

## Monoterpene chemodiversity of ponderosa pine in relation to herbivory and bark beetle colonization

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**Summary.** Ponderosa pine, *Pinus ponderosa* Laws. (Pinaceae), forests in Arizona have suffered from a nine-year period of drought and bark beetle, *Ips lecontei* Swaine (Coleoptera: Scolytidae), outbreaks. Abiotic and biotic stress in ponderosa pine results in the induced synthesis of certain monoterpenes that may in turn affect bark beetle behavior and survival. In this study, we investigate whether induced monoterpene production could result in a different monoterpene composition that remains stored in the needles or the trunk resin of the tree. Needle and resin samples in addition to trunk cores were collected from ponderosa pines at three locations in Arizona. Ungulate browsing induced a significant increase in limonene ( $P=0.010$ ) and in chemodiversity ( $P=0.009$ ), a measure of the evenness of distribution among the monoterpenes present in needles. We compared the level of ‘stress’ of the trees by measuring the thickness of annual rings in living trees and those that were killed by bark beetles. Where drought occurred, the spacing of annual rings from the last 10 years of trees killed by bark beetles was significantly smaller ( $P=0.020$ ) compared to living trees. There was no difference in the monoterpene composition between the core sections of closest spacing of annual rings (stressed years) compared to the sections of widest spacing, which indicates that monoterpenes are distributed evenly throughout the extended resin system. In the area where the degree of drought was less overall, none of the individual monoterpenes present in the resin was related to bark beetle killed trees. However, about half the living pines had resin in which one of the major monoterpenes ( $\alpha$ -pinene,  $\Delta^3$ -carene, and limonene) was absent, and these trees had a lower monoterpene chemodiversity compared to trees killed by bark beetles. Trees with these three major monoterpenes, corresponding to the average relative proportion in living pines at that location, may sustain higher selection and colonization by bark beetles.

**Key words.** Alpha-pinene – limonene – induction – *Ips lecontei* – bark beetles – ungulate browsing – *Pinus ponderosa*

### Introduction

Monoterpenes in conifers usually occur as mixtures that fulfil multiple ecological functions: defence against insect and mammalian herbivory and insect vectored fungi, attraction of pollinators, and allelopathic agents inhibiting seed germination and soil bacteria (White 1994; Langenheim 1994; Raffa & Smalley 1995; Vokou *et al.* 2003; Miller *et al.* 2005). Monoterpenes are highly variable in their relative composition within a single species as exemplified by the existence of five chemotypes for ponderosa pine, *Pinus ponderosa* Laws (Pinaceae), in continental North America (Smith 1977). Four of the five chemotypes have been sampled in Arizona (Latta *et al.* 2003). The different chemotypes are impacted by herbivory in different ways. For the highly specialised bark-feeding Albert’s squirrel (*Sciuridae alberti*) on ponderosa pine, the percentage composition of the major monoterpene  $\beta$ -pinene differed significantly between target and non-target trees (Snyder 1993). Sitka spruce trees that showed resistance to the white pine weevil had highly significant differences in the resin composition of the major monoterpenes  $\beta$ -pinene and  $\beta$ -phellandrene when compared with susceptible trees (Harris *et al.* 1983). A higher relative concentration of limonene in Scots pine (*Pinus sylvestris* L.) varieties was associated with resistance to pine moth *Dioryctria zimmermani* (Sadof & Grant 1997).

The production of monoterpenes within different tissues of ponderosa pine is determined by many types of genes, including those encoding the monoterpene synthases, the enzymes that catalyse the formation of all major monoterpenes from geranyl diphosphate (Bohlmann *et al.* 1998; Fäldt *et al.* 2003; Phillips *et al.* 2003; Theis & Lerdau 2003; Martin *et al.* 2004). The genetic coding refers to two distinct aspects of monoterpene synthesis: constitutive and induced. Constitutive production occurs under normal growth conditions, while induced synthesis is a result of stress as caused by, for example, herbivory (Litvak & Monson 1998) or drought (Johnson *et al.* 1997). However, neither the actual rate of production of individual monoterpene synthases within living tissues nor the contributions of constitutive and induced production are known as these depend on environmental conditions.

