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The publication is available from the publisher at:
http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1461-9563

Successful reproduction and pheromone production by the spruce bark beetle in evolutionary naïve spruce hosts with familiar terpenoid defenses

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Running title: Host suitability of evolutionary naïve spruces

Abstract

1 The European spruce bark beetle *Ips typographus* is a damaging pest on spruce in Europe. Beetle interactions with tree species originating outside the beetles' natural range are largely unknown and may be unpredictable, since trees without a co-evolutionary history with the beetle may lack effective defenses.

2 We compared the terpenoid composition and breeding suitability to *I. typographus* of the historic host Norway spruce *Picea abies* with two evolutionary naïve spruces of North American origin that are extensively planted in NW Europe, Sitka spruce *Picea sitchensis* and Lutz spruce *Picea glauca x lutzii*.

3 The bark of all three species had similar chemical composition and similar levels of total constitutive terpenoids, but Norway spruce had higher total induced terpenoid levels.

4 Beetles tunneling in the three spruce species produced similar amounts of aggregation pheromone. Controlled breeding experiments showed that *I. typographus* could produce offspring in all three species, with similar offspring length and weight across species. However, total offspring production was much lower in Sitka and Lutz spruce.

5 Overall, our results suggest that *I. typographus* will be able to colonize Sitka and Lutz spruce in European plantations and in native spruce forests in North America if introduced there.

Keywords: *Picea abies*, *Picea sitchensis*, *Picea lutzii*, terpenoids, naïve host

Introduction

The European spruce bark beetle *Ips typographus* (L.) is one of the most damaging forest pests in Europe and one of the few insects that can kill trees over large areas (Økland and Bjørnstad 2006). The beetles mass-attack timber, windthrows or healthy trees and oviposit in characteristic breeding galleries in the bark. The larvae tunnel through the bark as they develop, and the full development from egg to adult takes one to several months, depending on local climatic conditions. The main host of the spruce bark beetle in Europe is Norway spruce *Picea abies* (L.) H. Karst., but the beetle may occasionally reproduce in other spruce species (Økland et al. 2011). The relationship between the spruce bark beetle and its main host appears to have been shaped by an arms race of chemical warfare during their long co-evolutionary history (Franceschi et al. 2005). When the beetles tunnel into the bark, the trees defend themselves by constitutive defenses, which include storage of toxic terpenoids in premade resin ducts, and inducible defenses, such as mobilization of induced terpenoids (Keeling and Bohlmann 2006). Studies have shown that a tree's ability to effectively mobilize terpenoid defenses can be a reliable resistance marker, as trees with strong induced defenses are more resistant to bark beetle attack than trees with a weaker or slower response (Boone et al. 2011; Schiebe et al. 2012; Zhao et al. 2011b). The beetles use specific host terpenoids as precursors to produce the aggregation pheromones that coordinate their deadly mass-attacks (Blomquist et al. 2010). Because terpenoids have this dual role of defending trees against attack and promoting beetle mass-attacks, quantitative and qualitative aspects of the trees' terpenoids are important determinants of host suitability for the spruce bark beetle.

When bark beetles encounter novel host trees the outcome may be favorable for the beetles, since novel hosts without a co-evolutionary history with the beetles are evolutionary naïve and may lack effective defenses (Burke and Carroll 2016; Cudmore et al. 2010). Novel bark beetle-host tree associations form when trees are planted outside their native range and when beetles become invasive in other continents or undergo local range expansion, like the mountain pine beetle *Dendroctonus ponderosae* Hopkins in western Canada (Cudmore et al. 2010; Erbilgin et al. 2014). The two North American spruce species Sitka spruce *Picea sitchensis* (Bongard) Carrière and the Sitka/white spruce *P. glauca* hybrid Lutz spruce *Picea* × *lutzii* Little have been extensively planted within the spruce bark beetle's range in northern and western Europe since the 1960s. Plantations of Sitka spruce now exceed 1.2 million hectare (Mason and Perks 2011), mainly in coastal regions of the British Isles (1.07 million ha), Norway (50 000 ha), France (50 000 ha), and Denmark (35 000 ha). No outbreaks of the

spruce bark beetle are known on Sitka spruce in Europe, although attacks on individual trees have been observed in the British Isles and Sweden (Sean Murphy pers. comm.; Ulf Johansson pers. comm.). In addition to attacking Sitka and Lutz spruce in Europe, the spruce bark beetle could become introduced in the native ranges of these species in North America, where it has been intercepted 286 times between 1985 and 2000 (Haack 2001). It is thus of great practical interest to determine the spruce bark beetle's potential to colonize Sitka and Lutz spruce.

Like many other tree-killing bark beetles the spruce bark beetle carries a multitude of blue-stain fungi (Kirists 2004; Linnakoski et al. 2016) that are thought to help the beetles break down spruce defenses (Krokene 2015). One of the most virulent fungal associates is *Endoconidiophora polonica* (Siemaszko) C. Moreau, which can kill Norway spruce when experimentally inoculated into trees (Horntvedt et al. 1983). The ability of beetle-associated fungi to colonize and kill phloem tissues may be a critical factor determining beetle colonization success in different spruce hosts. The relationship between the spruce bark beetle, *E. polonica* and their historic host Norway spruce is well-documented (Krokene and Solheim 1996; Krokene and Solheim 1998; Krokene et al. 2003; Lahr and Krokene 2013). However, the interaction between the beetle-fungus complex and the potential hosts Sitka and Lutz spruce is almost unknown, except for a small-scale inoculation study on four trees indicating that *E. polonica* is relatively virulent to Sitka spruce (Christiansen and Solheim 1990).

As discussed above, conifers without a history of attacks from tree-killing bark beetles may be less resistant and have a bark chemistry that is more conducive to beetle colonization than trees that have co-evolved with such beetles. Evolutionary naïve trees may for example have a terpene composition that promotes beetle pheromone production and aggregation (Erbilgin et al. 2014; Raffa et al. 2013). Lack of co-evolved defenses is probably contributing to the devastating effects many invasive insects and pathogens have as they encounter "defense-free space" on novel hosts in their invasive range (Erbilgin et al. 2014; Gandhi and Herms 2009; Ploetz et al. 2013). However, evolutionary naivety goes both ways and bark beetles facing novel host trees are also evolutionary naïve and may be discouraged from colonizing the trees by different mechanisms of non-host resistance (Kaloshian and Walling 2016). Naïve beetles could be less successful in colonizing novel hosts because the beetles do not recognize the trees as a breeding substrate or because the trees' chemical composition interferes with beetle pheromone production and reproduction. Because

evolutionary naivety goes both ways novel bark beetle-conifer interactions are unpredictable with an outcome that could be beneficial to either the trees or the beetles.

The main objective of this study is to determine if evolutionary naïve Sitka and Lutz spruce are chemically suitable hosts for the spruce bark beetle, or if their terpenoid composition might be an obstacle for establishment of the beetles' symbiotic blue-stain fungus *E. polonica* and tree colonization and pheromone production by the beetles. To do this we compare the constitutive and induced terpenoid profile of Sitka and Lutz spruce with that of the co-evolved historic host Norway spruce. We also determine beetle pheromone production, beetle breeding success, and fungal colonization success in the bark of the three spruce species.

