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Constitutive and Induced Defenses in Long-lived Pines Do Not Trade Off but Are Influenced by Climate

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Abstract
Plants resist herbivores and pathogens by using constitutive (baseline) and inducible (change in defense after an attack) defenses. Inducibility has long been predicted to trade off with constitutive defense, reflecting the economic use of resources. However, empirical evidence for such tradeoffs is variable, and we still lack understanding about when and where defense trade-offs occur. We tested for tradeoffs between constitutive and induced defenses in natural populations of three species of long-lived pines (*Pinus balfouriana, P. flexilis, P. longaeva*) that differ greatly in constitutive defense and resistance to mountain pine beetle (MPB, *Dendroctonus ponderosae*). We also assessed how climate influenced constitutive and inducible defenses. At seven high-elevation sites in the western U.S., we simulated MPB attack to induce defenses and measured concentrations of terpene-based phloem defenses on days 0, 15, and 30. Constitutive and induced defenses did not trade off among or within species. Simulated MPB attack induced large increases in defense concentrations in all species independent of constitutive levels. MPB and its symbiotic fungi typically kill trees and thus could be selective forces maintaining strong inducibility within and among species. The contrasting constitutive concentrations in these species could be driven by the adaptation for specializing in harsh, high-elevation environments (e.g., *P. balfouriana* and *P. longaeva*) or by competition (e.g., *P. flexilis*), though these hypotheses have not been empirically examined. Climate influenced defenses, with the greatest concentrations of constitutive and induced defenses occurring at the coldest and driest sites. The interactions between climate and defenses have implications for these species under climate change.

Keywords Mountain pine beetle · *Pinus longaeva* · *Pinus balfouriana* · *Pinus flexilis* · Defenses

Introduction

A fundamental defense used by plants against herbivores and pathogens is the production of deterrent or toxic chemical compounds. These chemical defenses include pre-existing constitutive and plastic inducible concentrations that increase after an attack (Howe and Jander 2008; Zaynab et al. 2018; Karban 2020). Ecologists have long sought to understand inter- and intraspecific variation in constitutive and induced plant defenses and the factors shaping these traits. Explanations for the great variation seen in plant investment in defense – and for the evolution of inducible defenses itself – center on management and allocation by plants of limited resources (Stamp 2003; Karban 2020). Constitutive defenses are presumed to be favored in environments with abundant and consistent pressure from herbivores or pathogens – in these habitats, maintaining permanent high levels of baseline defenses (and the associated costs) should be optimal. By contrast, inducible defenses are thought to be the preferred strategy if herbivory or pathogen infection is infrequent and unpredictable, allowing plants to save resources by enacting defenses only when needed (Stamp 2003; Cipollini and Heil 2010; Karban 2011). Plant allocation to constitutive and induced defenses is also predicted to be modulated by abiotic factors, including climate and resource availability (Coley et al. 1985; Herms and Mattson 1992; Hahn and Maron 2016).

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Accordingly, it has long been hypothesized that constitutive and induced defenses should trade off (Rhoades 1979; Herms and Mattson 1992; Zangerl and Bazzaz 1992; Koricheva et al. 2004). This prediction follows from the ideas that defenses are costly and that plants that are investing in high levels of constitutive defenses should already incur minimal damage and benefit little by further induced resistance (Zangerl and Bazzaz 1992). However, support for this hypothesis has been mixed with studies finding both evidence for tradeoffs (e.g., Lewinsohn et al. 1991; Kempel et al. 2011; Moreira et al. 2014), against tradeoffs (e.g., Brody and Karban 1992; Thaler and Karban 1997; Rasmann and Agrawal 2011; Soderberg et al. 2022), or mixed results (López-Goldar et al. 2020). Several possible explanations have been posited for this ambiguity. First, it is unclear whether this tradeoff hypothesis applies to both within and among species (Agrawal and Hastings 2019), and tests for the existence of tradeoffs have variously been applied to either level (rarely both). Second, there has been confusion over the proper way to calculate inducibility and test for tradeoffs, with some methods potentially leading to spurious conclusions (Morris et al. 2006). Third, many studies that did not find a tradeoff were those that compared species or genotypes of cultivated plants that have been subjected to strong artificial selection that could have affected evolutionary tradeoffs (Brody and Karban 1992; Thaler and Karban 1997; Underwood et al. 2000). Fourth, if constitutive and induced defenses are not functionally redundant and instead provide different benefits to the plant, tradeoffs would not be expected (Koricheva et al. 2004). Finally, within species, the existence of tradeoffs can be contingent on the trait studied (Bingham and Agrawal 2010; Villari et al. 2014; López-Goldar et al. 2020), suggesting that defense traits can evolve independently (Koricheva et al. 2004). We still have much to understand about the relationship between constitutive and induced defenses and under what circumstances tradeoffs manifest.

The constitutive and induced defenses of conifers are well studied because they dominate many of the world's forests and are involved in some of the most spectacular plant-herbivore interactions on the planet – those between conifers and bark beetles (Franceschi et al. 2005; Keeling and Bohlmann 2006; Celedon and Bohlmann 2019; Kopaczyk et al. 2020). A hallmark of conifers is their copious production of resin-based defenses that protect trees from biotic (e.g., herbivores and pathogens) and abiotic (e.g., extreme temperature and light, drought) stresses (Kopaczyk et al. 2020). Studies investigating constitutive and induced chemical defenses in conifers have tended to find tradeoffs among species (Lewinsohn et al. 1991; Litvak and Monson 1998; Moreira et al. 2014; but see Soderberg et al. 2022) but less so within species (Lombardero et al. 2000; Villari et al. 2014; López-Goldar et al. 2020; Soderberg et al. 2022). However, we still lack the ability to make confident predictions about if and under what conditions constitutive and induced defenses trade off in conifers. The detection of tradeoffs varies in part because the biological scale examined across studies is variable (e.g., from within a population to across species and seedlings in greenhouses to mature trees in nature) (Howe et al. 2020). Further, several studies suffer from methodological issues related to measuring inducibilty, extending the difficulty of consistently predicting tradeoffs in conifers (Morris et al. 2006).

The abiotic environment, including climate, can influence concentrations of constitutive and induced defenses in conifers directly through effects on available resources (Sampedro et al. 2011; López-Goldar et al. 2020), which can vary with elevation or latitude (Moreira et al. 2014; Ferrenberg et al. 2017; Gray et al. 2019; Mullin et al. 2021). Resource availability is predicted to be more positive at lower latitudes and elevations with more favorable climate and growing conditions. These high resource environments are also theorized to select for fast growth or regrowth over defense, whereas low resource environments result in slow growth and better defended tissues (i.e., the resource availability hypothesis, RAH; Coley et al. 1985). Specifically, the RAH posits that inducible defenses should be more common in resource-rich environments, while a higher investment in constitutive defenses is expected in resource-poor environments (Endara and Coley 2011). Climate can also indirectly influence conifer defensive chemistry by altering herbivore and pathogen population pressure. Trees from lower latitudes and elevations with more favorable climate are predicted to experience greater and more consistent herbivore pressure than those from higher latitudes and elevations and thus should invest more in defenses (Salazar and Marquis 2012; Rasmann and Agrawal 2011; Moreira et al. 2018). The opposing predictions of these two hypotheses could explain the inconsistency among studies examining plant defense investment across latitudes and elevations both among species (Moreira et al. 2014) and among populations within a species (Hahn and Maron 2016; Hahn et al. 2019).

We measured induced and constitutive phloem defenses across populations of three five-needle pine species: *Pinus balfouriana* Grev. and Balf. (foxtail pine), *P. longaeva* Bailey (Great Basin bristlecone pine), and *P. flexilis* James (limber pine). These species grow at high elevations, are considered among the longest-lived organisms worldwide (Piovesan and Biondi 2021), and have varying attraction and susceptibility to a specialized herbivore, the mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB, Coleoptera: Curculionidae, Scolytinae) (Gray et al. 2015; Bentz et al. 2017; Eidson et al. 2018). MPB-caused mortality has

been found to be extensive in *P. flexilis* (Millar et al. 2007; Cleaver et al. 2015), low in *P. balfouriana* (Nesmith et al. 2019; Dudney et al. 2020), and historically rare in *P. longaeva* (Bentz et al. 2017) although hotter droughts associated with climate change may be altering this relationship (Bentz et al. 2022). The discovery that constitutive investment in defense varies tremendously across these species provides a unique opportunity to test for tradeoffs between induced and constitutive defenses, and to evaluate the role of environmental variation in the two defense traits. Bentz et al. (2017) found that *P. longaeva* and *P. balfouriana* had up to 8-fold greater constitutive terpene concentrations than *P. flexilis*, which often grows intermixed with the two other species. Although an induced response has not been investigated, these results highlight the potential for tradeoffs between induced and constitutive defenses among these species.

