

The simultaneous inducibility of phytochemicals related to plant direct and indirect defences against herbivores is stronger at low elevation

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Summary

1. Ecological theory indicates that warmer and more stable climates should result in stronger biotic interactions. Therefore, plant species growing at lower elevations and experiencing greater herbivore pressure should invest in higher levels of defences than those at higher elevations. Nonetheless, there are a number of studies that have found no effect of elevational gradients on plant defensive traits. Several factors might explain the lack of consistency for the altitude–defence relationships, including (i) the reduction of all defensive traits into one measure of resistance; (ii) not considering plant defence as the simultaneous expression of several defensive traits; and (iii) not considering the relative influence of biotic (e.g. herbivory) and abiotic (e.g. climate and soil conditions) factors associated with the ecological gradient.

2. Here, we present a comprehensive test of the effects of elevation and its associated biotic and abiotic factors on the individual and simultaneous expression of constitutive direct and indirect defences and their inducibility (i.e. expression of defences after herbivore attack). Specifically, we estimated climatic and soil variables and measured herbivore damage and constitutive and jasmonic acid-induced glucosinolate levels in the leaves as a proxy for direct defences, and volatile emission as a proxy for indirect defences in 16 *Cardamine* species naturally growing along the steep elevational gradient of the Alps.

3. Within a phylogenetic comparative framework, we found that species growing at lower elevations invested more in the simultaneous inducibility of both direct and indirect defences, whereas species growing at higher elevations invested more in constitutive direct defences. Although we found strong elevational gradients in herbivory and climatic and soil variables, these biotic and abiotic factors only partially explained elevational patterns in plant defences.

4. Synthesis. These results highlight that the complex regulation of multiple defence traits strongly vary across elevational gradients and build towards a better understanding of the multiple mechanisms underlying trait evolution and species interactions along ecological gradients.

Key-words: *Cardamine*, defence induction, elevational gradients, glucosinolates, plant defence syndromes, plant–herbivore interactions, resource availability hypothesis, volatile organic compounds

Introduction

For millions of years, plants have been exposed to herbivore attack (Labandeira 2007), and much of their success in

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dominating nearly all ecosystems is due to a wide range of highly effective anti-herbivore defences (Agrawal 2007, 2011). Plant defences can be broadly classified as direct and indirect (Karban & Baldwin 1997). Plant direct defences are conferred by chemical and mechanical traits that deter herbivores, reduce consumption or decrease their survival (Agrawal 2007; Carmona, Lajeunesse & Johnson 2011; Mithöfer & Boland 2012). Indirect defences, on the other hand, involve traits that provide shelter (e.g. domatia), reward (e.g. extrafloral nectar) or information on herbivore presence, such as the release of volatile organic compounds (VOCs), for the natural enemies of herbivores (i.e. predators and parasitoids; Kessler & Heil 2011). These indirect defensive traits have been shown to mediate herbivore–enemy interactions by increasing herbivore suppression, and ultimately potentially increasing plant fitness (Hairston, Smith & Slobodkin 1960; Schmitz, Hamback & Beckerman 2000; Romero & Koricheva 2011).

Variation in plant anti-herbivore defensive traits frequently follows ecological clines (Coley & Aide 1991; Schemske *et al.* 2009; Rasmann & Agrawal 2011; Moreira *et al.* 2014; Anstett *et al.* 2015; Lim, Fine & Mittelbach 2015). For instance, it has been observed that plant species at lower elevations, which should experience greater levels of herbivory, tend to invest more in defences (Scheidel & Bruelheide 2001; Zehnder *et al.* 2009; Rodríguez-Castañeda *et al.* 2010; Garibaldi, Kitzberger & Chaneton 2011; Pellissier *et al.* 2012; Rasmann, Pellissier & Alvarez 2014; Rasmann *et al.* 2014a). Despite these findings, studies reporting no effects of elevational gradients in direct and indirect defensive traits are also common (see review by Rasmann *et al.* 2014a).

Several understudied factors might explain the lack of consistency in altitude–defence relationships. First, both direct and indirect defences are constitutively expressed and can be induced after herbivore attack (Karban & Baldwin 1997). The costs and benefits of these two defensive strategies can vary depending on the abiotic and biotic conditions in which plants are embedded (Agrawal 2000). For example, as resistance is costly and constitutive and induced defences commonly trade off (Koricheva, Nykanen & Gianoli 2004; Kempel *et al.* 2011; Rasmann *et al.* 2011; Moreira *et al.* 2014), plants growing in environments with low herbivore pressure (e.g. high elevations; Pellissier *et al.* 2012) should select for high levels of induced defences and low levels of constitutive defences, and *vice versa* (Zangerl & Rutledge 1996; Moreira *et al.* 2014; Rasmann, Pellissier & Alvarez 2014). Secondly, previous studies investigating elevational gradients in direct and indirect defences have treated plant defences individually. However, plants are embedded into complex food webs, likely changing along ecological clines. For instance, low elevations are thought to bear greater species diversity, including higher numbers of predators (Hodkinson 2005b; Rasmann, Pellissier & Alvarez 2014), therefore prompting low-elevation plants to invest more in predator-recruitment traits (i.e. indirect defences), and less in direct defences. And thirdly, most studies investigating elevational gradients in plant defences have not evaluated the relative influence of both biotic (herbivory) and abiotic (e.g. temperature, precipitation, seasonal-

ity, soil fertility) factors. Indeed, plant defensive traits respond to herbivore pressure, but could also be adaptive depending on climatic conditions and resource availability (Pearse & Hipp 2012; Moreira *et al.* 2014). For instance, because soil fertility and temperature decline with elevation, along with associated reduction in growth, plants should be selected for greater investment in constitutive defences as long as herbivore pressure remains constant (i.e. resource availability hypothesis; Coley, Bryant & Chapin 1985). Finally, the combination of different defensive strategies leads to the evolution of ‘defence syndromes’ which are based on the prediction that synergistic interactions between multiple defensive strategies provide higher levels of defence than would be achieved by independent defensive strategies (Becerra 1997; Agrawal & Fishbein 2006; Agrawal 2007; Walters 2010).

