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Research

Do multiple herbivores maintain chemical diversity of Scots pine monoterpenes?

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A central issue in our understanding of the evolution of the diversity of plant secondary metabolites (PSMs) is whether or not compounds are functional, conferring an advantage to the plant, or non-functional. We examine the hypothesis that the diversity of monoterpene PSMs within a plant species (Scots pine *Pinus sylvestris*) may be explained by different compounds acting as defences against high-impact herbivores operating at different life stages. We also hypothesize that pairwise coevolution, with uncorrelated interactions, is more likely to result in greater PSM diversity, than diffuse coevolution. We tested whether up to 13 different monoterpenes in Scots pine were inhibitory to herbivory by slugs (*Arion ater*), bank voles (*Clethrionomys glareolus*), red deer (*Cervus elaphus*) and capercaillie (*Tetrao urogallus*), each of which attack trees at a different life stage. Plants containing more α-pinene were avoided by both slugs and capercaillie, which may act as reinforcing selective agents for this dominant defensive compound. Herbivory by red deer and capercaillie were, respectively, weakly negatively associated with δ^3-carene, and strongly negatively correlated with the minor compound β-ocimene. Three of the four herbivores are probably contributory selective agents on some of the terpenes, and thus maintain some, but by no means all, of the phytochemical diversity in the species. The correlated defensive function of α-pinene against slugs and capercaillie is consistent with diffuse coevolutionary processes.

Keywords: coevolution; diversity; plant secondary metabolites; selection

1. INTRODUCTION

Although several hypotheses address the distribution and occurrence of plant secondary metabolites (PSMs, [1]) relatively few attempt to specifically explain the diversity of PSMs. The widely accepted hypothesis of a coevolutionary arms race between the plant and its herbivores or pathogens [2,3] suggests that new defensive compounds evolve progressively by mutation and natural selection that favours new genotypes, until their enemies evolve counter-adaptations and the processes is repeated [4–6]. The 'screening hypothesis' suggests that phytochemical diversity evolves and is maintained because it maximizes the probability of evolving active compounds when defence requirements demand them [7,8]. Although these hypotheses differ in the emphasis placed on the functionality of the defensive attributes of PSMs, both lead to the expectation that many of the extant PSMs that contribute to overall phytochemical diversity would be redundant in terms of their efficacy against a plant's current suite of natural enemies. Under the screening hypothesis, redundancy exists as a consequence of diversity, whereas under the arms race hypothesis diversity results from redundancy. It is, therefore, important to our understanding of the evolutionary circumstances that lead to phytochemical diversity, to know whether defence, and any associated selective advantage or disadvantage, could be ascribed to possession of particular PSMs [9,10]. However, since any compound may be one among numerous compounds in a particular plant species, this task is not trivial [10]. This is especially so given that a plant may be involved in many ecological interactions, including with multiple herbivores [11,12], pathogens [13], mutualists [14] and competitors [15], and these operate at multiple trophic levels [16,17].

PSM diversity, which we consider here to mean simply the richness or number of compounds, may arise in a plant species if different compounds independently confer selective advantage in pairwise interactions with different herbivore or pathogen species. Coevolution can be shown not to be pairwise if the plant's susceptibility to multiple natural enemies is genetically correlated [18]. When considering PSMs as defensive traits, if a single compound determines

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the outcome of interactions with more than one herbivore, then susceptibility to those herbivores is likely to be genetically correlated [18] and is consistent with the occurrence of diffuse coevolution. Diffuse coevolution does not necessarily lead to greater chemical diversity in the plant, because multiple herbivores can simultaneously exert selection on only one or a few correlated compounds.

Monoterpenes are a widespread and diverse class of volatile compounds, which are prevalent in coniferous plants, some of which are biologically active against herbivores and/or pathogens (see references above). In this study, we have used field and laboratory experiments to test the hypothesis that different monoterpenes from Scots pine (*Pinus sylvestris*) needles are effective defences against different coevolved, high-impact herbivore species. Support for this hypothesis would provide an explanation for the diversity of monoterpenes found in this species. Using the range of natural qualitative and quantitative variation in needle monoterpenes, we sought deterrent effects in several simple ditrophic interactions: herbivory by large black slugs (*Arion ater*) on newly emerged seedlings; by bank voles (*Myodes glareolus*) on three to seven month-old seedlings, by red deer (*Cervus elaphus*) on 3 and 5 year-old saplings and by capercaillie (Aves: *Tetrao urogallus*) on mature trees. Each experiment or field survey also considered the effects of some variables in addition to needle chemistry. Some explanatory factors for herbivory were identified in each study, and although these were analysed along with the full methodological design, here we focus on identifying and reporting the effects of the monoterpenes.