Terpenes are the most abundant and diverse secondary metabolites in pines (Franceschi et al. 2005) and are known to be toxic to MPB (Raffa et al. 2005; Zhao et al. 2011; Chiu et al. 2017; Reid et al. 2017) and its symbiotic microorganisms (Adams et al. 2011). High investment in constitutive terpene defenses by *P. balfouriana* and *P. longaeva*, relative to *P. flexilis*, correlates with their purportedly low susceptibility and attraction to MPB (Bentz et al. 2017; Eidson et al. 2018). Constitutive levels in *P. longaeva* (and *P. balfouriana*), therefore, may provide sufficient protection against MPB, and these species would likely benefit little from additional induced defenses. Thus, we hypothesized that constitutive and induced defenses (to simulated MPB attack) would trade off among species with *P. flexilis* displaying greater inducible defenses than *P. balfouriana* and *P. longaeva*. Because tradeoffs have not generally been found within conifer species (Lombardero et al. 2000; Villari et al. 2014; López-Goldar et al. 2020; Soderberg et al. 2022), we predicted that no tradeoffs would be found across populations within these species. Further, we hypothesized that environments with a more favorable climate would be associated with greater induced defenses, and that trees in less favorable environments would invest more in constitutive defenses. The effects of environmental variation on inter- and intra-specific variation in defense traits were incorporated into our analyses using climate data for the geographic locations of each population sampled within the three species.

Materials and methods

Species and study sites

We sampled three five-needle pine species at sites reported in Bentz et al. (2017) (Fig. 1a; Table 1). *Pinus balfouriana* and *P. longaeva* are closely related species in the *Pinus* subsection Balfourianae. *Pinus balfouriana* is found at high elevations in two disjunct populations in California, either in the Klamath Mountains of northern California or the southern Sierra Nevada mountains (Eckert et al. 2008). *Pinus longaeva* is found in disjunct populations at high elevations across the Great Basin in Nevada, eastern California, and western Utah (Bentz et al. 2022). The distributions of *P. longaeva* and *P. balfouriana* do not overlap, but each species can be found growing with *P. flexilis*, which has an extensive distribution in western North America (Windmuller-Campione and Long 2016). Among conifers, the three species are among the longest-lived (Piovesan and Biondi 2021). *Pinus longaeva* is arguably the most famous, however, with individual trees aged to be more than 4000 yrs old, and one more than 5000 yrs (Salzer and Baisan 2013). We attempted to sample sites where *P. flexilis* and either *P. longaeva* or *P. balfouriana* intermixed. The three species grow at high elevations and are often found in isolated mountain ranges where access can be difficult. Where possible, sites were chosen based on the presence of the target pine species and accessibility, with a goal to capture the geographic extent of the two Balfourianae species. At the northern *P. balfouriana* site, we were not able to sample a site mixed with *P. flexilis*; and *P. flexilis* and *P. longaeva* were sampled separately on the Dixie and Fishlake National Forests in southern Utah (Table 1).

Treatments and phloem sampling

At each site, 15 un-attacked and seemingly healthy trees of each species were selected within a diameter at breast height (dbh) range of 31 to 46 cm (Table 1). The GPS location of each tree was recorded. Within each species, ten trees were randomly chosen to receive a simulated MPB attack treatment and the remaining five trees received a mechanical wounding only treatment. All samples were collected during the historical period of MPB flight and attack timing between mid-July and early September (Bentz et al. 2014, 2015). To assess constitutive chemistry, a 22.2 mm x 6.4 mm phloem plug was removed with an oblong punch (Osborne #2; UPC 01646; C.S. Osborne & Co., Harrison, NJ, USA) at dbh from east and west aspects of each tree bole. Phloem thickness (mm) was measured, and the tissue from each tree was pooled into a vial and immediately placed on dry ice for transport to the lab where samples were stored at -40 °C

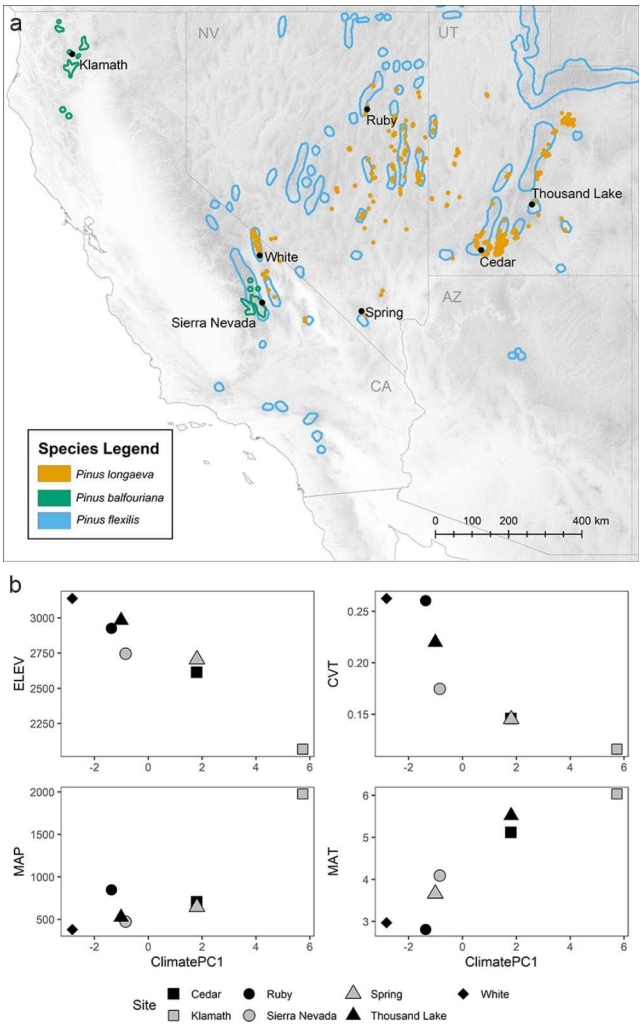

Fig. 1 (a) Study sites (points) and geographic ranges (only partial range shown for *P. flexilis*) of the three western North American *Pinus* species studied here. See Table 1 for species sampled at each site. (b) Mean coefficients of the first principal component axis (ClimatePC1) for trees at each site plotted against elevation (ELEV), coefficient of variation in mean annual temperature (CVT), mean annual precipitation (MAP), and mean annual temperature (MAT)

until processing. The ten randomly chosen trees receiving simulated MPB attack were immediately treated by placing a fungal inoculation of *Grosmannia clavigera* (University of British Columbia; M002-06-03-05, UC21G26; Canmore, AB, Canada), a fungal symbiont of MPB, spore side down into the two 22.2 mm x 6.4 mm wounds created by the constitutive sample (Fig. S1). *Grosmannia clavigera* was grown on 2% Malt Extract Agar (MEA) for 14 days at room temperature prior to inoculation. Multiple studies have shown that inoculating phloem with *G. clavigera*, the most aggressive and often most abundant pathogen vectored by MPB, induces a resin defense response that is similar to attacks by live beetles (Raffa and Berryman 1983; Keefover-Ring et al. 2016; Soderberg et al. 2022). Mechanically wounded

Table 1 Site and sample tree metrics. Fifteen trees of each species were sampled at each site

