Differential drivers of chemical defenses in three widespread pine species along a 1500-m elevational gradient: a test of the elevational gradient in plant defense hypothesis

Scott Ferrenberg¹,²*, Joseph M. Langenhan², Steven A. Loskot², Leonardo M. Rozal², Jeffry B. Mitton¹

¹Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA
²Department of Chemistry, Seattle University, Seattle, WA, USA

*Corresponding author: scott.ferrenberg@colorado.edu
Summary

1. The elevational gradient in plant defense (EGPD) hypothesis posits that natural enemy pressures increase positively alongside temperature across elevational climatic gradients, thereby selecting for enhanced defenses at lower elevations while leaving plants less defended at higher elevations. Phylogenetically constrained tests of this hypothesis in tree populations are exceedingly rare. Nevertheless, the presumed presence of poorly-defended trees has been invoked as an important driver of recent pest outbreaks at higher elevations than historically common.

2. Tree age, growth rate, and size have all been correlated with levels of tree defenses. Thus, we sought to disentangle the interacting influences of these properties from possible elevational climatic effects on monoterpane composition, concentrations, and diversity of constitutive resin within three widespread pines (Pinus contorta, Pinus ponderosa, and Pinus flexilis) across a 1532 m elevational transect in the Rocky Mountains, Colorado, USA.

3. Collectively, elevation and tree properties were relatively weak predictors of defenses in P. ponderosa and P. flexilis, but explained ~ 75% of variation in monoterpane concentration and ~ 50% of diversity in P. contorta.

4. Increasing tree age had the greatest positive influence on monoterpane concentration and diversity in P. contorta, while increasing tree size had a negative influence suggesting a potential lifelong tradeoff between tree growth and defense. Elevation had a significant, negative influence on monoterpane concentration but little to no effect on monoterpane diversity in P. contorta.

5. Overall, we found some support for the EGPD hypothesis within P. contorta, but no support within P. ponderosa or P. flexilis. Our results suggest the presence of divergent conifer defense allocation strategies and drivers even among congers growing in shared environments. An improved understanding of the controls on tree defenses, particularly possible influences of climate-based drivers, is necessary for predicting forest pest dynamics under global change scenarios.

Key words: chemical ecology, climate, conifer, elevational gradient, monoterpenes, Pinus, plant defenses, resin
Introduction

Coniferous trees possess defense and resistance mechanisms that enable some individuals to avoid or withstand pest attack (Trapp & Croteau 2001; Franceschi et al. 2005; Mumm & Hilker 2006). Tree defense phenotypes, while influenced by contemporary environmental conditions, are genetically controlled and have been shaped by abiotic and biotic selection pressures occurring at local and regional scales (Rosner & Hannrup 2004; Holeski, Kearsley & Whitham 2011; Sampedro et al. 2011; Moreira et al. 2015; Westbrook et al. 2015; Zinkgraf et al. 2015; Harper et al. 2016), as well as continental scales (Pearse & Hipp 2012; Moreira et al. 2014; Carrillo-Gavilán et al. 2015; Wang et al. 2016). As a result, tree defense phenotypes can vary substantially within and among populations (Moreira et al. 2014; Ferrenberg, Kane & Langenhan 2015; Vogan & Schoettle 2015; Zinkgraf et al. 2015; Harper et al. 2016). This spatial variation in tree defenses, particularly as it relates to landscape features, has important implications for pest outbreaks (Holdenrieder et al. 2004).

Spatial variation in tree defense phenotypes can result from many dynamic, interacting mechanisms. At the scale of individual trees, defenses can increase or decrease across stages of ontogeny and phenology (Boege & Marquis 2005; Barton & Koricheva 2010). For example, in lodgepole pines (Pinus contorta Dougl. ex Loud.), older stem material has been reported to be less chemically defended than younger materials (Goodman et al. 2013). In contrast, chemical defenses of white spruce (Picea glauca Moench) needles increase with age (Quiring 1992). With respect to phenology, variations in tree size and growth rate over time have been linked to changes in defenses and risk of mortality (Ireland et al. 2014; Bigler 2016). For example, larger diameter limber pines (Pinus flexilis James) suffered higher densities of bark beetle attacks (beetle attacks/m² bark surface) than smaller trees (Ferrenberg & Mitton 2014) and larger diameter lodgepole pines (Pinus contorta) were attacked at higher frequencies than smaller trees in some locations (Björklund & Lindgren 2009). At the same time, slower growth rates correlate with reduced production of resin ducts (specialized cells where resins are produced and stored) in the
stems of numerous pine species, which increases susceptibility to bark beetle attack and mortality (Kane & Kolb 2010; Gaylord et al. 2013; Ferrenberg, Kane & Mitton 2014; Moreira et al. 2015; Hood et al. 2015; Bentz et al. 2016). Importantly, Moreira et al. (2016) found that resin defenses of 17 pine species clustered into two divergent defensive-syndromes with slow-growing species (e.g., Pinus contorta and Pinus ponderosa Lawson) investing more heavily in constitutive defenses while fast growing species have greater inducible defenses regardless of phylogenetic relatedness. This finding suggests a hierarchy of influences on pine defenses, whereby an individual tree’s defenses are influenced by age, size, and growth rate within the context of species-level adaptive strategies.

