ECOLOGY LETTERS

Ecology Letters, (2019) **22**: 292–301 doi: 10.1111/ele.13190

LETTER

Environmental gradients and the evolution of tri-trophic interactions

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Abstract

Long-standing theory predicts herbivores and predators should drive selection for increased plant defences, such as the specific production of volatile organic compounds for attracting predators near the site of damage. Along elevation gradients, a general pattern is that herbivores and predators are abundant at low elevation and progressively diminish at higher elevations. To determine whether plant adaptation along such a gradient influences top-down control of herbivores, we manipulated soil predatory nematodes, root herbivore pressure and plant ecotypes in a reciprocal transplant experiment. Plant survival was significantly higher for low-elevation plants, but only when in the presence of predatory nematodes. Using olfactometer bioassays, we showed correlated differential nematode attraction and plant ecotype-specific variation in volatile production. This study not only provides an assessment of how elevation gradients modulate the strength of trophic cascades, but also demonstrates how habitat specialisation drives variation in the expression of indirect plant defences.

Keywords

Entomopathogenic nematodes, local adaptation, plant-mediated interaction, root herbivore, tri-trophic interactions, trophic cascade, volatile organic compounds.

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INTRODUCTION

Carnivores' effects on plant biomass and survival through the suppression of plant-eating insects (i.e. top-down control of herbivores) (Slobodkin *et al.* 1967; Polis 1999; Estes *et al.* 2011), are the best examples of how indirect interactions determine population dynamics and ecosystem functioning (Loreau 2010; Schmitz *et al.* 2015). While several examples have shown that carnivore presence has a positive effect on plant fitness, disentangling the mechanisms driving variation in the magnitude of such effects, both within and across ecosystems, remains a central question in ecology (Brett & Goldman 1996; Schmitz *et al.* 2000; Estes *et al.* 2011). Drivers of variation in the strength of trophic cascades include top-down forces such as intraguild predation (Vance-Chalcraft *et al.* 2007), multi-carnivore interactions (Sih *et al.* 1998), or the non-consumptive effects of carnivores on herbivores (Schmitz *et al.* 2000; Preisser *et al.* 2005). In addition, bottom-up forces such as diversity and productivity, predominantly mediate the magnitude of herbivore top-down control (Oksanen *et al.* 1981; Uriarte & Schmitz 1998; Pace *et al.* 1999; Polis 1999; Schmitz *et al.* 2000). For instance, resources have been shown to impact the expression of plant phytochemistry mediating multitrophic interactions (Mooney *et al.* 2010; Hunter 2016). Specifically, the release of volatile organic compounds (VOCs) by herbivore-infested plants facilitates carnivore attraction near the site of wounding and subsequent herbivore suppression, via so-called indirect plant defences, in both above- (Turlings *et al.* 1990) and belowground compartments of the ecosystem (Rasmann *et al.* 2005), but this depends on resource availability and biotic interactions (Dicke & Baldwin 2010; Kessler & Heil 2011). Therefore, we postulated that plants' adaptation to different biotic and abiotic environments selects for variation in the emission of VOCs, in turn regulating top-down control of herbivores.

Variation in plant VOCs production is mediated by changes in abiotic and biotic conditions (Gershenzon & Dudareva 2007). Abiotic factors such as climate and soil resources have been shown to affect VOCs production in plants (Copolovici & Niinemets 2016). For instance, along elevation gradients, plants growing at high elevation, colder and more nutrient-stressed sites are physiologically constrained in how many and how much volatiles they can produce (Pellissier *et al.* 2016). Secondly, the biotic environment, such as the abundance of herbivores and carnivores interacting with the plant, should be the main evolutionary driver of traits involved in indirect plant defences, including VOCs production (Dicke & Baldwin 2010). In other words, carnivore attraction should be the selective agent for specific VOCs blends that favour plant survival when under herbivore attack (Kessler & Heil 2011). Along these lines, Schuman *et al.* (2012) showed that in the presence of carnivores, VOCs production in *Nicotiana attenuata* had a positive effect on plant growth. Consequently, the simultaneous variation in abiotic and biotic factors along ecological clines is expected to strongly regulate phytochemical diversity but the subsequent outcome on herbivore top-down control has never been addressed.

Ecotypic adaptation along large-scale ecological clines should result in plants to display different phenotypes through a combination of phenotypic plasticity and adaptive genetic changes (Clausen *et al.* 1947). Therefore, by studying conspecific individuals that originated from different localities along ecological clines within common environments (i.e.

reciprocal transplants and common garden experiments), it is possible to deduce a heritable basis to trait variation mediated by variation in local biotic and abiotic conditions (e.g. Göte 1930; Endler 1977; Woods *et al*. 2011). For instance, variation in climatic conditions along geographical clines modifies the intensity of biotic interactions, ultimately dictating evolutionary changes in plant chemical defence trait expression (Hunter 2016). However, only an integrative approach that combines reciprocal transplant experiments using ecotypes of plants collected along ecological gradients, with mechanistic behavioural studies can address long-standing hypotheses of how plant strategies evolve within widely distributed organisms, and how they influence community dynamics.

