THE ROYAL SOCIETY
PUBLISHING

A below-ground herbivore shapes root defensive chemistry in natural plant populations

Meret Huber[1], Zoe Bont[3], Julia Fricke[3], Théo Brillatz[1], Zohra Aziz[1], Jonathan Gershenzon[2] and Matthias Erb[1,3]

[1]Root Herbivore Interactions Group, Department of Biochemistry, and [2]Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany
[3]Institute of Plant Sciences, University of Bern, Bern, Switzerland

Plants display extensive intraspecific variation in secondary metabolites. However, the selective forces shaping this diversity remain often unknown, especially below ground. Using *Taraxacum officinale* and its major native insect root herbivore *Melolontha melolontha*, we tested whether below-ground herbivores drive intraspecific variation in root secondary metabolites. We found that high *M. melolontha* infestation levels over recent decades are associated with high concentrations of major root latex secondary metabolites across 21 central European *T. officinale* field populations. By cultivating offspring of these populations, we show that both heritable variation and phenotypic plasticity contribute to the observed differences. Furthermore, we demonstrate that the production of the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) is costly in the absence, but beneficial in the presence of *M. melolontha*, resulting in divergent selection of TA-G. Our results highlight the role of soil-dwelling insects for the evolution of plant defences in nature.

1. Background

Heritable intraspecific variation is a common feature of many biological traits. Genetic variation results from heterogeneous selection pressures in relation to genetic architecture, population substructures and gene flow [1–4]. In plants, local differences in both abiotic and biotic factors may drive trait evolution [5]. Insect herbivores, the most abundant and diverse plant consumers, have long been suspected to play an important role in this context [6]. Recent studies demonstrate that herbivore abundance can covary with the expression of plant defence metabolites [7], that the exclusion of phytophagous insects can lead to a relaxation of defences within a few generations [8] and that defence genes are under differential selection across environments [9,10]. Together, these studies show that the temporal and spatial variation in above-ground herbivore communities can shape plant defensive chemistry.

In contrast with the ecological and evolutionary dynamics of above-ground plant–herbivore interactions, below-ground interactions have received little attention, despite the importance of roots for plant fitness and the high concentrations of secondary metabolites in below-ground organs [11–14]. The rhizosphere differs from the phyllosphere in both biotic and abiotic conditions [15], and the selective forces shaping variation in secondary metabolites may therefore differ between the two environments. The evolution of root secondary metabolites may for instance be driven by herbivores [11,12,16], pathogens [17] and symbionts [18], as well as nutrient availability [19], salt, drought and cold stress [20]. By comparing one mainland and two island populations, Watts *et al.* [21] showed that geographical isolation, including the escape from pocket gophers (Geomyidae), resulted in the evolutionary decline of root alkaloid concentrations of the host plant *Eschscholzia californica* (Papaveraceae). The specific potential of root herbivores to shape the abundance and geographical distribution of plant secondary metabolites, however, remains to be explored.

Among Europe's largest and most prevalent native root feeding insects are the larvae of the common cockchafer (*Melolontha melolontha*) (Coleoptera: Scarabaeidae). Owing to their pest status and conspicuous appearance in the adult stage, *M. melolontha* populations have been monitored closely by authorities, farmers and the public over recent decades [22]. *Melolontha melolontha* females lay their eggs close to their emergence site into meadows with high heat emission [23–25], resulting in stable geographical distribution patterns. Although the larvae are highly polyphagous, they preferentially oviposit close to and feed on the common dandelion (*Taraxacum officinale* agg., Flora Helvetica, 5th edn) during the final instar [26,27]. *Taraxacum officinale* is a perennial that relies heavily on its strong tap root for resprouting and flowering in spring. The plant is native to and widely distributed in Europe [28], and has recently gained a cosmopolitan distribution due to human dispersal. *Taraxacum officinale* is described as a species complex with sexual, outcrossing diploids in central and southern Europe and a multitude of apomictic, clonal triploids across the globe [29]. One of the factors that may explain the worldwide success of *T. officinale* is its capacity to produce latex in roots and other organs as a protection against herbivores. Latex is the cytoplasm of the specialized laticifer cells, and typically contains high concentrations of toxic and sometimes sticky secondary metabolites [30]. The latex of *T. officinale* is dominated by three secondary metabolite classes: phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) [31]. In a recent study, we showed that TA-G protects plants against *M. melolontha* attack, opening up the possibility that this compound may be under natural selection by the root herbivore [32].

In this study, we investigated whether *M. melolontha* shapes variation in latex secondary metabolites in *T. officinale*. We profiled the latex chemistry of natural *T. officinale* populations growing under different *M. melolontha* herbivore pressure, and tested for inducibility, heritable variation and differential selection of the metabolites in the offspring of the field populations. Our results provide evidence that below-ground herbivores drive the ecology and evolution of chemical defences in plants.

2. Material and methods

(a) Identification and characterization of *Taraxacum officinale* field populations

To investigate the influence of *M. melolontha* on the phenotypic variation of latex chemistry in natural *T. officinale* populations, we collected latex from a total of 252 *T. officinale* plants from 21 populations that evolved under different *M. melolontha* herbivory over the last 20 years. We identified two areas in which the presence of *M. melolontha* has been monitored by federal authorities: the Swiss Alps and Germany (figure 1a; electronic supplementary material, figure S2 and table S1). Within these two areas, we contacted local farmers who provided detailed information about the infestation histories of their fields over the last 20 years. Fields that repeatedly showed visible signs of *M. melolontha* feeding (reduction in plant growth and yield, brown patches, *M. melolontha* adult flights) were classified as historically *M. melolontha* infested (local herbivory). Fields at a distance of maximally 2 km that lacked visual signs of below-ground herbivory during the last 20 years were termed *M. melolontha* free (local control). As *T. officinale*

seeds and pollen may disperse over 200–500 m [33,34], we identified additional *T. officinale* populations under low *M. melolontha* herbivory within the same geographical areas at a distance of 50–100 km to the local herbivory and local control populations (regional controls). Regional controls were identified based on data from Swiss and German authorities. The different populations were arranged in triplets so that each herbivore population had a matching local and regional control population in the same area with similar environmental conditions, including altitude, exposition, inclination, land use and climate. Detailed information about the information sources for the individual populations can be found in electronic supplementary material, table S1.

The populations in Germany were shown earlier to consist mainly of triploids, while plants in the Swiss Alps are mostly diploid [29,35], allowing us to study the impact of *M. melolontha* on the evolution of secondary metabolites in both triploid apomictic and diploid obligate outcrossing plants. In both Germany and the Swiss Alps, we analysed three to four local herbivory, local control and regional control populations in spring 2013. For each population, we collected one fully ripened seed capsule per plant from 12 *T. officinale* individuals, with a minimal distance of 50 cm between plants. To characterize the present root herbivore community, we excavated a cube with 18 cm side length around each plant and recorded the identity and number of belowground herbivores in each cube. Furthermore, we collected latex from the main root of each plant into Eppendorf tubes and glass vials, which were immediately frozen in dry ice. After transport to the laboratory, samples were stored at −80°C and extracted as described below.

(b) Secondary metabolite quantifications

The latex of *T. officinale* is dominated by three classes of secondary metabolites: TA-G, PIEs and TritAcs [31]. To quantify TA-G and PIEs, 1 ml methanol containing 10 µg ml^{-1} loganin and 100 µg ml^{-1} salicin as internal standards for TA-G and PIEs, respectively, was added to the Eppendorf tubes containing the sampled root latex. Tubes were vortexed for 5 min, centrifuged, and the supernatant was stored at −80°C until analysis. Methanol samples were measured on a high pressure liquid chromatograph (HPLC 1100 series equipment, Agilent Technologies), coupled to a photodiode array detector (G1315A DAD, Agilent Technologies). For quantification, peak areas were integrated at 245 nm for TA-G and at 275 nm for PIEs, and quantified using internal standards. For extraction of the TritAcs, 1 ml hexane containing 0.1 mg ml^{-1} cholesteryl acetate as internal standard was added to the glass vials. Vials were shaken for 5 min, centrifuged and the supernatant stored at −80°C until analysis. Hexane samples were analysed on an Agilent series 6890 gas chromatograph coupled to a flame ionization detector (GC-FID). Individual triterpene acetates were quantified based on the internal standard. Methodological details for the analytical procedures are described in Huber *et al.* [31].