2. MATERIAL AND METHODS

(a) *Experimental methods*

(i) *Slugs*

We tested whether morphology, chemical characteristics and genetic origin of seedlings less than three weeks old influenced foraging by large black slugs. Three hundred and thirty-six Scots pine seedlings were grown from seeds from six open-pollinated maternal trees (56 randomly selected from each mother) from Ballochbuie Wood, Balmoral, Aberdeenshire, UK (latitude: 57° 3′ N, longitude: 3° 16′ E). Fourteen slugs were each randomly allocated to one of 14 separate enclosures (49 × 40 × 45 cm) made from seven sub-divided large plastic containers. Each enclosure contained a 10 cm depth of potting soil and two strips of shade cloth (5 × 50 cm) as refugia. Four seedlings from each of the six families (here and elsewhere in this paper, a family comprises half-sib seedlings sharing the same open-pollinated maternal tree) were planted in a regular 7 cm grid with six columns and four rows in each of the 14 enclosures, such that each row contained one seedling from each of the six families, randomly allocated to columns but with the constraint that no family was over-represented on the edges of the grid. The edges of the grids were 7 cm from the walls of the enclosures and from the refugia. At the same time, 24 control seedlings were planted in a separate tub (with no slug) to measure mass change during the course of the experiment.

Seedling stem length and diameter were measured at planting, and one needle was cut from each seedling and its length was measured (±0.01 mm). This needle was cut at its midpoint and placed in a 1.5 ml glass vial with extraction solvent (*n*-hexane) and an internal standard (isobutylbenzene), then stored in the dark at room temperature for 48 h to extract monoterpenes. After this time, vials were stored at −20°C for gas chromatography–mass spectrometry (GC-MS) analysis at a later date. Slugs were taken off their artificial diet [19] and 4 days later were placed in the freshly planted enclosures for seven nights. On each morning of the experiment, each seedling was scored as either entirely eaten or uneaten. Results reported here are from day 2 of the experiment, when much herbivory had taken place, but some seedlings remained intact. For detailed methods, see [19].

(ii) *Voles*

A field experiment tested the survival of planted seedlings in the summer of their germination, from age three to seven months, in relation to family, needle monoterpene concentrations at the time of planting out, accessibility to voles, local plant community and height of surrounding vegetation at the time of planting. Seeds were collected from five Scots pine trees in March 2004 and germinated in damp paper before being transplanted into individual cells 3 × 3 × 5 cm (LBS Group, Colne, Lancashire, UK), containing Levingtons C2a compost (intermediate nutrient level, coarse, acidic) with 10 per cent grit (Levingtons, Ipswich, Suffolk, UK) and a slow-release fertilizer (Ficote 180: Levingtons, Ipswich, Suffolk, UK; 4.5 kg m^{-3}). At three months, seedlings were planted out in Ballochbuie Wood (above) in a balanced factorial design at 12 sites across four distinct ground vegetation communities (*Vaccinium myrtillus* dominated, *Callluna vulgaris*, *Calluna*/grass and grass only). At each site, seedlings were planted in one 1 × 1 m exclosure that was small mammal-proof, and in another, similar, control ‘exclosure’, which was designed to be open to small mammals. Within each exclosure, 25 seedlings (5 replicates × 5 familes) were planted in each of two 50 × 50 cm areas, one of natural vegetation, and an adjacent one in which all living and green above-ground vegetation, including moss, was clipped to ground level prior to planting, but in which existing litter was retained. Immediately before planting out, a single needle was removed from each of the 1200 seedlings for solvent extraction of monoterpenes, as described above. Survival of the seedlings was assessed on 18th October, 111 days after planting.

(iii) *Red deer experiment 1: 3 year-old saplings*

A food choice experiment was conducted under intensive indoor conditions to test whether browsing red deer selected 3 year-old Scots pine saplings on the basis of morphology, nitrogen and/or monoterpene concentrations in needles and elongating buds.

Sixty saplings from each of five Ballochbuie Wood (above) families were germinated and propagated from seed collected in March 2003 as described above. The five maternal trees spanned a range of total monoterpene concentrations and included high, medium and low

δ^3-carene individuals. Seedlings were maintained in a glasshouse at Craigiebuckler, Aberdeen, UK (latitude 57° 09′ N: longitude 02° 05′ W) for six months, then transplanted to individual pots containing the same growing medium. They were re-potted after another six months into 2 l pots containing Scotts M2a compost (Scotts, Ipswich, Suffolk, UK), and were watered when necessary.