To test the hypotheses that plants' adaptation to different habitats shapes the chemical phenotypes, biotic interactions and ultimately the magnitude of herbivore top-down control, we used a belowground tri-trophic system that varies along elevation gradients. In the Alps, grasslands are characterised by thick grass cover, where roots can be damaged by generalist beetle larvae such as *Melolontha melolontha* (Coleoptera: Scarabeidae). Heavy plant infestations are mainly restricted to low-elevation areas with relatively high annual mean temperatures (Hann *et al*. 2015), where soil biodiversity (Fig. S1a) and carnivore activity (Fig. S1b) are generally higher. As many other insects with soil-dwelling stages, beetle larvae can be killed by entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae), which kill arthropods using their symbiotic bacteria to reproduce and survive. EPNs are widespread in soils of all continents except Antarctica, and are used to control several root pests, including beetle larvae (Poinar 1993). First, we conducted common garden experiments at two elevations to estimate the magnitude of herbivore top-down control at different elevations by manipulating plant ecotypes (i.e. high- and low-elevation plants), infestation of *M. melolontha* larvae and EPN presence. We focused on elevation-specific ecotypes of red fescue (*Festuca rubra*), one of the predominant plant species colonising the entire elevational spectrum of the Alps. Second, we performed olfactometer-based bioassays to test differential attraction of EPNs between uninfested and herbivore-infested plants originating from different elevations. Finally, we characterised volatile profiles released by *F. rubra* roots to uncover the underlying mechanisms of EPN attraction. In Table 1, we present the different questions raised by these three approaches and how they come together to address potential selective forces on phytochemical traits regulating top-down control of herbivore and, ultimately, the shape of trophic cascades in natural ecosystems. Specifically, we expected that plants' adaptation to higher elevation would diminish their ability to produce volatiles involved in predator (in this case EPN) recruitment and the resulting herbivore suppression.

MATERIALS AND METHODS

Study system

The red fescue, *F. rubra* (Poales: Poaceae), is a perennial plant with a widespread distribution that spans the collinean (low) to the alpine (high) environment (Landolt 2010). A relatively high degree of genetic isolation in *F. rubra* is ensured by restricted anthesis and low pollen dispersal (Kerguelen & Plonka 1989; Stojanova *et al*. 2018). This genetic isolation makes *F. rubra* a good candidate to test for local adaptation and evolutionary processes producing individual forms that match given environmental conditions, such as those encountered along mountain slopes (Münzbergová *et al*. 2017). In order to study variation in trophic cascades along elevation gradients, we used a generalist herbivore, the beetle, *Melolontha melolontha* (Coleoptera: Scarabaeidae). The larvae develop in soils by feeding on roots for 3 years, causing severe damage to plants, particularly in low-elevation grasslands, where population densities can reach 100 individuals per m^2 (Lewis & Clements 2008). Those soil-dwelling larvae are readily attacked by EPNs. Once inside the host, the third instar juvenile EPNs release pathogenic bacteria in the haemolymph, leading to the rapid host death (Dillman & Sternberg 2012). Species of EPNs in the genera *Steinernema* and *Heterorhabditis* are found in most habitats, including the Swiss Alps (Kergunteuil *et al*. 2016). Most EPNs actively seek their hosts in the soil and, while CO_2 can serve as a general cue for host searching, more specific plant-associated compounds have been shown to inform EPNs of the presence of a root insect herbivore in the surroundings (Turlings *et al*. 2012). For this study we used the generally widespread *Heterorhabtidis megidis* (Rhabditida: Heterorhabditidae), as it has been shown to rely on host–plant cues for several systems such as coniferous plants, poaceous plants and other angiosperms (Rasmann *et al*. 2012). Additionally, nematodes belonging to

Table 1 Workflow and experimental design for testing hypotheses related to plants' indirect defense adaptation along elevation gradients

Question	Hypothesis	Ecological approach	Experimental design
How phytochemicaly mediated tri-trophic interactions influence the top-down control of herbivores?	Phytochemical traits regulating plant performance through predator recruitment and herbivore suppression are under selective pressures.	Trophic cascade	Common garden experiment
Does ecotytpic divergence in phytochemical traits affect plant–herbivore–predator interactions?	Plant trait affecting tri-trophic interactions shape herbivore control by predators and plant performance.	Community ecology	Behavioural assays
Does ecotypic differentiation embrace phytochemical variability?	Phytochemical variability is the fundament for trait selection.	Chemical ecology	Phytochemical analysis

Heterorhabditis genera have been shown to result in relatively fast larval mortality (i.e. after 7 days) for different scarabaeidae species (Koppenhofer & Fuzy 2004). In this study, herbivore insects were collected in areas free of *F. rubra* while we used commercially available EPNs (Andermatt Biocontrol, Andermatt, Switzerland).

Common garden experiment

We collected individual plants of *F. rubra* at different sites located at low elevation (< 700 m above sea level) and alpine elevation (2000–2200 m asl) along three elevational transects during May and June 2016 (Fig. S2). At each site, 15 plants were sampled and potted in 3 L pots with a mixture of soil compost (Ricoter, Aarberg, Switzerland) and sand (Neogard, Gontenschwil, Switzerland) after removing rhizospheric soils to exclude natural nematodes and soil-dwelling larvae occurrence. In order to ensure that there was only one clone per pot, we took care of working with clonal tufts of each *F. rubra* from the field. Nonetheless, potential mixing of clones within one pot could have still happened. We therefore use the term 'ecotype' throughout for distinguishing plants collected at different sites.