(c) Analysis of field populations

Differences in the present root herbivore abundance between populations of different infestation histories were analysed with Kruskal–Wallis rank sum tests using the mean values of each population for each area separately. The effect of infestation history on the concentrations of TA-G, total PIEs and total TritAcs was analysed using mixed-effect models with maximum-likelihood estimations using the nlme package [36] for each area separately, with metabolite concentrations as response variable, infestation history as fixed effect and the population as a hierarchical random effect that includes region, village and plot number (electronic supplementary material, table S1). First, the hierarchical random effects were reduced stepwise by comparing two models with the original and simplified random effects using a likelihood ratio test. Second, two models—which after model
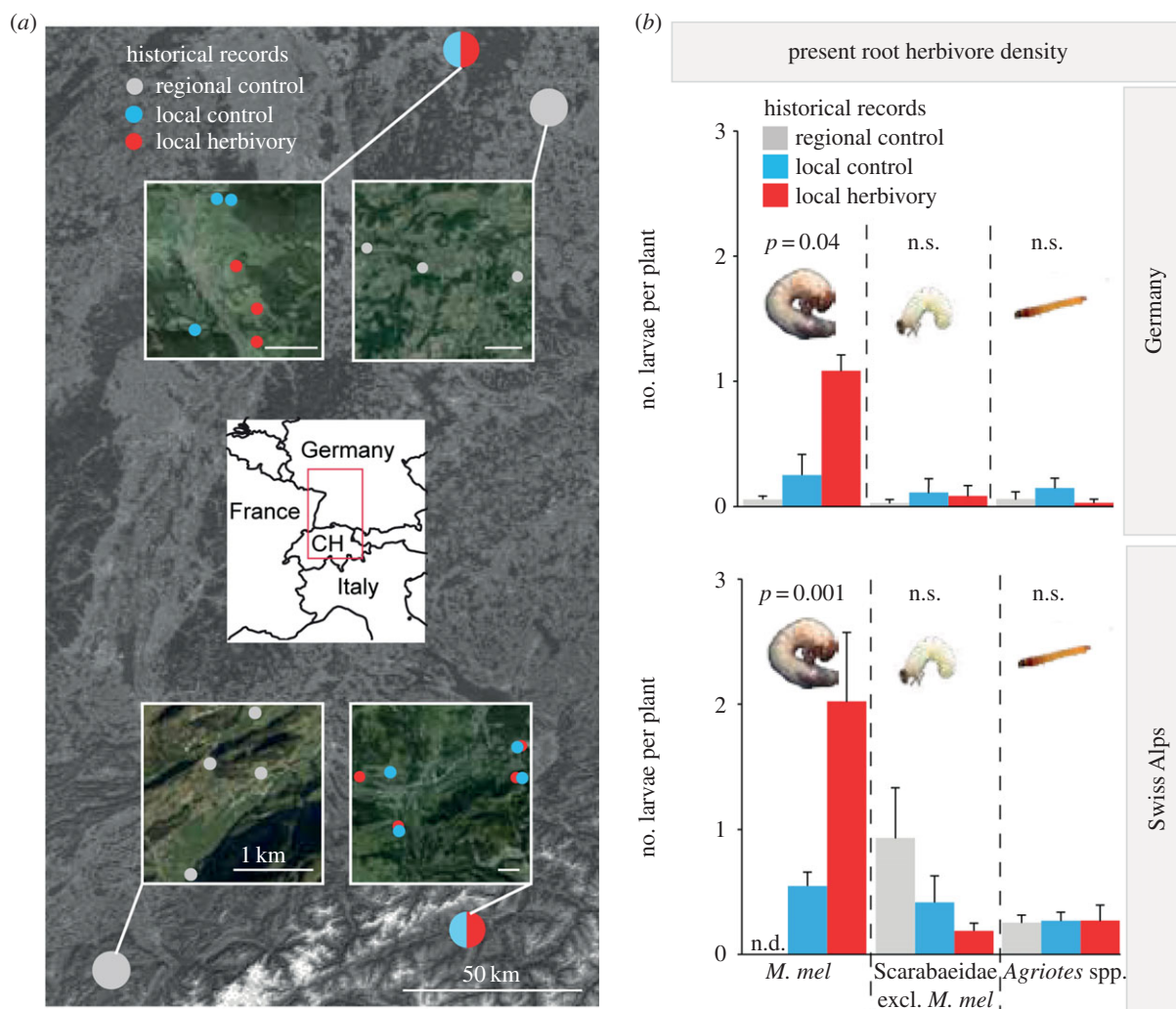
Figure 1. Distribution map and present root herbivore abundance of different *T. officinale* populations across central Europe. (*a*) Map of identified natural *T. officinale* populations evolving under high ('local herbivory') or low ('local control' and 'regional control') *M. melolontha* infestation levels over the last 20 years. Circles inside insets denote individual *T. officinale* populations. CH, Switzerland. (*b*) Mean number of root herbivore larvae per *T. officinale* plant found during a field survey in 2013. *M. mel*, *M. melolontha*. *p*-values of Kruskal–Wallis rank sum tests are shown. Error bars correspond to standard errors (+s.e.). n.s., not significant ($p > 0.10$).

simplification only contained the plot number as a random effect—with and without infestation history were compared with a likelihood ratio test for each area and metabolite separately. Summary statistics of the models are presented using the highly infested populations as the reference level. Results were visualized using ggplot2 [37] and gridExtra [38]. Maps were drawn with RgoogleMaps [39] and rworldmap [40]. All statistical analyses were performed in R v. 3.1.1 [41].

(d) Offspring growth and sampling

In order to investigate heritable variation, inducibility and fitness effects of latex secondary metabolites, as well as to verify the plant ploidy levels, we grew offspring of the plants from the field-sampled populations with and without *M. melolontha* larvae. To balance the number of populations between Germany and the Swiss Alps, the three populations with the most well-documented history of *M. melolontha* abundance for each infestation history were selected in both areas. Seeds were germinated in seedling trays in a growth chamber under standard conditions. After four weeks of growth in July 2013, the trays were placed outside for acclimatization (Jena, Germany: 50°54′34.8″ N; 11°34′00.1″ E). Each plant from the local herbivore populations was then potted together with either a plant from the matching regional control populations (regional pair) or a plant from the matching local control populations (local pair) inside a 2 l pot filled with soil (74% Klamann

substrate TS1, 26% 0.7–1.2 mm sand, 1 g l^{-1} Osmocote Exact 3–4 M EG fertilizer), resulting in 144 combinations for each of the regional and local pairs. Two identical pots of each combination were set up; one of them was infested with one third instar *M. melolontha* larva seven weeks after germination, and the other was not manipulated (control). *Melolontha melolontha* larvae were collected from meadows in Spessart (Germany), and reared individually in 200 ml plastic beakers filled with potting soil and grated carrots in a phytotron in darkness operating under the following conditions: 12 L : 12 D cycle; temperature: day 13°C, night 11°C; humidity: 70%. Plants were watered whenever necessary. At the end of November 2013, plants were moved to a greenhouse (4–10°C) to overwinter. Plants were moved outside again in early March 2014, and seeds of mature capitula were collected every day and weighed. Plants were harvested at the end of May when seed production had ceased. Latex from the main roots was collected in Eppendorf tubes and glass vials for secondary metabolite analysis and immediately frozen in liquid nitrogen. Latex in Eppendorf tubes and glass vials was extracted, analysed and quantified as described above. The ploidy level of each accession was furthermore determined by flow cytometry as described by Bubner *et al.* [42].