Beyond these influences, tree defenses are also hypothesized to have evolved in response to contemporary and historical climatic gradients (Moles et al. 2011a). For example, climatic suitability gradients for insects and pathogens are thought to influence the intensity of selection pressure placed upon plant defenses, shaping defenses as a function of time and biogeography (Schemske et al. 2009; Salazar & Marquis 2012; Anstett et al. 2016). This longstanding idea is encapsulated in the longstanding latitudinal gradient in plant defenses (LGPD) hypothesis (Moles et al. 2011a) and the related elevational gradient in plant defenses (EGPD) hypothesis (Rasmann et al. 2014). These hypotheses posit that trees from lower latitudes and elevations (warmer regions) experience more consistent pest pressures than trees at higher latitudes and elevations (colder regions). Thus, in forest ecosystems, these selection gradients should result in greater defenses as a function of warmer temperatures (Moles et al. 2011a; Mitton & Ferrenberg 2012; Pearse & Hipp 2012; Pellissier et al. 2012; Moreira et al. 2014; Wang et al. 2016). In general, the LGPD and EGPD hypotheses have received mixed support across a range of plants. This may be due in part to experimental designs that fail to control for phylogeny, variation in defense traits across tree species, and variable feeding strategies of different natural enemies (Anstett et al. 2016). The recent spread of pests from lower to higher elevation forests has rapidly increased interest in the EGPD hypothesis. In particular, this model might prove particularly applicable to coniferous tree...
species that often occur along relatively large elevational climatic gradients (Mitton & Ferrenberg 2012; Rasmann et al. 2014). Unfortunately, whether for conspecifics or congeners, comparisons of conifer defenses that examine a substantial elevational gradient are exceedingly rare (Hengxiao et al. 1999; Smith 2000). To our knowledge, there are no previous phylogenetically constrained studies of defenses across elevation that further parse the influences of elevation, tree age, and growth rate on defense patterns.

The possible interacting influences of tree growth rate, size, age, and climatic gradients inspired us to examine conifer defenses along a 1532 m elevational transect in the Rocky Mountains, Colorado, USA. We characterized the defenses of three widespread and ecologically important pine species of western North America: *Pinus ponderosa* var. *scopulorum* (Engelm), *Pinus contorta* var. *latifolia* (Engelm), and *Pinus flexilis* (James). Like all conifers, these species use resin as a primary defense against pests (Franceschi et al. 2005; Mumm & Hilker 2006), investing significantly in constitutive resin defenses, particularly in stem material targeted by specialized bark and wood-boring insects that can cause substantial pine mortality rates during outbreaks and droughts (Ferrenberg et al. 2014). Pine resin consists of a suite of secondary metabolites, primarily diterpene acids dissolved in sesquiterpenes and monoterpenes. Variation in tree monoterpane phenotypes strongly influences host selection and reproductive success of insect pests, as well as tree survival during pest outbreaks (Seybold et al. 2006; Raffa, Powell & Townsend 2013; Taft, Najir & Erbilgin 2015; Taft et al. 2015). Thus, in this study, we focused on the monoterpane components of constitutive stem resin. We hypothesized that monoterpane concentrations and diversity would be positively related to tree growth rates, and would decline with increasing tree age, size (stem diameter), and elevation. Our *a priori* prediction was that relatively faster-growing, younger trees at lower elevations would be the most defended within our sampling region, while slower-growing and older trees at higher elevations would be the least defended. However, given potential tradeoffs among tree defenses and growth (or other physiological processes) under optimal resource conditions, we considered an alternate hypothesis.
that higher growth rates would lead to decreased concentration of defenses as predicted by several plant defense theories (Stamp et al. 2003).

Materials and Methods

We tested our hypothesis regarding the influence of tree age, size, and growth on tree defenses along climatic gradients by sampling monoterpane chemistry of the constitutive resin of *P. ponderosa*, *P. contorta*, and *P. flexilis* across an elevational transect covering a total range of 1532 meters from the lowest to highest sampling sites. The highest sampling site was located at 3330 m above sea level (asl) (40.0375, -105.5739; -0.3°C mean annual air temperature) and the lowest sampling site at 1795 m asl (40.1113, -105.3076; 11.1°C mean annual air temperature). The highest, western-most site (located within the Niwot Ridge LTER, Boulder County, Colorado, USA) and the lowest, eastern-most site (located on private property near the city of Boulder, Colorado, USA) were separated by approximately 20 km of planar distance. The majority of sampling sites for intermediate elevations were located within a 10 km wide transect (North to South) lying between the high and low points of the East to West transect. Sampling sites were restricted to low and moderately sloping ridge-tops and benches with a south-southeast aspect in locations where access was permitted by private landowners, the Niwot Ridge LTER, the University of Colorado’s Mountain Research Station, the U.S Forest Service, and Boulder County Parks and Open Space. Previous studies along or near this elevation gradient have revealed a decreasing stand disturbance frequency as elevation increases (Schuster et al. 1995) leading to a higher proportion of trees >100 years for all three species in higher elevation stands. However, selective harvesting in the late 19th and early 20th century has led to a more homogenous age distribution in *P. contorta* compared to other conifers (Knowles & Grant 1983).

