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Time-resolved characterization of biotic stress emissions from Scots pines being fed upon by pine weevil by means of PTR-ToF-MS

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Insect herbivory of plants leads to the increased emissions of highly reactive volatile organic compounds (VOCs). We measured above-ground VOC emissions of Scots pine (*Pinus sylvestris*) saplings in the laboratory using a proton-transfer-reaction time-of-flight mass spectrometer (PTR-ToF-MS) before, during and after a 48-hour exposure to bark-feeding pine weevils. This study provides an in-depth investigation of the whole-plant VOC emission profile and its changes due to pine weevil feeding with a high time resolution. Monoterpenes dominated the emissions during and after herbivore feeding. The average monoterpene emission rate increase due to pine weevil feeding was 90 times and maximum monoterpene emission response was linearly proportional to the damaged bark area, demonstrating a dose-dependent response. Overall, the emission rates of more than ten VOC groups were increased due to herbivore feeding. These results increase knowledge about the effect of bark-feeding herbivores on VOC emissions from conifer forests and shed light on the important influence of herbivore ecosystem disturbance on atmospheric chemistry.

Introduction

Plants constitutively emit a wide variety of volatile organic compounds (VOCs) into the atmosphere. Due to herbivore feeding, a biotic stressor, plants increase the emissions of several VOCs. (Holopainen and Gershenzon 2010, Holopainen and Blande 2013). Conifer forests are important VOC emitters on a global scale; and boreal forests, dominated by conifer tree species, cover at least 30% (15 108 ha) of the total forest area on Earth (Steinbrecher *et al*. 2009, Taggart and Cross 2009). Previous studies have shown that due to herbivore feeding, the emissions of sev-

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eral VOCs of conifer tree species are increased (Litvak and Monson 1998, Blande *et al*. 2009, Heijari *et al*. 2011, Amin *et al*. 2012, Ghimire *et al*. 2013, 2016, 2017, Joutsensaari *et al*. 2015). There are multiple functions of increased VOC emissions in attacked plants, which include the protection and repellency against attacking herbivores, the attraction of natural enemies of the herbivore and the signaling to neighboring plants to prime their chemical defenses (Holopainen and Gershenzon 2010).

Many of the biotic stress-induced VOCs are highly reactive in the atmosphere (Atkinson and Arey 2003). Once released by plants, VOCs undergo chemical reactions in the atmosphere, which influences air quality and forest health. For example, the chemical reactions of VOCs emitted by plants can lead to the formation of secondary organic aerosol (SOA) (Joutsensaari *et al*. 2005, Virtanen *et al*. 2010, Faiola *et al*. 2018) and atmospheric oxidants, such as ozone (Atkinson and Arey 2003). Ozone is a phytotoxic gas, while SOA will scatter solar light back to space, act as cloud condensation nuclei (CCN) and diffuse solar radiation (Cohan *et al*. 2002, Ashmore 2005, Hallquist *et al*. 2009, Poschl *et al*. 2010, Zhao *et al*. 2017). It is predicted that global warming will increase the frequency and intensity of large-scale insect outbreaks in different types of forests, with particularly large effects expected in boreal forests (Niemela *et al*. 2001, Bale *et al*. 2002, Logan *et al*. 2003, Kurz *et al*. 2008, Ammunet *et al*. 2012). Therefore, it is likely that global warming will lead to increased biotic stress-induced VOC emissions, which could increase the production of SOA and atmospheric oxidants.

In the past, the biotic stress-induced emissions of plants have been studied using off-line techniques such as gas chromatography (GC) (Turlings *et al*. 1990, De Moraes *et al*. 2001, Kessler and Baldwin 2001, Holopainen 2011). With GC, both quantitative and qualitative analysis of VOCs emitted by plants can be achieved. However, GC measurement techniques are limited by a low time resolution, a possible need for sample pre-treatment before analysis (e.g. extraction or derivatization) and the limited number of different VOCs that can be measured with one column. During the last 15 years, the research community has started measuring biotic stress emissions from plants using a proton-transfer-reaction mass spectrometer (PTR-MS) — an analytical technique that enables rapid and continuous monitoring of plant emissions (Hansel *et al*. 1995). The PTR-MS measured clear differences in VOC emissions from a variety of plants before and after mechanical wounding (Tani *et al*. 2003, Brilli *et al*. 2011, Portillo-Estrada *et al*. 2015). PTR-MS data also clarified the effect of herbivore damage on belowground VOCs emitted from the roots of the plants (Crespo *et al*. 2012, Danner *et al*. 2012, van Dam *et al*. 2012, Danner *et al*. 2015). Furthermore, the rapid changes of herbivore-induced VOCs from leaves and branches were observed with the PTR-MS (von Dahl *et al*. 2006, Brilli *et al*. 2009, Schaub *et al*. 2010, Ghirardo *et al*. 2012, Maja *et al*. 2014, Danner *et al*. 2015, Giacomuzzi *et al*. 2016). These observed changes in VOC emissions could have been missed with off-line techniques, which have a lower time resolution.

Both above-mentioned techniques, on-line PTR-MS and off-line GC techniques, have different strengths. Thus, these techniques serve two very different purposes and it cannot be said that one is superior over the other. The PTR-MS provides on-line measurements of a broad range of VOC groups (from methanol to sesquiterpenes) while the GC cartridge samples provide more detailed molecular-level separation and identification for targeted compound classes. Therefore, these different measurement techniques nicely complement each other when plant VOC emissions are studied. Previous studies have shown the advantages of having a high time resolution PTR-MS included in the measurement setup, when plant stress VOC emissions are measured. After mechanical wounding of *Dactylis glomerata* leaves, the plants emitted a burst of methanol and the emissions of five-carbon (C5) compounds increased (Brilli *et al*. 2011). These emissions were not observed previously by a GC coupled with a mass spectrometer (GC-MS). In *Populus tremula*, mechanical wounding of leaves led to the emissions of different light-weight oxygenated compounds and lipoxygenase (LOX) products that were measured using a PTR-MS

(Portillo-Estrada *et al*. 2015). Moreover, a key sequence for VOC emissions that indicated a time-dependent elicitation of distinct pathways was found because of the high time resolution of the PTR-MS (Portillo-Estrada *et al*. 2015). In *Betula pendula*, PTR-MS measurements showed the temporal dynamics of green leaf volatile (GLV) emissions to be strongly dependent on the feeding activity of moth larvae (Maja *et al*. 2014). Observations of GLV bursts at uneven time intervals during the herbivore feeding period on *Populus tremula* observed with the PTR-MS also suggest that GLV emissions are indicative for feeding activity on deciduous trees (Schaub *et al*. 2010). These insights into VOC emission dynamics of different plants would have been missed with a GC-MS and other low time resolution off-line techniques.

The PTR-MS has been used to explore the effect of biotic stress on VOC emissions of several plant species. However, no study has explored the effect of herbivores on VOC emissions from conifer tree species using a PTR-MS. Conifer trees emit a great diversity of different groups of VOCs and previous studies with a GC-MS have shown that due to herbivore feeding, the emissions of monoterpenes, sesquiterpenes and GLVs are significantly increased (Litvak and Monson 1998, Blande *et al*. 2009, Heijari *et al*. 2011, Amin *et al*. 2012, Ghimire *et al*. 2013, 2016, 2017, Joutsensaari *et al*. 2015). Branch-level monoterpene emissions, after herbivore feeding, have been reported to increase from 2.8-fold up to 21-fold; sesquiterpene emissions from 2.9-fold up to 85-fold; and GLV emissions from 3.5-fold up to 13-fold, when compared with branches of intact control saplings (Blande *et al*. 2009, Heijari *et al*. 2011, Ghimire *et al*. 2013, 2017, Joutsensaari *et al*. 2015). Even though all the observed increases in emissions are measurement-condition dependent, all GC-MS results are demonstrating the same increasing trend of emission response to herbivore feeding. Due to the observed emission response, there is a need to explore this response using a PTR-MS because it enables real-time emission measurements over a wide range of different groups of VOCs before, during and after a herbivore exposure. Real-time measurements of VOC emissions from different tree species due to herbivore damage will fill in existing data gaps and improve modeling predictions of the impact of increasing insect herbivore outbreaks. This will improve our understanding about the impacts of these emissions on climate and forest health.

In this study, the experiments were performed sequentially as a rigorous longitudinal study to identify whole-plant emission trends from Scots pine (*Pinus sylvestris*) saplings in the laboratory before, during and after an active large pine weevil (*Hylobius abietis*) feeding. We repeated a paired control/treatment experiment four separate times to get four pseudo-replicates over the course of the summer of 2015. This is the first comprehensive study to look at herbivore stress VOC emissions with the high time resolution and broad chemical coverage of a PTR-MS with a time-of-flight mass analyzer (PTR-ToF-MS) using a rigorous longitudinal study design. The objectives of this study were to identify and quantify the major plant volatile stress emissions from Scots pine saplings associated with bark-feeding pine weevil herbivory, and assess how long the stress-related emissions remain elevated after the pine weevil feeding has ended. To more quantitatively distinguish the changes in VOC emission profile caused by pine weevil herbivory, we applied Exploratory Factor Analysis (EFA) to PTR-ToF-MS data (Rencher and Christensen 2012). We also aimed to characterize the dose-dependent monoterpene response of Scots pines due to pine weevil feeding. Moreover, as monoterpenes are the major group of compounds emitted by Scots pines, one of the objectives was to explore how pine weevil feeding affected the monoterpene emission profiles of Scots pine. Furthermore, because monoterpenes have a central role in atmospheric chemistry through their reactions with different oxidants, we also aimed to investigate the changes in OH^- and O_3^- pseudo-reactivities, which is an important factor when atmospheric and forest health implications are considered. The detailed atmospheric chemistry implications were investigated by generating aerosol from the plant emissions before, during and after herbivory; and these results are presented in a separate publication (Faiola *et al*. 2018).

