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### Authors

Karban, R  
Wetzel, WC  
Shiojiri, K  
[et al.](#)

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# CHEMOTYPIC Variation in Volatiles and Herbivory for Sagebrush

Richard Karban<sup>1</sup>  · Patrick Grof-Tisza<sup>2</sup> · James D. Blande<sup>3</sup>

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**Abstract** Plants that are damaged by herbivores emit complex blends of volatile compounds that often cause neighboring branches to induce resistance. Experimentally clipped sagebrush foliage emits volatiles that neighboring individuals recognize and respond to. These volatiles vary among individuals within a population. Two distinct types are most common with either thujone or camphor as the predominate compound, along with other less common types. Individuals respond more effectively to cues from the same type, suggesting that some of the informative message is contained in the compounds that differentiate the types. In this study, we characterized the chemical profiles of the two common types, and we examined differences in their microhabitats, morphologies, and incidence of attack by herbivores and pathogens. Analysis by gas chromatography coupled with mass spectrometry revealed that the camphor type had higher emissions of camphor, camphene, and tricyclene, while the thujone type emitted more  $\alpha$ -thujone,  $\beta$ -thujone, (*Z*)-salvene, (*E*)-salvene, carvacrol, and various derivatives of sabinene. We were unable to detect any consistent morphological or microhabitat differences associated with the common types. However,

plants of the thujone type had consistently higher rates of damage by chewing herbivores. One galling midge species was more common on thujone plants, while a second midge species was more likely to gall plants of the camphor type. The diversity of preferences of attackers may help to maintain the variation in volatile profiles. These chemical compounds that differentiate the types are likely to be informative cues and deserve further attention.

**Keywords** Camphor · Cue · Eavesdropping · Herbivore · Induced resistance · Information · Priming · Thujone · Volatile organic compound

## Introduction

When plants are damaged by herbivores, they emit a blend of volatile chemicals (Dudareva et al. 2006; Pichersky and Gershenzon 2002). The volatiles emitted by damaged plants are chemically diverse and often aromatic compounds (Dudareva et al. 2004). They tend to be lipophilic and can cross membranes freely. Some volatile compounds are produced constitutively (Pichersky and Gershenzon 2002), while others are emitted in greater concentrations, or exclusively, following physical damage or herbivory (Hare 2011; Pare and Tumlinson 1997).

These compounds also cause diverse physiological and ecological consequences (Karban 2015). Volatiles that are emitted following herbivory have been found to repel herbivores (e.g., DeMoraes et al. 2001; Dicke 1986; Kessler and Baldwin 2001; Khan et al. 2008), although they can also be attractants (e.g., Dicke and van Loon 2000; Horiuchi et al. 2003; Pallini et al. 1997). Natural enemies often use the cues emitted by damaged plants to locate herbivore prey (e.g., Amo et al. 2013; Drukker et al. 1995; Kessler and Baldwin 2001; Mäntylä et al. 2008; Mumm and Dicke 2010; Thaler 1999). In

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✉ Richard Karban  
rkarban@ucdavis.edu

- <sup>1</sup> Department of Entomology and Nematology, University of California, Davis, Davis, CA 95616, USA
- <sup>2</sup> Ecology Graduate Group, Department of Entomology and Nematology, University of California, Davis, Davis, CA 95616, USA
- <sup>3</sup> Department of Environmental Science, University of Eastern Finland, Kuopio, Finland

some cases, the natural enemies of herbivores respond to specific information that indicates their favored hosts (Alborn et al. 1997; De Moraes et al. 1998; Mattiacci et al. 1995), although this high level of specificity is not always observed (Agrawal and Colfer 2000; Takabayashi et al. 2006). Herbivore induced plant volatiles (HIPVs) may inhibit the germination of competing plants, although the commonness and significance of this function are less well known (Karban 2007a, b; Romagni et al. 2000). Volatiles also have strong effects on plant pathogens (Quintana-Rodriguez et al. 2015; Shulaev et al. 1997). Volatiles emitted by plant tissue damaged by herbivores signal to other tissues on the same plant, thus priming distant organs and causing systemic resistance (Frost et al. 2008; Heil and Silva-Bueno 2007; Karban et al. 2006; Rodriguez-Saona et al. 2009).

If plants that have been attacked by herbivores emit cues that reliably provide information about the location of herbivores or the risk of attack for neighboring individuals, then selection might favor neighbors that eavesdrop on these cues and adjust their defenses accordingly. Studies involving many plant species from diverse habitats have found evidence for eavesdropping that increases resistance against herbivores (Karban 2015). Preliminary evidence suggests that a blend of volatile compounds may be required to convey information in plant-plant communication. For example, chrysanthemum plants responded to volatiles from experimentally damaged neighbors by producing pyrethrin (Kikuta et al. 2011). They also produced pyrethrin when exposed to a blend of major volatiles that were emitted by damaged plants but not when they were exposed to the individual components of the blend (Kikuta et al. 2011).

Intraspecific variation in volatile production generally has been found among cultivated and wild plant species whenever researchers have looked for it (Hare 2011). Variation in chemical traits can be found among individuals that are morphologically indistinguishable; when individuals within a population show sharp discontinuities, they are referred to as chemotypes (Santesson 1968 in Keefover-Ring et al. 2008). For example, qualitative and quantitative variation in volatile emissions has been observed for accessions of *Tanacetum vulgare* (Holopainen et al. 1987), *Ocimum basilicum* (Grayer et al. 1996), *Chamaelirium uncinatum* (Egerton-Warburton et al. 1998), *Zea mays mexicana* (Gouinguene et al. 2011), *Datura wrightii* (Hare 2007), *Solanum carolinense* (Delphia et al. 2009), *Nicotiana attenuata* (Schuman et al. 2009), and *Asclepias syriaca* (Wason et al. 2013). Herbivore induced compounds often vary by 10-fold among accessions, and much of this variation appears to be heritable (Hare 2011). Chemotypes also have been described for several *Artemisia* species: *A. annua* (Wallaart et al. 2000; Wu et al. 2011), *A. judaica* (Ravid et al. 1992), and *A. pedemontana* (Perez-Alonso et al. 2003).

In this study we use the term “chemotype” to refer to plants that emit different volatile compounds after experimental damage.