The species used here have large, often overlapping geographic ranges, yet have elevation ranges partitioned by climate. For instance, while all three species can be found growing together between 2579 m asl and 2870 m asl, *P. ponderosa* dominates at lower elevations while *P. contorta*
and *P. flexilis* are more common at higher elevations. We attempted to sample each pine species across the majority of their elevational distributions, with *P. ponderosa* sampled from 1795 to 2854 m asl, *P. contorta* sampled from 2347 to 3330 m asl, and *P. flexilis* sampled from 2579 to 3330 m asl. Trees of each species were sampled between 2011 and 2014 from five or more locations along the elevational transect, with a diverse range of tree sizes included from along randomly oriented transects within each sampling site. The goal was to collect data on defenses from across a range of tree ages and growth rates while also effectively assessing elevational/climatic influences. In addition to characterizations of constitutive monoterpene chemistry, we measured tree diameter at breast height (DBH, 1.4 m above the ground surface) and collected an increment core from the majority of sampled trees with a 12 mm diameter borer (several trees of each species were lost after resin sampling but before physical measurements were taken due to wildfires or harvest). Increment cores were used to estimate tree age at breast height from annual ring counts and annual radial growth (mm·yr⁻¹) of the most recent 5 years which were measured from high resolution scans (9600 dpi) in *Image J*. Radial growth rates were then used to calculate each tree’s basal area increment (BAI) growth for the 5 year xylem ring interval. BAI in this study was calculated as the percentage of a tree’s total cross-sectional area (mm²) comprised by of the most recent five years of xylem growth.

**Resin chemistry**

Constitutive monoterpene types and concentrations were measured from resin collected in glass scintillation vials placed into a 65 mm diameter bore hole drilled at an upward 45° angle and approximately 25 mm deep into each tree’s xylem. Vials were tightly inserted to reduce contact between resin and the atmosphere, left in place for 48 hours, and then capped and stored at -30°C until analysis. For laboratory analysis, resin samples were allowed to warm to room temperature before an aliquot of each sample was transferred to a 3 dram glass vial which was promptly capped and weighed. Each sample was then dissolved into methyl tert-butyl ether (MTBE, C₅H₁₂O)
containing either 5 mM isobutylbenzene (C_{10}H_{14}) or 5 mM cyclohexylbenzene (C_{12}H_{16}) as an internal standard. A Hewlett-Packard HP6890 Series GC/MS equipped with a Restek Rtx-5Sil MS column was used to quantify monoterpene amounts. After split injection (2 µL, 34:1 ratio, 220 °C), the oven temperature increased from an initial value of 40 °C at 10 °C/min until 200 °C was reached (5 min hold). During this time, helium flow rate was maintained at 1.6 mL/min. Identification of peaks was accomplished by comparing retention times to those of authentic standards. Calibration curves for quantification for the following monoterpenes were generated using authentic (standard) α-pinene (Fluka), β-pinene (Fluka), α-phellandrene (TCI), β-phellandrene (supplied by Dr. Ken Keefover-Ring, University of Wisconsin), Δ^3-carene (Aldrich), limonene (Fluka), myrcene (Acros), sabinene (Indofine), and terpinolene (TCI).

Statistical analyses

Relationships among tree age and size (DBH) were examined for each tree species with linear regression. DBH for each species was log-transformed to meet the assumptions of normality prior to regression analysis. Monoterpene concentration (mg monoterpenes / mg resin) and diversity (α-diversity) calculated as the Shannon diversity index (H’) based on the composition and abundance of monoterpenes in each sample were compared among the three species using Kruskal-Wallis nonparametric tests followed by Steel-Dwass comparisons (a nonparametric multiple means comparison with sequential correction). Differences in the mean pair-wise dissimilarity of monoterpene diversity (β-diversity) calculated as the Bray-Curtis dissimilarity index of each species were compared to mean differences obtained by calculating all possible values of the test statistic under rearrangements of the labels on the observed data points, otherwise known as a permutation resample test. This approach determines whether observed differences in mean monoterpene dissimilarity among species differs from random differences; we used this approach to avoid violating assumptions of sample independence associated with other possible tests. In addition to simple assessments of monoterpene concentration and diversity, we treated...
monoterpene composition and abundances in each tree’s resin sample as ‘communities’ which were compared via multi-response permutation procedure (MRPP, a non-parametric method for comparing the hypothesis of no difference among groups/communities). We also used a stepwise logistic regression model to determine what proportion of resin samples could be correctly categorize to their species of derivation via monoterpene measures, with the best-fit logistic regression model determined via the Bayesian information criterion (BIC).

We examined hypothesized relationships of total monoterpene concentration, diversity, dissimilarity, and individual monoterpene concentrations with elevation, tree age, size, growth rate (entered into each model as radial growth and BAI of the most recent five years from tree increment cores) via generalized regression models. Generalized regressions were completed in JMP Pro 12.0.1 (2015) using the adaptive lasso estimation method, an approach that computes parameter estimates by penalizing a weighted sum of the absolute values of the regression coefficients (Zou 2006). Best-fit models were validated using the Corrected Akaike Information Criterion (AICc). Concentration, diversity, and dissimilarity data had variable distributions across the three species. Thus, *P. contorta* monoterpene concentration (untransformed) and diversity (cube-root transformed) were modeled as normal distributions, while monoterpene dissimilarity was modeled as a gamma distribution. For *P. ponderosa* and *P. flexilis* diversity and dissimilarity were modeled as a gamma distribution and monoterpene concentrations were modelled as a Cauchy-Lorentz distribution due to the larger number of outliers observed.

