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## Research paper

# Seasonal and elevational variability in the induction of specialized compounds from mountain birch (*Betula pubescens* var. *pumila*) by winter moth larvae (*Operophtera brumata*)

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**The mountain birch [*Betula pubescens* var. *pumila* (L.)] forest in the Subarctic is periodically exposed to insect outbreaks, which are expected to intensify due to climate change. To mitigate abiotic and biotic stresses, plants have evolved chemical defenses, including volatile organic compounds (VOCs) and non-volatile specialized compounds (NVSCs). Constitutive and induced production of these compounds, however, are poorly studied in Subarctic populations of mountain birch. Here, we assessed the joint effects of insect herbivory, elevation and season on foliar VOC emissions and NVSC contents of mountain birch. The VOCs were sampled in situ by an enclosure technique and analyzed by gas chromatography–mass spectrometry. NVSCs were analyzed by liquid chromatography–mass spectrometry using an untargeted approach. At low elevation, experimental herbivory by winter moth larvae (*Operophtera brumata*) increased emissions of monoterpenes and homoterpenes over the 3-week feeding period, and sesquiterpenes and green leaf volatiles at the end of the feeding period. At high elevation, however, herbivory augmented only homoterpene emissions. The more pronounced herbivory effects at low elevation were likely due to higher herbivory intensity. Of the individual compounds, linalool, ocimene, 4,8-dimethylnona-1,3,7-triene, 2-methyl butanenitrile and benzyl nitrile were among the most responsive compounds in herbivory treatments. Herbivory also altered foliar NVSC profiles at both low and high elevations, with the most responsive compounds likely belonging to fatty acyl glycosides and terpene glycosides. Additionally, VOC emissions from non-infested branches were higher at high than low elevation, particularly during the early season, which was mainly driven by phenological differences. The VOC emissions varied substantially over the season, largely reflecting the seasonal variations in temperature and light levels. Our results suggest that if insect herbivory pressure continues to rise in the mountain birch forest with ongoing climate change, it will significantly increase VOC emissions with important consequences for local trophic interactions and climate.**

**Keywords:** biotic stress, geometrid moth, global change, plant–insect interactions, secondary metabolites, volatile organic compounds.

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## Introduction

Plants are constantly under attack from herbivores and other biotic stresses (Trowbridge and Stoy 2013, Kutchan et al. 2015). To combat herbivory attack, plants have evolved a diverse arsenal of chemical defenses, including production of volatile organic compounds (VOCs) and non-volatile specialized compounds (NVSCs; Trowbridge and Stoy 2013, Kutchan et al. 2015). In response to herbivore attack, plants often increase biosynthesis and production of VOCs and NVSCs, and alter chemical compositions of these compounds (Kesselmeier and Staudt 1999, Howe and Jander 2008, Dicke et al. 2009, Tholl et al. 2011, Hartikainen et al. 2012, Hammond-Kosack and Jones 2015, Ameye et al. 2018).

Herbivore-induced chemical defenses are divided into direct and indirect defenses (Haukioja 2005, Turlings and Erb 2018). The direct defenses can be mediated by VOCs or toxic NVSCs, inhibiting the colonization, growth, reproduction, development and survival of herbivores (Trowbridge and Stoy 2013, Wang and Wu 2013, Hammond-Kosack and Jones 2015). As indirect defenses, VOCs can recruit natural enemies of herbivores, such as predators and parasitoids (Turlings and Erb 2018), and NVSCs might reduce the immunocompetence of insect herbivores, and thus impede herbivores' defenses against predators and parasitoids (Haukioja 2005). Volatile organic compounds and NVSCs can also protect plants from abiotic stresses (Loreto and Schnitzler 2010, Kutchan et al. 2015). For example, many VOCs such as isoprene and monoterpenes (MTs), and NVSCs such as phenolics, have been shown to operate as antioxidants and protect plants against oxidative stress caused by high temperatures, elevated tropospheric ozone levels and excess light exposure (Loreto and Schnitzler 2010, Kutchan et al. 2015). In addition to their ecologically relevant roles, VOCs are highly reactive in the atmosphere and can drive climate feedbacks via complex atmospheric chemistry (Peñuelas and Staudt 2010, Loreto and Fares 2013, Faiola and Taipale 2020).

Research on constitutive and herbivore-induced responses of specialized compounds often shows substantial seasonal variation in their magnitude (Peñuelas and Llusà 1999, Hakola et al. 2001). For example, the VOC emission patterns were observed to change substantially over the growing season in many plant species, such as *Quercus ilex* (Peñuelas and Llusà 1999), *Datura wrightii* (Hare 2010), *Betula* spp. (Hakola et al. 2001) and *Pinus edulis* (Trowbridge et al. 2014). Although VOC emission rates seem to increase progressively throughout the active growing season and decline during leaf senescence, large variabilities in the seasonal patterns occur between compounds and plant species. Such high seasonal variation has been thought to be caused by variation in environmental factors (e.g., solar radiation, temperature and humidity) that vary over the growing season and can influence production of specialized compounds, and/or by ontogenetic changes in leaf physiology (Hare 2010, Niinemets et al. 2010, Li et al. 2019).

Rising temperatures, due to climate change, are generally expected to increase VOC emissions, because their biosynthesis and release are temperature dependent (Holopainen et al. 2018). However, negative and neutral effects of temperature on VOC emissions have also been reported (Peñuelas and Staudt 2010, Holopainen et al. 2018). Moreover, the magnitude of the temperature influences on VOC emissions varies among compounds, depending on the physicochemical properties of the compounds, the presence of specialized storage pools and the co-occurrence of other environmental factors (Peñuelas and Staudt 2010, Li et al. 2019). For instances, previous studies have shown that some highly volatile compounds like isoprene and non-oxygenated MTs are more responsive to temperature than the less volatile compounds like oxygenated MTs and fatty acid-derived green leaf volatiles (GLVs), especially when they are released from plant species lacking storage structures (Peñuelas and Llusà 1999, Peñuelas and Staudt 2010). Likewise, the foliar contents of different classes of NVSCs may respond differently to warming (Holopainen et al. 2018). Furthermore, increasing temperatures may enhance defoliation pressure exerted by insect herbivore infestation (Bale et al. 2002). Higher latitude regions are expected to experience larger temperature increases compared with lower latitudes (IPCC 2014) and as such, higher latitude regions are likely to experience more pronounced increases in defoliation pressure (Bale et al. 2002) and VOC emissions (Rinnan et al. 2014). However, the simultaneous effects of rising temperatures and concomitant increased insect herbivore infestation pressure on plant chemical defense mechanisms and the susceptibility of plants to herbivores are poorly understood (Descombes et al. 2020), especially in the Arctic regions.

Here, we aimed to assess how the interactive effects of climate change and insect herbivory influence plant specialized compounds in the mountain birch [*Betula pubescens* var. *pumila* (L.)] forest that dominates the tree line zone in Subarctic Fennoscandia (Ammunet et al. 2015). We investigated in situ responses of VOCs and NVSCs to insect herbivory at two elevations during a growing season. Natural climatic conditions change with elevation and environmental variables, such as declining temperature with altitude, and allow us to investigate the effects of climate change on plant chemical defense mechanisms (Körner 2007, Moreira et al. 2018). We hypothesized that the VOC emission rates and foliar NVSC contents would (i) increase in response to herbivory in accordance with previous studies (Hartikainen et al. 2012, Ossipov et al. 2014, Li et al. 2019, Rieksta et al. 2020); (ii) differ between elevations in relation to temperature differences (Close and McArthur 2002, Körner 2007, Loreto and Fares 2013); and (iii) vary throughout the growing season as a result of seasonal variations in plant growth activity, phenology and the microclimate, such as air temperature and photosynthetically active radiation (Haukioja 2003, Loreto and Fares 2013).