Materials and methods

Study area and sampling of trees

This study was carried out in a 0.75 ha experimental stand established in 1963 near Prestebakke in Halden, SE Norway (N 58.999 E 11.522). The stand was planted with Norway spruce and different North American conifers, with 60 × 20 meter plots of trees in parallel rows with similar densities and growth conditions. On 14 June 2014, we selected 10 trees each of Norway spruce (local provenance), Sitka spruce (provenance Alaska 21-05) and Lutz spruce (Alaska 21-1.0), measured diameter at breast height (DBH), and removed debris from the bark surface of the lower stem using a plastic brush. To determine constitutive terpene levels in the trees, we collected four bark samples equally spaced around the stem circumference of each tree at 0.5 meter height using a 9 mm cork borer. These constitutive samples were pooled, wrapped in aluminum foil and flash frozen in liquid nitrogen. To determine induced terpene levels, we treated the same trees with the plant hormone methyl jasmonate (MeJA) by placing a filter paper (5 × 5 mm) soaked in a 50 mM MeJA solution in each of the four cork borer holes. Previous work has shown that MeJA application only affects terpenoid levels in bark tissues close to the application site (< 30 cm away; Zhao et al. 2010). The cork borer holes were sealed with a 9 mm bark plug taken from a neighboring "donor-tree" of the same species that was not used for terpene sampling. One month later (1 July 2014), new bark samples were collected immediately above the original sampling positions using a 9 mm cork borer, the four samples were pooled, wrapped in aluminum foil, and flash frozen in liquid nitrogen for later analysis of induced terpene levels ("induced samples"). All constitutive and induced samples were stored at -80 $^{\circ}$C until terpene analysis.

About 10 months after the final bark sampling (13 May 2015), we felled all the experimental trees, cut a 1.2 m long stem section from each tree between 1.0 and 2.2 m above ground and brought them to the institute. We sealed the cut ends with melted paraffin wax (VWR Chemicals) to reduce desiccation and stored the stem sections outdoors. Four days later we divided each stem section into three 0.4 m long bolts, sealed the freshly cut ends with melted paraffin wax, and stored the bolts indoors at 4 ˚C. Ten bolts per tree species were used to determine beetle reproductive performance, 10 bolts were used to determine fungal colonization success, and 10 bolts were used to quantify pheromone production by the spruce bark beetle.

Analysis of terpenes

Sample preparation and extraction. For each sample type (constitutive and induced) we split the four bark plugs from each tree in two, returned one half to -80 °C as a backup, and processed the other half for terpene analysis. The cork bark was removed, and the remaining phloem was submerged in 1 ml hexane (≥ 95% Sigma-Aldrich) containing 0.20 mg pentadecane (≥ 99% Sigma-Aldrich) as internal standard and 3-tert-butyl-4-hydroxyanisole (Sigma-Aldrich) as antioxidant. All samples were extracted in hexane at room temperature for 48 hours before the extracts were filtered, transferred to Agilent MS vials with crimp top, and stored at -80 °C. The phloem was dried at 80 °C for 24 hours and weighted for absolute amount calculation.

Terpene analysis by GC-MS. Terpene analysis was carried out on an Agilent 6890 N gas chromatograph (GC) connected to an Agilent 5973 mass spectrometer (MS) and fitted with an autosampler. The GC was operated in splitless mode at 250 °C with an injection volume of 1 µL, using a 30-m fused silica Agilent J &W Scientific DB-Wax separation column (Agilent Technologies) with an inner diameter of 0.25 mm and film thickness of 0.25 µm. A 2.5-ml methyl-deactivated pre-column (Varian Inc., Lake Forest, CA, USA) with the same inner diameter was coupled to the analytical column via a press-fit connector (BGB Analytik AG, Boeckten, Switzerland). After sample injection, the temperature was held at 40 °C for 2 min and subsequently raised 6.9 °C/min to 160 °C and then 21.5 °C/min to 250 °C. The temperature was then held constant at 250 °C for 3.6 min, giving a total running time of 27.18 min. The MS was operated in scan mode from m/z 40 to 550 with a threshold of 50 and 2.86 scans/s. The transfer line temperatures were set at 280 °C, the ion source temperature at 230 °C, and the quadrupole temperature at 150 °C. Volatile compounds were identified using a Deconvolution Reporting System (DRS, version A.02.00, Agilent Technologies), which combines an automatic mass spectral deconvolution and identification software (AMDIS version 2.62, NIST) with a mass spectral library (NIST08 database) and GC–MS software (ChemStation version D.03.00, Agilent Technologies). The AMDIS database contained 1100 mass spectra of volatile compounds, 180 of which were connected to Kovats retention indexes (Kováts 1958). To obtain comparable retention times for all samples, the retention time was locked and referenced according to the internal standard pentadecane at 10.748 min by use of the ChemStation retention time-locking program. Peaks that were present in the chromatogram but not identified by the DRS were manually interpreted using the NIST08 database. To ensure reliable identification, a match factor of at least 70 % similarity was used (Stein 1999). Compound identification was verified by comparing mass spectra and retention times with those obtained for synthetic standards on the same column. Terpenes were quantified as pentadecane equivalents by dividing the peak areas from the total ion chromatogram of single terpenes by the peak area of the internal standard pentadecane. The monoterpene α-pinene was provided by Yngve H. Stenstrøm (Norwegian University of Life Sciences, Ås, Norway), whereas all other compounds were acquired as standards from Aldrich, Fluka, Chiron AS, Supelco, and SAFC.

Chiral terpene analysis. The enantiomeric composition of α-pinene was determined in a second GC-MS run to test how precursor terpenes in the host trees influenced pheromone production by the spruce bark beetle (*Ips typographus*). Chiral separation was performed using the same Agilent GC-MS setup described above, but equipped with a 30-m Cyclodex-B column (Agilent Technologies) with an inner diameter of 0.25 mm and film thickness of 0.25 µm. After sample injection, the column temperature was held at 40 °C for 0.5 min and raised by 2 °C/min to 80 °C, then raised by 10 °C/min to 220 °C and held constant for 1 min, giving a total running time of 36 min. The injection volume, injector temperature and MS parameters were the same as for the non-chiral analysis.

Beetle pheromone quantification

On 10 July 2015, spruce bark beetles were introduced into 10 cut bolts per tree species to determine the beetles' ability to produce aggregation pheromones in the different spruce species. The beetles were collected in Ås, SE Norway (N 59.677 E 10.772) in early June 2015, using traps baited with Ipslure pheromone dispensers (Borregaard, Norway), and stored at 4 ˚C. Ten vigorous beetles were placed individually on the bark surface of each bolt. Each beetle was covered by a glass vial held tightly against the bark by a rubber band extending around the bolt. To facilitate beetle entry into the bark a superficial wound was made through the cork bark at each position. After 48 hours, beetles that had entered the bark were collected, their hind gut was removed, and the guts were analyzed by GC-MS to quantify pheromone compounds, following the procedure described above. Since males are the pheromone producing sex in the spruce bark beetle only male beetles were analyzed. The spruce bark beetle can only be reliably sexed by removing the subgenital plate and aedeagus, and during this process the hindgut of males was removed and pooled by bolt (n = 1 to 6 males per bolt) in 100 µl hexane containing 0.1 µg/ml pentadecane. To compare pheromone quantity on a per male basis we divided the total amount of each pheromone compound detected in a sample by the number of male hindguts in the sample.

