Similar local, but different systemic, metabolomic responses of closely related pine subspecies to folivory by caterpillars of the processionary moth

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ABSTRACT

Plants respond locally and systemically to herbivore attack. Most of the research conducted on plant–herbivore relationships at element and molecular levels have focused on the elemental composition or/and certain molecular compounds or specific families of defence metabolites showing that herbivores tend to select plant individuals or species with higher nutrient concentrations and avoid those with higher levels of defence compounds. We performed stoichiometric and metabolomics, both local and systemic, analyses in two subspecies of Pinus sylvestris under attack from caterpillars of the pine processionary moth, an important pest in the Mediterranean Basin. Both pine subspecies responded locally to folivory mainly by increasing relative concentrations of terpenes and some phenolics. Systemic responses differed between pine subspecies, and most of the metabolites presented intermediate concentrations between those of the affected parts and unattacked trees. Our results support the hypothesis that foliar nutrient concentrations are not a key factor for plant selection by adult female processionary moths for oviposition, since folivory was not associated with any of the elements analysed. Phenolic compounds generally did not increase in the attacked trees, questioning the suggestion of induction of phenolics following folivory attack and the anti-feeding properties of phenolics. Herbivory attack produced a general systemic shift in pines, in both primary and secondary metabolism, which was less intense and chemically different from the local responses. Local pine responses were similar between pine subspecies, while systemic responses were more distant.

INTRODUCTION

Carbon (C) to nitrogen (N) and C to phosphorus (P) biomass ratios are lower in herbivores than in plants (Fagan et al. 2002). Foliar nutrient concentration has been widely reported in recent decades as an important factor in the selection of foliage by insect herbivores, who usually choose plants with the highest nutrient concentrations for maintaining their internal C:N: P stoichiometric homeostasis (Elser et al. 2000; Sterner & Elser 2002; Ngai & Jeffries 2004; Cosme et al. 2011; Sardans et al. 2012) and for ensuring larval survival (Hódar et al. 2002). The location of oviposition is crucial for most herbivorous insects to ensure larval survival. However, the role of element composition on host selection by herbivores remains unclear (Rivas-Ubach et al. 2014; Jactel et al. 2015); other chemical and physical barriers could play even more significant roles (Tremmel & Müller 2013; Onodera et al. 2014). Onodera et al. (2014) reported that insects selected organs of a plant containing less defence compounds than plants with higher nutrient concentrations, thus demonstrating the importance of defence metabolites in host selection by herbivores. Plants have developed a wide array of resistance mechanisms against herbivores (Hanley et al. 2007; Heil 2009). Secondary plant metabolic compounds are examples of defence compounds (Herms & Mattson 1992; Kessler & Baldwin 2001) and can be constitutive or induced by a specific stressor. Herbivore attack induces the synthesis of defence compounds at both local and systemic levels (Karban & Baldwin 1997; Sticher et al. 1997; Heil & Bueno 2007; Heil 2009) through induced internal plant signalling (Howe & Jander 2008; Wu & Baldwin 2009) and production of reactive oxygen species (ROS) (Orozco-Cardenas & Ryan 1999; Wu & Baldwin 2010).

Studies of plant-induced chemical responses to herbivore attack have generally focused on a single compound or families of metabolites (Sardans et al. 2011). The role of volatile organic compounds, such as terpenes, in plant defence has been
extensively discussed and reviewed in recent decades (Kessler & Baldwin 2001; Mumm & Hilker 2006; Gershenzon & Dudareva 2007; Peñuelas & Sauter 2010). Low foliar terpene concentrations are often directly correlated with higher rates of herbivore attack, thus showing their role in constitutive defences (Kessler & Baldwin 2001; Hódar et al. 2004; Achotegui-Castells et al. 2013). Terpene synthesis, however, can also be induced by herbivore attack (Pare & Tumlinson 1997; Achotegui-Castells et al. 2013; Irmisch et al. 2014). Phenolics, a diverse group of plant secondary metabolites, are commonly considered one of the most important groups of defensive molecular compounds against folivores (Benett & Wallsgrove 1994), but evidence of their defence role in conifers is still very limited and unclear (Mumm & Hilker 2006; Hódar et al. 2015).

The metabolome is the complete set of metabolites present in an organism at a given time and is considered as the chemical phenotype of an organism (Fiehn 2002). Such metabolites include sugars, amino acids and nucleotides from primary metabolism, and terpenes and phenolics from secondary metabolism. The metabolome thus represents a large variety of complex physiological processes for maintaining homeostasis and function under diverse environmental conditions. The initial functional response of an organism to biotic and abiotic stressors produces shifts in metabolomes (Peñuelas & Sardans 2009). The most recently developed metabolomic techniques in the fields of plant physiology and ecology (ecometabolomics) has not only allowed differentiation of species-specific metabolomes (Deborde & Jacob 2014) or of specific metabolomes under different environmental situations (Robertson 2005; Bundy et al. 2009; Sardans et al. 2011; Macedo 2012; Rivas-Ubach et al. 2012; Fester 2015), but has also allowed understanding of intraspecific metabolic differences between organs (Gargallo-Garriga et al. 2014). Herbivores both increase the production of defensive chemical compounds and induce a general shift in the metabolome of the host plant (Leiss et al. 2009; Peñuelas & Sardans 2009; Rivas-Ubach et al. 2014). Sardans et al. (2014) recently reported a systemic shift in the Quercus ilex foliar metabolome after a few hours of simulated wounding. The suitability and sensitivity of ecometabolomics for detecting local and systemic metabolic shifts in plants under field conditions, however, are not well known but could provide an overview and understanding of how individual plants cope with herbivore attack both locally and systemically, taking into account simultaneous primary and secondary metabolism.