## Materials and methods

### Study area and experimental design

The mountain birch forest covers the ecotone between the coniferous forest zone and the treeless area beyond the Arctic, alpine and maritime tree lines (Wielgolaski 2005), ranging through climatic gradients from the mild oceanic regions of northwestern Norway, to cold and dry areas in northern Sweden and Finland (Ammunet et al. 2015). These mountain birch forests are exposed to defoliation pressure exerted by geometrid moth (Lepidoptera: Geometridae) species: the winter moth (*Operophtera brumata*), autumnal moth (*Epirrita autumnata*) and umber moth (*Agriopsis aurantiaria*). The three geometrid moth species are considered the most important natural disturbance in mountain birch forests influencing ecosystem dynamics and processes, such as carbon and nitrogen cycling (Tenow and Bylund 2000, Ammunet et al. 2015).

Our study was conducted in a mountain birch forest at Kvaløya in Tromsø, Norway, in the growing season of 2018. Tromsø has a Subarctic climate with a mean annual temperature of +2.9 °C and a mean annual precipitation of 1011 mm (1925–2013), with snow melt as late as the end of May (Opala et al. 2016). Mountain birch is the dominant vegetation in the tree layer (Opala et al. 2016) and winter moth, autumnal moth and umber moth all naturally occur in this area. The experiment consisted of two elevational transects (69°41'N, 18°47'E and 69°41'N, 18°49'E) on a southern slope, ~2.5-km apart. Each transect comprised two sites, one at the low elevation and the other at the high elevation, i.e., 50 and 240 m above sea level, respectively. The two sites at the low elevation, were established at the lower extent of the mountain birch forest, and the high elevation sites were established at the upper tree line.

In the beginning of the experiment in late May, we selected eight pairs of trees at each site, and on each tree, labeled a sun-lit branch with no visible sign of herbivory or disease ~1.5-m above ground. We then enclosed the distal 25–35 cm of the labeled branches in transparent mesh bags to protect them from natural insect herbivores. The branches were later assigned for experimental herbivory manipulation and specialized compound measurements. Because the whole birch stand at the low elevation sites suffered from an aphid infestation, aphids were gently removed with a soft paintbrush before the branches were enclosed in mesh bags. In total, we selected 64 branches (i.e., 2 transects × 2 elevations at each transect × 8 tree pairs at each elevation). The target parts of the branches contained on average  $71 \pm 3$  leaves [mean  $\pm$  standard error (SE),  $n = 64$ ] ranging from 30 to 126 leaves. To take into account the potential confounding effects of the large variation in branch size on herbivore-induced specialized compound production, we paired the branches with similar numbers of leaves and applied herbivory treatment randomly to the two branches within each pair [control branches:  $69 \pm 4$  leaves ranging from 30 to

123 ( $n = 32$ ); infested branches:  $73 \pm 4$  leaves ranging from 38 to 126 ( $n = 32$ )].

For the herbivory treatment, we used winter moth larvae that were collected in their first to second instar in northeast Finnmark (70°9'N, 28°51'E) and reared on detached birch branches in the laboratory. The winter moth populations in the mountain birch forest exhibit dramatic interannual variation. The number of larvae on a branch of ~60- to 80-cm long can vary from <1 during non-outbreak years to >100 during outbreak years (Pepi et al. 2017). In the beginning of the experiment, the low and high elevation sites differed in phenology; all leaves were unfolded at low elevation, whereas leaves had just started to unfold at high elevation. Due to the differences in leaf phenology between the elevations and in order to assess the herbivory effects over the entire feeding period, which usually lasts from late May/early June to late June/early July in the mountain birch forest, we applied three and five winter moth larvae to the high and low elevation sites, respectively, which fall well within the observed natural variation. The larvae were in their third to fourth instar and not starved when applied to the branches. The herbivory treatment lasted for ~3 weeks from mid-June to early July (see Table S1 available as Supplementary data at *Tree Physiology* Online for the exact start and end dates). During this feeding period, VOCs were collected three times (see below for detailed procedures). Before VOC collection, we gently removed mesh bags as well as larvae and larval feces from the leaves using a soft paintbrush. After VOC collection, we placed larvae back, along with the mesh bag, and replaced the dead or missing larvae with new ones so that the infested branches at high and low elevations still received three and five larvae, respectively. Different numbers of larvae were used corresponding to the differences in leaf and tree sizes at the two altitudes and targeting similar leaf area losses as a result of herbivory. Similar percentage leaf area losses were observed across elevations at the end of herbivory treatment period suggesting minimal confounding effects from the different larval numbers.

### Volatile organic compound sampling

Volatile organic compounds were sampled using the push–pull enclosure technique (Ortega and Helmig 2008). Enclosures were pre-cleaned (120 °C for 1 h) polyethylene terephthalate (PET) oven bags (35 × 43 cm), which were ventilated at a flow rate of 1000 ml min<sup>-1</sup> for a minimum of 10 min prior to VOC sampling. The inflow air was purified by a charcoal filter (F03-C2-100, Filter: MXP-95-054, Wilkerson Corp., Richland, Michigan, USA) and potassium iodide ozone scrubber (Ortega and Helmig 2008). During the 15-min sampling period, with an inflow rate of 300 ml min<sup>-1</sup> and outflow rate of 200 ml min<sup>-1</sup> (3-l sample volume), VOCs were trapped by stainless-steel adsorbent cartridges containing 150-mg Tenax TA and 200-mg Carbograph 1TD (Markes International Ltd, Llantrisant, UK).

After sampling, cartridges were sealed with Teflon-coated brass caps and stored at 5 °C until analysis. Blank measurements from empty PET bags were performed to obtain information on impurities originating from the sampling or analysis system. During VOC sampling, the air temperature and humidity at 1.5-m above ground and inside the PET bags was recorded every minute by shaded iButtons (Hygrochron, DS 1923-F5, Maxim Integrated Products Inc., San Jose, California, USA). Photosynthetic photon flux density (PPFD) was recorded every 10 s by S-LIA-M003 sensors (Onset Computer Corporation, Bourne, Massachusetts, USA) coupled with a Hobo Micro Station (Onset Computer Corporation, Bourne, Massachusetts, USA).

Five VOC sampling campaigns were conducted at each site in each elevation transect (see Table S1 available as Supplementary data at *Tree Physiology* Online). The first campaign was carried out 2–6 June, herein referred to as the ‘early’ measurement (see Table S1 available as Supplementary data at *Tree Physiology* Online). Herbivory treatments started 8–13 June and ended 1–5 July, comprising the defoliation period (see Table S1 available as Supplementary data at *Tree Physiology* Online). Three VOC campaigns were carried out during the defoliation period, herein referred to as the ‘mid-1’, ‘mid-2’ and ‘mid-3’ measurements (see Table S1 available as Supplementary data at *Tree Physiology* Online). The mid-1 measurement was carried out 13–17 June, the mid-2 measurement 25–30 June and the mid-3 measurement 1–5 July (see Table S1 available as Supplementary data at *Tree Physiology* Online). The final campaign was carried out 10–11 August, which was 5 weeks after the termination of the herbivory treatment, herein referred to as the ‘late’ measurement (see Table S1 available as Supplementary data at *Tree Physiology* Online). The VOC sampling took place over multiple days because it was not possible to complete all measurements in a single day and work sometimes was delayed by unfavorable weather conditions. At each transect, VOC sampling was carried out almost concurrently at the two elevations, except on 1 July when the measurements were first completed at the high and then the low elevation site.

The approximate leaf area (m<sup>2</sup>) per branch after the first four campaigns, as well as the leaf damage per branch, i.e., leaf area consumed by larvae, after each campaign within the defoliation period, was visually estimated as in Li et al. (2019). Branches were cut after the last campaign and leaf area was calculated as in Li et al. (2019). The VOC emission rates were calculated based on the estimated and calculated leaf areas per branch after each campaign. Leaf dry mass per unit area was  $50.3 \pm 0.8 \text{ g m}^{-2}$  ( $n = 18$ ).