Here, we present a comprehensive test of the effects of elevation and its associated biotic (herbivory) and abiotic (climate, soil conditions) factors on the individual and simultaneous expression of constitutive direct and indirect defences and their inducibility (i.e. the difference between induced and constitutive levels of defences). To test these objectives, we carried out a field study where we measured herbivory, climatic and soil variables, and correlated these variables to the constitutive and inducible levels of direct and indirect defence traits across 16 species of *Cardamine* (Brassicaceae: *Cardamine*) growing throughout the Alps, from 300 to 3000 m above sea level. Specifically, by using a phylogenetic comparative approach we addressed the following questions: (i) Is there interspecific variation in constitutive direct and indirect defences and their inducibility? (ii) Are there elevational gradients in constitutive direct and indirect defences and their inducibility across species? (iii) Is there an elevational gradient effect in the simultaneous expression of both direct and indirect defences across species? (iv) Do biotic and abiotic correlates of elevation explain ecological clines in plant defences? By addressing these questions, our work builds towards a better understanding of ecological factors driving multiple modes of plant defensive strategies across geographical gradients.

Materials and methods

NATURAL HISTORY, SPECIES DISTRIBUTION RANGE AND CLIMATIC VARIABLES

In order to assess natural variation in constitutive plant defences and their inducibility (i.e. expression of defence traits after herbivore attack) along elevational gradients, we sampled 16 species of *Cardamine*, out of 19 (including subspecies) currently growing in Switzerland (Aeschmann *et al.* 2004). Together, all species encompass almost a 3000-m elevational gradient of the Alpine ecosystem, growing as low as 300 m above sea level (m a.s.l.) (e.g. *C. bulbifera*) to up to more than 3000 m a.s.l. (e.g. *C. alpina*) (Fig. 1; Table 1). During the radiation of the group in rugged mountain landscapes, species have colonized variable habitats, including dry and wet alpine meadows, forests and riverbanks (Table 1). All species are classified as either geophyte (i.e. plant with resting buds below the ground,

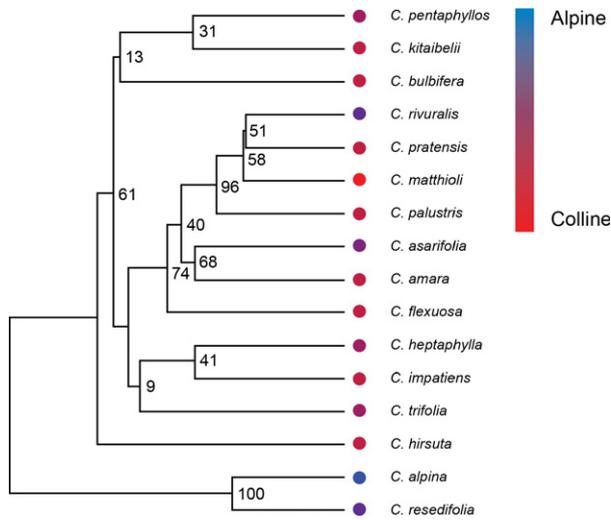


Fig. 1. Phylogenetic tree of the 16 *Cardamine* species. Habitat distribution, from alpine (colder colours) to colline (warmer colours). Bootstrap values above 50% are indicated on the branches.

including *C. amara*, *C. bulbifera*, *C. heptaphylla*, *C. kitaibelii*, *C. pentaphyllos*, *C. trifolia*), therophyte (i.e. plant surviving the winter as seed, including *C. hirsuta*, *C. impatiens*, *C. flexuosa*) or hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves, including the remaining species). At our field site, *Cardamine* species are predominantly attacked by leaf chewers such as Pieridae butterflies, leaf beetles and several slug species (S. Rasmann, personal observation) and some specialized aphid species. However, to date, a thorough analysis of the herbivore community feeding on this genus of plants is lacking. To construct the realized climatic niche of each species in Switzerland, we acquired occurrence data for each species from the National Data Center and Information on the Flora of Switzerland (www.infflora.ch). Occurrence data varied from a minimum of 18 points for rare species such as *C. asarifolia* to more than 1000 points for the widespread *C. pratensis*. For each accession, we extracted environmental data, including elevation, degree-days, solar radiation and potential evapotranspiration, which represent the most symptomatic niche values of elevation clines (Körner 2007). Values for degree-days and potential evapotranspiration indices were calculated from meteorological stations using a Digital Elevation Model (DEM) at 100 m resolution and interpolated following Zimmermann & Kienast (1999). Solar radiation values were calculated using the tool implemented in ArcGIS 10 (ESRI, Redlands, CA, USA). For each plot location, we extracted the values of environmental variables from associated environmental layers. Additionally, for each species we extracted ecological indicator values for soil fertility based on Landolt (2010) as shown in Table 1.