Sagebrush (*Artemisia tridentata*) that was experimentally damaged by herbivores or clipped with scissors emitted volatiles that provided many of the functions described above. Herbivore-induced volatiles attracted predatory bugs and coccinellid beetles, although these generalist predators were not found to be effective at reducing herbivory in this system (Karban 2007a). Sagebrush volatiles were potent germination inhibitors of both dicotyledonous species and grasses, but they did not inhibit sagebrush (Karban 2007b). Volatile cues were necessary for coordinating systemic induced resistance in the tissues of a single individual plant, and they also caused induced resistance in neighboring sagebrush and wild tobacco individuals (Karban et al. 2000, 2006). Blocking volatiles negated the effects of communication (Karban et al. 2006), and moving headspace volatiles between plants was sufficient to induce resistance in undamaged individuals (Karban et al. 2010).

The volatiles produced by individuals within a single meadow were highly variable; most individuals could be characterized as belonging to one of two “chemotypes” – either producing camphor or thujone as the major volatile constituent (Karban et al. 2014). Chemotypes can be assigned reliably to undamaged plants, but they are magnified by damage. Some rare individuals did not fit into these two groups, but they produced combinations of volatiles that were uncommon in the population. Chemotypes were stable over time, and analyses of mothers and their offspring indicated that these volatile types were highly heritable. Individuals communicated more effectively with other individuals of the same type, and less effectively with individuals of other types.

This current study examined the ecological correlates of the chemotypes of *Artemisia tridentata*. We asked the following questions: 1) What volatile compounds differ between experimentally damaged individuals of the two common chemotypes? 2) Are the chemotypes found in different habitats? 3) Are the chemotypes morphologically different? 4) Do herbivores and pathogens distinguish between the chemotypes? 5) Are results consistent over time and space?

## Methods and Materials

We characterized the volatile emissions of experimentally damaged sagebrush individuals from two populations. We next compared the morphologies and habitat associations of plants of the two common chemotypes along with other rare chemotypes in those populations. Finally, we quantified the levels of naturally occurring damage by herbivores and pathogens on plants of the abundant chemotypes.

**Chemical Characterization of the Chemotypes of Damaged Plants** We collected headspace volatiles from sagebrush plants (*Artemisia tridentata* Nutt. ssp. *Vaseyana* (Rybd.) Beetle) by using a dynamic flow-through system described in

**Table 1** Volatile organic compounds identified in the emissions of experimentally clipped sagebrush plants

CN	KRI	KRI <sup>L</sup>	RT	Compound name	Emission – Camphor CT (nmol g(DW) <sup>-1</sup> min <sup>-1</sup> )	Emission – Thujone CT (nmol g(DW) <sup>-1</sup> min <sup>-1</sup> )
1	853	854 <sup>a</sup>	12.180	2-hexenal	0.0030 ± 0.0030 ‡	0 ‡
2	854	859 <sup>a</sup>	12.190	(Z)-salvene	0 ‡	0.0762 ± 0.0154
3*	856	849 <sup>b</sup>	12.262	(Z)-3-hexen-1-ol	0.0361 ± 0.0126	0.0772 ± 0.0238
4	863	866 <sup>a</sup>	12.522	(E)-salvene	0 ‡	0.0093 ± 0.0017
5	866	863 <sup>a</sup>	12.591	(E)-2-hexen-1-ol	0.0032 ± 0.0016 ‡	0.0024 ± 0.0011 ‡
6*	867	867 <sup>a</sup>	12.650	1-hexanol	0.0030 ± 0.0020 ‡	0.0024 ± 0.0013 ‡
7	909	904 <sup>a</sup>	14.062	santolina triene	0.1288 ± 0.1288 ‡	0 ‡
8	929	924 <sup>a</sup>	14.731	tricyclene	0.0050 ± 0.0016	0.0028 ± 0.0008
9	932	932 <sup>a</sup>	14.816	α-thujene	0.0005 ± 0.0005 ‡	0.0022 ± 0.0005
10*	940	940 <sup>a</sup>	15.111	α-pinene	0.0322 ± 0.0122	0.0238 ± 0.0045
11*	957	953 <sup>a</sup>	15.663	camphene	0.2068 ± 0.0664	0.1091 ± 0.0281
12*	980	980 <sup>a</sup>	16.439	sabinene	0.0136 ± 0.0059	0.1201 ± 0.0251
13*	985	986 <sup>a</sup>	16.619	β-pinene	0.0197 ± 0.0081	0.0113 ± 0.0021
14	987	987 <sup>a</sup>	16.699	6-methyl-5-hepten-2-one	0.0008 ± 0.0004 ‡	0.0022 ± 0.0004
15*	993	993 <sup>a</sup>	16.876	myrcene	0 ‡	0.0020 ± 0.0015 ‡
16*	1006	1009 <sup>a</sup>	17.317	(Z)-3-hexenyl acetate	0.1157 ± 0.0378	0.3066 ± 0.1195
17	1011	1012 <sup>a</sup>	17.469	α-phellandrene	0 ‡	0.0004 ± 0.0004 ‡
18	1015	997 <sup>a</sup>	17.598	2-hexenyl acetate	0.0016 ± 0.0010 ‡	0.0022 ± 0.0011 ‡
19	1023	1024 <sup>a</sup>	17.876	α-terpinene	0.0006 ± 0.0006 ‡	0.0021 ± 0.0006
20	1031	1034 <sup>a</sup>	18.122	p-cymene	0.0120 ± 0.0067	0.0211 ± 0.0027
21*	1036	1032 <sup>a</sup>	18.278	limonene	0.0113 ± 0.0049	0.0067 ± 0.0013
22*	1039	1046 <sup>a</sup>	18.402	1,8-cineole	0.1979 ± 0.0919	0.2792 ± 0.0408
23	1050	1051 <sup>a</sup>	18.764	(E)-β-ocimene	0.0003 ± 0.0002 ‡	0.0005 ± 0.0002 ‡
24	1063	1061 <sup>a</sup>	19.176	artemisia ketone	0 ‡	0.0003 ± 0.0003 ‡
25*	1065	1067 <sup>a</sup>	19.235	γ-terpinene	0.0015 ± 0.0012 ‡	0.0050 ± 0.0009
26	1074	1060 <sup>c</sup>	19.534	(E)-sabinene hydrate	0.0061 ± 0.0040	0.0137 ± 0.0024
27	1086	1084 <sup>b</sup>	19.919	artemisia alcohol	0.0015 ± 0.0015 ‡	0.00004 ± 0.00004 ‡
28	1101	1081 <sup>c</sup>	20.416	α-terpinolene	0 ‡	0.0006 ± 0.0003 ‡
29*	1105	1108 <sup>a</sup>	20.534	nonanal	0.0023 ± 0.0018 ‡	0.0010 ± 0.0010 ‡
30*	1106	1103 <sup>a</sup>	20.550	linalool	0.0007 ± 0.0007 ‡	0 ‡
31	1106	1092 <sup>c</sup>	20.561	(Z)-sabinene hydrate	0.0064 ± 0.0043 ‡	0.0081 ± 0.0018
32*	1115	1101 <sup>a</sup>	20.834	α-thujone	0.0086 ± 0.0031	1.6892 ± 0.2508
33*	1118	1114 <sup>a</sup>	20.934	(E)-DMNT	0.0012 ± 0.0006	0 ‡
34*	1128	-	21.219	β-thujone	0.0007 ± 0.0005 ‡	0.1495 ± 0.0238
35	1135	1126 <sup>a</sup>	21.443	chrysanthenone	0.0063 ± 0.0028	0.0010 ± 0.0004 ‡
36	1149	1143 <sup>a</sup>	21.882	(Z)-sabinol	0 ‡	0.0330 ± 0.0109
37	1152	1140 <sup>d</sup>	21.968	(E)-pinocarveol	0.0063 ± 0.0038	0.0026 ± 0.0007
38*	1158	1161 <sup>a</sup>	22.161	camphor	0.7621 ± 0.2990	0.2990 ± 0.0463
39	1175	1164 <sup>a</sup>	22.690	pinocarvone	0.0068 ± 0.0051 ‡	0.0068 ± 0.0015
40*	1178	1188 <sup>a</sup>	22.779	borneol	0.0208 ± 0.0082	0.0210 ± 0.0064
41*	1186	1173 <sup>a</sup>	23.017	(Z)-3-hexenyl butyrate	0.0012 ± 0.0010 ‡	0.0075 ± 0.0040
42*	1188	1192 <sup>a</sup>	23.078	terpinen-4-ol	0.0021 ± 0.0016 ‡	0.0028 ± 0.0005
43*	1200	1203 <sup>a</sup>	23.433	α-terpineol	0.0003 ± 0.0003 ‡	0.0010 ± 0.0005 ‡
44	1207	1195 <sup>b</sup>	23.642	(Z)-dihydrocarvone	0 ‡	0.0008 ± 0.0008 ‡
45	1207	1202 <sup>b</sup>	23.652	(E)-dihydrocarvone	0.0082 ± 0.0036	0.0047 ± 0.0012
46	1210	1215 <sup>a</sup>	23.722	myrtenal	0.0020 ± 0.0020 ‡	0.0004 ± 0.0004 ‡
47	1273	-	25.551	lyratyl acetate	0.0255 ± 0.0255 ‡	0 ‡
48*	1297	1297 <sup>a</sup>	26.239	bornyl acetate	0.0219 ± 0.0122	0.0049 ± 0.0013