#### Volatile organic compound analysis

The VOCs were thermally desorbed (TD100-xr, Markes International Ltd, Llantrisant, UK) from the adsorbent cartridges at 250 °C for 10 min and analyzed on a gas chromatograph–mass spectrometer (7890A GC, 5975C VL MSD, Agilent Tech-

nologies, Santa Clara, California, USA). The VOCs were first cryofocused at –10 °C and injected into a HP-5MS capillary column (50 m × 0.2 mm, film thickness 0.33 μm), with helium as carrier gas at a flow rate of 1.2 ml min<sup>-1</sup>, and separated using the following temperature program: 40 °C, held 1 min; raised at 5 °C min<sup>-1</sup> to 210 °C; raised at 20 °C min<sup>-1</sup> to 250°, held 8 min.

Chromatograms were analyzed using PARADISE v. 3.8 (Johnsen et al. 2017). Compounds were identified using pure standards, when available, or tentatively identified using the Mass Spectral Library (NIST 2014). Compounds were categorized into the following classes: MTs, homoterpenes (HTs), sesquiterpenes (SQTs), GLVs, isoprene and other VOCs (OVOCs, i.e., compounds that were not included in any of the above-mentioned groups). When pure standards were not available, quantification was achieved using the following pure standards: for MTs and HTs: α-pinene; SQTs: caryophyllene; GLVs: hexenal; and OVOCs: toluene. Compounds were included in the dataset if they were present in a minimum of 50% of the samples in at least one treatment group and had a match factor above 800 against the Mass Spectral Library (NIST 2014). The VOC concentrations in blanks were subtracted from those in the samples. The leaf area-based emission rates were calculated in accordance with Ortega and Helmig (2008).

#### Non-volatile specialized compound sampling

From each branch, five leaves at the low and three leaves at the high elevation sites were harvested before and after the defoliation period, i.e., during the period 8–13 June and 2–6 July, respectively (see Table S1 available as Supplementary data at *Tree Physiology* Online). The leaves harvested before the defoliation period were picked from neighboring branches on the same tree, and the leaves harvested after the defoliation period were only picked if marked by larvae chewing. Leaf samples were kept on ice during local transportation to the laboratory and stored in a –80 °C freezer, but all samples were unintentionally defrosted for a maximum of 24 h during transportation to Copenhagen.

The effect of the defrosting event on foliar NVSC contents was assessed by comparing NVSC contents in different mountain birch leaves with and without a similar defrost treatment. The defrost treatment significantly affected the foliar contents of a considerable number of the tested NVSCs: 30% of NVSCs decreased and 7% increased ( $P < 0.05$ ,  $T$ -test). All the samples collected during our experiment were stored and transported together, under the same conditions and as such, we decided to investigate changes in the proportional foliar NVSC contents, rather than reporting the absolute quantities.

#### Non-volatile specialized compound analysis

Frozen leaves were ground in liquid nitrogen with a mortar and pestle and homogenized tissue (~0.03 g) was boiled (5 min

at 90 °C) in 500  $\mu$ l of extraction buffer (85% methanol, 0.1% formic acid and 250  $\mu$ M Amygdalin as internal standard), subsequently cooled on ice (10–20 min), and centrifuged (4 °C, 15,000 r.p.m., 5 min; dos Santos et al. 2019). The supernatant was diluted five times and filtered through a membrane filter (0.45  $\mu$ m, Merck Millipore, Burlington, Massachusetts, USA) before being stored at 4 °C until further analysis (dos Santos et al. 2019). Sample dry weight was obtained from the dried pellets (60 °C for 24 h).

Chromatographic separation of the filtered samples was performed on a Dionex Ultimate 3000RS ultra-high performance liquid chromatograph (UHPLC; Thermo Fisher Scientific, Waltham, Massachusetts, USA) system with a diode array detector, cooling autosampler at 10 °C, and column oven at 40 °C. Five microliters of every sample were separated, using a Phenomenex Kinetex<sup>®</sup> column (100 Å, 1.7  $\mu$ m C18, 150  $\times$  2.1 mm, Phenomenex Inc., Torrance, California, USA), by gradient elution, at a constant flow rate of 0.3 ml min<sup>-1</sup>. The gradient elution profile was as follows: 98% solvent A (0.05% formic acid in water) and 2% solvent B (0.05% formic acid in acetonitrile) for 3 min, linearly increasing to 40% solvent B over 12 min, then 60% solvent B over 5 min, 100% solvent B over 10 min and held for 10 min before solvent B was decreased to 2% over 5 min and the column was re-equilibrated for a further 5 min. The UHPLC system was coupled with a compact<sup>™</sup> mass spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA) with an electrospray ionization source. The mass spectrometer detected compounds in negative mode in the range *m/z* 50–1400 as in dos Santos et al. (2019). All samples were run in full scan mode followed by the auto tandem mass spectrometry (MS/MS) mode having a bbCID collision energy of 20 eV.

Data acquisition and calibration was performed using the Bruker Compass Data Analysis v. 4.3 (Bruker Daltonik GmbH 2014, Billerica, Massachusetts, USA) software. Raw chromatogram data were subsequently exported as .mzML format and further processed using MZmine2 (v.2.5.3; Pluskal et al. 2010). A principal component analysis was conducted that revealed narrow clustering of pooled samples and blanks, indicating overall reliable spectral data acquisition throughout the analysis (see Figure S1 available as Supplementary data at *Tree Physiology* Online). The chemical annotation pipeline was conducted as described in Gericke et al. (2020). An in-house MS/MS spectral database was generated including 18 commercially sourced reference compounds (see Table S2 available as Supplementary data at *Tree Physiology* Online). The three levels (1–3) of identification that describe confidence in dereplication events in this study are in line with the refined levels by the Metabolomics Standards Initiative of the Metabolomics Society (Schymanski et al. 2014). A detailed description of the MZmine2 processing as well as molecular networking parameters used in this study can be found in Table S3 and Figure S2, available as Supplementary data at

*Tree Physiology* Online. Individual metabolites were selected for further identification via SIRIUS (v4.5.1) under consideration of M<sup>-</sup>H<sup>-</sup> adducts while using default settings (Dührkop et al. 2015, 2019, 2020, Böcker and Dührkop 2016). A total of 449 NVSCs were detected in our untargeted UHPLC–MS/MS analysis. This resulted in the annotation of 63 metabolites based on levels 1 and 2 identification. Of the 449 NVSCs, 235 were included in the dataset after removing the compounds present in <6% of the samples.

### Statistical analyses

All statistical analyses were executed using R statistical framework version 3.6.1 (R Development Core Team 2019). We assessed the effects of herbivory, elevation, measurement time and their interactions on VOC emissions, foliar NVSC contents and leaf damage using mixed-effect models (LMM) fitted with maximum likelihood using the 'lmer' function from the *lme4* package (Bates et al. 2015). Treatment, elevation and measurement time were predictor variables and transect, site, branch pair and branch were random factors. Analysis of variance (ANOVA) was used to test for overall effects of the predictor variables on all of the response variables. *T*-tests were used on all of the response variables to test for differences between the control and herbivory treatments in each measurement time at each elevation, as well as differences between elevations in each measurement time, using the 'diffsmeans' function from the *lmerTest* package (Kuznetsova et al. 2017). *T*-tests were also used to test for significant differences between measurement times for the foliar NVSC contents. Tukey's post-hoc tests were used to test for significant differences in VOC emissions between the measurement times using the 'emmeans' function from the *emmeans* package (Russell 2019). Similar methods were used to test for differences in temperature and PPFD between treatments, elevations and measurement times. To fulfill the requirements of normal distribution and homogeneity of variance, a log(*x* + 1, raised to the power of the exponent of the lowest value not equal to zero) was applied on all the response variables, except for the enclosure temperature.