INDUCTION OF PLANT DEFENCES, SAMPLING, CHEMICAL ANALYSES AND HERBIVORY MEASUREMENTS

We measured constitutive direct (glucosinolates – hereafter GSLs) and indirect (volatile organic compounds – hereafter VOCs) defences and their inducibility (induced minus constitutive) across 16 species of *Cardamine* (i.e. species-level variation). Our approach – the macroevolutionary comparisons of traits among species – is common (e.g. Rasmann & Agrawal 2011; Moreira *et al.* 2014) and based upon the well-documented premise that among-species variation greatly

exceeds within species (microevolutionary) variation. Plants in the mustard family (Brassicaceae), including *Cardamine* species, contain GSLs that have been shown to be effective against generalist and to some extent against specialist herbivores (e.g. Louda & Rodman 1983; Daxenbichler *et al.* 1991; Montaut & Bleeker 2011). When in contact with myrosinase, an enzyme present in separate specialized cells, GSLs are degraded into toxic sulphur- and nitrogen-containing by-products (Hopkins, van Dam & van Loon 2009). Brassicaceae also produce VOCs after mechanical or herbivore damage, which have been shown to attract natural enemies of herbivores (e.g. Dudareva, Pichersky & Gershenzon 2004; Gols *et al.* 2009; Harvey *et al.* 2011).

For phytochemical analyses, all species were sampled at the flowering stage, from May until August, following the natural phenology of the plants. The phenological state was chosen in order to avoid ontogenetic effects on plant chemistry, while we chose to sample at the flowering stage since most species flower very rapidly, and for long periods of time, sometimes throughout the whole growing season. For each species, one or two populations growing in their optimal habitat (i.e. the centre of their elevational distribution; see Table S1 in Supporting Information) were chosen for a total of $n = 8–20$ plants per species. The individual plants were sampled requiring a minimum distance of 5 m. Only unchallenged plants with little to no herbivore damage were included. We considered our sampling at the centre of the distribution to be conservative in regards to within species variation (i.e. we considered the centre of the elevational distribution the optimal realized niche conditions), but we acknowledge that we likely underestimated the effect of elevational breadth variation across species, since niche breadth itself could influence investment in plant defences, but this remains to be addressed with future work.

For trait collection, 24 h prior to sampling, vegetative parts of the plants were bagged with Nalophan bags (1 L). Half of the plants were induced with methyl jasmonate (CAS number 39924-52-2, Sigma, St. Louis, IL, USA; hereafter JA induction) by applying 5 μL of the pure compound to a cotton bud, which was placed in the bags. Jasmonic acid (JA) is a plant hormone involved in defence signalling against necrotrophic pathogens, chewing herbivores and phloem-feeding insects (Thaler 1999). The other half of the plants were used as controls, which also received cotton buds, but without JA application. On the following day the bags were replaced with new bags to avoid sampling highly accumulated scent.

Plant VOCs were collected by pulling air through a charcoal filter trap (Orbo-32; Supelco, Bellefonte, PA, USA) at a flow rate of 200 mL min^{-1} for four hours, always between 10:00 AM and 2:00 PM. After collection, the traps were kept at 4 °C before elution with 350 μL of dichloromethane after the addition of 5 μL of tetralin (50 $\text{ng } \mu\text{L}^{-1}$) as an internal standard. Aliquots of 1 μL of each sample were subsequently analysed by gas chromatography–mass spectrometry (GC-MS Thermo GC coupled to a DSQ MS; Thermo Fisher Scientific, Waltham, MA, USA) equipped with a DB-5 MS column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness; Thermo Scientific). Helium was used as carrier gas at a constant flow of 1.2 mL min^{-1} and the following column temperature gradient: 40 °C for 5 min, increased to 180 °C at 10 °C min^{-1} , increased to 220 °C at 40 °C min^{-1} , followed by a final hold at 220 °C for 10 min. Peaks were identified by comparison of retention times and mass spectra with authentic standards when available. Volatile emissions were reported as equivalents of nanograms tetralin. Finally, all compounds were classified into terpenoids and non-terpenoids (Table S2).

Plant GSL profiles were obtained for three fully expanded leaves per sampled plant immediately after the volatile collection. Lyophilized plant material was ground to a fine powder using a Retsch ball

Table 1. Sampled *Cardamine* species. Shown are the optimum of elevation, temperature, moisture of each species, their altitudinal distribution based on biomes, and their characteristic habitat. Occurrence data for each species were obtained from the National Data Center and Information on the Flora of Switzerland (www.infoflora.ch). Habitat characteristics and soil nutrient requirements were extracted from (Landolt 2010)

Plant species	Elevation (m)	Degree-days	Potential evapotranspiration	Soil nutrients ¹	Herbivore damage (%) ²	Distribution	Habitat ³
<i>C. mathioli</i>	325	3169	280	4	24	warm colline	1.3 5.1 6.3 9.2
<i>C. palustris</i>	465	2808	-474	3	18.8	colline	1.3 6.3 9.2
<i>C. hirsuta</i>	512	2424	-77	4	14.4	colline to montane	2.1 2.2
<i>C. bulbifera</i>	581	2506	0.45	4	37.5	colline to montane	9.2
<i>C. impatiens</i>	701	2449	-136	4	15.5	colline to montane	2.6 3.2 9.2
<i>C. flexuosa</i>	744	2250	7	3	24.5	colline to montane	2.6 4.1 4.2 9.2
<i>C. pratensis</i>	767	2077	88	4	4.7	colline to montane	1.3 2.3 6.3 9.2
<i>C. trifolia</i>	820	2147	-3	3	4	montane	6.3 9.2
<i>C. kitaibelii</i>	860	2316	497	4	26.3	colline to montane	9.2
<i>C. amara</i>	954	2227	-147	3	8.7	colline to montane	4.1 4.2 9.2
<i>C. asarifolia</i>	999	2142	-264	3		montane to subalpine	4.1 4.2 6.3 7.2
<i>C. pentaphyllos</i>	1041	1939	190	4	18.5	montane	9.2
<i>C. heptaphylla</i>	1097	1893	230	4	10.8	montane	9.2
<i>C. rivularis</i>	1323	1672	304	2	1.1	montane	2.2 2.3 7.2
<i>C. alpina</i>	2450	681	678	2	3.4	alpine and nival	3.3 4.1 6.6
<i>C. resedifolia</i>	2517	583	588	2	7.1	subalpine to nival	3.2 3.3

¹Soil nutrient requirement varies from a minimum of 1 = infertile substrate poor in nutrients (e.g. siliceous rock or peat) to a maximum of 5 = fertile substrates rich in nutrients. Overall, the phylogenetic-corrected correlation indicates that soil fertility decrease with increasing elevation (Fig. 2a, pGLS; $r = -0.64$, $P = 0.004$).

