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Original Article

Herbivore-induced volatile emission in black poplar: regulation and role in attracting herbivore enemies

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ABSTRACT

After herbivory, plants release volatile organic compounds from damaged foliage as well as from nearby undamaged leaves that attract herbivore enemies. Little is known about what controls the volatile emission differences between damaged and undamaged tissues and how these affect the orientation of herbivore enemies. We investigated volatile emission from damaged and adjacent undamaged foliage of black poplar (*Populus nigra*) after herbivory by gypsy moth (*Lymantria dispar*) caterpillars and determined the compounds mediating the attraction of the gypsy moth parasitoid *Glyptapanteles liparidis* (Braconidae). Female parasitoids were more attracted to gypsy moth-damaged leaves than to adjacent non-damaged leaves. The most characteristic volatiles of damaged versus neighbouring undamaged leaves included terpenes, green leaf volatiles and nitrogen-containing compounds, such as aldoximes and nitriles. Electrophysiological recordings and olfactometer bioassays demonstrated the importance of nitrogenous volatiles. Under field conditions, parasitic Hymenoptera were more attracted to traps baited with these substances than most other compounds. The differences in volatile emission profiles between damaged and undamaged foliage appear to be regulated by jasmonate signalling and the local activation of volatile biosynthesis. We conclude that characteristic volatiles from damaged black poplar foliage are essential cues enabling parasitoids to find their hosts.

Key-words: *Populus nigra*; Braconidae; cytochrome P450; *Glyptapanteles liparidis*; herbivore-induced volatiles; indirect defence; *Lymantria dispar*; phytohormones; Salicaceae; terpene synthase.

INTRODUCTION

Plants under attack by insect herbivores release a blend of volatile compounds into the surrounding air. This phenomenon has been documented for numerous plant species in the past two decades and may be a nearly universal feature in vascular plants (Mumm & Dicke 2010). Volatile release occurs not only from damaged tissue but also from adjacent undamaged plant parts (Dicke *et al*. 1990; Heil & Ton 2008; Hiltpold *et al*. 2011). Typically, there are significant differences between damaged and undamaged organs in the spectrum of compounds released and their rate of emission (Turlings & Tumlinson 1992; Rose *et al*. 1996), creating a complex pattern of volatile plumes in the air surrounding herbivore-attacked plants (recently reviewed by Beyaert & Hilker 2014).

The importance of herbivore-induced volatile compounds has been investigated with respect to several possible functions in plants, including direct defence against herbivores (Unsicker *et al*. 2009), attraction of herbivore enemies, within plant signalling (Heil & Ton 2008) and communication between plants (Karban *et al*. 2013). The attraction of herbivore enemies has been the main concern of most researchers in this field, and numerous species of herbivore predators and parasitoids, including insects, mites, nematodes and birds, have been demonstrated to be attracted by herbivore-induced volatiles (Turlings & Wackers 2004; Mäntylä *et al*. 2008; Mumm & Dicke 2010).

Plant volatiles are considered reliable and readily detectible cues for indicating the presence of herbivores (Vet & Dicke 1992). However, the actual components responsible for herbivore enemy attraction have only rarely been identified (Kessler & Baldwin 2001; Kappers *et al*. 2005; Schnee *et al*. 2006). The complexity of herbivore-induced volatile blends makes it difficult to determine which components are most important for herbivore enemy attraction. Certain volatiles are abundant and widespread in plant species, such as the monoterpenes linalool and (*E*)-*β*-ocimene, the sesquiterpenes (*E*)-*β*-caryophyllene and (*E*,*E*)-*α*-farnesene, the green leaf volatiles (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenal, and the aromatic compound indole. Various terpenoids and green leaf volatiles are also emitted in minor amounts as are many aromatic and nitrogen-containing compounds. Although rarely studied, the less abundant volatiles may also be active as cues for herbivore enemies (D'Alessandro & Turlings 2005) given the high sensitivity of the insect olfactory system (Hansson *et al*. 1999; Angioy *et al*. 2003). Minor constituents could also contribute to the

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specificity of the volatile blend, providing detailed information to herbivore enemies with respect to the identities of the plant and the attacker as well as the age of the herbivore and its abundance (Dicke 1999; Clavijo McCormick *et al*. 2012 and references therein). Another dimension of complexity arises due to emission from both damaged plant parts and adjacent undamaged parts. Emission from undamaged organs has been interpreted as a way for plants to supplement production from damaged organs in order to create an amplified volatile plume, increasing the attractiveness to herbivore enemies (Vet & Dicke 1992). However, at close range, enemies need local cues that direct them to the exact location of their prey. To date, it is not known how specific plant volatiles contribute to local attraction against the background of volatiles from undamaged parts.

Knowledge of how volatile emission is regulated should help understand what causes the differences in emission profiles between damaged and undamaged foliage of individual plants. At a basic level, volatiles released after herbivore damage are usually products of *de novo* biosynthesis initiated after the onset of herbivore attack (Pare & Tumlinson 1997; Kollner *et al*. 2004), but it is unclear if *de novo* biosynthesis also occurs in connection with volatile release from undamaged leaves. Much information is available on the pathways involved in volatile biosynthesis (Dudareva *et al*. 2004). For example, in the formation of volatile terpenoids, including the monoterpenes, the sesquiterpenes and the homoterpenes, a key step is the conversion of ubiquitous diphosphate intermediates of terpenoid metabolism, geranyl diphosphate (GPP) or farnesyl diphosphate (FPP), to volatile olefins or alcohols via the action of terpene synthases (Degenhardt *et al*. 2009). Terpene synthases are often followed in volatile biosynthetic pathways by cytochrome P450 monooxygenases, leading to further oxidized products (Lee *et al*. 2010). Less is known about the herbivory-triggered pathways that activate biosynthesis of volatiles, but the role of jasmonic acid (JA) in inducing volatile formation has been well described (Dicke *et al*. 2009).

The vast majority of studies on herbivore-induced volatiles have been carried out with a limited number of genotypes of cultivated, mostly herbaceous species (Mumm & Dicke 2010 and references therein). Experiments on woody, non-cultivated species are rarer (e.g. Staudt & Lhoutellier 2007; Brilli *et al*. 2009; Rodriguez-Saona *et al*. 2009; Copolovici *et al*. 2011). Here, we characterized the volatile emission of different *Populus nigra* (black poplar) genotypes induced by *Lymantria dispar* (gypsy moth) herbivory to determine compounds released from damaged and neighbouring undamaged leaves. We investigated the behavioural response of the gypsy moth caterpillar parasitoid, *Glyptapanteles liparidis*, to different poplar leaf treatments and then tested individual volatiles in electrophysiological and behavioural studies in the laboratory as well as under field conditions to identify the compounds involved in herbivore enemy attraction. Finally, we explored some of the factors regulating volatile emission in damaged versus undamaged black poplar leaves such as phytohormones and gene transcription patterns.

MATERIALS AND METHODS

Plants and insects

P. nigra trees were propagated from monoclonal stem cuttings derived in 2010 from branches of 20 old-growth trees (30–60 years old) from a natural population growing in a floodplain forest along the Oder river in northeastern Germany (52°34′1″N, 14°38′3″E). The trees were grown in the greenhouse under summer conditions (24 °C, 60% relative humidity, 16 h/8 h light cycle) in a 1:1 mixture of sand and soil.

L. dispar caterpillars were hatched from egg clutches [kindly provided by the Animal and Plant Health Inspection Service (APHIS) from the United States Department of Agriculture (USDA)] and reared on artificial gypsy moth diet (MP Biomedicals LLC, Illkirch, France) until they were transferred to poplar leaves two days before the onset of the experiments. Fourth and fifth instar caterpillars were used for the experiments.

G. liparidis wasps were obtained from parasitized *L. dispar* larvae, kindly provided by Dr. Axel Schopf and Andrea Stradner [University of Natural Resources and Applied Life Sciences of Vienna (BOKU), Austria]. The wasps were fed with 10% honey solution and water dispensed in pieces of foam, and maintained in a climate chamber at 20 °C, 60% relative humidity and 16/8 h photoperiod. Emerging females were divided into two groups. One 'naïve' group had no previous experience with black poplar odour or potential hosts, whereas the second 'experienced' group was allowed to parasitize early instar *L. dispar* larvae, feeding on poplar leaves. Male parasitoids were not previously exposed to poplar odours.