Due to the temperature and light dependencies of VOC emissions (Loreto and Fares 2013), we assessed whether the temporal variations in the measured VOC emissions were connected to the temporal variation in the enclosure temperature and PPFD by Pearson's correlation test using the 'cor.test' function.

The machine learning algorithm Random Forest (RF) was used to assess whether the blends of VOC emission rates and the proportional foliar NVSC contents could be separated by the treatments, elevations and measurement times. The precision of RF to separate groups is assessed with the RF prediction error, i.e., out-of-bag error (OOB; Breiman 2001, Ranganathan and Borges 2010). Furthermore, RF was used to obtain the variable importance (E) table, which shows individual VOCs and NVSCs that are most important for separation between

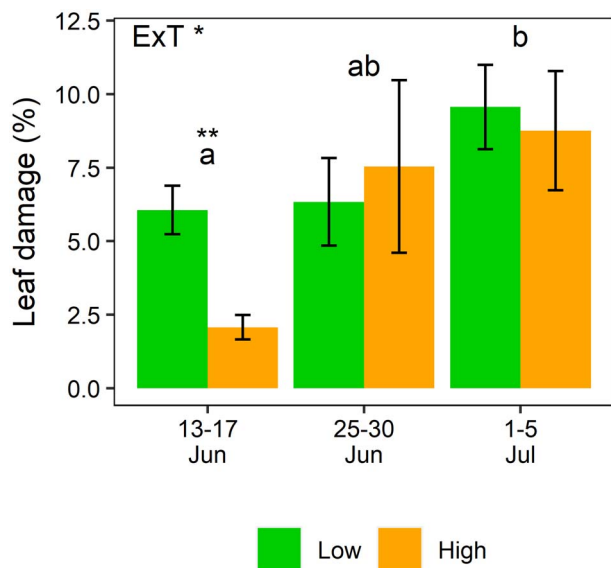


Figure 1. Leaf damage on herbivory treated branches at low and high elevations. The label (EXT \*) denotes a significant interaction between elevation and measurement time ( $P < 0.05$ , ANOVA). The asterisks above the bars indicates a significant difference between the elevations ( $P < 0.01$ ,  $T$ -test). Letters above bars denotes significant differences between measurement times ( $P < 0.05$ , Tukey's post-hoc test). Bars represent mean  $\pm$  SE ( $n = 16$ ).

treatments, elevations and measurement times (Breiman 2001, Ranganathan and Borges 2010). We used the 'randomForest' function from the *randomForest* package (Liaw and Wiener 2002) with an  $n_{\text{tree}} = 100.000$ , and an  $m_{\text{try}} = 12$  for VOCs and  $m_{\text{try}} = 16$  for NVSCs (Breiman 2001). The RF analyses were performed for each measurement time at each elevation for both VOCs and NVSCs.

## Results

### Leaf damage from herbivory treatment

The herbivory treatment progressively increased leaf damage over time, leading to  $\sim 9\%$  leaf area loss by the mid-3 measurement (Figure 1). There was a significant interaction effect between elevation and measurement time on leaf damage. Percentage leaf area losses were significantly higher at low than high elevation during the early herbivory period (i.e., 6% versus 2%), but similar between the two elevations both in the middle and the end of the herbivory period (Figure 1).

### Variation in temperature and PPFD

Air temperature differences between the two elevations were in general low (see Figure S3 available as Supplementary data at *Tree Physiology* Online). The temperature difference was the largest in the early growing season when the monthly air temperatures were  $9.1 \pm 0.5$  °C (mean  $\pm$  SE) for May and  $7.6 \pm 0.5$  °C for June at low elevation, and 1.1 °C cooler at the high elevation. In July, the monthly air temperatures were similar

at both elevations, on average  $16.3 \pm 0.7$  °C. In August and September, the temperature differences between the elevations for the monthly means were 0.7 °C.

The enclosure temperature in VOC measurements was 1.5 °C higher at the low compared with the high elevation during the early measurement ( $P < 0.01$  for elevation  $\times$  time interaction; see Figure S4A available as Supplementary data at *Tree Physiology* Online). There was no effect of elevation on PPFD (see Figure S4B available as Supplementary data at *Tree Physiology* Online). Both enclosure temperature and PPFD varied substantially across the season, being higher at mid-1, mid-3 and late measurement times, and lower at early and mid-2 measurement times ( $P < 0.05$ , Tukey's post-hoc test; Figure 2A; see Figure S4A and B available as Supplementary data at *Tree Physiology* Online).

### Herbivory effects on VOC emissions

A total of 130 VOCs were detected, consisting of 22 MTs, 49 SQTs, 2 HTs, 11 GLVs and isoprene, and 45 OVOCs containing alcohols, aldehydes, alkanes, alkenes, benzenoids, furans, ketones and nitrogen-containing compounds (see Table S4 available as Supplementary data at *Tree Physiology* Online). Sesquiterpenes, GLVs, OVOCs, MTs, isoprene and HTs accounted for 62, 17, 11, 8, 0.8 and 0.5% of total VOC emissions, respectively.

There were no overall significant herbivory effects on total VOCs or most VOC classes (Figures 2B; and 3; see Figure S5 available as Supplementary data at *Tree Physiology* Online). However, there were significant interaction effects between herbivory treatments, elevation and sampling time on the MT and HT emissions (Figure 3A and B). Furthermore, clear herbivory effects were seen on emissions of MTs and HTs during the defoliation periods, especially at low elevation (Figure 3A and B). At each of the three sampling times during the defoliation period, herbivory at low elevation significantly increased MT and HT emissions, which, when averaged across sampling times, were 300 and 590% higher in defoliated branches than control branches, respectively. At low elevation, the herbivory treatment also increased the proportional contributions of the MTs and HTs to the total VOC emission by an average of 230 and 400%, respectively, during the defoliation period (see Figure S6 available as Supplementary data at *Tree Physiology* Online). Moreover, at low elevation, herbivory substantially increased emissions of total VOCs, GLVs, SQTs and OVOCs, by 200, 300, 200 and 60%, respectively, at the end of the defoliation period (Figures 2B and 3C,D; see Figure S5A available as Supplementary data at *Tree Physiology* Online). By contrast, herbivory at high elevation sites significantly influenced only HT emissions, with 270% higher emissions observed in defoliated branches (Figure 3B). There was no effect of herbivory treatment on isoprene emissions (see Figure S5B available as Supplementary data at *Tree Physiology* Online).

