Published in "Ecography doi: 10.1111/ecog.00833, 2014"
which should be cited to refer to this work.

High elevation *Plantago lanceolata* plants are less resistant to herbivory than their low elevation conspecifics: is it just temperature?

Loïc Pellissier, Aurélien Roger, Julia Bilat and Sergio Rasmann

L. Pellissier, Univ. of Fribourg, Unit of Ecology and Evolution, Ch. du Musée 10, CH-1700 Fribourg, Switzerland. – A. Roger, Research station Agroscope ACW Changins-Wädenswil, route de Duillier 50, CH-1260 Nyon, Switzerland. – J. Bilat and S. Rasmann (srasmann@uci.edu), Dept of Ecology and Evolution, UNIL Sorge, Le Biophore, Univ. of Lausanne, CH-1015 Lausanne, Switzerland. Present address of SR: Ecology and Evolutionary Biology, Univ. of California, Irvine, CA 92697, USA.

Traits that mediate species interactions are evolutionarily shaped by biotic and abiotic drivers, yet we know relatively little about the relative importance of these factors. Herbivore pressure, along with resource availability and 'third-party' mutualists, are hypothesized to play a major role in the evolution of plant defence traits. Here, we used the model system *Plantago lanceolata*, which grows along steep elevation gradients in the Swiss Alps, to investigate the effect of elevation, herbivore pressure, mycorrhizal inoculation and temperature on plant resistance. Over a 1200 m elevation gradient, the levels of herbivory and iridoid glycosides (IGs) declined with increasing elevation. By planting seedlings at three different elevations, we further showed that both low-elevation growing conditions and mycorrhizal inoculation resulted in increased plant resistance to herbivores. Finally, using a temperature-controlled experiment comparing high- and low-elevation ecotypes, we showed that high-elevation ecotypes are less resistant to herbivory, and that lower temperatures impair IGs deployment after herbivore attack. We thus propose that both lower herbivore pressure, and colder temperatures relax the defense syndrome of high elevation plants.

Since plants are sessile organisms, plant trait differentiation, both within species and individuals, has been postulated to influence community composition within different environments due to changes in biotic and abiotic conditions (Ackerly and Cornwell 2007, Webb et al. 2010). Among the biggest threats for plants is herbivore attack, which has resulted in the evolution of physical and chemical defenses (Schoonhoven et al. 2005). Herbivory rates are not homogenous, and insects herbivores, particularly, are also influenced by changes in the abiotic environment (Hodkinson 2005). Therefore, plant traits should co-vary with the abiotic environment because the traits may simultaneously confer adaptations to tolerate variations in herbivory levels and a given environmental regime (Coley et al. 1985). Indeed, it has been shown that interaction between resources and herbivore pressure drives habitat specialization (Fine et al. 2004), and studies should simultaneously account for abiotic and biotic factors to evaluate their relative strength.

Strong environmental gradients (i.e. contrasting habitats), which are increasingly being used to measure the effects of varying abiotic and biotic factors on plant traits (Körner 2007), can influence plant–herbivore interactions in three distinct ways. First, the species composition and relative abundance within herbivore communities may shift among habitats because herbivores are affected by abiotic conditions (Novotny et al. 2005, Pellissier et al. 2012). Second, the variation in abiotic conditions, determining the plant metabolic activity, also determines the extent of defence that can be synthesized. Due to the differential costs of tissue replacement in low- and high-resource environments, the optimal defence allocation should be higher in low-resource habitats and lower in high-resource habitats (Coley et al. 1985, Herms and Mattson 1992). Despite strong evidence that soil resources mediate trade-offs between growth and defences (Coley et al. 1985, Fine et al. 2004), relatively little is known about whether other abiotic factors, such as temperature variation, directly affect plant defences (Rizhsky et al. 2004). Plant responses to thermal stress generally lead to rapid increases in reactive oxygen species and rapid protein turnover (Suzuki and Mittler 2006), which might ultimately impact secondary-metabolite production (Walker et al. 2012). Thus, growth at colder sites, such as montane environments, might impose physiological constraints on how plants can resist herbivore attack. And third, mutualistic interactions with soil microorganisms, such as arbuscular mycorrhizal fungi (AMF), known to modify resistance against herbivory (Gange and West 1994,

Bennett et al. 2006), vary along environmental gradients (Kernaghan 2005, Rasmann et al. 2011) and are thus expected to modify plant responses to herbivores.

The type of defence and the amount of energy that a plant allocates should represent an optimal strategy given the local biotic and abiotic conditions, such as climate, soil resources and composition, and the abundance of attackers and their impacts on plant fitness (Grime 1977, Herms and Mattson 1992, Züst et al. 2012). Nevertheless, little is known regarding how biotic and abiotic factors interact to shape the diversity and amount of plant defensive traits along ecological gradients. Classic examples clearly indicate strong plant-trait differentiation and local adaptation along steep ecological gradients (reviewed by Núñez-Farfán and Schlichting 2001), where both climate and biotic pressure are responsible for the maintenance of local variation. For instance, it was shown that along elevation gradients, strong genetic differentiation of *Polemonium viscosum* plants occurs simultaneously with differentiation in plant traits. Particularly, slow growth is associated with the harsher climate on the mountaintop, but higher resistance at the bottom of the range is associated with higher herbivore pressure near the timberline (Galen et al. 1991).

In this study, we investigated the factors influencing how the ribwort plantain *Plantago lanceolata*, Plantaginaceae resists leaf-chewing herbivores in the Alps. First, we observed natural herbivory levels and plant defensive traits in *P. lanceolata* throughout the plantain's full elevational distribution in the Swiss Alps (from 600 to 1800 m a.s.l.). Second, we investigated how elevation per se and mycorrhizal mutualists affect the plants' responses to herbivores using a field experiment and growing individuals at three different elevations with and without mycorrhizae. And third, we performed a temperature-controlled experiment with seeds from low- and high-elevation populations to directly measure the effect of temperature on plant resistance. Specifically, we addressed the following questions: 1) is the rate of herbivore attack declining along elevation gradients, and does this correlate with defense production? 2) Does growing conditions, including elevation and the presence of mycorrhizal mutualists, influence plant resistance and defenses? And 3) does temperature drive variation in resistance and secondary metabolite deployment?

Material and methods

Plants

Plantago lanceolata is a short-lived perennial rosette plant with adventitious roots that may reproduce via seeds or by vegetatively forming new rosettes from axillary buds (Sagar and Harper 1964). This plant produces the monoterpene-derived iridoid glycosides (IGs) aucubin and catalpol, known to influence feeding by both specialist and generalist insect herbivores (Bowers et al. 1992) as well as the growth of fungal pathogens (Biere et al. 2004). Aucubin is the biosynthetic precursor to catalpol (Damtoft et al. 1983), itself shown to be more heavily induced by herbivory than aucubin, especially by specialist herbivores (Bowers and Stamp 1993).