According to a full reciprocal transplant design along a single transect, half of the plants was placed at high elevation (site of 'Morcles', Switzerland, elevation: 1519 m; lat./long.: 46°12′54.97″N/7° 2′36.81″E), whereas the second half was transplanted at low elevation (site of 'Lavey', Switzerland, elevation: 437 m; lat./long.: 46°11′55.13″N/7° 1′31.47″E). Differences in climatic conditions between the two altitudes are shown in Fig. S3. On July 22, the plants were separated in three equal batches (i.e. 15 plants each) and assigned to three different treatments (Fig. S4). The first batch consisted of plants that were infested with two *M. melolontha* L2 larvae near the roots of the plants ('herbivore' treatment). The remaining plants were randomly paired and potted at the two extremities of a rectangular flowerpot (dim.: 60 × 17 × 14 cm; flowerpot Geli Standard, Hornbach, Sursee, Switzerland) filled with 10% moist sand in order to mimic olfactometer situation (Fig. S4a). In each flowerpot, one plant was infested with two *M. melolontha* L2 larvae, whereas the other plant remained uninfested. Both insects and roots were surrounded with a fine-mesh metal bag (diam.: 5 cm, height: 15 cm, pore size diameter of the mesh: 2 mm) to impede herbivores to move between plants. Three days after herbivore infestation, 2000 five-day-old infective *H. megidis* juveniles were released at the centre of the rectangular flowerpot. During 72 h, EPNs were allowed to move within the rectangular flowerpot towards either uninfested plants ('control' treatment) or infested plants ('herbivore + carnivore' treatment). After this bioassay period, all plants were placed back in their initial pots with the metal bag surrounding roots and the nematodes that moved inside (Fig. S4b). Immediately after repotting, shoots for all the plants were cut at the soil surface, a manipulation that mimics plant re-growth in natural settings after dormancy. Two month later, on October 5 and 6, we monitored plant survival in order to assess an increase in plant performance in attracting nematodes under root infestation (Fig. 1). Based on plant survival in the treatment i (n_i/N_i) and plant survival in the 'control' treatment ($n_{control}/N_{control}$), we estimated the effect of treatment on plant survival via the log incidence rate ratio: $IRR_i = \log(n_i/N_i) - \log(n_{control}/N_{control})$. For this purpose, we used the function 'escalc' implemented in the package 'metafor' (Viechtbauer 2010) in R (R Development Core Team 2017). Significant IRR differences between treatments were estimated with a Welch modified two-sample *t*-test (function 'tsum.test' from the package 'PASWR'; Arnholt 2012) after Benjamini and Hochberg *P*-value adjustment.

Belowground olfactometer bioassays

Plant collection for laboratory bioassays was conducted during July 2015 along three elevation transects (Fig. S2). For each transect, we collected three individual tufts of *F. rubra* at three samples sites located at low (400–700 m asl), mid (1200–1500 m asl) and high elevation (2000–2200 m asl). In total, 27 tufts were collected and potted in a 2 : 1 mixture composed by soil compost (Ricoter) and sand (Neogard). Plants were stored at the botanical garden of Neuchâtel (Switzerland) and watered twice per week. After 2 months of growth, the tufts were separated in half to obtain 27 clonal pairs of plants. The resulting 54 plants were potted in custom-made two-arm belowground olfactometer pots (Fig. 1; modified after Rasmann *et al.* (2005), one pair of clones per olfactometer and filled with the same mixture as previously described. One month later, one plant per olfactometer was infested with three *M. melolontha* L2 larvae previously starved for 3 days. Two days after plant infestation, the central pieces of the olfactometers were filled with 10% moist sand (Neogard) and assembled with each pair of plants (Fig. 1; $n = 9$ olfactometers per elevation). On day three after initial damage, 2000 infective juveniles of 6-day-old EPN (*H. megidis*) were released in the central chamber of the olfactometers (Fig. 1), and allowed to move towards either uninfested or infested plants (side arms in Fig. 1) during 24 h. Behavioural choices of nematodes were recorded by counting individuals collected in the arms connected to 'uninfested' and 'infested' treatments after Baermann funnel extraction (Rasmann *et al.* 2005). For each elevation, the effect of herbivore treatment on the number of nematodes collected in the two arms of the olfactometers was assessed through a generalised linear model (GLM) with 'quasi-poisson' distribution in order to take into account over-dispersion. Significant differences were estimated with a likelihood-ratio test.

Chemical analyses of VOCs

Immediately after the olfactometer bioassays described above, the 54 plants were removed from the pots, roots were carefully washed and ground to a fine powder in liquid nitrogen. The root material was then stored at −80°C before chemical analyses of VOCs. All analyses were done with a gas chromatograph (GC) (Agilent 7890A, Agilent, Santa Clara, CA, USA) coupled to a mass spectrometer detector (MSD) (Agilent 5975C). Samples were prepared and introduced into the GC with the use of robotic multipurpose samplers (Gerstel