(e) Offspring analysis

To investigate the inducibility of latex secondary metabolites, TA-G, total PIE and total TritAc concentrations were compared

between control and *M. melolontha*-infested plants with Kruskal–Wallis rank sum tests. We analysed the inducibility of TA-G (TA-G concentration in herbivore-infested plants divided by TA-G concentration in control plants) according to the infestation history using Kruskal–Wallis rank sum tests for the local and regional pairs separately. To test for heritable variation in latex secondary metabolite concentrations, we analysed constitutive TA-G, total PIE and total TritAc concentrations according to infestation history for each area separately with Kruskal–Wallis rank sum tests for both the local and the regional pairs. The diploid individuals from Germany (5 of 108 plants) were excluded from the analysis to avoid confounding effects caused by their different ploidy levels. To measure selection on latex secondary metabolite concentrations in the presence and absence of *M. melolontha* herbivory, we calculated directional and disruptive/stabilizing selection gradients using regression coefficients for each metabolite class separately [43–45]. To obtain the regression coefficients, relative fitness was calculated by dividing total seed mass of each individual by the mean total seed mass of the population. Total seed mass is a good approximation for plant fitness, as it reflects total reproductive investment by integrating both seed number and seed weight [46]. The directional selection gradient was calculated by linear regression of the relative fitness to the concentration of latex secondary metabolites. The selective effect of *M. melolontha* on the latex secondary metabolite was determined by comparing the slope of the selection gradients in the presence and absence of this herbivore (significant interaction between treatment and secondary metabolites in a linear regression). The robustness of the selection gradients and of the net effect of *M. melolontha* on the selection was investigated by excluding the two indivduals with the highest TA-G concentration from the analysis. An estimate for stabilizing/disruptive selection gradient was obtained by the second-order coefficient of a quadratic regression of the relative fitness on the latex secondary metabolites [43–45]. The offspring from Germany in the local pair were excluded from the analysis to avoid non-independence of data points due to the replication of clonal individuals that were already present in the regional pair. Individuals that contained other non-identified sesquiterpene lactones or that did not produce seeds were excluded from the analysis. Broad sense heritability (H^2), which we could estimate from different replicated asexual clones, was calculated using the replicated offspring from the German triploid populations (in the local and regional pairs). H^2 of latex secondary metabolites was calculated as $H^2 = V_g/V_T$, where V_g is the total genetic variance and V_T the total phenotypic variance (genetic and environmental variance) [47]. H^2 was estimated for the control and herbivore-infested plants separately. Differences in ploidy levels between infestation histories were analysed with χ^2-tests for each area separately. All statistical analyses were performed in R v. 3.1.1.

3. Results

To explore the influence of *M. melolontha* abundance on the phenotypic variation in latex chemistry in nature, we surveyed the root herbivore community and latex secondary metabolites profiles in *T. officinale* populations that evolved under different *M. melolontha* pressure over the last 20 years in Germany and the Swiss Alps (figure 1*a*). As expected, *M. melolontha* was the major insect root herbivore in both number and size, and its present abundance matched the historical records (figure 1*b*; electronic supplementary material, table S2). The abundance of the other root feeding insects, including other Scarabaeidae and *Agriotes* spp. larvae, did not differ significantly between populations with different infestation histories and did not covary with *M. melolontha*

abundance (figure 1*b*; electronic supplementary material, table S2). In both Germany and the Swiss Alps, all major latex secondary metabolite classes, TA-G, PIEs and TritAc, were positively associated with *M. melolontha* abundance (figure 2; electronic supplementary material, table S3). The concentration of TA-G was higher in the populations under strong *M. melolontha* pressure compared with both local and regional control populations with low *M. melolontha* abundance. Similarly, the total concentrations of PIEs and TritAcs were higher in the heavily infested populations than in either the regional or the local control populations, although the patterns were weaker than for TA-G. Taken together, these data indicate that *M. melolontha* shapes intraspecific phenotypic variation in the concentration of latex secondary metabolites in natural *T. officinale* populations.