Natural population survey

In the Swiss Alps, *P. lanceolata* individuals grow from the lowland (approximately 500 m a.s.l.) up to the subalpine stage (approximately 1800 m a.s.l.). We measured herbivory on *P. lanceolata* individual plants growing from 600 to 1800 m a.s.l. at elevation increments of 100 m, which resulted of a total of 13 different elevations (see site coordinates in Supplementary material Appendix 1, Table A1). At each elevation site, we sampled 10 fully expanded, randomly chosen leaves on three plants growing separated from each other by at least 30 m. Each leaf was scanned to quantify the level of herbivory on the plant using the image-analysis software ImageJ (<http://rsbweb.nih.gov/ij/>). Every missing part of the leaf was redrawn and used to quantify the percentage of damage, expressed as the missing area on the leaf/total area of leaf (including the estimated missing parts) $\times 100$ and averaged across 10 leaves. Finally, we averaged % damage across the three plants per site to obtain site-specific effects. We used Pearson's correlation test to measure variation of plant damage along the elevation gradient. The percentage herbivory levels were arcsin (square-root)-transformed prior to analysis.

Of the 13 population sampled, we measured IGs of plants coming from seven elevations, distributed at about 670, 980, 1180, 1290, 1500, 1690, and 1750 m a.s.l. Quantification of catalpol and aucubin was done following a modified Bowers (1996) protocol. Between 50 and 100 mg dried tissue powder was extracted in 3 ml methanol overnight. Following solvent evaporation, 1 ml of Phenyl-β-D-glucopyranoside (= PBG, CAS 1464-44-4, Sigma-Aldrich) (0.5 mg ml^{-1}), and 3 ml additional deionized H_2O were added to the dry residue. After ether-rinsing the solution three times, a 0.1 ml aliquot was evaporated and added with 0.1 ml TMSI + PYRIDINE, 1:4 (Sylon TP, CAS 8077-35-8, Sigma-Aldrich), as a silylation agent, and heated at 70°C for 20 min. Finally, 1 µl of this solution was analysed using gas chromatography-mass spectrometry (GC-MS) with a Thermo Trace GC Ultra Lan GC system coupled to a quadrupole-typemass-selective detector (DSQ II from Thermo Fisher Scientific, Waltham, MA, USA; transfer line 220°C, source 220°C, ionization potential 70 eV). The sample was injected on an apolar column (Zebron ZB-5MS, 30 m, 0.25 mm internal diameter, 0.25 µm film thickness; Phenomenex, Torrance, CA, USA). Helium at a constant flow (1.2 ml min^{-1}) was used as the carrier gas. After injection through split injector (split flow 24 ml min^{-1}, splitless time 1 min), the column temperature was maintained at 140°C for 1 min and then increased to 280°C at 6°C min^{-1} followed by a final stage of 5 min at 300°C. The IG level was quantified by comparing the area of IGs with the area of the internal standard. Absolute values were obtained based on calibration curves of catalpol and aucubin and expressed in µg mg^{-1} of leaf dry weight. The relationship between herbivory levels and elevation and between herbivory and total IGs were analysed with Pearson correlation tests.

Elevation and mycorrhizae experiment

To determine the effects of elevation and arbuscular mycorrhizal fungi (AMF) on plant performance and resistance

traits, we planted *P. lanceolata* plants at three different elevations (500, 1000, and 1500 m a.s.l.) and applied a mycorrhizal treatment. With this manipulation we aimed at measuring responses of plants to local environment when growing at different elevations. Therefore, seeds from plants distributed throughout the elevation gradient were pooled and germinated on wet paper at room temperature. The seedlings were planted in 12 cm diameter plastic pots filled with an autoclaved soil mix (120°C, 120 min, 1 atm) with a low P concentration (Substrat 4, Klasmann-Deilmann, Geeste, Germany) and sand (ratio 1:1, v/v). Immediately after planting, an inoculum of approximately 250 AMF spores (*Rhizophagus irregularis,* previously known as *Glomus intraradices*, Glomygel from Mycovitro S.L., Albolote, Granada, Spain) in 2 ml water solution was added to half of the plants. *Rhizophagous irregularis* isolates have been shown to form symbioses with *P. lanceolata* in natural conditions (Croll et al. 2008). A second similar inoculation was performed after four days, and the next day, potted plants were placed outside at three elevations, and under 20% UV-removal shading to protect unaccustomed small seedling from intense UV light. In total we planted 25 plants × 3 elevations × 2 treatments (with and without mycorrhizae) = 150 plants. Of these, 60 plants were used to measure growth-related traits, and the remaining 90 plants were used to measure resistance and defenses. After seven weeks of growth, the 60 plants were scored for plant traits relating to growth and nutritive value (root and shoot biomass, chlorophyll levels, water content, and specific leaf area [SLA]). The aboveground biomass was measured after drying all the leaves and flowering stems at 60°C for four days. The root biomass was measured by washing soil from roots and drying the material as described above, using a subset of five randomly chosen plants. Root sampling also showed that plants were not root-bound, similarly to previous experiments in the system (Roger et al. 2013). The chlorophyll levels were measured using a Konica Minolta SPAD-502 chlorophyllometer (Konica Minolta Sensing, Osaka, Japan) as the average value of three different leaves per plant. The water content was calculated as the fresh leaf weight minus the weight of the leaves left drying for four days at 60°C. The SLA (expressed in $mm^2\ mg^{-1}$) was obtained by cutting a 1 cm diameter leaf disc and measuring its dry weight after four days at 60°C.