Volatile collection and analysis

To investigate the local and systemic emission of volatiles from *P. nigra* after *L. dispar* feeding, two individual trees (approximately 1.20 m in height and 1 year old) of each of 20 different genotypes were selected (40 trees altogether). Twenty trees were then infested with gypsy moth caterpillars and the other 20 functioned as controls. Thus, each treatment contained 20 tree genotypes as replicates. During the experiment, trees were kept in a climate chamber (humidity: 60%, day/night temperature: 20 °C/16 °C; 16 h light). The experiment was performed approximately 2 months after leaves started flushing. The young foliage of each tree was divided into two sections, basal and apical, based upon the position in the tree. Each section had 20–30 leaves and was enclosed with polyethylene terephthalate (PET) foil (Toppits® Bratschlauch, Minden, Germany) (Fig. 1a). Seven fourth instar *L. dispar* caterpillars were released in the PET bags covering the basal section and allowed to feed for 41 h before volatile collection started. Caterpillars were allowed to remain on the trees during volatile collection to avoid possible mechanical damage to the tree caused by bag removal. As a control, we also collected volatiles released from the insects themselves (and their frass) for 4 h right after these were removed from the trees (Supporting Information

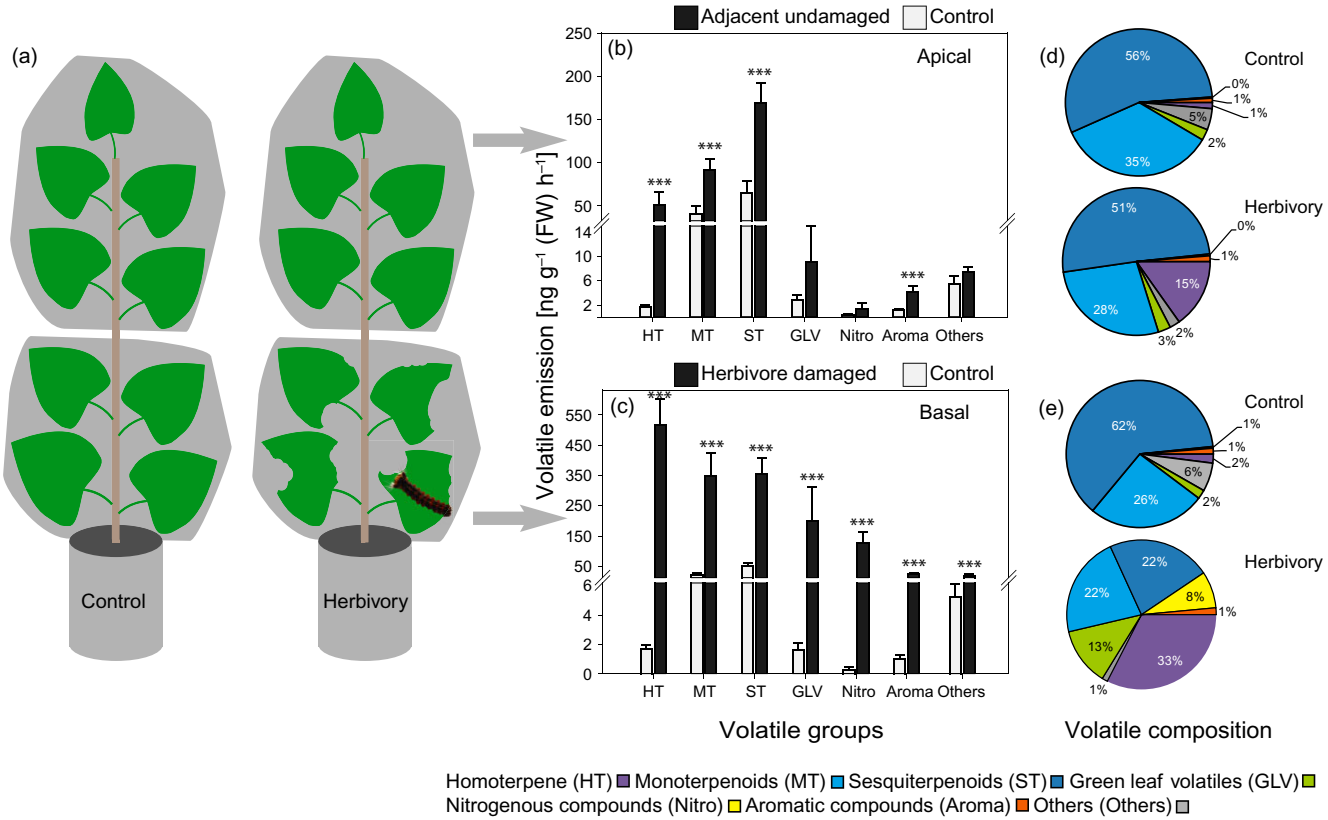

Figure 1. Emission of volatile compounds from herbivore-damaged and adjacent undamaged leaves of *Populus nigra* (black poplar) trees infested with *Lymantria dispar* (gypsy moth) caterpillars. (a) The foliage of young trees established from cuttings of 20 different genotypes of old-growth *P. nigra* was divided into basal and apical sections with polyethylene terephthalate (PET) foil and seven 4th-instar caterpillars were released into the basal section. A second set of PET foil-divided trees was left as controls without caterpillars. After 41 h of caterpillar feeding, volatiles were collected using a dynamic headspace collection system and analysed by GC-MS and GC-FID. (b, c) Emission of major groups of volatiles was recorded from herbivore-damaged and adjacent undamaged foliage in relation to control emission from corresponding regions of trees not subject to *L. dispar* herbivory. Data are presented as mean ± SE, $n = 20$ (one representative of each of 20 genotypes). Asterisks indicate significant differences between herbivore-infested trees and the controls: $*P < 0.05$; $***P < 0.001$; Mann–Whitney *U*-tests. (d, e) Relative proportion of the major groups of volatiles presented with respect to the full *P. nigra* odour blend.

Table S1 shows the results). All measurements were performed in May 2011. Herbivory and following volatile collections were both carried out in a climate chamber (humidity: 60%, day/night temperature: 20 °C/16 °C; 16 h light). As the 40 plants could not be treated at the same time, the experiment was split up into four blocks, each block containing trees from each treatment.

Volatiles were collected using a dynamic push-pull system. Charcoal-filtered air was pumped into the PET bags at a flow rate of 1.5 L min^{-1}. At the same time, a portion of the air was pumped out of the bags with a second pump at a flow of 1 L min^{-1}. The outgoing air passed through a trap packed with 20 mg Super Q adsorbent (ARS, Inc., Gainesville, FL, USA) to retain the volatile compounds. Preliminary experiments detected no breakthrough of poplar volatiles from the traps under these conditions. Volatiles were collected for 4 h the second day after herbivore release on the plants. Volatile compounds were desorbed by eluting the filter twice with 200 μL of dichloromethane containing nonyl acetate as an internal standard (10 ng μL^{-1}).

Analysis of *P. nigra* volatiles was carried out using gas chromatography–mass spectrometry/flame ionization detector (GC-MS/FID) analysis (as described in detail in the Supporting Information).

Olfactometer bioassays for parasitic wasps

To test the preference of *G. liparidis* towards poplar volatile blends, a two-choice arena was employed consisting of a 21 × 27 × 17 cm plastic box, with two holes at the top and cylindrical traps 10.5 × 4.5 cm (L × ID) with entrance funnels attached to it. The headspace of the appropriate plant treatment enclosed in a PET bag was pumped into the arena through Teflon tubing at 0.5 L min^{-1}. Treatments were offered pairwise as follows: control versus *L. dispar* damage, mechanical damage versus *L. dispar* damage, and *L. dispar* caterpillars plus feces (in a PET bag) versus clean air.

The behaviour of naïve *G. liparidis* males and naïve and experienced females towards odours of damaged leaves, adjacent undamaged leaves and individual volatile

compounds was tested in a four-field arena (Supporting Information Fig. S1). The arena was located inside a white box ($40 \times 25 \times 35$ cm) equipped with light-emitting diode (LED) lights (LED light flex 15 high performance; Joseph Barthel GmbH & Co., Nürnberg, Germany, under the arenas, and white LED A4 light foil, Zigan Displays, Apelnstedt/Sickte, Germany, on top of the arena) supplying homogeneous light conditions for video recording (digital camera C510/C310, Logitech, Munich, Germany). For comparing parasitoid behaviour towards volatiles released from damaged versus adjacent undamaged leaves, trees were prepared as described earlier. Air was pumped into the headspace at 1 L min^{-1} and into the olfactometer at a rate of 0.2 L min^{-1}. At the center of the arena, air was pumped away constantly at 0.8 L min^{-1}. Air from the headspace of damaged leaves was pumped into three fields of the arena, whereas air from the adjacent undamaged leaves of the same tree was pumped into the remaining field. Parasitoid behaviour was recorded for 5 min and the videos were analysed using EthoVision XT® (Noldus Information Technology, Wageningen, the Netherlands). For tests with individual compounds, substances were dissolved in dichloromethane (10 $\mu g\ \mu L^{-1}$) and individually dispensed (10 μL) onto filter paper disks. These were placed inside a 100 mL flask with a screw cap top (Schott-Duran, Wertheim/Main, Germany). Air pumped at 0.2 L min^{-1} through the Teflon tubing inserted into the cap entrained the tested volatile into one field of the olfactometer. The other three fields of the arena released air previously pumped through a flask containing filter paper with the solvent only. Behaviour was recorded and analysed as described earlier.