Materials and methods

Plant and insect material

In this study, eight 7-year-old Scots pine (*Pinus sylvestris*) saplings (hereafter referred to as the trees) were used. The trees were grown in 7.5-liter plastic pots in a 1:1:1 mixture of quartz sand, garden soil and natural peat in the Kuopio campus research garden at the University of Eastern Finland (UEF). During the preceding spring of the measurement campaign, the trees were moved from the garden to the roof of the UEF. On the roof, the trees were fertilized (0.5 l of 0.1% fertilizer solution Turve-Superex, N:P:K 12:5:27, Kekkilä Oy, Finland) once per week and watered when needed.

The large pine weevil, *Hylobius abietis*. (Coleoptera: Curculionidae), is found in conifer forests from the Mediterranean area to northern Scandinavia and is one of the most serious pests of conifer plantations (Bentz and Jönsson 2015). The weevil larvae develop in conifer stumps where they do not cause any economic damage. Adult weevils develop in early summer and attack young conifer seedlings and often causing their death. The main damage to the conifer seedling is caused by the weevil feeding on the bark and phloem under the bark (Heijari *et al*. 2011). For this study, the large pine weevils were collected from a pine sawdust storage at a sawmill (Iisveden Metsä Oy) located in Suonenjoki, Finland. The collected pine weevils were kept at +8 °C, and they were fed with freshly cut pine branches. Prior to the start of the feeding period of the experiment, the pine weevils were kept without food for 24 hours to promote feeding during the experiment.

Experimental setup for Scots pine saplings

Four experiments were conducted during the summer of 2015 (Table 1). The trees used for the experiment were brought to the Aerosol Physics laboratory at UEF at least 24 hours prior to the start of the experiment to allow the trees to acclimatize to laboratory conditions. For each experiment, two trees were individually placed inside a ~70-liter Tedlar bag with two stainless steel barb fittings (Johnson Inert Products, USA), herein referred to as the plant enclosures. The Tedlar bags were enclosed around the trees with cable ties around the plant stem such that all biomass, except the roots, was within the enclosures. Of the two trees brought to laboratory conditions each time, one tree was exposed to pine weevils (referred to as the treatment tree) during the experiment while the other tree stayed undamaged (referred to as the control tree). The temperature inside the plant enclosures was monitored continuously with thermocouples and purified air was constantly flushed through the plant enclosures at a flow rate of 3.4–4.0 l min^{-1}. Purified air was generated from laboratory compressed air using a custom-built air cleaning system. The system consisted of an activated charcoal cartridge to remove VOCs; a purafil cartridge to remove NOx, SO_2 and HCl; and a High Efficiency Particulate Air (HEPA) filter to remove particles. Furthermore, to simulate a natural daily light cycle during the experiment, four high-output LED lamps (Valoya model B100-NS1, Valoya Oy, Helsinki, Finland) illuminated each tree and were set on a timer to turn on from 06:00–18:00. The PAR (photosynthetically active radiation) level of the lamps were set between 300–400 μmol m^{-2} s^{-1}. An illustration of the complete experimental design of the study's measurement campaign can be found in Faiola *et al*. (2018).

Experimental procedure

Four experiments that lasted from 8 to 16 days, excluding the time the trees acclimatized to laboratory conditions, were conducted during the measurement campaign. After the trees acclimated to laboratory conditions for 12–24 hours inside the plant enclosures, their baseline VOC emission rates were monitored with the PTR-ToF-MS. This period was called the pre-treatment period that lasted for at least three days for each experiment. After the pre-treatment period, the treatment tree was exposed to four pine weevils for approximately 48 hours. This period was called the treatment period. Pine weevil exposure was conducted by placing four weevils

inside a mesh enclosure that was attached to the treatment tree trunk. An empty mesh enclosure without pine weevils was attached to the trunk of the control tree. Placing an empty mesh enclosure on the trunk of the control tree accounted for any change in emissions that occurred due to the handling of the tree during the treatment procedure. After 48 hours of the treatment period, the pine weevils were removed and VOC emissions monitoring continued in order to observe how long the emissions remained elevated. This period was called the post-treatment period and it lasted for 2–7 days.

PTR-ToF-MS measurements of VOC groups emitted by Scots pine saplings

The PTR-MS technology and working principle have been described in detail in several previous publications (Hansel *et al*. 1995, Lindinger *et al*. 1998, Blake *et al*. 2009, Jordan *et al*. 2009). Consequently, only the key details of the PTR-ToF-MS used in this study can be found in Appendix 1. In this study, the PTR-ToF-MS (PTR-TOF 8000, Ionicon Analytik, Austria) with a mass resolution of > 5000, semi-continuously measured emissions from the control and treatment trees. The PTR-ToF-MS sampling location was varied with an automated valve switching system. The valve switching system alternated with 10–20 min time intervals between the control tree plant enclosure, the treatment tree plant enclosure and activated charcoal that was used to measure the background noise of the instrument. The sampling time from plant enclosures was set at 10 min. In addition to these sampling locations, the PTR-ToF-MS measured from the inlet and outlet of an oxidation flow reactor (OFR) was conducted to characterize the production of atmospheric aerosol from the Scots pine-emitted VOCs. The results from these additional samplings were reported in a separate study (Faiola *et al*. 2018).

The PTR-ToF-MS was operated under the following conditions: 2.3 mbar drift tube pressure, 600 V drift tube voltage, 130 Td E/N and 60 °C temperature of the drift tube. Sample air from the plant enclosures was introduced into the PTR-ToF-MS drift tube via a 1.5-m-long, heated (50 °C) Teflon tubing (i.d. 4 mm) and a 1-m-long heated (60 °C) PEEK tubing (i.d. 1 mm) at a flow rate of 200 ml min^{-1}. PTR-ToF-MS data were pre-processed (including mass scale calibration and peak fitting) by PTR-MS Viewer software ver. 3.2 (Ionicon Analytik), and further analyzed by MATLAB ver. 2016b (MathWorks). After data pre-processing, the background noise of the instrument was subtracted from the signals, before further analysis of the data.

The chemical formulas of the groups of VOCs were identified from the PTR-ToF-MS mass spectra based on their exact mass. A mass calibration was performed with three compounds that were always present in the mass spectra of the tree emissions: $H_{318}O^+$ at *m/z* 21.0226, NO^+ at *m/z* 29.998, and $C_{10}H_{17}^+$ at *m/z* 137.133. Moreover, the PTR-ToF-MS signal intensities were corrected for the transmission efficiency of ions with different molar masses using a calibration gas standard containing eight aromatic compounds with mixing ratios ~100 ppbV in nitrogen (BOC, United Kingdom). Furthermore, even though the soft ionization technique is used in the PTR-MS, many VOCs including monoterpenes, undergo a substantial fragmentation due to a high electric field used inside the drift tube (Kari *et al*. 2018). Therefore, for the quantitative analysis, the response of the PTR-ToF-MS against different monoterpenes was calibrated.

Table 1. Important dates of the experiments conducted during the summer of 2015.

Experiment Number	Experiment start date	Pine weevil application date	Pine weevil removal date	Experiment end date
1	1 June	8 June 14:40	10 June 13:30	12 June
2	22 June	29 June 11:30	1 July 13:30	3 July
3	3 July	8 July 12:00	10 July 16:00	16 July
4	16 July	22 July 11:00	24 July 15:15	31 July

The details of the calibration of the PTR-ToF-MS against different monoterpenes emitted by the trees can be found in Appendix 2 and in Kari *et al*. (2018).

From the calibration-corrected concentration of monoterpenes, the emission rate of monoterpenes was calculated according to Eq. 1:

$$E = \frac{c_{\mathrm{MTF}} R T F_{\mathrm{m}}}{A}\ , \qquad (1)$$

where E is the emission rate in units of μg m^{-2} hour^{-1}, c_{MTF} is the calibration-corrected concentration of monoterpenes in μg m^{-3}, R is the gas constant in L atm mol^{-1} K^{-1}, T is the temperature in K, F_{m} is the molar flow rate of emission in mol hour^{-1}, and A is the surface area of needles in m^2, estimated by the methods described in Kivimäenpää *et al*. and following Flower-Ellis and Olsson (Flower-Ellis and Olsson 1993, Kivimäenpää *et al*. 2016).

Terpene emission rates have a known temperature-dependence (Guenther *et al*. 1993). Thus, all monoterpene emissions were normalized to the temperature of 303 K using Eq. 2 to calculate basal emission rates (BER) for comparisons between enclosures and between days.

$$\mathrm{BER} = \frac{E}{\exp\left[\beta\left(T - T_{\mathrm{s}}\right)\right]}\ , \qquad (2)$$

where E is the emission rate of monoterpenes at sampling temperature T, Ts is the standard temperature of 303 K and β is an empirical coefficient. For monoterpenes, the value of $\beta = 0.1$ K^{-1} was selected according to Guenther *et al*. (2012).