Site	National Forest	Species	Elevation (m)	DBH ± SE (cm)	Phloem thickness (mm)
White Mountains	Inyo	*P. longaeva*	3135	38.9 ± 1.57	6.2 ± 0.22
		P. flexilis	3141	37.1 ± 0.79	3.8 ± 0.13
Ruby Mountains	Humboldt-Toiyabe	*P. longaeva*	2933	38.4 ± 1.15	6.1 ± 0.22
		P. flexilis	2920	38.5 ± 0.82	3.8 ± 0.16
Thousand Lake Mountain	Fishlake	*P. flexilis*	2982	36.8 ± 0.98	5.4 ± 0.22
Sierra Nevada Mountains	Inyo	*P. flexilis*	2751	39.1 ± 1.49	3.9 ± 0.15
		P. balfouriana	2742	36.6 ± 1.20	7.4 ± 0.32
Cedar Mountains	Dixie	*P. longaeva*	2615	37.9 ± 0.93	3.6 ± 0.11
Spring Mountains	Humboldt-Toiyabe	*P. longaeva*	2722	37.4 ± 1.01	3.7 ± 0.17
		P. flexilis	2690	38.1 ± 1.04	3.1 ± 0.08
Klamath Mountains	Klamath	*P. balfouriana*	2069	40.2 ± 0.85	6.0 ± 1.13

trees received the constitutive sampling but were not treated with fungal inoculum. Wounds on all trees were sealed with Parafilm (Bemis™; PM999) to minimize desiccation and deter environmental contamination. Induced responses on each tree were measured twice, ~ 15 and ~ 30 days following the constitutive wounding. A Trephor tool was used to collect microcores of 2 mm diameter and 12 mm length (Rossi et al. 2006). The 15- and 30-day induced samples were taken 1 cm above and 1 cm below each constitutive sample on both MPB simulated attack and mechanically wounded trees. Samples were pooled by tree into vials and kept on dry ice for transport and stored at -40 °C until processing. All tools used in sampling were cleaned with 95% ethanol between trees.

Extraction and analysis of phloem chemistry

We extracted and analyzed the monoterpenoids, sesquiterpenoids, and low molecular weight benzenoids, and hydrocarbons (hereafter collectively called volatile resin) in phloem using gas chromatography following methods in Powell and Raffa (2011) and Soderberg et al. (2022). Approximately 25 mg of frozen phloem, sampled from both east and west aspects of each tree, was cut into small pieces (ca. 2 mm^3), and placed in 1 ml of 95% *n*-Hexane (Sigma-Aldrich, St. Louis, MO; ACS reagent grade) in 2-ml glass gas chromatograph (GC) vials with PTFE screw caps (Agilent Technologies, Wilmington, DE) and agitated on a shaker plate for

24 h (20 °C, 250 rpm). After shaking, the solvent was transferred to new GC vials using a pipette with low retention and extra fine tips (Denville Scientific Inc., Metuchen, NJ). The phloem vial was rinsed twice with 0.25 ml of hexane for a final volume of 1.5 ml and 100 µg of 2-nonanone was added as the internal standard. Phloem was dried in an oven at 30 °C for one week and then weighed.

Samples were analyzed using gas chromatography and mass spectrometry (Agilent 7890 A GC coupled with a 5975 C mass spectrometer) and separated on a chiral Cyclodex-B column (Agilent; 30 m x 0.25 mm i.d, 0.25 µm film thickness). Helium was used as the carrier gas (flow rate of 1.0 ml min^{-1}). One microliter of each sample was injected using splitless mode (injector 225 °C) with the GC oven maintained at 60 °C for 10 min, then increased 2.5 °C min^{-1} to 160 °C, then 30 °C min^{-1} to 225 °C. Quantifications were made relative to the internal standard by using software (ChemStation, Agilent Technologies, Wilmington, DE, USA). Compounds were identified by comparison of retention times and mass spectra with those of commercially available standards, when available (Sigma-Aldrich, St. Louis, MO, USA for all standards except for (E)-β-farnesene, Bedoukian Research, Inc., Danbury, CT, USA). For compounds without available standards, we used mass spectra and NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD, USA) to classify unidentified compounds as monoterpenoids (MT), sesquiterpenoids (ST), benzenoids (B), or hydrocarbons (HC). The occurrence of unidentified compounds across species was verified by comparing retention times, mass spectra, and using the NIST 08 Mass Spectral Search Program. Compound concentrations were analyzed on a dry phloem weight basis (mg g^{-1} phloem d.w.). The enantiomers of β-pinene co-eluted in many samples and were combined and reported as (+/-)-β-pinene.

Climate data

The influence of climate on defense traits was analyzed using modeled annual monthly PRISM climate data between 1981 and 2020 at 800 m resolution (PRISM Climate Group). For the location of each tree at all sites we calculated mean annual summer precipitation (SUMP), mean annual precipitation (MAP), mean annual temperature (MAT), mean annual temperature of the warmest month (MTWM), mean annual temperature of the coldest month (MTCM), coefficient of variation of annual temperature among years (CVT), climatic water deficit (CWD), and actual evapotranspiration (AET). CWD, a metric of water stress experienced by plants (Stephenson 1990), was modeled using a Thornthwaite water balance model (Lutz et al. 2010). CWD is a measure of evaporative demand that exceeds available water and is computed as potential evapotranspiration minus actual evapotranspiration. CWD ranges from zero, when soils are fully saturated, to positive values with no upper limit. Higher values indicate soils depleted of water and water increasingly unavailable to meet transpiration demand. CWD and actual evapotranspiration (AET) were calculated using monthly average temperature and summed precipitation, latitude, folded aspect, slope, soil available water storage, and modeled hydrological processes, including snow and rain accumulation and snow water storage (Lutz et al. 2010; Redmond 2019). Soil available water storage for each site, representing the amount of water that the soil can store in the top 1 m, was acquired from the USDA soil survey gridded SSURGO database (Natural Resources Conservation Service 2013). These variables were summarized using standardized principal component (PC) analyses (prcomp package in R, R Core Team 2021). The first two PCs explained about 86% of the variability among sites. PC2 explained 25.7% of variability, but with no strong trends among sites for any variable. PC1 (hereafter ClimatePC1) explained 60.5% of the variation and was largely composed of variables related to temperature and elevation (Fig. S2). Increasingly positive ClimatePC1 values were associated with warmer locations, lower elevations, and lower variation among years in mean temperature (CVT) (Fig. 1b). The most positive PC1 value was associated with the site with the greatest precipitation (MAP). ClimatePC1 coefficients for each tree were included in generalized linear mixed models to estimate the influence of climate variation among trees and sites on constitutive and induced defense traits.

Data analyses

We initially tested total volatile resin concentrations for differences between the species and treatments (i.e., simulated MPB attack and mechanical wound alone) on each sample day (i.e., day 0, 15, 30). A generalized linear mixed effects model with a gamma distribution and a logistic link function (glmmTMB package in R) was used to model a three-way interaction of the fixed effects (species, sample day, treatment), and we included an autoregressive random effect of the correlation between sample day and individual tree (Brooks et al. 2017; R Core Team 2021). Inducibility is the difference between induced concentrations at day 15 or day 30 and constitutive concentrations (Morris et al. 2006), and it was calculated in post-hoc linear contrasts using estimated marginal means because day 0 concentrations are not independent of inducibility at day 15 and 30 (Lenth 2021). A post-hoc Tukey's HSD test (emmeans package) was used to examine whether constitutive concentrations differed among species and treatments, and if inducibility at day 15

and day 30 differed among species and treatments. A similar analysis was used to test the influence of species and ClimatePC1 on constitutive (day 0) and induced (days 15 and 30) concentrations of volatile resin compounds using a three-way interaction between the fixed effects of species, sample day, and ClimatePC1, after accounting for the random effects mentioned above. This glmm model type was used to account for the lack of independence between individual trees sampled repeatedly across time and space, and to account for a non-normal distribution.

To test if treatment (MPB simulated attack or mechanical wounding) changed the composition of volatile resin, we ran a permutational analysis of variance (PERMANOVA) for each species ('adonis' in the vegan package, Oksanen et al. 2020), based on Bray-Curtis dissimilarities, focusing on differences between day 0 (constitutive) and day 30. Volatile resin compound concentrations (see Table 2 for each compounds analyzed) were log-transformed to meet the assumption of multivariate homogeneity of group dispersions. Significant PERMANOVAs ($\alpha < 0.05$) were followed with pairwise comparisons ('pairwise.adonis' function from the 'pairwiseAdonis' package). We used similarity percentage tests (SIMPER) to identify specific compounds that contributed the most to differences in volatile resin composition between day 0 and day 30 for each treatment (Oksanen et al. 2020).