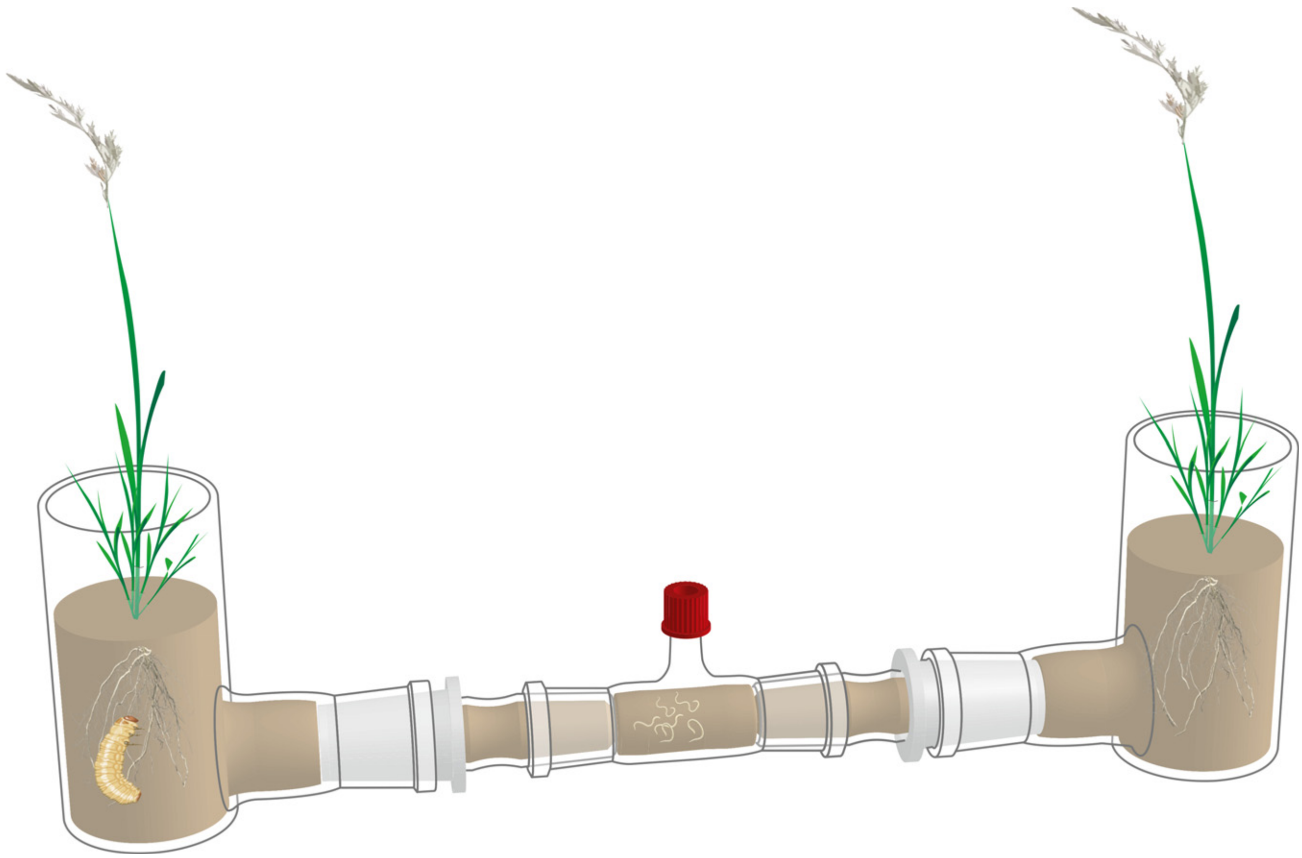

Figure 1 Two-arm belowground olfactometer used for measuring entomopathogenic nematode behaviour. All parts of the olfactometers were filled with moist sand (10% humidity). Two clones of the same plant were placed on each side of the olfactometer and one plant received three *Melolontha melolontha* larvae. *Heterorhabditis megidis* infective juveniles (2000 individuals) were released at the centre of the olfactometer. After 24 h, sand contained in two arms of the olfactometer (separated by 24 cm) was collected and transferred to Baermann funnels in order to assess the number of nematodes that moved towards uninfested or root herbivore-infested plants.

GmBH, Mülheim an der Ruhr, Germany). Compounds were separated on Agilent HP-5MS columns (30 m length × 0.25 mm i.d., and 0.25 μm film thickness). In all cases, the MSD transfer line temperature was set at 280°C and the ion source and quadrupole temperatures were set at 230°C and 150°C respectively. Electron impact mode was used with a scanning over the mass range of 33–250 m/z. Chemical compounds were trapped with the use of solid-phase microextraction (SPME) headspace technique. Samples were incubated in a 20 mL glass vial for 3 min at 35°C before inserting a 100 μm polydimethylsiloxane coated fibre (Supelco, Bellefonte, PA, USA) into the headspace during 20 min. Afterwards, compounds were thermally desorbed from the fibre during 210 s (splitless mode, 250°C, 6.5 psi pressure, 210 mL min^{-1} purge flow, helium carrier gas) before injection onto the GC column. The initial column temperature of 50°C was held for 1 min, then was ramped 6°C min^{-1} until 250°C (hold time 1 min), and finally a 3 min post run at 260°C. The helium flow rate was 0.9 mL min^{-1} (constant flow mode).

The analysis of root-emitted volatiles was based on 20 compounds that were not detected in blank samplings. We retained organic compounds released by a minimum of 10 plants. While three volatiles were identified using pure standards, the additional volatiles were tentatively identified by comparing mass spectra with the NIST05 mass spectra library (Table S1). Peak area for each detected compound was divided by the peak area of the internal standard (Tetralin®; Sigma-Aldrich, St. Gallen, Switzerland), and root emissions of volatiles were finally given as tetralin equivalent nanograms of compound released by gram of root biomass collected (Rasmann *et al*. 2011). For further statistical analyses, root emissions were log-transformed to normalise the distribution of the variables (function 'decostand' from the package 'vegan'; Oksanen *et al*. 2013). First, in order to conduct discriminant analyses to determine to what extent volatile profiles can predict elevation assignment for each plant, we first computed the within-group covariance matrices of explanatory variables (function 'betadisper' from the package 'vegan') based on Anderson's method (Anderson 2006), and then applied a permutation test to verify the homogeneity of multivariate dispersions (function 'permutest' from the package 'vegan'; permutations = 999, $F = 0.73$, $P = 0.50$). This procedure provides a multivariate analogue of Levene's test for homogeneity of variances. Since the application conditions were respected, we performed a linear discriminant (LD) analysis based on elevation groups (function 'lda' from the package 'MASS'; Venables & Ripley 2002). The quality of the resulting model was assessed through the classification success derived from a jackknife-based cross-validation (i.e. leave-one-out process) using the 'CV' argument of the 'lda' function. Overall, 72% of samples were correctly classified although this proportion varied between elevation groups: 89% for low, 56% for intermediate and 72% for high-elevation plants respectively. The first LD1 of the model accounted for 66% of between-group variance. The differences in the distribution of root volatile profiles along LD1 were tested with a pairwise Wilcoxon test coupled with a p-value adjustment based on the Benjamini and Hochberg method. Differences in volatile profiles released by each elevation group of plants were confirmed with a permutational multivariate analysis of variance (permanova) based on distance matrices (function 'adonis' from the package 'vegan'; permutations = 999, $P < 0.02$). Finally, we tested for quantitative differences in single compound emissions between plants using linear mixed models (function 'lmer' from the package 'lme4'; 58) with both 'elevation' and 'treatment' as fixed factors and 'individual tuft' (i.e. genetic background of the mother plant) as random factor. Significant differences between treatments were assessed with least square means multiple comparisons (function 'emmeans' from the package 'emmeans'; Lenth 2016) after Benjamini and Hochberg correction.