We used Structural Equation Modeling (SEM; Grace 2006) to parameterize and evaluate the fit of hypothetical, causal models linking the monoterpene concentration and monoterpene diversity of constitutive resin to factors found to be significant in generalized regressions (i.e., tree age, size, growth rate, and elevation). Similar to generalized regressions, monoterpenes measures were only weakly explained at best by tree properties and elevation for *P. ponderosa* and *P. flexilis* resulting in poorly supported SEMs for these species, leading us to discard models for these species. SEMs were performed in R version 3.1.2 (R Core Development Team 2014) with the
the lavaan package (Rosseel 2012). With the exception of monoterpane concentration (normally distributed without transformation in P. contorta) most variables used in the model were not normally distributed so we used maximum likelihood parameter estimates (MLM) and a mean adjusted Satorra-Bentler χ² (Satorra & Bentler 1988) to fit and test model fit, respectively. Because data used in the model were comprised of measures with various units (i.e., monoterpane concentration per mg of resin, tree age in years, tree size/growth in mm, and meters above sea level) we calculated standardized coefficients as indicators of the magnitudes of the various paths in our SEM.

Results

We attempted to collect samples of constitutive resin from 106 individual trees of P. contorta, 118 individuals of P. ponderosa, and 106 individuals of P. flexilis across the elevational gradient. Of these trees, 60 P. contorta (57%), 105 P. ponderosa (89%), and 106 P. flexilis (100%) yielded a sufficient quantity of resin for further analysis. For trees yielding samples, the mean age of P. contorta was 104 years with a range of 23 to 186, P. ponderosa mean age was 135 years with a range of 21 to 424, and P. flexilis mean age was 118 with a range of 29 to 375. Mean tree size, measured as diameter at breast height (DBH) in P. contorta was 20 cm with a range of 11.3 to 36.1, in P. ponderosa mean DBH was 20.2 with a range of 10.1 to 43.8, and in P. flexilis mean DBH was 16.5 with a range of 3.7 to 39.2. Tree size was negatively related to tree age for P. contorta (R² = 0.14, P = 0.009), but positively related to tree age in P. ponderosa (R² = 0.14, P = 0.0004) and P. flexilis (R² = 0.22, P < 0.0001) (Fig. S1).

Regardless of tree properties (age, growth rate, or size) or sample elevation, monoterpane concentration within constitutive resin (mg monoterpane / mg resin) was significantly greater in P. contorta and P. ponderosa than in P. flexilis (H = 58.8, d.f. = 2, P < 0.0001; Fig. 1a). Total monoterpane ‘communities’ of constitutive resin significantly differed among all three species (MRPP T = -142.5, A =0.38, P < 0.0001; Fig. 2). Logistic regression with a subset of six
monoterpane concentrations (in order of their log worth for categorizing the pine species: β-
phellandrene, limonene, sabinene, β-pinene, Δ^3-carene, and terpinolene) correctly categorized 98.2
% of resin samples by their tree species or origin (Generalized $R^2 = 0.99$, $\chi^2 = 557.5$, $P = 0.0001$; Fig. 3) with an area under the receiver operating characteristic (ROC) curve ≥ 0.99 for all three
species (a value of 1.0 indicates a perfect grouping test, a value of 0.5 indicates a poor test that
performs no better than random assignment).

The diversity of resin monoterpenes (calculated as Shannon’s $H'$, a measure of within
sample diversity or α-diversity) significantly differed among all three species (Kruskal-Wallis $H$
= 101.3, d.f. = 2, $P < 0.0001$; Fig. 1b), with the highest diversity found in P. flexilis (1.57 ± 0.03),
followed by P. ponderosa (1.31 ± 0.03) and P. contorta (1.09 ± 0.04). Mean pair-wise dissimilarity
in monoterpane diversity (a measure of average differences in monoterpane composition among
samples or β-diversity) was also significantly different among the three species ($P < 0.0001$; Fig.
1c), with the lowest level of monoterpane dissimilarity found in P. ponderosa (0.48 ± 0.007),
followed by P. flexilis (0.51 ± 0.009), and P. contorta (0.63 ± 0.010).

Generalized regressions using the adaptive lasso estimation method revealed that the
combination of elevation and tree properties (age, size, and growth rates) were strong determinants
of monoterpane concentration ($R^2 = 0.76$) and diversity ($R^2 = 0.50$) in P. contorta (Table 1).
However, these factors are very weak influences on monoterpane concentration and diversity in
P. ponderosa ($R^2 = 0.08$ and $R^2 < 0.01$, respectively) and moderate to weak influences on
concentration and diversity in P. flexilis ($R^2 = 0.17$ and $R^2 = 0.11$, respectively) (Table 1).
Monoterpane dissimilarity among trees was not influenced by tree properties or elevation for P.
contorta ($R^2 = 0.01$) but was weakly influenced by tree properties (primarily age) in P. ponderosa
($R^2 = 0.12$), and by tree age and elevation in P. flexilis ($R^2 = 0.10$).

Generalized regressions were also used to test the relationships of concentrations of
individual monoterpenes (as opposed to total monoterpane concentration described above) with
elevation and tree properties. Overall, generalized regressions suggested that variation in the
concentrations of 7 of the 10 identified monoterpenes were significantly linked to one or more tree properties in *P. contorta*, with a significant influence of elevation apparent for only two monoterpenes (*P* < 0.05; Table S1). Importantly, tree age was the most influential factor in the variation of three of the four most abundant monoterpenes on average found in *P. contorta* (Table S1). Full generalized regression models (i.e., age plus all other factors retained by adaptive lasso estimation) resulted in an $R^2 = 0.71$ for $\beta$-phellendrene (the most abundant monoterpane of *P. contorta*), $R^2 = 0.14$ for $\beta$-pinene (the third most abundant monoterpane in *P. contorta*), and $R^2 = 0.58$ for $\alpha$-pinene (the fourth most abundant monoterpane in *P. contorta*); with fits ranging from $R^2 = 0.69$ to 0.10 for the remaining less abundant monoterpenes in *P. contorta* (Fig. 2, Table S1).