The additional 15 plants per elevation and mycorrhizal treatment (n = 90) were set aside for measuring plant resistance against the generalist herbivore *Spodoptera littoralis* (Lepidoptera; Noctuidae), obtained from Syngenta, Stein, Switzerland. *Spodoptera littoralis* is a generalist caterpillar known to feed on plants of at least 40 different families (Brown and Dewhurst 1975), including *Plantago* spp., and is widely used to perform plant resistance bioassays. We also chose a non-adapted herbivore to remove the confounding effect of possible local adaptation. After seven weeks of field growth, all plants were brought into a light- and temperature-controlled room (24°C; 16:8 h light period) for the insect bioassay. The next day, 10 neonate larvae were placed on 10 plants per treatment and per elevation, previously enclosed with a fine mesh white cloth, and allowed to feed for seven days (n = 10 plants per elevation and mycorrhizal treatment). The remaining five, control, plants were also enclosed with fine mesh cloth veil but left herbivore-free for measuring constitutive plant chemistry. Recollected larvae were dried for four days at 60°C before being weighed. Herbivore-induced IGs were measured as described above on six randomly chosen herbivore-damaged plants per treatment, and on the five additional undamaged plants. On the same plants, the carbon-to-nitrogen ratio (C/N) was analysed with an elemental analyser. Finally, we measured AMF-colonization rates at different elevations on a subset of plants (n = 4 undamaged plants per elevation) by clarifying fine root tissue (10 cm in length and approximately 50 mg) with 10% KOH for 10 min and staining in 0.05% trypan blue in a 1:1:1 mixture of water:glycerine:lactic acid (Phillips and Hayman 1970). The roots were mounted on slides and scored at ×40 magnification. All the spores and arbuscules were counted and pooled to obtain the mycorrhizal colonization level per cm of root.

To assess the effects of growing conditions on plant resistance and defenses, we tested whether elevation, herbivore treatment and mycorrhizal treatments influence larval growth using two-way ANOVAs and IGs production using three-way ANOVAs. All the IG parameters (catalpol, aucubin, total amounts and the ratio between aucubin and catalpol) were square-root-transformed prior to analysis. We also quantified the effect of treatments on several plant growth traits (biomass, chlorophyll content, SLA, C/N, root:shoot ratio) using three-way ANOVAs.

Controlled temperature experiment

To determine possible elevation-driven local adaptation in resistance and chemical defences as well as environment-driven response to a distinct temperature regime, we conducted a growth chamber experiment using high and low elevation *P. lanceolata* populations, grown at two temperature regimes of 12°C and 20°C. Based on meteorological data from stations in the study area extracted from the Meteosuisse IDAWEB database (<www.meteoschweiz.admin.ch/>), 12°C and 20°C are the average daily temperatures of the plant-growing season at 1500 m and 500 m, respectively. It should be noted however that the variance in growing season temperature is approximately 10% greater at 1500 m compared to 500 m. We collected seeds from five populations situated between 500–600 m a.s.l., whereas the high-elevation individuals were obtained by collecting seeds from five populations situated between 1500–1700 m a.s.l., all from different alpine valleys (Supplementary material Appendix 1, Table A2). The two batches of seeds (high and low elevation) were germinated as described above, transplanted in 10 cm pots, and placed in two different temperature-controlled chambers (14:10 h light regime) at 12°C and 20°C (n = 26 plants per elevation and per temperature regime). After seven weeks of growth, twenty plants from high and low elevations and the two temperature regimes were used to measure the resistance against 10 *S. littoralis* larvae per plant. Plants were enclosed with fine mesh cloth and placed in the greenhouse with the same parameters as described above to remove the confounding effect of temperature on the insect development.

Six randomly chosen, herbivore-damaged plants were used for measuring herbivore-induced IG levels as described above. The remaining six plants were used for measuring constitutive levels of IGs.

Plant growth (biomass) and IGs were analysed using three-way ANOVAs including the elevation of origin (high or low elevation), herbivore treatment (with and without herbivore) and temperature treatments as factors. All the IG parameters (catalpol, aucubin, total amount and the ratio between aucubin and catalpol) were square-root-transformed prior to analysis. The larval biomass was analysed with two-way ANOVA including the elevation of origin and temperature regimes as treatments.

Results

Natural population survey

Across almost 1200 m of elevation, *P. lanceolata* plants display strong variation in how much herbivory they experience (Fig. 1a, $F_{12,26} = 3.68$, $p = 0.003$), ranging from a maximum of 6% damage in lowland populations to about 0.2% damage for the high elevation populations. Paralleling this result, we found a significant relationship between IGs production and elevation (for aucubin, $F_{7,36} = 2.95$, $p = 0.01$; for catalpol $F_{7,36} = 2,35$, $p = 0.04$; and for the sum $F_{7,36} = 2.50$, $p = 0.03$; but not for the ratio of aucubin to catalpol $F_{7,36} = 1,35$, $p = 0.25$). As a consequence, the herbivory levels were positively correlated with the total IG levels in the plants (Supplementary material Appendix 1, Fig. A1, Pearson correlation, $n = 7$, $r = 0.79$, $p = 0.02$). We note here that total IGs content, and particularly for catalpol (data not shown), is low compared to previous published studies (ranging from 1–7%) (Bowers et al. 1992, Stamp and Bowers 1994, Marak et al. 2003). We speculate this to be derived from a delayed processing of the field-collected material. Nevertheless, all plants underwent the same handling and processing, which would validate the comparison among sites as done here.

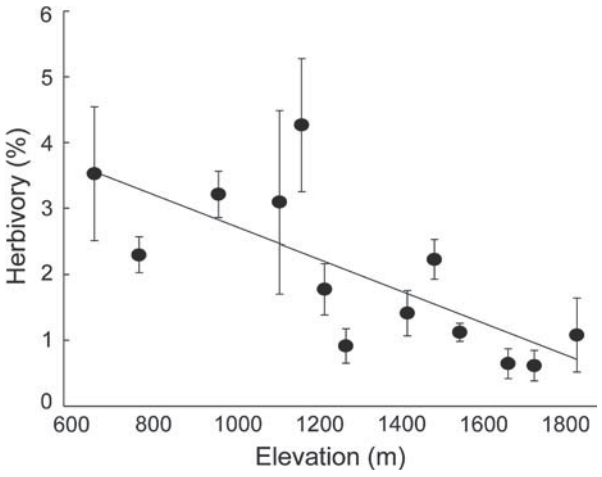

Figure 1. Elevation effect on the levels of herbivory (measured as the number of feeding holes per plant) on 13 *P. lanceolata* populations. Each dot is the average of three plants per elevation (± 1 SE).