Beetle reproductive performance

To assess beetle reproductive performance we introduced beetles into 10 cut bolts per tree species at a controlled low density (~0.25 colonization sites per dm^2 bark surface) to minimize any effects of competition for breeding substrate on reproductive performance. On the morning of 27 May 2015, we hung the bolts from the ceiling in the institute's insectarium and

made a superficial wound through the outer bark to facilitate entry of beetles collected in pheromone traps a few days earlier. The insectarium is a shed with open wire mesh walls providing similar light and temperature conditions as those outside in the shade. We covered each bark wound by a glass vial containing four beetles. The vials were held in place by rubber bands extending around the bolt. Depending on the circumference of the bolt, 3-4 glass vials were evenly spaced around the upper and lower part of the bolt, distributed to minimize competition between galley systems. The following morning, we introduced additional beetles into each glass if some of the original beetles had not entered the bark. The spruce bark beetle cannot be accurately sexed without dissecting the genitalia, and four or more beetles were added at each colonization site to ensure that there were enough females (each colonizing male can accommodate up to four females). After three days, we removed all glass vials and covered each bolt by an emergence net with a collection bottle underneath. The bottles were emptied biweekly for emerging offspring that were counted and stored at 4 ^{o}C. On 12 October 2015, we cleared all bark from the bolts, collected and counted all remaining live beetles under the bark, and recorded the number of breeding galleries on each bolt. We dried all beetles in an oven at 70 ^{o}C for 50 hours before we determined dry weight and length for 6-75 beetles per bolt (267 beetles in total from Norway spruce, 109 from Sitka spruce, and 180 from Lutz spruce).

Performance of Endoconidiophora polonica

Ten bolts per tree species were inoculated with the bark beetle-associated blue-stain fungus *Endoconidiophora polonica* at four evenly spaced positions around the middle part of each bolt. Inoculations were made by removing a 5 mm diameter bark plug, placing fungal inoculum into the hole, and replacing the bark plug. Inoculum consisted of malt agar colonized for 21 days at a 20 ^{o}C by *E. polonica* isolate no. 193-208/115 from the culture collection of the Norwegian Institute of Bioeconomy Research. This is a virulent isolate that has been used in many previous inoculation studies with Norway spruce (e.g. Krokene & Solheim 1998; Krokene et al. 2003). The fungus was allowed to colonize the bolts for 90 days before the cork bark was removed and the length of the necrotic lesions in the inner bark was measured. Two measures were taken: (1) the length of the outer necrosis, which represents the full extent of fungal colonization, and (2) the length of the darker inner necrosis, which represents the active host defense area.

Data analyses

All statistical analyses were carried out in R (v.3.3.1) (R Core Team 2016) using the packages Vegan (v 2.3-5) for Detrended Correspondence Analysis (DCA), Nonmetric Multidimensional Scaling (NMDS) and Procrustes analysis (Oksanen et al. 2016) and the package ggplot2 (Wickham 2009) for plotting. We used NMDS to visualize differences in chemical composition among tree species and treatments (constitutive vs. induced samples) based on Bray-Curtis dissimilarities of square-root-transformed concentration data for 107 compounds from constitutive samples and 127 compounds from induced samples. The NMDS model was set to two dimensions, plots were centered, rotated to principal components, and axes were rescaled to half-change units. For both constitutive and induced samples convergence was found after 20 iterations with a goodness-of-fit measure (called 'stress') of 0.09, which indicates a good fit (Kruskal and Wish 1978). To find the optimal configuration we used NMDS in parallel with DCA and Procrustes analysis to see if the two different methods revealed the same structure. Since we found no structural differences between NMDS and DCA, and since NMDS gave an acceptable representation of the data structure, we only present the NMDS results here.

Mean constitutive and inducible terpenoid amounts were compared between tree species, both for individual compounds and for total terpenoids. Treatments were compared using one-way ANOVA followed by Tukey honest significant difference test (Tukey HSD) at $p = 0.05$.

Because (-)-α-pinene in the host tree is the main precursor for the beetle pheromone component cis-verbenol (Birgersson et al. 1988), we tested four hypotheses for how cis-verbenol production might vary with host terpene levels. Data from all three spruce species were considered together, as we assumed that effects of terpene levels on pheromone production are independent of other host tree qualities. H_0: cis-verbenol production is independent of the amount of (-)-α-pinene in the bark and there is no correlation between cis-verbenol and (-)-α-pinene within the range of (-)-α-pinene concentrations found in our samples. H_1: cis-verbenol production increases with the amount of (-)-α-pinene in the bark (positive correlation). H_2: cis-verbenol production varies with the relative amount of (-)-α-pinene in the bark (amount of (-)-α-pinene divided by the total amount of all other terpenes). H_3: cis-verbenol production varies with the amount of (-)-α-pinene minus the total amount of all other terpenes. H_2 and H_3 takes into consideration that the beetles must detoxify other host terpenoids in addition to (-)-α-pinene. H_2 assumes a linear effect of other terpenes, whereas H_3 assumes that other terpenes must be detoxified first before the beetles can use (-)-α-pinene to

produce cis-verbenol. The function cor.test in R was used to calculate Spearman's rho statistics between cis-verbenol and terpene amounts when testing hypotheses H_0-H_3.

We tested for differences between spruce species in beetle reproductive performance in cut bolts, bolt properties, length and weight of beetle offspring, beetle pheromone production, and fungal lesion lengths by using one-way ANOVA followed by Tukey HSD at $p = 0.05$. Variables with multiple measurements for each bolt were averaged before analysis (offspring length and weight, fungal lesion length). Correlations between beetle offspring production and terpenoid quantities in each bolt were calculated using the function cor.test (R Core Team 2016).

Results

Overall differences in terpenoids between Sitka, Lutz and Norway spruce

A total of 148 unique terpenoids were identified with >70% certainty in one or more of the three spruce species, of which 107 compounds were detected in constitutive samples and 127 in induced samples. There was no significant difference in total terpenoid levels between spruce species in constitutive samples ($F_{2,27} = 1.23$, $p = 0.31$), but Norway spruce had significantly higher induced levels than the other species ($F_{2,27} = 7.57$, $p = 0.0025$) (Figure 1). All three spruce species had more than 20-fold higher terpenoid concentrations in induced samples than in constitutive samples (Figure 1).

The NMDS plot (Figure 2) shows the overall chemical similarity between tree species, i.e. the closer the species are in the plot, the more similar is their terpenoid composition. The axis range spanned by the data (ca. 1.5 half-change units), indicates that there were only moderate differences between tree species in terpenoid composition, i.e. the tree species mostly shared the same compounds. The data for Sitka and Lutz spruce (blue and red areas) and their mean site scores (black dots) moved in the same direction from constitutive to induced samples, indicating that the terpenoid composition of these species changed in a similar way after MeJA treatment. Norway spruce (green areas and arrow) moved away from Sitka and Lutz following induction, becoming slightly more different in terpene composition after MeJA treatment.

Differences in abundant terpenoids between Sitka, Lutz and Norway spruce

Most of the 148 terpenoids that were identified only occurred in a few samples from each tree species and in trace amounts, and were therefore assumed to be biologically less important.