For *P. ponderosa*, tree properties and elevation were poor predictors of concentrations for any of the individual monoterpenes; the model explaining the most variation in any monoterpane yielded an $R^2 = 0.08$ (camphene), while models for the four most abundant monoterpenes of *P. ponderosa* ($\delta^3$-carene, $\beta$-pinene, $\alpha$-pinene, and terpinlene) all had an $R^2 \leq 0.03$ (Fig. 2, Table S1). Finally, for *P. flexilis*, tree basal area increment growth, tree age, and elevation were significant factors (*P* < 0.05) which combined to explain a moderate amount of variation ($R^2 = 0.17$) in the second most abundant monoterpane (limonene); while tree growth rates and elevation were significantly linked (*P* < 0.05) to models explaining a modest amount of variation in terpinolene ($R^2 = 0.10$) and myrcene ($R^2 = 0.21$) both of which were moderately abundant in *P. flexilis* (Fig. 2, Table S1).

The strong relationship of monoterpane concentration and diversity in *P. contorta* to tree properties and elevation in generalized regression models led us to propose and test a hypothetical casual model (i.e., a structural equation model, SEM) of the interactive effects of tree age, size, growth rate, and elevation on *P. contorta* monoterpane concentration and diversity. Unlike generalized regressions where collinear predictors can lead to inflated model fit values, SEMs allow for predictor terms to interact via multiple paths (hence the alternate name ‘path models’). In our case, both SEMs were considered to be well fit to the observed data with both models having a Satorra-Bentler $\chi^2 = 0.043$, d.f. = 1, and *P* = 0.836 (a small $\chi^2$ and a large *P*-value indicate an
SEM that is well fit to observed data. The SEMs for *P. contorta* explained 75% ($R^2 = 0.752$) of the variation in monoterpene concentration (Fig. 4a) and 49% ($R^2 = 0.493$) of the variation in monoterpene diversity (Fig. 4b). The SEM for monoterpene concentration in *P. contorta* resin revealed: 1) elevation has the strongest, negative direct-effect on monoterpene concentration, 2) tree age has a strong, positive direct-effect on total monoterpene concentration, as well as two paths of indirect influence via tree size and growth rate (basal area increment represented by the most recent five years of annual growth), and 3) increasing tree growth rates and sizes have conflicting direct-effects on monoterpene concentrations, with faster growing trees having greater concentrations and larger trees having lower concentrations (Fig. 4a). The SEM for monoterpene diversity was similar to that for monoterpene concentrations with regard to the effects of tree age, but differed in finding no significant links of diversity and elevation. Increasing tree size appeared to have the strongest, negative direct-effect on monoterpene diversity (Fig. 4b). Taken together, the SEMs would suggest that smaller, older *P. contorta* trees with larger growth rates have the greatest levels of constitutive monoterpene defenses. At the same time, trees with these properties would also be more defended (in terms of total monoterpene concentration) at lower elevations.

**Discussion**

Pests rank among the leading causes of tree mortality and forest disturbance, with their impacts expected to increase due to global change pressures (Dietze & Matthes 2014; Haynes, Allstadt & Klimetzek 2014; Kozlov et al. 2014; Anderegg et al. 2015; Jules et al. 2016). Spatiotemporal patterns in tree defenses are an important feature of the landscape that affects forest pest dynamics (Ferrenberg 2016). Indeed, range expansion by insects has been linked to greater susceptibility of host plants within novel versus historical ranges (Desurmont et al. 2011). Also, studies ranging in focus from herbaceous plants to coniferous trees suggest that a diverse set of secondary chemicals is important for resisting natural enemies (Keefover-Ring & Linhart 2010; Iason et al. 2011; Roach et al. 2014; Richards et al. 2015). Previous work has suggested potentially
strong influences of tree properties on risk of pest attack and mortality (Iason et al. 2014). Resource
and climatic gradients have also been shown to influence tree defenses in some species (O’Neill
et al. 2002; Moreira et al. 2015; Ferrenberg 2016). At the same time, warming temperatures can
interact with conifer defense chemistry to affect the relative risk of forest insect pest outbreaks
(Kollberg et al. 2015). A greater understanding of the underlying drivers of spatiotemporal patterns
in tree defenses is necessary to improve predictions of forest pest dynamics in relation to global
change pressures (Holdenrieder et al. 2004; Ferrenberg 2016). We employed a study design that
characterized resin defenses of three congeners occurring within shared environments across a
1500 m elevational gradient. The design supported our goal of disentangling potentially interacting
influences of age, growth, size, and climate on defenses of *P. ponderosa*, *P. contorta*, and *P.
flexilis*.

We found that the combined influences of tree properties and elevation explained a
substantial amount of the variation in monoterpane concentration and diversity within *P. contorta*
(Tables 1 and S1, Fig. 4), but explained a relatively modest to small amount of the variation for
both *P. ponderosa* and *P. flexilis* (Tables 1 and S1). Specifically, for *P. contorta*, tree properties
and elevation collectively explained roughly 75% of the variation in monoterpane concentrations
and 50% of the variation in monoterpane diversity depending on the modeling approach utilized
(i.e., generalized regression vs. SEM) (Fig. 4, Tables 1 & S1). The same set of factors were
relatively weak predictors of variation in these measures for *P. flexilis* and *P. ponderosa* (Tables
1 & S1) leading us to reject SEMs for these species due to poor model fit. Similarly, monoterpane
dissimilarity was poorly explained by elevation or tree properties for all three species (i.e., $R^2 \leq
0.12$ for all). These results suggest that resin defense chemistry is determined by divergent
mechanisms among these pine species, with climate and tree properties varying greatly in their
overall influence at both the individual and species level. This result presents challenges for
predicting spatiotemporal patterns in tree secondary chemistry across coniferous species.

