1 **Variable Effects on Growth and Defence Traits for Plant Ecotypic Differentiation and**
2 **Phenotypic Plasticity along Elevation Gradients**

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16 Running head: plant ecotypic variation and phenotypic plasticity along elevation

17 **Abstract**

18 Along ecological gradients, ecotypes generally evolve as the result of local adaptation to a
 19 specific environment to maximize organisms' fitness. Alongside ecotypic differentiation,
 20 phenotypic plasticity, as the ability of a single genotype to produce different phenotypes
 21 under different environmental conditions, can also evolve for favouring increased organisms'
 22 performance in different environments. Currently, there is a lack in our understanding of how
 23 varying habitats may contribute to the differential contribution of ecotypic differentiation and
 24 plasticity in growth versus defence traits. Using reciprocal transplant-common gardens along
 25 steep elevation gradients, we evaluated patterns of ecotypic differentiation and phenotypic
 26 plasticity of two coexisting but unrelated plant species, *Cardamine pratensis* and *Plantago*
 27 *major*. For both species, we observed ecotypic differentiation accompanied by plasticity in
 28 growth related traits. Plants grew faster and produced more biomass when placed at low
 29 elevation. In contrast, we observed fixed ecotypic differentiation for defence and resistance
 30 traits. Generally, low elevation ecotypes produced higher chemical defences regardless of the
 31 growing elevation. Yet, some plasticity was observed for specific compounds, such as indole
 32 glucosinolates. We speculate that ecotypic differentiation in defence traits is maintained by
 33 costs of chemical defence production, while plasticity in growth traits is regulated by
 34 temperature driven growth response maximization.

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36 **Key-words:** caffeoyl phenylethanoid glycoside, glucosinolates, iridoid glycosides, plant
 37 defence, plant-herbivore interaction.

38 Introduction

39 Species with extensive geographical ranges tend to exhibit large intraspecific variation in
 40 most functional and phenotypic traits. Such geographic variation can lead to the evolution of
 41 morphologically and functionally different genotypes or ecotypes (Hufford and Mazer, 2003;
 42 Kawecki and Ebert, 2004; Savolainen *et al.*, 2007). Ecotypes are comprised of genetically
 43 distinct population of a given species retaining traits that maximize fitness leading to local
 44 adaptation to particular local abiotic and biotic conditions (Kawecki and Ebert, 2004).
 45 Phenotypic variation within a species due to heritable ecotypic differentiation is further
 46 distinguished in different habitats by phenotypic plasticity. Phenotypic plasticity refers to the
 47 ability of a single genotype to produce different phenotypes under different environmental
 48 conditions. Plasticity itself can also be selected for and evolve differently for different
 49 developmental, physiological, and reproductive traits or in different habitats in order to
 50 optimize organisms' performance (Bradshaw, 1965; Gotthard *et al.*, 1995; Lortie and Aarssen,
 51 1996; Murren *et al.*, 2015; Scheiner, 1993; Sultan, 1987; Sultan, 2003). Species with greater
 52 adaptive plasticity respond more acutely to environmental changes, and may be better able to
 53 survive in novel environments allowing their rapid geographical spread inhabiting a broad
 54 range of environmental conditions (Baker, 1974; Oliva *et al.*, 1993; Spencer *et al.*, 1994), thus
 55 promoting local adaptation (Baldwin, 1896; Ghalambor *et al.*, 2007; Price *et al.*, 2003).

56 As sessile organisms, plants should experience strong local adaptation to local climate that
 57 strongly affects plants' fitness. For instance, with temperature transitions across species'
 58 latitudinal ranges or altitudinal niches- spanning low to high elevations-, plants tend to evolve
 59 to produce smaller seeds, to have earlier phenology, slower growth rates, and display greater
 60 investment in clonal reproduction (e.g. Chapin and Chapin, 1981; Körner, 2003; Moles *et al.*,
 61 2007; Montague *et al.*, 2008; Pilon *et al.*, 2003). At the community level, the emergence of
 62 interspecific interaction clines out of biogeographical clines is also expected. Since the initial
 63 Dobzhansky's postulate of a potential correlation between biotic interaction strength and trait
 64 values for traits mediating such interactions (Dobzhansky, 1950), a large volume of literature
 65 has focused on plant-herbivore interaction (Bolser and Hay, 1996; Coley and Aide, 1991;
 66 Schemske *et al.*, 2009). More specifically, it is expected that increased herbivory pressure in
 67 the tropics should favour the evolution of more potent defences in plants (Coley and Barone,
 68 1996; Moles *et al.*, 2011; Pellissier *et al.*, 2014; Pennings *et al.*, 2001; Rasmann and Agrawal,
 69 2011; Siska *et al.*, 2002; Woods *et al.*, 2011). Furthermore, a decrease in species diversity at
 70 high altitude can also be associated to a reduction in species interaction, and in turn, a