For further comparisons of terpene composition between tree species we included only the most abundant terpenoids, i.e. compounds making up 1% or more of the total terpenoid volume in constitutive or induced samples in at least one tree species (16 compounds in total). In Norway, Sitka and Lutz spruce these abundant terpenoids made up 95, 96 and 96% respectively of the total constitutive terpenoid volume and 92, 93 and 92% of the total induced terpenoid volume. Fourteen of the 16 compounds were detected in all three spruce species.

The total concentration of the 16 most abundant terpenoids was higher in constitutive Norway spruce bark than in Sitka spruce (25% higher) and Lutz spruce bark (51% higher), but the differences were not statistically significant ($F_{2,27} = 1.20$, $p = 0.32$). The concentration of individual terpenoids in constitutive bark was quite similar across species, but Norway spruce had significantly higher concentrations than the two other species for β-pinene, thunbergol, germacrene-d, and longifolene and significantly lower concentrations than either Sitka or Lutz spruce for β-phellandrene, epimanool, and sabinene (Figure 3A).

The total concentration of the 16 most abundant terpenoids increased in all three spruce species in response to MeJA-treatment, with 20- to 25-fold increases in the different species. Total induced terpenoid levels in Norway spruce were significantly higher than in Sitka (55%) and Lutz spruce (102%) ($F_{2,27} = 7.27$, $p = 0.003$). There was no significant difference between the two North American species. For seven of the 16 most abundant compounds Norway spruce had significantly higher concentrations than Sitka and Lutz spruce (α-pinene, β-pinene, thunbergol, germacrene-d, longifolene, manooyloxide and 3-carene; Figure 3B). Concentrations of the cis-verbenol precursor (-)-α-pinene was significantly higher in Norway spruce than in the two North American spruce species in induced samples ($F_{2,27} = 9.50$, $p = 0.008$) but not in constitutive samples ($F_{2,27} = 0.57$, $p = 0.57$).

Beetle pheromone production

Beetle pheromone production was highly variable between bolts and there were no significant differences in pheromone production between males tunneling in the different spruce species (cis-verbenol: $F_{2, 20} = 1.73$, $p = 0.20$; 2-methyl-3-buten-2-ol (methylbutenol): $F_{2, 23} = 1.52$, $p = 0.24$) (Figure 4). The ratio between methylbutenol and cis-verbenol production was significantly higher in Lutz spruce (34.4 : 1) than in Norway spruce (2.4 : 1) and Sitka spruce (10.1 : 1) ($F_{2, 17} = 12.11$, $p = 0.0005$).

Because there were no significant differences in cis-verbenol production by tunneling beetles between tree species, the influence of terpenes on the production of cis-verbenol was

analyzed using data from all tree species in the same analysis. We found no significant correlations between the amount of cis-verbenol produced by the beetles and any of the combinations of terpene amounts tested in hypotheses H_1-H_3 (Table 1). Thus, our results supported the null hypothesis H_0, stating that the amount (-)-α-pinene in the host does not influence beetle cis-verbenol production within the range of (-)-α-pinene concentrations found in our samples.

Beetle reproductive performance

More spruce bark beetle offspring developed successfully in Norway spruce than in Sitka spruce (~5-fold more) and Lutz spruce (~3-fold more), but the difference was significant only between Norway spruce and Sitka spruce (Table 2). The first offspring emerged from the bolts on 27 July, and only a small proportion of the total brood had emerged from the bolts when the breeding experiment ended 12 October (14% of the total in Norway spruce, 22% in Sitka and Lutz spruce). The rest of the live offspring were recovered from under the bark of the bolts. The higher offspring production in Norway spruce than in Sitka and Lutz spruce corresponded with a significantly higher number of breeding galleries in Norway spruce (Table 2). Interestingly, the number of offspring produced per breeding gallery did thus not differ significantly between spruce species (Table 2). There was no statistically significant difference in mean offspring length between Norway spruce (4.87 ±0.04 mm (±SE)), Sitka spruce (4.76 ±0.05 mm) and Lutz spruce (4.77 ±0.05 mm) ($F_{2,16} = 1.82$, $p = 0.19$). Mean offspring weight was also similar across spruce species (5.18 to 5.25 ±0.2 mg (±SE) in the three species; $F_{2,16} = 0.03$, $p = 0.97$). Bolt properties such as length, diameter, bark surface area, and bark thickness were similar for Norway, Sitka and Lutz spruce and a similar number of beetles were introduced to each spruce species (Table 2).

To test if beetle offspring production varied with spruce defense chemistry we investigated the relationship between offspring production and terpenoid levels in the bark of each bolt. Offspring production was positively correlated with constitute levels of thunbergene and thunbergol. Offspring production was also positively correlated with the inducibility of thunbergene ($r = 0.6$, $p < 0.0001$), thunbergol ($r = 0.6$, $p < 0.0001$) and α-longipinene ($r= 0.5$, $p = 0.004$), i.e. with the difference in terpenoid concentration between bark where defenses had been induced by MeJA and non-induced control bark. There were no other significant relationships between offspring production and terpene chemistry. The terpenoids that showed a significant relationship with offspring production made up < 1% of

total terpenoids in the different spruce species, except thunbergol which made up about 15% of the total in Norway spruce.

Necrosis lengths induced by Endoconidiophora polonica

Inoculation with *E. polonica* produced longer necrotic lesions in the bark of Norway spruce than in Sitka and Lutz spruce, but the difference was statistically significant only for outer necrosis lengths ($F_{2,27} = 5.62$, $p = 0.009$ (outer); $F_{2,27} = 1.86$, $p = 0.18$ (inner)) (Figure 5). There were no significant differences in necrosis lengths between Sitka and Lutz spruce (Figure 5).

Discussion

Our results show that the bark of evolutionary naïve Sitka and Lutz spruce have different sets of properties that could make the trees either more or less suitable hosts for the spruce bark beetle. Properties that would be beneficial for beetle reproduction included aspects of the trees' terpenoid chemical defenses, such as lower induced terpene concentrations in the bark (Figure 2) and a terpenoid composition resembling that of the beetles' historic host Norway spruce (Figure 1). On the negative side, offspring production was lower in the novel hosts (Table 2) and growth of the mutualistic beetle symbiont *E. polonica* was inhibited (Figure 5). We also found that although individuals that oviposited produced a similar number of offspring in all three spruce species, a much lower proportion of beetle attacks on the novel hosts led to oviposition and successful reproduction compared to Norway spruce (Table 2). This suggests that physical or chemical properties of the bark of the novel hosts may have interfered with host recognition and acceptance by the spruce bark beetle. In the following, we discuss the various positive and negative properties of Sitka and Lutz spruce bark as a breeding substrate for the spruce bark beetle in more detail.

Constitutive terpenoid chemistry and host suitability

Sitka and Lutz spruce had lower total constitutive terpene concentrations than Norway spruce, although the differences were not significant. Because terpenoids have negative effects on attacking bark beetles (Erbilgin et al. 2006; Wallin and Raffa 2000; Zeneli et al. 2006; Zhao et al. 2011b), these results suggest that Sitka and Lutz spruce are equally or more suitable hosts for the beetles than Norway spruce. However, host suitability to the spruce bark beetle is probably also influenced by physical or chemical characteristics of the bark that we did not

quantify, such as the abundance of stone cells and the concentration of nutrients and secondary metabolites other than terpenoids (Schiebe et al. 2012; Whitehill et al. 2016).