The observed differences in latex secondary metabolite concentrations in natural *T. officinale* populations may have resulted from different ploidy levels, phenotypic plasticity and/or heritable variation. To test these hypotheses, we analysed offspring from the field-sampled plants for ploidy, latex secondary metabolites and seed production in the presence and absence of *M. melolontha* larvae; 95% of the plants from the German populations were found to be triploid apomicts, while all plants from the Swiss Alps were diploid outcrossers. Plant ploidy levels did not differ between populations within Germany (herbivore : local control: $\chi^2_{(1)} = 0.86$, $p = 0.35$; local herbivore : regional control: $\chi^2_{(1)} = 0$, $p = 1.0$; χ^2-tests) or the Swiss Alps ($\chi^2_{(1)} = 0$, $p = 1.0$ for both local herbivore : local control and local herbivore : regional control, χ^2-tests), showing that the herbivore-associated patterns were not confounded by differences in plant ploidy levels and that *M. melolontha* pressure had no influence on the abundance of diploids and triploids. Under controlled conditions, *M. melolontha* infestation increased TA-G concentration by 36% ($\chi^2_{(1)} = 21.3$, $p < 4 \times 10^{-6}$, Kruskal–Wallis rank sum test), while total PIE and total TritAc concentrations were reduced by 9–16% (figure 3; PIEs: 16%, $\chi^2_{(1)} = 12.7$, $p = 0.0004$, TritAcs: 9%, $\chi^2_{(1)} = 4.3$, $p = 0.04$, Kruskal–Wallis rank sum tests). Inducibility of TA-G concentration did not differ between infestation histories (electronic supplementary material, figure S1). Inducibility of TA-G may therefore have contributed to the patterns observed in the field. Nevertheless, we also observed heritable variation of latex secondary metabolite concentrations among *T. officinale* populations with different infestation histories: in the triploid apomictic populations in Germany, constitutive TA-G concentrations were 60% higher in the populations with high *M. melolontha* abundance than the regional controls (figure 4; $\chi^2_{(1)} = 5.6$, $p = 0.02$, Kruskal–Wallis rank sum test). No difference in constitutive TA-G concentrations was found between the populations with high *M. melolontha* abundance and the local control populations (figure 4; $\chi^2_{(1)} = 1.3$, $p = 0.26$, Kruskal–Wallis rank sum test). Constitutive total TritAc concentrations were 20–25% higher in the heavily infested populations compared with both local and regional controls (local controls: $\chi^2_{(1)} = 8.7$, $p = 0.003$; regional controls: $\chi^2_{(1)} = 3.1$, $p = 0.08$, Kruskal–Wallis rank sum tests). No difference in total PIE concentrations was observed between offspring populations (local controls: $\chi^2_{(1)} = 0.1$, $p = 0.71$; regional controls: $\chi^2_{(1)} = 1.4$, $p = 0.24$). In the sexual, obligate outcrossing populations from the Swiss Alps, the concentrations of the three latex metabolite classes did not differ between offspring populations (figure 4; TA-G: local controls:
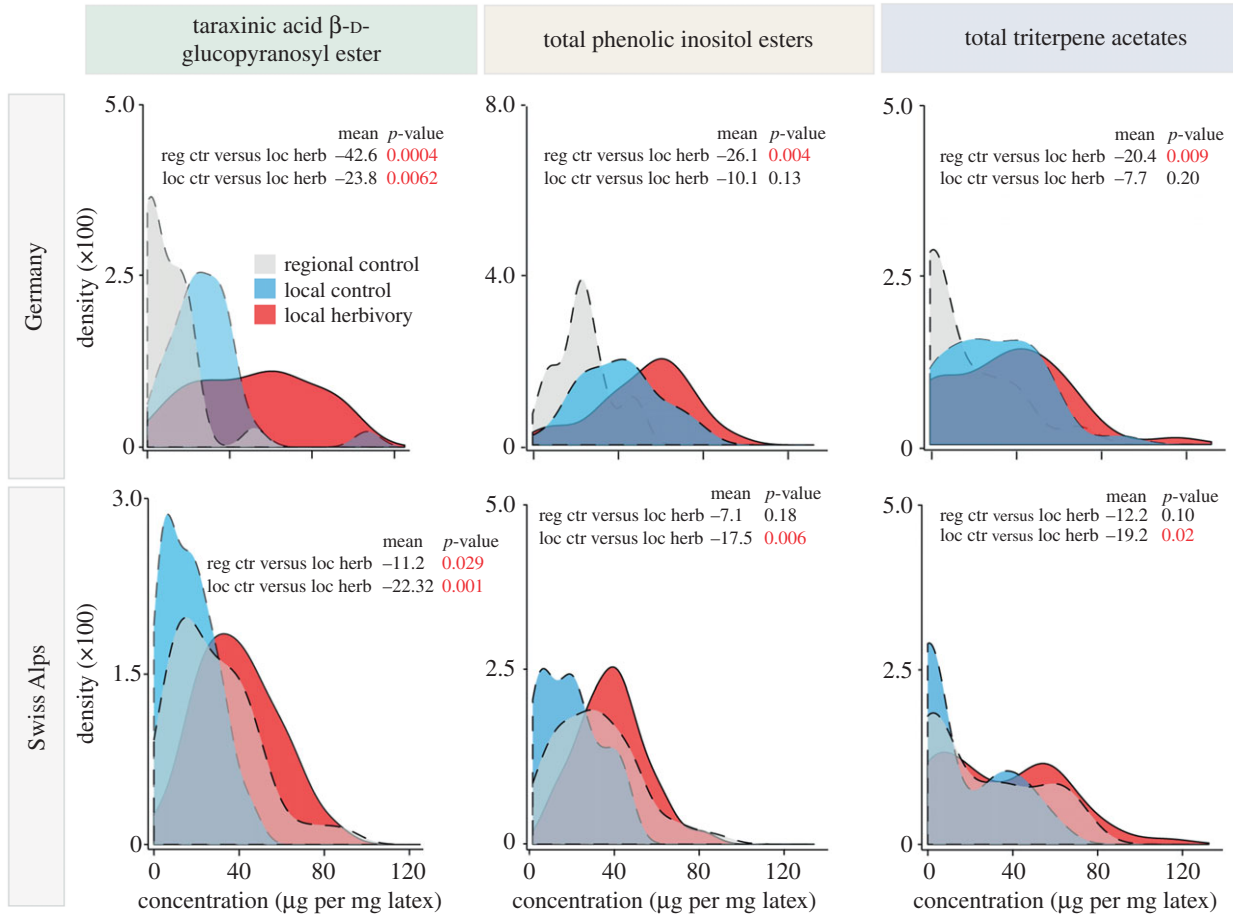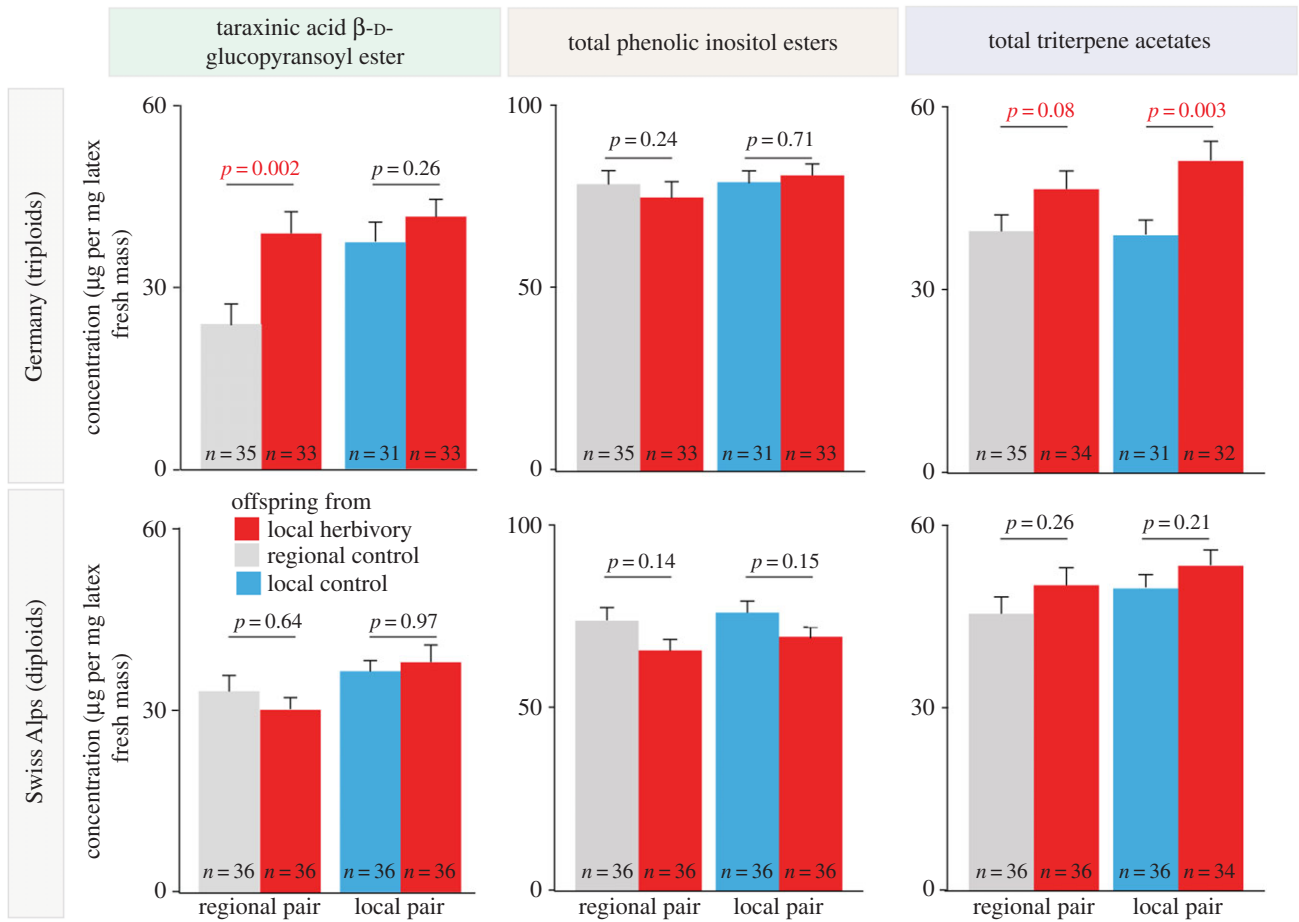
Figure 2. Latex secondary metabolite concentrations in *T. officinale* field populations. High latex secondary metabolite concentrations were associated with high *M. melolontha* abundance in both Germany and the Swiss Alps. Distributions show density estimates of latex secondary metabolite concentrations of *T. officinale* populations. *p*-values of linear mixed effect models are shown. reg ctr, regional control; loc ctr, local control; loc herb, local herbivory.
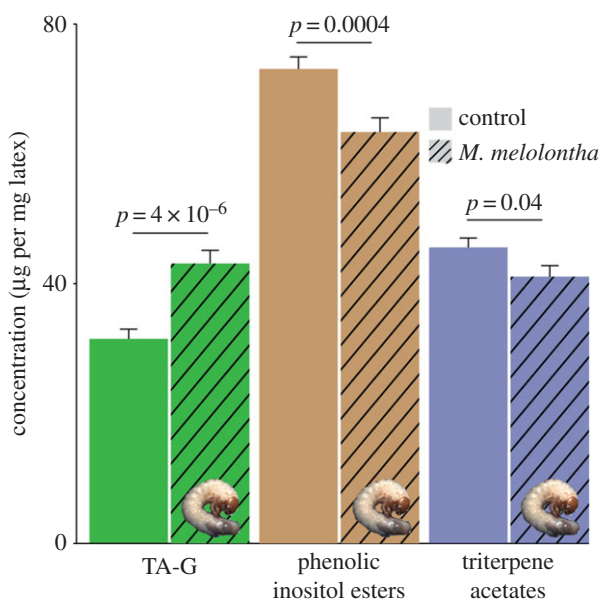
Figure 3. Induction of latex secondary metabolites upon *M. melolontha* infestation. Taraxinic acid β-D-glucopyranosyl ester (TA-G) concentration was induced, whereas total phenolic inositol ester and total triterpene acetate concentrations were reduced upon *M. melolontha* feeding in the offspring of the field populations. *p*-values from Kruskal–Wallis rank sum tests are shown for each metabolite class separately. Error bars correspond to standard errors (+s.e.). (Online version in colour.)