**Table 1** (continued)

CN	KRI	KRI <sup>L</sup>	RT	Compound name	Emission – Camphor CT (nmol g(DW) <sup>-1</sup> min <sup>-1</sup> )	Emission – Thujone CT (nmol g(DW) <sup>-1</sup> min <sup>-1</sup> )
49	1299	1298 <sup>a</sup>	26.279	sabinyol acetate	0 ‡	0.0370 ± 0.0121
50	1305	1307 <sup>a</sup>	26.450	carvacrol	0 ‡	0.0035 ± 0.0007
51*	1395	1398 <sup>c</sup>	28.902	α-copaene	0.0017 ± 0.0006	0.0034 ± 0.0006
52	1407	1406 <sup>c</sup>	29.204	β-bourbonene	0.0078 ± 0.0032	0.0118 ± 0.0023
53*	1445	1436 <sup>c</sup>	30.177	( <i>E</i> )-caryophyllene	0.0352 ± 0.0099	0.0282 ± 0.0037
54*	1462	1448 <sup>c</sup>	30.611	( <i>E</i> )-β-farnesene	0.0002 ± 0.0002 ‡	0 ‡
55*	1479	1465 <sup>c</sup>	31.059	α-humulene	0.0012 ± 0.0004	0.0009 ± 0.0002
56	1492	-	31.386	unknown sqt 1	0.0115 ± 0.0041	0.0102 ± 0.0023
57	1495	-	31.457	unknown sqt 2	0.0050 ± 0.0012	0.0052 ± 0.0007
58	1505	1489 <sup>c</sup>	31.718	germacrene d	0.0092 ± 0.0030	0.0125 ± 0.0027
59	1521	-	32.103	unknown sqt 3	0.0038 ± 0.0022	0.0045 ± 0.0016
60	1542	1524 <sup>c</sup>	32.600	δ-cadinene	0 ‡	0.0001 ± 0.00008 ‡
61	-	1576 <sup>c</sup>	34.328	caryophyllene oxide	0.0001 ± 0.0001 ‡	0 ‡
62	-	-	39.538	Homomenthyl salicylate	0.0006 ± 0.0006 ‡	0.0009 ± 0.0004 ‡

Compounds for which an external standard was used for identification and quantification are highlighted with an asterisk

<sup>a</sup> Nist Chemistry WebBook (values for an HP-5 or HP-5MS non-polar column with a temperature ramp)

<sup>b</sup> Jirovetz et al. 2003 (values for a nonpolar FSOT-RSL-200 column with a temperature programme)

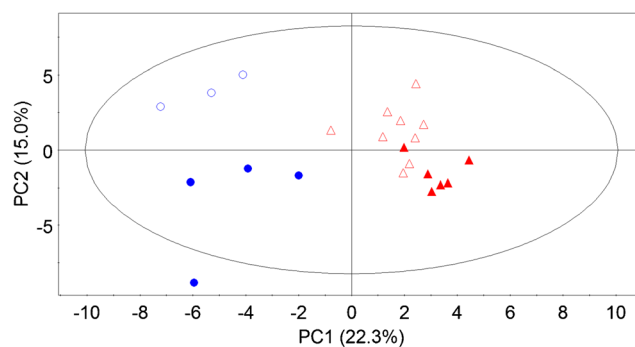
<sup>c</sup> Davies 1990 (values for a methyl silicone column with a temperature programme)

<sup>d</sup> Veličković et al. 2012. (values for an HP-5 non-polar column with a temperature programme)