Electrophysiological recordings from parasitic wasps

Twelve compounds were tested for electrophysiological responses with parasitoid antennae. Supporting Information Table S2 shows the source of the compounds, degree of purity and vapour pressure values. 2-Methylbutyraldoxime and 3-methylbutyraldoxime were synthesized by condensation of the respective aldehyde with hydroxylamine (Schwetlick 2009) as recently described in Irmisch *et al.* (2013, a detailed description of the aldoxime synthesis can be found in the Supporting Information). Antennae from naïve female *G. liparidis* were excised, positioned between two electrodes using Spectra 360 electrode gel (Parker, Fairfield, NJ, USA) connected to an EAG PRG-2 probe (Syntech, Kirchzarten, Germany). Antennal signals were digitized by an IDAC-4 acquisition controller (Syntech), recorded on a personal computer (PC) and evaluated using the software EAGPro version 1.1 (Syntech). The antennae were constantly flushed with charcoal-filtered and humidified air at a rate of 200 mL min^{-1}. All compounds tested were diluted in dichloromethane (100, 10, 1, 0.1 and 0.01 $\mu g\ \mu L^{-1}$), and 10 μL of each dilution pipetted on filter paper disks (1 cm diameter) placed in clean Pasteur pipettes. Dichloromethane was used as the solvent control. (*E*)-2-Hexenal at a concentration of 10 $\mu g\ \mu L^{-1}$ was used as the reference stimulus as preliminary tests showed that there was a constant response of parasitoid antennae at this concentration. Parasitoid antennae were exposed to each compound by injecting air from the odour laden Pasteur pipette into the main flow of clean humidified air. Each puff had 0.5 s duration at a flow rate of 0.2 L min^{-1} (Stimulus controller unit, Syntech). Recordings started 2 s before stimulation and lasted for 10 s. Antennae were given a recovery period of 60 s minimum between stimulations and trials lasted up to 15 min. To determine physiological activity, five randomly selected compounds per antenna were used at a dose of 100 μg. Stimulations were performed in random order until 10 replicates were achieved for each compound. Dose-dependent responses were established at 0.1, 1, 10, 100 and 1000 μg starting at the lowest dose. Experiments were repeated once per compound and antenna on a total of five antennae per compound. The solvent control, a clean paper filter and the reference compound were presented at the beginning and at the end of each trial. Responses (μV) to the individual volatiles were normalized to the reference stimulus after subtraction of responses elicited by the solvent control.

Hymenopteran parasitoid attraction to single volatiles in the field

Parasitoid attraction to nine individual poplar volatiles and a solvent control (hexane) was tested in the lower canopy of four old-growth trees within the black poplar population described earlier. The choice of compounds for this experiment was based upon the results from the electrophysiological and behavioural experiments with parasitoids conducted in the laboratory, as well as on the results from the random forest analysis of volatiles released from 20 tree genotypes. Vials (2 mL) with 10 μL glass micropipette pierced through the screw tops were used as dispensers containing compounds dissolved in hexane at a concentration of 10 $\mu g\ \mu L^{-1}$ (release rates for the dispensers are shown in Supporting Information Fig. S2). Each dispenser was located at the bottom of a rectangular yellow sticky trap (10×15 cm) which was pasted vertically onto a 'hat trap' (KLP+; C. Salomon, Budapest, Hungary, Supporting Information Fig. S3), confining insects that moved upwards from the dispenser. In each of the four trees, three replicates per compound and solvent control were installed, resulting in a total of 120 traps. Traps were randomly distributed in the lower crown spaced a minimum of 1 m apart from each other. After 5 days, the yellow sticky traps were collected, and the captured insects separated by order. For the Hymenoptera, specimens of the superfamily Ichneumonoidea were further classified into the subfamilies Braconidae and Ichneumonidae.

Defence hormone analysis

For analysis, 20 mg of finely ground lyophilized leaf material was extracted with methanol containing 40 ng of 9,10-D2-9,10-dihydrojasmonic acid, D4-salicylic acid (Sigma-Aldrich, St. Louis, MO, USA), 8 ng of jasmonic acid-$^{13}C_6$-isoleucine conjugate and 40 ng of D6-abscisic acid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as internal standards.

The extracts were analysed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source. Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent) with a gradient of formic acid (0.05%) in water and acetonitrile. The mass spectrometer was operated in negative ionization mode in multiple reactions monitoring (MRM) mode. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Hormones were quantified relative to the signal of their corresponding internal standard.

Effect of hormones on volatile emission

Single similar-sized *P. nigra* leaves from 2-year-old monoclonal individuals were detached from the petiole and immediately transferred to aqueous solutions of 200 μM JA, 200 μM salicylic acid (SA) or a water control. Half of the leaves of the JA and control treatments were mechanically wounded with a razor blade, making five cuts parallel to the mid-vein 18 h before and once again prior to the beginning of the experiment. All leaves (5 replicates per treatment) were incubated overnight. The next day (18 h after the start of treatment), volatiles were collected from individual leaves by wrapping a PET bag around the leaf and the container holding the solution. A dynamic push-pull system was used for volatile collection as described earlier with an incoming flow rate of 1 L min^{-1} and an exiting flow rate of 0.6 L min^{-1}. Volatiles were collected over 6 h with 20 mg Super-Q traps and elution and volatile analysis were carried out as described earlier.

Isolation of genes of volatile biosynthesis

A set of 14 1-year-old monoclonal *P. nigra* trees (genotype GTK2) were selected and 7 were subjected to herbivory with *L. dispar* caterpillars for 24 h. Thereafter, volatiles were measured in the seven herbivore-damaged and seven non-damaged control trees. The experiment was performed in the same way as described earlier. Leaf material was collected and immediately frozen in liquid nitrogen. Total RNA was isolated from leaf powder using the Invisorb Spin Plant RNA Mini Kit (Invitek GmbH, Berlin, Germany) according to manufacturer's instructions. RNA concentration, purity and quality were accessed using a spectrophotometer (Nano Drop 2000c, Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany). Prior to cDNA synthesis, 0.75 μg RNA was DNase-treated using 1 μL DNase (Fermentas GmbH, St. Leon Roth, Germany). Single-stranded cDNA was prepared from the DNase-treated RNA using Super Script TM III reverse transcriptase and oligo d (T12–18) primers (Invitrogen, Carlsbad, CA, USA).

The complete open reading frames of two putative terpene synthase genes were amplified from cDNA made from herbivore-damaged *P. nigra* leaves using the primers listed in the Supporting Information Table S3. The genes were inserted as a *Bsa*I fragment (*PnTPS1*) and a blunt-end fragment (*PnTPS2*) into the expression vector pASK-IBA7-plus (IBA GmbH, Göttingen, Germany) and pET100/D-TOPO (Invitrogen), respectively, and fully sequenced. Sequences were deposited in GenBank with the accession numbers BankIt1702420-KJ490918 (*Pntps1*) and BankIt1702420-KJ490919 (*Pntps2*). The two amplicons contained open reading frames of 1782 and 1587 nucleotides, respectively. Their encoded proteins possessed all typical features of terpenes synthases, such as the highly conserved DDxxD motif and the NSE/DTE motif both known to be involved in metal co-factor binding (Supporting Information Fig. S4). Due to a predicted N-terminal signal peptide (ChloroP), a truncated version of PnTPS1 (Δ39 aa) was used for expression. The terpene synthases were expressed in *Escherichia coli* and purified following the procedure described in Danner *et al.* (2011). To determine their catalytic activity, enzyme assays containing 50 μL of the bacterial extract and 50 μL assay buffer [10 mM Tris–HCl (pH 7.0), 1 mM dithiothreitol, 10% (v/v) glycerol] with 10 μM substrate [GPP or (*E*,*E*)-FPP] and 10 mM $MgCl_2$ in a Teflon-sealed, screw-capped 1 mL GC glass vial were performed. Products were trapped with an SPME (solid phase microextraction) fibre consisting of 100 μm polydimethylsiloxane (Supelco, Bellefonte, PA, USA) placed in the headspace of the vial that was incubated at 30 °C for 1 h. GC/MS analysis and product identification was performed as described earlier.

qRT-PCR

Gene-specific primers for *PnTPS1* and *PnTPS2* were designed to give a predicted melting temperature of about 60 °C, a primer length in the range of 20–25 nucleotides (nt) and an amplicon length between 100 and 150 base pair (bp). For expression analysis of the *P. nigra CYP79D6* and *D7*, we used a primer pair designed for the amplification of both *CYP79D6v3* and *CYP79D7v2* from Populus *trichocarpa* (Supporting Information Table S3; Irmisch *et al.* 2013). Primer specificity was confirmed by agarose gel electrophoresis, melting curve analysis, and standard curve analysis and sequence verification of cloned PCR amplicons (see Supporting Information Table S3 for primer information). Specific primers for *ubiquitin* were used as reference genes (Ramirez-Carvajal *et al.* 2008). The following PCR conditions were applied for all reactions: Initial incubation at 95 °C for 3 min followed by 40 cycles of amplification (95 °C for 20 s, 60 °C for 20 s). Reactions were measured during the annealing and the extension step of each cycle. Data for the melting curves were gathered at the end of the 40 cycles from 55 to 95 °C. Samples were run in triplicate using Brilliant® III SYBR® Green QPCR Master Mix (Stratagene, Agilent) with ROX as reference dye. Reactions were set up according to manufacturer's instructions. Each cDNA was diluted 1:3 and 1 μL of the diluted cDNA was used in qRT-PCR reactions. All samples were run on the same PCR machine (MxPro – Mx3000P, Stratagene, Agilent) in an optical 96-well plate.

Sequence analysis

An amino acid sequence alignment of PnTPS1 and PnTPS2 with two previously described TPS from *P. trichocarpa*

(Danner *et al*. 2011) was constructed and visualized using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the Clustal W algorithm. A signal peptide prediction was performed using the online prediction server ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), which revealed the presence of a putative N-terminal signal peptide for PnTPS1 (Supporting Information Fig. S3).