The concentration of individual terpenoids in constitutive bark suggests that Sitka and Lutz spruce should be suitable hosts for the spruce bark beetle, as we found relatively small differences in terpenoid composition between the exotic spruces and the historical host. Spruce oleoresin is a complex mixture of individual terpenoids and most of these have unknown effects on bark beetle behavior (Andersson et al. 2009; Phillips and Croteau 1999). Specific volatile terpenoids are used by the beetles as cues in host identification and selection, in combination with other visual and olfactory cues from both host and non-host plants (Andersson et al. 2009; Campbell and Borden 2006a; Campbell and Borden 2006b). Because the exact relationship between the spruce bark beetle and most host terpenoids is unknown it is difficult to predict accurately how qualitative differences in terpene chemistry will affect beetle colonization biology (Erbilgin et al. 2007b). However, four of the most abundant constitutive compounds in all spruce species [(-)-α-pinene, ß-pinene, ß-phellandrene, myrcene] elicit strong antennal responses in the spruce bark beetle and are probably important in the host selection process (Andersson et al. 2009; Kalinova et al. 2014). The relatively high concentrations of these compounds also in Sitka and Lutz spruce suggests that the spruce bark beetle will be able to detect and colonize these species under natural conditions.

Sitka and Lutz spruce had a very similar constitutive terpenoid composition (Figure 1). This was expected, since Lutz spruce is a natural hybrid between Sitka spruce and white spruce. Norway spruce, that belongs to a different clade in the spruce phylogeny (Lockwood et al. 2013), had a somewhat different constitutive terpenoid composition from Sitka and Lutz spruce.

Induced terpenoid chemistry and host suitability

All three spruce species showed a strong induced response to MeJA treatment, with 20- to 25-fold increases in total terpenoid levels one month after treatment. This massive accumulation of terpenoids in the bark probably strongly reduces tree suitability to the spruce bark beetle. Previous studies of the spruce bark beetle and other tree-killing bark beetles have shown that the beetles tend to avoid trees with very strong or rapid terpene accumulation (Boone et al. 2011; Schiebe et al. 2012; Zhao et al. 2011a). Norway spruce had higher total induced terpene concentrations than Sitka and Lutz spruce (55% and 102% higher, respectively), suggesting that the novel hosts should be a more favorable substrate for the spruce bark beetle than

Norway spruce. However, as we discuss below the opposite was true, as the beetles produced more offspring in Norway spruce than in Sitka and Lutz spruce.

Several individual terpenoids, such as α-pinene, β-pinene, 3-carene and thunbergol, increased more in Norway spruce than in Sitka and Lutz spruce following MeJA treatment. Because these compounds can be toxic or repellent to the beetles and their fungal associates the lower levels in Sitka and Lutz spruce suggest that these species may be chemically more favorable host trees for the spruce bark beetle. High concentrations of α-pinene, β-pinene and limonene have for example been found to reduce establishment of the pine engraver *Ips pini* (Say) on its host trees (Wallin and Raffa 2000). Limonene, myrcene, and 3-carene have also been shown to act as repellents and attractants for various bark beetles (Erbilgin et al. 2007a; Miller and Borden 2000; Wallin and Raffa 2000) and may also be toxic (Raffa and Berryman 1982).

Thunbergol, thunbergene and α-longipinene were much more abundant in induced and constitutive bark of Norway spruce than in Sitka and Lutz spruce. In fact, thunbergol and thunbergene were not detected at all in Sitka spruce (Figure 3). The effect of thunbergene and α-longipinene on the spruce bark beetle is unknown, but thunbergol has been suggested to inhibit growth of the beetle's fungal associate *E. polonica* (Zhao et al. 2011a; Zhao et al. 2010). Considering the negative effects of thunbergol we were surprised to find a positive relationship between constitutive levels of thunbergol and offspring production.

Pheromone production

Spruce bark beetle males produced comparable amounts of the two main components of their aggregation pheromone, methylbutenol and cis-verbenol, in the novel hosts Sitka and Lutz spruce as in the historical host Norway spruce. Methylbutenol is by far the most abundant component in the spruce bark beetle pheromone blend. It is produced *de novo* by the beetles (Birgersson et al. 1988) and released in substantial amounts in the early phase of tree colonization, equaling >3% of beetle body weight over the first week of an attack (Birgersson and Bergstrom 1989). This substantial metabolic cost probably means that only physiologically fit males are able to produce methylbutenol in high amounts. The fact that the beetles produced high amounts of methylbutenol in all three spruce species suggests that the chemical defenses of these species represented a comparable physiological challenge for the beetles.

Cis-verbenol and methylbutenol play different roles in spruce bark beetle aggregation: cis-verbenol is used for long-range orientation towards attacked trees, whereas

methylbutenol is as short-range orientation or arrestment stimulus that concentrates beetles on an attacked tree. Different methylbutenol to cis-verbenol ratios are believed to affect beetle orientation, attraction and ability to mass attack trees, with higher ratios being more attractive to flying beetles (Schlyter et al. 1987). Thus, the methylbutenol to cis-verbenol ratios we observed in our experiments suggest that beetles colonizing Sitka and Lutz spruce produce a more attractive pheromone blend (10.1 and 34 : 1 ratios, respectively) than the blend produced in Norway spruce (2.4 : 1). Cis-verbenol is produced from (-)-α-pinene ingested by the males as they tunnel into the host tree (Birgersson et al. 1988). However, even though Norway spruce contained more (-)-α-pinene than Sitka and Lutz spruce we found no significant differences in cis-verbenol production in beetles colonizing different tree species. Furthermore, we did not find support for any of the three hypotheses we tested of how concentrations of (-)-α-pinene and other terpenes in the host bark might affect cis-verbenol production. This lack of correlation between pheromone production and host tree chemistry contrasts with the results of Burke and Carroll (2016) on the mountain pine beetle. They found a significant correlation between pheromone production and the ratio of (-)-α-pinene to other terpenes and thus support for the equivalent of our hypothesis H_2. We suggest that terpene levels in the host influence cis-verbenol production also in the spruce bark beetle, but only when (-)-α-pinene levels are either very low or very high, and thus beyond the levels detected in our experiment.

Beetle reproductive performance

The total number of offspring produced in the novel hosts Sitka and Lutz spruce was much lower than in Norway spruce because much fewer of the beetles entering Sitka and Lutz spruce established successful breeding galleries (Table 2). In Norway spruce one in two entrance holes led to successful breeding, but in Sitka and Lutz spruce the ratio of breeding galleries to entrance holes were as low as 1 : 7.8 and 1 : 4.5, respectively. The lower establishment success and offspring production in Sitka and Lutz spruce suggests that terpenoids or other chemical or physical defenses made these hosts a less suitable breeding substrate for the beetles. It is also possible that the beetles did not recognize these exotic spruces as good hosts, perhaps due to a lack of positive host signals such as thunbergene and thunbergol. However, offspring quality was equally good in the novel hosts as in the beetle's historic host Norway spruce with respect to offspring length and weight. The fact that offspring size was similar across spruce species indicates that the bark of the different spruce species offered comparable nutritional quality for the beetles. Thus, the beetles appeared to

behave naively in their interaction with these species. Because beetles choosing to oviposit in Sitka and Lutz spruce produced as many offspring as beetles ovipositing in Norway spruce we speculate that over time the beetles may increase their preference for Sitka and Lutz spruce in areas where the beetle co-occur with these spruce species.