The caterpillar of the pine processionary moth Thaumetoeca pityocampa (Denis and Schiffermüller; hereafter PPM) is an important defoliating pest of pines in the Mediterranean region. PPM caterpillars feed on several pine and other coniferous species (Battisti 1988; Hódar et al. 2003). The caterpillars develop through various stages from the end of summer to the beginning of spring and present intense folivore activity that peaks in winter (Battisti et al. 2005). The PPM is geographically limited mainly by low winter temperatures (Huchon & Déomolin 1971). Scots pine (Pinus sylvestris) grows at high altitudes and is exposed to low temperatures and was consequently not the usually a host for the PPM (Huchon & Déomolin 1971; Hódar et al. 2003), but several recent studies have shown that the global increase in temperature has allowed geographic and demographic expansion of the PPM, which is thus now able to access Scots pine and other pine species naturally occurring at higher altitudes (Benigni & Battisti 1999; Hódar et al. 2003; Battisti et al. 2005, 2006). Sierra Nevada Natural Park (alongside Sierra de Baza Natural Park) in Spain is the southernmost limit of distribution of Scots pine in Western Europe (Boratinski 1991). Two sympatric subspecies of P. sylvestris, P. sylvestris subsp. nevadensis (hereafter nevadensis) and P. sylvestris subsp. iberica (hereafter iberica), are currently seriously affected by the PPM (Hódar et al. 2002) to the point that PPM caterpillars constitute a serious problem for the conservation of pine populations in Sierra Nevada, especially for nevadensis (Blanca et al. 1998). The rising temperatures (IPCC 2013) threatens these pines indirectly by favouring climate conditions for the expansion and activity of the PPM.

The present study is an initial exploration of the local and systemic shifts in element concentrations and metabolomes induced by PPM attack in the above two wild pine subspecies coexisting in the same environment. This analysis elucidates which metabolic pathways are altered as a consequence of herbivore attack. Moreover, the elemental analyses shed light on the still unclear role of foliar element concentrations and C:N:P ratios in host selection by herbivores. We sampled needles of both subspecies of Scots pine (nevadensis and iberica) in winter, when PPM folivorous activity is highest, in Sierra Nevada Natural Park where pine populations are now naturally exposed to PPM attack. The foliar element compositions and untargeted metabolomes were analysed in non-attacked trees, branches of attacked trees and in non-attacked branches of attacked trees of both subspecies.

MATERIAL AND METHODS

Study site

Samples were collected in March 2011 (late winter) on Collado de Matasverdes in Sierra Nevada National Park (Granada, SE Spain; 37.05°N, 3.27°W, 1900 m a.s.l.), a site where nevadensis coexists with iberica (Robledo-Arnuncio et al. 2009). The climate is Mediterranean, with hot summers, cold winters and usually a severe summer drought. The mean annual temperature is 9.8 °C, and mean annual precipitation is 945 mm. January is the coldest month, with a mean minimum temperature of −0.1 °C, and July is the warmest, with a mean maximum temperature of 30.1 °C. Rainfall is concentrated mainly in autumn and spring. See Achotegui-Castells et al. (2013) for more details.

Experimental design and sampling of needles

Twenty-four adult iberica and nevadensis trees, >45 years old and >5-m high, were randomly selected as study subjects (total n = 48), 12 with no signs of caterpillar attack and 12 with caterpillars in the canopy, which are easily located by their winter tents (two to four per tree). A small branch exposed to the sun was removed from the non-attacked trees, from the non-attacked area of the attacked trees and from the attacked area of the attacked trees using a pole (see Fig. S1). The needles of non-attacked trees thus served as controls (hereafter; Control-Ns), the non-attacked needles of the attacked trees and the attacked needles of the attacked trees were used for determining systemic and local responses to folivory (hereafter; Systemic-Ns and Local-Ns, respectively), referred to as folivory levels (FLs) throughout this article. We acknowledge that
metabolomes of plants can shift due environment conditions, for this reason, in order to obtain robust comparative metabolomic data, needle samples were collected in a narrow timeframe (from 10:30 to 14:30 h) under sunny, non-windy conditions with little temperature variation. A bunch of the youngest well-developed needles (>100) from each sampled branch was collected, packed in plastic bags and quickly frozen and stored in liquid nitrogen. It took often less than 1 min from branch sampling to needle freezing.