²Overall, the phylogenetic-corrected correlation indicates that herbivore damage decreases with increasing elevation (Fig. 2b, pGLS; $r = -0.54$, $P = 0.022$).

³Habitat names are adopted from (Delarze & Gonseth 2008). 1.3 = seeps and sources; 2.1 = vegetated shores; 2.2 = low marsh; 2.3 = wet meadows; 3.2 = alluvium and moraines; 3.3 = screes; 4.1 = rocky slabs; 4.2 = thermophilic dry grasslands; 5.1 = herbaceous edges; 6.3 = hardwood forests; 6.6 = high elevation coniferous forests; 7.2 = anthropogenic rocky environments; 9.2 = buildings.

mill MM400 (Haan, Germany), and GSLs were extracted from 50 mg plant powder in 1.0 mL 70% methanol (MeOH) in water (v/v). After heat-deactivating the myrosinase at 90 °C for 5 min tubes were placed in an ultrasonic bath for 15 min and centrifuged at 58 g in an Eppendorf centrifuge for 10 min. The extraction was repeated twice except for the boiling step, and both supernatants were added to a DEAE-Sephadex A-25 column (5 × 10 mm) and washed twice with 1 mL 70% MeOH, once with 1 mL Milli-Q (Merck, Darmstadt, Germany) water and then twice with 1 mL 20 mM NaOAc buffer (pH 5.5). A total of 20 µL of aryl sulfatase (Type H-1 of *Helix pomatia*; CAS 9016-17-5, Sigma) was added to the columns and flushed down with 50 µL NaOAc buffer (pH 5.5) to break the sulphur bonds of GSLs. The columns were covered with aluminium foil and incubated overnight at room temperature. Thereafter, desulfoglucosinolates were eluted from the columns twice with 0.75 mL Milli-Q water and freeze-dried. The residue was dissolved in 1.0 mL of Milli-Q water and stored at -20 °C until liquid chromatography analysis. Glucosinolate analysis was done according to van Dam, Witjes & Svatos (2004). The desulfoglucosinolate extract was separated by means of a reversed-phase C18 column (Alltima C18 3 µm, 150 mm × 4.6 µm) using a Dionex Ultimate 3000 HPLC with a CH₃CN-H₂O gradient (2–35% acetonitrile from 0 to 30 min; flow 0.75 mL min⁻¹). GSLs were detected with a photodiode-array detector at 229 nm (Dionex, Sunnyvale, CA, USA). Desulfoglucosinolate peaks were identified by comparing the retention times and UV spectra to authentic standards (progointrin, gluconapin, glucoiberin, glucobrassicinapin, glucotropaeolin, gluconasturtiin, glucoraphanin, glucoerucin, glucobrassicin, sinalbin; Phytoflan, Heidelberg, Germany) or to the in-house retention time and UV spectra library. For unknown GSLs the different shapes of the UV spectra (between 200 and 300 nm) were compared to known standards to identify the GSL class. GSL concentrations were calculated by using an external sinigrin calibration curve (50–

650 µM) and dividing by dry mass. GSLs were categorized as aromatic, indole or aliphatic (Table S3).

Finally, during each field sampling date, we recorded the amount of chewing herbivore damage on each plant using a percentage scale from 0% = no visible damage to 100% = all leaves were completely eaten, and using percentage increments as follows 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 80%, 90%, 100%.

PHYLOGENETIC ANALYSES

In order to control phylogenetic non-independence among *Cardamine* species, we built a phylogenetic tree of all species by pruning it from a more comprehensive phylogeny. The initial phylogenetic tree was reconstructed based on a plastid intergenic region (*trnL-trnF*), and two nuclear genes (*ndhF* and 5.8S-ITS). Sequences from GenBank were compiled and supplemented with the 16 species generated here. For those, DNA was extracted and amplified using the primers Poac ndhF1, Poac ndhF 728-rev, ndhF 1403, ndhF 2110 and the same PCR protocols as in Christin *et al.* (2007). A total of 120 species in the genera *Armoracia*, *Barbarea*, *Cardamine*, *Iodanthus*, *Leavenworthia*, *Nasturtium*, *Rorippa* and *Syrenia* were sampled (GenBank accessions in Table S4). The DNA matrix was aligned using MAFFT (Katoh *et al.* 2002). Poorly aligned positions were removed using Guidance software, with a threshold of 0.93, resulting in a final matrix with 2207 bp (Penn *et al.* 2010). The GTR + Γ model of nucleotide substitution was selected using the Akaike information criterion (AIC) for all DNA regions. A maximum likelihood analysis was conducted with PhyML (Guindon & Gascuel 2003) using SPR branch swapping algorithm and 1000 bootstrap replicates. The resulting tree (Fig. S1) was pruned to the 16 investigated species (Fig. 1).

STATISTICAL ANALYSES

Treatment and plant species effects on direct and indirect defences

Differences in VOC and GSL amount and composition among species, treatments (control vs. JA-induced) and their interaction were tested using a permutational ANOVA (PERMANOVA) using the Adonis function in the vegan package in R (Oksanen *et al.* 2013). The Bray–Curtis metric was used to calculate the dissimilarity matrix of all compounds across species.