Previous breeding experiments by Økland et al. (2011) in Norway and Sweden have demonstrated that the spruce bark beetle can develop successfully in several North American spruce species, including Sitka and Lutz spruce. However, because these experiments did not control the beetles' colonization density they could not conclude whether offspring productivity in the different spruce species was determined by differences in substrate quality or differences in intra-specific competition due to different colonization densities. In the present study we standardized the colonization density by caging beetles onto the bark and could therefore determine beetle reproductive performance more precisely. However, since such no-choice conditions may cause bark beetles to enter different hosts in equal numbers (Raffa et al. 2013) it is possible that we have overestimated the beetles' propensity to enter Sitka and Lutz spruce.

Performance of E. polonica

Endoconidiophora polonica is an important associate of the spruce bark beetle that can colonize healthy bark and sapwood and kill Norway spruce trees when experimentally inoculated into the stem (Krokene and Solheim 1996). The fungus is associated with Norway spruce and a few other Eurasian spruce species and has never been recorded outside Eurasia (Kirisits 2004). In non-host Sitka and Lutz spruce the fungus performed much more poorly than in Norway spruce, inducing necrotic lesions that were less than half as long as those in Norway spruce (Figure 5). This contrasts with the results of Christiansen & Solheim (1990), who found *E. polonica* to be relatively virulent to Sitka spruce and produce comparable symptoms as in Norway spruce. The explanation for the relatively low virulence that we found in Sitka and Lutz spruce could be that the innate immunity of these non-host spruce species more efficiently confined the fungus through mechanisms of non-host resistance (Gill et al. 2015).

If the spruce bark beetle gets little assistance from *E. polonica* in breaking down tree resistance in Sitka and Lutz spruce the beetles may be disadvantaged if they attack these species. However, the spruce bark beetle carries many other fungal associates that may assist in breaking down host tree defenses (Krokene and Solheim 1996; Linnakoski et al. 2016) and it is possible that some of these are more virulent to Sitka and Lutz spruce than *E. polonica*.

Conclusion

Sitka and Lutz spruce appear to be chemically suitable hosts for the spruce bark beetle with a similar terpenoid composition as Norway spruce, including the presence of key compounds that are attractive to the spruce bark beetle (Andersson et al. 2009; Kalinova et al. 2014). The spruce bark beetle bred successfully in both Sitka and Lutz spruce and produced offspring of comparable quality as that produced in the historic host Norway spruce. However, the beetles established fewer galleries and produced fewer offspring in Sitka and Lutz spruce, possibly because they did not recognize these spruce species as good hosts due to a lack of positive host signals or because host suitability was reduced by physical or chemical properties we did not quantify. Similarly, the beetles' phytopathogenic fungal associate *E. polonica* performed worse in Sitka and Lutz spruce than in Norway spruce. Thus, both the beetles and the fungus appeared to be evolutionary naïve in their interaction with the exotic spruce species.

Our results suggest that the spruce bark beetle will be able to mass attack and reproduce in Sitka and Lutz spruce also under field conditions. This European bark beetle may thus be attacking spruce forests in North America (Økland et al. (2011), where the beetle has been intercepted many times (Haack 2006) but has not yet established. In Europe, range shifts facilitated by climate change may bring the beetle into contact with Sitka and Lutz spruce in north-western maritime climates where these tree species have been extensively planted. This could lead to substantial economic damage unless traditional forest management practices to reduce the impact of the spruce bark beetle are implemented in high-risk Sitka and Lutz spruce plantations.

Acknowledgment

We thank Ole Brække and Skogselskapet for letting us use trees from their experimental plots and for helping us fell the trees. We thank Torstein Kvamme for help with identifying male *Ips typographus* and extracting hind guts, and Pia Krokene for field work and lab assistance.

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Tables

Table 1 Spearman's rho and P-value for rank correlations between the amount of cis-verbenol produced by the spruce bark beetle and H_1: the amount of (-)-α-pinene, H_2: the amount of (-)-α-pinene divided by the amount of other terpenes, and H_3: the amount of (-)-α-pinene minus the amount of other terpenes in the bark of Norway, Sitka and Lutz spruce

Hypotheses	Rho	P
H_1: (-)-α-pinene concentration influences cis-verbenol production	0.06	0.75
H_2: (-)-α-pinene/other terpenes influences cis-verbenol production	-0.31	0.10
H_3: (-)-α-pinene - other terpenes influences cis-verbenol production	0.02	0.92

Table 2 Bolt properties and reproductive performance of the spruce bark beetle in cut bolts from three different spruce species. All values are given as mean per bolt (n = 10 bolts per spruce species) and SE. Means with different letters are significantly different following ANOVA and Tukey HSD ($p < 0.05$).

Species	Bolt length (cm)	DBH (cm)	Surface area (dm^2)	Bark thickness [1] (mm)	Number of beetles introduced	Number of entrance holes	Number of offspring	Number of offspring per surface area (dm^2)	Number of gallery systems	Number of offspring per gallery system
Norway spruce	41.1 ±0.7	22.7 ±0.9	29.4 ±1.4	5.3 ±0.2	32.5 ±3.6	14.3 ±1.60a	179.6 ±48.8a	6.2 ±1.7a	6.7 ±1.5a	25.9 ±2.6
Sitka spruce	40.7 ±0.9	20.9 ±0.9	26.8 ±1.2	4. 7±0.2	27.0 ±2.3	10.1 ±1.04b	37.1 ±16.4b	1.5 ±0.7b	1.3 ±0.6b	27.1 ±5.6
Lutz spruce	41.5 ±0.5	20.7 ±.0.8	27.1 ±1.4	4.8 ±0.3	25.6 ±2.3	10.4 ±1.09b	65.7 ±32.3ab	2.6 ±1.3ab	2.3 ±0.8b	29.7 ±8.1
$F_{2,27}$	0.26	1.78	1.16	1.45	1.71	3.37	4.61	3.58	8.07	0.14[2]
P	0.77	0.19	0.33	0.25	0.20	0.05	0.02	0.04	0.002	0.87

[1] Measured on the bolts used to determine fungal colonization success. We measured the thickness of four bark plugs removed during fungal inoculation using a caliper.