Six adult female red deer were individually penned and fed a maintenance diet of timothy (*Phleum pratense*) hay supplemented by Scots pine branches for 5 days before the trial. Hay was offered to the deer twice daily while branches were offered only in the morning. Each animal had access to water ad libitum. Animals were allowed individually into an arena with a concrete floor (18 × 10 m) for food choice tests. The experiment was run over 4 days (2 consecutive days in one week and 2 consecutive days the following week) in May 2006. On each day, three deer were used sequentially after being randomly allocated a running order of 1, 2 or 3, on each day. Each deer was tested twice, once in each week. Twenty-five saplings, in individual pots, were arranged in the feeding arena as five heterogeneous patches, set up around the perimeter of the arena. Each patch contained five saplings (one from each of the five families) spaced evenly around the circumference of a circle of diameter 1.5 m. Families were allocated randomly to the five positions in each patch. All patches were separated by at least 4 m from each other. A feeding session was terminated when the deer had visited and left all five patches, or after a minimum time of 10 min, whichever was the longer. The maximum time allowed per session was 30 min. This regime precluded depletion of all saplings. Fresh saplings were used for each session. On each day of the experiment, deer that were used that day received only half of their usual morning maintenance feed of hay before the experiment and the remainder after the session with their regular second feed.

Five morphological measurements were made on each sapling prior to use (table 1). In addition, three fully expanded needles and one elongating lateral bud were cut from each sapling. The needles were cut into 1 cm pieces, and the bud was cut in half longitudinally. Half of each sample was extracted for monterpene analysis as above and half was frozen for later nitrogen analysis. Saplings were weighed (g) in their pots each day immediately before being placed in the arena, and were re-weighed (g) and had their height re-measured (cm) once the feeding session was complete.

(iv) *Red deer experiment 2: 5 year-old saplings*

A second set of experiments investigated the effect of monterpene composition on feeding behaviour by two groups of three red deer on 5 year-old Scots pine saplings in a natural moorland setting over the course of several hours. In preparation for these experiments, the non-chiral needle monoterpene chemistry of 100 trees from each of the same five families used in the previous red deer experiment was analysed by gas chromatography and trees were selected from these for two sub-experiments. The first was designed to test the effect of the presence/absence of the diterpene alcohol, isoabienol and the second, the effect of the presence/absence of the monterpene δ^3-carene on deer feeding behaviour. These two compounds were chosen as the basis for the experiment, because they are major needle constituents, and Scots pines show clear chemotypic differentiation for both. Each experimental replicate presented 10 trees from a single family, arranged in a symmetrical array measuring 4 m across, positioned in the centre of a 50 × 50 m fenced plot on *Calluna* moorland, to a group of three adult red deer hinds. Five of the trees possessed the test compound (either isoabienol or δ^3-carene) and the five remaining trees did not. As far as possible, total monoterpene concentrations were matched between the two sets of five trees. For each experiment, five replicates were conducted with each of two groups of hinds. Across both experiments, 200 trees were presented to the deer. Each morning, morphometric measurements were recorded from each tree (table 1) and a terminal branch section 5 cm in length was sampled and frozen for subsequent chemical analysis. Hinds were released into the experimental arena for 5 h, after which time the trees were recovered and re-measured if herbivory had occurred. Each sub-experiment was analysed separately to test the hypothesis that concentrations of the test compound had influenced whether or not trees were browsed and/or the amount or proportion of the tree that had been eaten (measured as the total length and proportion of leader and lateral stems browsed).

(v) *Capercaillie*

We assessed the extent of usage of natural, mature Scots pine trees for foraging and roosting by capercaillie and compared the chemical differences between those used by capercaillie and those which were not. Individually marked trees along 20 different 2 km long transects in Abernethy Forest, Scotland, UK (Latitude 57° 13′ N: Longitude 3° 18′ W), were monitored each spring for 6 years: 1991–1994, 2004 and 2005. Twenty one trees used by foraging capercaillie during winter were identified by an accumulation of droppings below the crown in at least 3 years, signs of foraging damage to shoots in 2004 or 2005, and droppings distributed beneath the crown, rather than in a single heap in either of these years. Each tree was paired with a nearby tree of similar size and morphology that had not been used by capercaillie. Morphological measurements (table 1) were taken and needle samples collected from the southern aspect of each tree in the highest and middle third of each crown during winter dormancy in December 2004. Needles were analysed for monoterpenes and total nitrogen as described below, and for calcium, magnesium, sodium and potassium using flame photometry.