Our selection of trees in the wild was based on the presence/absence of natural defoliation, so the pines were not assigned to the different levels of this factor completely randomly. However, this problem should not affect the reliability of our results. While many studies analysed between-species host selection by PPM, none established a clear pattern of individual tree selection within species based on nutritional and/or chemical cues (see Jactel et al. 2015 for a recent review). Rather, it is usually admitted that moths in monospecific stands, as in our case, base their selection on visual cues to focus on isolated or taller trees that are more likely to provide optimal microclimate conditions (high solar radiation) for egg survival and successful development of larvae, rather than on chemical differences between individuals (Jactel et al. 2015). The assignment of attacked/non-attacked levels by female moths when ovipositing can thus be reliably considered as a random selection of the prior chemistry of the trees.

Foliar processing for element and metabolome analyses

The foliar processing is described in detail in Rivas-Ubach et al. (2013). Briefly, pine needles frozen in liquid nitrogen were lyophilised and stored in plastic cans at −20 °C. The samples were ground with a ball mill at 1600 rpm for 8 min (Mikrodismembrator-U; B. Braun Biotech International, Melsungen, Germany). The fine homogeneous powder produced was stored at −80 °C until extraction of metabolites for analyses with liquid chromatography-mass spectrometry (LC-MS).

Elemental analysis

The C and N concentrations were determined in 1.4 mg sample powder with elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy). P and K were extracted by acid digestion in a MARSXpress microwave reaction system (CEM, Matthews, NC, USA) under high temperature and pressure (Sardans et al. 2010). Briefly, 250 mg sample powder were placed in a Teflon tube with 5 ml nitric acid and 2 ml H2O2. The digested material was transferred to 50-ml flasks and resuspended in Milli-Q water to a final volume of 50 ml. After digestion, P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma; Perkin-Elmer, Norwalk, CA, USA). See Elemental Analyses section of the supporting information for more detail.

Extraction of metabolites for LC-MS analysis

Polar and semi-polar metabolites were extracted as described in t’Kindt et al. (2008) with some modifications. Briefly, two sets of 2-ml centrifuge tubes were labelled: set A for metabolite extractions and set B for extracts from set A. An aliquot of 100 mg needle powder for each sample was weighed into each tube of set A, and 1 ml extractant (MeOH/H2O, 80:20) added. All tubes were vortexed for 15 min, sonicated for 5 min at 24 °C and then centrifuged at 23,000 g for 5 min. After centrifugation, 0.6 ml of supernatant from each tube of set A was transferred to the corresponding 2-ml centrifuge tube of set B. This procedure was repeated to perform two extractions of each sample. The tubes of set B were centrifuged at 23,000 g for 5 min, and the extracts collected in crystal syringes, filtered through 0.22-μm microfilters and transferred to a labelled set of high performance liquid chromatography (HPLC) vials. Extracts were stored at −80 °C until LC-MS analysis.

Liquid chromatography-mass spectrometry analysis

Liquid chromatography was performed with a reverse-phase C18 Hypersil gold column (150 × 2.1 mm, 3 μm particle size; Thermo Fisher, Waltham, MA, USA) and a Dionex Ultimate 3000 HPLC system (Thermo Fisher/Dionex RSLC, Wien, Austria) at a constant temperature of 30 °C and a flow rate of 0.3 ml·min−1; 5 μl of each sample were injected. We used water (0.1% acetic acid) (A) and acetonitrile (B) as mobile phases. Both A and B were previously filtered and degassed for 10 min in an ultrasonic bath. The elution gradient was initiated at 90% A to 10% B and held for 5 min, then the solvent was linearly changed from 90% A (10% B) to 10% A (90% B) from 5 to 20 min. The gradient was maintained for 5 min and then returned to the starting conditions. The gradient was held at these conditions for 5 min to re-equilibrate the chromatographic system prior to analysis of the next sample.

The HPLC was coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher) equipped with an HESI II (heated electrospray ionisation) source for mass spectrometry. All samples were injected twice, once with the HESI operating in positive ionisation mode (+H) and once in negative ionisation mode (−H). The mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with high-mass resolution (60,000) and a mass range of 50–1000 m/z. For both ionisation modes, capillary temperature was set at 275 °C, sheath and auxiliary gas flow rates were operated at 35 and 5, respectively (arbitrary units). Heater temperature was 250 °C for +H and 150 °C for −H. Capillary voltage operated at 4 and 125 V for +H and −H, respectively. Tube lens operated at 100 and −125 V for +H and −H ionisation modes, respectively. A caffeine standard was injected every ten samples to monitor resolution and sensitivity of the spectrometer. The resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. Auto sampler temperature was set at 4 °C. See LC-MS analyses section of supporting information for more details.