Statistical analysis

For all statistical analyses, we used the open source software R Studio (http://www.r-project.org/; Crawley 2007) and SPSS for Windows (SPSS, Chicago, IL, USA). If necessary, data were transformed to meet the statistical assumptions of normality and homogeneity of variances. Mann–Whitney *U*-tests were performed to test for differences in volatile emission between the treatments (caterpillar infested and undamaged control leaves) in the different sections (basal and apical leaves) on the trees.

Poplar volatile classification

To identify compounds that distinguished the volatile blends of damaged versus adjacent undamaged foliage (caterpillar treatment) to the blend released from the two different sections of the control trees, we used the machine learning algorithm 'Random Forest' (Breiman 2001), a multivariate statistical tool. $n = 100\,000$ bootstrap samples were drawn with seven volatiles (variables) randomly selected at each node (number of variables selected is based upon the square root of all variables). The importance of each compound for the distinction is expressed as the mean decrease in accuracy (MDA) and the chance of the compound being improperly classified is expressed as the out-of-bag (OOB) error rate. The 'Random Forest' algorithm is especially suited to analyse datasets with more variables than sample size and variables of autocorrelated nature, such as plant volatiles with common biosynthetic pathways (Ranganathan & Borges 2010).

Parasitoid electrophysiological and behavioural experiments

To compare the electrophysiological response of parasitoids to different volatiles, we performed a Kruskal–Wallis ANOVA on ranks, followed by a Student–Newman–Keuls (SNK) test for comparison of means ($P < 0.05$). To establish if electrophysiological responses behaved in a dose-dependent manner, we applied a Kendal (τ) correlation. For the behavioural assays in the four-arm olfactometer, a Friedman test was performed in which we averaged the time spent in clean air arms, since the arms were randomized, and compared it to the time spent in the odour-treated arm of the arena. A paired sample *t*-test was performed to compare the parasitoids attracted to individual volatiles and solvent controls in the field. Defence hormone data, gene expression data and corresponding volatile emission of their products, as well as the effect of JA, SA and mechanical damage on volatile emission, were analysed with linear mixed effects models.

RESULTS

Volatile emission from black poplar leaves after gypsy moth herbivory

We identified over 75 volatile compounds in the headspace of young black poplar (*P. nigra*) trees by GC-MS after they were fed upon by gypsy moth (*L. dispar*) caterpillars for almost 2 d (Supporting Information Table S4). The volatiles emitted included homoterpenoids, monoterpenoids, sesquiterpenoids, green leaf volatiles, nitrogen-containing volatiles and aromatic compounds (Fig. 1). Herbivory increased emission from damaged leaves over 20-fold compared to emission from corresponding leaves of control trees not fed upon (Fig. 1c). Every class of compounds showed a significant increase in emission rate. Undamaged leaves adjacent apically to the site of herbivore damage also showed a significantly greater total emission compared to equivalent leaves on control trees (Fig. 1b).

Response of herbivore parasitoids to poplar volatile blends

To determine how herbivore enemies orient to black poplar volatiles, we investigated the behaviour of *G. liparidis*, a parasitic wasp that specializes on gypsy moth caterpillars. When tested using an olfactometer, naïve female *G. liparidis* were more attracted to gypsy moth-damaged trees than to either undamaged or mechanically damaged trees ($\chi^2_1 = 26.273$, $P = 0.000$ and $\chi^2_1 = 6.721$, $P = 0.010$, respectively, $n = 50$), but did not show a preference for the odour of gypsy moth caterpillars themselves and their faeces over charcoal-filtered air ($\chi^2_1 = 1.455$, $P = 0.228$) (Fig. 2a).

G. liparidis was also able to distinguish the odours of leaves damaged by gypsy moth caterpillars from the odours of adjacent undamaged leaves in the same tree. In a four-field olfactometer, naïve females showed a preference for the volatile blend of damaged versus undamaged leaves (Friedman's test, $n = 10, \chi^2 = 0.579, P = 0.014$), whereas males exhibited no preference (Friedman's test, $n = 10$, $\chi^2 = 1.060$, $P = 0.787$). When females were first allowed to parasitize gypsy moth caterpillars with the odour of damaged black poplar in the background, their preference for the odour blend of damaged leaves, expressed as the time spent in the odour field, increased even more (Friedman's test, $n = 10$, $\chi^2 = 11.842$, $P = 0.008$; Fig. 2b).

Identification of the most characteristic volatiles of damaged leaves

To investigate the chemical basis for *G. liparidis* selection of damaged leaf volatiles over those of adjacent undamaged leaves, we employed the Random Forest algorithm (Breiman 2001) to identify the most important compounds that distinguish the two blends. This multivariate statistical approach has been previously applied to plant volatile studies (Ramachandran & Norris 1991; Junker *et al*. 2011). Among the most important compounds distinguishing damaged leaves from undamaged adjacent leaves and from leaves on

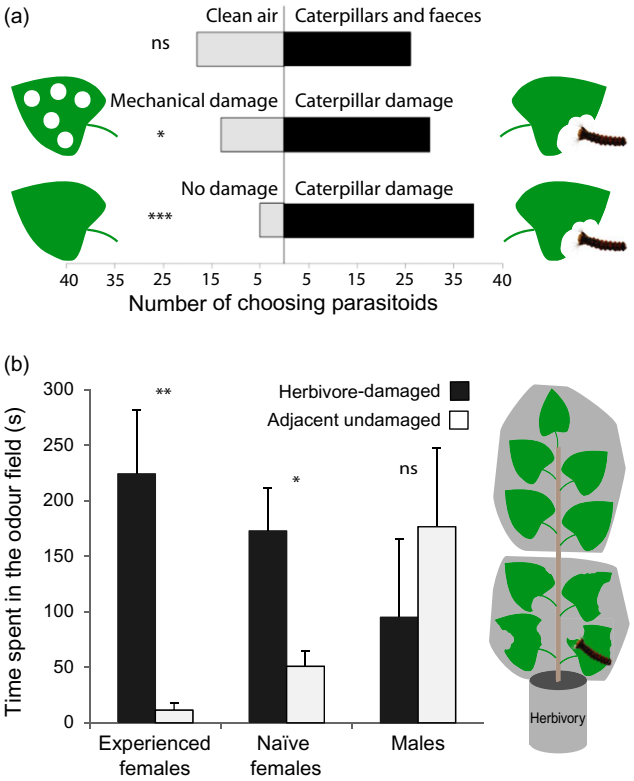

Figure 2. Preference of the parasitic wasp *Glyptapanteles liparidis* for odour blends associated with its host *Lymantria dispar* feeding on young *Populus nigra* trees. (a) Preference in three binary choice tests: Undamaged versus *L. dispar*-damaged tree (bottom), mechanically damaged versus *L. dispar*-damaged tree (middle) and *L. dispar* caterpillars and feces versus clean air (top). Bars indicate total number of parasitoids making each choice ($n = 50$). Parasitoids used were all females and tested individually. Number of non-responding parasitoids was 6, 7 and 6 for bottom, middle and top tests, respectively. Asterisks indicate significance level: $***P < 0.001$; $**P < 0.01$; ns, not significant, after a chi-squared test. χ^2 values were 26.27, 6.72 and 1.45 for bottom, middle and top graphs, respectively. (b) Preference for odour blends associated with *L. dispar*-damaged laves (black bars) and adjacent undamaged leaves (grey bars). Choice assays were conducted in an olfactometer and the time spent in each odour field recorded, mean ± SE ($n = 10$). Experienced females had been previously allowed to parasitize *L. dispar* larvae fed on *P. nigra*. Asterisks indicate significant differences in the time spent in the two different odour fields (Friedman's test; $*P < 0.05$; $**P < 0.01$).

undamaged trees, several compounds were characteristic of damaged leaves, including a widespread C_{11} homoterpene, 4,8-dimethylnona-1,3,7-triene (DMNT), a well-known green leaf volatile, (*Z*)-3-hexenol, and a group of nitrogen-containing volatiles, (*Z*)- and (*E*)-2-methylbutyraldoxime and (*Z*)- and (*E*)-3-methylbutyraldoxime, indole and benzyl cyanide (Table 1). Aldoximes are imine-type compounds derived from amino acids, in which the nitrogen atom is substituted with a hydroxyl group and the olefinic carbon is bonded to one hydrogen atom and one carbon chain. With the exception of indole, these N-containing substances have only rarely been reported as plant volatiles (Knudsen *et al.* 2006).

Electrophysiological and behavioural response of parasitoid to poplar volatiles

Electrophysiological recordings from antennae of naïve female *G. liparidis* were carried out to establish which compounds of the damaged leaves were detected at the receptor level. Thirteen representative compounds were tested at a concentration of 10 $\mu g\ \mu L^{-1}$ versus a solvent control and the reference compound (*E*)-2-hexanal, often found to be electrophysiologically active for both herbivores and herbivore enemies. The compounds eliciting the greatest response amplitudes were the N-containing volatiles, 2-methylbutyraldoxime (*E* : *Z*, 3:1), 3-methylbutyraldoxime (*E* : *Z*, 2:1) and benzyl cyanide. The green leaf volatiles (*Z*)-3-hexenol and (*E*)-2-hexenol also elicited strong responses (Fig. 3). The terpenoids to which the antennae of *G. liparidis* were most responsive were DMNT and (*E*)-β-ocimene. Most of these substances also showed clear dose-dependent responses over a range of 0.01–100 $\mu g\ \mu L^{-1}$, with the nitrogen-containing compounds showing especially elevated responses to the high doses in comparison to other compounds (Fig. 4).