$\chi^2_{(1)} = 0.001$, $p = 0.97$; regional controls: $\chi^2_{(1)} = 0.2$, $p = 0.64$; PIEs: local controls: $\chi^2_{(1)} = 0.2$, $p = 0.15$; regional controls: $\chi^2_{(1)} = 2.2$, $p = 0.14$; TritAcs: local controls: $\chi^2_{(1)} = 1.6$, $p = 0.21$; regional controls: $\chi^2_{(1)} = 1.3$, $p = 0.26$, Kruskal–Wallis rank sum tests).

The fixation of phenotypic variation among populations requires divergent selection and heritability. We determined selection gradients by correlating secondary metabolite concentrations with total seed mass in the offspring from the field-sampled plants. For TA-G, the directional selection gradient (β) was positive in the presence ($\beta = 0.013 \pm 0.007$ (mean $\pm$ s.e.), $p = 0.05$) of *M. melolontha* and negative in its absence (figure 5; $\beta = -0.025 \pm 0.008$, $p = 0.002$), resulting in a strong effect of herbivory on the selection of TA-G (table 1). As the selection gradient in the presence of *M. melolontha* may be affected by two individuals with high TA-G concenration, we removed them from the analyses. Although the selection gradient of TA-G in the presence of *M. melolontha* was affected by the two individuals ($\beta = -0.007 \pm 0.006$, $p = 0.25$), the net effect of herbivory on the selection of TA-G was robust to their removal (electronic supplementary material, table S4). For total PIEs and TritAcs, no significant directional selection gradients and no influence of herbivory were observed (table 1). For all major latex secondary metabolites, no signature of stabilizing or disruptive selection was found (electronic supplementary material, table S5). We further measured

Figure 4. Latex secondary metabolite concentrations in the offspring of the field populations. Heritable variation in the constitutive concentrations of taraxinic acid β-D-glucopyranosyl ester (TA-G) and total triterpene acetates were observed in the triploid, clonal offspring of the German populations but not in the diploid, sexual offspring of the populations of the Swiss Alps. Two offspring from either regional control and local herbivory (regional pair) or local control and local herbivory (local pair) were grown together in one pot. *p*-values from Kruskal–Wallis rank sum tests are shown. Sample size is shown inside each bar. Error bars correspond to standard errors (+s.e.).

broad-sense heritability (H^2) for all three metabolite classes. H^2 ranged between 0.34 and 0.65 for the three metabolite classes in the absence and presence of *M. melolontha*, with highest heritability estimates found for TA-G (figure 5). Together, these experiments show that in *T. officinale* the concentrations of the three latex secondary metabolite classes are heritable, and that TA-G concentration but not total PIE or total TritAc concentrations are under *M. melolontha*-imposed divergent selection.

4. Discussion

Plant roots display high concentrations and substantial intraspecific variation in secondary metabolites [12]. Despite the importance of roots for plant fitness, the function and evolution of these metabolites is often unknown [11]. We previously showed that the latex-derived sesquiterpene lactone TA-G is a major defensive compound in *T. officinale* roots that protects plants against *M. melolontha* attack [32]. Here, we demonstrate that *M. melolontha* can drive intraspecific variation of TA-G concentration in natural *T. officinale* populations. Three parallel lines of evidence support this hypothesis, as discussed below.

Taraxacum officinale populations that were growing under high *M. melolontha* pressure over the last 20 years had higher

concentrations of the three major secondary metabolites in their root latex than plants growing under low *M. melolontha* herbivory. The pattern was most pronounced for the sesquiterpene lactone TA-G. As control and herbivore-infested populations of each area experienced similar abiotic and biotic conditions, and as no other root herbivore covaried with *M. melolontha* abundance, this screen suggests that *M. melolontha* can shape phenotypic variation of plant chemistry in nature. Such herbivore-associated patterns have often been predicted [48], but rarely been shown in nature either above [7,9] or below ground [21]. The positive association between TA-G concentration and *M. melolontha* abundance could be driven by two processes: first, TA-G may act as a defence and may be positively selected or induced by *M. melolontha*. Second, *M. melolontha* may preferentially occur on high TA-G containing plants. Previous experiments show that TA-G deters rather than attracts *M. melolontha* [32]. Furthermore, the data presented here provide evidence for both positive selection and induction of TA-G under *M. melolontha* attack. Therefore, the association between TA-G concentration and *M. melolontha* abundance in the field is most probably due to the defensive properties of TA-G.

We found that heritable variation contributed to the phenotypic differences of latex secondary metabolites in the field. TA-G and total TritAc levels were higher in the herbivore-infested populations compared with control
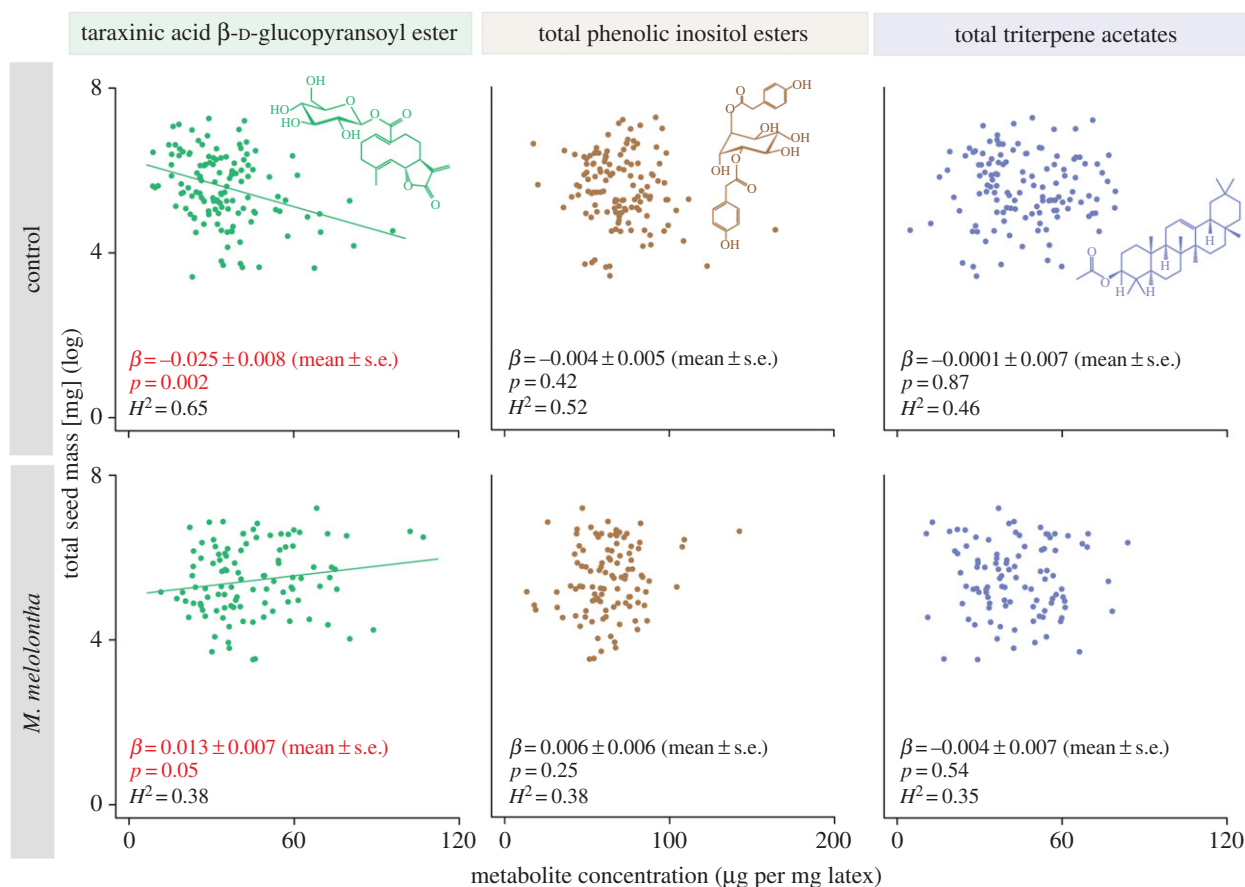
Figure 5. Herbivory-dependent, divergent selection patterns of taraxinic acid β-D-glucopyranosyl ester (TA-G) concentrations. Correlations of TA-G, total phenolic inositol ester and total triterpene acetate concentrations with total seed mass (log-transformed) of different *T. officinale* genotypes in the absence (upper panel) and the presence (lower panel) of *M. melolontha* are shown. Broad-sense heritability (H^2) and *p*-values of directional selection gradients (β) are shown. Representative chemical structures are depicted. s.e., standard error. (Online version in colour.)