(b) ***Chemical analyses***

The direct collection of needles into extraction solvent is described above. When needles were frozen after collection and stored whole (red deer experiment 2 and capercaillie tree-sampling), needles were later thawed and chopped into 1 mm lengths. Five hundred milligrams of fresh needles was weighed into a glass scintillation vial

Table 1. The dependent and explanatory variables entered into the analysis of results from each study.

herbivore	pine life stage	dependent variable modelled	explanatory variables
slugs	less than 3 weeks	seedling mortality (alive = 0, dead = 1)	*monoterpenes*: α-pinene, β-pinene, δ^3-carene, terpinolene, myrcene, total monoterpenes camphene, limonene *other chemical traits*: needle nitrogen *morphological traits*: stem diameter (mm), stem length (mm), needle length (mm) *experimental design parameters*: family, slug × array row (row) × array column (col)
voles	three to seven months	seedling mortality (alive = 0 dead = 1)	*monoterpenes*: α-pinene, camphene, β-pinene, myrcene, δ^3-carene, α-terpinene, limonene, γ-terpinene, terpinolene *other chemical traits*: — *morphological traits*: no. of needles, needle length (mm) *experimental design parameters*: family, plant community (PC) × access to voles (AV) × vegetation cleared (C)
red deer experiment 1	3 years	% height loss of saplings owing to deer browsing	*monoterpenes*: needle and elongating bud (−) α-pinene, (+) α-pinene, tricyclene, (−) camphene, myrcene and (+) camphene (coeluting), (+) β-pinene and an unidentified monoterpene (coeluting), (−) β-pinene, δ^3-carene, (−) limonene, β-ocimene, (+) limonene and β-phellandrene (coeluting), γ-terpinene and terpinolene *other chemical traits*: needle and elongating bud nitrogen *morphological traits*: total number of new buds, total height of the sapling (cm), length of the leading bud (cm), average length of three lateral buds (cm), stem diameter under the leading bud (mm) and stem diameter at the base of the sapling (mm) *experimental design parameters*: family, day of experiment, deer, deer × day, patch number, location in patch
red deer experiment 2	5 years	proportional length of available leader and laterals eaten	*monoterpenes*: as for red deer experiment 1, plus bornyl acetate *other chemical traits*: — *morphological traits*: mean length of five needles (mm), diameter at base of leading shoot (mm), diameter of main stem at ground level (mm), total length of leading + lateral shoots (mm), total height of sapling (cm), number of laterals at uppermost whorl (count), number of laterals below uppermost whorl (count). *experimental design parameters*: —
capercaillie	mature trees	trees used by capercaillie for foraging (0 = non-caper, 1 = caper)	*monoterpenes*: as for red deer experiment 1 *other chemical traits*: needle concentrations of calcium, magnesium, sodium, potassium, nitrogen, condensed tannin, total phenolics *morphological traits*: tree height (m), girth (cm), crown diameter (m), nearest neighbour distance (m), proportion of total height occupied by crown

and extracted overnight in 2.5 ml of *n*-hexane-containing isobutylbenzene as an internal standard.

Monoterpene extractions and analyses were based on the methods reported in Thoss *et al.* [20]. The main exceptions were that for the Scots pines in the two red deer and the capercaillie studies a chiral analysis was carried out using a 30 m RT BetaDEXsm column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (Thames Restek, UK) operated with helium as a carrier gas at a flow rate of 1 ml min^{-1}. One microlitre of extract was injected into a split/splitless inlet operating at 180°C with a split ratio of 40 and an initial oven temperature of 60°C. The initial temperature was held for 2 min before increasing at a rate of 3.5°C min^{-1} to 130°C and then at 10°C min^{-1} to 210°C. The FID was operated at 250°C. For the second deer experiment, 200 mg of the silica gel dehydration agent was replaced with sodium sulphate to allow the complete quantification of bornyl acetate.