The high electrophysiological antennal activity of many of these components of the black poplar volatile blend prompted us to test a subset of them individually for their attractiveness to *G. liparidis* females in the olfactometer. Four substances elicited a significant behavioural response compared to solvent controls. (*E*)-β-Ocimene and (*Z*)-3-hexenol had a

Table 1. *Populus nigra* volatiles that distinguish between the blends of *Lymantria dispar*-damaged and adjacent undamaged leaves as determined by the Random Forest algorithm (Breiman 2001)

Rank	Compound	MDA
1	β-Cubebene	27.95
2	(*E*)-DMNT	23.64
3	Germacrene D	23.33
4	2-Methylbutyraldoxime	23.06
5	Benzyl cyanide	21.66
6	(*Z*)-3-Hexenylisovalerate	20.52
7	(*Z*)-3-Hexenol	18.83
8	3-Methylbutyraldoxime	18.22
9	(Z)-DMNT	16.45
10	(*E*)-β-Caryophyllene	14.75
11	α-Cubebene	14.49
12	Unidentified STOH	13.08
13	(*Z*)-3-Hexenyl acetate	12.72
14	Indole	11.40
15	(*Z*)-3-Hexenyl benzoate	9.46
16	Unidentified STOH	9.10
17	β-Pinene	9.00
18	Methyl salicylate	8.53
19	(*Z*)-Linalool oxide	8.51
20	Limonene	8.09
	OOB error rate	32.50%

The 20 most diagnostic volatiles are listed in decreasing relevance based upon mean decrease in accuracy (MDA). The mean out of bag (OOB) error rate for each classification is presented at the end of the column.
ST, sesquiterpene; STOH, sesquiterpene alcohol.

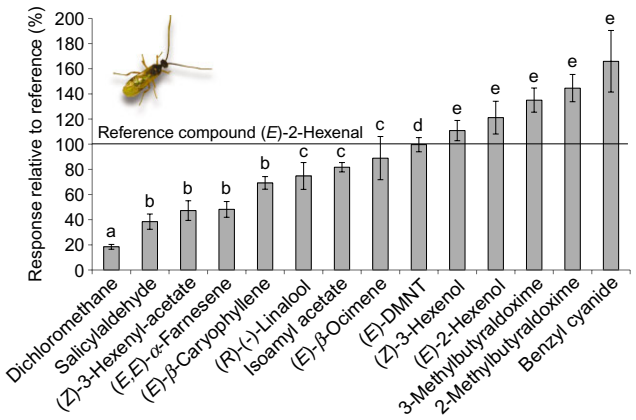

Figure 3. Electroantennogram (EAG) recordings of *G. liparidis* responses to *Populus nigra* volatiles expressed relative to the reference compound (*E*)-2-hexenal, mean ± SEM ($n = 5$). For each compound, 100 μg dissolved in dichloromethane were pipetted onto a filter paper disk and exposed to antennae via a 0.5 s puff of air swept over the disk. Different letters indicate significant differences in antennal response to compounds after a Kruskal–Wallis ANOVA on ranks, followed by a Student–Newman–Keuls test for comparison of means ($P < 0.05$).

repellent effect at the tested dose, whereas the two aldoximes 2-methylbutyraldoxime and 3-methylbutyraldoxime were attractive (Fig. 5).

Recruitment of parasitic wasps to individual volatile compounds in the field

Individual black poplar volatiles were also tested as attractants in a natural old-growth stand. Sticky traps baited with dispensers releasing one of nine selected compounds and a solvent control caught over 8000 individual insects (data not shown). Our attention focused upon the catch of two families of parasitic wasps, the Braconidae and the Ichneumonidae. Among the Braconidae trapped, a significantly higher number of captures occurred in traps baited with the N-containing compound, 2-methylbutyraldoxime, and the green leaf volatile, (*Z*)-3-hexenol as compared to the solvent baited control traps (Fig. 6). For the Ichneumonidae, (*Z*)-3-hexenyl acetate, benzyl cyanide and linalool attracted significantly more individuals than the control traps. None of the other tested compounds showed significant wasp attraction in comparison to the solvent control.

Volatile emission and hormone signalling

Among the defence-related hormones, there was a significant increase in JA and the JA-isoleucine conjugates, (−)-jasmonoyl-L-Ile and (+)-7-*iso*-jasmonyl-L-Ile, as well as SA and abscisic acid (ABA) in herbivore-damaged leaves (the results from the statistical analysis are shown in Table 2) (Fig. 7; Table 2).

As jasmonates and SA were significantly elevated in herbivore-damaged poplar leaves, we tested if these hormones or simple mechanical damage would increase volatile emission when applied directly to detached leaves. JA treatment induced the emission of nitrogen-containing compounds, aromatics and all three classes of terpenoids, but mechanical damage only induced green leaf volatiles strongly and caused a minor increase in monoterpenoid emission (Fig. 8). SA treatment had no effect. These results suggest that jasmonates are a prime cause of volatile synthesis in and emission from herbivore-damaged leaves, but other factors must be operating in adjacent undamaged leaves where volatile emission increased substantially without any parallel increase in jasmonates or SA.

Volatile emission and the site of volatile biosynthesis

Undamaged leaves might conceivably emit volatiles without any increase in defence signalling if these compounds were

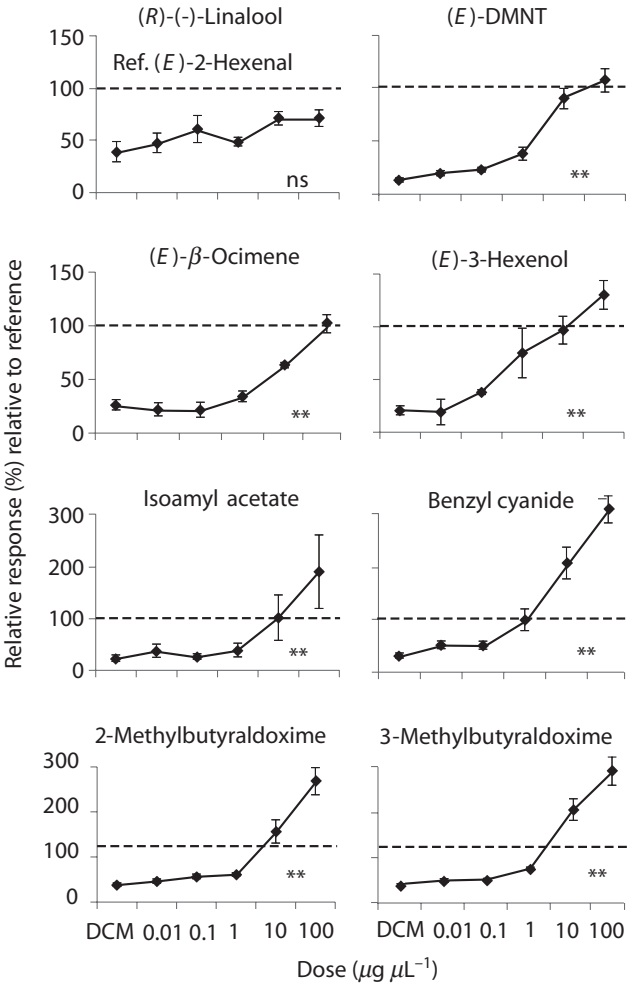

Figure 4. Relative EAG responses of *Glyptapanteles liparidis* females to five different concentrations of *Populus nigra* volatile compounds. Values expressed as relative response with respect to the reference compound (*E*)-2-hexenal (concentration: 10 μg μL^{-1}) here represented by dotted lines, DCM = solvent (dichloromethane). Mean ± SE, $n = 6$. Asterisks indicate significance level after Kendal (τ) correlation: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$; ns, not significant.