Elevation and mycorrhizae experiment

We found significant effects of the treatments on the plant resistance and palatability parameters (Fig. 3). *Spodoptera littoralis* caterpillars feeding on high elevation grown plants were on average 26% heavier than the caterpillars that fed on low elevation grown plants (Fig. 2a, Table 1). Interestingly, we found that mycorrhizae decreased the *S. littoralis* mean larval weight by 25% in the low and high elevation

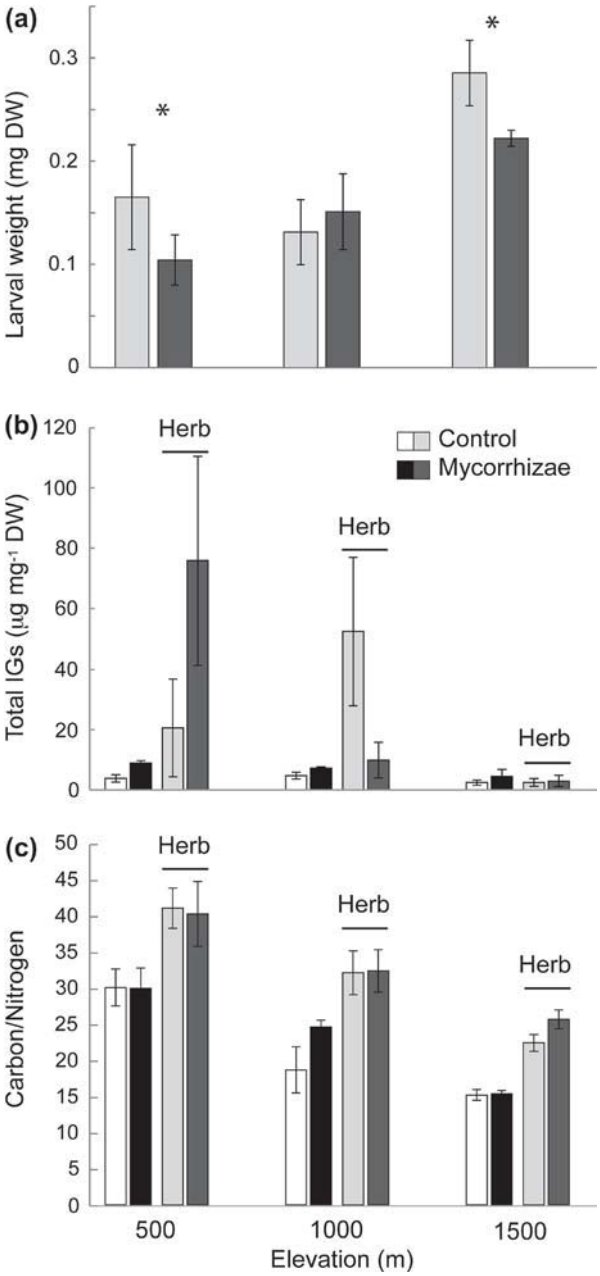

Figure 2. *Plantago lanceolata* resistance and palatability parameters, including (a) *Spodoptera littoralis* growth rate, (b) total iridoid glycosides (IGs), and (c) carbon to nitrogen ratio of the plants that were grown at three elevation sites (500, 1000, and 1500 m a.s.l.). Half of the plants were either inoculated with mycorrhizae (black and dark grey bars) or left uninoculated (open and light grey bars). The light grey and dark grey bars represent plants that were treated with *S. littoralis* for 7 d. The bars represent averages ± 1 SE. Asterisks indicate a significant difference between pairs of bars (t-test, $p < 0.05$).

Table 1. Two-way and three-way interaction ANOVAs for *P. lanceolata* plant resistance and defense, respectively. Plants were grown at three different elevations, and half of the plants were inoculated with the AM fungi *R. irregularis*. *Spodoptera littoralis* larval weight after 7 d of growth was measured on the same plants placed in a glasshouse to remove the effect of elevation on insect growth. Total iridoid glycosides (IGs) represents the sum of catalpol and aucubin. Denominator degrees of freedom for larval weight is 36, and for total IGs is 49.

Response variable	Factor	DF_{num}	F
Larval weight	Elevation (E)	2	4.61**
	Mycorrhizal treatment (M)	1	6.06**
	M × E	2	2.54
Total IGs	E	2	5.12***
	M	1	0.15
	Herbivore treatment (H)	1	0.36
	E × M	2	0.53
	E × H	2	0.78
	M × H	1	0.64
	E × M × H	2	0.94

**$p < 0.01$, ***$p < 0.001$.

sites (Fig. 2a, Table 1). IGs were overall only slightly affected by mycorrhizae, but we found strong elevation and herbivore effects. Plants growing at low elevation had higher total levels of IGs (Fig. 2b, Supplementary material Appendix 1, Table A3). This result was mainly driven by the fact that IGs, and particularly the IG catalpol, were highly induced by *S. littoralis* feeding in plants growing at the low-elevation sites (Fig. 2b, Supplementary material Appendix 1, Table A3). In addition, growing at high elevation decreased the total C/N, while the presence of herbivores increased the C/N ratio (Fig. 2c, Supplementary material Appendix 1, Table A3). We never found an interaction between the elevation and mycorrhizal treatment, suggesting similar colonization rates across elevation sites. Note that we found no differences in AMF colonizing the roots of plants from the three elevation sites (sum of spores plus arbuscules = 23.75 ± 3.71, 21.21 ± 2.98, and 24.5 ± 0.5, respectively from low to middle to high elevation; elevation effect $F_{2,11} = 0.38$, $p = 0.69$).

Finally, variation in growing conditions across elevations and mycorrhizae influenced the studied plant traits (Fig. 3, Supplementary material Appendix 1, Table A4). Growing at high elevation increased the plant chlorophyll levels by 10% (Fig. 3a), decreased the above- and below-ground plant biomass by 73 and 84%, respectively (Fig. 3b), and increased the SLA by 29% (Fig. 3c), and the water content by 10% (Fig. 3d). In contrast, mycorrhizae did not increase plant biomass, SLA or chlorophyll levels but significantly increased the mean water content by 5% (Fig. 3d).

Controlled temperature experiment

The controlled chamber experiment showed the plastic effect of temperature and the effect of elevation driving