In general, support for hypothesized climatic gradients in plant defenses, where plants are
predicted to have greater defenses in warm regions, is mixed and a majority of studies refute their existence (Moles et al. 2011a, 2011b). However, phylogenetically constrained experiments—i.e., those performed within the same species or genus—lend stronger support for the LGPD, including support from studies completed on defenses of coniferous trees (O’Neill et al. 2002; Pearse & Hipp 2012; Moreira et al. 2014; Wang et al. 2016; Zadworny et al. 2016). Support for the EGPD hypothesis is also mixed, with support available from both community-level studies (Pellissier et al. 2014; Rasmann et al. 2014b; Descombes et al. 2017) and from phylogenetically constrained studies suggesting a general decline in plant defenses with elevation (Hengxiao et al. 1999; Smith 2000; O’Neill et al. 2002; Anderson et al. 2015). However, phylogenetically constrained studies of tree defenses completed across substantial elevational gradients in field settings are exceedingly rare, possibly limited to our present study and those by Hengxiao et al. (1999) and Smith (2000). Importantly, these previous studied diverge in their findings as Hengxiao et al. (1999) found support for the EGPD, while Smith (2000) found no change in the average monoterpene composition and concentrations in *P. ponderosa* (var. *ponderosa* Lawson) sampled across a 1430 m elevation transect of California’s Sierra Nevada. Additionally, phylogenetically constrained studies of insect damage on conifers, while also rare, suggest strong influences of soil properties on pest impacts (Kosunen et al. 2016). Conflicting results in cross-gradient studies, combined with evidence of alternative drivers of defenses, highlights the need for additional research to justify broad claims—such as those of Raffa et al. (2014) whose study did not include an elevation gradient—that climatic conditions have led to trees primarily found at high elevation being poorly defended against pests compared to low elevation trees.

A strong influence of tree age on the number of pests attacking trees and in the amount of damage they cause has been reported in numerous studies (Boege, Barton & Dirzo 2011). A meta-analysis by Barton & Koricheva (2010) found that mammalian herbivores consumed more tissue from older trees than younger ones, but there was no clear preference across studies for phytophagous insects possibly due to variation in plant defense strategies in relation to diverse
insect feeding types. We found that tree age was consistently a significant factor in best-fit models of monoterpane diversity, dissimilarity, and total concentration for all three species (Table 1), as well as in models for the concentrations of seven individual monoterpenes within *P. contorta* and three monoterpenes within *P. flexilis* (Table S1). For *P. contorta*, where models explained a substantial portion of variation in defenses, we found monoterpane concentration and diversity significantly increased with tree age—i.e., older trees have higher monoterpane concentrations than younger trees (Table 1, Fig. 4). Previous studies have also found that chemical defenses—particularly terpenoid compounds in conifer needles—increased with age in various conifer species (Barton & Koricheva 2010; Boege et al. 2011). However, Goodman et al. (2013) found that monoterpane concentrations were greater in younger xylem tissues than in older tissues of individual *P. contorta* trees—i.e., lower stem material was less defended than upper stem material. In addition to variation in tree ages and local environmental pressures across study sites, a plausible explanation for these seemingly contradictory results of our study with that of Goodman et al. (2013) likely stems from their focus on phloem defenses versus our focus on xylem defenses. Indeed, dramatic variation in levels and types of defenses across plant structures is a common feature of nearly all natural plant populations (Moore et al. 2014; Hahn & Maron 2016). At the same time, we found a significant negative effect of *P. contorta* tree size on monoterpane concentration and diversity, which initially seems to be in conflict with an age-related increase in defenses since older trees are generally expected to be larger than younger trees within a given population. Surprisingly, we found that the relationship among tree age and size was negative for *P. contorta* trees in our study, while the relationship of tree age and size were positive for *P. ponderosa* and *P. flexilis* (Fig. S1). This negative relationship among tree age and size for *P. contorta* is likely the result of late 19th and early 20th century mass harvests that targeted *P. contorta* over other species for building material (Knowles & Grant 1983). This negative relationship among age and growth in *P. contorta* in our study location might further explain the increase in monoterpane concentration and diversity with age, since slow growing conifers tend to produce
higher concentrations of constitutive chemical defenses (Moreira et al. 2016). Also, evidence indicates that conifers exhibiting slower growth rates in early years of their lifespans tend ultimately to be smaller in size but live longer (Black, Colbert & Pederson 2008; Rötheli, Heiri & Bigler 2012; Bigler 2016) and suffer less pest damage later in life than trees with rapid early growth rates (Ruel & Whitham 2002).