Table 1. Analysis of directional selection of *M. melolontha* on latex secondary metabolites. Relative fitness was analysed as a function of latex secondary metabolites in the absence and the presence of *M. melolontha*. Significant interaction terms indicate that *M. melolontha* exerts selection on the metabolite.

	estimate	s.e.	*p*-value
herbivory	−1.1	0.72	0.14
TA-G	−0.024	0.008	0.003**
total PIEs	0.0046	0.006	0.44
total TritAc	9.2^{-5}	0.006	0.99
herbivory : TA-G	0.034	0.01	0.001**
herbivory : total PIEs	−0.002	0.009	0.83
herbivory : total TritAc	−0.004	0.01	0.69

populations in the offspring of the apomictic, triploid populations when grown in a common garden. Although confounding effects due to the geographical distance between the populations cannot fully be discounted, these data indicate that *M. melolontha* can affect heritable variation in TA-G concentration in the triploid populations. By contrast, offspring populations of the diploid populations did not differ in the concentration of any of the three metabolite classes. The lack of heritable variation between diploid populations may be due

to outcrossing of the parental plants from heavily infested populations with plants growing under low *M. melolontha* pressure. *Taraxacum officinale* is visited by a broad range of pollinators [34]; thus, pollen transport over several kilometres is likely to occur [49]. By contrast, *M. melolontha* pressure can differ dramatically between sites that are only a few metres away from each other. Furthermore, recombination during meiosis in the diploid individuals may have led to the loss of allelic combinations that were responsible for the high TA-G concentrations in the mother plants. Our observation suggests that beneficial traits, such as protection against herbivores, can be selected faster in asexual than in sexual populations. This is in line with the general notion that differentiation between populations is established more rapidly in asexual than sexual populations [50], and highlights the importance of the plant reproductive strategy for herbivore-dependent natural selection.

The establishment of heritable variation between populations requires differential selection and heritability. Selection gradient analyses of the three metabolite classes in the presence and absence of *M. melolontha* showed negative selection of TA-G in the absence of *M. melolontha* and positive selection of TA-G in the presence of this herbivore, suggesting that *M. melolontha* attack leads to divergent selection of this sesquiterpene lactone. Negative correlations between the concentration of defensive metabolites and plant performance in the absence of herbivory are usually taken to indicate the potential fitness costs of compounds. However,

the assumption that secondary metabolites are costly for plants is very controversial, with few examples of negative correlations between growth and defence [51,52] and many other examples with no apparent correlation [53,54]. Fitness costs can include both directs costs (i.e. costs of production of the metabolites) and indirect costs, such as decreases in mutualistic interactions [55]. Arbuscular mycorrhizal fungi for instance—which are often associated with *T. officinale* in the field [56]—may be hidden players in this context, as they affect plant fitness and resistance, and modulate primary and secondary metabolites [57–59]. Although mycorrhization was not measured in the present study, the common garden experiment is unlikely to be confounded by variation in mycorrhization, as the plants were grown simultaneously in the same soil and environment. Furthermore, TA-G accumulates specifically in the lacticifers [31], which are associated with the vascular tissues [60] and are very unlikely to come into direct contact with mycorrhizal fungi, which colonize the outer cell layers [61]. This spatial separation suggests that direct feedback effects between mycorrhizal colonization and TA-G abundance are unlikely. Although our experiments do not allow for a detailed assessment of the specific mechanisms behind the negative correlation of TA-G concentration and total seed mass in the absence of *M. melolontha* herbivory, they strongly suggest that TA-G production is costly for *T. officinale* growing under low root herbivore pressure.

The positive correlation between TA-G concentration and plant fitness indicates that the costs of TA-G are outweighed by its beneficial effect under high root herbivore pressure. Although this pattern was influenced by two individuals with high TA-G concentrations, the net effect of *M. melolontha* herbivory on the selection gradient was highly significant and robust to their removal. The beneficial effects of TA-G against root herbivores is further supported by our previous manipulative experiments demonstrating that TA-G deters *M. melolontha* feeding and thereby protects roots and improves plant performance [32]. Contrary to our previous observation that TA-G is not induced in different *T. officinale* genotypes after 11 days of *M. melolontha* herbivory in the greenhouse [32], we show here that TA-G is induced by 36% in plants that are attacked for 1 year under field conditions. Differences in infestation timing, growth conditions and plant genetic backgrounds may account for this result. The induction of TA-G upon prolonged *M. melolontha* infestation fits its role as a potentially costly defence, as inducibility is commonly regarded as a cost-saving strategy in the absence of herbivory [62]. Apart from divergent selection of TA-G in the presence and absence of *M. melolontha*, we also found high broad-sense heritability of TA-G concentration in the triploid plants. These data indicate that variation in *M. melolontha* abundance can lead to evolutionary changes of TA-G concentration over time.

Taken together, the association of TA-G concentration with *M. melolontha* abundance in the field, the heritable differences in TA-G concentration among triploids, the patterns of divergent selection of TA-G concentration in the absence and presence of *M. melolontha*, the relatively high heritability estimates of TA-G concentration and the defensive properties of TA-G provide evidence that *M. melolontha* shapes variation in *T. officinale* root latex chemistry in nature. As root herbivory can also affect plant growth and resistance indirectly via soil feedbacks [14], the evolution of plant defences in nature may be modulated by both direct and indirect effects of root

herbivores on plant performance. Additional experiments involving reciprocal transplant experiments, population genetics data to track gene flow and the identification of potential TA-G-associated genes that are under selection may shed further light on the mechanistic underpinning of the observed patterns.