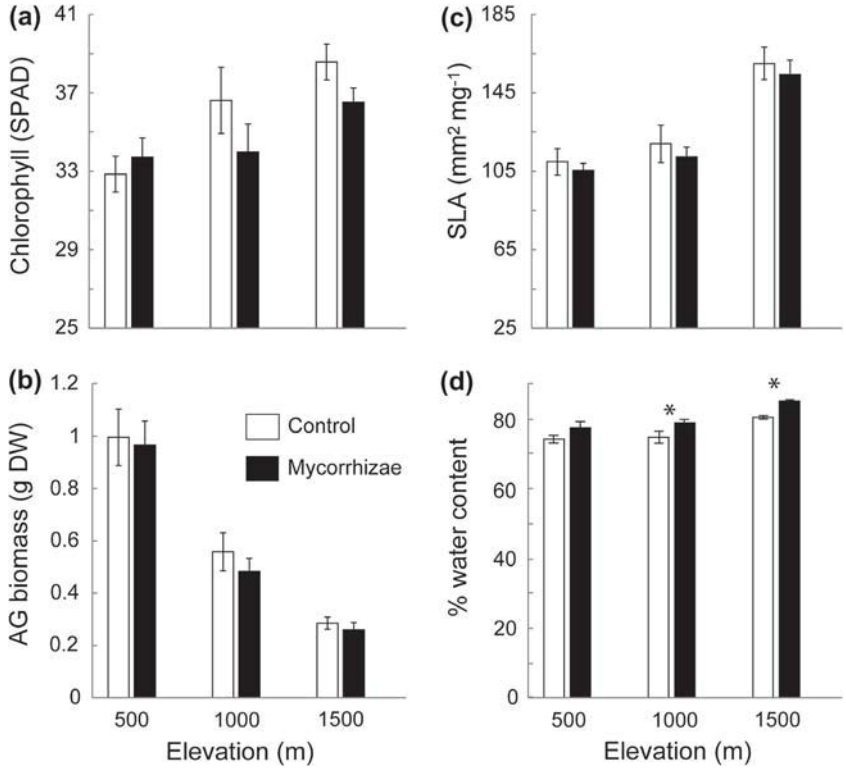

Figure 3. *Plantago lanceolata* traits, including (a) chlorophyll content, (b) aboveground biomass, (c) specific leaf area (SLA), and (d) water content. The plants were grown at three elevation sites (500, 1000, and 1500 m a.s.l.). Half of the plants were either inoculated with mycorrhizae (black bars) or left uninoculated (open bars). The bars represent averages ±1 SE. Asterisks indicate a significant difference between pairs of bars (t-test, $p < 0.05$).

local adaptation on the resistance, and chemical defence production in *P. lanceolata*. Plants were overall 60% smaller when growing at the colder (12°C) temperature (Fig. 4a, temperature effect; $F_{1,22} = 50.30$, $p < 0.0001$), regardless of the original elevation (elevation of origin effect: $F_{1,22} = 0.003$, $p = 0.96$, and interaction between temperature and elevation of origin: $F_{1,22} = 0.01$, $p = 0.91$). Despite this pattern, the larvae grew 22% larger on the high-elevation plants than on the low-elevation ones (Fig. 4b, elevation of origin effect; $F_{1,63} = 4.05$, $p = 0.048$), independently of the temperature at which the plants were growing (temperature effect: $F_{1,63} = 1.03$, $p = 0.31$, and interaction term: $F_{1,63} = 0.10$, $p = 0.75$). In contrast, IGs responded differently to temperature depending on the elevation of origin (Fig. 4c, Table 2). In particular, the total amounts of IGs were 60% higher in the low-elevation populations than in the high-elevation ones (Table 2). Overall, growing at a lower temperature decreased the levels of IGs by 89% (Table 2). This effect was more pronounced on the low-elevation individuals (see the significant interaction between the ecotype and temperature treatment in Table 2). Herbivory increased the total amount of IGs only in the low-elevation individuals when growing at 20°C (see the significant temperature by herbivore treatment interaction in Table 2). This effect was driven by changes in aucubin levels and to a lesser extent by catalpol (Table 2, see the individual IGs effect and the effect of the ratio between aucubin and catalpol).

Discussion

Our study suggests that on-site plant resistance to herbivores is a combination of short-term responses to prevailing temperature conditions and the cumulative effect of long-term adaptive responses to local herbivory pressure. First, we showed that the growth and defence traits of *P. lanceolata* plants respond to variation in environmental conditions. When growing at high elevations, the plants became more susceptible to herbivores, and the presence of mycorrhizae increased resistance. Paralleling those results, the climate-controlled experiment isolated a direct temperature effect on plant defence deployment and suggested that local growing conditions impose physiological constraints that modulate a plastic response in plant defences. Second, we showed that populations from low and high elevations have distinct resistance responses against herbivores, likely a consequence of the shift in herbivore pressure documented in the field, and temperature-driven physiological adaptations.

Trait variation along elevation gradients

Since the pioneering work of Clausen et al. (1947), elevation gradients have been shown to shape plant trait variation (Núñez-Farfán and Schlichting 2001, Pellissier et al. 2010). These findings indicate that abiotic factors that co-vary with elevation (e.g. temperature, Körner 2007) shape plant physiology to maximize fitness at different elevations (Linhart and Grant 1996, Joshi et al. 2001).

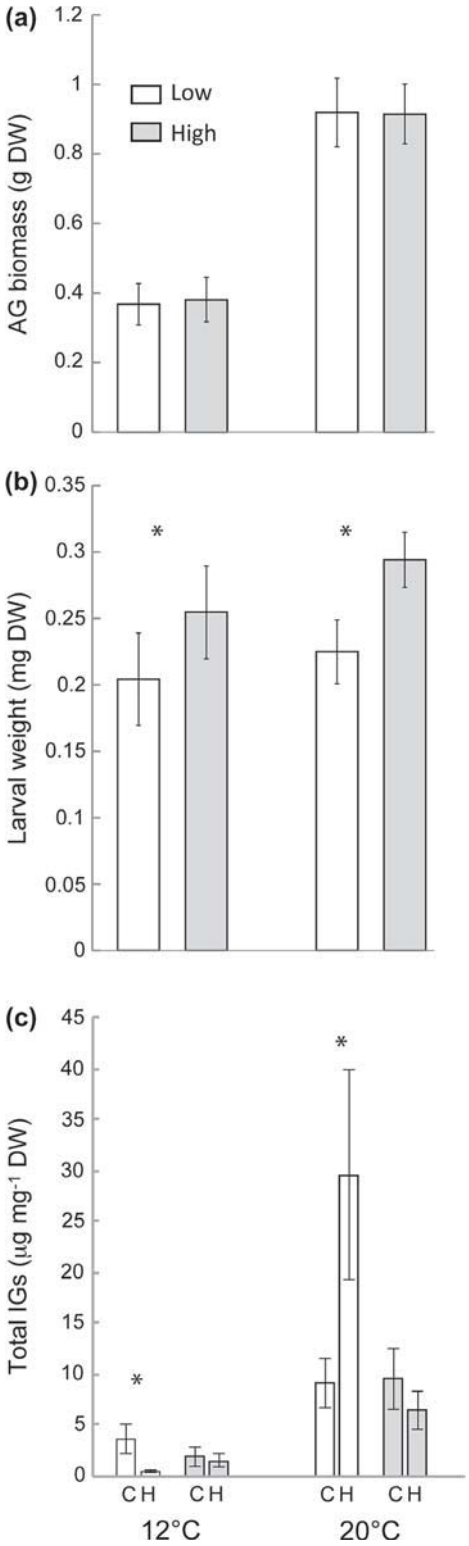

Figure 4. Temperature by elevation of origin interaction on (a) the aboveground biomass of *P. lanceolata* plants, (b) the *S. littoralis* weight, and (c) the total amount of iridoid glycosides on the same plants after 7 d of feeding. The plants were grown at 12°C or at 20°C and originated from either low- (~500 m a.s.l., open bars) or high- (~1500 m a.s.l., grey bars) elevation populations. Iridoid glycosides were measured on healthy plants (C) and on plants damaged by *S. littoralis* (H). The bars represent averages ± 1 SE. Asterisks indicate a significant difference between pairs of bars (t-test, $p < 0.05$).