Trade-offs between mean total constitutive and inducible concentrations of volatile resin were tested both among species and among sites within each species. Among species, a tradeoff was assumed if one species had lower constitutive concentrations and greater inducibility than other species. To test for tradeoffs among sites within a species, we used a Monte Carlo procedure to account for the lack of independence between the response and explanatory variables and for measurement error (Morris et al. 2006; R Core Team 2021). This analysis was conducted for *P. flexilis* and *P. longaeva*. Because *Pinus balfouriana* was only sampled at two locations, we were not able to run the Monte Carlo procedure. Instead, we tested for differences in the mean inducibility concentrations between sites using a one-tailed *t*-test and examined the relationship for a trade-off (i.e., that trees at the site with lower mean constitutive concentration were more inducible than trees at the site with higher mean constitutive concentration).

Results

Constitutive phloem chemistry

A total of 59 compounds were identified using GC-MS analysis from phloem of *Pinus balfouriana, P. longaeva*, and *P. flexilis* (Table 2). The composition was mainly monoterpenoids (30 compounds) followed by sesquiterpenoids (25 compounds), two benzenoids, and two hydrocarbons. The total concentration was also dominated by monoterpenoids (74–91% of total concentration, depending on species) followed by sesquiterpenoids (9–26%) with benzenoids and hydrocarbons making up < 0.5% of total concentrations. The dominant compounds in all species were (+)-α-pinene and (-)-α-pinene, which together made up 55–69% of total concentrations, depending on species (Table 2). The most abundant compounds were present in all species, but there were qualitative differences among species for some compounds present in trace amounts (Table 2).

Constitutive (day 0) volatile resin concentrations and composition did not differ between trees randomly selected for MPB simulated attack and mechanical wounding alone treatments in any species (Fig. S3; Tables 3a,b). By comparison, total concentrations in MPB simulated attack trees were significantly greater than the mechanically wounded trees on day 15 and day 30 in each species ($p < 0.0001$) (Fig. S3; Tables 3a,b). Therefore, analyses of constitutive concentrations included all trees, and only MPB simulated attack trees were included in analyses of induced responses.

Total volatile resin concentrations differed among sample days, species, and ClimatePC1 (Table 4a). As expected, phloem constitutive concentrations were significantly lower in *P. flexilis* (3.1 ± 0.3 mg g^{-1}) than *P. balfouriana* (67.8 ± 10.2 mg g^{-1}) and *P. longaeva* (36.6 ± 3.7 mg g^{-1}) (Fig. 2; Table 4b). *Pinus balfouriana* also had greater constitutive concentrations than *P. longaeva*.

Induced phloem chemistry

Simulated MPB attack induced large increases in total concentrations of volatile resins 15 and 30 days after treatment (Fig. 3). *Pinus balfouriana* had greater inducibility than *P. flexilis* on day 15 and day 30, although *P. longaeva* and *P. flexilis* did not differ in inducibility on either day (Fig. 2b; Table 4b). *P. balfouriana* and *P. longaeva* differed in inducibility on day 15 but not on day 30 (Fig. 2b; Table 4b). Day, treatment, and the interaction between day and treatment significantly affected volatile resin composition in each species (Table S1), but on day 0, there were no differences in volatile resin composition between trees of any species assigned to the two treatments, MPB simulated attack or mechanical wound ($p > 0.60$) (Table S2). In all three species, resin composition changed from day 0 to day 30 in MPB simulated attack trees, but not mechanically wounded trees except in *P. flexilis*. On day 30, however, volatile resin composition differed between the MPB simulated attack and mechanically wounded trees in all species ($p \leq 0.006$) (Table S2). The compositional differences between day 0

Table 2 Total constitutive (day 0) proportion (%) and concentration (mg/g, raw mean ± se) of volatile resin compounds in phloem of *P. balfouriana*, *P. longaeva*, and *P. flexilis*. Compounds are grouped by their secondary metabolite class (monoterpenoids, sesquiterpenoids, benzenoids, and hydrocarbons) and sorted from most to least concentrated for *P. balfouriana*. If a compound was not detected, the proportion and concentration was recorded as 0

	P. balfouriana		*P. longaeva*		*P. flexilis*	
	%	Abs.	%	Abs.	%	Abs.
	mean ± se	mean ± se	mean ± se	mean ± se	mean ± se	mean ± se
Total Secondary Metabolites						
(Total)	100	60.449 ± 1.042	100	56.28 ± 0.606	100	4.109 ± 0.082
Monoterpenoids						
(Total)	74.35 ± 1.264	45.125 ± 6.02	83.31 ± 0.889	47.023 ± 3.957	90.693 ± 1.281	3.715 ± 0.579
(+)-α-pinene	28.409 ± 3.404	20.473 ± 4.119	48.593 ± 1.829	27.452 ± 2.608	41.666 ± 2.566	1.637 ± 0.267
(-)-α-pinene	35.666 ± 3.279	19.101 ± 2.238	20.829 ± 1.492	11.462 ± 1.328	12.859 ± 0.988	0.454 ± 0.072
(+/-)-β-pinene	2.238 ± 0.241	1.153 ± 0.149	2.836 ± 0.159	1.575 ± 0.156	9.16 ± 0.769	0.491 ± 0.118
(-)-limonene	1.804 ± 0.779	0.715 ± 0.289	0.255 ± 0.017	0.128 ± 0.012	4.96 ± 1.024	0.2 ± 0.048
3-carene	1.55 ± 0.27	0.697 ± 0.104	2.421 ± 0.931	1.637 ± 0.677	7.484 ± 1.679	0.236 ± 0.065
bornyl acetate	0.853 ± 0.03	0.529 ± 0.075	1.073 ± 0.038	0.617 ± 0.056	0.276 ± 0.046	0.022 ± 0.008
β -phellandrene	0.472 ± 0.073	0.362 ± 0.158	2.194 ± 0.277	1.262 ± 0.187	4.719 ± 0.854	0.204 ± 0.063
α-terpinene	0.492 ± 0.068	0.317 ± 0.068	0.095 ± 0.037	0.039 ± 0.012	0.042 ± 0.007	0.001 ± 0
terpinolene	0.503 ± 0.042	0.295 ± 0.044	0.967 ± 0.183	0.539 ± 0.136	1.059 ± 0.141	0.042 ± 0.009
β-myrcene	0.491 ± 0.029	0.275 ± 0.034	0.612 ± 0.039	0.362 ± 0.038	5.365 ± 0.648	0.288 ± 0.076
(+)-limonene	0.457 ± 0.015	0.269 ± 0.035	0.413 ± 0.019	0.217 ± 0.017	0.455 ± 0.019	0.019 ± 0.003
(-)-camphene	0.395 ± 0.03	0.229 ± 0.031	0.227 ± 0.015	0.125 ± 0.012	0.203 ± 0.029	0.006 ± 0.001
(+)-camphene	0.277 ± 0.007	0.17 ± 0.026	0.238 ± 0.01	0.13 ± 0.012	0.239 ± 0.045	0.02 ± 0.009
thymol methyl ether	0.117 ± 0.054	0.156 ± 0.115	0.947 ± 0.15	0.563 ± 0.105	0.726 ± 0.108	0.03 ± 0.006
α-terpineol acetate	0.219 ± 0.019	0.145 ± 0.025	0.359 ± 0.035	0.214 ± 0.031	0 ± 0	0 ± 0
γ-terpinene	0.141 ± 0.015	0.071 ± 0.008	0.125 ± 0.028	0.069 ± 0.02	0.113 ± 0.012	0.004 ± 0.001
MT5	0.064 ± 0.009	0.046 ± 0.011	0.066 ± 0.006	0.037 ± 0.004	0 ± 0	0 ± 0
MT1	0.064 ± 0.006	0.044 ± 0.009	0.427 ± 0.058	0.207 ± 0.036	0.1 ± 0.012	0.003 ± 0.001
MT7	0.052 ± 0.012	0.022 ± 0.004	0.071 ± 0.032	0.048 ± 0.023	0.063 ± 0.01	0.003 ± 0.001
terpinene-4-ol	0.034 ± 0.005	0.021 ± 0.004	0.037 ± 0.009	0.012 ± 0.002	0.018 ± 0.004	0.001 ± 0
MT6	0.018 ± 0.004	0.016 ± 0.007	0.023 ± 0.006	0.015 ± 0.004	0.013 ± 0.003	0 ± 0
MT4	0.011 ± 0.001	0.006 ± 0.001	0.009 ± 0.001	0.005 ± 0.001	0.023 ± 0.009	0 ± 0
MT2	0.009 ± 0.003	0.005 ± 0.002	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MT3	0.007 ± 0.001	0.004 ± 0.001	0.018 ± 0.004	0.012 ± 0.003	0.014 ± 0.008	0 ± 0
cis-β-ocimene	0.009 ± 0.001	0.004 ± 0.001	0.022 ± 0.007	0.01 ± 0.002	0.272 ± 0.046	0.012 ± 0.005
tricyclene	0 ± 0	0 ± 0	0.032 ± 0.003	0.015 ± 0.002	0.038 ± 0.009	0.001 ± 0
sabinene	0 ± 0	0 ± 0	0.35 ± 0.108	0.228 ± 0.082	0.7 ± 0.152	0.036 ± 0.016
terpinene	0 ± 0	0 ± 0	0.032 ± 0.009	0.019 ± 0.006	0.086 ± 0.026	0.002 ± 0
linalool	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.038 ± 0.01	0.002 ± 0.001
MT8	0 ± 0	0 ± 0	0.037 ± 0.01	0.024 ± 0.007	0 ± 0	0 ± 0
Sesquiterpenoids						
(Total)	25.646 ± 1.264	15.322 ± 2.325	16.385 ± 0.882	9.08 ± 0.855	9.073 ± 1.275	0.386 ± 0.1
ST7	18.501 ± 1.257	11.305 ± 1.793	10.294 ± 0.773	5.449 ± 0.574	3.339 ± 0.917	0.126 ± 0.044
ST1	1.914 ± 0.134	1.18 ± 0.195	0.257 ± 0.035	0.133 ± 0.017	0.059 ± 0.023	0.001 ± 0.001
caryophellene	2.32 ± 0.72	0.929 ± 0.336	0 ± 0	0 ± 0	1.138 ± 0.09	0.039 ± 0.006
ST4	0.754 ± 0.051	0.467 ± 0.076	0.483 ± 0.034	0.254 ± 0.027	0.179 ± 0.051	0.007 ± 0.002
ST9	0.464 ± 0.127	0.434 ± 0.233	3.736 ± 0.238	2.303 ± 0.253	0 ± 0	0 ± 0
ST5	0.525 ± 0.036	0.325 ± 0.054	0.258 ± 0.022	0.139 ± 0.016	0.072 ± 0.026	0.002 ± 0.001
β-farnesene	0.312 ± 0.024	0.202 ± 0.039	0.253 ± 0.013	0.149 ± 0.015	0.155 ± 0.018	0.007 ± 0.001
α-humulene	0.372 ± 0.11	0.149 ± 0.052	0 ± 0	0 ± 0	0.141 ± 0.018	0.005 ± 0.001
ST11	0.141 ± 0.028	0.107 ± 0.028	0.159 ± 0.012	0.083 ± 0.009	0.073 ± 0.016	0.002 ± 0.001
ST3	0.118 ± 0.016	0.08 ± 0.017	0.024 ± 0.003	0.013 ± 0.002	0.218 ± 0.072	0.013 ± 0.007
ST12	0.084 ± 0.017	0.062 ± 0.016	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ST13	0.073 ± 0.014	0.056 ± 0.015	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ST8	0.066 ± 0.013	0.027 ± 0.006	0 ± 0	0 ± 0	0.307 ± 0.045	0.012 ± 0.003