The small needles and concomitant small quantities of monoterpenes in the slug experiment required the sensitivity of detection provided by a Trace GC 2000 gas chromatograph paired with a Trace DSQ mass spectrometer operated in single ion monitoring mode for fragments of mass 93.2 µl of extract was injected in splitless mode onto a SolGel 10–25 (30 m length and 0.25 mm internal diameter; SGE, Australia) column. The initial temperature, 50°C, was held for 1 min then increased at 3°C min^{-1} to 75°C, then at 30°C min^{-1} to 200°C. The monoterpenes analysed in each experiment are listed in table 1.

(c) *Data analysis*

The data analyses were specific to each experiment but were based on the sequential elimination of terms not significant at $p < 0.05$ from multiple linear regression models that initially included all appropriate variables, including plant chemical and morphological variables. Response and explanatory variables are listed in table 1. As a precursor to model selection, candidate explanatory variables were transformed if necessary to improve the linear response with monoterpenes. All variables were examined for multi-collinearity by inspection of the full matrix of Pearson's correlation coefficients, and this information was used subsequently to inform exploration of the model space. Those variables that had been used to structure the experimental design, such as family, and that had been eliminated before the final model was reached, were re-entered into the final model to check their significance, again in order to guard against possible effects of multi-collinearity. Logistic regression with a logit link function was used to model binary response variables in the slug, vole and capercailie experiments. In deer experiment 2, observations were combined across both sub-experiments and rather than multiple linear regression, linear mixed models with the random term (family + unique replicate identifier) were used to model total stem length eaten. In order to assess the correlations among monoterpenes at both the family level and the individual level, we fitted a random effects model having only the two random terms—the factor indicating family and the factor indicating individual. Correlations could then be derived from the model covariance matrices, and this ensured the family-level correlations were not contaminated by within-family variation. Each set of coefficients is adjusted to remove the effects of the other random factor. We illustrate this for one example study (deer experiment 2; table 3), which has the family-level (genetic) correlations in the upper-right of the table, and the individual-level correlations in the lower left part of the table. The statistical significance level indicated in table 3 derived from likelihood ratio tests between the full model with both random terms and the simpler models obtained by dropping each term.

(d) *Induced phytochemical effects*

The collection of plant material for subsequent phytochemical characterization is hypothesized to lead to induction of phytochemical defences [21]. In order to test the induced response of Scots pine saplings and seedlings, we removed single needles from 24 additional control seedlings in the slug experiment, three needles from 20 additional saplings in red deer experiment 1 and clipped a single tip from a lateral branch of all saplings in deer experiment 2. Needles from the first clip, and the re-sampling event of each plant that took place 24 h later, at the time of the experiment, were analysed for monoterpenes. There was no induced effect on seedling monoterpene concentration after removing needles or branches in any of these cases (J. M. O'Reilly-Wapstra, B. D. Moore & G. R. Iason 2009, unpublished data). Previous research has also shown that needle terpene levels do not respond to simulated defoliation in Scots pine [22], that two sawflies did not induce chemical defence in Scots pine [23], and that monoterpene cyclase activity was not induced by stem damage in other species of *Pinus* [24]. Consequently, induced chemical effects are unlikely to influence the monoterpenes of Scots pine, and it is unlikely that removal of the needles and buds 1 day prior to the feeding trial influenced the chemical profile of the Scots pine seedlings and saplings offered to the animals in these experiments. However, a slight monoterpene induction effect has recently been shown in newly growing Scots pine needles after application of methyl-jasmonate for 2 years [25].

3. RESULTS

Plants containing more of the dominant monoterpene, α-pinene, were avoided by both slugs and capercaillie, but the minor compound β-ocimene was also strongly negatively associated with use of Scots pines by capercaillie (table 2). Mortality of three to seven month-old seedlings was not related to exposure to bank voles (table 2). Red deer showed no preference for nor avoidance of particular monoterpenes when foraging on 3 year-old Scots pine saplings but there was a weak negative relationship between the amount of herbivory on 5 year-old saplings and the concentration of δ^3-carene (table 2).

The individual slugs and red deer differed in their propensity to feed on the pines; two of 14 slugs failed to eat any seedlings at all and were excluded from the analysis. The slugs also showed spatial preferences within the arenas (column × slug interaction) and foraged from more seedlings with thicker stems and with needles containing lower nitrogen concentrations, which was itself a correlate of larger seedling size. In red deer experiment 1, deer ate a greater proportion of plants that had longer buds and in red deer experiment 2, deer ate less from the saplings with larger stem diameters and/or shorter needles. Both strategies maximize deer intake of needles relative to woody plant tissue. The capercaillie clearly selected trees with higher needle magnesium levels but their preference was modified by the monoterpenes.