RESULTS

Common garden experiment

At low elevation, root-herbivore infestation dramatically reduced plant survival independently from EPN presence or altitudinal origins of plants (Fig. 2a). Surprisingly, infested plant survival tended to be lower in the presence of EPNs as compared with infested plants free of predators. In contrast, at high elevation, we observed differences in plant survival among plant ecotypes and tri-trophic treatments (Fig. 2b). Specifically, at the higher elevation site, the impact of herbivore infestation in the absence of carnivores on plant survival did not differ significantly between low- and high-elevation plant ecotypes (Fig. 2; log IRR $\pm$ SE = -0.63 ± 0.46 and 0.83 ± 0.50 respectively; Welch modified two-sample t-test: $t = 0.80$, df = 10.32, $P = 0.22$). This suggests that plant direct tolerance to herbivory is similar between high- and low-elevation ecotypes. On the other hand, in line with differential carnivore-recruitment predictions, the addition of EPNs to the system only benefitted non-native low-elevation plants (Fig. 2; IRR = 0.18 ± 0.43; Welch modified two-sample t-test: $t = -2.12$, df = 14.67, $P = 0.05$). In addition, as suggested by results displayed in Fig. 2, the presence of EPNs can restore low-elevation plant survival to the same levels as uninfested plants. IRR for high-elevation plants remained similar whether or not EPNs were present in the system (IRR = -1.39 ± 0.65; Welch modified two-sample t-test: $t = 1.29$, df = 3.26, $P = 0.17$).

Belowground olfactomer biossays

Overall, we found that across elevations, a similar amount of nematodes moved through the olfactometer arms (Fig. 3; GLM, likelihood-ratio test, $\chi^2 = 3.88$, df = 1, $P = 0.14$). However, and in line with the results from the common garden experiment, we observed that only low-elevation plants, when infested, attracted more EPNs than uninfested plants (Fig. 3; GLM, likelihood-ratio test, $\chi^2 = 3.94$, df = 1, $P < 0.05$). For mid- and high-elevation plant ecotypes, while EPN movement away from the centre of the olfactometers was generally high (43 ± 3% and 28 ± 5% of nematodes responding to cues from mid- and high-elevation plant ecotypes respectively), EPNs could not discriminate between infested and uninfested plants.

Root VOCs

We collected root volatiles from the experimental plants using SPME, and analysed them with GC coupled with mass spectrometry (GC-MS) (see Table S1). LD analyses showed a net differentiation between volatiles released by low- and high-elevation plants (Fig. 4a). Overall, the total volatile emissions and the diversity of volatile profiles decrease with elevation (Fig. S5). More specifically, of the more than 20 volatiles detected across the three elevation chemotypes, six compounds were differentially expressed according to elevation of origin or infestation treatments (Table S1). This pattern was specifically driven by the differential production of terpenes, in which, for example high-elevation plants produced high levels of β-chamigren, whereas low-elevation plants were characterised by the production of a wider variety of specific terpenoids such as 1,6-dihydrocarveol, α-caryophyllene and eucalyptol (Fig. 4b). But more importantly, paralleling the behavioural observations, only low-elevation plants displayed significant differences in the emission of single volatiles between uninfested and herbivore-infested plants. Infestation of low-elevation plants significantly enhanced the production of 1-octen-3-ol and 3-octanone (Table S1), two metabolites deriving from the oxylipin pathway. To test whether 1-octen-3-ol and 3-octanone were indeed attractive to *H. megidis*, an authentic standard of each compound (Sigma-Aldrich, more than 98% pure) was tested in a four-arms olfactometer (i.e. the same as described above but with four perpendicular arms in total) (Rasmann *et al.* 2005). For this purpose, the system was entirely filled with clean moist sand and a 0.2-mL dose of each compound was injected in the centre of two opposite pots, whereas the two remaining pots received no such treatment (only humid sand). Nematodes were released in the middle of the olfactometer and on the next day nematodes were recovered from the six arms. The arm attached to the pot that had received 1-octen-3-ol and 3-octanone contained 2.2 and 1.8 times more nematodes than the average control arms respectively (Fig. S6).

DISCUSSION

Long-standing theory predicts that herbivores and predators act as the main agents of selection on plant defences, such as the production of VOCs (Kessler & Heil 2011). Habitats where herbivore and predator abundances are low (i.e. high-elevation habitats) should select for a relaxation of defences (Pellissier *et al.* 2012). Our reciprocal transplant experiment