Table 2 (continued)

	P. balfouriana		*P. longaeva*		*P. flexilis*	
β-elemene	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.14 ± 0.021	0.006 ± 0.002
ST6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.091 ± 0.031	0.006 ± 0.003
ST10	0 ± 0	0 ± 0	0.026 ± 0.002	0.016 ± 0.002	0.318 ± 0.039	0.012 ± 0.003
ST12	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.274 ± 0.04	0.012 ± 0.003
ST13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.752 ± 0.526	0.106 ± 0.049
ST14	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.105 ± 0.029	0.006 ± 0.003
ST15	0 ± 0	0 ± 0	0.42 ± 0.029	0.255 ± 0.029	0 ± 0	0 ± 0
ST16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.713 ± 0.087	0.024 ± 0.004
ST17	0 ± 0	0 ± 0	0.113 ± 0.013	0.067 ± 0.01	0 ± 0	0 ± 0
ST18	0 ± 0	0 ± 0	0.093 ± 0.005	0.056 ± 0.005	0 ± 0	0 ± 0
ST19	0 ± 0	0 ± 0	0.244 ± 0.018	0.15 ± 0.017	0 ± 0	0 ± 0
ST2	0 ± 0	0 ± 0	0.026 ± 0.003	0.014 ± 0.002	0 ± 0	0 ± 0
Benzenoids						
(Total)	0.004 ± 0	0.002 ± 0	0.095 ± 0.012	0.062 ± 0.011	0 ± 0	0 ± 0
α,p-dimethylstyrene	0.004 ± 0	0.002 ± 0	0.007 ± 0.001	0.003 ± 0.001	0 ± 0	0 ± 0
B1	0 ± 0	0 ± 0	0.088 ± 0.012	0.059 ± 0.011	0 ± 0	0 ± 0
Hydrocarbon						
(Total)	0 ± 0	0 ± 0	0.21 ± 0.023	0.115 ± 0.017	0.235 ± 0.019	0.008 ± 0.001
HC1	0 ± 0	0 ± 0	0.21 ± 0.023	0.115 ± 0.017	0 ± 0	0 ± 0
HC2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.235 ± 0.019	0.008 ± 0.001

and day 30 in MPB simulated attack trees were quantitative rather than qualitative. Importantly, the quantitative differences we observed were due to changes in relative abundances (blend ratios) of pre-existing compounds, and

Table 3a Results from a generalized linear mixed effects model (glmm) testing how total concentration of volatile resin compounds varied as a function of a three-way interaction between the fixed effects of species, sample day, and treatment (i.e., MPB simulated attack or mechanical wound), after accounting for an auto regressive random effect of the correlation between sample day and individual tree. Species tested were *Pinus longaeva*, *P. balfouriana*, and *P. flexilis*. Sample days were day 0 (constitutive) and days 15 and 30 following either a MPB simulated attack or mechanical wounding only

Fixed Effect	X^2	df	*p*
Intercept	5370.8	1, 472	< 0.0001
Treatment	133.7	1, 472	< 0.0001
Sample Day	584.2	2, 472	< 0.0001
Species	239.1	2, 472	< 0.0001
Treatment x Sample Day	148.9	2, 472	< 0.0001
Treatment x Species	8.06	2, 472	0.018
Sample Day x Species	214.9	4, 472	< 0.0001
Treatment x Sample Day x Species	11.2	4, 472	0.024

Table 3b Post-hoc Tukey's HSD results testing for differences between the MPB simulated attack and mechanical wounding treatments in total concentration of volatile resin compounds by species and sample day. Degrees of freedom for all contrasts = 1, 472

Species	Sample Day	t	*p*
P. longaeva	0	0.136	1.0
	15	6.695	< 0.0001
	30	8.305	< 0.0001
P. balfouriana	0	0.045	1.0
	15	4.616	0.0007
	30	5.495	< 0.0001
P. flexilis	0	-0.08	1.0
	15	10.714	< 0.0001
	30	13.176	< 0.0001