[2] $F_{2,16}$ because some bolts had no breeding galleries

Figures

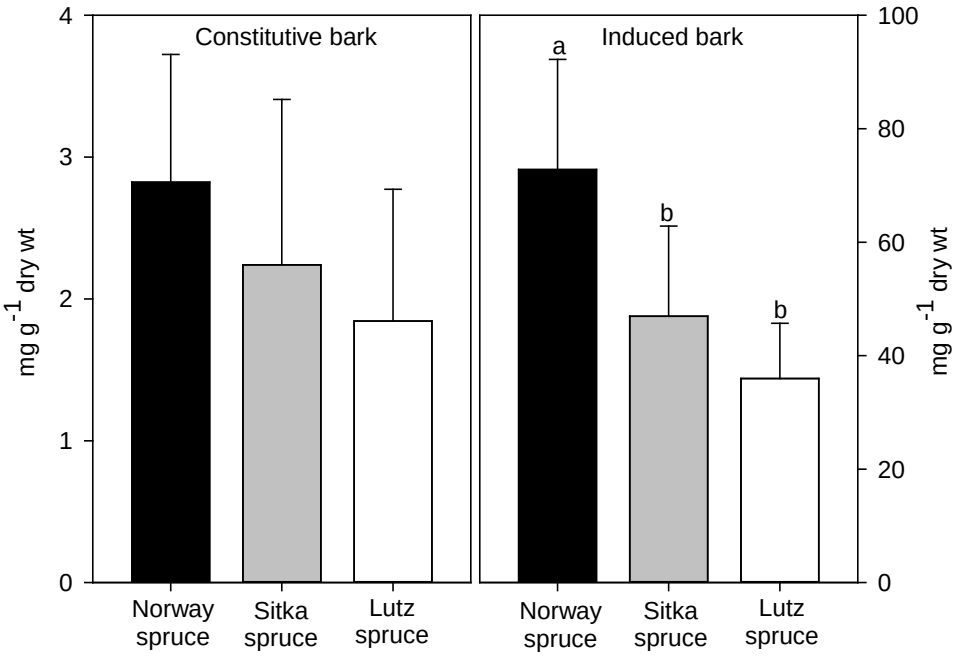

Figure 1. Total terpenoid concentrations in constitutive and induced bark of Norway, Sitka and Lutz spruce. Data are mean + 95% confidence intervals for n = 10 trees per spruce species. Bars with different letters are significantly different following ANOVA and Tukey HSD ($p < 0.05$).

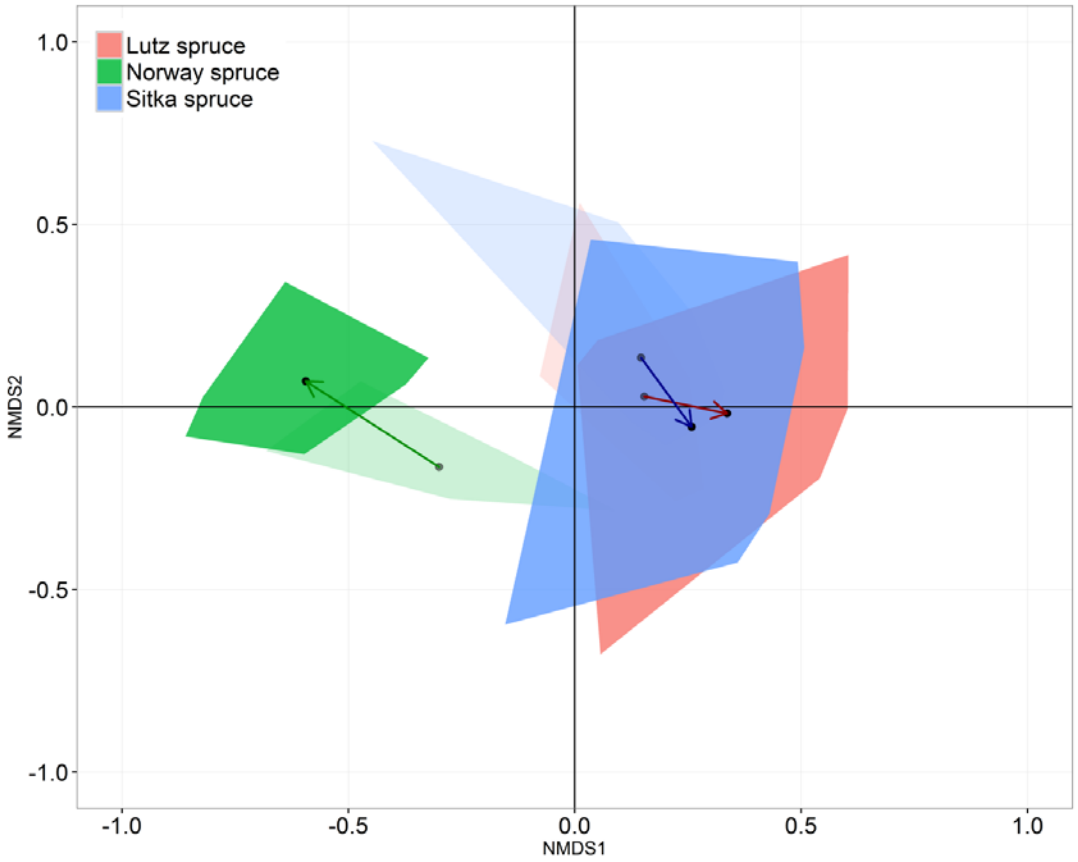

Figure 2. Non-metric multidimensional scaling (NMDS) showing the overall compositional terpenoid similarity between Norway, Sitka and Lutz spruce in constitutive bark samples (tint colors; 107 individual compounds) and induced bark samples (darker colors; 127 compounds). Black dots indicate mean site scores and arrows indicate direction of change from constitutive to induced samples. The polygons circumscribe the distribution of n = 10 samples for each tree species and bark sample type.