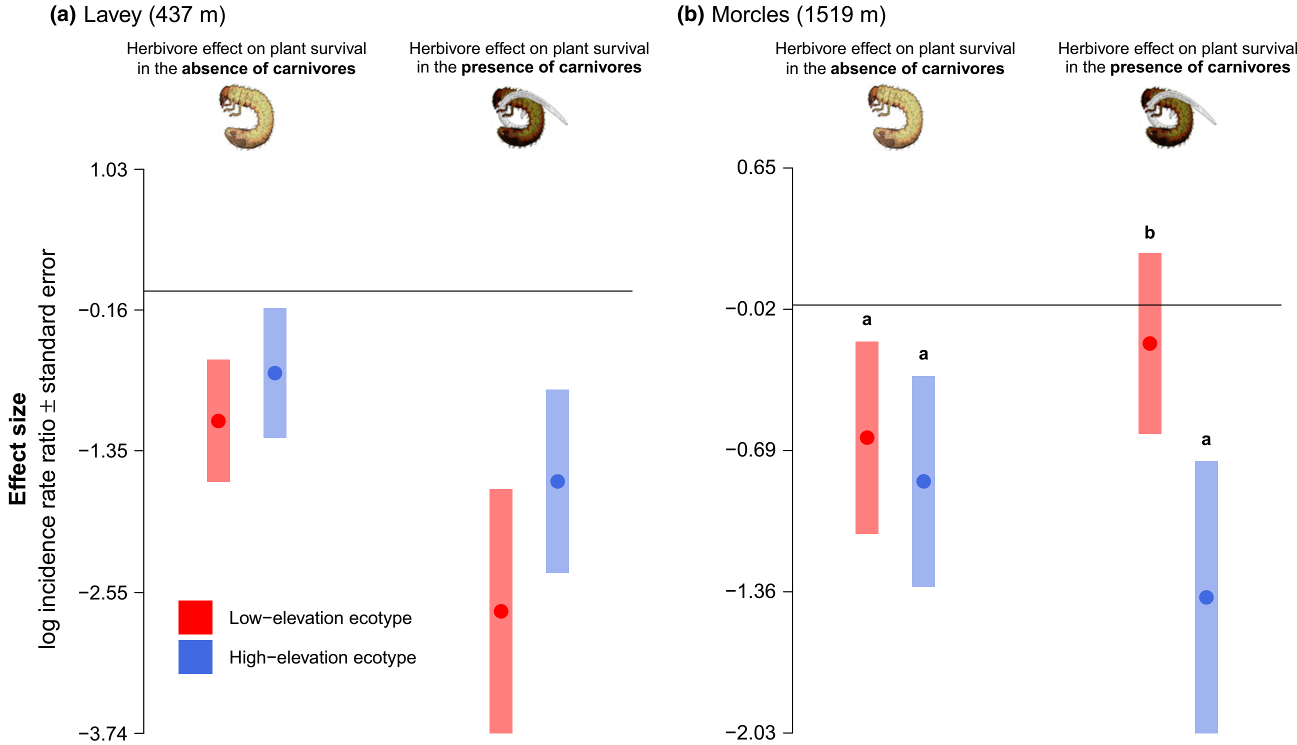

Figure 2 Effect size for the influence of herbivores on plant survival in the absence or the presence of carnivores at low elevation (a) and high elevation (b). Effect size in red and blue are estimated for plants originating from low or high elevation respectively. At low-elevation pairwise comparison in effect sizes does not result in significant differences between treatments. At high elevation, different effect sizes are represented with different letters (Welch modified two-sample t-test, $P \leq 0.05$ after Benjamini and Hochberg correction). Effect sizes of −1.38, −0.69 and −0.29 indicate that treatments reduced survival rates by 75, 50 and 25%, respectively, as compared to uninfested plants. An effect size equals to zero (horizontal line) indicates that the associated treatment restored plant survival as compared to uninfested plants.

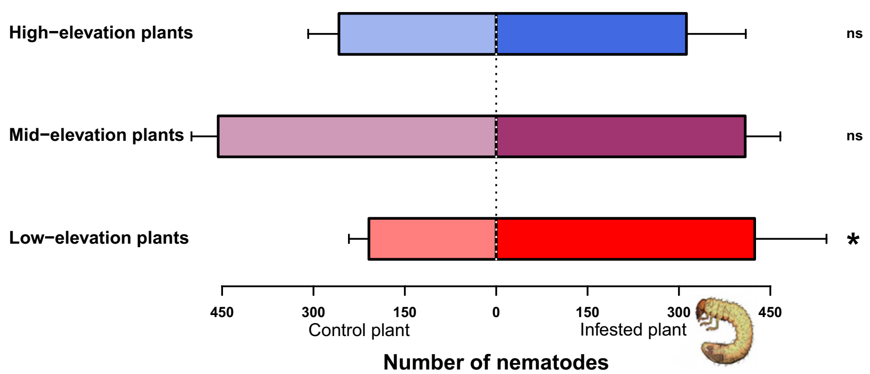

Figure 3 Variation in belowground indirect plant defences along elevation gradients. Dark (right) and light (left) bars indicate mean number of nematodes (± SEM) that moved towards *Melolontha melolontha*-infested plants or towards uninfested plants respectively. Plants were collected at three elevational zones (red = low elevation, magenta = mid elevation and blue = high elevation). Significant differences in nematode attraction between treatments are indicated by an asterisk (GLM, quasi-poisson distribution, likelihood-ratio chisquare test, *: $P < 0.05$, 'ns': non-significant).

results are in line with these expectations, and highlight a relaxation of chemically mediated trophic cascades for high-elevation *F. rubra* ecotypes. Additional laboratory experiments allowed for the detection of plant ecotypic differentiation in the strength of herbivore top-down control along elevation gradients. This difference was dependent on inherent differences in the plant's ability to recruit EPNs near their roots through differential expression of VOCs.