Katoh & Croteau (1998) found a mismatch between the enzymes isolated from stem tissue of grand fir and their monoterpene products compared to the actual blend of monoterpenes present in that tissue. This led them to conclude that: 'the particular mixture of synthase activities were not correlated with the level of oleoresin or total biosynthetic activity', and that 'the classical determination of a chemotype provides only a historical record of secondary metabolism'. Since the resin pool within individual ponderosa pines increases continually, the monoterpene resin composition might reflect the growing conditions during the life of the tree, which in effect would make pine resin an archive of a tree's life history. In ponderosa pine forests of Arizona, a severe drought extending over the preceding nine years (Breshears *et al.* 2005) is believed to be contributing to large outbreaks of the bark beetle *Ips lecontei* Swaine (Coleoptera: Scolytidae). Because drought triggers induced monoterpene production in ponderosa pine (Johnson *et al.* 1997), the products of induction could have remained in the resin. It is feasible that bark beetles recognize induced monoterpenes (Pureswaran *et al.* 2004a) as indicative of a stressed tree that is a weakened and more susceptible host.

It is well known that bark beetles kill living trees, and living trees produce resin to defend against attacking bark beetles, so natural selection would favour trees with toxic monoterpenes and bark beetles able to detoxify these compounds (White & Hobson 1993; Byers 1995, 2000, 2004; Wibe *et al.* 1998; Pureswaran *et al.* 2004a). Pines in a population exhibit significant variation in monoterpene composition of their oleoresin (Smith 1977; Katoh & Croteau 1998; Pureswaran *et al.* 2004b). Some monoterpene compositions of resin might be more toxic or deterrent to bark beetles and allow the tree to escape attack. Because ponderosa pine outcrosses by wind pollination (Latta *et al.* 1998), the genetically-derived monoterpene composition of trees is continually randomized during reproduction. Thus, the possible frequency-dependant diversifying selection mediated by bark beetles and outcrossing by wind pollination could maintain monoterpene variability. This process could contribute to the broad distribution and evolutionary success of pine species based on the genetic diversity of oleoresin production (Sturgeon 1979; Katoh & Croteau 1998; Richardson & Rundel 1998; Theis & Lerdau 2003; Martin *et al.* 2004).

In this study we focus on the monoterpene proportions of ponderosa pine and not on the absolute amounts produced. The background rationale for this approach was that herbivores recognize a blend of monoterpenes comprising the individual compounds while the absolute concentration relates to the intensity (dosage) of the signal. Therefore, we introduce a term "chemodiversity", which represents the contribution of individual monoterpenes to the overall composition. Chemodiversity increases in response to either (1) higher numbers of different monoterpenes being present or (2) the individual monoterpenes being more evenly proportioned (Iason *et al.* 2005). Compositional data reflects changes in energy allocation to different biochemical pathways and is thus not a statistical artefact of relative amounts but reflects changes among the absolute concentrations of various monoterpenes (Latta *et al.* 2003).

Because *I. lecontei* attack mature ponderosa pines, the ultimate aim of directly following hypothesized changes in the monoterpene composition of pine resin in response to stress is probably unattainable. Therefore, we have investigated the following three hypotheses that would support the idea that prior stresses over an extended period of time leave their mark on trunk monoterpene resin composition:

1) Browsing of needles by ungulates results in an altered monoterpene composition compared to physically flawless needles that remains stored in the needles after browsing.

2) Resin monoterpene composition within the trunk depends on the growing conditions of the tree at the time that the resin was formed.

3) The monoterpene composition has influenced tree selection by bark beetles.

## Methods and materials

### Sampling.

The sampling area fell within the ponderosa pine Mogollon region that is characterised by a predominance of the monoterpenes  $\alpha$ -pinene and  $\Delta^3$ -carene. Trees growing on sites below the Rim were strongly affected by drought (Table 1). Trees growing on sites above the Rim were far less affected by drought. In addition, severe losses of pines due to *I. lecontei* attack have occurred below the Rim, but were less apparent above the Rim. The forests in the Rim areas consisted predominantly of ponderosa pines that were more closely spaced than at Walnut Canyon, where samples were also collected (Table 1). The vegetation at Walnut Canyon consisted of an open forest of mixed ponderosa pine and juniper (*Juniperus monosperma*). All samples were collected in March 2004. Living as well as bark beetle killed trees (killed in 2002-2003 seasons as judged by remaining reddish needles) were sampled on and below the Mogollon Rim by taking a core (5-mm diameter by up to 30 cm in length) at breast height from a southern aspect with an increment borer. A second core sampled the latest 2 mm woody tissue (after phloem and bark removal). Since Latta *et al.* 2000 found that resin and wood were highly correlated in their composition and due to the lack of resin exudation at the time of sampling, wood was chosen for solvent extraction. For each living tree, five needles were selected from up to five different branch ends. From these needles, 5-mm-long sections were collected both at the base of the needle and close to the needle tip. These sections were pooled and extracted using 0.5 ml of diethylether to which ethylheptanoate (10 ng per  $\mu$ l) was added as internal standard. Two mm of the newest woody tissue was extracted by 0.2 ml of the same extractant. In order to obtain a measure of 'stress' for individual trees, the annual rings were counted and lengths of 10-year increments were recorded.