Processing of LC-MS chromatograms

The raw data files obtained from the spectrometer were processed with MZmine 2.17 (Pluska et al. 2010). The chromatograms of the positive and negative modes were always treated separately. All chromatograms were first baseline-corrected and then ion chromatogram lists were extracted. Those ion chromatograms were then deconvoluted, retention time normalised, aligned and automatically assigned (see Table S1 for parameter details). Metabolite assignment is putative since...
it was based on total exact mass of the metabolite, exact mass of the fragments and retention time using measurements of standards in the LC-MS Orbitrap system (See Table S2 for assigned metabolites). However, high resolution and RT reduced the number of false positives considerably. The numerical data sets were then exported to CSV format and filtered. Due to the chromatogram builder and deconvolution, diverse ions with the same mass may present slightly different retention times, hence all identified variables assigned to a same molecular compound were summed to obtain only one variable per metabolite. With the used chromatographic method, certain groups of carbohydrates with the same molecular mass co-elute at the same retention time, making it impossible to differentiate them at MS³, hence, different carbohydrates were classified into groups according their mass and retention time (hexoses: glucose, fructose, mannose and galactose; pentoses: arabinose, ribose and xylose; disaccharides: saccharose and maltose; group of sugars 1 (S1): deoxy-glucose, deoxi-galactose and D-fucose; group of sugars 2 (S2): sorbitol and mannitol; and group of sugars 3 (S3): xylitol and arabitol). Outliers and variables present in fewer than eight individuals were removed from the data set. Outlier variables were defined as measurements three-fold higher than the third quartile or three-fold lower than the first quartile of each cell factor. The numerical values of variables of the data sets correspond to absolute peak areas of the chromatograms detected with the spectrometer. The area value of deconvoluted chromatograms is directly proportional to concentration of the variable, so it is a suitable value for comparative analyses, as demonstrated in several metabolomics studies (Rivas-Ubach et al. 2012, 2013, 2014; Lee & Fiehn 2013; Leiss et al. 2013; Mari et al. 2013; Gargallo-Garriga et al. 2014), although it does not reflect the real concentration in terms of weight of metabolite per weight of sample. Hence, we use the term relative concentration when referring to differences in amount of metabolites among the studied factors (subspecies and FLs).

Statistical analyses

We first performed Shapiro and Levene’s tests on all variables to assess normality and homogeneity of variances, respectively. All identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set to comply with assumptions of the statistical tests. After processing and filtering of chromatograms, 43 (0.57%) unidentified variables were not normally distributed in the data set. The main data set of this study is composed of two categorical independent variables, subspecies (iberica and nevadensis) and FLs (Control-Ns, Systemic-Ns and Local-Ns), and 7595 dependent continuous variables, nine of which were element concentrations and stoichiometric variables (C, N, P, K, C:N, N:P, C:P, N:K and K:P) and 7586 were metabolomic variables, including 64 identified from our plant metabolite library. The whole data set, including the assigned and non-assigned metabolomic variables, (7595 variables in total) of the P. sylvestris needles was subjected to PERMANOVA using the Bray-Metabolomic variables, (7595 variables in total) of the chiometric variables (C, N, P, K, C:N, N:P, C:P, N:K and K:P) Systemic-Ns and Local-Ns, and 7595 dependent continuous variables, subspecies (this study is composed of two categorical independent variables, (i) number of metabolomic variables the Control-Ns had that were intermediate values between those of the Systemic-Ns and Local-Ns, (ii) number of metabolomic variables the Systemic-Ns had that were intermediate values between those of the Control-Ns and Local-Ns; and (iii) number of metabolomic variables the Control-Ns had that were intermediate values between those of the Control-Ns and Systemic-Ns. The data were subsequently analysed by chi-square tests to detect if any of the FLs (Control-Ns, Systemic-Ns or Local-Ns) presented overall intermediate metabolomes between those of the other two. The expected probability under the assumption of equal probability of intermediate values for each of the three FLs should thus be 1/3 of the total studied variables. The whole data sets of each of the subspecies, including assigned and unassigned metabolomic variables, were subjected to principal components analysis (PCA) to identify shifts in foliar stoichiometry and metabolome between FLs for nevadensis and iberica separately. The score coordinates of variables of the PCAs were subjected to one-way ANOVAs to identify statistical differences among the groups (see Supporting Information in Rivas-Ubach et al. 2013).

All statistical analyses were performed with R (R Core Team 2013). Benjamini-Hochberg P-value corrections and Shapiro and chi-square tests were performed with the p.adjust, shapiro.test and Chisq.test functions, respectively, in ’R stats’. Levene’s test was performed with the leveneTest function in ‘car’ (Fox & Weisberg 2011). The PERMANOVA was conducted with the adonis function in ‘vegan’ (Oksanen et al. 2013). Heat maps were constructed with the heatmap.2 function in ‘gplots’ (Gregory et al. 2015). The PCAs were performed with the pca function of R ‘mixOmics’ (Dejean et al. 2013). The matrix data included in the PCAs was scaled by setting the parameter SCALE = T of the pca function in R.