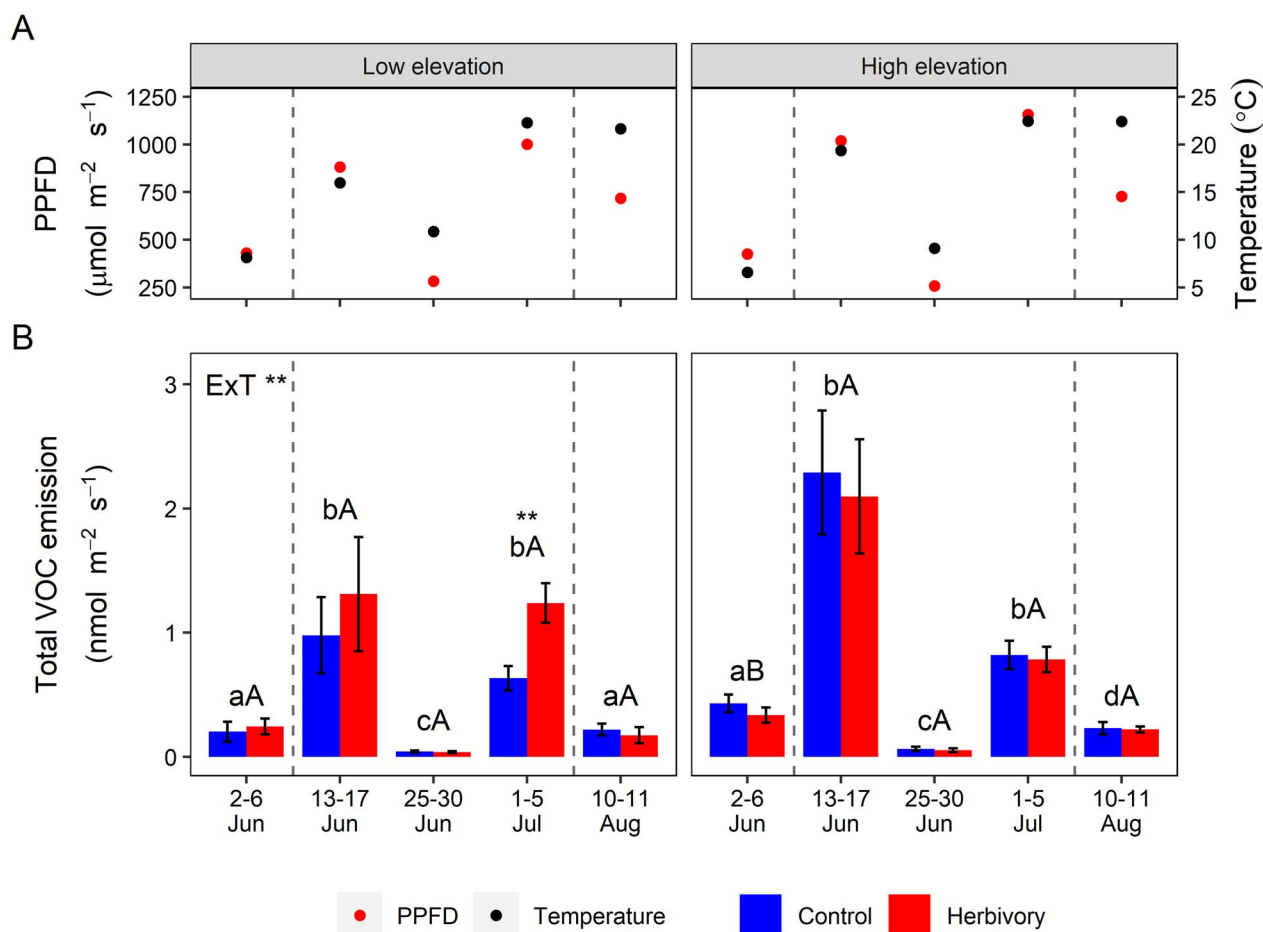


Figure 2. Total VOC emissions from control and herbivore-damaged branches at low and high elevations. The mean PPFD and enclosure temperature (A) and total VOC emission from control and herbivory treated branches (B) measured five times during the growing season at low and high elevations. The label (Ext \*\*) denotes a significant interaction between elevation and measurement time ( $P < 0.01$ , ANOVA). The asterisks above the bars indicate a significant difference between the control and herbivory treatment within each measurement time and elevation ( $P < 0.01$ , *T*-test). Bars labeled with different lowercase letters indicate significant differences between measurement times at the same elevation ( $P < 0.05$ , Tukey's post-hoc test). Bars labeled with different uppercase letters denotes significant differences between elevations within each sampling time ( $P < 0.05$ , *T*-test). Bars represent mean  $\pm$  SE ( $n = 16$ ;  $n = 15$  for the control at high elevation 2–6 June). Dashed lines indicate the defoliation period.

According to the RF analysis, differences in the VOC blends between herbivory and control treatments were most evidently separated at the end of the defoliation period (mid-3) at the low elevation (OOB: 18.75%; Figure 4A). Ten compounds were ranked as most important for discriminating between control and herbivory treatments, including five MTs, two HTs, one SQT and four OVOCs, all of which were released in significantly higher amounts from defoliated branches (Table 1).

#### Herbivory effects on foliar NVSC contents

Our untargeted UHPLC–MS/MS analysis detected 235 NVSCs, of which 63 were annotated based on levels 1 and 2 identification according to community standards. Identified metabolites consisted of carboxylic acids and derivatives, flavonoids, organic oxygen compounds, prenol lipids and tannins (see Figure S2

and Table S5 available as Supplementary data at *Tree Physiology* Online). Random Forest (RF) could not separate the NVSC blends by the herbivory treatment before the defoliation period, whereas it could after the defoliation period (Figure 5A). Of the 10 most important NVSCs for the separation between the herbivory and control treatments, four belonged to the NVSC subclass of fatty acyl glucosides and one to the NVSC subclass of carbohydrates and carbohydrate conjugates, based on the feature-based molecular networking analysis (Table 2; see Figure S7 available as Supplementary data at *Tree Physiology* Online). The structure of the remaining five compounds was predicted using the spectral annotation software SIRIUS, which showed their chemical nature related to the class of terpene glycosides as well as 1-hydroxy-2-unsubstituted benzenoids (Table 2). The foliar contents of seven of these NVSCs