Supplementary VOC measurements

The focus of this manuscript is the high, time-resolved PTR-ToF-MS measurements to investigate the temporal dynamics of VOC emissions across a broad range of VOC classes, which is why the manuscript focuses on the PTR-ToF-MS description. However, the PTR-ToF-MS lacks chromatographic separation and cannot distinguish between different structural isomers, and thus, the GC-MS data provides a complementary dataset for identification of individual monoterpene compounds. For the purposes of this paper, the GC-MS data were used to implement a rigorous PTR-ToF-MS quantitation procedure that accounts for different fragmentation patterns in the PTR-ToF-MS due to differences in monoterpene structures. This level of analytical rigor is higher than is applied to most PTR-ToF-MS publications: and our previous work has shown that ignoring differences in monoterpene fragmentation patterns can introduce almost a 20% error in estimated monoterpene mixing ratios (Kari *et al*. 2018). The procedure for collecting, analyzing, identifying and quantitating the GC-MS data is described in detail in Faiola *et al*. (2018). Briefly, duplicate cartridge samples (Tenax TA adsorbent, MARKES international, United Kingdom) were collected from each plant enclosure twice per day: once in the morning and once in the afternoon. The sampling time for each cartridge sample was 15–20 minutes with an air flow of ~200 ml min^{-1} through the sample tube. The trapped compounds were desorbed from the sample tube with a thermal desorption unit (TD, Perkin-Elmer ATD 400 Automatic Thermal Desorption system, USA) and introduced into a HP-5MS UI column (60 m × 0.25 mm, film thickness = 0.25 μm, Agilent Technologies, USA) with a helium carrier gas for gas chromatography-mass spectrometer (GC-MS, Hewlett Packard, GC 6890, MSD 5973, USA) measurements. The temperature program consisted of an initial temperature of 40 °C for 1 min, which was increased 5 °C min^{-1} until 125 °C. After, the temperature was increased 10 °C min^{-1} until 260 °C, when it was kept for an additional 3.5 min. The data were analyzed by MSD ChemStation software (Agilent Technologies) and Igor Pro (Wavemetrics, Inc.).

The emission profiles of monoterpenes were analyzed from the GC-MS data. In addition to monoterpenes, the GC-MS analysis gave information about sesquiterpene emissions during different experimental periods. The GC-MS analysis of mono- and sesquiterpenes were used more exhaustively in a separate publication by Faiola *et al*. (2018). In this study, monoterpene emission profiles were used to calculate the pseudo-reactivities for the reactions between monoterpenes and OH radicals (hereafter referred to as OH) and ozone (O_3). This was conducted during

different experimental periods because the pine weevil feeding may affect these reactions due to changes in the monoterpene emission profile. The pseudo-reactivities were calculated following the principles presented in Faiola *et al*. (2015). With this approach, we were able to explore the change to the concentration-normalized atmospheric oxidant reactivity instead of the absolute reactivity values.

Estimation of the damaged bark area of Scots pine saplings due to pine weevil feeding

The total damaged bark area of the trees due to pine weevil feeding was estimated from photographs of damaged bark and we used a tape measure for scale. A MATLAB script was created to estimate the damaged bark area from the photographs in a replicable manner. In the script, the user determines a unit length scale and the vertices for each feeding site in the tree by clicking the appropriate pixel in the photograph. The vertices form polygons whose area can be calculated using MATLAB's *polyarea* function. The total damaged stem area was determined by summing up the areas of individual polygons from damaged locations.

Statistical analysis

Exploratory Factor Analysis (EFA) (Rencher and Christensen 2012) is a type of dimension reduction technique. Factor analysis techniques are used to compress information from a large number of observed variables to a small number of latent variables. Generally, factor analysis methods solve the bilinear matrix equation: $\mathbf{X} = \boldsymbol{WH}$, where $\mathbf{X}$ is the original data matrix, $\boldsymbol{W}$ includes the factor time series and $\boldsymbol{H}$ includes the factor loadings that can be interpreted as a contribution of a variable to a specific factor (Tabachnick and Fidell 2013). In this study, Positive Matrix Factorization (PMF) (Paatero and Tapper 1994) and Principal Component Analysis (PCA) (Wold *et al*. 1987) were also tested, but EFA gave the most physically interpretable results. Interpretability of the results of these methods was assessed by inspecting the factor behavior as a time series. In addition, the contribution of different compounds in each factor was examined; provided we were able to identify the compounds in question. The compounds in PCA were not distributed as clearly as in EFA, causing some properties of the factors to be mixed, which made the identification and naming of those factors impossible. PMF, on the other hand, would have needed more factors to explain the data, but we were not able to interpret the extra factors, as they included a lot of unidentified compounds and their time series did not show any patterns that could have been used in the identification. The physical interpretability of the factors is very crucial when using dimension reduction techniques, as these methods do not take into account any real physical or chemical phenomena. Interpretability of the factors was also assessed when the selection of the number of factors is completed.

EFA solves the matrix equation using the correlations between the original variables. The factor analysis model expresses each variable as a linear combination of latent factors $f_1, f_2, \ldots, f_m$. For $y_1, y_2, \ldots, y_p$ in the observation vector y, the factor model can be expressed as:

$$\begin{aligned} y_1 - \mu_1 &= h_{11} f_1 + h_{12} f_2 \cdots + h_{1m} f_m + \varepsilon_1 \\ y_2 - \mu_2 &= h_{21} f_2 + h_{22} f_2 + \cdots + h_{2m} f_m + \varepsilon_2 \\ &\vdots \\ y_p - \mu_p &= h_{p1} f_1 + h_{22} f_2 + \cdots + h_{2m} f_m + \varepsilon_2 \end{aligned} \quad , \quad (3)$$

where $m << p$, h_{ij} are the loadings and ε_i are the error terms to account for unique variance. There are multiple iterative factorization algorithms, and in this study, maximum likelihood (de Winter and Dodou 2012) was applied with orthogonal varimax-rotation (Rencher and Christensen 2012). The statistical analysis was performed using R ver. 3.3 (Rewelle 2017, R Core Development Team 2017). More details about EFA are found in Appendix 3. As the data did not meet the normality and homogeneity assumptions, we applied the Mann-Whitney *U* test for comparing the emissions from control and treatment trees, and the Wilcoxon rank-sum test for correlated samples for comparing the differences in emissions between pre- and post-treatment periods. The calculations were performed using MATLAB.

Results

The primary objectives of this study were to: (1) identify the major compounds and groups of compounds in Scots pine volatile emissions, that are associated with the pine weevil herbivory treatment — including a rigorous, multivariate statistical evaluation; (2) quantify the emission rates of monoterpenes in real-time before, during and after pine weevil herbivory treatment; (3) assess how long the stress emissions remained elevated during the post-treatment period; (4) assess the atmospheric relevance of changing emission profiles for atmospheric reactivity; and (5) characterize the dose-dependence of the maximum monoterpene emission rate after the treatment tree was exposed to pine weevils.

Identification of stress volatile emissions from Scots pine saplings due to pine weevil feeding

Comparison of mass spectra

One of the primary objectives in this study was to identify the major compounds and groups of compounds in the whole-plant, Scots pine volatile emissions, that are associated with the pine weevil herbivory treatment. To do that, we first looked at the emission profile during the different experimental periods. The unit mass resolution spectra of the PTR-ToF-MS (Fig. 1) summarizes the difference between the emission profiles before pine weevil exposure (24-hour average of each experiment before the exposure) and during the treatment period when pine weevils were actively feeding (4-hour average of each experiment from the end of the treatment period). Figure 1a shows the difference for the treatment trees to explore the effect of pine weevil feeding on overall VOC group emissions. The compound group emissions that increased the most after pine weevil feeding were monoterpenes and two peaks, originated from monoterpenes, which dominated the difference between the emission profiles: $C_{10}H_{17}^+$ (monoterpenes) and $C_6H_9^+$ (the main fragment of monoterpenes). In addition to increased monoterpene emissions, there were also changes in several other

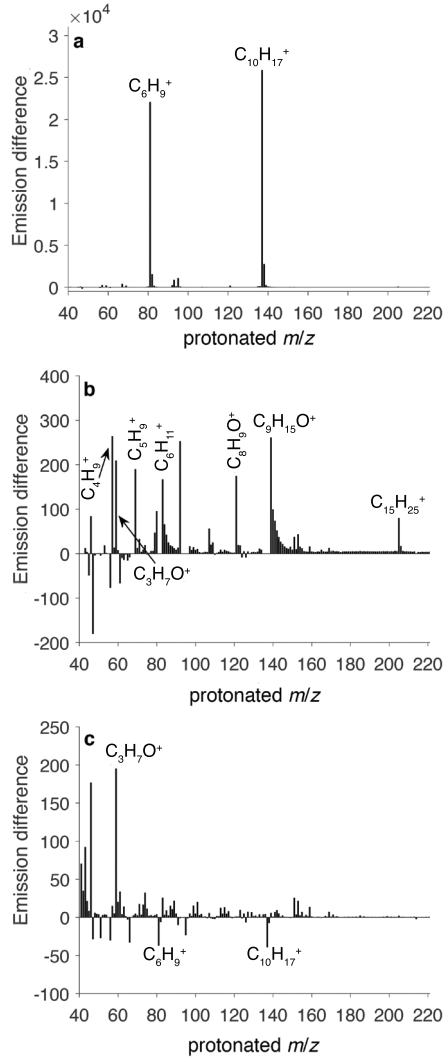

Fig. 1. The Scots pine emission differences (normalized signals during the treatment period minus the pre-treatment period) due to pine weevil feeding measured by the PTR-ToF-MS for **a**) treatment trees, **b**) treatment trees where monoterpene peaks are excluded, and **c**) control trees. The spectra are average of all four experiments.

VOC groups. To better visualize the emission changes of other VOC groups with lower concentrations, monoterpenes and their fragment ions were removed (Fig. 1b). Also other VOC emissions, such as sesquiterpenes ($C_{15}H_{25}^+$) and potentially 2-methyl-3-buten-2-ol (MBO) fragments ($C_5H_9^+$) and nopinone ($C_9H_{15}O^+$) were clearly increased during the treatment period;

even if the magnitude of the increased emissions was significantly smaller in comparison with monoterpenes (Fig. 1b). Figure 1c shows the emission profile differences of the control trees before and during the treatment period. After the treatment period began (i.e., the empty mesh enclosure was attached to the trunk of the control tree), the control trees increased their emissions of several VOC groups, but the magnitude of the increase was much smaller in the control trees for many VOC groups compared with treatment trees (Fig. 1c). However, the increase (e.g., in some oxygenated VOC group emissions) was in the same magnitude in both control and treatment trees. This indicates that not all increased VOC group emissions (cf. Fig. 1a and b) were due to pine weevil feeding, but that the increase of some VOC groups were most likely caused by the handling of the trees during the treatment procedure. By using the Mann-Whitney *U* test and comparing the emissions of control trees and treatment trees, we were able to distinguish the compound groups whose emissions changed significantly due to the pine weevil feeding from the emissions that increased due to the handling of the tree. The potentially-identified VOCs and VOC groups that clearly responded to the pine weevil feeding are listed in Table 2. As monoterpenes clearly dominated the emission profile of Scots pines during and after pine weevil feeding, monoterpenes were unarguably the most important group of VOCs emitted by Scots pines due to biotic stress. Therefore, the main focus of this study was on monoterpene emissions and other groups of VOCs that were elevated due to pine weevil feeding are only briefly discussed.