RESULTS

The PERMANOVA of the entire data set identified significant differences in the overall stoichiometry and metabolomes among all the different levels of the studied factors (subspecies and FLs) and their interactions (Table 1). One-way ANOVAs of all 73 known variables identified several significant differences (P < 0.05) after Benjamini-Hochberg correction between Control-Ns, Systemic-Ns and Local-Ns in both pine subspecies; 32 (43.84%) for iberica and 31 (42.5%) for nevadensis (Fig. 1, Table S3). The heat map with the relative concentrations between FLs of the 73 known variables showed that Systemic-Ns of both subspecies was stoichiometrically and metabolically closer to Control-Ns than to Local-Ns (Fig. 1). Chi-square tests
on the number of intermediate relative concentrations of each variable of each FL within each subspecies and season showed that Systemic-Ns had intermediate relative metabolite concentrations between those of Control-Ns and Local-Ns in 3486 of 7595 (45.9%) metabolomic variables in iberica and in 3259 of 7595 (42.9%) metabolomic variables in nevadensis, indicating that the overall intermediate response was not a random effect ($\chi^2 = 543.44, P < 0.0001$ for iberica and $\chi^2 = 332.58, P < 0.0001$ for nevadensis; Table 2).

The PCAs of each subspecies clearly separated the FLs (Fig. 2). The first four PCs of the PCA for iberica explained 31.7% of total variance, 14.4% by PC1 and 7.3% by PC2 (Fig. 2). For nevadensis, the first four PCs of the PCA explained 30.6% of the total variance, 12.3% by PC1 and 7.8% by PC2 (Fig. 2). Case plot of PCAs in both subspecies represented Systemic-Ns in an intermediate position between Control-Ns and Local-Ns, but closer to Control-Ns (Fig. 2A and C).

The foliar stoichiometry of both subspecies did not differ among the FLs, except Control-Ns of nevadensis, which had the highest K:P ratio. The Control-Ns and Systemic-Ns of both subspecies generally had higher relative foliar concentrations of amino acids. Adenine and guanine also tended to be higher in Control-Ns and Systemic-Ns of both subspecies, but adenosine was highest only in Control-Ns of nevadensis. Relative concentrations of the various sugars also differed as a function of FL and subspecies, but Control-Ns and Systemic-Ns of both subspecies generally had higher relative concentrations of hexoses and xylitol/arabitol (the latter two categorised as group 3 sugars), and Local-Ns had higher relative concentrations of disaccharides. The identified organic acids typically related to the tricarboxylic acid cycle did not show major shifts among FLs of either subspecies, but especially nevadensis, none of them changed significantly after Benjamini-Hochberg correction. Succinic acid increased significantly in Local-Ns (Figs 1 and 2). Control-Ns and Systemic-Ns of both subspecies had higher relative concentrations of most phenolics, but Local-Ns of iberica had higher relative concentrations of catechin, epicatechin, epigallocatechin and vitexin, while Local-Ns of nevadensis only had higher relative concentrations of vitexin. Local-Ns also had higher relative concentrations of δ-tocopherol and eugenol in both subspecies. Caryophyllene and carvone (terpenes) were also at higher relative concentrations in the Local-Ns of both subspecies. Control-Ns and Systemic-Ns in both subspecies had the highest relative concentrations of growth factors such as abscisic acid.

**DISCUSSION**

Our results show clearly that pine subspecies and folivory levels presented different metabolome structures (Table 1, Figs 1 and 2).

Commonly, responses of plants to folivory have focused on changes in concentrations of defence compounds (Karban & Baldwin 1997; Sticher et al. 1997; Heal & Bueno 2007; Heal 2009); however, our results showed that those shifts are also produced in the whole metabolome at both local and systemic levels of the plant.

**Element composition of needles**

The PPM caterpillars only feed in the trees in which they hatch. The concentrations of N, P and K in pine needles were not related to PPM oviposition since the FLs did not differ significantly in either pine subspecies (Figs 1 and 2). Some studies have reported herbivore preference for plants with higher concentrations of N (Cosme et al. 2011; Loaiza et al. 2011) or P (Cosme et al. 2011), even within the same plant species. If element composition of needles were a key factor for stand selection, we would expect to find differences between needles of attacked and non-attacked trees. PPM is able to feed on different species of conifer (Battisti 1988; Hödar et al. 2003), which may differ in foliar element concentrations, however, our study was performed in a monospecific forest with two subspecies of Scots pine. The lack of significance in our element and stoichiometric results could thus be mainly interpreted through two different hypotheses: (1) PPM females were not able to discriminate foliar concentrations of C, N, P and K between individuals of either subspecies for oviposition, in agreement with other studies performed with this Lepidopteran (Hödar et al. 2002; Jactel et al. 2015). This could be due the very short reproductive life of adult female moths, often mating and ovipositing within the first 24 h after pupal emergence (Hödar et al. 2003). (2) Although adult female PPM could discern element differences among individuals, other factors may play more important roles for stand selection since there were no differences in foliar concentrations of C, N, P and K, as also reported in other studies (Tremmel & Müller 2013; Onodera et al. 2014). However, although further research is still necessary regarding the role of elements in plant selection by folivores, our results of wild pine populations and recent literature on PPM suggest that the concentrations of C, N, P and K are not a key factor in stand selection for female PPM, at least in selection of stands within the same plant host species.