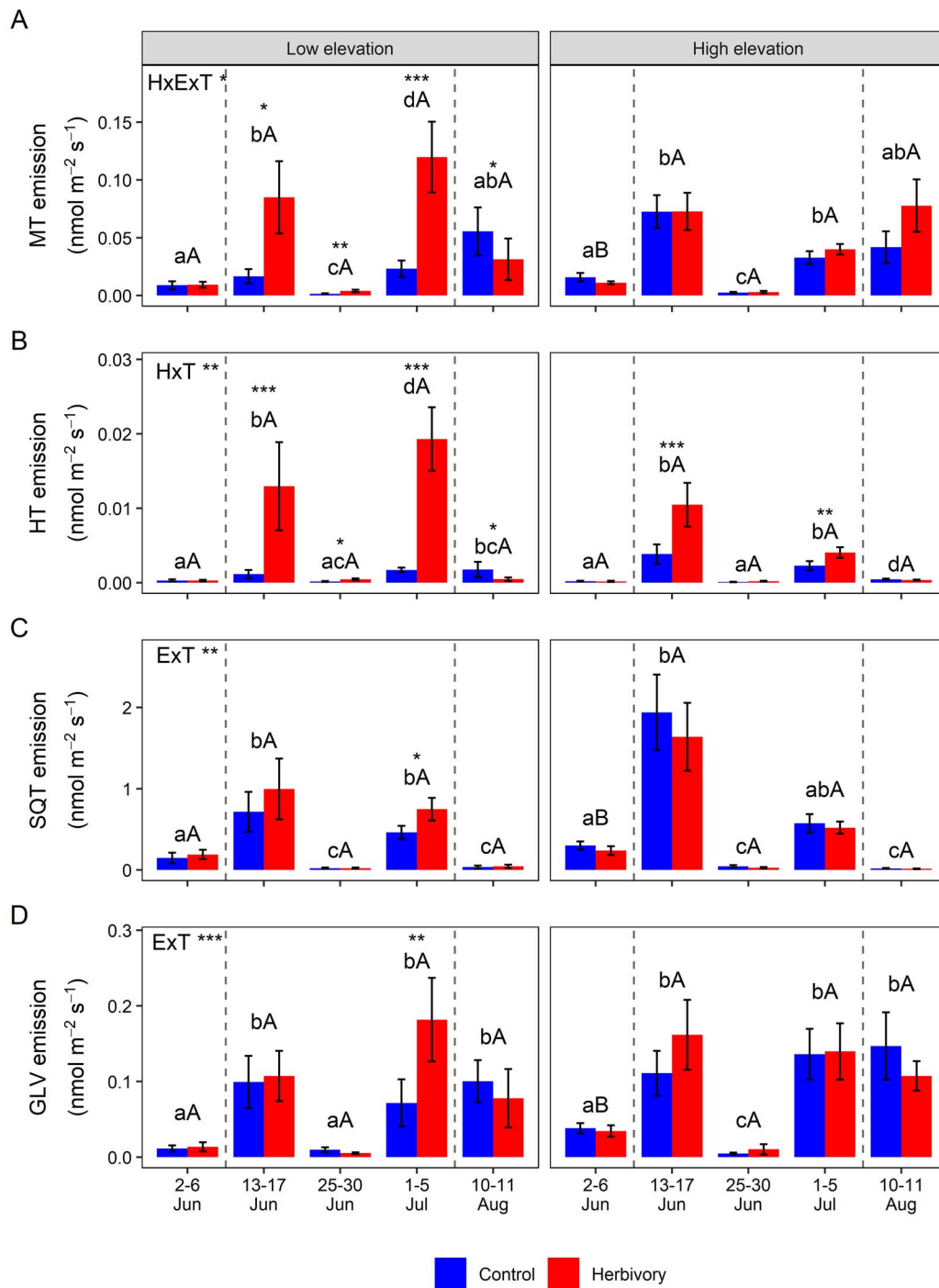


Figure 3. MT, HT, SQT and GLV emissions from control and herbivore-damaged branches at low and high elevations. MT (A), HT (B), SQT (C) and GLV (D) emissions from control and herbivory treated branches at five measurement times during the growing season at low and high elevations. Significant effects and interactions for herbivory (H), elevation (E) and measurement time (T) are shown (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ANOVA). The asterisks above the bars indicate a significant difference between the control and herbivory treatment within each sampling time and elevation (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $T$ -test). Bars labeled with different lowercase letters indicate significant differences between measurement times at the same elevation ( $P < 0.05$ , Tukey's post-hoc test). Bars labeled with different uppercase letters denotes significant differences between elevations within each sampling time ( $P < 0.05$ ,  $T$ -test). Bars represent mean  $\pm$  SE ( $n = 16$ ;  $n = 15$  for the control at high elevation 2–6 June). Dashed lines indicate the defoliation period.



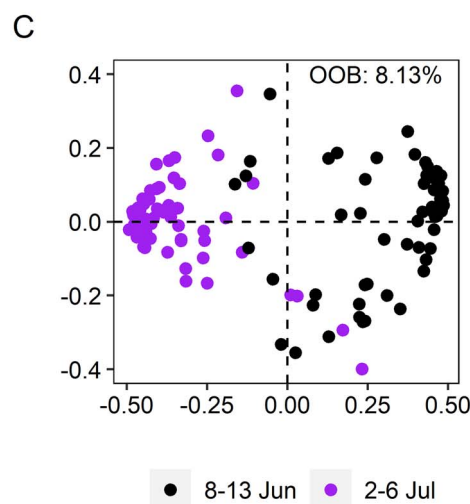
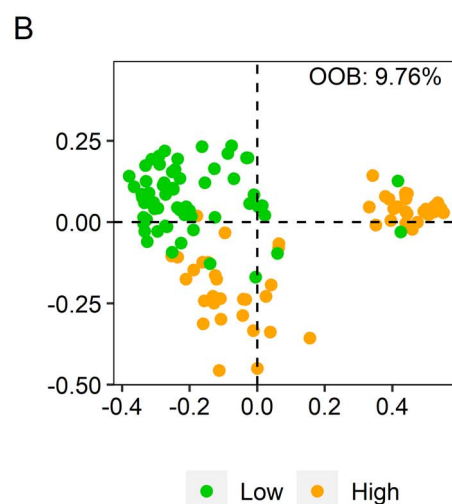
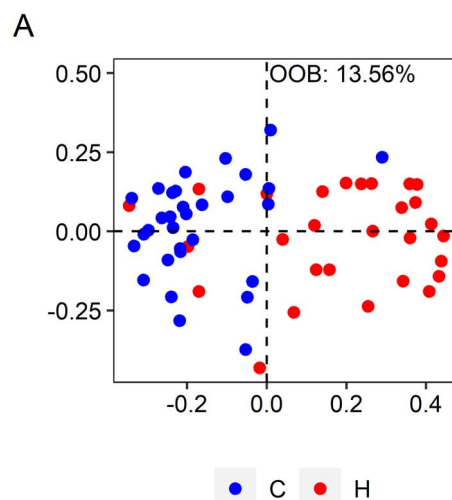
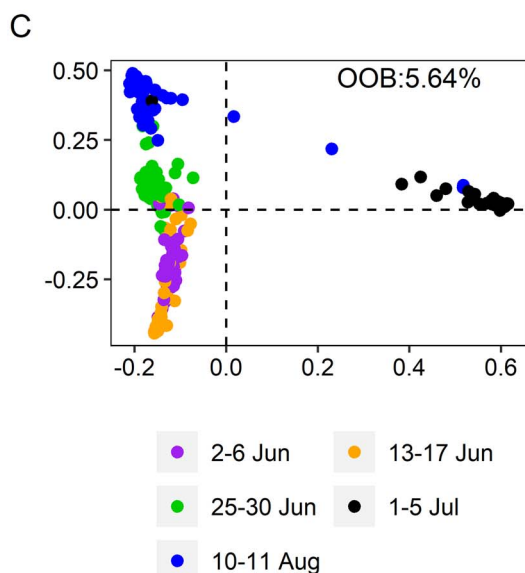
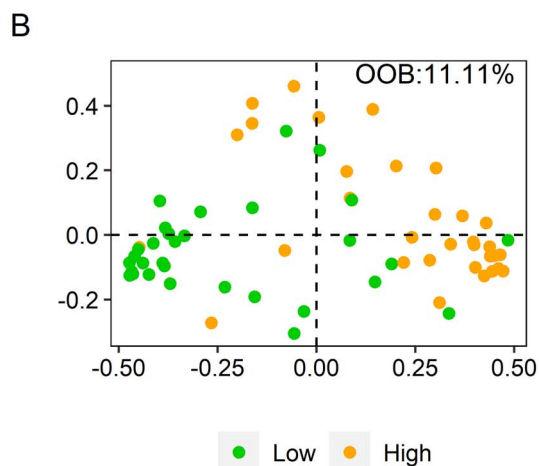
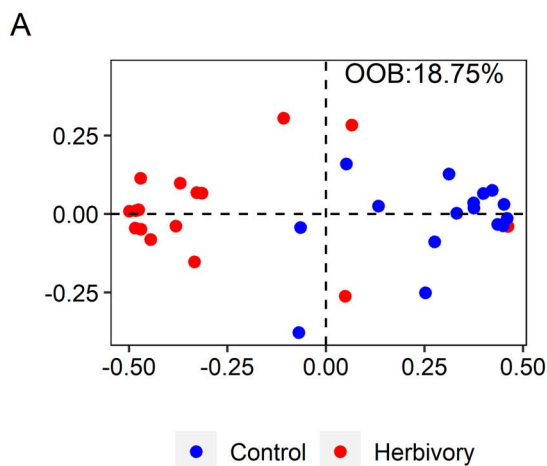


Figure 4. Metric multi-dimensional scaling (MDS) plots of the results of the RF analyses on VOCs. MDS plots of the proximity matrices showing the separation of the samples according to the VOC blends, between the control and herbivory treatment in the end of the defoliation period, i.e., 1–5 July, at the low elevation (A), between low and high elevations across herbivory treatments in the early growing season, i.e., 2–6 Jun (B), and among measurement times across both herbivory treatments and elevations (C).

Figure 5. Metric multi-dimensional scaling plots of the results of the RF analyses on NVSCs. MDS plots of the proximity matrices showing the separation of the samples according to the NVSC blends, between the control and herbivory treatment after the defoliation period, i.e., 2–6 July, at the low elevation (A), between low and high elevations across both herbivory treatments and measurement times (B), and between measurement times across both herbivory treatments and elevations (C).

Table 1. Ranking of the 10 VOCs that best distinguish the blends of the control and herbivory treatments as determined by the RF analysis. The compounds are listed in descending order based on decrease in variable importance (E).