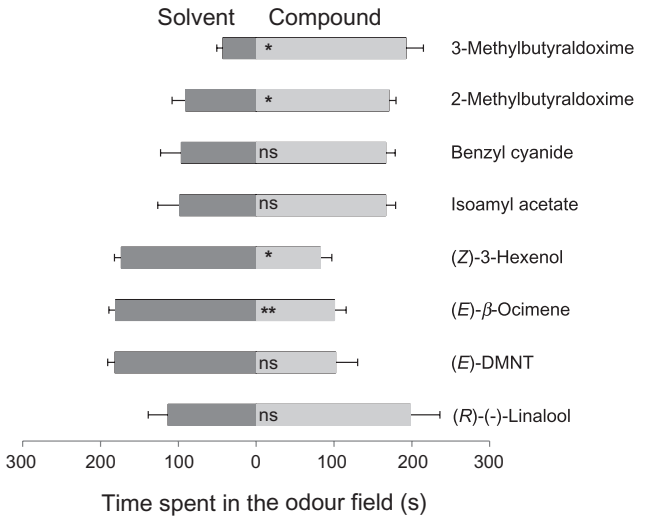

Figure 5. Attraction of *Glyptapanteles liparidis* parasitoids to *Populus nigra* volatiles in an olfactometer. For each compound, 100 μg dissolved in the solvent dichloromethane was transferred to a filter paper disk, and air pumped over the disk at 0.2 L min^{-1} entrained the compound into the olfactometer. The average time spent by parasitoids in odour fields of volatiles (black bars) versus odour fields of solvent (grey bars) was recorded, mean ± SE (n = 10). Asterisks indicate significance level: $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant, after a non-parametric Friedman's test.

actually biosynthesized in damaged leaves and then transported to adjacent undamaged leaves before emission. To determine the location of volatile biosynthesis, we isolated genes encoding key enzymes for the formation of terpenes and nitrogen-containing volatiles. Volatile terpenes are usually formed from the ubiquitous prenyl diphosphate substrates, GPP and FPP by the catalysis of terpene synthases (TPS). Using primers based upon the sequence of predicted terpene synthase genes in the genome of *P. trichocarpa* (http://www.phytozome.net/), two genes were amplified from cDNA from herbivore-damaged *P. nigra* leaves. After heterologous expression in *E. coli*, their catalytic activity was determined in assays with GPP and FPP (Supporting Information Fig. S5). One enzyme, *P. nigra* TPS1 (PnTPS1), produced a mixture of five cyclic monoterpenes from GPP dominated by camphene and β-pinene, whereas the other enzyme (PnTPS2) converted FPP into the sesquiterpene (*E*)-nerolidol, known as an intermediate in DMNT formation.

The monoterpene synthase gene *PnTPS1* did not show any significant change in transcript abundance after herbivore treatment in damaged leaves or adjacent undamaged leaves (Fig. 9, Table 3). We also isolated the sequences from *CYP79D6v3* and *CYP79D7v2*, two P450 monooxygenase genes recently reported to be involved in volatile aldoxime and nitrile formation in *P. trichocarpa* (Irmisch *et al.* 2013). In contrast, the relative transcript abundance of the sesquiterpene synthase gene *PnTPS2* and the P450 genes *CYP79D6* and *CYP79D7* were significantly increased in herbivore-damaged leaves compared to controls, but not in adjacent undamaged leaves relative to their controls. These observed expression patterns correlated closely with the emission of their corresponding volatile products, camphene (product of PnTPS1), (*E*)-DMNT [oxidized metabolite of (*E*)-nerolidol, product of PnTPS2] and 2- and 3-methylbutyraldoxime (product of CYP79D6 and CYP79D7), in damaged versus adjacent leaves, indicating a direct relation between biosynthetic activity and emission (Fig. 9; Table 3).

DISCUSSION

Herbivory alters volatile emission from damaged leaves and adjacent undamaged leaves

The blend of black poplar (*P. nigra*) volatiles released from leaves fed upon by gypsy moth (*L. dispar*) larvae differed qualitatively and quantitatively from that of neighbouring undamaged leaves and that of leaves on undamaged control trees. Although the same major groups of volatiles were emitted by these different leaf pools, there were significant differences in individual compounds and damaged leaves emitted over 20 times that from control leaves on unattacked trees (Fig. 1). The major volatile compounds detected have all been previously reported from other poplar species

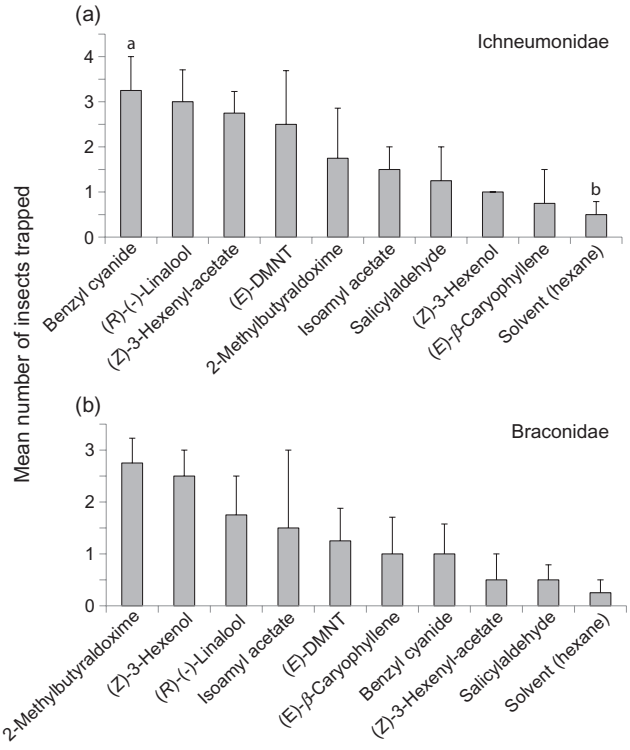

Figure 6. Attraction of parasitic Hymenoptera to *Populus nigra* volatiles in an old-growth *P. nigra* stand. Compounds dissolved in hexane as solvent were offered individually from dispensers attached to sticky traps (release rates are given in Supporting Information Fig. S2). Trapped insects were assigned to insect orders and families. Shown are the mean numbers of parasitic wasps belonging to the Ichneumonidae (a) and Braconidae (b) trapped by each compound, mean ± SE (n = 4). Bars with asterisks are significantly different with respect to the solvent control (paired samples t-test, $^{**}P < 0.01$, $^{*}P < 0.05$).

Table 2. Results of linear mixed effect models on changes in the concentrations of black poplar defence hormones in response to herbivory and leaf position

	Treatment		Leaf position		Treatment × leaf position	
	L ratio	*P*	L ratio	*P*	L ratio	*P*
Jasmonic acid	30.850	<0.001	20.333	<0.001	44.137	<0.001
Salicylic acid	6.641	<0.05	8.696	<0.01	10.307	<0.01
(−)-Jasmonic acid isoleucine	24.068	<0.001	31.178	<0.001	46.936	<0.001
(+)-Jasmonic acid isoleucine	23.536	<0.001	18.114	<0.001	31.278	<0.001
Abscisic acid	4.967	0.026	4.701	0.030	0.97	0.325

Herbivory was carried out on basal leaves and apical leaves sampled to determine the effects on leaves adjacent to herbivory. For both herbivore-damaged leaves and adjacent undamaged leaves, control samples (basal and apical, respectively) were taken from trees without herbivore damage. $n = 20$ (Fig. 7). Explanatory variables were included in the model stepwise. The results of likelihood ratio tests are presented in the table with L ratio values assessing model improvement and *P* values showing statistical significance of the explanatory terms.

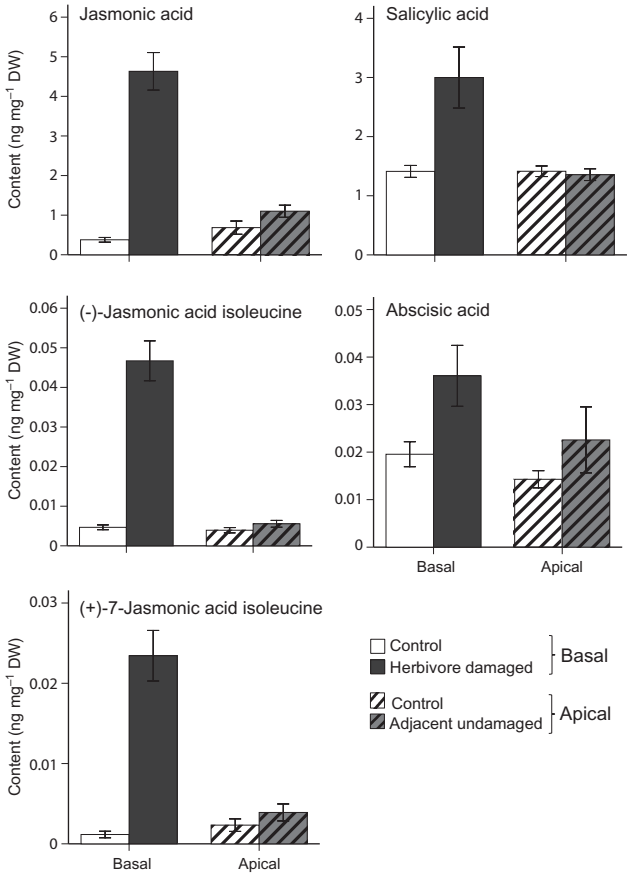

Figure 7. Effect of *Lymantria dispar* gypsy moth herbivory on defence-related hormones in *Populus nigra* leaves. Samples were taken from herbivore-damaged and adjacent undamaged foliage in relation to controls from corresponding regions of trees not subject to herbivory. Data are presented as mean ± SE, $n = 20$ (one representative of each of 20 genotypes). The results of the linear mixed effects models are shown in Table 2. '(-)-Jasmonic acid isoleucine' is the conjugate (−)-jasmonoyl-L-Ile, while '(+)-7-Jasmonic acid isoleucine' is the conjugate (+)-7-isojasmonoyl-L-Ile.