The strength of herbivore top-down control decreases with elevation

Classical food web theory predicts that the level of productivity determines the number of trophic levels that persist in a given system (Oksanen *et al.* 1981). For instance, as productivity increases, such as when moving from high to low elevation, we should observe an increase in the degree to which plants are limited by herbivores (Oksanen *et al.* 1981). In

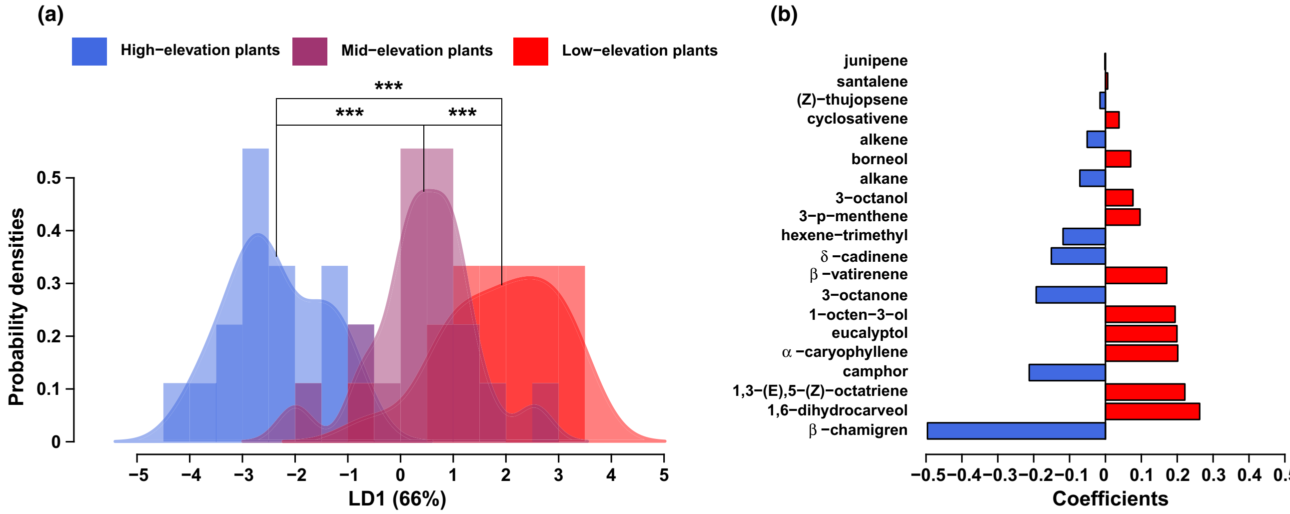

Figure 4 Linear discriminant (LD) analysis and change of root volatile profiles along elevation gradients. (a) Histogram showing the distribution of discriminant scores of root volatile profiles released by plants originating from different elevations. Significant differences between groups of plants are indicated by asterisks (pairwise Wilcoxon test; $P < 0.05$ after Benjamini and Hochberg correction). The first LD1 explains 66% of the between-group variance. (b) Discriminant coefficients for each compound included in the overall volatile blend. Compounds with negative coefficients (in blue) reflect negative discriminant scores of root volatile profiles (high-elevation plants), whereas compounds with positive coefficient (in red) reflect positive discriminant scores (low-elevation plants).

turn, we should observe a correlated increase in how predators can limit herbivore populations and help plants survive. Hence, in highly productive environments, carnivores should exert an indirect mutualistic effect on plants via top-down control of herbivore populations (Uriarte & Schmitz 1998; Schmitz *et al*. 2000). Here, we tested for the effect of plant ecotypic differentiation on the strength of plant-mediated herbivore top-down control with a full reciprocal transplant experiments that included low- and high-elevation plants (i.e. different ecotypes) growing in native and non-native environments. We measured survival of uninfested, herbivore-infested and herbivore-infested plus EPNs *F. rubra* plants using outdoor, custom-made, olfactometers that allowed EPNs to choose between infested and uninfested plants during 3 days. We found that only low-elevation ecotypes of *F. rubra* gained survival advantage from recruiting EPNs. Because *F. rubra* plants are perennials that regrow after every winter season from underground storage organs, our experimental design is close to real natural history conditions. The observed differences in survival thus represent the herbivore/predator effect on different phenotypes expressed by ecotypes. In addition, variation in herbivore effects on plant survival is consistent with a recent meta-analysis testing for the effect herbivore exclusion on plant communities (Jia *et al*. 2018). The equivalent effect size ranges between our study and Jia *et al*. (2018) suggest that the root-herbivore-EPNs effect on *F. rubra* survival we observed is equivalent to the aboveground effects on entire plant communities (Jia *et al*. 2018).

In addition, our reciprocal transplant common garden experiment aimed at testing for local ecotypic adaptation. In other words, if true local adaptation existed, high-elevation ecotypes should have survived better in a tri-trophic context at high elevation and vice-versa, low-elevation ecotypes should have survived better at low elevation. Our results indicated that genetic differentiation along the gradients favours only low-elevation ecotypes, particularly, when climatic conditions are milder. Indeed, the 'high elevation' site was at about 1500 m asl, which is still below the tree-line in the Alps. At this vegetation stage, and during a very hot summer, plants experience optimal growing conditions, which might have favoured plant survival compared to the low-elevation site under the same, but more intensified hot conditions. Low-elevation experimental plants suffered extremely high daily mean temperatures and low precipitation (Fig. S3). Heat might have stimulated root herbivore feeding activity on the drought-stressed plants, leading to high plant mortality at low elevation. These results therefore cannot test potential local adaptation to local climatic conditions, but indicate adaptation to variation in biotic interactions. Abundant herbivore and predator communities lead plants to invest more in defence production. Whether defence investment depends on elevation-dependent resources available (Mooney *et al*. 2010) vs. solely responding to biotic pressure still needs to be fully teased apart. One additional caveat of our field experimental design resides in the need of translocating plants from one container to a smaller one after nematode choice. This might have caused slightly heightened mortality for these plants (see Fig. 2).