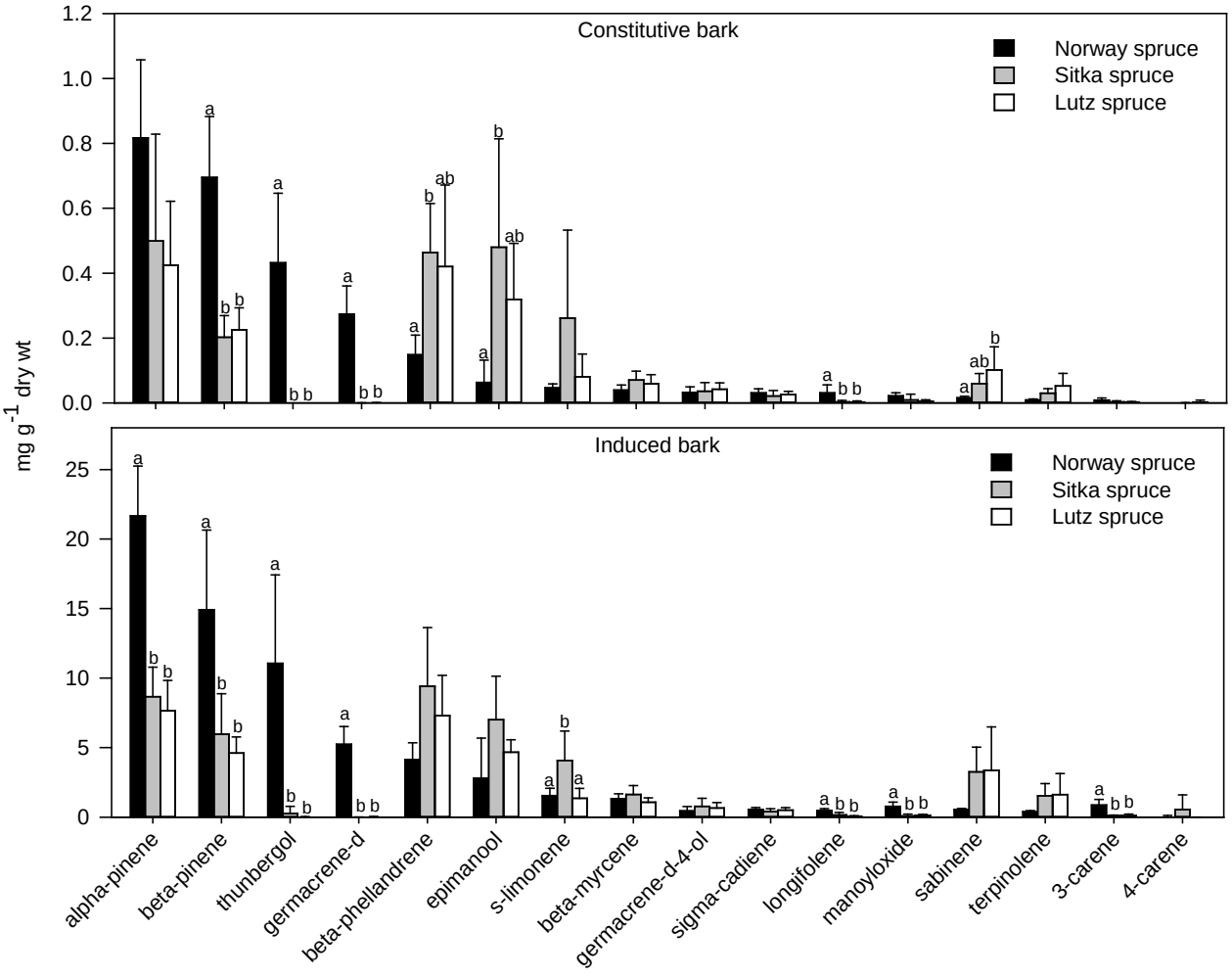

Figure 3. Concentrations of the 16 most abundant terpenoids in constitutive and induced bark of Norway, Sitka and Lutz spruce. Data are mean + 95% confidence intervals for n = 10 trees per spruce species. For each compound and panel bars with different letters are significantly different following ANOVA and Tukey HSD ($p < 0.05$).

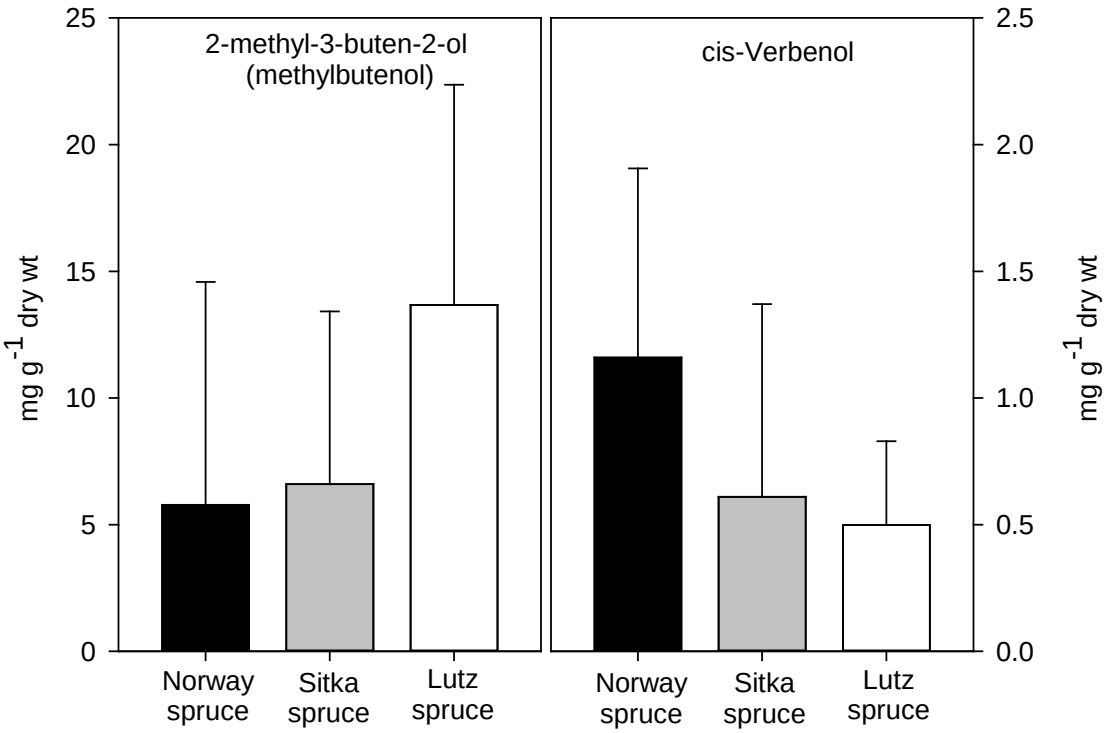

Figure 4. Mean pheromone content in hind guts of spruce bark beetles tunneling in cut bolts from three different spruce species. Error bars are + 95% confidence intervals, n = 10 bolts per spruce species.

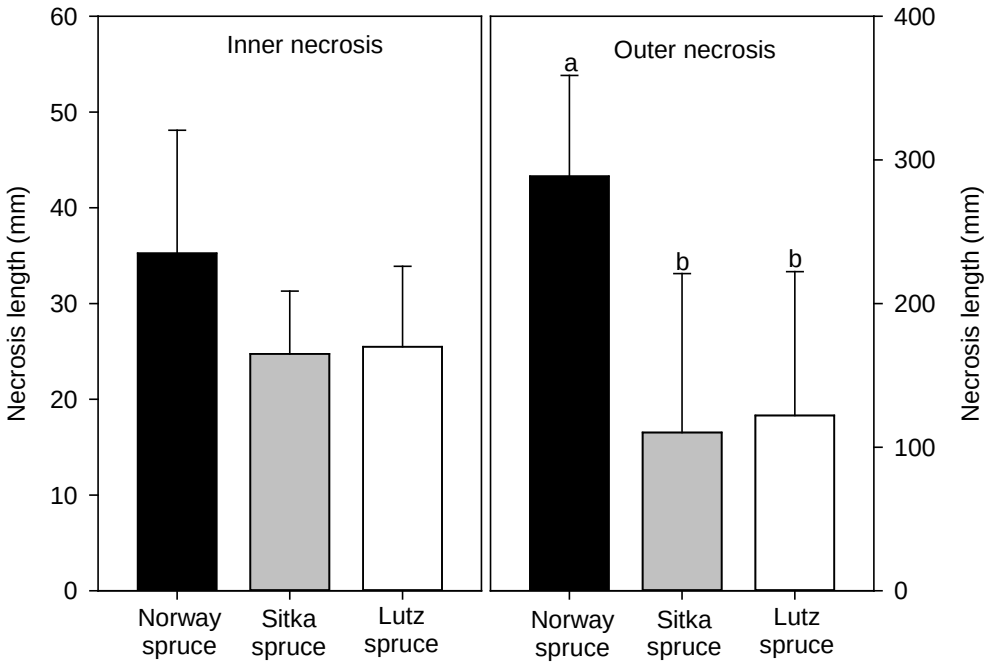

Figure 5. Mean lengths of necrotic lesions in the inner bark of cut bolts from three different spruce species 90 days after inoculation with the blue-stain fungus *Endoconidiophora polonica*. Inner necrosis represents the maximum extent of active host defenses, whereas outer necrosis represents the full extent of fungal colonization. Error bars are + 95% confidence intervals, n = 10 bolts per spruce species. Bars with different letters are significantly different following ANOVA and Tukey HSD ($p < 0.05$).