The tremendous diversity of plant secondary metabolites both within and between species has fascinated biologists for decades. Recent studies have shown that the heterogeneous distribution of herbivores can shape plant defensive chemistry above ground [7–9]. Moreover, it was found that heterogeneity in herbivore communities maintains intraspecific variation in defence genes [10]. By contrast, the potential of below-ground herbivores to shape chemical defences has remained elusive [21]. We show that variation in the abundance of a single root herbivore can contribute to the intraspecific variation in the concentration of a root secondary metabolite. We speculate here that secondary metabolites may be under strong selection by root herbivores for several reasons. First, many perennial plants depend on the roots as overwintering organs [63], which probably results in a strong selection for root defences. Second, herbivore avoidance strategies such as tissue shedding or nutrient reallocation may be deployed to a smaller extent in roots than in leaves due to the immediate requirement of roots for the survival of the plant (but see [64,65]). Third, roots are considered as an inferior food source compared with shoots, especially regarding nitrogen content [12]. Chemial defences that reduce the nutritional quality of the food source may therefore be especially effective in the roots. In general, the specific abiotic and biotic conditions that distinguish root herbivore interactions from leaf herbivore interactions [66] may result in distinct secondary metabolite selection patterns below ground. As the soil is rich in microbes [67], secondary metabolites that are active against both herbivores and pathogens may be under particularly strong positive selection, for instance. Integrating such additional factors will be important to further advance our understanding of natural variation in root secondary metabolite diversity and abundance. Taken together, the present study highlights the potential of root herbivores as evolutionary drivers of the chemical defences of their host plants. Thus, one of the central paradigms in plant–herbivore interactions can now be extended to below-ground environments.

References

1. Rainey PB, Travisano M. 1998 Adaptive radiation in a heterogeneous environment. *Nature* **394**, 69–72. (doi:10.1038/27900)

2. Lenormand T. 2002 Gene flow and the limits to natural selection. *Trends Ecol. Evol.* **17**, 183–189. (doi:10.1016/S0169-5347(02)02497-7)

3. Newton AC, Allnutt TR, Gillies ACM, Lowe AJ, Ennos RA. 1999 Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends Ecol. Evol.* **14**, 140–145. (doi:10.1016/S0169-5347(98)01555-9)

4. Sinervo B, Svensson E. 2002 Correlational selection and the evolution of genomic architecture. *Heredity* **89**, 329–338. (doi:10.1038/sj.hdy.6800148)

5. Turner TL, Bourne EC, Von Wettberg EJ, Hu TT, Nuzhdin SV. 2010 Population resequencing reveals local adaptation of *Arabidopsis lyrata* to serpentine soils. *Nat. Genet.* **42**, 260–263. (doi:10.1038/ng.515)

6. Mauricio R, Rausher MD. 1997 Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* **51**, 1435–1444. (doi:10.2307/2411196)

7. Züst T, Heichinger C, Grossniklaus U, Harrington R, Kliebenstein DJ, Turnbull LA. 2012 Natural enemies drive geographic variation in plant defenses. *Science* **338**, 116–119. (doi:10.1126/science.1226397)

8. Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen JP. 2012 Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science* **338**, 113–116. (doi:10.1126/science.1225977)

9. Prasad KVSK *et al.* 2012 A gain-of-function polymorphism controlling complex traits and fitness in nature. *Science* **337**, 1081–1084. (doi:10.1126/science.1221636)

10. Kerwin R *et al.* 2015 Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. *eLife* **4**. (doi:10.7554/eLife.05604)

11. Rasmann S, Agrawal AA. 2008 In defense of roots: a research agenda for studying plant resistance to belowground herbivory. *Plant Physiol.* **146**, 875–880. (doi:10.1104/pp.107.112045)

12. van Dam NM. 2009 Belowground herbivory and plant defenses. *Annu. Rev. Ecol. Evol. Syst.* **40**, 373–391. (doi:10.1146/annurev.ecolsys.110308.120314)

13. Rasmann S, Erwin AC, Halitschke R, Agrawal AA. 2011 Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *J. Ecol.* **99**, 16–25. (doi:10.1111/j.1365-2745.2010.01713.x)

14. Sonnemann I, Hempel S, Beutel M, Hanauer N, Reidinger S, Wurst S. 2013 The root herbivore history of the soil affects the productivity of a grassland plant community and determines plant response to new root herbivore attack. *PLoS ONE* **8**, e56524. (doi:10.1371/journal.pone.0056524)

15. Brown VK, Gange AC. 1990 Insect herbivory below ground. *Adv. Ecol. Res.* **20**, 1–58. (doi:10.1016/S0065-2504(08)60052-5)

16. Erb M, Huber M, Robert CAM, Ferrieri AP, Machado RAR, Arce CCM. 2013 The role of plant primary and secondary metabolites in root-herbivore behaviour, nutrition and physiology. In *Behaviour and physiology of root herbivores,* Advances in Insect Physiology, vol. 45 (eds SN Johnson, I Hiltpold, TCJ Turlings), pp. 53–95. San Diego, CA: Elsevier Academic Press Inc.

17. Sarwar M, Kirkegaard JA, Wong PTW, Desmarchelier JM. 1998 Biofumigation potential of brassicas. III. *In vitro* toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant Soil.* **201**, 103–112. (doi:10.1023/A:1004381129991)

18. Mandal SM, Chakraborty D, Dey S. 2010 Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signal. Behav.* **5**, 359–368. (doi:10.4161/psb.5.4.10871)

19. Dixon RA, Paiva NL. 1995 Stress-induced phenylpropanoid metabolism. *Plant Cell.* **7**, 1085–1097. (doi:10.1105/tpc.7.7.1085)

20. Ramakrishna A, Ravishankar GA. 2011 Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* **6**, 1720–1731. (doi:10.4161/psb.6.11.17613)

21. Watts SM, Dodson CD, Reichman OJ. 2011 The roots of defense: plant resistance and tolerance to belowground herbivory. *PLoS ONE* **6**, e0018463. (doi:10.1371/journal.pone.0018463)

22. Keller S. 2004 Engerlingsvorkommen und Bekämpfung in der Schweiz. *Nachrbl. Deut. Pflanzensch.* **56**, 88–90.

23. Pener MP. 2013 Hormonal effects on flight and migration. In *Endocrinology II*, vol. 8 (ed. GA Kerkut), pp. 491–550. Amsterdam, The Netherlands: Elsevier.

24. Blunck H. 1938 Über die Möglichkeit zur Bekämpfung der Maikäferengerlinge mittels landwirtschaftlicher Kulturmassnahmen. *Z. Pflanzenkrank. Pflanzensch.* **48**, 253–272.

25. Hasler T. 1986 Abundanz- und Dispersionsdynamik von *Melolontha melolontha* (L.) in Intensivobstanlagen. PhD thesis, Eidgenössische Technische Hochschule Zürich, Zurich, Switzerland.

26. Hauss R, Schütte F. 1976 Experiments on polyphagous habits of white grubs *Melolontha melolontha* on plants of grassland. *Anz. Schädlingsk. Pflanzensch. Umweltsch.* **49**, 129–132. (doi:10.1007/BF01984983)

27. Hauss R, Schütte F. 1978 Über die Eiablage des Maikäfers (*Melolontha melolontha* L.) in Abhängigkeit von den Wirtspflanzen des Engerlings. *J. Appl. Entomol.* **86**, 167–174.

28. Scheenen TWJ, Vergeldt FJ, Heemskerk AM, Van As H. 2007 Intact plant magnetic resonance imaging to study dynamics in long-distance sap flow and flow-conducting surface area. *Plant Physiol.* **144**, 1157–1165. (doi:10.1104/pp.106.089250)

29. Verduijn MH, Van Dijk PJ, Van Damme JMM. 2004 Distribution, phenology and demography of sympatric sexual and asexual dandelions (*Taraxacum officinale* s.l.): geographic parthenogenesis on a small scale. *Biol. J. Linn. Soc.* **82**, 205–218. (doi:10.1111/j.1095-8312.2004.00325.x)

30. Agrawal AA, Konno K. 2009 Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. *Annu. Rev. Ecol. Evol. Syst.* **40**, 311–331. (doi:10.1146/annurev.ecolsys.110308.120307)

31. Huber M *et al.* 2015 Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.). *Phytochemistry* **115**, 89–98. (doi:10.1016/j.phytochem.2015.01.003)

32. Huber M *et al.* 2016 A latex metabolite benefits plant fitness under root herbivore attack. *PLoS Biol.* **14**, e1002332. (doi:10.1371/journal.pbio.1002332)

33. von Hofsten CG. 1954 *Studies on the genus* Taraxacum *Wigg with special reference to the group vulgaria DT in Scandinavia*. Stockholm, Sweden: LTs Förlag.