Multivariate analysis: exploratory factor analysis

We also tested the applicability of a rigorous, multivariate statistical evaluation to distinguish the changes in the emission profile due to the pine weevil feeding. Different variations of EFA, positive matrix factorization (PMF) and principal component analysis (PCA) were tested on the PTR-ToF-MS measurement data. All mass signals common to each experiment that were differing significantly from the background noise of the PTR-ToF-MS were selected for the analysis and the data from all the experiments were combined to one data set before it was fed into the model. By combining the data, we were able to increase the efficiency of the used methods and more precisely detect similar patterns from the four experiments. The best results, meaning the most interpretable and the clearest separation of factors, were gained with EFA using orthogonal rotation. The purpose of EFA and other

Table 2. VOCs whose emissions from Scots pine saplings were increased due to pine weevil feeding.

m/*z* + 1	Chemical formula[1]	Possible compound identification[2]	Average magnitude of the maximum increase in emissions due to pine weevil feeding (*x*-fold)
33.034	CH_5O^+	Methanol[a)]	2
69.034	$C_4H_5O^+$	—	5
69.070	$C_5H_9^+$	MBO fragment[a)]	8
71.050	$C_4H_7O^+$	Methyl vinyl ketone[a)]	4
83.050	$C_5H_7O^+$	Methylfuran[a)]	4
83.086	$C_6H_{11}^+$	Fragment of C6-products[a)]	7
107.050	$C_7H_7O^+$	—	4
121.065	$C_8H_9O^+$	—	12
135.117	$C_{10}H_{15}^+$	*p*-cymene[b),c)]	21
137.133	$C_{10}H_{17}^+$	Monoterpenes[a),c)]	90
139.112	$C_9H_{15}O^+$	Nopinone[b)]	14
153.128	$C_{10}H_{17}O^+$	Camphor[b),c)]	5
205.196	$C_{15}H_{25}^+$	Sesquiterpenes[a),c)]	2

[1)]Chemical formulas were obtained using the PTR-ToF-MS measured exact mass.
[2)]The name of the compound according to: a) Rantala *et al.* (2015), b) Kim *et al.* (2010) or c) positive identification from GC-MS analysis.

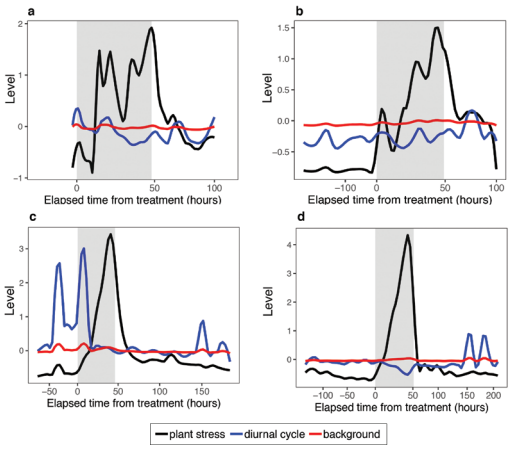

Fig. 2. Timeseries of different factors separated from PTR-ToF-MS data using EFA. Different experiments are shown in different panels: (**a**) Exp1, (**b**) Exp2, (**c**) Exp3, and (**d**) Exp4. The grey area indicates the treatment period.

dimension reduction techniques is to find variables (here, mass loadings), which behave similarly during the experiment. The factors gained from this grouping are named according to their behavior during the experiments. The factor levels can be thought as weighted sums of the grouped mass loadings and these sums can be presented as time series. Note that because EFA uses the correlations between the compounds in the calculation of the factors, the score level may become negative, due to negative correlations. Instead of inspecting the levels of the factors in detail, we should focus on the form of the time series, which shows the general structure of the data.

The first factor showed a significant increase in every experiment after the active feeding period started and recovery to the original level after the pine weevils were removed (Fig. 2). Thus, this factor is hereafter named, the plant stress factor. The plant stress factor contained mainly groups of VOCs that demonstrated increased emissions during pine weevil feeding, including monoterpenes, monoterpene fragments, oxygenated monoterpenes ($C_{10}H_{16}O$ and $C_9H_{14}O_2$), the main fragment of *p*-cymene ($C_7H_9^+$), MBO fragments ($C_5H_9^+$) and methyl vinyl ketone. EFA was able to distinguish some of the compound groups we identified from analyzing the mass spectral changes, but some of the compound groups were not included in the plant stress factor.

The second factor shows a diurnal cycle in the plant emissions; the most visible in the experiments 1 and 2 (Fig. 2). This factor demonstrated an oscillation that was unrelated to the treatment phase but was clearly associated with natural plant circadian rhythms. Therefore, this factor is hereafter named, the diurnal cycle factor. As trees have some natural variation in their temporal behavior, the signal of the diurnal cycle factor varies (Fig. 2). In addition, some uncertainty always exists in the factorization, and some variation might not be captured with EFA that may also cause some variation in the diurnal cycle factor. The compound groups related to the diurnal cycle factor included different oxygenated hydrocarbons and hydrocarbons, such as C_6H_{12}, $C_7H_{14}O$, $C_6H_{12}O_2$, $C_{10}H_{12}$, $C_7H_{10}O_3$, $C_9H_{18}O$ and $C_8H_{16}O_2$. The third factor does not show drastic change throughout the experiments (Fig. 2). Hence, the third factor is hereafter named, the background factor. The background factor consisted mostly of VOC groups that are not affected by this type of plant stress and do not have a diurnal cycle. However, the background factor contained traces of some VOCs, such as *p*-cymene ($C_{10}H_{14}$), nopinone ($C_9H_{14}O$) and camphor, which had a temporal variation or were affected by the treatment. This can be seen as a minor elevation during the active feeding period (Fig. 2).

Temporal trends of major volatile emissions influenced by pine weevil feeding

Monoterpenes

The pine weevil feeding caused the greatest increase in monoterpene emissions from Scots pine saplings (Fig. 1). Therefore, one of the objectives in this study was to quantify the emission rates of monoterpenes in real-time before, during and after pine weevil herbivory treatment using the PTR-ToF-MS. Moreover, another aim was to assess how long monoterpene emissions were elevated during the post-

treatment period (i.e., after the pine weevils were removed). The treatment trees exhibited drastically increased monoterpene BERs during the treatment period compared with baseline level emission rates during the pre-treatment period (Fig. 3). On average, the maximum monoterpene emission rate increase due to pine weevil feeding was 90-fold, from 28 ± 12 μg h^{-1} m^{-2} to 2560 ± 360 μg h^{-1} m^{-2}. A variance existed in monoterpene BERs between the experiments (Table 3, and Table A1 and Fig. A1 in Appendix 4). The greatest increase in monoterpene BERs occurred during Experiment 3 (Table 3 and Fig. 3c); during this experiment, the baseline BER was lower than 20 μg h^{-1} m^{-2} and increased up to 2900 μg h^{-1} m^{-2} due to pine weevil feeding, which resulted in more than a 180-fold increase in BER. After the pine weevils were removed, monoterpene emissions remained elevated during the whole post-treatment period of each experiment (2–7 days). The statistical significance of the difference between pre- and post-treatment periods was confirmed with Wilcoxon rank sum test for correlated samples. Monoterpene emissions began to decline after the post-treatment period was initiated (Fig. 3 and Table 3). However, the rate of decline was different for each experiment. In Experiment 2, we had the shortest post-treatment period of 44 hours. Hence, Table 3 shows monoterpene BERs of each experiment after 44 hours from pine weevil removal for as a reference. Compared with pre-treatment monoterpene BERs after 44 hours from pine weevil removal, monoterpene BERs were 6–37-fold higher, depending on the experiment. This highlights the great variability in the rate of decline of monoterpene BERs between the experiments.

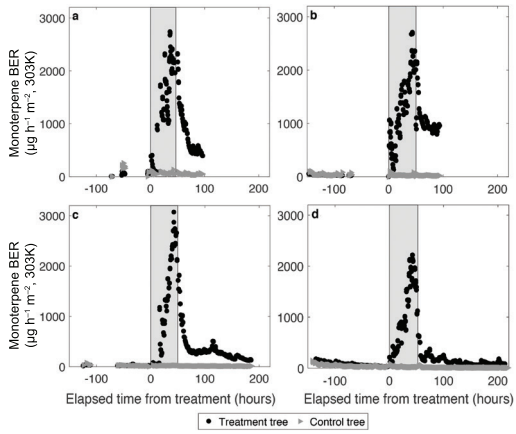

Fig. 3. The effect of pine weevils on monoterpene basal emission rates measured by the PTR-ToF-MS during different experiments. The panels **a**)–**d**) show experiments 1–4, respectively. The grey area indicates the treatment period. Pre-treatment and post-treatment periods took place before and after the treatment period, respectively.

Since the emissions remained elevated until the end of the post-treatment period in all experiments, we cannot quantify the duration of the prolonged effect of elevated emissions. In order to do this, longer post-treatment period measurements are required. The control trees did not significantly increase monoterpene emissions during the experiments (Fig. 3, and Fig. A1 and Table A1 in Appendix 4); the only time when monoterpene emissions of the control trees were slightly increased was during the treatment procedure when the empty mesh enclosure was attached to the trunk of the control tree. Moreover, there were differences in time lags between the start of the treatment period and the observed increase in monoterpene emissions (Fig. 3).