To investigate whether 'stressed' versus 'lesser stressed' periods of growth resulted in differing monoterpene composition, cores collected from Walnut Canyon (Table 1) were categorized into four sections based on the two smallest spacings of annual rings for 10-year periods as well as comparable segments for the two largest spacings of rings. As the drought had occurred for the past nine years, 10-year intervals were relevant and practical. The core sections were cut out and extracted using 0.5 ml of ether to which ethylheptanoate (10 ng per  $\mu$ l) was added as internal standard. For 21 out of 40 sections with the largest spacings, only sections of 5 years were collected. The overall length of a particular section was up to 30 mm.

To investigate whether herbivory resulted in an altered pattern of monoterpene composition, ten pines at Walnut Canyon that had suffered from large ungulate browsing were selected. From each tree, branch ends were chosen that showed either visual signs of feeding, or which were apparently unbrowsed, meaning no bite marks were visible on the branch as a whole. From five different browsed branch ends, five browsed needles were selected and

**Table 1** Description of sampling areas and number of tress samples

	Area location	Altitude (m) <sup>1</sup>	Trees sampled
On the Mogollon Rim	N 34° 30' 11 to 30 s W 111° 21' and 17 to 45 s	2100 to 2170	n <sub>killed</sub> = 18 n <sub>living</sub> = 17
Below the Mogollon Rim	N 34° 25' 25 to 44s W 111° 15' 40s to 16' 35s	1825 to 1910	n <sub>killed</sub> = 18 n <sub>living</sub> = 18
Walnut Canyon	N 35° 6' 7 to 26s W 111° 32' 3 to 50s	2168 to 2211	n <sub>cores</sub> = 20 <sup>2</sup> n <sub>browsing</sub> = 10 <sup>3</sup>

<sup>1</sup> height above sea level<sup>2</sup> two most closely spaced and two most widely spaced intervals were collected per core<sup>3</sup> two samples of browsed and two samples of unbrowsed needles were collected per tree

5-mm long sections were collected located next to the site of injury, but omitting any needle material that was discoloured. Samples were collected in duplicate. The same procedure was applied to the unbrowsed needles where sections were collected close to but not including the tip of whole needles.

#### Chemical Analysis

The samples were extracted as described above and kept at -20 °C once returned from the field until analysed by gas chromatography (GC) within 2 months. Initial temperature was 70 °C followed by a temperature gradient of 3 °C min<sup>-1</sup> up to 160 °C. The following monoterpenes in order of retention were separated and identified:  $\alpha$ -pinene, camphene,  $\beta$ -pinene, sabinene,  $\Delta^3$ -carene, myrcene and  $\alpha$ -phellandrene (co-eluting),  $\alpha$ -terpinene, limonene,  $\beta$ -phellandrene,  $\gamma$ -terpinene, and terpinolene. The GC used was a Carlo Erba MFC 500 GC with FID (Flame Ionisation Detector) (T = 250 °C) and was equipped with a WCOT fused silica 25 m  $\times$  0.25 mm i.d. column coated with CP WAX 52 CB df = 0.25 (Varian). For data capture and processing ChromQuest version 2.53 Chromatography workstation was used. The identifications of monoterpenes were confirmed with a Varian 3900 GC coupled to a Varian Saturn 2100D mass spectrometer (MS) by comparing spectra and retention times to authentic standards (Aldrich and Fluka).

#### Statistical Analysis

For statistical analysis relating to the relative monoterpene proportion, only the monoterpenes that contributed more than 1 % of total monoterpenes were included before converting the area ratio into the relative proportion of the remaining monoterpenes. A *paired t-test* was used for 'browsed' versus 'physically flawless' needles since each set of needles was collected from the same individual. 'Most widely spaced' versus 'most closely spaced' core sections were compared using a *paired t-test* because these came from the same individual. For the comparison of spacing of annual rings for the cores collected above and below the Mogollon Rim, a *two-sample t-test* was used. The chemodiversity derived from the three major monoterpenes was used in a *two-sample t-test* for 'bark beetle killed' versus 'healthy' trees on the Mogollon Rim. To test whether there was an effect of location, a multiple linear regression analysis was performed with 'on the Rim' and 'below the Rim' as groups. GenStat for Windows version 6.2.0.235 was used for the multiple linear regression while all other statistical analyses were performed using Minitab® Release 14. Chemodiversity (CD) of a tree was defined using the relative monoterpene proportions from the following formula:

$$CD = - \sum_{m=1}^n P_m \cdot \ln(P_m) \quad (1)$$

where  $P_m$  is the proportion of each monoterpene and  $n$  is the number of monoterpenes (Iason *et al.* 2005). Where results are given as 'number  $\pm$  number', it uses the average  $\pm$  standard error.