Monoterpene concentrations were mostly positively correlated across trees, and some were genetically correlated across the five families in deer experiment 2 (table 3). The concentrations of (+) α-pinene and (−) α-pinene were positively correlated with concentrations of most other monoterpenes across plants but only the latter was involved in strong genetic correlations. The concentration of another dominant monoterpene, δ^3-carene, was least often correlated with those of other monoterpenes, and the weak associations that did occur were mostly negative (table 3). Over 80 per cent of the total mass of monoterpenes was attributable to the four main monoterpenes, which were (+) and (−) α-pinene (−) β-pinene and δ^3-carene (table 3).

4. DISCUSSION

In order to rigorously assess the ecological significance of plant chemical diversity, individual compounds should be tested for activity against as many of the

Table 2. Statistical models explaining most variation in the dependent variables of each study—see text for model selection.

herbivore	response variable	explanatory variable	coefficients for covariates (s.e.)	statistic (d.f.)	p
slugs	seedling mortality (alive = 0, dead = 1)	slug	—	$\chi^2 = 4.10$ (1,265)	0.045
		slug × col	—	$\chi^2 = 3.22$ (5,265)	0.007
		stem diameter	3.06 (1.08)	$\chi^2 = 8.07$ (1,265)	0.005
		α-pinene	−0.714 (0.357)	$\chi^2 = 7.46$ (1,265)	0.006
		needle nitrogen	−0.1765 (0.0717)	$\chi^2 = 6.23$ (1,265)	0.031
voles	seedling mortality (alive = 0, dead = 1)	PC × AV × C	—	$\chi^2 = 9.45$ (3,1154)	<0.001
red deer experiment 1	% height loss of saplings owing to deer browsing	day	—	$F = 23.19$ (1,286)	<0.001
		deer	—	$F = 39.80$ (5,286)	<0.001
		bud length	2.611 (0.4894)	$F = 28.46$ (1,286)	<0.001
		deer × day	—	$F = 8.73$ (5,286)	<0.001
red deer experiment 2	proportional length of available leader and laterals eaten	mean needle length	0.00513 (0.00127)	$F = 15.33$ (1,142)	<0.001
		leader diameter	−0.0658 (0.01539)	$F = 17.00$ (1,148)	<0.001
		δ^3-carene	−0.0409 (0.0207)	$F = 3.89$ (1,149)	0.05
capercaillie	trees used by capercaillie for foraging (0 = non-caper, 1 = caper)	magnesium	0.0148 (0.0056)	$\chi^2 = 7.56$ (1,38)	0.006
		β-ocimene	−19.58 (7.78)	$\chi^2 = 12.78$ (1,38)	<0.001
		(−) α-pinene	−0.790 (0.0.39)	$\chi^2 = 8.01$ (1,38)	0.005

plant's natural enemies as possible [10], and any accrued benefits should be balanced against any detrimental effects, such as the attraction of specialist herbivores [6]. In the absence of existing examples of such thorough testing, our efforts bring this goal somewhat closer. Although many monoterpenes were screened for herbivore deterrence in these experiments, relatively few were found to be involved in the defence of Scots pine from the four herbivores. Only one or two monoterpenes were associated with defence in three of five studies of different life stages of Scots pine, and across all studies only three monoterpenes were retained in statistical models of herbivore impact. The dominant monoterpene, α-pinene, was associated with the defence against both slugs and capercaillie. The models we present contained relatively few explanatory variables, and were readily identified by our procedures. They are not, however, the only plausible models describing the observed patterns of herbivory, and multicollinearity between many monoterpenes (table 3) means that compounds in addition to those identified here may also possess anti-herbivore activity, which was not detected statistically.

If herbivory is to act as a selective force for plant resistance trait(s), three requirements must be met: (i) there must be phenotypic variability in the plant population in the resistance trait(s); (ii) the trait(s) conferring resistance must be under genetic control; and (iii) the herbivore damage incurred by plants in the absence of the resistance trait(s) must negatively impact the overall fitness of the plant [26,27]. Monoterpenes are typically very variable within conifer populations, including those of Scots pine [28,29]. This variation is exemplified by the concentrations of single monoterpenes in the needles of trees sampled in the capercaillie study; the mean of the coefficients of variation of monoterpene concentrations calculated across the 42 trees was 57 per cent, in contrast to 11 per cent for needle nitrogen and 18 per cent for needle magnesium concentrations. Constitutive variation in monoterpenes within and between pine populations is genetically determined to a large degree [30–33]. All herbivore species studied here exert large impacts on Scots pine and all eat needle tissue. Those that target the early life stages of the plant either always (slugs) or frequently (voles and red deer) cause mortality of attacked plants. Because capercaillie repeatedly use the same trees for foraging on pine needles, they are likely to reduce the net primary productivity of trees over a long period. Consequently, like the other herbivores they are also likely to negatively impact the lifetime fitness of individual Scots pine trees. We expect, therefore, that the long coexistence of Scots pine with these herbivores should have led to the selective retention of PSMs that mediate defence against them. Across all the plant–herbivore interactions, we expected to find negative rather than positive effects of monoterpenes on herbivory, and this was indeed what the results showed.