Similar to results from previous comparisons of monoterpane defenses in these species (Smith 2000; West et al. 2016), we found that overall, monoterpane ‘communities’ of these pine species had little overlap (Figs 2 and 3), with interspecific variation exceeding intraspecific variation regardless of elevational position or tree properties. The large differences among species was illustrated by the ability of logistic regression (based on monoterpane compositions and concentrations) to be more than 98% accurate for grouping resin samples within the proper species. Additionally, we found that monoterpane concentration and diversity were highest on average in P. contorta (Fig. 1). Specifically, monoterpane concentration was significantly lower in P. flexilis than in the other two species, while P. contorta had a significantly greater level of monoterpane diversity than both P. flexilis and P. ponderosa. Increasing levels of both of these measures have been linked to greater resistance to natural enemies (Keefover-Ring & Linhart 2010; Raffa 2014). Thus, our results suggest that P. contorta has greater levels of defense against natural enemies relative to P. ponderosa and especially to P. flexilis. Our finding that P. flexilis (subgenus Strobus) appears to be less defended against natural enemies than co-occurring congeners of the subgenus Pinus joins a result by Bentz et al. (2016) who found P. flexilis to be less defended than co-occurring Great Basin bristlecone pines (Pinus longaeva) and foxtail pines (Pinus balfouriana) despite these three species being members of the same subgenus. However, we found that 43% of P. contorta, compared to 11% of P. ponderosa and 0% of P. flexilis, failed to produce a sufficient quantity of resin for monoterpane extraction upon wounding in our experiment. Smith (2000) reported similarly low levels of resin yield in several populations of P. contorta suggesting weak responses to mechanical wounding in the absence of pathogens. Whether the failure to ‘pitch’ resin
upon mechanical wounding indicates susceptibility to natural enemies is not entirely clear based on conflicting outcomes across tree and pest species. For example, while low resin flow could indicate poor production, trees can also over-produce defensive resins and chemicals in response to relatively minor pest damage, thereby inducing their own mortality (Hulcr & Dunn 2011).

While interspecific variation in monoterpane concentrations and diversity were far greater, intraspecific variation in monoterpane chemistry was still substantial over the relatively small spatial scale of our study. Specifically, monoterpane dissimilarity—measured as the mean pairwise difference in monoterpane ‘communities’ among each tree of a species—was roughly two-fold greater in P. contorta than in P. ponderosa and P. flexilis (Fig. 1c). In other words, P. ponderosa and P. flexilis display less variation in defense from tree to tree than did P. contorta. Previous work has unveiled similar levels of intraspecific variation in resin chemistry of the species studied here, but the majority of this work was completed over larger spatial scales than present in our study (Forrest 1980; Smith 2000; Pollack & Dancik 1985; Lindström et al. 1989; Zavarin, Cool & Snajberk 1993; Pureswaran, Gries & Borden 2004; Clark et al. 2010). However, substantial variation in secondary resin chemistry in P. ponderosa was previously shown to occur in trees near the lower end of the same elevational gradient used in the current study (Latta et al. 2003).

Given that congeners occurring in the same environment must deal with similar abiotic and biotic pressures, large interspecific variation in resin chemistry suggests continued influences of deep evolutionary pressures alongside contemporary pressures on tree secondary chemistry. Elucidating the relationship between compositions and concentrations of constitutive and induced secondary chemicals is challenged by conflicting selection pressures at large and fine scales (Talluto & Benkman 2014). Local adaptations and low gene-flow have been previously demonstrated for both P. ponderosa and P. flexilis populations along the same elevational transect used in our study (Schuster & Mitton 2000). Such local adaptation is likely driven by numerous factors. Evidence indicates that pressure by different natural enemies or abiotic factors can select for diverse monoterpane profiles (Iason et al. 2011) but also lead to divergent plant defense.
strategies (Carmona & Fornoni 2012; Moreira et al. 2016). Meta-analytical results also indicate that secondary chemicals can have both detrimental and beneficial effects on insect pests of woody plants leading to conflicting adaptive influences over spatiotemporal scales (Smilanich, Fincher & Dyer 2016). Additionally, profiles of monoterpenes and other chemicals in various coniferous tree species have been associated with a range of interactions with mammalian herbivores and seed dispersers, insect pollinators, and resistance to fungal pathogens (Snyder 1993; Langeheim 1994; Latta & Mitton 1997; Litvak & Monson 1998; Witzell & Martín 2008). Similarly, the presence of ‘poorly defended’ pine species (e.g., P. flexilis) at higher elevations could be linked to physiological responses in response to low atmospheric pressure, high UV irradiance, and other factors unrelated to pest-selection pressures (Bentz et al. 2016). Finally, a focus on temperature in the absence of consideration for precipitation regime is potentially at the heart of incongruence results among studies testing the LGPD and EGPD hypotheses in coniferous trees. Work by O’Neill et al. (2002) found both constitutive and inducible defenses of spruce trees (Picea spp.) decreased with elevation and latitude in western Canada, but increased with aridity. Understanding the drivers of interspecific and intraspecific variation in defense chemistry of the species studied here and others will require additional experimental and observational efforts to disentangle influences of multiple, interacting pressures on conifer defense expression.

Authors’ Contributions
SF conceived and designed the study; SF, JL, and JM implemented the study and collected samples and data; SF, JL, SL and LR implemented laboratory procedures to quantify monoterpe concentrations; SF analyzed the data and led the writing of the manuscript; JL and JM edited drafts. All authors gave final approval for publication.

Acknowledgements
We thank Boulder County Parks and Open Space, the Indian Peaks Wilderness Alliance, the John Marr Ecology Fund, the University of Colorado’s Department of Ecology and Evolutionary Biology for support.

Data Accessibility
Data from this study will be made publicly available from FigShare pending acceptance.
References


Ferrenberg, S., Kane, J. M., & Langenhan, J. M. (2015) To grow or defend? Pine seedlings grow less but induce more defences when a key resource is limited. Tree physiology, 35(2), 107-111.