## Results

#### Resin and needle monoterpene composition

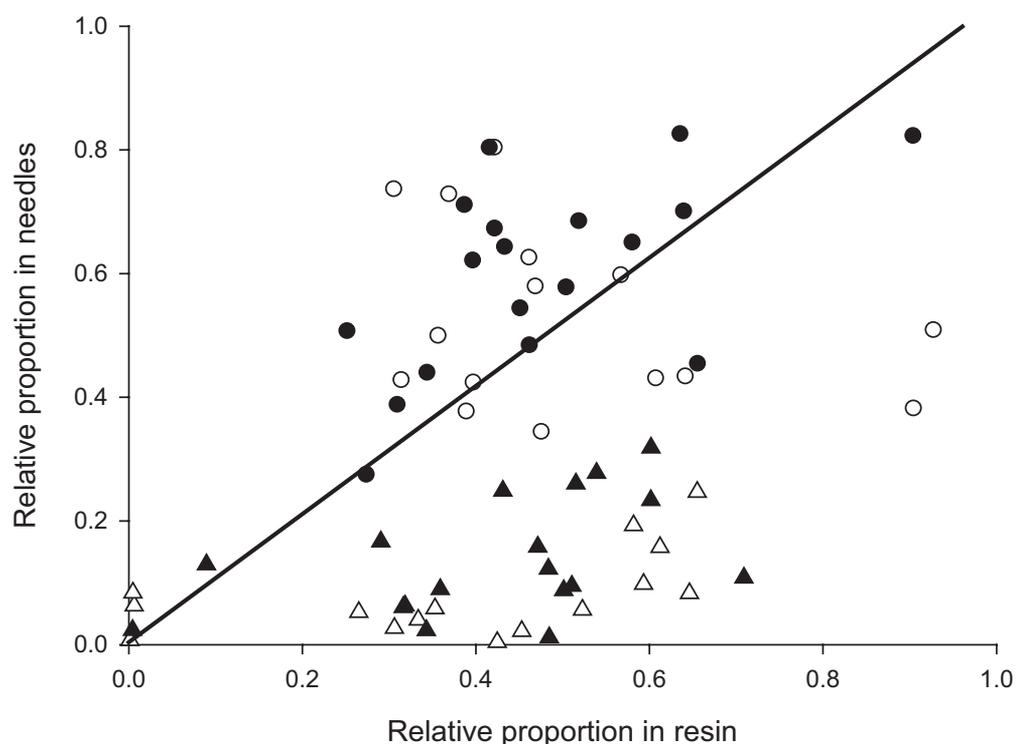
The relative proportions of all monoterpenes in needles and resin of ponderosa pine are shown in Table 2. Based on the monoterpene composition in the resin, there were three different chemotypes present for living trees with the majority of trees (n=28 of 32 total) showing the chemotype in which  $\alpha$ -pinene and  $\Delta^3$ -carene dominated. Needle chemodiversity was higher indicating that needles have a more balanced composition of monoterpenes compared to resin. In particular the relative composition of camphene and  $\beta$ -pinene was on average a magnitude higher in needles compared to resin. The relative proportions of myrcene and  $\alpha$ -phellandrene (co-eluting peaks) and  $\beta$ -phellandrene were also higher in needles compared to resin.  $\Delta^3$ -Carene and limonene increased in proportion from needles to trunk resin. There seems to be little difference in the average composition of  $\alpha$ -pinene between needles and resin. However, on the basis of individual trees the proportion of  $\alpha$ -pinene was usually higher in the needles compared to the resin (Fig. 1). There was a weak ( $r^2 = 0.12$ ) and insignificant ( $P = 0.503$ ) correlation between the relative proportion of  $\alpha$ -pinene in needles and resin. For  $\Delta^3$ -carene the relative proportion was lower in the needles compared to the resin in nearly all trees, with a weak ( $r^2 = 0.23$ ) but significant ( $P=0.005$ ) correlation. There was no effect of location, 'below' or 'on' the Mogollon Rim, since the relative proportions of both  $\Delta^3$ -carene and  $\alpha$ -pinene overlapped.