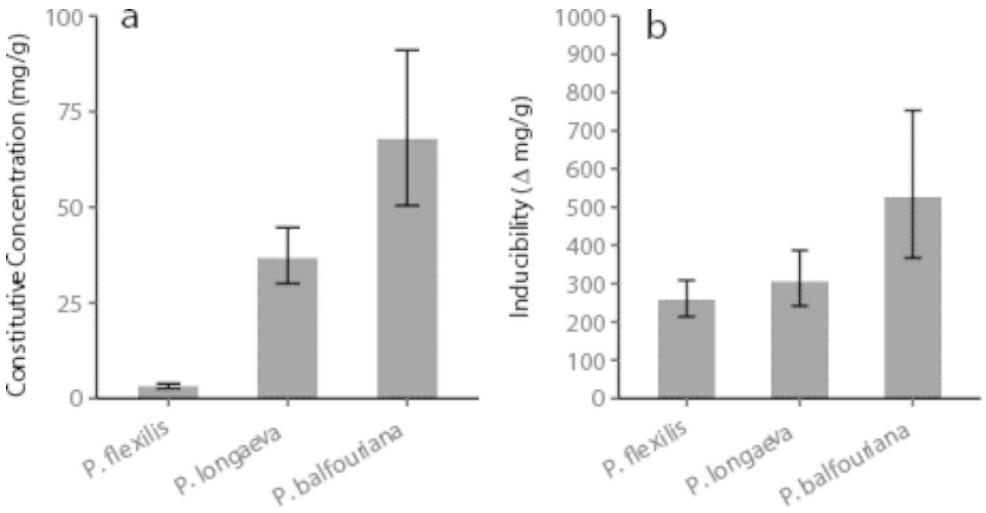

Fig. 2 (a) Constitutive concentrations and (b) inducibility 30 days after simulated mountain pine beetle attack of volatile resin in phloem of *Pinus flexilis, P. longaeva*, and *P. balfouriana*. Estimated marginal means across all sites are shown from the glmm model in Table 4a. Note different scales of y-axes. Error bars represent 95% confidence intervals. Differences among groups should be interpreted using Tukey's HSD results in Table 4b

no *de novo* compounds were detected. The compounds contributing most to overall dissimilarity of volatile resin composition between day 0 and day 30 in MPB simulated attack trees were the same for all species: (+)-α-pinene accounted for the most dissimilarity, followed by (-)-α-pinene and (+/-)-β-pinene (Table S3). The mechanical wound treatment induced relatively small increases in compound concentrations that were lower than MPB simulated attack trees on both 15 and 30 days after treatment (Fig. S3; Table 3b).

Tests for constitutive and induced tradeoffs

Among species we found no evidence for constitutive and induced tradeoffs. *Pinus flexilis* had the lowest constitutive

Table 4a Results from glmm testing how total concentrations of volatile resin compounds varied as a function of a three-way interaction between the fixed effects of species, sample day, and ClimatePC1. Species tested were *Pinus longaeva*, *P. balfouriana*, and *P. flexilis*. Sample days were day 0 (i.e., constitutive) and days 15 and 30 following a MPB simulated attack (i.e., induced). ClimatePC1 is shown in Fig. 1b

Fixed Effect	X^2	df	*p*
Intercept	9206.94	1, 364	< 0.0001
Sample Day	1111.45	2, 364	< 0.0001
Species	212.08	2, 364	< 0.0001
ClimatePC1	36.05	1, 364	< 0.0001
Sample Day x Species	225.49	4, 364	< 0.0001
Sample Day x ClimatePC1	6.10	2, 364	0.047
Species x ClimatePC1	3.28	2, 364	0.194
Sample Day x Species x ClimatePC1	11.79	4, 364	0.019

Table 4b Post-hoc Tukey's HSD results testing for differences between species in total concentration of volatile resin compounds at day 0 (constitutive) and inducibility (Δ) on days 15 and 30 following a MPB simulated attack. Degrees of freedom for all contrasts = 1, 364

Species contrast	Sample day	t	*p*
P. balfouriana - P. longaeva	0	-9.10	< 0.0001
P. balfouriana -P. flexilis	0	-6.36	< 0.0001
P. longaeva -P. flexilis	0	-2.90	0.0039
Δ *P. longaeva* -Δ *P. flexilis*	30	1.03	0.30
Δ *P. longaeva* -Δ *P. flexilis*	15	0.79	0.42
Δ *P. balfouriana* -Δ *P. longaeva*	30	1.94	0.052
Δ *P. balfouriana* - Δ *P. longaeva*	15	2.09	0.037
Δ *P. balfouriana* -Δ *P. flexilis*	30	2.47	0.014
Δ *P. balfouriana* -Δ *P. flexilis*	15	2.50	0.013

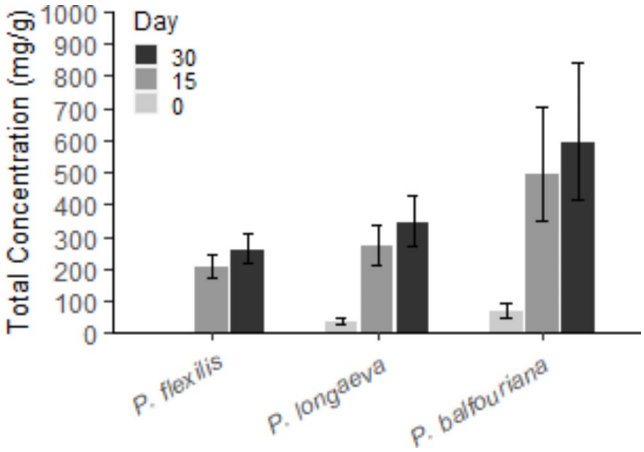

Fig. 3 Total concentrations of volatile resin in phloem of *Pinus flexilis, P. longaeva*, and *P. balfouriana* at 0, 15 and 30 days following a simulated mountain pine beetle attack. Estimated marginal means across all sites are shown from the glmm model in Table 4a. Error bars represent 95% confidence intervals. Differences among groups should be interpreted using Tukey's HSD results in Table 4b

defense concentrations among the three species, and equal or lower inducibility by day 30 (Fig. 2; Table 4b). This pattern held across all sites in which *P. flexilis* co-occurred with *P. longaeva* or *P. balfouriana* ($p < 0.0001$) (Fig. 4; Tables S4-6).

We also found no evidence for constitutive and induced tradeoffs among populations within each species. Because constitutive and induced axes are not independent (potentially leading to incorrect conclusions; Morris et al. 2006), we used a bias-corrected Monte Carlo procedure that confirmed no evidence for within-species trade-offs in *P. flexilis* ($n = 5$, Monte Carlo procedure $r = -0.58$, $p = 0.17$) or in *P. longaeva* ($n = 4$, Monte Carlo procedure $r = -0.31$, $p = 0.4$). These tests could not be performed for *P. balfouriana* because only two sites were sampled. However, no trade-off was detected, because the site with the greatest constitutive concentrations (Klamath) also had the greatest inducibility ($t_{(1, 17)} = -2.52$, $p = 0.01$).

Climate influence on constitutive and induced chemistry

We found evidence that ClimatePC1 and a three-way interaction between species, sample day, and ClimatePC1 described total volatile resin concentrations (Fig. 5; Table 4a). These results suggest an influence of climate on both constitutive and inducible volatile resin defenses in the three species. Constitutive (day 0) concentrations were negatively related to ClimatePC1 in *P. balfouriana* and *P. longaeva*, but not *P. flexilis*, which generally had very low concentrations across all sites (Fig. 5). The relationship between ClimatePC1 and inducibility at Day 30 also trended negative for all three species (Fig. 5). Sites with greater constitutive and induced defense concentrations were at higher elevations with the coolest temperatures, and also had the greatest variability among years in annual temperature (i.e., low ClimatePC1 values) (Figs. 1b and 5).

Discussion

We found no evidence that constitutive and induced concentrations of terpene-based phloem defenses trade off among the three pine species investigated. This is contrary to our prediction that a tradeoff would exist between *P. flexilis* and both *P. balfouriana* and *P. longaeva*. *Pinus flexilis* has very low levels of constitutive defenses that may not sufficiently protect it from MPB attacks, and *P. balfouriana* and *P. longaeva* have higher constitutive levels that presumably provide sufficient protection. In this scenario, *P. flexilis* should rely on and be more inducible to simulated MPB attack than *P. balfouriana* and *P. longaeva*. However, defenses of *P. flexilis* were not more inducible than either species at any site.