Table 3. Basal emission rates (BERs) of monoterpenes during different experimental periods. BERs are calculated as a 10 minute average from PTR-ToF-MS data.

Experiment Number	BER before treatment period (μg h^{-1} m^{-2})	BER maximum during treatment period (μg h^{-1} m^{-2})	BER 44 hours after pine weevil removal (μg h^{-1} m^{-2})	BER at the end of an experiment (total length of the post-treatment period, hours) (μg h^{-1} m^{-2})
1	46 ± 2	2570 ± 132	447 ± 0.8	393 ± 0.4 (46 h)
2	25 ± 4	2650 ± 115	955 ± 2	955 ± 2 (44 h)
3	16 ± 6	2960 ± 115	301 ± 3	129 ± 0.6 (135 h)
4	27 ± 0.2	2080 ± 67	199 ± 0.3	79 ± 0.1 (159 h)

These differences in time lags reflect differences in pine weevil feeding activity.

Other VOC groups

In addition to monoterpenes, one objective of this study was to identify other stress-related VOC groups and to determine how long the emissions of these compound groups remained elevated during the post-treatment period. Other stress-related VOCs and groups of VOCs found from Scots pine emissions using the PTR-ToF-MS are listed in Table 2. The VOCs listed in Table 2 were tentatively identified based on their *m/z* ratios and literature (Kim *et al*. 2010, Rantala *et al*. 2015). Moreover, for some VOCs, a positive identification was obtained from GC-MS analysis. In Table 2, the average magnitude of the maximum increase of each VOC group during the treatment period is given. The response of VOCs and groups of VOCs to pine weevil feeding varied from a 2-fold increase in methanol and sesquiterpene emissions up to a 14-fold increase in tentative nopinone ($C_9H_{15}O^+$) and a 21-fold increase in *p*-cymene emissions (Table 2). The increases were calculated from the normalized signal values of the protonated molecules. This was because the reliable molecular structure identification was not possible for all VOCs that showed increased emissions due to pine weevil feeding. Hence, the calibration of the PTR-ToF-MS against these unidentified compound groups was not possible and data for these fragments is presented with a semi-quantitative approach using normalized signals. Without the calibration, the calculation of concentrations and emission factors is not possible due to the substantial fragmentation of some VOCs inside the drift tube of the PTR-ToF-MS. Therefore, the normalized signals of the protonated molecules were also used to generate Fig. 4, which shows the temporal evolution of selected VOC groups. The usage of normalized signal values of the protonated molecules does not prevent the comparison of the magnitude of the increase of the VOC group emissions due to pine weevil feeding, because the compound always fragments similarly inside the PTR-MS as long as the operating conditions do not change.

Figure 4 exemplifies the observed increases of the selected VOC groups listed in Table 2.

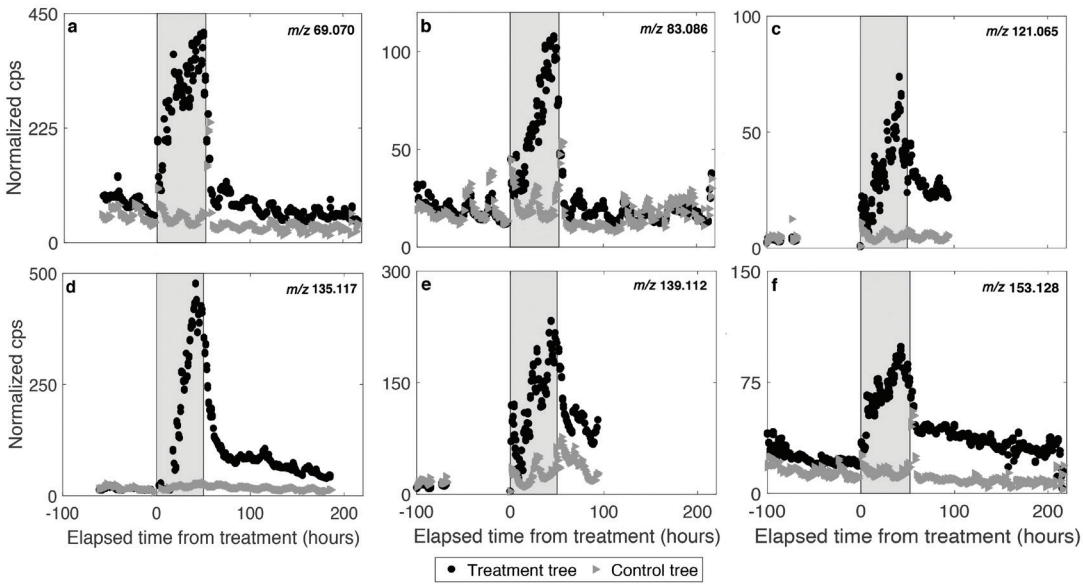

Fig. 4. The temporal evolution of selected VOCs due to pine weevil feeding during different experiments measured by the PTR-ToF-MS. **a**), **b**) and **f**) are taken from Experiment 4, **b**) and **e**) from Experiment 2, and **d**) from Experiment 3. The grey area indicates the treatment period. Pre-treatment and post-treatment periods took place before and after the treatment period, respectively.

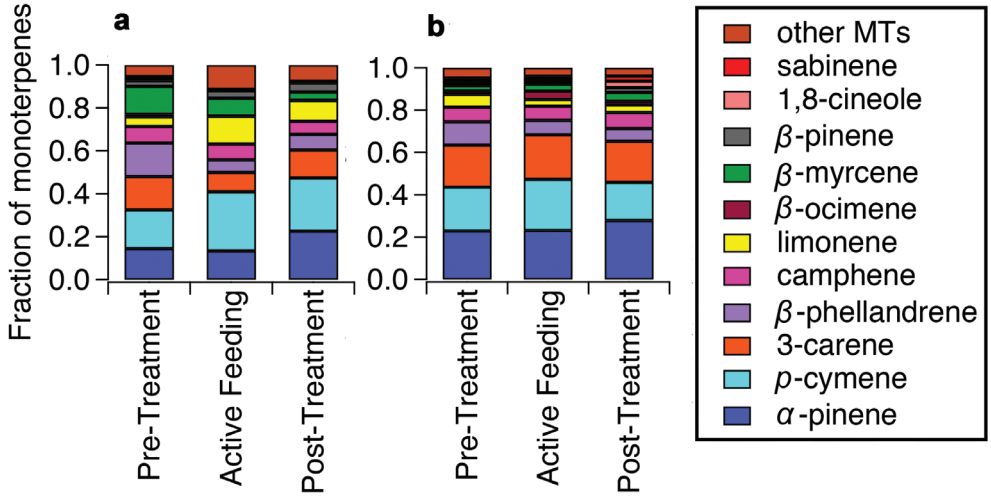

Fig. 5. Monoterpene (MT) emission profiles of (**a**) treatment trees and (**b**) control trees. Emission profiles are averaged GC-MS results from all four experiments. The "other" category includes 23 monoterpenoid peaks that never individually contributed more than 1% of the total monoterpene emissions.

This demonstrates the clear changes observed in each experiment and for different types of VOCs. After the pine weevils were removed, the selected VOC groups showed elevated emissions for more than 48 hours (Wilcoxon rank sum test for correlated samples) (Fig. 4). The Wilcoxon rank sum test for correlated samples was also applied to verify the increase of other VOC group emissions due to pine weevil feeding. The test revealed largely different results compared with the baseline emissions measured during the pre-treatment period.

Changes in monoterpene emission profiles due to pine weevil feeding

The average monoterpene emission profile for all the four experiments was measured by the GC-MS for the treatment tree (Fig. 5a) and the control tree (Fig. 5b). No systematic change in monoterpene emission profiles, due to pine weevil feeding, was observed between different experiments. The emission profile of the treatment tree was dominated by the same seven monoterpenes during each experimental period. This finding suggests that the main mechanism causing increased emissions was the mechanical damage of stem tissue exposing terpene pools into the atmosphere, as opposed to a biochemical induction leading to the emission of new volatile compounds. More information about the monoterpene emission profiles from each experiment can be found in Faiola *et al*. (2018).

To characterize the dose-dependence of the maximum monoterpene emission rate after the treatment tree was exposed to pine weevils (see primary objectives above), we estimated the area of the bark damaged by pine weevils from the photographs taken from the trunk of the treatment tree after each experiment. Figure 6 indicates that the maximum monoterpene emission response can be related to the damaged bark area, demonstrating a dose-dependent response. The damaged bark area correlated well with the maximum monoterpene BER measured by the PTR-ToF-MS, having a slope of 228 $\mu g\ h^{-1}\ m^{-2}$ per cm^2 of damaged bark (Fig. 6). This finding supports our earlier statement that the main mechanism causing increased emissions was the mechanical damage of stem tissue, thus exposing terpene pools into the atmosphere.