#### Herbivory and needle monoterpene composition

Limonene significantly increased ( $P=0.010$ ) in response to browsing on needles (Table 3). Chemodiversity also increased in response to browsing on needles ( $P=0.009$ ). This indicates that the various monoterpenes in the browsed needles were more uniformly present in response to browsing as no additional monoterpenes were detected.

**Table 2** Ranges, average and standard error for the relative proportions of monoterpenes in needles and trunk of ponderosa pine sampled at the Mogollon Rim in Arizona (March 2004)

	Needles		Trunk	
	Range	Average $\pm$ se (n = 33)	Range	Average $\pm$ se (n = 32)
$\alpha$ -Pinene	0.255 – 0.767	0.490 $\pm$ 0.022	0.241 – 0.909	0.453 $\pm$ 0.030
Camphene	0.006 – 0.124	0.040 $\pm$ 0.006	0.000 – 0.009	0.004 $\pm$ 0.000
$\beta$ -Pinene	0.017 – 0.518	0.207 $\pm$ 0.026	0.004 – 0.564	0.026 $\pm$ 0.017
Sabinene	0.000 – 0.016	0.010 $\pm$ 0.001	0.000 – 0.014	0.009 $\pm$ 0.001
$\Delta^3$ -Carene	0.004 – 0.276	0.098 $\pm$ 0.014	0.001 – 0.624	0.371 $\pm$ 0.031
Myrcene and $\alpha$ -phellandrene	0.018 – 0.258	0.058 $\pm$ 0.008	0.010 – 0.052	0.023 $\pm$ 0.002
Limonene	0.010 – 0.159	0.046 $\pm$ 0.007	0.004 – 0.339	0.068 $\pm$ 0.013
$\beta$ -phellandrene	0.007 – 0.211	0.025 $\pm$ 0.006	0.000 – 0.050	0.011 $\pm$ 0.001
Terpinolene	0.002 – 0.026	0.012 $\pm$ 0.001	0.001 – 0.041	0.026 $\pm$ 0.002
Chemodiversity	0.884 – 1.693	1.342 $\pm$ 0.036	0.373 – 1.308	0.999 $\pm$ 0.037

**Fig. 1** Relative proportions of  $\alpha$ -pinene (circles) and  $\Delta^3$ -carene (triangles) in trunk resin and needles of ponderosa pine sampled on the Mogollon Rim (empty shapes) and below the Rim (solid shapes) in March 2004. Points lying above the inserted line indicate that a higher relative proportion was found in the needles, while points below the line indicate a higher relative proportion in the resin

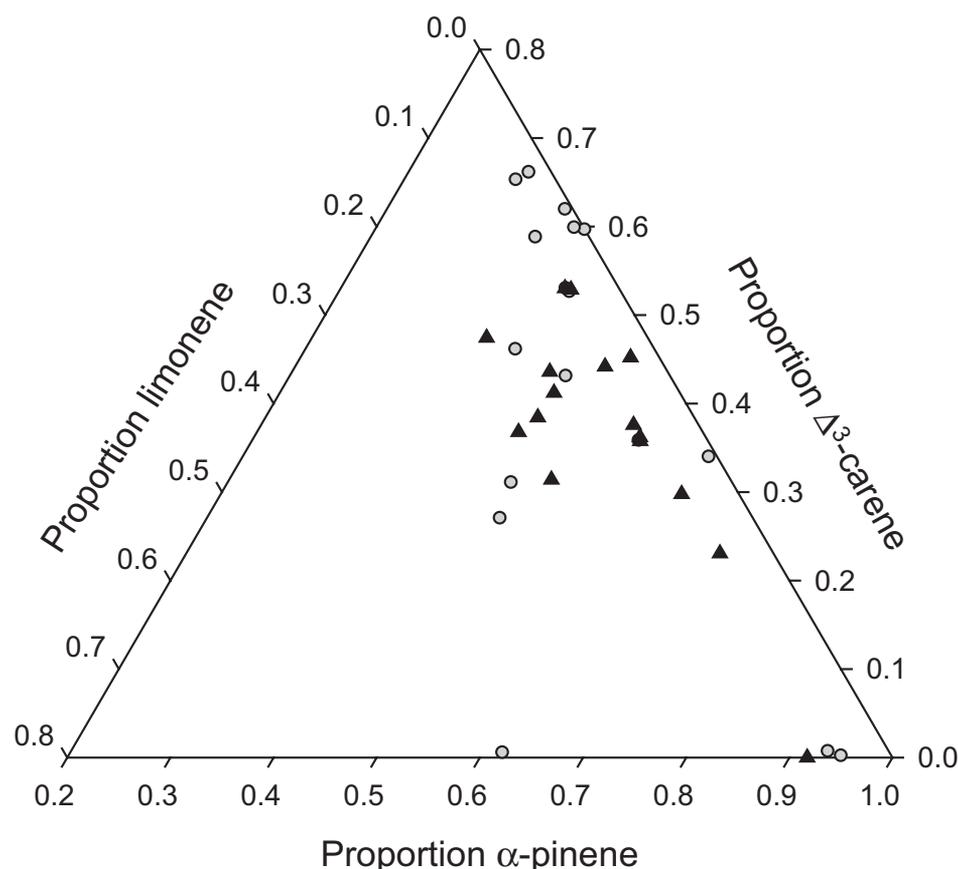
#### Resin composition throughout the trunk radius