71 relaxation of plant defences across scales, such as at the community level (Callis-Duehl *et al.*,
72 2017; Descombes *et al.*, 2016; Kergunteuil *et al.*, 2018), at the interspecific level (Defossez *et*
73 *al.*, 2018; Pellissier *et al.*, 2012), as well as at the intraspecific level (Pellissier *et al.*, 2014;
74 Scheidel and Bruelheide, 2004; Zehnder *et al.*, 2009). In analogy with latitudinal gradients,
75 elevation gradients are emerging as optimal tools for studying plant trait variation along
76 ecological clines that occur over short geographic distances (Körner, 2007). Indeed, plant
77 adaptation to habitat-specific abiotic and biotic factors can be studied along elevation
78 transects regardless of biogeographic history, gene-flow barriers, and within homogenous
79 macroclimatic conditions (Rasmann *et al.*, 2014; Sundqvist *et al.*, 2013). Along
80 environmental gradients, trait-mediated local adaptation of plant ecotypes is the result of
81 selection for fitness maximization given the local biotic and abiotic conditions. Within
82 genetically determined trait differences between ecotypes, variation emerges from phenotypic
83 plasticity if plasticity for such trait expression does not come with relative costs to fitness
84 (Gratani *et al.*, 2003; Van Tienderen, 1989).

85 Plant growth and defence related traits have been shown to vary in response to different
86 conditions. For instance, high and low elevation *Plantago lanceolata* ecotypes growing at two
87 temperature regimes (15 and 25 °C) showed strong plasticity in growth (i.e. both genotypes
88 grew similarly within each environment), while their resistance to generalist herbivores
89 reflected genetically-fixed patterns; high-elevation ecotypes were always less resistant,
90 independently of the temperature regimes (Pellissier *et al.*, 2014). Such differences in
91 plasticity would suggest that ecotypes that, at high elevation, produce lower amounts of
92 constitutive defences were favoured by selection, and growing in warmer temperatures could
93 not modulate this pattern of defence production. Similar reciprocal transplant experiments
94 have been classically used to measure the extent of ecotypic differentiation and phenotypic
95 plasticity (Nahum *et al.*, 2008). The predictions being that ecotypes adapted to one
96 environment should change their phenotypes when place in a novel environment given their
97 genetic constraints. Coupling reciprocal transplant with common garden experiments is
98 critical because phenotypic plasticity of growth and defence traits in response to growing
99 conditions can also generate clines, and such plasticity can obscure genetically based trait
100 expression.

101 With this study, we aimed at measuring the magnitude of ecotypic differentiation and
102 plasticity in growth and defence traits of two unrelated plant species with similar
103 geographical distribution along elevation gradients in the Alps (Supplementary Fig. S1).

Specifically, we collected seeds of four populations of *Cardamine pratensis* (Brassicaceae) and six populations of *Plantago major* (Plantaginaceae); half of the populations were native to low elevation and the other half to high elevation. We grew high and low elevation ecotypes at both their native or non-native elevation range using two common gardens along a mountain transect, and we assessed variation in growth and defence (secondary metabolite) related traits. Based on the theoretical framework as shown in Fig. 1 (Leggett *et al.*, 2014; Schlichting and Pigliucci, 1998), we expected five contrasting scenarios: 1) to observe no ecotypic variation or plasticity when the traits remain constant across ecotypes and environments (Fig. 1A). 2) To observe ecotypic differentiation (ecotype effect only) with no plasticity when trait variation remains constant across elevations for a given ecotype but different ecotypes would exhibit different trait values (Fig. 1B). 3) To detect plasticity without ecotypic differentiation (elevation effect only) when both ecotypes show trait variations across different growing elevation, without significant difference between ecotypes (Fig. 1C). 3) To observe ecotypic effect accompanied by plasticity if different ecotypes exhibit differential values both from one another and at different growing elevation (elevation and ecotype effects) (Fig. 1D). Finally, we would expect to observe plasticity through genotype by environment effect when the interaction of ecotype and elevation explains the traits value (elevation $\times$ ecotype effect) (Fig. 1E). Overall, this study builds towards a better understanding of the ecological and evolutionary drivers of pathways mediating plant phenotypic variation in growth versus defence traits along ecological clines.