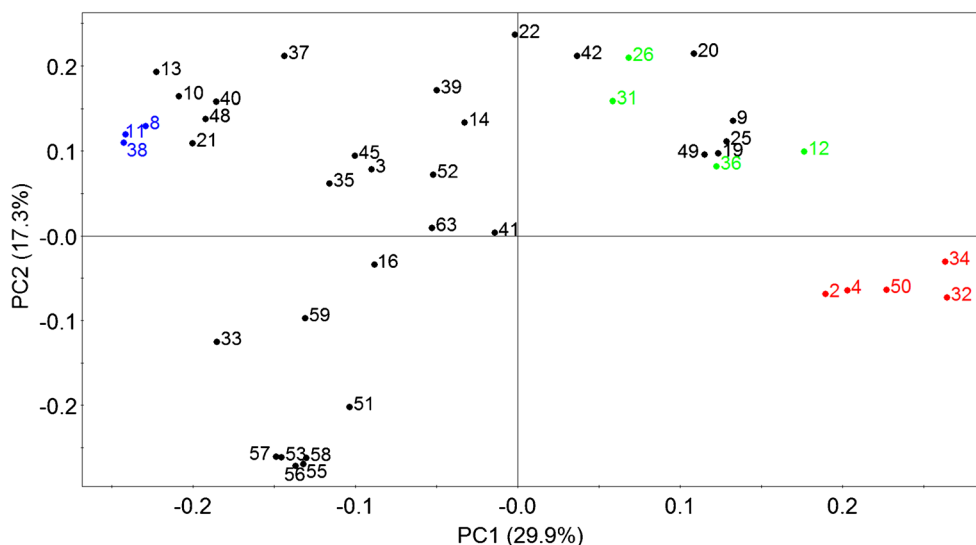
\*Compounds that were present in less than 50 % of plants of a chemotype are indicated with the double dagger symbol ‡. If compounds were present in less than 50 % of both chemotypes, they were excluded as individual compounds in the principal component analysis (PCA). The compound numbers (CN) correspond to the numbers used as labels in the PCA. Compounds removed as individual data points in the PCA were included in the analysis as 'other compounds', which are denoted by the number 63. Kovats Retention Indices (KRI) were calculated with a series of n-alkanes (C7-C16); where possible KRIs reported in the literature (KRI<sup>L</sup>) are also provided. Letters (a-d) represent the references

detail in a previous paper (Karban et al. 2014). We sampled volatiles from 10 plants located in Taylor meadow at UC Sagehen Natural Reserve, north of Truckee, California (N 39.433 W 120.237) and from 11 plants from a second site 25 km to the southeast, near the Donner Party picnic area north of Alder Creek (N 39.377 W 120.181). Five leaves of each plant were damaged experimentally with scissors. The headspace samples were analyzed using gas chromatography coupled with mass spectrometry (GC-MS), and compounds were identified by comparison with external standards. Volatile samples were analyzed by GC-MS (Agilent 7890 A GC and 5975C VL MSD; New York, NY, USA). Trapped compounds were desorbed with an automated thermal desorber (TD-100; Markes International Ltd) at 250 °C for 10 min, cryofocused at -10 °C, and then transferred in a split mode to an HP-5 capillary column (50 m × 0.2 mm; film thickness, 0.33 μm). Helium was used as a carrier gas. The oven temperature was held at 40 °C for 2 min, increased by 5 °C/min to 210 °C, and then by 20 °C/min to 250 °C, and held for 5 min. Column flow was maintained at a rate of 1.2 ml/min. The column effluent was ionized by electron impact at 70 eV. Mass spectra were acquired by scanning from 35 to 350 m/z with a scan rate of 5.38 scans/s. Compounds for which standards were not available were identified tentatively

by comparing their mass spectra with those in the Wiley library (John Wiley & Sons, Hoboken, NJ, USA). Retention indices were calculated using an external standard containing a series of n-alkanes C7-C16 (Restek Corp., Bellefonte, PA, USA). This analysis allowed us to categorize plants as either camphor or thujone chemotypes, based on the dominance of



**Fig. 1** Principal component scores for plants of the two chemotypes. Individuals of the camphor chemotype are represented by blue circles and individuals of the thujone chemotype by red triangles. Filled symbols represent plants from Taylor meadow at Sagehen Creek and open symbols represent plants from Donner Party Picnic area at Alder Creek. The first principal component (horizontal axis) separates the chemotypes and the second principal component (vertical axis) separates the sites



**Fig. 2** The chemicals that separate the camphor and thujone chemotypes. The loading values of volatile compounds that were emitted when plants were damaged arrayed along the two principal component axes. The thujone chemotype was associated with relatively large amounts of  $\alpha$ -thujone,  $\beta$ -thujone, (*Z*)-salvene, (*E*)-salvene, and carvacrol (red) and the camphor chemotype was associated with relatively large amounts of camphor, camphene, and tricyclene (blue). Sabinene and sabinene-

derived compounds (green) were more abundant in the thujone chemotype, although this was also affected by site. See Table 1 for a list of the compounds represented by each number. Several compounds that were emitted by one or very few plants were summed as “other compounds” represented by the number 63. This reduced the influence of occasional outliers in the analysis

these particular compounds in the volatile blends. Plants not conforming to either chemotype were also identified, however, not specifically classified.