Comparing resin found within the most widely spaced annual rings with that of the most closely spaced showed no significant differences for any of the relative proportions of individual monoterpenes or chemodiversity. The twenty trees sampled by taking cores were between 50 and 220 years old and had a circumference of more than 1.2 m (average = 2.2 m). During the selection we tried to collect both seemingly healthy trees and trees with apparent injuries incurred during the life of the tree, in particular lightning or leaning trunks. There were four chemotypes present within this collection. The chemotype in which  $\alpha$ -pinene and  $\Delta^3$ -carene dominated was present eleven times. The chemotype with primarily  $\alpha$ -pinene occurred six times.  $\Delta^3$ -Carene,  $\alpha$ - and  $\beta$ -pinene were all present above 15% relative composition in two trees. The last chemotype occurred once

and was dominated by  $\alpha$ - and  $\beta$ -pinene with the relative concentration of  $\Delta^3$ -carene being less than 5% (chemotypes used were described in Latta *et al.* 2003).

#### Bark beetles and drought

Drought seemed to have had an effect on pine susceptibility to bark beetles below the Mogollon Rim, since the average spacing of annual rings of the last ten years of growth was significantly smaller ( $P=0.020$ ) for pines killed by bark beetles ( $13.4 \pm 1.1$  mm) compared to nearby living pines ( $22.8 \pm 3.7$  mm). However, on top of the Rim drought had apparently less effect, with rings over the last ten years averaging  $23.7 \pm 3.1$  mm for bark beetle killed trees that was slightly less than for living trees with  $26.2 \pm 2.3$  mm, but the difference was not significant.



**Fig. 2** Ternary diagram of the proportion of the three major resin monoterpenes of ponderosa pines either killed by bark beetles (black triangles) or living (grey circles) from the Mogollon Rim, Arizona (March 2004)

**Table 3** Results of paired t-test on browsed versus physically intact needles sampled from the same tree for the relative concentration of individual monoterpenes and chemodiversity

	Browsed		Intact		Relative proportion (p-value)
	Range	Average $\pm$ se (n = 20)	Range	Average $\pm$ se (n = 20)	
$\alpha$ -Pinene	18.4 – 67.2 %	37.8 $\pm$ 3.3 %	17.8 – 69.3 %	38.6 $\pm$ 4.0 %	0.502
$\beta$ -Pinene	3.3 – 50.8 %	34.2 $\pm$ 3.2 %	2.1 – 57.4 %	36.5 $\pm$ 3.8 %	0.114
$\Delta^3$ -Carene	0.0 – 41.9 %	14.2 $\pm$ 2.8 %	0.0 – 41.8 %	13.3 $\pm$ 3.0 %	0.370
Myrcene + $\alpha$ -Phellandrene	0.0 – 9.5 %	4.0 $\pm$ 0.5 %	1.7 – 5.2 %	3.7 $\pm$ 0.3 %	0.544
$\beta$ -Phellandrene	2.6 – 11 %	5.8 $\pm$ 0.6 %	1.5 – 8.8 %	4.8 $\pm$ 0.5 %	0.082
Limonene	1.4 – 10.3 %	4.0 $\pm$ 0.5 %	1.8 – 6.7 %	3.2 $\pm$ 0.3 %	0.010
Chemodiversity	1.029 – 1.480	1.291 $\pm$ 0.040	0.974 – 1.501	1.209 $\pm$ 0.046 %	0.009

#### Bark beetles and resin composition

For the samples collected on top of the Mogollon Rim it was possible to compare resin composition between bark beetle killed and living pines, as opposed to the samples collected below the Rim where resin residues were too low in about two thirds of the bark beetle killed trees. Bark beetle killed pines, with one exception, seem to have  $\alpha$ -pinene,  $\Delta^3$ -carene, and limonene present as opposed to living pines where in half of the pines one of these monoterpenes was absent (Figure 2). Omitting one outlier for the resin collected from bark beetle killed trees, which could have been caused by an attack spillover at high populations (Anderbrant *et al.* 1988), the difference in resin composition was significant ( $P=0.045$ , including the outlier  $P=0.190$ ). In addition most bark beetle killed trees clustered around certain proportions of about

54 %  $\alpha$ -pinene, 37 %  $\Delta^3$ -carene and 9 % limonene, which is close to the average relative proportions in the resin of living ponderosa pines at that location (Table 2).