Elevational gradients in biotic and abiotic factors

We performed simple linear regressions (using species means) to evaluate the relationship between herbivore damage, temperature, moisture, solar radiation and soil nutrients with elevation. To account for phylogenetic non-independence among species, these simple regressions were performed using phylogenetic correct generalized least square analyses (pGLS) using the *pgls* function in caper (Orme 2013).

Elevational gradients in constitutive direct and indirect defences and their inducibility

To investigate whether elevation was related to constitutive direct and indirect defences and their inducibility, we first performed simple linear regressions (using species means) to evaluate the relationship between constitutive amount of VOCs and GSLs and their inducibility (*i.e.* species means of induced levels minus constitutive levels) with elevation.

Subsequently, we performed multiple regression analyses relating elevation, herbivore damage, temperature, moisture, solar radiation and soil fertility to the total amount of constitutive defences and their inducibility. The aim of performing these multiple regression analyses was to test which biotic and/or abiotic factors still drive variation in secondary metabolite concentrations once correlations among these factors are accounted for, for example between elevation and temperature. Basically, we consider that if the elevational gradient effect *per se* remains significant when accounting for other factors, then we can

conclude that other unmeasured niche factors also drive ecological clines in defence investment (e.g. Moreira *et al.* 2015). Both simple and multiple regressions were performed using phylogenetic correct generalized least square analyses (pGLS) using the *pgls* function in caper (Orme 2013). The phylogenetic signal (lambda value, λ), the R^2 and coefficients of each trait are provided in Table 2.

Elevational gradients in the simultaneous expression of constitutive direct and indirect defences and their inducibility

We tested for a shared structure between production of the different VOC and GSL compounds, which would represent a coupled direct–indirect defence syndrome, using a coinertia analysis. In other words, here we tested whether the matrices of VOC and GSL production (including abundance and diversity of compounds) vary across species. If this is the case, it would lead us to conclude that species vary in the simultaneous expression of both direct and indirect defences. We first assessed the correspondence between constitutive VOCs and constitutive GSLs (coinertia 1), then between the inducibility of VOCs and inducibility of GSLs (coinertia 2). The coinertia analysis indicates whether the group of species producing a similar structure of an indirect chemical defensive trait also produces a similar structure of another direct chemical defensive trait (*i.e.* both types of defences are expressed similarly within one species). The coinertia analyses were performed using the *ade4* package in the R environment (Dray, Chessel & Thioulouse 2003; Dray & Dufour 2007), and the significance of the shared variance was assessed using a Monte Carlo test as implemented in *ade4*.

When the coinertia analysis was significant (*i.e.* there is variation across species; see Table 2 in the Results section), we performed linear regressions (using species means) to investigate whether the main axis of the coinertia analysis representing the shared covariation or syndrome between VOCs and GSLs was significantly associated with elevation. Next, we performed multiple regression analyses to simultaneously evaluate the effect of elevation, herbivore damage, temperature, moisture, solar radiation and soil nutrients on the main axis of coinertia analysis as described above. Both simple and multiple regressions were performed using phylogenetic-corrected generalized least square analyses (pGLS) using the *pgls* function in caper (Orme

Table 2. Multiple regression analyses between constitutive glucosinolates (GSLs) and volatile organic compounds (VOCs) and their inducibility and the environmental axes of the elevational gradient (elevation, temperature in degree-days, moisture as potential evapotranspiration, solar radiation, herbivore damage and soil fertility)

Variable	Constitutive GSLs					Inducibility of GSLs					Inducibility of VOCs				
	$\lambda = 0$					$\lambda = 1$					$\lambda = 0$				
	$F_{1,8}$	<i>P</i>	R^2	Coef.	VIF	$F_{1,7}$	<i>P</i>	R^2	Coef.	VIF	$F_{1,7}$	<i>P</i>	R^2	Coef.	VIF
Elevation	15.28	0.004	0.49	−0.08	57.82	4.80	0.065	0.30	−0.01	57.60	7.09	0.028	0.38	0.006	80.15
Degree-days	0.50	0.498	0.02	−0.09	47.15	0.56	0.478	0.03	−0.01	47.24	2.30	0.168	0.01	0.005	94.04
Moisture	0.64	0.446	0.02	−0.03	3.08	0.37	0.559	0.02	0.01	3.61	15.04	0.004	0.48	−0.006	9.46
Radiation	1.88	0.207	0.06	−0.007	1.81	4.12	0.082	0.26	0.004	2.19	0.10	0.756	0.001	0.0003	2.91
Herbivore damage	0.005	0.943	0.001	1.2	2.86	5.07	0.059	0.32	0.42	3.42	0.08	0.785	0.01	−0.11	3.52
Soil fertility	12.68	0.007	0.41	−31.95	5.36	0.91	0.371	0.06	7.11	5.96	0.58	0.467	0.13	1.92	5.42

Significant ($P < 0.05$) *P*-values are typed in bold. We did not include constitutive VOC production in the analyses since elevation alone was not a predictor. Lambda (λ) values fluctuate between phylogenetic signal ($\lambda = 1$) and no phylogenetic signal ($\lambda = 0$). Averages (\pm) 1 SE of total VOC and GSL production across species are reported in Table S2 and Table S3, respectively. Variance inflation factors (VIFs) were also included to show high collinearity among ecological variables (IF = 1: not correlated; 1 < VIF < 5: moderately correlated; VIF > 5–10: highly correlated).

2013). The phylogenetic signal (lambda value, λ) of the correlation between each trait and the coinertia axis is provided in Table 3.

