0**	**0.6**	**5.720 ***	**0.05**	**0.03**	**4.155 ns**
1024	limonene	2.6	2.1	0.766 ns	0.13	0.12	0.068 ns
1025	β-phellandrene	7.5	6.3	0.588 ns	0.38	0.37	0.012 ns
1044	(E)-β-ocimene	0.2	0.1	nt	t	t	nt
1054	γ-terpinene	0.5	0.5	nt	<0.05	<0.05	nt
1065	cis-sabinene hydrate	0.1	0.1	nt	t	t	nt
1086	terpinolene	1.3	1.2	0.011 ns	0.07	0.07	0.005 ns
1098	trans-sabinene hydrate	0.2	0.2	nt	t	t	nt
1100	n-nonanal	t	t	nt	t	t	nt
1112	methyl butanoate, 3-methyl-3-butenyl-, 3-	0.5	0.4	0.694 ns	0.02	0.02	0.052 ns
1122	α-campholenal	0.2	0.1	nt	t	t	nt
1136	trans-p-menth-2-en-1-ol	0.6	0.4	1.129 ns	0.03	0.02	0.644 ns
1141	camphor	1.4	1.0	0.994 ns	0.12	0.05	2.951 ns
1144	neo-isopulegol	0.9	0.5	1.936 ns	0.05	0.03	1.468 ns
1145	camphene hydrate	t	t	nt	t	t	nt
1158	trans-pinocamphone	0.2	0.2	nt	t	t	nt
1165	borneol	0.2	0.2	nt	t	t	nt
1172	cis-pinocamphone	0.1	0.1	nt	t	t	nt
1174	terpinen-4-ol	0.5	0.5	nt	t	t	nt
1179	p-cymen-8-ol	0.2	0.1	nt	t	t	nt
1186	α-terpineol	0.2	0.5	nt	t	t	nt
1193	(4Z)-decanal	0.1	0.1	nt	t	t	nt
1195	cis-piperitol	0.2	0.2	nt	t	t	nt
1207	trans-piperitol	0.5	0.3	nt	t	t	nt
1215	trans-carveol	0.1	0.1	nt	t	t	nt
1235	trans-chrysanthenyl acetate	0.2	0.1	nt	t	t	nt
1249	piperitone	0.2	0.2	nt	t	t	nt
1258	trans-myrtanol	t	t	nt	t	t	nt
1274	pregeijerene B	2.8	2.7	0.030 ns	0.16	0.15	0.052 ns
1284	bornyl acetate	0.7	0.6	0.626 ns	0.04	0.03	1.444 ns

1289	thymol	0.4	0.2	nt	t	t	nt
1298	carvacrol	t	t	nt	t	t	nt
1315	(2E,4E)-decadienal	t	0.3	nt	t	t	nt
1396	duvalene acetate	t	t	nt	t	t	nt
1417	(E)-caryophyllene	t	t	nt	t	t	nt
1489	β-selinene	t	t	nt	t	t	nt
1498	α-selinene	t	t	nt	t	t	nt
1500	α-muurolene	t	t	nt	t	t	nt
1517	nootkatene	0.2	0.2	nt	t	t	nt
1533	trans-cadina-1,4-diene	0.1	0.1	nt	t	t	nt
1548	elemol	1.6	1.4	0.068 ns	0.08	0.08	0.008 ns
1629	eremoligenol	0.3	0.2	nt	t	t	nt
1630	γ-eudesmol	1.1	0.9	0.984 ns	0.06	0.05	0.281 ns
1640	epi-α-muurolol	t	t	nt	t	t	nt
1649	β-eudesmol	3.7	2.8	1.189 ns	0.19	0.17	0.517 ns
1652	α-eudesmol	2.7	2.2	0.715 ns	0.14	0.13	0.088 ns
1688	shyobunol	t	t	nt	t	t	nt
1746	8-α-11-elemodiol	0.6	0.5	2.538 ns	0.03	0.03	0.556 ns
1792	8-α-acetoxyelemol	1.2	1.0	0.001 ns	0.07	0.07	0.001 ns