#### Discussion

Ponderosa pine needles are newly formed each year so needles are the only tissue type in pines that, if physically flawless, represent only constitutive monoterpene production. Latta *et al.* (2000) compared the absolute and relative concentration of monoterpenes in ponderosa pine needles on the northern versus the southern side of individual pines. They found the absolute concentration to vary while the relative composition was unchanged. Changes in composition in needles could occur in response to biotic or abiotic stress

(Litvak & Monson 1998), of which the former was chosen by studying ungulate browsing. The difference found in monoterpene patterns between browsed and physically flawless needles located on the same tree suggests that induced monoterpene production utilises monoterpene synthases in different proportions compared to those constitutively present (Barnolo *et al.* 1994; Raffa & Smalley 1995; Nebeker *et al.* 1995; Katoh & Croteau 1998 for grand fir; Litvak & Monson 1998). Our main question was whether a difference in monoterpene composition caused by a blending of constitutive and induced production remains within needles, because losses via volatilisation could have occurred (Martin *et al.* 2003). The increased relative composition of limonene and increased chemodiversity in browsed needles suggests that effects of induction remain for some time.

Pine resin is thought to have constitutively a different monoterpene composition compared to needles, as was found for Sitka spruce (Martin *et al.* 2002). Working with long-lived conifers does not allow assessing constitutive monoterpene composition in resin as the monoterpene pattern determined in resin is likely to be a combination of constitutive and induced production (Katoh & Croteau 1998 for grand fir). Previous studies that investigated the composition of monoterpenes in needles and resin of individual pines remarked upon the need to analyse the specific tissue of interest because there was no predictive relationship (Borg-Karlson *et al.* 1993 for *Picea abies*; Sjödin *et al.* 1996 for *Pinus sylvestris*; Latta *et al.* 2000), and our results support this contention.

Since there are 367 insect species that attack different tissues of ponderosa pines, with 106 species attacking wood (de Groot & Turgeon 1998), induction should take place regularly. Assuming that during years of slow growth induction is more frequent than during years of fast growth, the monoterpene composition within different parts of the trunk could have showed variability. However, we found no variation in monoterpene composition depending on growth conditions within individual trees, indicating significant mixing of monoterpenes within the extensive resin canal system of ponderosa pine (Lewinsohn *et al.* 1991a,b). The stable composition of resin, independent of the position within the tree (Latta *et al.* 2000; Smith 1968), and lack of seasonal or inter-annual variation (Smith 1964) was noted previously. This indicates that monoterpenes act as a solvent for resin acids in oleoresin (Trapp & Croteau 2001). Factors contributing to this even monoterpene distribution could be mixing in response to changing temperature and resin exudation after physical injury or in response to fungal elicitors (Lieutier & Berryman 1988). Losses of monoterpenes from the resin pool could occur due to catabolism, which was estimated to be less than 5 % (Gershenson 1994), or due to volatilisation (Byers 1981; Martin *et al.* 2003). In order to maintain a relatively constant absolute concentration of oleoresin in the trunk, the increasing size of the trunk with increasing age of the tree requires an overall accumulation of monoterpenes.

Bark beetles choose ponderosa pine as a host, in part, if the resin pressure exuding in response to attack is low (Wood 1962). Slower growth rate has been associated with lesser resin pressure in ponderosa pine (Latta & Linhart 1997). During drought conditions, turgor pressure is lowered which in turn lowers resin flow rate (Hodges & Lorio 1975). In this study we found a significantly smaller spacing of annual rings for bark beetle killed pines below the Mogollon

Rim. While the cause of the slower growth is unclear (with drought the most likely contributing factor) weakened ponderosa pines seem to be more susceptible to being attacked by bark beetles. Miller and Keen (1960) summarized a number of published and unpublished reports by US Forest Service researchers (pp. 154–161) that showed bark beetles killed ponderosa pines with a slower annual radial growth than living trees, or that a higher percentage of trees were killed in stands with the slowest average growth rates. They also summarized reports that indicated drought contributed to outbreaks of Western pine beetles (pp. 134–137).