Material and methods

Plant materials

Cardamine pratensis is a rhizomatous perennial herb that grows in a variety of habitats including nutrient-rich meadows, pastures, and forests and is common throughout Europe and in Central and Eastern Asia (Hultén and Fries, 1986). *C. pratensis* populations cover a wide elevation range, from sea level to 1600 meters above sea level (Aeschmann *et al.*, 2004), and flowers from April to June. Flowers are self-incompatible, and plants generally produce clonal offspring as new rosettes, especially under moist conditions (Lövkvist, 1956), and are considered hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves). *Cardamine pratensis* contain glucosinolates (GLS), which, when in contact with myrosinases, enzymes present in separate compartments of the cells, are degraded into glucose and

sulphate, along with various nitrile, isothiocyanate, and thiocyanate molecules that are toxic or deterrent to generalist insect herbivores and some pathogens (Giamoustaris and Mithen, 1995; Hopkins *et al.*, 1998; Kliebenstein *et al.*, 2002; Lambrix *et al.*, 2001). GLS are often classified into three classes of compounds depending on their side-chain: aliphatic, indole and aromatic, several of which have been shown to be effective against generalist and, to some extent, against specialist herbivores (Daxenbichler *et al.*, 1991; Louda and Rodman, 1983; Montaut and Bleeker, 2011). GLS are known to vary quantitatively and qualitatively (Kliebenstein *et al.*, 2001; Mauricio, 1998). In addition, phenotypic plasticity in GLS production has been previously observed in wild brassicaceous species (Agrawal *et al.*, 2002). For instance, GLS profiles of *Boechera stricta* were strongly plastic, both among habitats and within habitats, and patterns of GLS plasticity varied greatly among genotypes (Wagner and Mitchell-Olds, 2018).

Plantago major is an annual or facultative perennial rosette-forming herbaceous plant. Not being very competitive, *P. major* generally grows in ruderal areas especially along paths or roadsides and near gateways, where grass is short or absent (Warwick and Briggs, 1980). Native to Eurasia, *P. major* is a cosmopolitan species. It reproduces both sexually (self-compatible wind pollinated) and asexually through rosettes formation. Low genetic diversity within population of *P. major* has been shown to favour ecotypic and phenotypic differentiation (Halbritter *et al.*, 2015; Van Dijk *et al.*, 1988; Warwick and Briggs, 1980). *Plantago major* can cover a very wide elevation range: from the sea level to the alpine ecosystems all the way up to 3'000 meters above sea level (Ren *et al.*, 1999). *Plantago major* produces important amounts of secondary metabolites belonging to the class of cyclopentanoid monoterpenes called iridoids glycosides (IGs) and caffeoyl phenylethanoid glycoside (CPG) compounds (Pankoke *et al.*, 2013), which act as herbivore deterrents against generalist chewing insect (Fuchs and Bowers, 2004). IGs and CPG display a relatively high degree of variation in plant tissues depending on plant population, plant phenology and environmental factors (Barton, 2008; Bowers and Stamp, 1993; Darrow and Bowers, 1999; Darrow and Deane Bowers, 1997; Miehe-Steier *et al.*, 2015; Pellissier *et al.*, 2014), and they have been shown to display phenotypic plasticity (Bowers and Stamp, 1992; Halbritter *et al.*, 2015; Kuiper and Smid, 1985; Lotz and Blom, 1986).

Experimental design

Cardamine pratensis seeds were collected from four different natural populations: two low elevation and two high elevation populations along elevation gradients of Jura Mountains in Switzerland in 2016. *Plantago major* seeds were collected from six different natural populations along three elevation gradients in the Swiss Alps during summer 2016 (Supplementary Table S1). Seeds were collected on randomly selected plants (*C. pratensis*, $n = 6$ plants /population; *P. major*, $n = 10$ plants / population) within a 100 m radius for each population. We here did not track the maternal genetic background as is classically done in selection experiment studies, because we were principally interested at ecotypic variation and not at genotypic variation. Therefore seeds within one population were pooled to obtain elevation-specific ecotypes. Seeds were germinated in Petri dishes lined with humid filter paper. One week after germination, 25 seedlings of *C. pratensis* per population (total of 100 plants) and 24 seedlings of *P. major* per population (total of 144 plants) were transplanted independently into plastic potting pots (13 cm width $\times$ 10 cm height) filled with 500 ml of sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. Plants were immediately transferred to a climate-controlled chamber and kept at 16h/22°C - 8h/16°C day-night, and 50% relative humidity conditions for two weeks. Plants received nutrients twice a week until the beginning of reciprocal transplant experiment.