Table 2. Three-way interaction ANOVAs for the total amount of iridoid glycosides, catalpol only, and aucubin only in *P. lanceolata* plants grown at 12°C or at 20°C. The plants originated from either high or low elevation. Additionally, the plants were either left undamaged or were treated with the generalist herbivore *S. littoralis* for 7 d. For all the factors, the degrees of freedom are $F_{1,39}$.

Response variable	Factor	F value
Total IGs	Origin (elevation) (O)	6.19*
	Temperature (T)	36.72***
	Herbivore treatment (H)	0.47
	O×H	1.21
	O×T	6.50*
	H×T	4.86*
	O×H×T	4.56*
Catalpol	O	1.24
	T	14.36**
	H	0.02
	O×H	0.16
	O×T	0.48
	H×T	1.02
	O×H×T	1.94
Aucubin	O	4.74*
	T	28.16***
	H	0.66
	O×H	0.97
	O×T	5.22*
	H×T	4.83*
	O×H×T	3.58
Aucubin/catalpol	O	1.16
	T	5.35*
	H	0.26
	O×H	0.01
	O×T	2.09
	H×T	0.04
	O×H×T	0.002

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Here, we expand the paradigm of trait differentiation along elevation by integrating herbivore and plant defence traits. Because insects are ectothermic, it is generally expected that insect abundance and herbivory rates will decrease with increasing elevation and thus modify the selective pressure on plant defence traits (Hodkinson 2005). Here, we showed a clear decrease of herbivory pressure with increasing elevation in *P. lanceolata*. In line with our expectations, the high-elevation individuals were less resistant to caterpillar feeding and produced lower amounts of IGs before and after herbivory than the low-elevation individuals. However, it might be argued that high elevation-growing plants contained lower amounts of IGs solely due to a decreased, temperature-mediated, development. Indeed, previous work on *P. lanceolata* showed that early stages of development contain lower amounts of IGs (Darrow and Bowers 1997, Barton 2007), a general rule for secondary metabolite production in herbaceous plants (Barton and Koricheva 2010). Plants from both the natural population and the field experiment were all at the flowering stage, thus ontogenic differences were minimized. Additionally, the growth chamber experiment showed that low temperature does reduce plants' growth rate, but this is independent of the site of origin (Fig. 4a), and despite this, we still observe high elevation individuals showing lower levels of total IGs (Fig. 4c). We thus suggest that reduction of chemical defenses in high elevation *P. lanceolata* plants results from a combination of a decrease in herbivore pressure and physiological and ontogenic constraints.

Interestingly, although a phylogenetically controlled paired experiment recently showed that high-elevation plants are less resistant to a generalist caterpillar than their congeneric low-elevation relatives (Pellissier et al. 2012), reports on secondary metabolite concentration along elevation gradients often contain contrasting results and document both an increase and a decrease of plant defences with elevation (see the meta-analysis in Rasmann et al. 2014). Why is the decrease of plant defence capacity with increasing elevation as documented in this study not universal? Particular classes of secondary metabolites, such as phenolic-based compounds, known to reduce herbivore performance (Forkner et al. 2004), might also be selected for increased levels at high elevations as protection against photodamage or to act as antioxidants (Close and McArthur 2002). Therefore, reduced biotic selective pressure at high elevations by herbivores may be compensated by an increase in abiotic selective pressure for particular, but not all, molecules. A study found that the phenol-derived compound verbascoside was increased when *P. lanceolata* plants were grown under high UV-B light conditions (McCloud and Berenbaum 1999). Future studies should therefore scale-up to take into account the full breadth of *P. lanceolata* defensive traits, including for example phenol-based compounds or other physical traits such as trichomes (Levin 1973).

Temperature effects on plant resistance and defences

We showed that at higher elevation and in colder temperatures, plants produce lower levels of IGs and are less resistant to herbivory. Temperature is known to strongly affect primary and secondary plant metabolism, in particular under high light conditions. For example, in *Arabidopsis thaliana*, high temperatures increase isoprene emissions, which in turn reduce cellular-membrane denaturation (Loreto et al. 2006). However, for metabolites not related to plant tissue repair, it might be argued that lower temperatures have a negative impact on production by limiting metabolic activity. Walker et al. (2012) showed in *Bituminaria bituminosa* that the amount of furanocoumarins, supposed to protect against infection and herbivory, was regulated by short-term variations in temperature conditions. Here, we demonstrated that growing-temperature conditions also affect secondary metabolite production in *P. lanceolata*, in particular showing that low temperatures constrain the induction of IGs after herbivory. Alongside this temperature-driven secondary metabolite suppression, we also observed a reduction in resistance to herbivores at lower temperatures in the field. Interestingly, plants from low elevations, when grown at 20°C, showed strong induction compared to high elevation adapted plants. However, plants from low elevation growing at 12°C showed null or negative induction of IGs after herbivory. These results first corroborate previous findings showing strong

genotypic and context-dependent variation in IGs induction in *P. lanceolata* (Bowers and Stamp 1993, Marak et al. 2002), and second, they suggest phenotypic adaptation to the harsh growing environment of Alpine meadows, as was shown for *P. lanceolata* and its pathogen host *Podosphaera plantaginis* (Laine 2008). Additionally, the genetically-based effect of increased susceptibility for high elevation plants might derive from maternal or epigenetic effects (Bossdorf et al. 2010, Rasmann et al. 2012), and future work would need to tease apart potential genetically-based local adaptation to environmental driven phenotypic differences.

Taken together, these results provide insights into the responses of plant species to climate change. Increased temperature under climate change is expected to promote a higher levels of herbivory at high elevations (Rasmann et al. 2014). However, because plant defence expression is plastic and reacts to the growing temperature, our results also suggest that increased temperature at higher elevation is expected to promote plant defences, therefore mitigating the impact of herbivores under climate change (Pratt and Mooney 2013). Finally, other factors may explain the difference in palatability along elevation. Plants may become more palatable for herbivores due to higher water content as shown here, or to the higher photosynthetic activity-enhancing nitrogen content in high-elevation individuals. Indeed, we found a strong negative phenotypic correlation between chlorophyll levels and C/N ratios across all the plants in the field (Supplementary material Appendix 1, Fig. A2).