The failure of voles to negatively impact seedling survival or to discriminate among seedlings on any phenotypic basis, including monoterpene concentrations, means that the first and third requirements for natural selection by these herbivores on plant chemistry were not met. However, herbivory by slugs, capercaillie and red deer may impose positive selection upon at least three different monoterpenes. Slugs and capercaillie provide reinforcing selection upon the single dominant defensive compound in Scots pine, α-pinene. The plant's susceptibility to these herbivores is, therefore, probably genetically correlated and coevolution is diffuse [34,35]. Most attention has been paid to plant interactions with communities of herbivores at one point in time. However, diffuse coevolution may also involve sets of herbivores, such as slugs and capercaille, which do not compete or directly interact in any way, but which each interact with a plant at different times, as long as the phytochemical trait under selection is constitutive and is expressed across time periods. Such interactions might

Table 3. Genetic (family level) and among-tree correlations for the monoterpenes in deer experiment 2. The top right part of the table shows correlation coefficients of the family means (20–60 plants per family), calculated for groups of half sibs, across the five families in the experiment. The bottom left part of the table shows correlations among individual monoterpenes calculated across all 200 plants in the experiment. Statistical significance is derived from likelihood ratio tests (see text). The mean, maximum and minimum needle concentrations are shown for each monoterpene.

	(+) α-pinene	(−) α-pinene	tricyclene	(−) β-pinene	δ^3-carene	(−) limonene	(+) β-pinene & uid[a]	β-ocimene	(+) limonene & β-phellandrene	terpinolene	(−) camphene	myrcene & (+) camphene	γ-terpinene
(+) α-pinene	—	0.70	1.00***	0.55	−0.59	0.98*	0.98*	0.41	0.99**	−0.74	0.99**	1.00***	0.83
(−) α-pinene	0.46***	—	0.93	0.95	−0.98*	0.75	0.87	−0.04	0.98*	−0.97	0.85	0.90	1.0000*
tricyclene	0.90***	0.47***	—	0.51	−0.93	0.97*	0.90*	0.22	0.97*	−0.54	1.00***	1.00**	0.70
(−) β-pinene	0.58***	0.38***	0.46***	—	−0.54	0.67	0.87	0.76	0.93	−0.32	0.49	0.32	1.00*
δ^3-carene	−0.15*	−0.11	0.04	0.05	—	−0.61***	−0.67***	0.77***	−0.95***	1.00***	−0.74	−0.87	−0.96
(−) limonene	0.50***	0.45***	0.53***	0.48***	0.01	—	1.00	0.48	0.77	−0.66	0.98*	0.99**	0.04
(+) β-pinene & uid	0.64***	0.36***	0.75***	0.50***	0.58***	0.46***	—	0.56	0.01	−0.01	0.96*	0.98*	0.88
β-ocimene	0.54***	0.31***	0.52***	0.41***	0.10	0.39***	0.51***	—	−0.18	0.65	0.18	0.10	0.53
(+) limonene & β-phellandrene	0.65***	0.45***	0.64***	0.67***	0.27***	0.48***	0.76***	0.25***	—	−0.77	0.97*	0.98*	0.92
terpinolene	−0.03	−0.01	0.16*	0.10	0.95***	0.08	0.68***	0.17*	0.36***	—	−0.72	−0.64	−0.92***
(−)camphene	0.88***	0.39***	0.97***	0.44***	0.02	0.51***	0.76***	0.50***	0.63***	0.15*	—	1.00***	0.67
myrcene & (+) camphene	0.83***	0.42***	0.91***	0.48***	0.16*	0.54***	0.85***	0.54***	0.71***	0.31***	0.95***	—	0.72
γ-terpinene	0.16*	0.07***	0.17*	0.14	0.32***	0.21**	0.34***	0.26	0.30***	0.32***	0.16*	0.23**	—
mean concentration ($\mathrm{mg\ gDM^{-1}}$)	1.8901	0.7924	0.0923	0.3512	0.5320	0.0180	0.0595	0.0638	0.0330	0.0554	0.3800	0.1670	0.0080
minimum concentration ($\mathrm{mg\ gDM^{-1}}$)	0.1144	0.0899	0.0065	0.0331	0.0034	0.0023	0.0195	0	0.0075	0.0063	0.0338	0.0405	0.0026
maximum concentration ($\mathrm{mg\ gDM^{-1}}$)	5.5986	3.9301	0.3490	1.2503	3.3971	0.11305	0.1623	0.28073	0.1352	0.29305	1.5160	0.5591	0.0431