After two weeks of growth in the climate chamber, 25 *C. pratensis* plants per population and 24 *P. major* plants per population were equally distributed in two common gardens placed along the same mountain slope: La Neuveville (N: 47°06'84.28", E: 7°10'43.9", elevation: 450 m), and Chasseral (N: 47°07'03.36", E: 7°01'45", elevation: 1600 m). The plants were left growing for a period of two months during summer 2017.

Plant growth-related traits

For both plant species, the aboveground plant parts were separated from roots at the end of the experiment, oven-dried at 40°C for 48h and weighted to determine their dry biomass. Furthermore, in *P. major* plants, two additional growth-related traits were measured. The chlorophyll content of the plant was measured as the average of three fully expanded leaves per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta (China) Investment Ltd). Specific leaf area (SLA) was measured as the area (calculated using ImageJ software) of one fully expanded leaf per plant divided by their oven-dried (40°C for 48h) biomass ($\text{mm}^2 \text{mg}^{-1}$ DW). Higher SLA levels and chlorophyll content tend to positively correlate with potential

relative growth rate across species, photosynthetic rate, or leaf nitrogen (N) (Garnier and Laurent, 1994; Poorter and Garnier, 2007). In general, species in resource-rich environments tend, on average, to have a higher SLA than do those in resource-poor environments (Garnier and Laurent, 1994; Poorter and Garnier, 2007).

Chemical defences

All leaves were harvested immediately at the end of the field experiment prior to removal of plants from the field sites, while leaf preparation for each species followed two different methods due to the different secondary metabolite extractions and analyses.

Cardamine pratensis leaves were immediately frozen in liquid nitrogen and stored at -80 °C; ground to powder using mortars and pestles in liquid nitrogen, and a 100 mg aliquot was weighed for GLS extraction. The extraction solvent (1.0 ml methanol: H₂O: formic acid (70:29.5:0.5, v/v)) was added to the tubes along with 5 glass beads, shaken in a tissue lyser (Retsch GMBH, Haan, Germany) for 4 min at 30 Hz, and centrifuged at 12800 rpm for 3 min. The supernatant was diluted 20 times with 70% methanol and transferred to an HPLC vial. GLS identification and quantification was performed using an Acquity ultra-high pressure liquid chromatography (UHPLC) from Waters (Milford, MA) interfaced to a Synapt G2 quadrupole time-of-flight mass spectrometry (QTOF) from Waters with electrospray ionization, using the method as described in (Glauser *et al.*, 2012).

Plantago major leaves were oven-dried at 40 °C for 48 h prior being ground to powder using stainless steel beads in the tissue lyser, a 10 mg aliquot was weighed and a 1.5 ml methanol were added to the tubes along with 5 glass beads. The tubes were shaken 4 min at 30 Hz and centrifuged at 14000 rpm for 3 min. The supernatants were diluted five times by adding 800 µl of MilliQ water to 200 µl of pure extract. IGs and CPG were separated by UHPLC-QTOF using an Acquity BEH C18 column from Waters (50x2.1mm, 1.7 µm particle size) at a flow rate of 0.4 ml/min. The following gradient of water + formic acid 0.05% (phase A) and acetonitrile + formic acid 0.05% (phase B) was applied: 2-9 % B in 1.5 min, 9-50 % B in 3.5 min, 50-100% B in 1.5 min, held at 100% B for 1.5 min, back to 2% B and held for 2.0 min. The column was maintained at 25 °C. The injection volume was 1 µl. Detection was achieved in negative electrospray using the deprotonated ions or the formate adducts as quantification ions. Quantification ions and retention time of the two standards were: aucubin m/z 391.124 (formate adduct), retention time 1.17 min, and verbascoside m/z 623.198 (deprotonated ion), retention time 3.16 min. Absolute amounts of IGs and CPG were determined by external

calibration using five standard solutions of aucubin at 0.2, 0.5, 2, 5 and 10 µg/land verbascoside at 0.2, 0.5, 2, 5 and 20 µg/ml. Concentrations were normalized to plant weight and expressed as µg/mg. Other IGs and CPG were putatively identified based on their retention time and chemical formula by comparing them to previous detection in *P. major* or in species of *Plantago* genus (Rønsted *et al.*, 2000) and database (Dictionary of Natural Products, CRC Press, USA, version 6.1. on DVD) containing information on known IGs and CPGs and quantified as aucubin or verbascoside equivalents. IGs named with the code IGs followed by numbers represent molecular formula corresponding to potential IGs for which several isomers exist in the literature and thus cannot be unequivocally annotated.