### Table 1. Generalized regression models* of monoterpene concentration (mg monoterpenes / mg resin), diversity (Shannon H*), and dissimilarity (Bray-Curtis dissimilarity) relationships to tree age, size (DBH), growth rate, and elevation in *Pinus contorta* (lodgepole pine), *Pinus ponderosa* (ponderosa pine), and *Pinus flexilis* (limber pine)

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* $R^2$ are for full generalized regression models including all factors not listed as "removed". Factors retained in each model were determined with the adaptive lasso estimation method and best-fit models were validated by AICc scores.
Figure Legends

Figure 1: Monoterpene measures from constitutive stem resin of *P. contorta* (PICO), *P. ponderosa* (PIPO), and *P. flexilis* (PIFL): (a) Monoterpene concentration (mg of monoterpenes per mg of resin), (b) monoterpene diversity (H’), and (c) monoterpene dissimilarity (pairwise Bray-Curtis distance among each tree; i.e., higher dissimilarity values indicates larger differences in chemistry among trees). Box plots show the median (center line) and the 1st and 3rd quartiles, and whiskers represent 1.5 inter-quartile range (IQR). The *P*-value is from a Kruskal-Wallis test; boxes with different lettering are significantly different (*P* < 0.05) via Steel-Dwass nonparametric pairwise comparison.

Figure 2: Mean relative abundance of 10 identified monoterpenes and unidentified (other) monoterpenes in the constitutive resin of lodgepole pines (*P. contorta*; *P. CONTORTA*), ponderosa pines (*P. ponderosa*, PIPO), and limber pines (*P. flexilis*, PIFL). Monoterpene relative abundances significantly differ among all three species (*χ^2*=203.7, *P* < 0.0001).

Figure 4: Canonical correlation plot illustrating the differences in monoterpene chemistry among lodgepole pine (*P. contorta*; red triangles), ponderosa pine (*P. ponderosa*; grey circles), and limber pine (*P. flexilis*; blue squares) trees. For each species, the larger open concentric circle shows the 50% contour (the region in ordination space that contains ~50% of observations), while the smaller open concentric circle indicates the 95% confidence ellipses. Vectors indicate the strength and influence of specific monoterpenes on point locations relative to canonical axis 1 and axis 2.

Figure 5: Structural equation models relating resin monoterpene concentration (a) and diversity (b) to tree properties and elevation. Values are standardized coefficient estimates, lines widths are scaled to represent effects sizes with solid lines indicating significant paths (*P* < 0.05) and dashed lines nonsignificant paths. Measures of tree age, tree growth increment (basal area...
increment, BAI, represented by the most recent five years of annual growth), tree size (diameter at breast height, DBH), and monoterpene content of constitutive resin (mg monoterpenes/mg resins) were determined from samples (increment cores and liquid resin) collected 1.4 meters above the ground surface.
Figure 1
Figure 2
Figure 3
Figure 4
### Table S1. Generalized regression models* of monoterpene relationships to tree age, size (DBH), growth rate, and elevation across *Pinus contorta* (lodgepole pine), *Pinus ponderosa* (ponderosa pine), and *Pinus flexilis* (limber pine)

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* $R^2$ are for full generalized regression models with all factors not listed as "removed". Best-fit models were validated by AICc scores.
Figure S1

Relationship of tree size (diameter at breast height, DBH) to tree age (# annual xylem rings at DBH) in *Pinus contorta* (a), *Pinus ponderosa* (b), and *Pinus flexilis* (c). DBH was log-transformed for all three species to meet linear model assumptions of normality.
R script for SEM

## Structural Equation Model(SEM) code for pine resin monoterpenoid concentration and diversity in relation to tree age, size, growth rate, and elevation.

## scott.ferrenberg@colorado.edu

###############
# Set your working directory (in this example a folder named SEM on the C drive)
setwd("C:/SEM")

#Load library
library(lavaan)

#Load data
ResChem <- read.csv("ResinChemistryPICO.csv")

#Examine contents of data file
names(ResChem)
head(ResChem)

##MODEL 1
##Monoterpene Concentration
mod1 <- 'Mt_conc ~ Age + DBH + BAI5 + Elev
       DBH ~ Age + BAI5
       BAI5 ~ Age + Elev'

#Note that this model was fit with Fml, but, due to multivariate non-normality
#we scaled the test of fit and the standard errors
#monoterpene concentrations were normally distributed
mod1Fit <- sem(mod1, data=ResChem, estimator="MLM")
summary(mod1Fit)
varTable(ResChem)

#standardized coefficients are in Fig. 5 of the model
standardizedSolution(mod1Fit)
modindices(mod1Fit)

#variation explained
inspect(mod1Fit, "r2")

#model fit BIC
fitMeasures(mod1Fit, "bic")

##################################################################

##Model 2
##Monoterpene Diversity
mod2 <- `Mt_div ~ Age + DBH + BAI5 + Elev`

DBH ~ Age + BAI5

BAI5 ~ Age + Elev`

# Note that this model was fit with Fml, but, due to multivariate non-normality
# we scaled the test of fit and the standard errors
# monoterpane diversity was cube-root transformed to better meet the assumption of normality

mod2Fit <- sem(mod2, data=ResChem, estimator="MLM")
summary(mod2Fit)
varTable(ResChem)

# Standardized coefficients are in Fig. 5 of the model
standardizedSolution(mod2Fit)
modindices(mod2Fit)

# Variation explained
inspect(mod2Fit, "r2")

# Model fit BIC
fitMeasures(mod2Fit, "bic")