Even though no specific "stress-induced" monoterpene was found from the average emission profiles and the monoterpene emission profile did not drastically change during or after

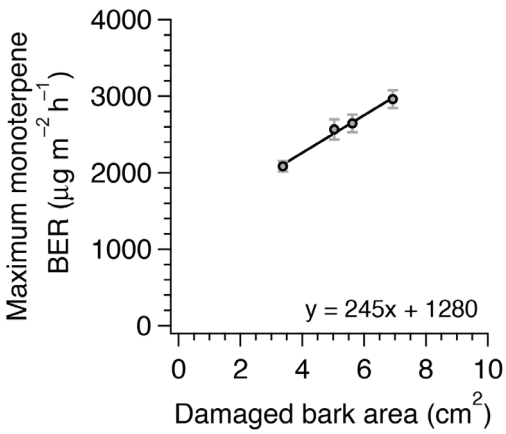

Fig. 6. The maximum monoterpene basal emission rate (10-minute average) as a function of the damaged bark area of Scots pine saplings. The damaged bark area was estimated from photographs taken after each experiment.

herbivory (Fig. 5), one of the objectives of this study was to assess the atmospheric relevance of even small changes in the emission composition for atmospheric reactivity. The OH reaction rate constants for different monoterpenes measured in this study can range from 5.30×10^{-11} cm^3 molecule^{-1} s^{-1} for camphene up to 2.52×10^{-10} cm^3 molecule^{-1} s^{-1} for ocimene, and O_3 reaction rate constants can vary from 9.00×10^{-19} cm^3 molecule^{-1} s^{-1} for camphene up to 5.40×10^{-16} cm^3 molecule^{-1} s^{-1} for ocimene (Atkinson 1997, Seinfeld and Pandis 2016). Therefore, even small changes in the monoterpene emission profile could produce large changes in reactivity. Based on monoterpene emission profiles, the OH and O_3 pseudo-reactivities of monoterpenes were calculated to explore if there are any changes in reactivities between different experimental periods. Additionally, they were calculated to evaluate whether or not these changes have any effect on atmospheric reactivity. Figure 7 shows the average OH and O_3 pseudo-reactivities of monoterpenes averaged for all four experiments. During the pine weevil feeding (i.e., the active feeding period), OH pseudo-reactivity was dominated by β-myrcene, limonene, and 3-carene, that together, contributed to over 50% of the total OH pseudo-reactivity. Meanwhile, during the pre-treatment period, more than 60% of the OH pseudo-reactivity was dominated by β-myrcene and β-phellandrene (Fig. 7a). Despite this pseudo-reactivity profile change, the change in atmospheric lifetimes of emitted monoterpenes for reaction with OH-radicals was small (Atkinson and Arey 2003).

The change in the O_3 pseudo-reactivity profile of monoterpenes due to pine weevil feeding was more substantial than the change in OH pseudo-reactivity profile. The total OH pseudo-reactivity of the treatment trees changed from 2.7 s^{-1} to 2.0 s^{-1} from the pre-treatment period to the active feeding period; and decreased to 1.8 s^{-1} during the post-treatment period (Fig 7a). The total O_3 pseudo-reactivity of the treatment trees changed from 3.0×10^{-6} s^{-1} to 2.2×10^{-6} s^{-1} from the pre-treatment period to the active feeding period; and from the active feeding period it decreased to 1.8×10^{-6} s^{-1} during the post-treatment period (Fig. 7c). The O_3 pseudo-reactivity was dominated by β-myrcene, limonene, and α-pinene, covering more than 70% of the total O_3 pseudo-reactivity due to herbivore damage (active feeding period in Fig. 7c). Before the pine weevil exposure, the O_3 pseudo-reactivity was only dominated by β-myrcene. During the active feeding period, monoterpenes with shorter atmospheric lifetimes reacting with O_3 were emitted into the atmosphere compared with the post-treatment period. This was mainly due to the decreased emission of β-myrcene and increased emissions of limonene and α-pinene during the post-treatment period (Fig. 7c). Furthermore, the changes in pseudo-reactivity profiles were not observed with the control trees (Fig. 7c and d). This demonstrates that the pine weevil feeding caused the observed changes in monoterpene pseudo-reactivity profiles. Even though the total pseudo-reactivities (white circles in Fig. 7) of the treatment trees seemed to change between experimental periods, paired-samples from the Wilcoxon signed rank test applied for the individual reactivities of each experiment showed that the changes in the total pseudo-reactivities were not statistically significant either in treatment trees or control trees. This result is unexpected based on the total pseudo-reactivity shown in Fig. 7c for O_3, but it can be explained by a small number of data points (four for each experimental period) and

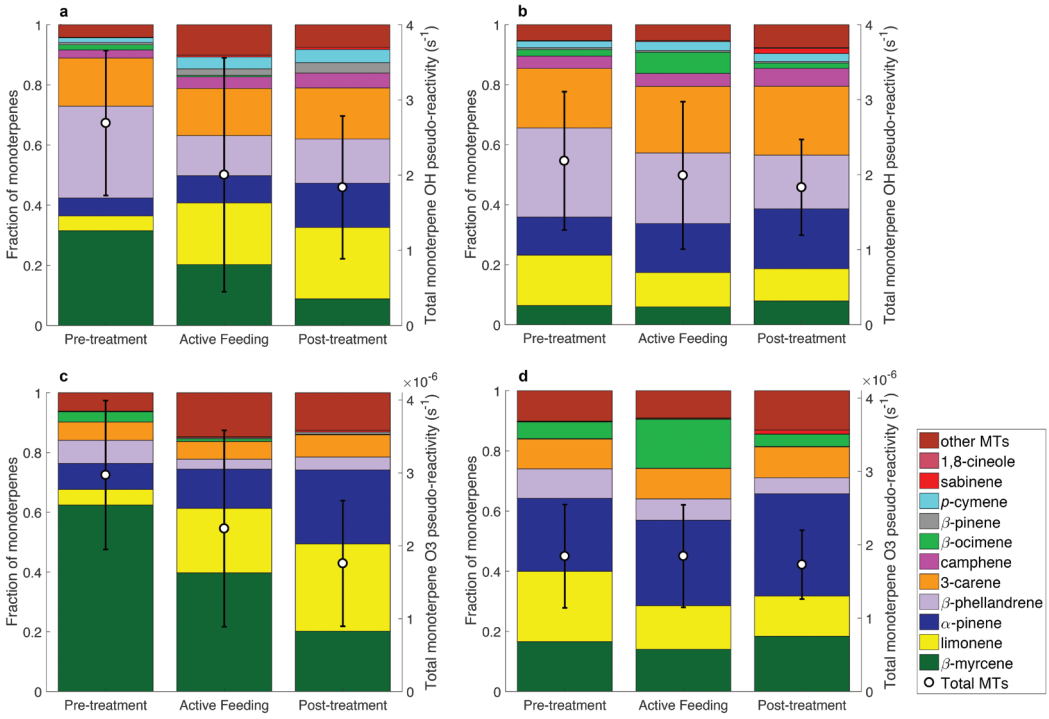

Fig. 7. Monoterpene (MT), OH and O_3 pseudo-reactivity of treatment trees for OH **a**) and for O_3 **c**); and of control trees for OH **b**) and for O_3 **d**). Results were averaged in all four experiments. Pseudo-reactivities were calculated by normalizing total monoterpene emissions obtained from GC-MS analysis to 1 ppbV. Error bars were estimated using a propagation of uncertainty.

the large difference between the total reactivities of individual experiments during the same experimental period (see error bars in Fig. 7).

Discussion

In this work, the major compound groups of Scots pine, above-ground volatile emissions associated with the pine weevil herbivory treatment were identified using the PTR-ToF-MS and EFA. With the PTR-ToF-MS, we observed that Scots pine saplings increased their emissions of several VOC groups as a response to the feeding of pine weevils, which includes monoterpenes, sesquiterpenes and oxygenated VOCs. These measured emissions well-represented the whole-plant emissions, because a previous study showed that bark-feeding of Scots pine saplings does not influence significantly upon the root system VOC emissions (Tiiva *et al*. 2018).

The PTR-ToF-MS has some limitations that make qualitative and quantitative data analysis complex. First, the PTR-ToF-MS with H_3O^+ ionization is not able to separate isomers from each other, so all compounds with the same formula are detected as a single mass peak. Second, even though the soft ionization technique is used in the PTR-ToF-MS, the high electric field used inside the drift tube may lead to some degree of fragmentation depending on the structure of the compound. This fragmentation leads to more complex mass spectrum measured with the PTR-ToF-MS. Moreover, the fragmentation prevents the determination of the concentration of the measured compounds using only the measured counts of the protonated molecule; provided that the PTR-ToF-MS is not calibrated against the specific compounds. Due to these limitations in PTR-ToF-MS data analysis, we identified the VOCs and groups of VOCs listed in Table 2 based on earlier studies, and GC-MS analysis of this study.

Most of these VOCs and groups of VOCs with an identical chemical formula were detected earlier during the field measurements of different pine forests (Kim *et al*. 2010, Rantala *et al*. 2015). Moreover, from the GC-MS analysis, we received a positive identification with some VOCs. This provides additional confidence in our chemical structure identification for most of the VOCs and groups of VOCs reported. The observation that herbivore damage increases the VOC emissions is in agreement with previous studies conducted with Mountain birch, Scots pine, Ponderosa pine, Lodgepole pine, and Norway spruce (Litvak and Monson 1998, Blande *et al*. 2009, Heijari *et al*. 2011, Amin *et al*. 2012, Ghimire *et al*. 2013, Joutsensaari *et al*. 2015, Ghimire *et al*. 2016, Yli-Pirilä *et al*. 2016, Ghimire *et al*. 2017). The most drastic effect was observed for monoterpenes — monoterpene emissions increased on average, monoterpene emissions increased 90-fold from the baseline emissions; and as much as 180-fold from the baseline emissions due to pine weevil feeding during experiment 3. Furthermore, some of the VOC groups identified as pine weevil feeding-related stress emission were also separated by the statistical analysis method, EFA to plant stress factor, which included potential MBO fragments, the main fragment of *p*-cymene, monoterpenes and oxygenated monoterpene derivatives. These were the VOC and VOC group emissions that showed the highest increases due to pine weevil feeding. Hence, even though EFA was not able to separate all VOCs and groups of VOCs that showed increased emissions due to pine weevil feeding into plant stress factor, EFA demonstrated to be a valuable tool when complex data sets produced by plants are analyzed — as it was able to separate the major VOC groups related to pine weevil treatment into its own factor.