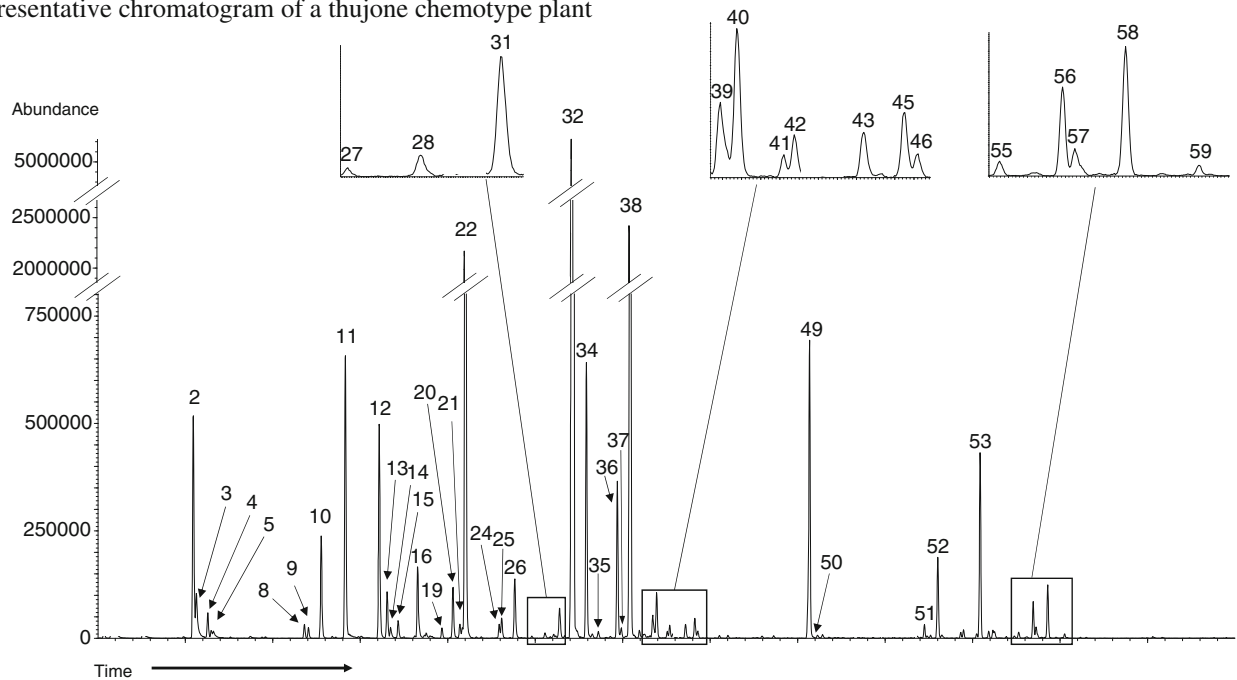
When standards were available (16 monoterpenes, 4 sesquiterpenes, and 6 green leaf volatiles, all obtained from Sigma-Aldrich, St. Louis, MO, USA), each individual compound was used to calibrate the natural amounts of the particular compound. (See Table 1). Compounds for which a standard was not available, were quantified by comparing the areas of the peaks in the samples with the peak area and quantity of the camphor standard. Camphor was selected as a general standard due to it being at a central point in the chromatogram RT22.161 min and being a compound that was present in all chromatograms. The emission rate for each compound was calculated in  $\text{nmol g(DW)}^{-1} \text{min}^{-1}$ . The volatile profiles for each plant were subjected to a principal component analysis (PCA) to characterize differences between the two common chemotypes (Gotelli and Ellison 2004). PCA and partial least squares discriminant analysis (PLS-DA) then were used to evaluate which of the individual chemical components were consistently different between the chemotypes. Both analyses were conducted with the SIMCA-P14 software package (Umetrics, Umeå, Sweden).

**Biological Attributes of the Chemotypes** We determined the chemotypes of 99 damaged plants at Taylor meadow and 40 plants from the Donner Party picnic area using methods described previously (Karban et al. 2014). Chemotypes were marked in the field, and we visually tested many potential

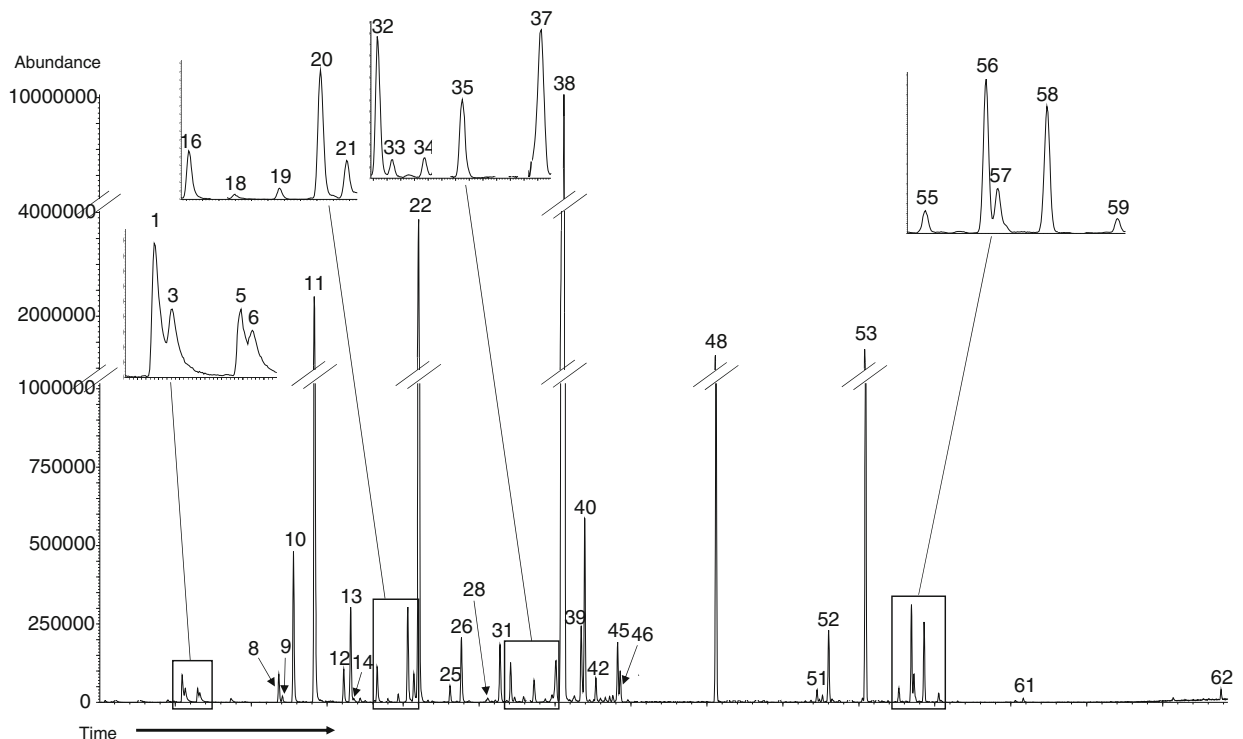
environmental and morphological correlates (listed in a supplemental table in the online resources) by looking for any traits that might be different among the three groups (camphor, thujone, and other). Only those traits that informally appeared to differ among the chemotypes were formally evaluated as described below. From field observations, it appeared that plants of the thujone type may have been more heavily shaded than those of the other two types. We tested this hypothesis by recording whether each plant was in the sun or shade between 16:00 and 17:00 on 16 May 2014 in Taylor meadow. We measured plant height on 19 May. Height was measured from the soil to the top of the vegetation (not including inflorescences). This is a vertical distance, not the length of the stem once it has been straightened. We collected one branch from each plant and measured the mean length of the five longest internodes and the mean length and width of the five largest leaves. We compared leaf color by photographing 5 leaves from each plant in a dark room with a flash. These images were analyzed using the RGB mode in Adobe Photoshop, which quantified the amount of red, green, and blue frequencies contained in each image on a scale of 0 to 255.