While there is a consistent decrease in temperature with elevation, many other environmental factors co-vary with elevation including precipitation and winds. Also, alpine environments at high elevations are often characterized by stronger fluctuations in physical factors, such as greater daily and seasonal climatic variability (Körner 2007).

Mycorrhizal effect on plant resistance: are AMF important for the local adaptation of plants?

AMF are present worldwide and form symbioses with approximately 80% of land plants (Smith and Read 2008). The relationship of AMF with elevation has been studied extensively, and the general findings confirm the low sensitivity of AMF to this parameter, although some decreasing diversity patterns have been observed at very high elevations (Chaurasia et al. 2005, Lugo et al. 2008, Gai et al. 2009). Here, we did not observe any change of colonization levels along elevation, implying that local environmental conditions (in our case mainly air temperature change) did not influence the mycorrhization of *P. lanceolata*. This result corroborates the findings of Väre et al. (1997), who observed that AMF colonization within plant species did not vary along an elevation gradient of 600 to 900 m. We found that the AMF colonization had a significant effect on the plant water content (a 5% increase compared to non-mycorrhizal plants) and on the larval biomass (a 25% decrease compared to larvae feeding on non-mycorrhizal plants). Because we did not detect any other plant trait that was modulated by AMF colonization that could explain the lower larval fitness, we could hypothesize that AMF colonization is an alternative way to improve plant resistance via yet-unmeasured plant defensive traits. Indeed, *P. lanceolata* has been shown to produce phenolic-based compounds, including for instance verbascoside, with putative anti-fungal (Nikonorova et al. 2009) and anti-herbivore (Fajer et al. 1992, Adler et al. 1995, McCloud and Berenbaum 1999, Sutter and Mueller 2011) properties.

The effect of AMF on plant resistance to herbivores can vary greatly and depends on the identity and combinations of AMF species and/or populations simultaneously colonizing plant roots (Roger et al. 2013). Given the lower herbivory pressure at high elevation, the need for AMF for increased defence may be reduced, and this shift could also explain the generally increasing relative coverage of non-mycorrhizal plants at higher elevations (Ruotsalainen et al. 2004). However, future studies will be needed to test the relative levels of multitrophic mutualism across elevation gradients.

Conclusion and perspectives

Local adaptation by natural selection is a fundamental process in population differentiation and speciation (Kawecki and Ebert 2004). Here, we observed that within *P. lanceolata* populations, alpine individuals have lower resistance to herbivores than low-elevation relatives, in addition to lower herbivore damage, and this trend can be generalized to many alpine plant species (Pellissier et al. 2012). In our case, both the high- and low-elevation populations of *P. lanceolata* may have adapted to the local biotic and abiotic conditions, including shifts in temperature and in herbivory, potentially promoting different ecotypes through differential selection. We therefore propose that along elevation gradients, variation in herbivory regimes and the abiotic environment affect overall resistance strategies. If this promotes plant fitness, it may favor divergence among high- and low-elevation lineages, thus finally promoting the exceptional radiation of the Alpine flora.

Acknowledgements – We are grateful to Julien Leuenberger and Quentin Aeberli for help with data collection and to Will Petry, Scott McArt, and Anurag Agrawal for commenting on previous versions of the manuscript. This work is supported by a National Science Foundation Ambizione grant PZ00P3_131956/1 to SR. LP was supported by the Danish FNU grant no. 12-126430.

References

Ackerly, D. D. and Cornwell, W. K. 2007. A trait-based approach to community assembly: partitioning of species trait values into within- and among-community components. – Ecol. Lett. 10: 135–145.

Adler, L. S. et al. 1995. Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the