Despite finding no among-species tradeoffs in our study, constitutive and induced defenses have been found to trade off among other pine species (Lewinsohn et al. 1991; Litvak and Monson 1998; Moreira et al. 2014). Past studies, however, differed from ours in several ways: (1) seedlings grown in a greenhouse were used rather than mature trees in nature, (2) defenses of needles were examined rather than phloem chemistry (Lewinsohn et al. 1991; Litvak and Monson 1998; Moreira et al. 2014), and (3) non-volatile resins and phenolics were quantified rather than volatile terpenoids (Moreira et al. 2014). Perhaps most importantly, the three species we examined represent a much narrower phylogenetic and environmental range than, for example, the 18 species sampled by Moreira et al. (2014), wherein source populations of seedlings varied greatly across elevation and latitude. Tradeoffs are thought to more likely be detected across species that have evolved to grow in different habitats with varying resources and herbivore/pathogen pressure (Agrawal and Hastings 2019). We focused on *P. balfouriana*, *P. longaeva*, and *P. flexilis* because their distinct strategies regarding constitutive defense levels offered a unique test for tradeoffs with defenses induced by their shared herbivore MPB. Recently, a lack of tradeoff between constitutive and induced volatile resin was also found between *P. flexilis* and another Balfourianae species, Rocky Mountain bristlecone pine (*P. aristata* Engelm.), despite intraspecific differences in constitutive concentrations (Soderberg et al. 2022). Thus, for these four high-elevation *Pinus* species in western North America, it's clear that constitutive levels can vary dramatically, but this does not entail a tradeoff with

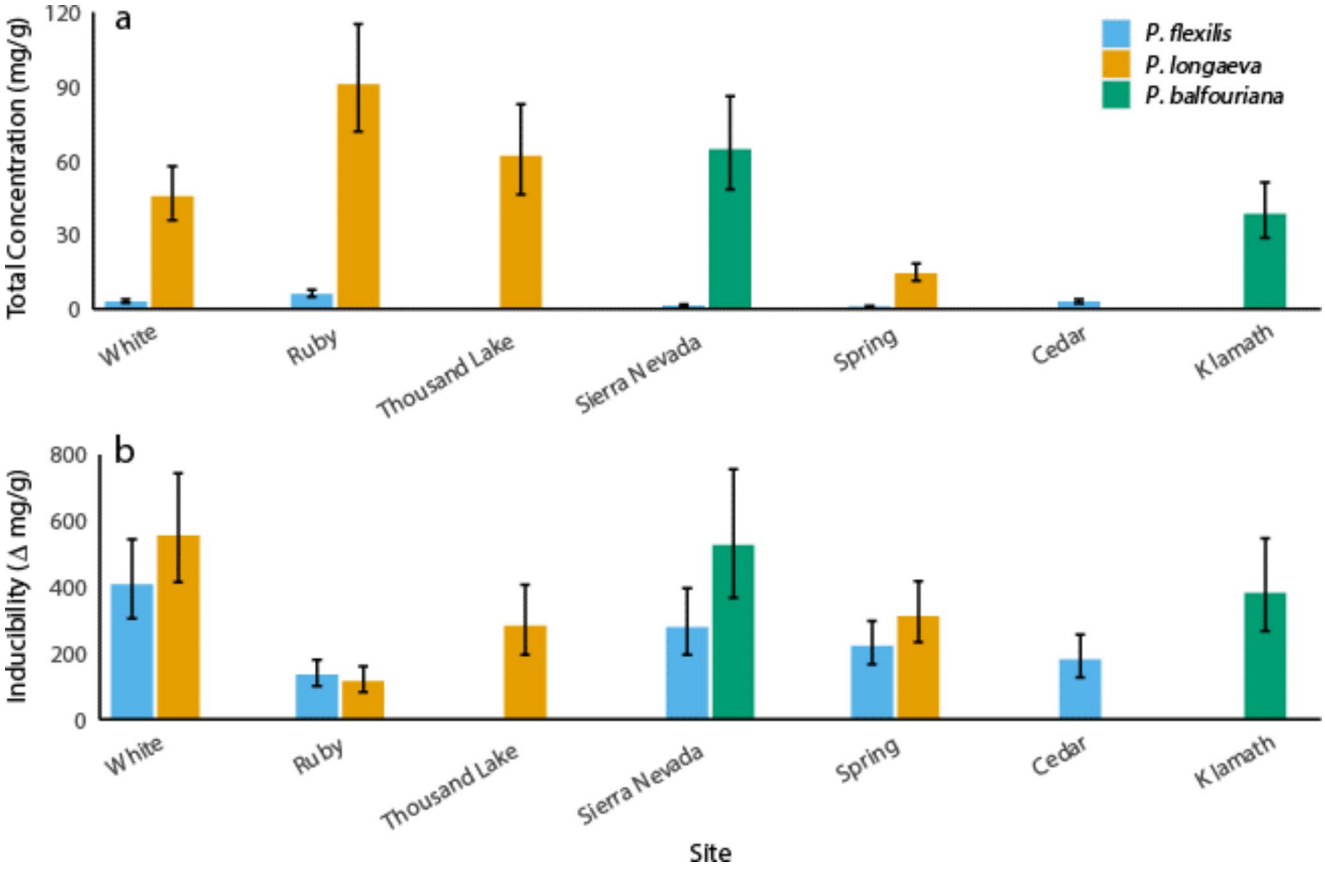

Fig. 4 Constitutive and inducible concentrations of volatile resin in phloem of *Pinus flexilis, P. longaeva*, and *P. balfouriana* across sites. (a) constitutive concentrations and (b) inducibility at day 30 following simulated mountain pine beetle attack. Sites are ordered by ClimatePC1 with more negative (harsher) sites on left and more positive on right (see Fig. 1b). Estimated marginal means are shown from the glmm model in Table 4a. Note different scales of y-axes. Error bars represent 95% confidence intervals

induced volatile resin defenses. Each species maintains the ability for a delayed (i.e., 15 to 30 days post-induction) but significant increase in concentrations of chemical defenses in response to simulated MPB attack.

As expected, we also found no evidence for a tradeoff in allocation to constitutive and induced defenses within species. Constitutive and induced tradeoffs have generally not been found within conifer species (Lombardero et al. 2000; Villari et al. 2014; Howe et al. 2020; Soderberg et al. 2022), the exception being phenolics (but not volatile resin terpenes) in a maritime pine (*P. pinaster* Ait.; López-Goldar et al. 2020). Although constitutive and inducible concentrations varied across the sites within each species, the strategy to be highly inducible to simulated MPB attack did not vary. As expected, constitutive concentrations of *P. balfouriana* and *P. longaeva*, but not *P. flexilis*, were the greatest at the highest elevations and coldest and driest sites with the highest interannual variations in temperature. Cold environments are typically associated with slow growth, which is hypothesized to increase allocation to constitutive defenses (RAH, Coley et al. 1985). Contrary to our expectations however, because no tradeoffs were found, inducible defenses were also generally greater at the highest elevation and coldest and driest sites rather than the more climatically favorable sites. For example, the northern (Klamath) *P. balfouriana* site was warmer and received > 5x the precipitation of the southern (Sierra Nevada) *P. balfouriana* site, yet both inducible and constitutive defenses at the northern site were less than both defense types at the southern site. The White Mountain site had the greatest inducible response in both *P. flexilis* and *P. longaeva* and was also the most climatically stressful of all sites. Our results suggest that for these pine species, harsher climatic conditions and potentially lower resources result in greater overall volatile resin defenses, not just constitutive concentrations as predicted by the RAH.

MPB and its symbiotic fungi could be selective forces maintaining strong inducibility within and among species (Raffa et al. 2005; Erbilgin et al. 2014). MPB will attack most pine species in western North America and a successful mass attack often kills the tree (Raffa et al. 2008). Thus, unlike many herbivore species, failure of defense against MPB results in tree death, and the strategies that many plants