Some of the VOC emissions that increased due to pine weevil feeding, such as monoterpenes, sesquiterpenes, possible MBO fragments, *p*-cymene, tentative nopinone and camphor. These emissions can undergo atmospheric chemical reactions resulting in the formation of ozone and SOA; with the latter pollutants affecting the climate (Joutsensaari *et al*. 2015, Faiola *et al*. 2018). The production of ozone, a phytotoxic gas, is known to have a negative effect on the vegetation-climate feedback by adversely affecting, for example, the forest growth carbon-sink strength of forests and plant-plant interactions (Ashmore 2005, Wittig *et al*. 2009, Blande *et al*. 2010). SOA is a major component of atmospheric aerosols, and it affects climate by influencing the size distribution, chemical composition, and radiative and cloud formation properties of the atmospheric particle population (Kanakidou *et al*. 2005, Hallquist *et al*. 2009). A recent report (Rap *et al*. 2018) suggested that SOA produced from biogenic VOCs will enhance global primary production via diffusion of solar radiation, which has a fertilization effect on plant growth. Their model simulations show that there is a strong positive ecosystem feedback between biogenic VOC emissions and plant productivity through plant-canopy light-use efficiency. Therefore, biotic stresses, such as insect outbreaks increasing forest scale BVOC emissions, may improve light-use efficiency. Moreover, because biogenic SOA can act as CCN (Poschl *et al*. 2010, Zhao *et al*. 2017), increased biogenic SOA leads to increased precipitation and shading of the ecosystem by clouds. Therefore, ecosystems are affected by increased amount of water received (precipitation), and decreased amount of solar light (shading). Due to the above-mentioned effects that secondary air pollutants have on climate and vegetation, it is important to have information about the VOC groups released as a response to herbivore feeding so that future models can take these into account when regional secondary air pollutant production is estimated.

The PTR-ToF-MS results from this study did not only show that pine weevil feeding significantly increased the pooled bark and foliage emissions of several VOC groups, but also that VOC group emissions remained elevated for several days after pine weevils had been removed. The results show that even if the active feeding period would not be long, pine weevils still have a significant cumulative effect on daily VOC emissions from Scots pines because emissions from 13 VOC groups remained elevated after the feeding had ended. The prolonged effect of pine weevil feeding on increased VOC group emissions was observed for each experiment. This is the first study investigating the prolonged emissions after the herbivore feeding has ended with

a high time resolution. Earlier studies have been conducted using off-line methods and having a significantly lower time resolution; hence, information on the detailed temporal evolution of emissions after exposure is missing (Copolovici *et al*. 2011, Ghimire *et al*. 2013, Yli-Pirilä *et al*. 2016). In previous studies where the PTR-MS was used, post-treatment emissions were not included in the experimental design.

Herbivore effects on monoterpene emissions from deciduous trees have previously been measured with a PTR-MS (Schaub *et al*. 2010, Ghirardo *et al*. 2012, Maja *et al*. 2014, Giacomuzzi *et al*. 2016). A 4-fold increase in monoterpene emissions from Pedunculate oak was observed due to *Tortrix viridana* feeding (Ghirardo *et al*. 2012); monoterpene emissions from Silver birch have been observed to increase 20-fold due to *Erannis defoliaria* feeding (Maja *et al*. 2014); A 22-fold increase in monoterpene emissions from Hybrid aspen was reported due to *Epirrita autumnata* feeding (Schaub *et al*. 2010); and a 93-fold increase of monoterpenes from apple trees due to *Pandemis heparana* feeding was observed (Giacomuzzi *et al*. 2016). These values are smaller than the highest increase we observed with Scots pines due to pine weevil feeding (180-fold in experiment 3). Major differences between our study and previous studies that could explain the observed discrepancies include the tree species (resin-storing vs. non-storing), herbivore species, type of herbivory (defoliator vs. bark borer) and the experimental set-up. Notwithstanding, all the previously-conducted experiments studied only part of the tree (branches or leaves) rather than placing the whole tree in a single enclosure as was done in this study. Furthermore, all the previous experiments that used a PTR-MS measured with a shorter duration (from less than 24 hours to 72 hours) than experiments in this study. Therefore, this study clarifies the overall effect of herbivores on VOC emissions from Scots pines in real-time. Our results can be used to estimate the total monoterpene emission increase in conifer forests due to herbivore outbreaks, which are predicted to increase in different types of forests due to global warming (Niemela *et al*. 2001, Bale *et al*. 2002, Logan *et al*. 2003, Kurz *et al*. 2008, Ammunet *et al*. 2012).

To put our results into context, it is a useful exercise to compare the results of our off-line GC-MS VOC analysis with similar studies using the GC-MS conducted with potted Scots pine and Norway spruce (*Picea abies*). In this study, the GC-MS monoterpene emissions (presented in Fig. 5) illustrated a 36-fold increase in total monoterpene emissions from the pre-treatment to the active feeding periods. Please note that this value is much lower than the 90-fold average maximum increase observed with the PTR-ToF-MS measurements, because GC-MS results are averaged over the whole active feeding periods to make it comparable with previous GC-MS studies. Our GC-MS results of more than a 36-fold increase in monoterpene emissions in response to pine weevil feeding is much higher than an earlier report from pine weevil-damaged Scots pine (18-fold increase) and Norway spruce (12-fold increase) in laboratory conditions (Joutsensaari *et al*. 2015). In outdoor conditions, only a 3.4-fold increase in monoterpene emissions from potted Scots pine was observed, which was due to pine weevil feeding (Heijari *et al*. 2011). Overall, the results of this study are consistent with the trends observed in previous studies — pine weevils significantly increase monoterpene emissions from conifer tree species, especially when the experiments are conducted under laboratory conditions. However, there were some discrepancies in the magnitude. This could be due to the nature of this type of off-line sampling and analysis technique, where sampling cartridges collect VOC emissions during a small sub-set of the entire stressed period, and do not necessarily represent the actual average emissions throughout the entire stress period. Thus, differences between measurements using this type of off-line technique could simply be due to the timing of sample collection. These results strongly suggest that we need more studies using real-time, continuous measurement approaches representing different conifer tree species and bark-feeding herbivores to better clarify the effect of herbivore feeding on plant VOC emissions. This would improve the accuracy of future models that predict the effect of herbivore outbreaks on climate and forest health.

As was mentioned above in the Results section, any specific "stress-induced" monoterpene

emission was not found from the GC-MS analysis. This indicates that the mechanism causing increased emissions was mainly the mechanical damage of stem tissue exposing stored terpene pools into the atmosphere. However, with the experimental setup of this study, the increase of monoterpene emissions due to synthesis by needles during the pine weevil feeding cannot be ruled out. It should be noted that if the elevated monoterpene emissions in response to the feeding were due to synthesis in the needles, the amounts of monoterpenes emitted by the needles would have been significantly smaller than the emissions from terpene pools of the stem (Heijari *et al*. 2011), as the emissions were linearly dependent on damaged surface area. Moreover, it should be noted that in extreme cases, a completely defoliated pine branch will emit more VOCs than a branch with full-grown needles, because herbivore-defoliated needle stumps release small resin droplets (Ghimire *et al*. 2013), which strongly supports the observation of Eller *et al*. (2013).

One of our objectives of this study was to characterize the dose-dependence of the maximum monoterpene emission rate after the treatment tree was exposed to pine weevils. Our analysis shows that there is a clear correlation between the maximum monoterpene emission rate and the damaged bark area. This is an important finding and reported for the first time for Scots pine saplings and bark-feeding pine weevils. Therefore, based on the observed correlation, we suggest that this result could be used as the first approximation for models to estimate the increase in magnitude of monoterpene emissions from conifer forests due to stem damage caused by bark-feeding herbivores. In the future, field studies should take into account the damaged bark area of trees that will be compared with the measured emission rates of monoterpenes. Studying the relationship between the biotic stress severity and increased VOC emissions is highly important (Niinemets *et al*. 2013). Without knowing the plant response patterns against the stress dose, plant stress responses in the field under fluctuating stress levels cannot be predicted and the lack of this information prevents the quantitative model development of plant stress response (Niinemets *et al*. 2013). Emissions of different groups of compounds, such as LOX, monoterpenes and sesquiterpenes from deciduous trees, have been related to the strength of herbivore damage (Niinemets *et al*. 2013, Copolovici *et al*. 2017). Moreover, total VOCs emitted by Scots pine have been related to pine sawfly feeding activity (Ghimire *et al*. 2017). The results of our study, like previous studies, have shown clear dose-dependent responses, but more experimental work is required from this topic to better quantify the temporal evolution of these responses caused by various biotic stresses and to separate the contributions of local and systemic-induced emissions (Niinemets *et al*. 2013).

One of the objectives in this study was to assess the relevance of changing emission profiles for atmospheric reactivity. Based on our results and statistical analysis, we can conclude that even though the pseudo-reactivity profiles of monoterpenes changed between experimental periods, there were no statistical differences in the total pseudo-reactivities of monoterpenes between the experimental periods. There may be two reasons: first, the reactivities of different monoterpenes were too close to each other to create statistically significant difference between the experimental periods: and second, our sample size was simply too small and the variability between samples too large to evaluate statistical significance. Therefore, only the drastic change in monoterpene BERs caused by pine weevil feeding increases the atmospheric reactivity of monoterpenes. The result that total pseudo-reactivities did not significantly change due to herbivore feeding is an interesting result, particularly when it is compared with results from a previous study where the effect of a different type of stress on coniferous plant emissions was studied (Faiola *et al*. 2015). The previous study demonstrated that treating the coniferous plants with methyl jasmonate substantially changed the total OH and O_3 pseudo-reactivities of monoterpenes. Such difference between the studies indicates that different types of stresses will have very different effects on the atmospheric reactivity of the plant emission profile.