**Local plant responses to PPM attack**

Local PPM attack induced several different metabolomic responses in both subspecies (Table 1). Phenolic compounds have usually been associated as important defence molecular compounds in response to herbivore attack, especially in conifers (Swain 1977; Franceschi et al. 2005). However in this study, Control-Ns and Systemic-Ns of both subspecies were the groups presenting the highest relative concentrations of most of the 18 phenolic compounds identified in our metabolomic analyses (Fig. 1). Vitexin, epicatechin, catechin, luteolin, robinetin, quercetin, epigallocatechin and myricetin are examples of phenolics that changed significantly amongst the different FLs in one or both subspecies (Fig. 1). Local-Ns of both subspecies had the highest relative concentrations of vitexin and Local-Ns of iberica also had higher relative concentrations of catechin, epicatechin and epigallocatechin in Local-Ns (Figs 1 and 2, Table S3). All these compounds are flavonoids.
Fig. 1. Heat maps (hierarchical variable relationships) of the stoichiometric and assigned metabolomic data for the three folivory levels (Control-Ns, Systemic-Ns and Local-Ns) for iberica (A) and nevadensis (B). The colors represent the relative metabolite concentrations between folivory levels. Red represents the highest concentration, and greater color differences for a variable indicate larger concentration differences between folivory levels. Metabolites marked by an asterisk or a cross had statistically significant ($P < 0.05$) or marginally significant ($P < 0.1$), respectively, relative concentration differences between folivory levels in one-way ANOVAs after Benjamini-Hochberg correction of $P$ values (Table S3).
with strong antioxidant properties that protect lipid membranes and other cellular structures from peroxidation. They decrease oxidative stress produced by the accumulation of cellular H₂O₂ and other ROS (Rice-Evans et al. 1996; Kim et al. 2005) and may be directly induced by folivory (Orozco-Cárdenas & Ryan 1999; Wu & Baldwin 2010). The fact that most phenolics did not increase in Local-Ns suggests that these compounds are not necessarily induced by PPM attack and supports the premise that phenolics have multiple and diverse, even more significant, functions in plants rather than only defence properties against biotic stressors (Treutter 2006). In agreement with our results, some studies with lepidopteran folivores did not detect direct relationships between folivory rate and phenolic allocation (Zou & Cates 1997; Hódar et al. 2004, 2015). Other plant–herbivore studies have reviewed a wide variety of phenolic functions diverging from defence roles (Close & McArthur 2002; Treutter 2006; Rivas-Ubach et al. 2011). Our metabolomic results, however, indicated that local-Ns of both subspecies activate metabolic pathways related to oxidative stress. PMM attack induced increases in tocopherol (vitamin E) concentrations in needles, with the highest values in needles of Local-Ns of both subspecies (Fig. 1 and 2). Tocopherols are among the most important antioxidants, protecting the stability of biomembranes from the effects of ROS (Munné-Bosch & Peñuelas 2004; Falk & Munné-Bosch 2010) by reacting with them and forming a tocopheryl radical that is then reduced by hydrogen donors (Traber & Stevens 2011). The higher concentrations of tocopherols and some end-product flavonoids (epicatechin, catechin, vitexin) in Local-Ns of both subspecies support the idea of antioxidant requirement of the attacked needles. However, other end products, such as luteolin, robinetin, quercetin and myricetin, had lowest concentrations in Local-Ns in one or both subspecies, which thus questions their induction by herbivore attack and, consequently, the anti-feeding role of these flavonoids (Fig. 1). Even though the association of phenolics with deterrent function against herbivores is common (Swain 1977; Franceschi et al. 2005), our results suggest that phenolics should not be considered only as a group of compounds with defence properties. Further research more focused on the anti-feeding properties of phenolics is still required (Close & McArthur 2002), especially in conifers (Mumm & Hilker 2006).

Metabolomic analyses also suggested certain non-phenolic compounds related to herbivore attack. Eugenol was found at higher relative concentrations in needles of Local-Ns of both subspecies (Figs 1 and 2). Eugenol is a secondary metabolite described as an essential oil with toxic properties against nematodes and insects (Sangwan et al. 1990; Isman 2000) and acts as an inhibitor of acetylcholine esterase (Maffei et al. 2011). On the other hand, Local-Ns had the highest relative concentrations of the two identified terpenes, carvone and caryophyllene (Figs 1 and 2), suggesting that their presence was induced by local attack. Terpenes are a varied class of organic secondary metabolites produced by diverse plants and are typically associated with direct and indirect defence against insect attack (Peñuelas & Llusia 2001; Mumm & Hilker 2006; Gershenzon & Dudareva 2007; Achotegui-Castells et al. 2013). Terpene production is a principal constitutive and induced defence chemical mechanism, together with the production of phenolics, against insect folivory, especially in pines and other conifers (Mumm & Hilker 2006). Carvone is an oxygenated monoterpene with some repellent and anti-feeder properties in conifers against coleopterans and lepidopterans (Klepzig & Schlyter 1999; Schlyter et al. 2004). Increases in caryophyllene, a volatile sesquiterpene, however, have been reported in wild plants in response to herbivore damage (Gouiuguené et al. 2001). Caryophyllene has been described as attracting parasitoids or predators and thus acts as an indirect defence compound in both above- and belowground parts of the plant in response to injury by foliviores (Rasmann et al. 2005; Kollmer et al. 2008).