Results

TREATMENT AND PLANT SPECIES EFFECTS ON DIRECT AND INDIRECT DEFENCES

In total, we found 27 individual volatile organic compounds (VOCs) of which 19 belonged to the class of terpenoids and the remaining eight were of different origin and include a number of green leaf volatiles (Table S2). We also found 23 individual glucosinolates (GSLs) belonging to aliphatic (12 compounds), aromatic (3 compounds) or indole (8 compounds) GSLs (Table S3).

Volatile organic compound and GSL composition (the identity and relative abundance of each) differed largely between species, and across induction treatments (*i.e.* JA induction *versus* control). Specifically, we found that *Cardamine* species varied in the composition of VOCs, and induction treatment shifted foliar VOC composition in a species-specific manner (PERMANOVA; species effect: $F_{15,95} = 14.63$, $P < 0.001$; induction treatment effect: $F_{1,95} = 23.80$, $P < 0.001$; species \times treatment effect: $F_{15,95} = 4.07$, $P < 0.001$). Similarly, *Cardamine* species varied in foliar GSL composition, and induction treatment also shifted GSL composition in a species-specific manner (PERMANOVA, species effect: $F_{13,72} = 22.00$, $P < 0.001$; induction treatment effect: $F_{1,72} = 1.67$, $P = 0.07$; species \times treatment effect: $F_{13,72} = 1.41$, $P = 0.003$).

ELEVATIONAL GRADIENTS IN BIOTIC AND ABIOTIC FACTORS

Landolt indicator values highlighted that species growing at high elevation experience poorer soil conditions (*i.e.* lower fertility) than those growing at low elevation (Table 1; Fig. 2a). The field survey indicated that species at higher elevation are less eaten by chewing herbivores than lowland species (Table 1 and Fig. 2b). The reconstruction of the climatic niche for all species confirmed that temperature decrease

linearly with elevation (pGLS, degree-days; $r = -0.97$, $P < 0.001$), while potential evapotranspiration increases with elevation ($r = 0.66$, $P = 0.003$), but there was no relationship between elevation and solar radiation ($r = -0.11$, $P = 0.378$).

ELEVATIONAL GRADIENTS IN CONSTITUTIVE DIRECT AND INDIRECT DEFENCES AND THEIR INDUCIBILITY

Simple (univariate) regressions showed that constitutive concentration of total GSLs (Fig. 3a) was higher at higher elevations, whereas their inducibility (Fig. 3b) significantly increased at lower elevations. The effect of elevation on the constitutive production of GSLs remained significant once biotic and abiotic factors were accounted for in the multiple regression analysis (Table 2), suggesting that other biotic and/or abiotic factors must be accounted for to fully explain these elevation patterns. Nevertheless, in the multiple regression analysis, we found a significant effect of soil fertility on constitutive GSLs, in which constitutive GSLs were higher in species adapted to poor soils (Fig. S2a; Table 2), but the elevational gradient effect remained significant nonetheless, suggesting that soil fertility itself is not the only explanatory variable of the investment in constitutive GSLs at high elevation.

In the multiple regression analysis, we found only marginal significance for the elevation effect on the inducibility of GSLs, and a marginal significant effect of herbivore damage on the inducibility of GSLs, in which inducibility of GSLs was positively associated with higher levels of herbivore damage (Fig. S2b; Table 2).

Simple regression showed that constitutive emission of VOCs was not significantly related to elevation (Fig. 3c). These results remained qualitatively unchanged based upon the multiple regression analysis (Table 2). Finally, simple regressions also showed that the inducibility of VOCs significantly increased at lower elevations (Fig. 3d). Elevation effect on the induction of VOCs remained significant once biotic and abiotic factors were accounted for in the multiple regression analysis (Table 2), suggesting that the measured biotic and abiotic factors did not fully explain these elevation

Table 3. Results of the coinertia analyses between constitutive glucosinolates (GSLs) and constitutive volatile organic compound emission (VOCs) and between and jasmonic acid (JA) inducibility of VOCs and inducibility of GSLs

Coin.	Defence (coinertia) axis	$P_{\text{Coinertia}}$	Variable	$F_{1,11}$	P	R^2	Coef. (\pm SE)
1	Constitutive VOCs–Constitutive GSLs	0.675	N/A				
2	Inducibility of VOCs–Inducibility of GSLs	0.042	Elevation	7.09	0.029	0.27	0.004 (0.002)
			Degree-days	2.35	0.167	0.01	0.003 (0.002)
			Moisture	15.04	0.005	0.57	−0.004 (0.001)
			Radiation	0.10	0.756	0.004	−0.0001 (0.0001)
			Herbivore damage	0.08	0.785	0.003	−0.007 (0.04)
			Soil fertility	0.58	0.467	0.02	0.41 (0.53)

The table shows i) the significance value of the coinertia analysis and ii) the phylogenetic controlled multiple regression analyses (pGLS) results of the correlations between the coinertia axes and the environmental axes of the elevational gradient (elevation, temperature in degree-days, moisture as potential evapotranspiration, solar radiation, herbivore damage and soil fertility). Significant ($P < 0.05$) P -values are typed in bold. Lambda (λ) values are either 0 or 1. Averages (\pm) 1 SE of total VOC and GSL production across species are reported in Table S2 and Table S3, respectively.