34. Lázaro A, Totland O. 2010 Population dependence in the interactions with neighbors for pollination: a field experiment with *Taraxacum officinale*. *Am. J. Bot.* **97**, 760–769. (doi:10.3732/ajb.0900263)

35. Menken SBJ, Smit E, DenNijs HCM. 1995 Genetical population structure in plants: gene flow between diploid sexual and triploid asexual dandelions (*Taraxacum* section *Ruderalia*). *Evolution* **49**, 1108–1118. (doi:10.2307/2410435)

36. Pinheiro J, Bates D, DebRoy S, Sarkar S, orpteam Rc. 2014 nlme: Linear and nonlinear mixed effects models. R package version 3.1–117 ed. See http://CRAN.R-project.org.

37. Wickham H. 2009 *ggplot2: elegant graphics for data analysis*. New York, NY: Springer.

38. Auguie B. 2012 gridExtra: Functions in grid graphics. R package version 0.9.1 ed. See http://CRAN.R-project.org/package=gridExtra.

39. Loecher M. 2014 RgoogleMaps: overlays on Google map tiles in R. See http://cran.r-project.org/web/packages/RgoogleMaps/index.html.

40. South A. 2011 rworldmap: a new R package for mapping global data. *R J.* **3**, 35–43.

41. R Core Team. 2014 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

42. Bubner B, Gase K, Berger B, Link D, Baldwin I. 2006 Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. *Plant Cell Rep.* **25**, 668–675. (doi:10.1007/s00299-005-0111-4)

43. Rausher MD. 1992 The measurement of selection on quantitative traits—biases due to environmental covariances between traits and fitness. *Evolution* **46**, 616–626. (doi:10.2307/2409632)

44. Lande R, Arnold SJ. 1983 The measurement of selection on correlated characters. *Evolution* **37**, 1210–1226. (doi:10.2307/2408842)

45. Mitchell-Olds T, Shaw RG. 1987 Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution* **41**, 1149–1161. (doi:10.2307/2409084)

46. Venable DL. 1992 Size-number trade-offs and the variation of seed size with plant resource status. *Am. Nat.* **140**, 287–304. (doi:10.1086/285413)

47. Lynch M, Walsh B. 1998 *Genetics and analysis of quantitative traits*. Sunderland, MA: Sinauer.

48. Bernhardsson C, Robinson KM, Abreu IN, Jansson S, Albrectsen BR, Ingvarsson PK. 2013 Geographic structure in metabolome and herbivore community co-occurs with genetic structure in plant defence genes. *Ecol. Lett.* **16**, 791–798. (doi:10.1111/ele.12114)

49. Chifflet R, Klein EK, Lavigne C, Le Feon V, Ricroch AE, Lecomte J, Vaissière BE. 2011 Spatial scale of insect-mediated pollen dispersal in oilseed rape in an open agricultural landscape. *J. Appl. Ecol.* **48**, 689–696. (doi:10.1111/j.1365-2664.2010.01904.x)

50. Heywood JS. 1991 Spatial analysis of genetic variation in plant populations. *Annu. Rev. Ecol. Syst.* **22**, 335–355. (doi:10.1146/annurev.es.22.110191.002003)

51. Züst T, Joseph B, Shimizu KK, Kliebenstein DJ, Turnbull LA. 2011 Using knockout mutants to reveal the growth costs of defensive traits. *Proc. R. Soc. B* **278**, 2598–2603. (doi:10.1098/rspb.2010.2475)

52. Paul-Victor C, Züst T, Rees M, Kliebenstein DJ, Turnbull LA. 2010 A new method for measuring relative growth rate can uncover the costs of defensive compounds in *Arabidopsis thaliana*. *New Phytol*. **187**, 1102–1111. (doi:10.1111/j.1469-8137.2010.03325.x)

53. Koricheva J. 2002 Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology* **83**, 176–190. (doi:10.1890/0012-9658(2002)083[0176:MAOSOV]2.0.CO;2)

54. Almeida-Cortez JS, Shipley B, Arnason JT. 1999 Do plant species with high relative growth rates have poorer chemical defences? *Funct. Ecol.* **13**, 819–827. (doi:10.1046/j.1365-2435.1999.00383.x)

55. Strauss SY, Rudgers JA, Lau JA, Irwin RE. 2002 Direct and ecological costs of resistance to herbivory. *Trends Ecol. Evol.* **17**, 278–285. (doi:10.1016/S0169-5347(02)02483-7)

56. Becklin KM, Galen C. 2009 Intra- and interspecific variation in mycorrhizal associations across a heterogeneous habitat gradient in alpine plant communities. *Arct. Antarc. Alp. Res.* **41**, 183–190. (doi:10.1657/1938-4246-41.2.183)

57. Gange AC, Brown VK, Sinclair GS. 1994 Reduction of black vine weevil larval growth by vesicular-arbuscular mycorrhizal infection. *Entomol. Exp. Appl.* **70**, 115–119. (doi:10.1111/j.1570-7458.1994.tb00739.x)

58. Vannette RL, Rasmann S. 2012 Arbuscular mycorrhizal fungi mediate below-ground plant–herbivore interactions: a phylogenetic study. *Funct. Ecol.* **26**, 1033–1042. (doi:10.1111/j.1365-2435.2012.02046.x)

59. Cameron DD, Neal AL, van Wees SCM, Ton J. 2013 Mycorrhiza-induced resistance: more than the sum of its parts? *Trends Plant Sci.* **18**, 539–545. (doi:10.1016/j.tplants.2013.06.004)

60. Razak A, Bahri SB. 2000 Development and distribution of laticifers in plants. Doctoral thesis, Durham University. See http:etheses.dur.ac.uk/4485.

61. Smith SE, Read D. 2008 *Mycorrhizal symbiosis*, 3rd edn. London, UK: Academic Press.

62. Baldwin I, Hamilton WIII. 2000 Jasmonate-induced responses of *Nicotiana sylvestris* results in fitness costs due to impaired competitive ability for nitrogen. *J. Chem. Ecol.* **26**, 915–952. (doi:10.1023/A:1005408208826)

63. Crawley M. 2009 The structure of plant communities. In *Plant ecology*, vol. 2 (ed. M Crawley), pp. 475–531. New York, NY: Wiley.

64. Robert CAM *et al.* 2014 Induced carbon reallocation and compensatory growth as root herbivore tolerance mechanisms. *Plant Cell Environ.* **37**, 2613–2622. (doi:10.1111/pce.12359)

65. Robert CAM, Schirmer S, Barry J, Wade French B, Hibbard BE, Gershenzon J. 2015 Belowground herbivore tolerance involves delayed overcompensatory root regrowth in maize. *Entomol. Exp. Appl.* **157**, 113–120. (doi:10.1111/eea.12346)

66. Johnson SN, Erb M, Hartley SE. In press. Roots under attack: contrasting plant responses to below- and aboveground insect herbivory. *New Phytol*. (doi:10.1111/nph.13807)

67. Curtis TP, Sloan WT, Scannell JW. 2002 Estimating prokaryotic diversity and its limits. *Proc. Natl Acad. Sci USA* **99**, 10 494–10 499. (doi:10.1073/pnas.142680199)