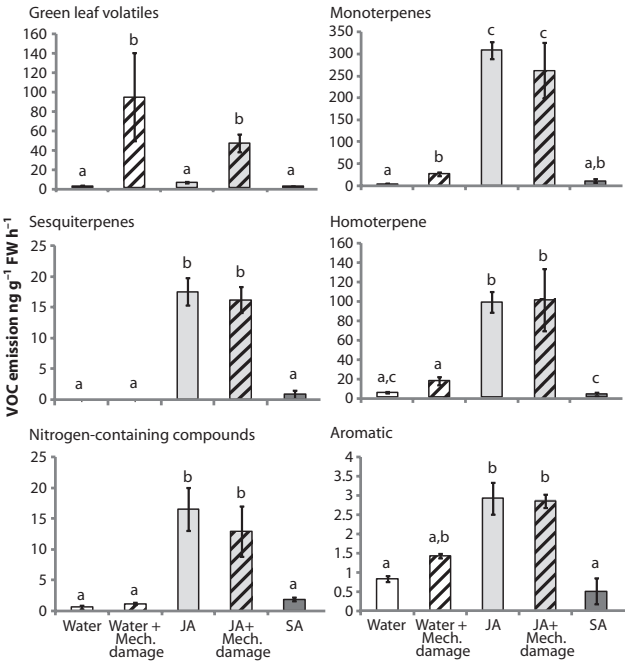

Figure 8. Effect of jasmonic acid (JA), salicylic acid (SA) and mechanical damage on volatile emission from *Populus nigra* leaves. Individual leaves were detached from young trees and left in a 200 μM solution of JA, SA or a water control. Mechanical damage was imposed by cutting with a razor blade. Different letters indicate significant differences among treatments after an ANOVA followed by a Student–Newman–Keuls test for comparison of means. Only a subset of *P. nigra* volatiles was recorded in these measurements including green leaf volatiles: (Z)-3-hexenyl acetate and (Z)-3-hexenol; nitrogen-containing compounds: 2- and 3-methylbutyraldoximes, indole and benzyl cyanide; monoterpenes: α-pinene, β-pinene, camphene, limonene and (*E*)-β-ocimene; homoterpenes: (*E*)-DMNT; sesquiterpenes: germacrene D, α-humulene and (*E*)-β-caryophyllene; aromatic: benzaldehyde.

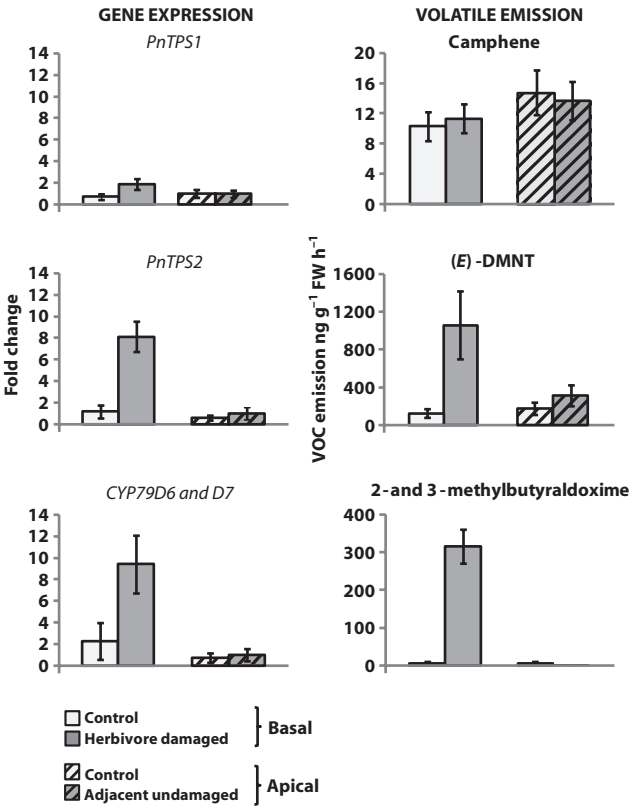

Figure 9. Comparison between transcript abundance of volatile biosynthetic genes and volatile emission from leaves of *Populus nigra*. Measurements are from *Lymantria dispar*-damaged leaves, adjacent undamaged leaves and their respective controls from trees without herbivory. Depicted are relative transcript levels of two terpene synthases and two cytochrome P450 genes and their principal volatile products either produced directly by the enzyme or by a subsequent step. *L. dispar* caterpillars were allowed to feed for 48 h before the volatiles were measured. mRNA abundance was determined using qRT-PCR in leaf material harvested immediately after volatile collection. Shown are means ± SE ($n = 6$ for qRT-PCR, $n = 7$ for volatile emission). The results of the linear mixed effects models are shown in Table 3.

(Arimura *et al*. 2004; Frost *et al*. 2007; Danner *et al*. 2011), although in this study with black poplar we detected far more minor compounds from herbivore-damaged leaves than previously seen in the other species. Earlier work on *P. trichocarpa* x *deltoides* also found increased emission from undamaged leaves adjacent to leaves fed upon by the herbivore, in this case *Malacosoma disstria* caterpillars, but unlike in the present study no qualitative differences were found between the volatile blends of damaged and undamaged foliage (Arimura *et al*. 2004). In our study, the undamaged foliage of control trees emitted mostly terpenes with a decline from apical to basal leaves (Fig. 1b,c). Constitutive monoterpene emission has previously been documented from the foliage of other poplar species (Hakola *et al*. 1998; Brilli *et al*. 2009) with only very low amounts emitted from older leaves. Another type of terpene, the hemiterpene isoprene, has been shown to be a major volatile released from poplar trees (for review, see Schnitzler *et al*. 2010) and may play a role in the attraction of herbivores and herbivore enemies (Laothawornkitkul *et al*. 2009; Loivamäki *et al*. 2008). The volatile analysis methods we employed were not able to measure isoprene, but this compound should be investigated in future experiments to determine if emission is herbivore-induced and affects the attraction of herbivores and their enemies.

Signalling of volatile emission in damaged versus undamaged leaves

The difference in the volatile profiles of herbivore-damaged versus adjacent undamaged black poplar leaves point to divergence in the underlying signal cascades operating in these organs. Such differences are likely due to changes in jasmonate levels as these compounds are the best known herbivore-induced signals simulating volatile emission (Wasternack 2007; Dicke *et al*. 2009; Erb *et al*. 2012). Damaged leaves of black poplar were found to accumulate much higher levels of jasmonates than adjacent undamaged foliage or

Table 3. Results of the linear mixed effect models on changes in the relative transcript abundance of three black poplar volatile synthase genes and the corresponding emission of their products in response to herbivory treatment

	Treatment		Leaf position		Treatment × leaf position	
	L ratio	*P*	L ratio	*P*	L ratio	*P*
Relative transcript abundance						
PnTPS1	1.898	0.168	0.536	0.464	7.710	<0.01
PnTPS2	5.796	<0.05	7.851	<0.01	14.45	<0.01
CYP79D6 and D7	4.201	<0.05	8.308	<0.01	10.192	<0.0
Volatile emission						
Camphene	0.042	0.837	3.880	<0.05	0.395	0.53
DMNT	8.221	<0.01	1.051	0.305	3.522	0.061
2- and 3-methylbutyraldoxime	6.58	<0.05	8.541	<0.01	25.294	<0.001

Herbivory was carried out on basal leaves and apical leaves sampled to determine the effects on leaves adjacent to herbivory. For both herbivore-damaged leaves and adjacent undamaged leaves, control samples (basal and apical, respectively) were taken from trees without herbivore damage. $n = 5$ for the control trees and $n = 4$ for the caterpillar infested trees (Fig. 9). Explanatory variables were included in the model stepwise. The results of likelihood ratio tests are presented in the table with L ratio values assessing model improvement and *P*-values showing statistical significance of the explanatory terms.

foliage in undamaged control trees, and direct application of JA induced the emission of most of the classes of black poplar volatiles. The lack of jasmonate induction in the adjacent undamaged leaf suggests that increased volatile emission results from another signalling pathway. Or, perhaps the volatile compounds themselves or their immediate precursors are transported from damaged to undamaged leaves for release. To investigate this possibility, we determined the location of volatile biosynthesis at the leaf level for several major components of the black poplar blend. Terpene synthases are ubiquitous enzymes in the formation of volatile monoterpenes or sesquiterpenes usually catalysing the final step in the conversion of GPP or FPP to a volatile olefin or alcohol (Degenhardt *et al*. 2009). A terpene synthase (PnTPS2) was isolated that catalysed the conversion of FPP to the sesquiterpene (*E*)-nerolidol, the precursor of DMNT, a volatile compound very characteristic of damaged, but not neighbouring undamaged, leaves. The terpene synthase PnTPS2 was found to be expressed at much higher levels in damaged as compared to undamaged leaves. But what about terpenes found in both damaged and neighbouring undamaged leaves? A group of cyclic monoterpenes, including camphene and β-pinene, was emitted at very similar rates from both damaged and neighbouring undamaged leaves. The terpene synthase forming this mixture from GPP (PnTPS1) was found to be expressed in all leaves, indicating that these compounds are likely not transported among leaves but are synthesized *de novo* in each one. Evidence from other species supports the proposition that volatile terpenes and immediate precursors are not transported around the plant, but are rather synthesized at the sites of emission (Pare & Tumlinson 1997; Arimura *et al*. 2004; Schnee *et al*. 2006; Danner *et al*. 2011; Hiltpold *et al*. 2011; Köllner *et al*. 2013). The same conclusion was also found to apply to another group of black poplar volatiles, the aldoximes and nitriles. The CYP79 enzymes that convert amino acids to these nitrogen-containing volatiles (Irmisch *et al*. 2013) were found to be expressed only at the sites of release, which in this case are restricted to damaged leaves.