Variation in VOCs production and EPN recruitment is mediated by ecotypic differentiation

Within food webs, patterns in trophic interactions are non-randomly associated to functional traits emerging at the organism levels. Numerous traits of animals have been shown to influence the strength of trophic cascades. Asymmetrically, plant traits remain poorly studied (Schmitz *et al*. 2004), despite clear evidence indicating that genetic variations driving plant trait expression can alter trophic-level interactions (Bailey *et al*. 2006). This study brings insights in how adaption of plants to different biotic and abiotic conditions results in phytochemical divergence, in turn modifying predator recruitment. Using belowground olfactometer bioassays, we showed that low-elevation plants were more attractive to EPNs when attacked by root herbivores. This is in line with earlier work showing that EPNs are generally attracted to herbivore-induced emissions of volatiles, particularly terpene-derived compounds, in several systems (Rasmann *et al*. 2012), and that plants that release such volatiles are less damaged than non-producing plants (Degenhardt *et al*. 2009). For higher elevation plant ecotypes, we observed that EPNs could not discriminate between infested and uninfested plants. For all plants, EPN movement away from the centre of the olfactometers was generally high (43 $\pm$ 3% and 28 $\pm$ 5% of nematodes responding to cues from mid- and high-elevation plant ecotypes respectively). Since root emissions of CO_2 can enhance the attraction of EPNs (Turlings *et al*. 2012), the high production of root biomass recorded for mid- and high-elevation plants (Fig. S7) could have favoured a general, but untargeted, EPN movement towards roots. While this could have favoured higher herbivore mortality in the field, we speculate that untargeted predator recruitment such as this one acted as confusion factor that inhibited EPNs efficacy (Smith 1996).

Paralleling EPNs behavioural observations, only low-elevation plants showed differences in the emission of volatiles between uninfested and infested plants. Over the 20 volatiles detected, six compounds were differentially released across plant treatments. Specifically, infestation of low-elevation plants significantly increases oxylipins, such as 1-octen-3-ol and 3-octanone, which can act as potential attractants for EPNs (Rasmann *et al*. 2012). This family of compounds has been shown to represent by-products of lipoxygenase enzymes in response to wounding of plant tissues and, more interestingly, plays a central role the recruitment of natural enemies (Halitschke & Baldwin 2003). Our follow-up experiment using pure standards indeed confirmed that those two compounds were attractive to *H. megidis* nematodes. We, however, acknowledge that potentially the synergistic role of compound mixture for enhancing EPN attraction might exist (Turlings *et al*. 2012) and needs to be addressed further in future chemical fractionation bioassays.

Reciprocal transplant experiments are the most rigorous way of demonstrating adaptive clines, because the genetic and environmental basis of trait expression can be tied to fitness components in matched and mismatched environments (Clausen *et al*. 1947; Chapin & Chapin 1981). However, phenotypic plasticity in response to growing conditions can also generate clines, and such plasticity can obscure genetically based trait expression. Along the elevational transect, the predominant abiotic factor that varies is temperature (Körner 2007). Temperature is also broadly controlling volatile emissions (Holopainen & Gershenzon 2010), in which volatile production increases with increasing temperatures (Guenther *et al*. 1995). Therefore, if temperature-based phenotypic plasticity was the main driver of volatile production, moving different ecotypes to a colder growth chamber or the common garden at high elevation, would result in low-elevation adapted plants decreasing, whereas high-elevation-adapted plants increasing volatile production. In spite, we found that low-elevation plants produced more abundant and diverse volatile blends overall, suggesting that phenotyptic plasticity in VOCs production played a minimal role on shaping the observed patterns.

Finally, this study also challenges the generally accepted principle of adaptive trade-offs in the expression of plant defensive traits (Agrawal *et al*. 2010). When no predators are inoculated in the rhizospheric soil, survival rate of infested plants is independent from the altitudinal origin of plants, despite the expression or not of indirect defences for low- and high-elevation plants respectively. This result suggests that direct resistance against root herbivores is equal between low- and high-elevation ecotypes (i.e. roots were eaten at equal rates since we added the same amount of roots in the field pots) confuting the postulated trade-off between direct and indirect resistance (Ballhorn *et al*. 2008), and suggests that volatile production for indirect defences is independent to the production of direct physical and chemical defences.

CONCLUSIONS

Despite four decades of research on plant volatiles structuring tri-trophic interactions and potential trophic cascades, evidence for selection on traits governing indirect defences remains quasi non-existent (Schuman *et al*. 2012). Proving that volatiles are effectively under selection indeed requires first demonstrating that traits involved in the recruitment of carnivores result in fitness advantages for plants exposed to herbivores (Kessler & Heil 2011). Our approach that combined natural history observations, field experiments with manipulation of trophic interactions at the community level, and behavioural assays associated to chemical analyses, points exactly in the direction that variation in herbivore pressure and predator abundance are among the chief selective agents of volatile production mediating top-down control of herbivores in nature. At low elevation, where herbivore pressures and carnivore abundances are high, natural selection likely favoured the establishment of effective indirect plant defence traits via positive effects of carnivore recruitment on plant survival. On the other hand, at high elevation, fitness benefits of maintaining effective volatiles production could have been outweighed by ecological costs related to change in biotic patterns, ultimately leading to a relaxation of trophic cascades.

ACKNOWLEDGEMENTS

We thank Ludovico Formenti for field assistance and Anurag Agrawal, Kailen Mooney and Adrienne Godschalx for insightful comments on previous drafts of the manuscript. This work was financed by Swiss National Science Foundation grant 31003A_159869 to SR.

AUTHORSHIP

SR and AK conceived and design the ideas and experiments. GR and AK performed the chemical analyses. AK analysed the data. All authors contributed to the writing.

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Editor, Vojtech Novotny
Manuscript received 9 July 2018
First decision made 14 August 2018
Second decision made 15 October 2018
Manuscript accepted 31 October 2018