Within the two populations sampled, three (Mogollon Rim) or four (Walnut Creek) of the five chemotypes described for ponderosa pine occurred (Smith 1977; Latta *et al.* 2003). Selective herbivory has been suggested to contribute to different chemotypes having evolved (Sturgeon 1979; Katoh & Croteau 1998; Pureswaran *et al.* 2004b). For ponderosa pine, areas that historically suffered increased bark beetle predation showed more variability for monoterpene composition (Sturgeon 1979). We were interested whether bark beetle host selection is dependent on the chemotype, and we found that monoterpene composition of killed trees was mostly representative of average monoterpene composition at that location, supporting the hypothesis that it is advantageous for individual ponderosa pines to have a different monoterpene pattern from the norm. Monoterpene enantiomers of  $\alpha$ -pinene and limonene are likely to vary in ponderosa pine as well so further work is needed regarding the chemodiversity since bark beetles can respond to different monoterpene enantiomers (Pureswaran *et al.* 2004a; White & Hobson 1993; Byers *et al.* 2000). Other pine species present on the sites, Southwestern white pine (*P. strobiformis*) and pinyon pine (*P. edulis*), do not contain  $\Delta^3$ -carene or limonene, respectively (Ekundayo 1988; Byers unpublished). Hence a ponderosa pine that did not contain limonene could seem to an approaching bark beetle like the non-host pinyon pine. Bark beetles also might select pines of average monoterpene composition since the detoxification enzymes within the bark beetle should have evolved optimally for the particular mixture of monoterpenes most frequently encountered. Lower monoterpene chemodiversity, assuming total monoterpene quantity was similar among trees, would mean higher concentrations of fewer monoterpenes that could overload detoxification enzymes and repel or kill the bark beetle.

The presence of  $\alpha$ -pinene in all bark beetle killed ponderosa pines was expected, as  $\alpha$ -pinene is a precursor of the pheromone component *cis*-verbenol (Byers 1981, 1995). The presence of limonene as part of the blend was surprising as limonene had previously been implicated as being more toxic than other monoterpenes to different bark beetle species (Smith 1965; Werner 1995; Cook & Hain 1998). However, the differences in toxicity between monoterpenes may be little or insignificant (Byers 1981; Raffa & Smalley 1995). The significant increase in the relative concentration of limonene in response to browsing opens an intriguing hypothesis: Could bark beetles use the presence of limonene as a cue to indicate higher induced monoterpene production in response to abiotic and biotic stress in ponderosa pine which in turn suggests a weakened target? Apart from a historic accumulation of limonene, its relative concentration could also indicate a more recent induction in for example

response to other attacking bark beetles. That limonene is produced in ponderosa pine resin in response to biotic and abiotic stress is hinted at by Latta *et al.* (2000) who described the relative concentration of limonene in resin to be particularly poorly correlated to that in wood. Latta & Linhart (1997) found a negative correlation between the relative concentration of both  $\Delta^3$ -carene and limonene with growth rate in ponderosa pine.

It can be questioned whether the monoterpene composition of bark beetle killed trees remained stable during and after the colonization period (sampling occurred from five to nine months after attack). Induction might have taken place within each tree either due to bark beetles themselves or associated fungi. After death of the tree, it can be expected that monoterpenes would be volatilised first from areas closest to the surface near holes created by the beetles. This might lead to an apparent lowering of chemodiversity due to differential volatilisation of the most volatile monoterpenes (e.g.,  $\alpha$ -pinene) compared to slightly less volatile monoterpenes (limonene). However, our study does not support this but rather the composition of monoterpenes in killed trees was more chemodiverse with more types of monoterpenes than in living trees. Byers (1981) found that as the concentration of monoterpenes declined in a ponderosa pine log infested with bark beetles, the ratio of  $\alpha$ -pinene:limonene did not change. Therefore, changes in ratios of monoterpenes upon death may have been insignificant, but it is possible that chemodiversity was altered by induction prior to death of the tree.

In summary, this study found that monoterpenes in ponderosa pine needles showed an altered composition after ungulate browsing with the relative concentration of limonene and chemodiversity having increased. The composition of monoterpenes throughout the trunk of ponderosa pine was uniform and independent of annual growth rate, which does not support the notion that trunk resin is an archive of a tree's history of defensive reactions to stress. Ponderosa pines killed by bark beetles were apparently more chemodiverse having an average monoterpene composition representative of the location.

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