Some studies have reported increases in glucose (hexose) in wounded plants (Widarto et al. 2006; Lafta & Fugate 2011; Peñuelas et al. 2013), which may be involved in increases in assimilation and efficiency of photosynthetic C (Seco et al. 2011; Sardans et al. 2014) and changes in carbohydrate metabolism produced by defence responses to wounding (Ehness et al. 1997; Seco et al. 2011). Hexoses did not increase in needles of the Local-Ns in our study, but the relative concentrations of disaccharides were highest in Local-Ns in both subspecies (Figs 1 and 2). Ness (2003) reported a stimulation of rates of sucrose excretion in leaves damaged by foliviore that attracted insect predators, indicating an indirect defence mechanism. The attraction of other insect visitors through increases in disaccharides in our study could indicate an indirect defence strategy in Scots pine, but the roles of the various sugars released under herbivore attack still remain unclear and warrant further research.

**Systemic plant responses to PPM attack**

The heat maps (Fig. 1) and PCAs (Fig. 2) identified significant differences in several metabolites among folivory levels in both

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**Table 2.** Chi-square analyses comparing the expected and observed number of variables with intermediate means for each folivory level (FL), using an expected value of 33.3% of the total observed compounds. This expected value was based on the neutral supposition that each of the three levels of folivory should have the same probability of having intermediate concentrations of each variable (2530 variables for each FL). The proportion of metabolites for each group with intermediate means with respect to the total is represented in bold.

<table>
<thead>
<tr>
<th></th>
<th>Control-Ns</th>
<th>Systemic-Ns</th>
<th>Local-Ns</th>
<th>Expected for each folivory level</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iberica</td>
<td>1985 (26.1%)</td>
<td>3486 (45.9%)</td>
<td>2124 (28%)</td>
<td>2530 (33.3%)</td>
<td>543.44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nevadensis</td>
<td>2326 (30.6%)</td>
<td>3259 (42.9%)</td>
<td>2010 (26.5%)</td>
<td>2530 (33.3%)</td>
<td>332.58</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Fig. 2. Principal component 1 (PC1) vs. PC2 of the principal component analysis (PCA) of the metabolomic and stoichiometric variables for the iberica and nevadensis subspecies. PCA is performed with the whole set of variables but unassigned metabolites are not represented in the graph. Case (A) and variable (B) plots for iberica. Case (C) and variable (D) plots for nevadensis. Carbon (C), nitrogen (N), phosphorus (P) and potassium (K) ratios are shown in red. Metabolomic families are indicated by different colors: blue, sugars; green, amino acids; cyan, nucleotides; orange, organic acids related to the tricarboxylic acid cycle; violet, phenolics; yellow, terpenes; deep purle, other metabolites. Most of metabolites are identified by abbreviations: disaccharides (Disacch), hexoses (Hex), pentoses (Pent), group 1 sugars representing deoxyglucose, deoxygalactose and D-fucose (S1), group 2 sugars representing sorbitol and mannitol (S2), group 3 sugars representing xylitol and arabitol (S3), alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), glycine (Gly), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), α-Ketoglutaric acid (α-Keto.ac), citric acid (Cit.ac), malic acid (Mal.ac), oxaloacetic acid (Oxa.ac), pyruvic acid (Pyr.ac), succinic acid (Succ.ac), carvone (Carv), caryophyllene (Caryoph), trimethoxyflavone (Flavone), apigenin (Apig), catechin (Catech), cinnamic acid (Cinn.ac), epicatechin (Epicut), epigallocatechin (Epigallocatechin), ferulic acid (Fer.ac), kaempferol (Kaemp), myricetin (Mylan), quercetin (Quer), resveratrol (Resv), robinetin (Rob), taxifolin (Tax), vanillic acid (Van.ac), abscisic acid (ABA), ascorbic acid (Vit.C), α-tocopherol (Tocoph), pantotenic acid (Vit B5), quinic acid (Quin.ac), shikimic acid (Shik.ac), gibberellic acid 3 (GA3) and salicylic acid (Sal.ac). Folivory level (FL) is represented by different colors: blue, Control-Ns; green, Systemic-Ns and red, Local-Ns. The colored arrows indicate the means for PC1 and PC2 of the corresponding FLs. Different letters beside the arrows indicate significant differences between the FLs of each subspecies detected by least significant difference post hoc tests (P < 0.05).
pine subspecies, demonstrating a general systemic response induced by PPM attack. Interestingly, chi-square tests on the number of intermediate metabolite relative concentrations in both subspecies (Table 2) indicated that Systemic-Ns tended to have intermediate metabolomes between those of Control-Ns and Local-Ns. This result is corroborated by dendograms of FLs in the heat-map analyses (Fig. 1) and case plots of PCAs, in which Systemic-Ns are represented between Control-Ns and Local-Ns (Fig. 2). Furthermore, Systemic-Ns in both subspecies clustered closer to Control-Ns than to Local-Ns in the case plot of PCAs, thus showing major induced metabolomic shifts in Local-Ns but not in Systemic-Ns. Even so, metabolomic shifts between Systemic-Ns and Control-Ns were still significantly different (Fig. 2A and C). These results support the premise that local PPM attack is able to trigger significant responses in Scots pine systemically by shifting a large proportion of the overall pine metabolome (Sticher et al. 1997; Heil & Bueno 2007; Heil 2009).