Our initial results from Taylor meadow suggested that plants of the thujone chemotype were more likely to grow in shaded locations. We evaluated the association between chemotypes and sun exposure by selecting 40 plants near the Donner Party picnic area, 20 that received afternoon sun and 20 that received afternoon shade during the summer of 2015. We determined their chemotype by collecting volatiles and analyzing them with GC-MS as described above.

## a) Representative chromatogram of a thujone chemotype plant



## b) Representative chromatogram of a camphor chemotype plant



**Fig. 3** Representative chromatograms from (a) a thujone chemotype plant and (b) a camphor chemotype plant. Numbers correspond to the chemicals listed in Table 1

Plants at our study sites were attacked by herbivores and pathogens. We recorded the herbivore damage on a marked branch for each of the 99 plants at Taylor meadow on 17 May, 15 June, 3 July, 5 August, and 26 September 2014. These collections spanned the growing season for this montane species.

We repeated our survey of damage by herbivores and pathogens on the 99 plants once at the end of the 2015 season on 23 September. Chewing damage was assessed by recording the percentage of leaves that showed any signs of chewing. The most common chewing herbivore at our study site was the

**Table 2** The distribution of the 99 plants of the different chemotypes from Taylor meadow with regard to sun and shade

	Sun	Shade	Total
Camphor	19	10	29
Other	1	4	5
Thujone	27	38	65
Total	47	52	99

grasshopper *Cratypedes neglectus* (Orthoptera: Acrididae) although damage by chewing caterpillars and browsing by mammals also was observed. Estimates of chewing damage from our single survey in 2015 were reduced relative to multiple surveys in 2014 but provided an unbiased measure. In 2014, aphids were abundant, particularly during the July survey. There were too few aphids recorded in our 2015 survey to analyze. There also were three species of gall makers that were found in sufficient abundance to include in analyses – a stem gall made by *Eutreta diana* (Diptera: Tephritidae), a brain-like leaf gall made by *Rhopalomyia* sp. (Diptera: Cecidomyiidae), and a small leaf gall made by a second *Rhopalomyia* sp. (Diptera: Cecidomyiidae). These gall makers are univoltine, so that single estimates of these organisms were as accurate as repeated surveys. In 2015, densities of gall makers were not as high as in 2014, and only the small leaf gall was sufficiently abundant to subject to analysis. Leaves also had black spots caused by fungal infections made by *Cladosporium* sp. (Capnodiales: Davidiellaceae). We estimated the percentage of leaves with symptoms (black spots) caused by this fungus in both years.

We recorded the percentage of leaves with chewing damage, the number of small leaf galls, and the percentage of leaves with fungal infections for the 40 plants near the Donner Party picnic area on 7 June, 1 July, 7 August, and 9 September, 2015.

**Statistical Analyses of Biological Attributes of the Chemotypes** We examined any differences between environmental and morphological characteristics of the chemotypes by using general linear models of individual response variables. Since we were testing multiple response variables without *a priori* expectations, univariate tests inflate the probability of type I error, in which we might conclude that an effect was statistically significant when observed differences were actually

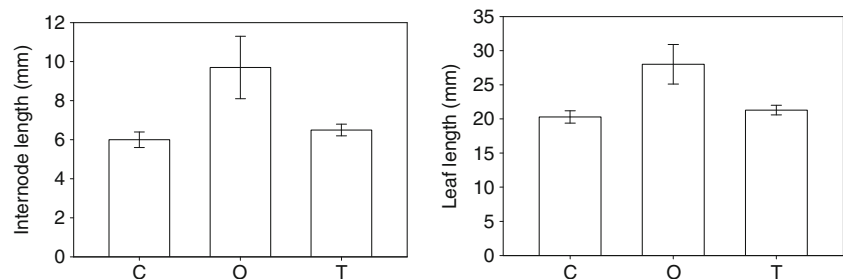
due to chance (Scheiner et al. 2001). We addressed this problem in a variety of ways. First, when our variables met the assumptions, we used multivariate analysis of variance (MANOVA). This was the case for the morphological variables that we measured. We calculated Roy's greatest root from the MANOVA because it provides the most statistical power (Scheiner et al. 2001). When MANOVA indicated an overall effect of the chemotypes on our morphological measures, we conducted protected univariate analyses to examine which variables were different. We were specifically interested in testing whether the camphor and thujone chemotypes were different, since those two chemotypes had larger sample sizes and had less intra-type variation. For environmental variables that were binary, and for estimates of herbivore and pathogen numbers that violated assumptions of normality, we were unable to use MANOVA. Relationships between binary response variables and chemotypes were evaluated using *G*-tests with Yates' continuity correction for small values.

Counts of plants with symptoms of damage by herbivores and the pathogen were best fit by Poisson distributions, and we used GLM with log link functions to compare plants of the two common chemotypes (JMP 11.1). In those instances where Poisson variances were overdispersed, we used GLMM with a negative binomial distribution and log link function in R (glmmADMB package, Fournier et al. 2012, R Core Development Team 2008, Skaug et al. 2012). Chemotype was included as a fixed effect, and year and site were modeled as random effects. Likelihood ratio tests were used to evaluate each model fit relative to a null model that included intercept and random effects only.

## Results

**Chemical Characterization of the Chemotypes of Damaged Plants** The chemical data were best fit by a model that included three principal components that together accounted for 58.9 % of the total variation. The first principal component explained 29.9 % of the overall chemical variance in the volatile profiles and separated plants of the two common chemotypes (Fig. 1). The second component explained 17.3 % of the variance and separated plants of the two sites. Plants of the

**Fig. 4** Internode and leaf length in mm for plants of the camphor (C), thujone (T), and other (O) chemotypes at Taylor meadow. Means  $\pm$  1 s.e. are presented in the figure and statistical analyses are presented in Table 3





**Table 3** Analysis of relationships between sagebrush chemotypes and morphological traits, Taylor Meadow, 2014

	Protected	Univariate	Model	Contrast:	Camphor	v thujone
Trait	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Plant height	0.94	2,94	0.39	-	-	-
Internode length	4.18	2,94	0.02	0.59	1,94	0.45
Leaf length	4.37	2,94	0.02	0.70	1,94	0.40
Leaf width	2.55	2,94	0.08	-	-	-
Leaf color						
Red	2.29	2,94	0.11			
Green	2.03	2,94	0.14			
Blue	1.35	2,94	0.26			

thujone chemotype had more  $\alpha$ -thujone,  $\beta$ -thujone, (*Z*)-salvene, (*E*)-salvene, and carvacrol at both sites (Fig. 2). Another group of related compounds, sabinene, (*E*)-sabinene hydrate, (*Z*)-sabinene hydrate, sabinyl acetate, and (*Z*)-sabinol, also were relatively overrepresented in the thujone chemotype, although these also were affected by site. Plants of the camphor chemotype had higher emissions of camphor, camphene, and tricyclene. The PLS-DA analysis gave a similar separation with the same compounds most responsible (data not shown). A full list of emission rates for each compound recorded is given in Table 1, and representative chromatograms for each chemotype are presented in Fig. 3.