In this study, we have provided the longest PTR-ToF-MS experiments related to VOCs and groups of VOCs emitted by herbivore-damaged

trees reported in literature. In addition, we have been able to show four times the temporal emission dynamics of the single Scots pine before, during and after pine weevil treatment. Our results demonstrated that monoterpene emissions can be elevated substantially and they will stay elevated after the pine weevils have finished feeding. However, because this study was performed as a longitudinal study where the focus was on the exploration of the temporal dynamics of plant VOCs, this study lacks true replicates. In Appendix 4, we present the results of the statistical analyses for which the trees from temporally separate experiments were used as pseudo-replicates. The results show additional evidence that pine weevil feeding significantly increases the mean monoterpene BER treatment tree, which remains elevated after pine weevils have finished feeding. Due to the lack of true replicates, there is a need for future studies to understand the response of several trees measured concurrently to get a more general picture of the emission dynamics of Scots pine saplings before, during and after pine weevil feeding. However, this kind of study with several true replicates requires several PTR-MS instruments, otherwise the data time resolution suffers; leading to an incomplete study of the temporal dynamics of plant VOCs.

Conclusions

This study demonstrated the advantage to have a PTR-ToF-MS included for experiments where the temporal evolution of different plant VOCs are expected. In this study, we provided the longest PTR-ToF-MS experiments related to VOC groups emitted by herbivore-damaged trees reported in literature (8–16 days). We also provided an in-depth investigation of the VOC emission profile and its changes during and after the 48-hour exposure with a high time resolution. We identified more than 10 VOCs or groups of VOCs that were associated with the pine weevil herbivory treatment. These VOC groups included monoterpenes, sesquiterpenes and oxygenated VOCs, such as monoterpene derivatives. Moreover, in this study, we showed that a dose-dependence at the maximum monoterpene emission rate exists. This result could possibly be used as a first approximation for models, when the increased monoterpene emission rates due to bark-feeding herbivore outbreaks are estimated. Furthermore, this study was the first to show the prolonged effect herbivores have on plant VOC emissions in real-time. VOC group emissions that were increased due to pine weevil feeding remained elevated for several days after pine weevils were removed. Overall, the results of this study showed that increased herbivory outbreaks in conifer forests increase the emissions of several VOC groups that will go through chemical reactions in the atmosphere, which will ultimately affect the climate. However, more research is needed with different conifer tree species and herbivores using real-time, continuous measurement approaches with true replicates to better clarify the responses of conifer trees from herbivore damage.

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Appendix 1

The PTR-ToF-MS is made up of four main parts: (1) a hollow cathode discharge ion source that produces H_3O^+ ions from water vapor; (2) a drift tube reaction chamber, where the sample VOCs are introduced and ionized by proton-transfer reactions with H_3O^+ ions; (3) a transfer lens system that guides the ions into the mass spectrometer; (4) a reflectron time-of-flight mass spectrometer, where the ionized compounds are separated with high mass resolution based on their mass-to-charge (m/z) ratios. After the separation, the ions are detected by a multi-channel plate (MCP) detector. With the PTR-MS, H_3O^+ ions are used as a reagent gas, such that all VOCs possessing higher proton affinity than water are ionized by a proton-transfer reaction. Many trace VOCs in the atmosphere have a higher proton affinity than water and can therefore be measured with the PTR-MS.

Appendix 2

We describe here how the PTR-ToF-MS was calibrated against monoterpenes emitted by Scots pine saplings. During the calibration, the calibration factor of monoterpenes was determined using a dynamic dilution system with Eq. A1) (Faiola *et al*. 2012, Kari *et al*. 2018a):

$$C_f = \sum (C_i \times f_i) , \quad \text{(A1)}$$

where the final monoterpene calibration factor (C_f) of the complex mixture is calculated from the sum of the individual calibration factors of the monoterpene components (C_i) multiplied by their relative fraction in the mixture (f_i). C_i values were determined for the protonated molecule of monoterpenes during the calibrations of pure standards. The fraction of an individual monoterpene in the mixture was determined with adsorbent cartridge sampling analyzed with thermo-desorption gas chromatography mass spectrometry (TD-GC-MS). The calibration factor for monoterpenes was calculated as a sum of the weighted averages of individual calibration factors of the major monoterpenes in the plant enclosures. The major monoterpenes were α-pinene, camphene, β-myrcene, β-pinene, 3-carene, δ-limonene and β-phellandrene. All these compounds except camphene and β-phellandrene were calibrated with pure standard compounds. For camphene and β-phellandrene, proxy calibration factors of β-pinene and limonene were used, respectively; because of structural similarity.

The calibration-corrected concentration of monoterpene mixture emitted by the trees was then calculated using Eq. A2):

$$C_{MTF} = C_f \times C_{MT} , \quad \text{(A2)}$$

where C_f is the final monoterpene calibration factor and C_{MT} is the concentration of the protonated molecule of monoterpenes measured by the PTR-ToF-MS at m/z 137.133.

Appendix 3

The factor analysis technique used in this study (in addition to PCA) was Exploratory Factor Analysis (EFA), which attempts to uncover the complex underlying patterns from the data (e.g., common factors) (Rencher and Christensen 2012). EFA takes advantage of the correlations between the original variables as it creates the factor model. The factors are generated to explain the correlation between the measured variables. Rotations to the factors can be performed to enhance the interpretation.

The EFA factors can be extracted using different methods. In this study, the maximum likelihood factor analysis (hereafter ml-EFA) was used in the final analysis. The ml-EFA assumes that all errors

are sampling errors and the estimation itself is derived from the normal distribution theory. The function to be minimized in ml-EFA can be approximated with Eq. A3):

$$F = \sum_i \sum_j \frac{(s_{ij} - \sigma_{ij})^2}{u_i^2 u_j^2}\ , \tag{A3}$$

where u_i and u_j are the variances for the variables i and j (de Winter and Dodou 2012, Rencher and Christensen 2012).

In addition, orthogonal varimax rotation was used to clarify the interpretation of the factors. Varimax rotation tries to maximize the variance of the squared loading values. In other words, varimax rotation attempts to increase the large loading values and decrease the small values; thus, it clarifies the classification of the variables to the different factors (Rencher and Christensen 2012). Compared to oblique rotations, the formed orthogonal basis created by ml-EFA is preserved when varimax rotation is used to keep the components orthogonal and the factors are not allowed to correlate (Harman 1976).

Appendix 4

Table A1 and Figure A1 show mean monoterpene basal emission rates (BERs) from all four experiments using each experiment as an independent replicate. Before the feeding period, BER was at the same level in both seedlings, but the BER became 26–65-fold higher during the insect feeding period and 20-fold higher during the post-treatment period. This was observed for seedlings used for the treatment compared with control seedlings. Large uncertainties exist in mean BERs, which is expected because the four experiments conducted during this study were not true replicates, but temporal pseudo-replicates (Hulbert 1984, see also Oksanen 2001), because the experiments were performed sequentially as a longitudinal study and there are known seasonal influences on plant emission rates and on plant-shoot and plant-needle development (Bäck *et al*. 2005, Vanhatalo *et al*. 2018). In addition, the damaged area in the experiments varied depending on the pine weevil feeding intensity/activity (see Fig. 6) that affects monoterpene BER during the treatment period. Pine weevil feeding increased significantly the mean monoterpene BER (Table A1 and Fig. A1), and the Wilcoxon rank sum test for correlated samples verified ($p = 0.0003$) that the mean monoterpene BER remained higher during the post-treatment period compared with mean pre-treatment BER. The Mann-Whitney U test

Table A1. Mean monoterpene BERs ($n = 4$, ± S.D.) during different experimental periods.

Experimental period[1]	Treatment tree BER (μg h^{-1} m^{-2}, 303K)[2]	Control tree BER (μg h^{-1} m^{-2}, 303K)[2]
Pre-treatment	32 ± 24	29 ± 30
Treatment period 1	1060 ± 339	41 ± 24
Treatment period 2	2030 ± 444	31 ± 19
Post-treatment 1	548 ± 276	29 ± 16
Post-treatment 2	504 ± 319	24 ± 19

[1]Pre-treatment period corresponds to the time just before the start of the treatment period. Treatment period 1 and 2 correspond to times after the treatment period have lasted one day and at the end of the treatment period, respectively. Post-Treatment period 1 and 2 correspond to times after the post-treatment period have lasted one day and two days, respectively.

[2]BERs are calculated as 3-hour means of all four experiments for different periods of the experiments. Stated uncertainties represent one standard deviation of the mean BER.

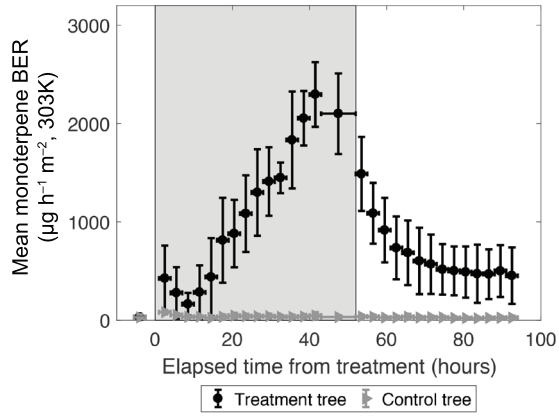

Fig. A1. The effect of pine weevils on mean monoterpene BER. Mean monoterpene BERs are calculated as 3-hour means of all four experiments. The exception is the last data point at the end of the treatment period, which is longer due to uneven lengths of the treatment periods during this study (cf. Table 1 for the lengths of the treatment periods). Stated uncertainties represent one standard deviation of the mean BER. The grey area marks the treatment period. Pre-treatment and post-treatment periods took place before and after the treatment period, respectively. The important information about temporal dynamics was lost due to averaging; compared with the high time resolution data shown in Fig. 3.

verified that there were no significant difference ($p > 0.05$) between treatment tree mean BER and control tree mean BER during the pre-treatment period, but after the pine weevil treatment, a significant difference existed during the treatment ($p = 0$) and post-treatment ($p = 0$) periods.