specialist herbivore *Junonia coenia* (Nymphalidae). – Oecologia 101: 75–85.
Barton, K. E. 2007. Early ontogenetic patterns in chemical defense in *Plantago* (Plantaginaceae): genetic variation and trade-offs. – Am. J. Bot. 94: 56–66.
Barton, K. E. and Koricheva, J. 2010. The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. – Am. Nat. 175: 481–493.
Bennett, A. E. et al. 2006. Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: hypotheses and synthesis. – Am. Nat. 167: 141–152.
Biere, A. et al. 2004. Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? – Oecologia 140: 430–441.
Bossdorf, O. et al. 2010. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. – Evol. Ecol. 24: 541–553.
Bowers, M. D. 1996. Variation in iridoid glycosides in a population of *Plantago patagonica* Jacq. (Plantaginaceae) in Colorado. – Biochem. Syst. Ecol. 24: 207–210.
Bowers, M. D. and Stamp, N. E. 1993. Effects of plant age, genotype and herbivory on *Plantago* performance and chemistry. – Ecology 74: 1778–1791.
Bowers, M. D. et al. 1992. Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. – Oecologia 91: 201–207.
Brown, E. S. and Dewhurst, C. F. 1975. The genus *Spodoptera* (Lepidoptera, Noctuidae) in Africa and the Near East. – Bull. Entomol. Res. 65: 221–262.
Chaurasia, B. et al. 2005. Distribution, colonization and diversity of arbuscular mycorrhizal fungi associated with central Himalayan rhododendrons. – For. Ecol. Manage. 207: 315–324.
Clausen, J. et al. 1947. Heredity of geographically and ecologically isolated races. – Am. Nat. 81: 114–133.
Close, D. C. and McArthur, C. 2002. Rethinking the role of many plant phenolics – protection from photodamage not herbivores? – Oikos 99: 166–172.
Coley, P. D. et al. 1985. Resource availability and plant antiherbivore defense. – Science 230: 895–899.
Croll, D. et al. 2008. Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*. – New Phytol. 178: 672–687.
Damtoft, S. et al. 1983. The biosynthesis of iridoid glycosides from 8-epi-deoxyloganic acid. – Biochem. Soc. Trans. 11: 594–595.
Darrow, K. and Bowers, M. D. 1997. Phenological and population variation in iridoid glycosides of *Plantago lanceolata* (Plantaginaceae). – Biochem. Syst. Ecol. 25: 1–11.
Fajer, E. D. et al. 1992. The effect of nutrients and enriched Co_2 environments on production of carbon-based allelochemicals in *Plantago* – a test of the carbon nutrient balance hypothesis. – Am. Nat. 140: 707–723.
Fine, P. V. A. et al. 2004. Herbivores promote habitat specialization by trees in amazonian forests. – Science 305: 663–665.
Forkner, R. E. et al. 2004. Feeny revisited: condensed tannins as anti-herbivore defences in leaf-chewing herbivore communities of *Quercus*. – Ecol. Entomol. 29: 174–187.
Gai, J. P. et al. 2009. Occurrence and distribution of arbuscular mycorrhizal fungal species in three types of grassland community of the Tibetan Plateau. – Ecol. Res. 24: 1345–1350.
Galen, C. et al. 1991. Ecotypic divergence in alpine *Polemonium viscosum*: genetic structure, quantitative variation, and local adaptation. – Evolution 45: 1218–1228.
Gange, A. C. and West, H. M. 1994. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. – New Phytol. 128: 79–87.
Grime, J. P. 1977. Evidence for existence of 3 primary strategies in plants and its relevance to ecological and evolutionary theory. – Am. Nat. 111: 1169–1194.
Herms, D. A. and Mattson, W. J. 1992. The dilemma of plants – to grow or defend. – Q. Rev. Biol. 67: 283–335.
Hodkinson, I. D. 2005. Terrestrial insects along elevation gradients: species and community responses to altitude. – Biol. Rev. 80: 489–513.
Joshi, J. et al. 2001. Local adaptation enhances performance of common plant species. – Ecol. Lett. 4: 536–544.
Kawecki, T. J. and Ebert, D. 2004. Conceptual issues in local adaptation. – Ecol. Lett. 7: 1225–1241.
Kernaghan, G. 2005. Mycorrhizal diversity: cause and effect? – Pedobiologia 49: 511–520.
Körner, C. 2007. The use of 'altitude' in ecological research. – Trends Ecol. Evol. 22: 569–574.
Laine, A. L. 2008. Temperature-mediated patterns of local adaptation in a natural plant-pathogen metapopulation. – Ecol. Lett. 11: 327–337.
Levin, D. A. 1973. The role of trichomes in plant defense. – Q. Rev. Biol. 48: 3–15.
Linhart, Y. B. and Grant, M. C. 1996. Evolutionary significance of local genetic differentiation in plants. – Annu. Rev. Ecol. Syst. 27: 237–277.
Loreto, F. et al. 2006. On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. – Plant Cell Environ. 29: 1820–1828.
Lugo, M. A. et al. 2008. Arbuscular mycorrhizal fungi and rhizospheric bacteria diversity along an altitudinal gradient in South American puna grassland. – Microb. Ecol. 55: 705–713.
Marak, H. B. et al. 2002. Systemic, genotype-specific induction of two herbivore-deterrent iridoid glycosides in *Plantago lanceolata* L. in response to fungal infection by *Diaporthe adunca* (Rob.) niessel. – J. Chem. Ecol. 28: 2429–2448.
Marak, H. B. et al. 2003. Fitness costs of chemical defense in *Plantago lanceolata* L.: effects of nutrient and competition stress. – Evolution 57: 2519–2530.
McCloud, E. S. and Berenbaum, M. 1999. Effects of enhanced UV-B radiation on a weedy forb (*Plantago lanceolata*) and its interactions with a generalist and specialist herbivore. – Entomol. Exp. Appl. 93: 233–247.
Nikonorova, A. K. et al. 2009. Antifungal activity of phenolic glicozide verbascoside from *Plantago major* seeds. – Mikol. Fitopatol. 43: 52–57.
Novotny, V. et al. 2005. An altitudinal comparison of caterpillar (Lepidoptera) assemblages on *Ficus* trees in Papua New Guinea. – J. Biogeogr. 32: 1303–1314.
Núñez-Farfán, J. and Schlichting, C. D. 2001. Evolution in changing environments: the "synthetic" work of Clausen, Keck, and Hiesey. – Q. Rev. Biol. 76: 433–457.
Pellissier, L. et al. 2010. Plant traits co-vary with altitude in grasslands and forests in the European Alps. – Plant Ecol. 211: 351–365.
Pellissier, L. et al. 2012. Shifts in species richness, herbivore specialization, and plant resistance along elevation gradients. – Ecol. Evol. 2: 1818–1825.
Phillips, J. M. and Hayman, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. – Trans. Br. Mycol. Soc. 55: 158–161.
Pratt, J. D. and Mooney, K. A. 2013. Clinal adaptation and adaptive plasticity in *Artemisia californica*: implications for

the response of a foundation species to predicted climate change. – Global Change Biol. 19: 2454–2466.
Rasmann, S. et al. 2011. Predicting root defence against herbivores during succession. – Funct. Ecol. 25: 368–379.
Rasmann, S. et al. 2012. Ecological role of transgenerational resistance against biotic threats. – Plant Signaling Behav. 7: 447–449.
Rasmann, S. et al. 2014. Climate-driven change in plant–insect interactions along elevation gradients. – Funct. Ecol. 28: 46–54.
Rizhsky, L. et al. 2004. When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. – Plant Physiol. 134: 1683–1696.
Roger, A. et al. 2013. Identity and combinations of arbuscular mycorrhizal fungal isolates influence plant resistance and insect preference. – Ecol. Entomol. 38: 330–338.
Ruotsalainen, A. L. et al. 2004. Root fungus colonization along an altitudinal gradient in north Norway. – Arct. Antarct. Alp. Res. 36: 239–243.
Sagar, G. R. and Harper, J. L. 1964. Biological flora of the British Isles. *Plantago major* L., *P. media* L., *P. lanceolata* L. – J. Ecol. 52: 189–211.
Schoonhoven, L. M. et al. 2005. Insect–plant biology. – Oxford Univ. Press.
Smith, S. E. and Read, D. R. 2008. Mycorrhizal symbiosis. – Academic Press.
Stamp, N. E. and Bowers, M. D. 1994. Effects of cages, plant-age and mechanical clipping on plantain chemistry. – Oecologia 99: 66–71.
Sutter, R. and Mueller, C. 2011. Mining for treatment-specific and general changes in target compounds and metabolic fingerprints in response to herbivory and phytohormones in *Plantago lanceolata*. – New Phytol. 191: 1069–1082.
Suzuki, N. and Mittler, R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. – Physiol. Plant. 126: 45–51.
Väre, H. et al. 1997. Shifts in mycorrhiza and microbial activity along an oroarctic altitudinal gradient in northern Fennoscandia. – Arct. Alp. Res. 29: 93–104.
Walker, D. J. et al. 2012. Accumulation of furanocoumarins by *Bituminaria bituminosa* in relation to plant development and environmental stress. – Plant Physiol. Biochem. 54: 133–139.
Webb, C. T. et al. 2010. A structured and dynamic framework to advance traits-based theory and prediction in ecology. – Ecol. Lett. 13: 267–283.
Züst, T. et al. 2012. Natural enemies drive geographic variation in plant defenses. – Science 338: 116–119.