#### Abundance, Distribution, Morphology of the Chemotypes

Plants of the thujone chemotype ( $N = 65$ ) were more common in Taylor meadow than those of camphor ( $N = 29$ ) or other rare chemotypes ( $N = 5$ ). Plants of the thujone chemotype were more likely to be found in the shade, and plants of the camphor chemotype were more likely to be found in locations that received afternoon sun (Table 2, *G*-test of independence:  $X^2 = 7.37$ ,  $df = 1$ ,  $P = 0.007$ ). Plants from the rare (other) chemotypes were not included in this analysis to maintain the value of all cells  $>5$ .

Overall, plants of the three chemotypes differed in the morphological traits that we measured (Fig. 4, MANOVA Roy's greatest root = 0.185,  $F_{7,89} = 2.36$ ,  $P = 0.03$ ). Most of this effect was caused by the rare chemotypes (neither camphor nor thujone) being bigger and having larger leaves. Protected univariate GLMs showed that the two common chemotypes (camphor and thujone) were not significantly different from one another in terms of any of the morphological traits that we considered (Table 3). We did not observe any differences in the plant communities that surrounded individuals of the different chemotypes or characteristics of the soils in which they were growing (data not shown).

In the sample from the Donner Party picnic area, 31 individuals were of the camphor chemotype, 9 were of the thujone chemotype, and there were no individuals that failed to fit into these two categories. Plants of the two chemotypes were not

more likely to be found in sun or shade (Table 4, *G* test of independence = 2.12,  $df = 1$ ,  $P = 0.15$ ). This result was inconsistent with the trend we observed at Taylor meadow.

#### Herbivore and Pathogen Abundance on the Chemotypes

Plants of the two common chemotypes supported consistently different levels of damage by some herbivores (Fig. 5, Table 5). Plants of the camphor chemotype experienced less chewing damage during both years and at both sites. Plants of the camphor chemotype also received fewer small leaf galls. In contrast, plants of the thujone chemotype received fewer brain galls, although they were only sufficiently abundant to analyze from Taylor meadow in 2014. The abundance of black spots caused by *Cladosporium* fungus did not differ between plants of the two chemotypes based on the samples that we collected.

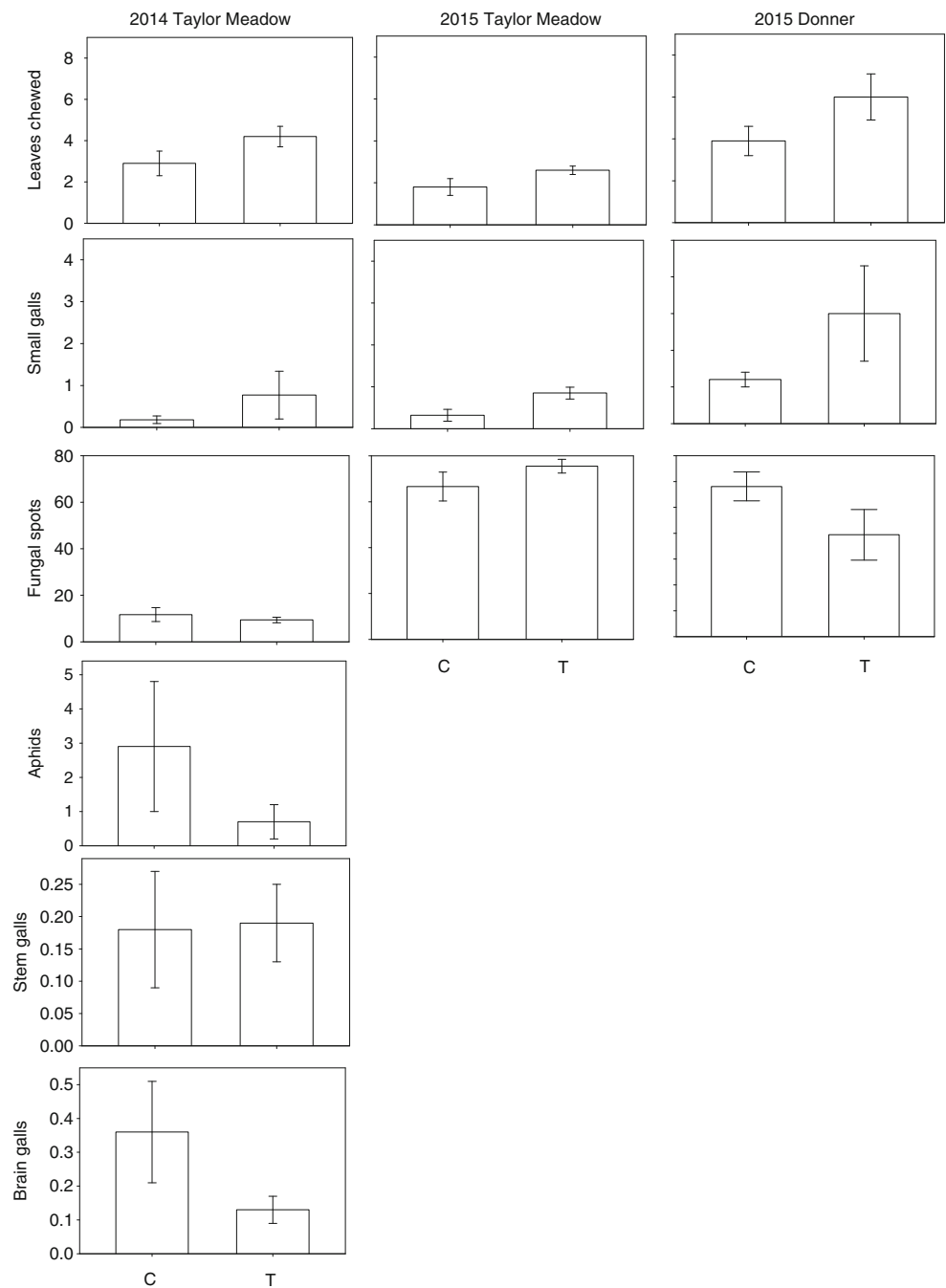
#### Discussion

As mentioned earlier, several key compounds that were emitted by mechanically damaged sagebrush differed for the two most common chemotypes. Since sagebrush emits a large number of compounds following damage, the number of possible informative candidates perceived by receiver plants also is great. However, some information is specific to the chemotypes (Karban et al. 2014), and future researchers may want to focus their search for biologically active cues on these compounds that are similarly specific to the chemotypes. Our