[a] uid, unidentified monoterpene.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

take place in different seasons or involve different plant life stages. A herbivore's involvement in a diffuse coevolutionary interaction does not preclude its simultaneous involvement in a strict pairwise one. Thus, slugs, red deer and capercaillle may also select for other, independently varying, monoterpenes in Scots pine, thereby promoting the evolution of greater chemical diversity. The contribution of these processes to the expansion of chemical diversity would be diminished if the complement of monoterpenes changed significantly, or was independent between the life stage of the tree; however, there is evidence to the contrary. Although there are some phenological shifts in relative monoterpene concentrations during Scots pine needle maturation, the composition of mature needles is consistent within individuals from year to year [20]. This consistency reflects the high degree of genetic control of monoterpene biosynthesis and was the reason that monoterpenes were traditionally used as proxy genetic markers for taxonomic studies in conifers [36,37].

We identified several monoterpenes with no apparent function against the four conspicuous, high-impact herbivores that we studied. This is consistent both with the screening hypothesis [7] and with the explanation (to paraphrase [38]) that many of these compounds are 'the ghosts of herbivory past'. Specifically, many inactive compounds may have served as active deterrents of current or now-extinct herbivores in earlier iterations of the coevolutionary process, but have since become redundant. A recent study, which takes a phylogenetic approach, confirms that chemical diversity increases through evolutionary time [4]. This suggests that the retention or disappearance of redundant compounds may well be a key determinant of PSM diversity. If selective advantage is gained by possession of a compound, the necessary intermediary metabolites and their synthetic machinery will also be selected for, even though particular intermediaries may themselves be without apparent ecological or evolutionary benefit, as long as the evolutionary costs of their maintenance remain lower than the benefits. Although selection by the four high-impact herbivores tested cannot explain the entire diversity of monoterpenes in Scots pine, three of the four herbivores are likely to exert selective forces on three of the 13 monoterpenes tested. Consideration of pathogens and other organisms that interact with this species, and possible interactive effects between the monoterpenes [39] indicate that we may ultimately uncover adaptive explanations for more of the compounds. For example, the identities and concentrations of monoterpenes are also likely to be influenced to varying degrees by some of the 172 species of phytophagous insects and mites that are associated with Scots pine in Britain [40]. We have tested an important part of coevolutionary theory regarding the functionality of PSMs: what proportion of compounds serve a defensive purpose or are most of them incidental or just defences in waiting, as predicted by the screening hypothesis? Although the degree of apparent redundancy supports the screening hypothesis, it also supports the predictions of the coevolutionary arms race hypothesis. Distinguishing hypotheses and testing the screening hypothesis remain challenges for the future, which we suggest will centre around the conditions favouring retention versus deletion of redundant or apparently redundant compounds.

In conclusion, the defence of Scots pine against at least two of the four herbivores tested here, slugs and capercaillie, is mediated by a single compound (α-pinene), consistent with diffuse coevolutionary processes, involving two herbivores that interact with different life stages. If α-pinene is advantageous in defending against at least two herbivores, and potentially by others unstudied, then the selection pressure this generates may explain why it is the most abundant monoterpene in Scots pine. The evolutionary relationship between effectiveness of particular PSMs, particularly those that act against generalist herbivores in a dose-dependent manner, and their relative concentrations in nature, has not, to our knowledge, been explored. Two other monoterpenes are associated with defence, one against red deer and another against capercaillie, consistent with pairwise coevolution. In none of our experiments did monoterpenes form the sole basis for selection or avoidance of Scots pine. In those cases where relationships between mortality or feeding parameters and monoterpenes were identified, although they were always negative, they were always operational in association with other plant and community parameters. This indicates the complexity of the environment in which PSMs function and evolve.

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