Ecometabolomics has proved an excellent tool for the simultaneous detection of general shifts of metabolomes induced by herbivore attack, including primary and secondary metabolism rather than only molecular compounds directly linked to systemically acquired resistance (Gör latch 1996; Sticher et al. 1997; Heil & Bueno 2007; Erb et al. 2011). From the assigned metabolites, we did not detect significant shifts of Systemic-Ns compared to Control-Ns in iberica (Table S3). An increase in relative concentrations of flavones (Kim et al. 2005) was one clear systemic response in iberica. Although not statistically significant due its metabolomic proximity to Control-Ns, Systemic-Ns in iberica showed increases in relative concentrations of eugenol, catechin, vitexin, epigallocatechin (Rice-Evans et al. 1996), and terpenes (Rasmann et al. 2005) (Figs 1 and 2, Table S3), compounds that increased significantly in Local-Ns in iberica, supporting again the presence of systemic acquired resistance. The systemic response in nevadensis nevertheless differed with respect to iberica and consisted of significant higher relative concentrations of choline, robinetin and flavones relative to Control-Ns (Figs 1 and 2, Table S3). Choline is proven to act as an osmolite after membrane injury (McNeil et al. 2001), and robinetin is a flavonol with strong antioxidant properties (Sroka 2005). Similar to Systemic-Ns of iberica, Systemic-Ns of nevadensis also showed slight increases in terpenes and eugenol with respect to Control-Ns, although still not significant (Fig. 1). Interestingly, the relative concentrations of several amino acids were higher in the Systemic-Ns of nevadensis, e.g., proline, a multifunctional amino acid with important antioxidant properties (Szabados & Savouré 2010). This overall amino acid shift did not occur in Systemic-Ns of iberica (Figs 1 and 2, Table S3).

CONCLUSIONS

- None of the concentrations of elements analysed (N, P or K) differed between PPM attacked and non-attacked trees. Although, there is no evidence of within-species selection in adult female PPM for oviposition, our results support the hypothesis that foliar concentrations of N, P or K are probably not key components of within-species selection by PPM.
- Each folivory level (Control-Ns, Systemic-Ns, Local-Ns) showed increases in different phenolic compounds, which questions whether their induction was produced by folivory, or if they have role as a general group with deterrent properties.
- Local-Ns had higher relative concentrations of terpenes such as carvone and carvophyllene, which were likely more directly involved in defence against foliviros.
- The non-attacked branches of the attacked trees (Systemic-Ns) had metabolomes intermediate between those of the non-attacked trees (Control-Ns) and the attacked branches of attacked trees (Local-Ns), demonstrating an induced gradual response of metabolomes of the entire plant (systemic plant response) after herbivore attack.
- There were more metabolomic similarities between Local-Ns of both subspecies than between those of Systemic-Ns.
- The metabolomic techniques were sufficiently sensitive to distinguish between local and systemic responses in both primary and secondary metabolism of the trees, demonstrating their power as excellent tools for ecological studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Details on methods used for element analysis.
Table S1. LC-MS chromatogram processing.
Table S2. Retention time (RT) and mass to charge ratio (m/z) of the deconvoluted ions in both negative and positive ionisation modes assigned to metabolites with MZmine v.2.1.7.
Table S3. Data for one-way ANOVAs of all stoichiometries and assigned metabolites extracted from Scots pine needles for FLs (Control-Ns, Systemic-Ns, Local-Ns).
Table S4. Data for one-way ANOVAs of the 200 most changed detected ions between Systemic-Ns vs. Control-Ns and between Local-Ns vs. Mass (m/z) and retention time (RT) of each of the ions.
Figure S1. Schematic of needles selected for non-attacked trees (Control-Ns), non-attacked branches of attacked trees (Systemic-Ns) and attacked branches of attacked trees (Local-Ns).
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study of host plant resistance to Western flower thrips in cultivated, biofortified and wild carrots. Phytochemistry, 93, 63–70.