**Table 4** The distribution of plants of the different chemotypes from the Donner Party Camp with regard to sun and shade

	Sun	Shade	Total
Camphor	13	17	30
Thujone	7	2	9
Total	20	19	39

**Fig. 5** Damage caused by various herbivores and a pathogen to plants of the camphor (C) and thujone (T) chemotypes at Taylor meadow during the 2014 and 2015 seasons and at Donner party camp during the 2015 season. Means  $\pm$ 1 s.e. are presented in the figure and statistical analyses are presented in Table 5



chemical characterization of the chemotypes identified a relatively small number (10–12) of these specific compounds.

We examined many different environmental and morphological traits associated with the chemotypes (see supplemental table in online resources), as well as rates of damage by herbivores and pathogens. We failed to detect any differences in the size, shape, or color of plants or leaves associated with the chemotypes. At one of our field sites, Taylor meadow, plants of the thujone chemotype were more often in locations that received afternoon shade and plants of the camphor chemotype were found more often in the sun. However, this

spatial association was not observed at our second field site. We were not able to identify any other differences in the abiotic or biotic environments where the chemotypes were encountered.

Herbivores apparently distinguish between the chemotypes, even if we cannot. Plants of the thujone chemotype received more chewing damage than those of the camphor chemotype at both sites and years (Fig. 5). Thujone plants were not universally preferred by all herbivores since camphor plants received more brain galls at Taylor meadow in 2014. The midges causing brain galls were uncommon in

**Table 5** Analysis of relationships between sagebrush chemotypes and the prevalence or damage caused by various herbivores and pathogens

Species	Distribution	$\chi^2$	$df$	$P$
Chewing damage	Neg. Binomial	9.04	1	0.003
Aphids	Neg. Binomial	1.31	1	0.25
Stem galls	Poisson	0.01	1	0.93
Brain galls	Poisson	4.87	1	0.03
Small leaf galls	Neg. Binomial	5.26	1	0.02
Fungal spots	Neg. Binomial	1.23	1	0.27

2015. A second midge that caused small leaf galls showed the opposite pattern and was more frequent on the thujone chemotype. These chemotypic differences in herbivory were characterized by large effect sizes. For example, even if we subject our interpretations to Bonferroni corrections, which many ecological statisticians consider to be unnecessarily conservative (Gotelli and Ellison 2004), chewing damage was still more common on plants of the thujone chemotype than one would expect.

Intraspecific variation in the emissions of plants is a common observation. Although the ecological consequences of chemotypes are not well understood, there are several notable exceptions. For example, seven genetically determined volatile chemotypes of *Thymus vulgaris* have been identified and these segregate depending upon habitat (Gouyon et al. 1986; Vernet et al. 1986). Non-phenolic chemotypes are more tolerant of freezing, and phenolic chemotypes are more tolerant of drought (Amiot et al. 2005; Thompson et al. 2007). The chemotypes have different effects on competitors with phenolic chemotypes showing stronger inhibition of germination (Linhart et al. 2015). Phenolic chemotypes also are more resistant to generalist molluscan and insect herbivores (Linhart and Thompson 1995, 1999). Another example involves natural populations of *Arabidopsis thaliana* that vary in their glucosinolate profiles across Europe (Zust et al. 2012). These chemotypes provide varying levels of resistance to different herbivores, and the herbivores exert selection that maintains chemotypic variation (Bidart-Bouzat and Kliebenstein 2008; Zust et al. 2012).

The results with sagebrush have three implications. First, they indicate that the compounds that differ among the chemotypes are involved in determining levels of attack by important herbivores. Both camphor and thujone are oxygenated monoterpenes, derived from the same precursor by different synthases (Foster et al. 2013; Grausgruber-Groger et al. 2012). Their roles as volatile signaling molecules are better understood in animal systems than in plants. Camphor binds to muscarinic acetylcholine receptors that are important in neuronal signaling, and desensitizes neurons by acting on receptor potential (Moqrich et al. 2005; Xu et al. 2005). Camphor also is an effective olfactory repellent for many insects (Dethier 1947).

Thujone binds to HT<sub>3</sub> and GABA receptors, which are known to be involved in plant responses to herbivory and other stresses (Deiml et al. 2004; Michaeli and Fromm 2015; Mirabella et al. 2008). Since the association between the chemotypes and herbivory are correlations, it also is possible that another compound or compounds (including non-volatile substances) that are produced in conjunction with camphor and thujone are responsible for the activity. For example, carvacrol is another oxygenated monoterpene that is produced more by plants belonging to the thujone chemotype than the camphor chemotype. Carvacrol affects phosphorylation of mitogen activated protein kinases, which are implicated in plant defenses against pathogens (Lloyd et al. 2014; Yin et al. 2012).

Second, these volatiles cause responses that have idiosyncratic effects on different insect species. The volatile bouquet associated with high levels of attack by chewing insects and *Rhopalomyia* sp. midges that cause small leaf galls also resulted in low incidence of attack by *Rhopalomyia* sp. midges that cause brain-like leaf galls. This opposite pattern of susceptibility may place limits on the ability of plants to be constitutively defended; effective defense against one herbivore may make plants more susceptible to other species.

Third, the specificity of chemotypic communication suggests that the compounds that differentiate the chemotypes may contain information that is a part of the message. Previous work indicated that plants of the camphor chemotype communicate more effectively with other plants of the camphor chemotype, while thujone plants communicate more effectively with other thujone individuals (Karban et al. 2014). Since there are relatively few compounds that differ between the two chemotypes, it is likely that these unique differences are responsible for at least some of the biological effects.

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