Rank	Compound	VOC class	E
1	2-Methyl butanenitrile	OVOC	110
2	(E)-4,8-Dimethylnona-1,3,7-triene <sup>1</sup>	HT	81.52
3	Benzyl nitrile	OVOC	79.96
4	2-Butanone	OVOC	76.04
5	(E)- $\beta$ -Ocimene <sup>1</sup>	MT	66.5
6	(Z)-4,8-Dimethylnona-1,3,7-triene <sup>1</sup>	HT	63.01
7	Linalool <sup>1</sup>	MT	61.33
8	1-Penten-3-ol	OVOC	58.27
9	(Z)- $\beta$ -Ocimene <sup>1</sup>	MT	57.81
10	Unidentified SQT 7	SQT	54.99

<sup>1</sup>Compound identification was verified by available pure standards. All compounds were released in significantly higher amounts from infested than control branches.

Table 2. Ranking of the 10 NVSCs that best distinguish the blends of the control and herbivory treatments as determined by the RF analysis. The subclass of the individual compounds resulting from the feature-based molecular networking analysis is listed for each NVSC. The compounds are listed in descending order based on decrease in variable importance (E).

Rank	ID	NVSC subclass	E
1	226 <sup>↑</sup>	Fatty acyl glycosides	137.51
2	81 <sup>↑</sup>	Terpene glycosides <sup>1</sup>	93.44
3	136 <sup>↑</sup>	NA	77.57
4	133 <sup>↑</sup>	1-Hydroxy-2-unsubstituted benzenoids <sup>1</sup>	74.69
5	84 <sup>↓</sup>	NA	63.58
6	127 <sup>↑</sup>	Fatty acyl glycosides <sup>2</sup>	63.28
7	104 <sup>↓</sup>	Carbohydrates and carbohydrate conjugates	61.24
8	97 <sup>↑</sup>	Fatty acyl glycosides	57.14
9	58 <sup>↓</sup>	Fatty acyl glycosides	50.34
10	211 <sup>↑</sup>	Terpene glycosides <sup>1</sup>	48.03

Arrows beside ID numbers indicate the direction of the herbivory effect (<sup>↑</sup>: increase; <sup>↓</sup>: decrease). The tandem mass spectra (MS/MS) of the 10 NVSCs are given in Figure S7, and details of the class, subclass and identity of each NVSC are shown in Table S5, both available as Supplementary data at Tree Physiology Online. NA: not available metabolite annotation/identification within the molecular network.

<sup>1</sup>Identified via SIRIUS.

<sup>2</sup>Tentatively identified as 7-epi-12-hydroxyjasmonic acid glucoside.

increased, whereas three decreased, significantly in response to herbivory treatment after the defoliation period (Table 2).

### Differences in VOC emissions and foliar NVSC contents between elevations

The emissions of total VOCs, MTs, GLVs, OVOCs and SQTs were significantly higher at high elevation, compared with low elevation, in the early measurement (Figures 2B and 3; see

Figure S5A available as Supplementary data at Tree Physiology Online). Furthermore, they were all having higher emissions at the high compared with low elevation when averaged across all measurement times. However, the HT emissions were higher at low elevation compared with high elevation when averaged across all measurement times. The VOC blend was best separated between the elevations during the early measurement (Figure 4B), whereas the NVSC blend was generally well separated between the elevations (Figure 5B).

### Temporal variation of VOC emissions and foliar NVSC contents

VOC emissions varied substantially over the season, following the temporal variation of enclosure temperature and PPFD (Figures 2 and 3). Total VOC emissions were, indeed, positively correlated with the measured enclosure temperature ( $R^2 = 0.209$ ) and PPFD ( $R^2 = 0.308$ ;  $P < 0.001$ , Pearson's correlation test). For example, the lowest constitutive and induced emissions of total VOCs and individual compounds were observed on 25–30 June when both the enclosure temperature and PPFD were very low (Figures 2 and 3). Generally, the RF analysis separated the VOC and NVSC blends between measurement times (Figures 4C and 5C).

## Discussion

### Herbivory effects on VOC emissions and foliar NVSC contents

The herbivory treatment, feeding of mountain birch foliage by winter moth, increased emission rates of total VOCs, most of the VOC classes and individual VOCs. MTs, HTs and GLVs responded most strongly to herbivory. Furthermore, the herbivory treatment also increased foliar NVSC contents. Overall, the herbivory effects were strongest towards the end of the defoliation period and stronger at low elevation compared with high elevation. This is most likely due to accumulative leaf damage nearer the end of the defoliation period and the higher herbivore pressure (i.e., greater number of larvae applied at the low elevation site). The VOC emission rates increase linearly with the severity of insect herbivory, as shown in many studies (as reviewed by Niinemets et al. 2013), including a recent field study on mountain birch (Rieksta et al. 2020).

The increase in herbivore-induced VOC emissions we observed in our study corresponds well with previous studies (Kesselmeier and Staudt 1999, Dicke et al. 2009, Tholl et al. 2011, Hartikainen et al. 2012, Ameye et al. 2018). However, only a few studies have investigated herbivore-induced VOC emission patterns in natural settings (Faiola and Taipale 2020), and even fewer located in the Subarctic Fennoscandia, as we did here (Mäntylä et al. 2008, Li et al. 2019, Rieksta et al. 2020). These studies report strong increases of isoprenoid VOCs and GLVs, in agreement with our study (Mäntylä et al. 2008, Li et al. 2019, Rieksta et al. 2020). The importance of these studies is

further emphasized by in-field experiments generally indicating that herbivore-induced VOC emissions differ greatly between natural and controlled settings (Faiola and Taipale 2020). In our study, we advance the knowledge of herbivore-induced VOC emissions in natural settings by assessing the effects of herbivory throughout the defoliation period of the insects and the growing season, compared with previous studies commonly measuring for a couple of days to 1 week (Faiola and Taipale 2020).

Herbivore-induced VOCs have been suggested to function as a 'cry for help' by attracting herbivores' natural enemies, though evidence from natural conditions remain rare (Turlings and Erb 2018). Our study detected several VOCs that are involved with indirect defenses against insect herbivores. For example, MTs such as linalool and ocimene, as well as the HTs, are known to be involved in indirect plant defenses by attracting natural enemies of herbivores (Tholl et al. 2011, McCormick et al. 2012, Turlings and Erb 2018, McCormick et al. 2019). Furthermore, the nine most important VOCs for the separation between treatments were compounds typically released for the attraction of natural enemies, such as the OVOCs, 2-methyl-butanenitrile and benzyl nitrile (McCormick et al. 2014), as well as 1-penten-3-ol and 2-butanone (Pinto-Zevallos et al. 2013). In mountain birch forest, earlier field studies have shown that birds could exploit VOCs released by mountain birch damaged by autumnal moth larvae to locate their prey (Mäntylä et al. 2008). In this regard, it is likely that in the present study, mountain birch altered VOC emissions in response to winter moth larval feeding to attract moths' predators, such as parasitoids and birds.

Birches are assumed to lack inner specific storage structures, meaning that many VOCs from birches are either released directly after de novo synthesis or from temporary non-specific storage (Ghirardo et al. 2010, Ormeño et al. 2011). The increased MT and HT emissions observed in our study are therefore most likely due to increased de novo synthesis in response to herbivory (Donath and Boland 1994, Pare and Tumlinson 1997, Ghirardo et al. 2010, Pinto-Zevallos et al. 2013, Ghirardo et al. 2020). Increased GLV emissions, on the other hand, are most likely due to GLVs bursting out upon foliar cell disruption caused by herbivore chewing (Heil 2009, Amey et al. 2018).