The mechanism by which volatile emission is induced in undamaged leaves of black poplar adjacent to herbivore-damage sites still needs clarification. One possibility is that jasmonates produced in damaged leaves move to undamaged leaves. Jasmonates and other octadecanoids have been proposed as mobile signals triggering systemic defences in species of the Solanaceae (Schilmiller & Howe 2005; Wasternack *et al*. 2006; Thorpe *et al*. 2007). Another possibility is that electrical signals, such as those recently discovered in Arabidopsis involving glutamate-like receptors, are involved (Mousavi *et al*. 2013), or changes in xylem hydraulic pressure (Koo *et al*. 2009) could also be responsible. It is also conceivable that volatiles released from damaged leaves serve as airborne signals to trigger emission in neighbouring leaves (Frost *et al*. 2007; Ton *et al*. 2007; Rodriguez-Saona *et al*. 2009). In the experiments where we collected volatiles separately from damaged and adjacent leaves, there was no volatile contact between these leaf types. However, in preliminary experiments in which damaged and adjacent foliage were not separated by PET bags, there was no evidence that volatiles of herbivore-damaged leaves stimulated volatile emission in adjacent leaves (S. Unsicker, unpublished results).

Herbivore parasitoids are more attracted to herbivore-damaged leaves

The distinctions between the volatile blends of damaged versus undamaged leaves were perceived by the parasitoid *G. liparidis*. Odours from leaves damaged by gypsy moth caterpillars were more attractive to *G. liparidis* in an olfactometer than odour from general undamaged leaves or from undamaged leaves adjacent to the site of herbivory. However, this choice was only made by females and was even more decisive for females that had been allowed to parasitize gypsy moth caterpillars in the presence of the odour of damaged black poplar prior to the olfactometer experiment. Thus, *G. liparidis*, in common with other herbivore parasitoids, exhibits an innate response to plant volatiles released by the feeding of its host that is reinforced by an oviposition experience associated with those volatiles (Vet & Dicke 1992; Steidle & van Loon 2003).

Parasitoids are especially attracted to nitrogenous volatiles

Multivariate statistical analysis revealed a small group of volatiles to be important in distinguishing the blends of damaged black poplar leaves from adjacent undamaged foliage. These compounds, together with some of the most abundant black poplar volatiles, were tested in electrophysiological and behavioural assays to determine which are responsible for *G. liparidis* attraction. Multivariate statistical approaches are well-suited to find general patterns in plant volatile emission in different experimental settings (van Dam & Poppy 2008; Hare 2011).

Electrophysiological recordings of *G. liparidis* antennae demonstrated perception of various compounds, including terpenes and green leaf volatiles that are constituents of many herbivore-induced blends and have been shown to be detected by other hymenopteran parasitoids of herbivorous insects Gouinguene *et al*. 2005; (Chen & Fadamiro 2007; Ngumbi *et al*. 2010, for review see Mumm & Dicke 2010). However, the compounds eliciting the strongest electrophysiological responses in the antennae were, in fact, minor constituents of the herbivore-induced black poplar volatile blend, nitrogen-containing compounds, the nitrile benzyl cyanide and 2- and 3-methylbutyraldoxime. Substances that elicit strong olfactory electrophysiological activity are often assumed to serve as important behavioural cues. In our study, the two aldoximes, but not benzyl cyanide, were also attractive to *G. liparidis* in olfactometer bioassays in the laboratory.

Aldoximes and nitriles are known to be released from several species after foliar damage (Takabayashi *et al*. 1991; Van Den Boom *et al*. 2004; Wei *et al*. 2006) as well as from flowers (Knudsen *et al*. 2006), and are typically minor

constituents of plant volatile blends dominated by terpenes and green leaf volatiles. Benzyl cyanide has already been detected from *P. trichocarpa* (Danner *et al*. 2011) and *P. trichocarpa* x *deltoides* (Arimura *et al*. 2004), but so far aldoximes have not been reported until now. In the present study, aldoximes and nitriles were shown to be widespread in *P. nigra* and were not confined to a single tree genotype. We found at least traces of them emitted from all 20 of the trees investigated in our study population.

As minor constituents of plant volatile blends released from damaged foliage, aldoximes and nitriles have been given much less experimental attention than other classes of plant volatiles as attractants for herbivore enemies. However, there are scattered reports of olfactory electrophysiological activity of 3-methylbutyraldoxime (Wei & Kang 2006) and benzyl cyanide (Smid *et al*. 2002; Ngumbi *et al*. 2010) to parasitic wasps. In addition, there are scattered reports of aldoximes (Wei *et al*. 2007) and nitriles (Kugimiya *et al*. 2010) acting as attractants for hymenopteran parasitoids of herbivores in behavioural studies.

Attraction of *G. liparidis* to aldoximes and nitriles is advantageous to the parasitoid as these substances are highly characteristic volatiles of herbivore-damaged poplar foliage and are almost completely absent from the volatile blend of undamaged leaves on either damaged or undamaged trees. Thus, they should function as honest cues to lead parasitic wasps to potential hosts. On the contrary, volatiles emitted from both damaged and neighbouring undamaged black poplar leaves may serve as long range cues to attract *G. liparidis* into the vicinity of a herbivore infestation. The emission pattern of nitrogen-containing volatiles may be diagnostic for attracting herbivore enemies in other systems as well. For example, the aldoximes characteristically released by older cucumber leaves infested by the spider mite *Phytoseiulus persimilis* have been suggested to be involved in the discrimination between old and new leaves by the predatory mite *Tetranychus urticae* (Takabayashi *et al*. 1994). Additionally, a group of *O*-methylated aldoximes was found to be more abundant in the headspace of maize plants damaged by early rather than late instar *Pseudaletia separata*; the parasitoid *Cotesia kariyai* prefers early instar damage (Takabayashi *et al*. 1995). Finally, the nitrile benzyl cyanide has been reported to be used by *Cotesia vestalis* to distinguish current from formerly infested *Brassica rapa* plants (Kugimiya *et al*. 2010). Thus, despite their low abundance compared to the many terpenes or green leaf volatiles, nitrogen-containing compounds may be valuable cues in the orientation of a range of parasitoid and predatory herbivore enemies.

Parasitoids orient towards nitrogenous black poplar volatiles in the field

To investigate the response of parasitic Hymenoptera to aldoximes and nitriles under more natural conditions, we tested individual compounds in an old-growth, floodplain forest containing over 300 black poplar trees. This gave access to a large community of native herbivore enemies. Such field tests also have the advantage of being performed against a background of volatiles from vegetation and other odour sources in the habitat as well as other major poplar volatiles, including isoprene. It has been shown that individual compounds are sometimes only attractive host finding cues as part of mixtures or against an appropriate odour background (Mumm & Hilker 2005; van Wijk *et al*. 2011). In our field tests, the aldoxime 2-methylbutyraldoxime (*E* : *Z*, 3:1), the nitrile benzyl cyanide plus two green leaf volatiles and a terpene all attracted significantly more hymenopteran parasitoids than solvent controls. Previous tests of specific plant volatiles as lures for herbivore enemies in the field have highlighted the attractiveness of green leaf volatiles (James & Grasswitz 2005; Zhu *et al*. 2005; Yu *et al*. 2008) and methyl salicylate (Hatano *et al*. 2008; Blande *et al*. 2010). However, no previous field experiments were performed with aldoximes or nitriles, although another type of nitrogen-containing volatiles, isothiocyanates, was shown to attract braconid parasitoids that exploit hosts feeding on Brassicaceae, whose glucosinolate defences are hydrolysed on tissue damage to isothiocyanates (Titayavan & Altieri 1990; Murchie *et al*. 1997).

CONCLUSION

Our study demonstrates that nitrogenous compounds rather than terpenes or green leaf volatiles best characterize the volatile blend of herbivore-damaged poplar foliage. Electrophysiological and behavioural experiments conducted in the laboratory and the field indicate that nitrogenous compounds are key attractants of parasitic Hymenopterans that serve as herbivore enemies. Future experiments are needed to determine if they participate, like other damaged-induced volatiles, within processes such as direct plant defence and intra- and inter-plant signalling.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Four-field arena for parasitoid preference tests.
Figure S2. Hourly release rates of dispensers with *P. nigra* herbivore-induced volatiles used for field tests of parasitoid attraction.
Figure S3. Design of the traps used for field experiments on parasitoid attraction.
Figure S4. Sequence comparison of PnTPS1 and PnTPS2 with a monoterpene synthase (PtTPS3) and a sesquiterpene synthase (PtTPS2) from *Populus trichocarpa*.
Figure S5. GC-MS analysis of enzyme products from recombinant PnTPS1 and PnTPS2.
Table S1. Volatile compounds collected from *Lymantria dispar* caterpillars and faeces.
Table S2. Source, purity and vapour pressure of the standards used for electrophysiological recordings (EAG).
Table S3. List of oligonucleotides used in this study.
Table S4. List of volatiles released from *Populus nigra*.