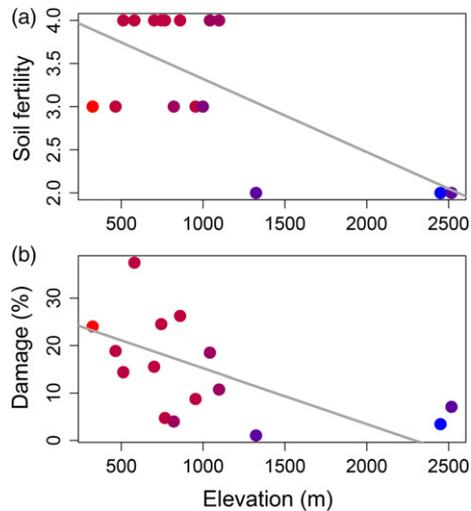


Fig. 2. Relationship between elevation and (a) nutrient availability in the soil, and (b) the average herbivore damage per species. Species are colour-coded based on their average altitudinal range; cold (blue) colours indicate high elevation, while warm (red) colours indicate low elevation. The grey line indicates significant correlation (pGLS; $P < 0.05$; see also Table 1).

patterns. In the multiple regression analysis, we however found a significant effect of moisture regimes (evapotranspiration) in the atmosphere on inducibility of VOCs, in which drier climate favour VOC inducibility (Fig. S2c; Table 2).

ELEVATIONAL GRADIENTS IN THE SIMULTANEOUS EXPRESSION OF CONSTITUTIVE DIRECT AND INDIRECT DEFENCES AND THEIR INDUCIBILITY

The coinertia analyses showed no congruent structure between constitutive VOCs and constitutive GSLs (coinertia 1 in Table 3), but we observed significant correlation between inducibility of VOCs and inducibility of GSLs (coinertia 2 in Table 3). In accordance with the results described above, the simple regression analysis showed that the main axis of the coinertia analysis between inducibility of VOCs and inducibility

of GSLs (coinertia 2) was significantly and negatively associated with elevation (Fig. 4). The elevation effect on the main axis of the coinertia analysis between inducibility of VOCs and GSLs remained significant once biotic and abiotic factors were accounted for in the multiple regression analysis (Table 3), suggesting that both biotic and abiotic factors did not fully explain these elevation patterns. Nevertheless, in the multiple regression analysis we also found a significant effect of evapotranspiration on the simultaneous induction of VOCs and GSLs, in which drier climate (lowland) favour the simultaneous induction of VOCs and GSLs (Table 3).

Discussion

Elevation, with strong clines in climatic and soil conditions, is largely associated with biotic changes such as species abundance and diversity. Shifts in abiotic conditions may also contribute to impact plant defensive phenotypes and the functional traits related to anti-herbivore defence (Rodríguez-Castañeda *et al.* 2010; Rasmann *et al.* 2014a). Traditionally, theory predicts that the strength of plant–herbivore interactions increases towards the sea level (Rodríguez-Castañeda *et al.* 2010; Pellissier *et al.* 2012), leading to evolution of greater investment in plant defences at lower elevations due to higher herbivore abundance and diversity (Raffa, Powell & Townsend 2013). Accordingly, in this study we found that the investment in constitutive direct and indirect defences and their inducibility in *Cardamine* species was strongly driven by elevational gradients, but these responses vastly varied depending on the plant defensive strategy that was considered. Specifically, we found that species growing at lower elevations invest more in the inducibility of both direct and indirect defences (VOCs and GSLs), whereas species growing at higher elevations invest more in constitutive direct defences (GSLs). These results support previous findings of studies analysing single and multiple species of herbaceous and woody plants (e.g. Moreira *et al.* 2014; Rasmann *et al.* 2014b). For example, Moreira *et al.* (2014) found that constitutive production of resins in the stems of pine species

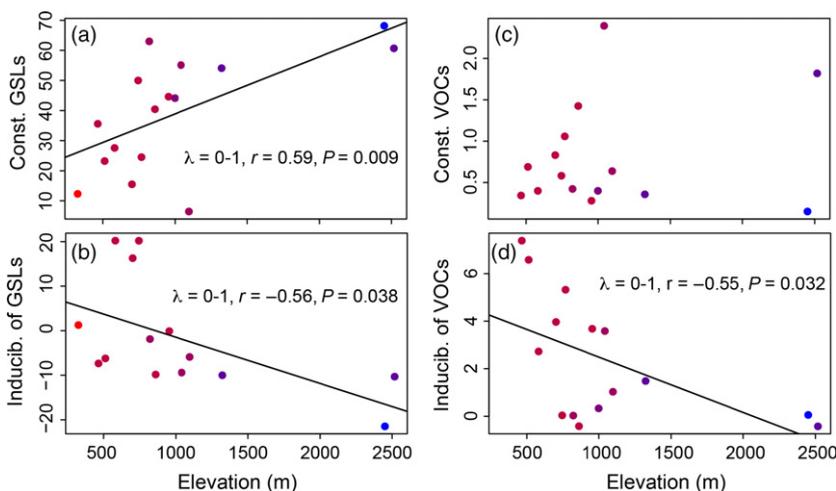


Fig. 3. Correlations between elevation and (a) constitutive glucosinolates (GSLs), (b) inducibility of GSLs by jasmonic acid (JA), (c) constitutive volatiles (VOCs) and (d) JA inducibility of VOCs. Colours correspond to the optimal elevational distribution of each species (see Fig. 1). Black solid lines represent significant correlations (pGLS; $P < 0.05$). Lambda (λ) values indicate intermediate levels of phylogenetic signal ($\lambda = 0$, and $\lambda = 1$ (0,1)).