We used an untargeted liquid chromatography–mass spectrometry (LC–MS) approach to investigate herbivory effects on the foliar NVSC contents and to identify compounds substantially affected by herbivory. Our untargeted LC–MS approach is distinctive compared to most other studies that only focus on a few selected NVSC classes, such as phenolics (Descombes et al. 2020). We investigated the 10 most important NVSCs driving the separation of the herbivory and control treatments. Four of the NVSCs were characterized as fatty acyl glycosides, of which one was specifically annotated as 7-epi-12-hydroxyjasmonic

acid glucoside, whereas the remaining structures resembled jasmonic acid derivatives. These compounds are likely responding to the herbivory treatment as the jasmonic acid pathway is known to be strongly elicited upon leaf damage by tissue-chewing insects (Seto et al. 2011). One of the NVSCs was identified as a carbohydrate/carbohydrate conjugate whilst two of the compounds were identified as terpene glucosides. The terpene glucosides could potentially be glucosidically bound volatiles, facilitating the transportation and storage of VOCs (Song et al. 2018). In general, the glycosylation of NVSCs can be particularly advantageous for defense responses, as glycosylation can aid metabolite translocation, prevent phytotoxicity, or increase the toxicity to herbivores (Neilson et al. 2011). One compound was identified as a 1-hydroxy-2-unsubstituted benzenoid. Benzenoids have previously been shown to increase in response to herbivory (Lavergne et al. 2020). Volatile benzenoids can attract natural enemies of the herbivores (Liu et al. 2018, Lavergne et al. 2020).

In summary, the NVSC response to herbivory suggests that different classes of NVSCs, particularly jasmonic acid derivatives, are substantially affected by herbivory. Furthermore, our results support the usefulness of untargeted LC–MS analyses for detecting NVSCs important for plant-herbivore interactions in mountain birch, beyond the often-studied phenolics. However, interpretation of the full chemical profile is challenging due to the high number of unknown compounds detected, leaving most of the data unexplored (Stavrianidi 2020). This opens future opportunities for investigating the metabolomic response of mountain birch and other forest trees to biotic and abiotic factors, and the development of new machine learning approaches based on mass spectral libraries would aid in such ventures (Blazenovic et al. 2018).

During the first campaign, it was noted that trees at the low elevation experienced aphid infestation. Even though aphids were removed from target branches before the experiment commenced, we cannot rule out potential legacy effects of previous aphid infestation on the target branches, nor the possibility of systemic effects caused by ongoing aphid feeding on adjacent branches of the measured trees. The co-occurrence of natural aphid infestation might interfere with the responses of VOCs and NVSCs to our experimental herbivory treatment and may thus have obscured the elevational effects on herbivore-induced responses, especially considering that aphid infestation has been shown to antagonize plant defense responses to attacks by chewing herbivores (Dicke et al. 2009). Future studies in natural settings are needed to assess to what extent local herbivore feeding triggers systemic responses, and how plant defense chemistry would respond to simultaneous or sequential attack by multiple herbivores of different feeding guilds (e.g., tissue-chewing versus phloem-sucking arthropods) as well as past insect and disease outbreaks.

### Differences in VOC emissions and foliar NVSC contents between elevations

We compared the chemical defenses in mountain birch at two elevations, expecting that as temperature generally decreases with altitude, we could compare the elevations as two temperature treatments functioning as proxies for climate warming (Körner 2007, Moreira et al. 2018). Indeed, the air temperature was slightly higher at the low compared with high elevation primarily in the early growing season and in the autumn. However, the difference was too small to translate into differences in plant defense chemistry and we only observed weak elevation effects due to temperature.

The VOC emissions were generally higher at high elevation compared with low elevation, mainly driven by the high emissions during the early measurement. The NVSC blend was generally well separated between elevations, suggesting a difference in the foliar NVSC contents between low and high elevations. These results are most likely due to elevation related phenological differences; all leaves were unfolded at low elevation whereas they had just started to unfold at high elevation. As birch leaf chemistry changes rapidly in the early season (Riipi et al. 2002), the different developmental stages in the two elevations were naturally accompanied by large differences in specialized compounds. Our study suggests that unfolding young leaves of mountain birch may have stronger VOC emission capacities than older leaves. This could be due to a higher level of chemical defense in young, compared with mature, leaves (Bracho-Nunez et al. 2011, Vedel-Petersen et al. 2015). Previously, it has been suggested that leaf development regulates VOC emissions, with for example, decreased emissions of MTs and SQTs at leaf maturity (Bracho-Nunez et al. 2011). Vedel-Petersen et al. (2015) also recorded higher emissions of MTs and SQTs from dwarf birch in the early growing season that decreased over time. However, other elevation-related environmental variables (e.g., air humidity, soil moisture, solar radiation and wind) might also have played a role in shaping elevational variation in plant specialized compounds, as has been found in previous studies (Niinemets et al. 2010). To better elucidate elevational effects on plant chemistry and determine the main drivers behind them, future studies should use elevation gradients with contrasting thermal conditions, measure different environmental and biotic (e.g., foliar herbivory and disease, soil microbial diversity) factors, and statistically assess their relative importance. We also recommend assessing photosynthetic performance of the studied plants in order to better distinguish responses in plant physiology from those in secondary chemistry.

### Temporal variations in VOC emissions and foliar NVSC contents

Temporal variations in VOC emissions largely followed the variation of the enclosure temperature and PPFD. This was expected because VOC emissions are known to be temperature-

and light-dependent (Loreto and Fares 2013, Ghirardo et al. 2020). However, leaf developmental stage and variation in environmental factors, such as water and nutrient availability, could potentially have influenced temporal variations in VOC emissions (Hakola et al. 2001, Chen et al. 2020). Generally, the NVSC blend changed from the early measurement to mid measurement, suggesting a seasonal change in the foliar NVSC contents. This is in agreement with previous studies reporting changes in foliar NVSC contents with leaf development (Riipi et al. 2002, Haukioja 2003, Zidorn 2018). However, foliar NVSC contents also follow the variation of other factors, such as light, temperature, water and nutrient availability (Gobbo-Neto and Lopes 2007, Zidorn 2018).

### Conclusions

We have, for the first time, assessed the combined effects of insect herbivory, elevation and season on mountain birch VOC emissions and NVSC production in a Fennoscandian birch forest. Our results show that feeding by winter moth larvae strongly alters birch VOC emissions and foliar NVSC contents, while elevation exerts little effect, which was most likely due to relatively similar temperatures between our low and high elevation sites. Furthermore, by using an untargeted LC-MS approach, we detected NVSC classes substantially affected by herbivory beyond the often-studied phenolics. The VOC emissions vary considerably over the growing season, mainly driven by seasonal variation in temperature and light intensity, whereas seasonal variations in foliar NVSC contents seems to be primarily driven by leaf development. Further studies employing distinct temperature differences along elevation gradients are needed to investigate how warming and herbivory interact to influence plant defense chemistry.

### Supplementary data

Supplementary Data for this article are available at *Tree Physiology* online.

### Data and materials availability

All VOC and NVSC data that support the findings of this study are available in Figshare (doi: 10.6084/m9.figshare.13370240) after the manuscript is accepted for publication.

The molecular networking job can be publicly accessed at <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ac7bb0838b544d4c96f81a59aa9f5c32>

The network annotation propagation job can be publicly accessed at <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7f48f4c1f9804e6aaec5b7e4cade9b4c>

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### Conflict of interest

None declared.

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### Authors' contributions

T.L. and R.R. designed the experiment. T.L., J.R. and R.R. designed the methods for VOC analysis. E.H.J.N. and B.M.S. designed the methods for NVSC analysis. OG conducted the untargeted UHPLC–MS/MS spectral data processing as well as subsequent molecular networking and SIRIUS-based dereplication pipeline. JUJ provided the experimental sites and laboratory resources at the Norwegian Institute for Nature Research (NINA) in Tromsø, and collected the insects. T.L., I.R., J.R., L.R.H.B. and H.S.H. executed the experiment and collected the data. I.R. and T.L. performed data analyses and wrote the manuscript with contributions from all authors.

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