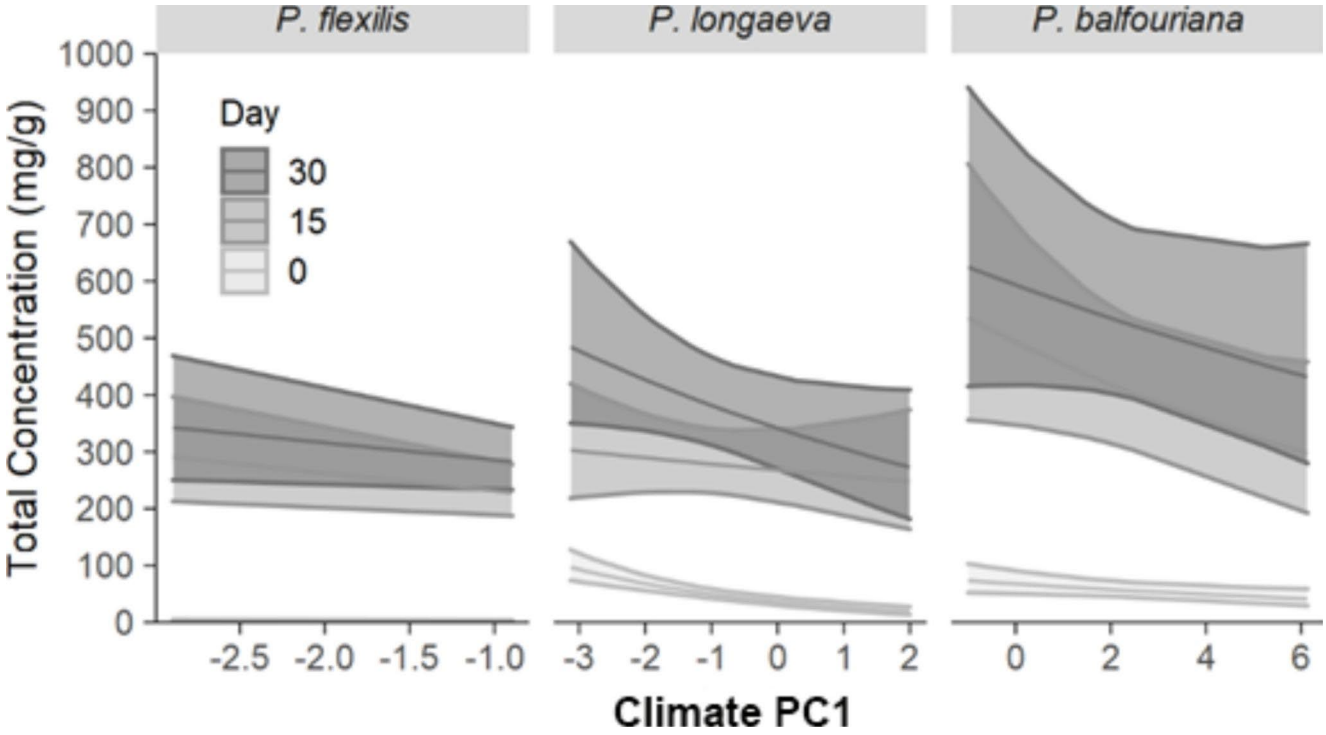

Fig. 5 Total concentrations of constitutive (day 0) and induced (day 15 and 30) volatile resin in phloem of *Pinus flexilis*, *P. longaeva* and *P. balfouriana* in relation to the mean coefficients of ClimatePC1 for trees at each site. Estimated marginal means are shown from the glmm model in Table 4a. Error bars represent 95% confidence intervals. Differences among groups should be interpreted using Tukey's HSD results in Table 4b

use to tolerate herbivory (e.g., reallocating resources to regrowth; van der Meijden et al. 1988; Strauss and Agrawal 1999) do not apply. This could explain the seemingly all-or-nothing strategy these species use to respond chemically to MPB and its associated fungi and why this response is independent of constitutive levels. Costs underly all hypotheses predicting tradeoffs (Koricheva et al. 2004; Agrawal et al. 2010), but when facing MPB the benefits of allocating all available resources to defense should outweigh the costs. Thus, for hosts to MPB (and perhaps other tree-killing beetles) an absence of tradeoffs between constitutive and induced defenses might be expected. Moreover, the two defense types may have evolved independently, and factors other than constitutive concentrations, including environmental variation as we found, may be a greater influence on induced levels (Koricheva et al. 2004). In addition, delayed (e.g., > 4 days post-induction) induced responses appear to be more targeted at MPB and its fungal symbionts compared to constitutive defenses and immediate induced responses (Soderberg et al. 2022). The high constitutive concentrations in *P. balfouriana* and *P. longaeva* could be an adaptation for maintaining extreme longevity and survival in the harsh abiotic conditions at high elevations (LaMarche 1969; Brutovska et al. 2013; Bentz et al. 2017) rather than targeted specifically at potential herbivores. For example, secondary chemicals can protect plants from a variety of abiotic stressors, including cold temperatures, intense solar (UV) radiation, and low soil moisture (Vickers et al. 2009). For these high-elevation species, investment in both defense types likely helps to reduce injury due to a wide range of biotic and abiotic threats.

Pinus balfouriana and *P. longaeva* often occur as the dominant species in open, low-diversity stands at or near treeline (Rundel et al. 1977; Beasley and Klemmedson 1980; Lloyd 1998; Bruening et al. 2017). Little competition in these harsh environments could further influence evolved traits of high investment in both constitutive and induced defenses (Herms and Mattson 1992). *Pinus flexilis*, by contrast, has a much broader elevational and geographic range (Schoettle and Rochelle 2000; Windmuller-Campione and Long 2016), and has likely evolved traits driven by high competition, especially in mid-elevation forests with greater tree density and diversity (Windmuller-Campione and Long 2016). Competition should require faster growth (Craine and Dybzinski 2013) which is predicted to trade off with defense investment (Herms and Mattson 1992; Fine et al. 2006). Because high constitutive levels may consume resources needed by *P. flexilis* (and other *Pinus* species) to compete for light and nutrients in denser forests, low investment in constitutive defenses may have evolved.

Adaptations of the high-elevation species *P. longaeva* and *P. balfouriana* to extreme climate stress may indirectly

confer increased resistance to the effects of a changing climate. Both species invest in high levels of constitutive and induced terpene defenses and are relatively less likely than co-occurring pine species to be attacked by bark beetles (Bentz et al. 2017; Nesmith et al. 2019; Dudney et al. 2020; but see Bentz et al. 2022). In addition to overall concentrations, individual compound(s) within their constitutive and induced volatile resin profiles could provide a level of protection and potential tolerance to attack and subsequent mortality. For example, both species have high concentrations of (+)-α-pinene, a compound known to be toxic to MPB adults (Chiu et al. 2017). Results from laboratory studies show that MPB feeding in *P. longaeva* die prior to becoming an adult (Eidson et al. 2018). In the first recorded *P. longaeva* mortality in the field due to bark beetles, Bentz et al. (2022) found that although MPB attacked and entered *P. longaeva*, the majority of developing brood in the phloem died prior to the adult stage and emergence from the host trees. The co-occurring *P. flexilis* trees were also attacked, and in contrast to *P. longaeva*, the majority of MPB brood survived and emerged. Climate metrics indicate that both tree species were experiencing water stress and high temperatures, highlighting climatic changes currently threatening high-elevation ecosystems. Taken together with laboratory findings, these results also highlight that volatile resin compounds in *P. longaeva* likely provide a level of protection from bark beetle-induced mortality. Additional research on *P. longaeva* phloem characteristics that influence mortality of developing MPB is needed to understand the evolutionary role of defense metrics that confer resistance to bark beetles.

In conclusion, we found no evidence for a tradeoff between constitutive and induced volatile resin defenses at either the macroevolutionary (among species) or microevolutionary (within species) scale. Despite a large disparity in constitutive concentrations, all species and populations were highly inducible to simulated MPB attack. Successful attacks by MPB necessarily kill the tree, which likely selects for a vigorous induced response across species and populations regardless of constitutive status or costs of this defense. Differences in constitutive concentrations among species could be driven by factors other than MPB. For example, *P. flexilis* likely experiences more competition than *P. balfouriana* and *P. longaeva*, and a growth-defense tradeoff could select for low constitutive concentrations in this species. Common garden experiments could be used to test for growth-defense tradeoffs and evaluate the costs of constitutive defenses across these three *Pinus* species. The high constitutive concentrations found in *P. balfouriana* and *P. longaeva* may be an adaptation allowing specialization in harsh, high elevation environments. That the greatest concentrations of constitutive defenses occurred at the harshest (coldest and driest) sites could offer support for this. However, before we can explain the patterns in constitutive and induced chemistry in these species, and why they do not trade off, we must better understand the function that terpenes play in biotic (e.g., resistance to MPB) and abiotic (e.g., tolerance to cold and drought) stressors.

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Author contributions JBR and BJB conceived and designed the experiments and performed the experiments. JBR, BJB, CAQ analyzed the data. JBR and BJB wrote the manuscript; CAQ provided editorial advice.

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Data Availability The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability The code for data analysis generated during the current study is available from the corresponding author on reasonable request.

Declarations

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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