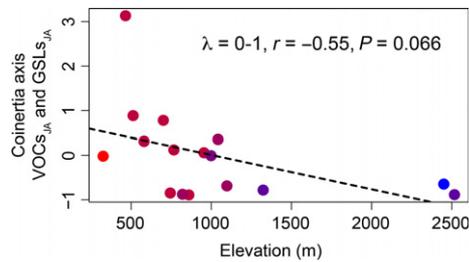


Fig. 4. Correlation between elevation and the correlated structure (i.e. first axes of the coinertia analysis) of jasmonic acid (JA) inducibility of volatile organic compounds (VOCs) and JA inducibility of glucosinolates (GSLs) along the elevation axis. Colours correspond to the optimal elevational distribution of each species (see Fig. 1). A black dashed line represents a marginally significant correlation (pGLS, $P < 0.10$). Lambda (λ) values indicate intermediate levels of phylogenetic signal ($\lambda = 0$, and $\lambda = 1$ (0–1))

increased at higher altitudes and latitudes, whereas inducibility of these direct defences was greater at lower latitudes. A similar elevation-driven trade-off between deployment of defences was reported for *Vicia sepium* (Fabaceae) (Rasmann, Pellissier & Alvarez 2014). The most plausible explanation for these findings is that constitutive defences evolve in the direction proposed by the resource availability hypothesis (Coley, Bryant & Chapin 1985; Endara & Coley 2011), in which more stressful environments (e.g. higher elevations) are commonly associated with slow-growing species with high tissue value. Consequently, plant species adapted to more stressful abiotic environments increase allocation to constitutive plant defences, as the cost of replacing tissues consumed by herbivores is higher under low resource conditions (Coley, Bryant & Chapin 1985; Endara & Coley 2011). In contrast, fast-growing species growing in resource-rich environments (e.g. lower elevations) should be selected for having increased induced defences, consistent with greater resource availability and with the constitutive–induced defences trade-off (Bingham & Agrawal 2010; Rasmann *et al.* 2011; Moreira *et al.* 2014).

Our results also showed that elevation was associated with variation in the simultaneous expression of direct and indirect defences. In particular, we found that the simultaneous inducibility of both direct and indirect defences was stronger at lower elevations. There are several plausible explanations for these findings. First, abundance and richness of herbivores have been commonly described to be higher under milder climatic conditions (e.g. lower elevations) (Pellissier *et al.* 2012, 2014). Therefore, in these habitats, synergistic interactions between multiple defensive strategies might be fundamental to provide effective resistance. Secondly, previous studies have documented a reduction in specialized herbivore pressure at higher elevations (Pellissier *et al.* 2012), which might in turn determine the effectiveness of plant defensive traits at different elevations. Unique or highly toxic secondary compounds might be enough to cope with generalist herbivores, but in contrast, specialist herbivores are able to overcome toxic effects of such defences (Ali & Agrawal 2012). Therefore, plants may need the involvement of higher trophic

levels to effectively reduce herbivore loads. Furthermore, while plant defences against generalist herbivores directly increase herbivore mortality (i.e. direct defences), those against specialist herbivores will prolong the time spent in feeding and indirectly increase herbivore mortality through suppression by natural enemies of herbivores (i.e. the synergistic combination of direct and indirect defences) (Volf *et al.* 2015). Therefore, at lower elevations, the effectiveness of plant defences against the complex herbivore assemblages should result from diffuse co-evolution selecting for multiple defensive traits (including both direct and indirect defences) rather than pairwise co-evolution selecting for a single defensive trait or strategy. Thirdly, as secondary metabolism is costly for the plant, the simultaneous expression of different defensive strategies should trade off (Stamp 2003). However, resource availability has been predicted to be higher under favourable conditions (e.g. lower elevations) so the emergence of associated costs to the production and maintenance of anti-herbivore defences might be more evident at higher elevations. Finally, as the abundance of invertebrate predators and parasitoids drastically decreases at higher elevations (Hodkinson 2005a; Rodríguez-Castañeda *et al.* 2011), we predict that indirect defences through VOC emission should be virtually useless at high elevation, at least in the Alps.

Although biotic (herbivory) and abiotic (climate and soil conditions) factors associated with elevation are considered as important drivers of plant defence allocation (Moreira *et al.* 2014; Anstett *et al.* 2015), we observed that they did not fully explain elevational gradients on the deployment of individual and simultaneous expression of constitutive direct and indirect defences and their inducibility. In particular, we found that after accounting for these factors in the multiple regressions, elevation still significantly predicted plant defence allocation in all analyses. Therefore, although we found strong elevational gradients for herbivore damage, climate and soil conditions, our findings clearly highlight the fact that elevational gradients in biotic and abiotic factors are not always associated with corresponding patterns of plant defence. Supporting our results, a recent study by Abdala-Roberts *et al.* (2015) found that although both leaf herbivory and fruit attack in the tropical herb *Ruellia nudiflora* varied latitudinally, this did not result in concomitant gradients in leaf and seed phenolics. We encourage further studies which will investigate alternative underlying factors driving geographical variation in plant defences (e.g. natural enemies of herbivores, Björkman, Berggren & Bylund 2011; Johnson & Rasmann 2011). In this sense, some recent investigations have demonstrated that predation and parasitism by arthropods is commonly stronger at lower latitudes (Stireman *et al.* 2005; Marczak *et al.* 2011), and this might in turn modify the allocation of defences.

Overall, our results highlight the importance of accounting for the simultaneous expression of several defensive strategies, as well as the relative influence of abiotic and biotic factors, in order to provide a better understanding of the multiple mechanisms and ecological factors underlying geographical gradients in plant defences.

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Data accessibility

Data from this paper can be accessed through the Dryad data repository (<http://dx.doi.org/10.5061/dryad.50kc9>) (Rasmann 2016).

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Supporting Information

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

Table S1. Collection sites for all *Cardamine* species.

Table S2. Volatile organic compounds (VOCs) found in 16 *Cardamine* species.

Table S3. Glucosinolates detected in 16 *Cardamine* species.

Table S4. GenBank sequences of brassicaceous plants.

Figure S1. Comprehensive phylogram of brassicaceous plants.

Figure S2. Relationship between *Cardamine* direct and indirect defences and ecological indicators.