Herbivore bioassay

To measure plant resistance against insect herbivores (resistance is defined as the effect of plant defence traits on herbivore performance (Karban and Baldwin, 1997)); we used a generalist herbivore, *Spodoptera littoralis* (Lepidoptera: Noctuidae; obtained from Syngenta, Stein AG, Switzerland). *Spodoptera littoralis* is known to feed on species belonging to more than 80 families of plants (Brown and Dewhurst, 1975), and is widely used for performing plant resistance bioassays. Newly hatched larvae were reared on corn-based artificial diet for 7 days before the beginning of the bioassay. Immediately after removal of plants from the field, both plant species were placed in a climate-controlled chamber (24 / 18 °C, 16/8 hr, day/night regime, and 55 % R.h.) to homogenize the condition for herbivores feeding on both species during bioassay performance. For *C. pratensis*, one fully expanded new leaf from 12 plant per ecotype and per population that were growing at the two elevation common gardens (n = 48) was cut and separately placed in a Petri dish on a filter paper moisten with one drop of distilled water. One 7-days old *S. littoralis* larva was added to each petri dish. For *P. major* instead, we performed a whole plant bioassay. We placed two 7-day old *S. littoralis* larvae on 24 plant per ecotype population that were growing at the two elevation common gardens (n = 96). Plants were covered with nylon nets to avoid escaping of caterpillars. After five days of herbivory for *C. pratensis* and three days for *P. major*, the insects were retrieved from individual Petri dishes and plants, respectively and their weights were measured and recorded. We consider the larval gain weight using the formula $\ln(\text{final weight} - \text{initial weight})$. For *P. major* the larval gain weight represent the average of the two caterpillar placed on each plant. Lower weight gains indicate that plants are more resistant (Humphrey *et al.*, 2018).

265

266 *Statistical Analyses*

267 All statistical analyses were performed within the R environment (R Development Core
268 Team, 2017).

269 For chemical data, we calculated the sum of glucosinolate compounds (GLS total) for *C.*
270 *pratensis* and the sum of iridoids glycosides (IGs total) and caffeoyl phenylethanoid
271 glycoside (CPG total) for *P. major*, as well as a measure of chemical diversity for both plant
272 species using the Shannon-Weaver diversity indices (Hill, 1973) with *diversity* function in
273 the *vegan* package in R (Oksanen *et al.*, 2017).

274 To measure the interactive effect of transplant site and elevation of origin of the plant
275 ecotypes on plant growth and defence traits, we used two-way ANOVAs by including
276 transplant sites (high and low), ecotypes (high and low) and their interaction as fixed factors.
277 We also included the term population nested within ecotypes in the model to assess
278 variability across populations within a given elevation of origin. The response variables
279 were; AG biomass, larval weight gain, total GLS, total indole, total aliphatic, and chemical
280 diversity for *C. pratensis*, and AG biomass, chlorophyll content, SLA, larval weight gain,
281 total chemistry, total IGs, total CPG and chemical diversity for *P. major*. All chemical traits
282 were log-transformed prior analyses to meet normality and homoscedasticity assumptions. A
283 significant effect of site of growth (i.e. elevation) would indicate a plastic response to
284 different environmental conditions. A significant effect of ecotype would indicate
285 differentiation in traits among populations belonging to different ecotypes. A significant
286 effect of population would indicate differentiation in traits among populations. A significant
287 elevation $\times$ ecotype term would indicate ecotype-specific selection for plasticity for a given
288 trait.

289 To address the multivariate nature of plant secondary compounds, we also ran a full-factorial
290 model including the individual secondary metabolites abundance matrix as response variable
291 and plant ecotype and elevation as factors using permutational analysis of variance
292 (PERMANOVA) with the *adonis* function in the package *vegan* in R (Oksanen *et al.*, 2017).
293 We also included plant biomass as covariate to control for potential direct effect of biomass
294 on plant chemistry (Züst *et al.*, 2015). The Bray–Curtis metric was used to calculate a
295 dissimilarity matrix of all compounds among samples for the PERMANOVA. We visualized
296 ecotypic differentiation of the secondary metabolites using an NMDS ordination analysis of

the chemical compounds based on Bray Curtis distance (package *vegan* in R) (Oksanen *et al.*, 2017).

Finally, to visualize and calculate the magnitude of plasticity of the plant growth and defence related traits when plants were placed in their non-native habitat, we calculated effect sizes for all traits as the log-response-ratio $LLR = \log\left(\frac{\text{non-native elevation}}{\text{native elevation}}\right)$ using the *effsize* function in the *effsize* package in R (Torchiano, 2017), and when significant, we reported them as standardized mean difference (SMD) values. The figure constructed based on effect size aims at representing the plastic response of traits, G×E effects, as well as the magnitude of responses. A 95% of confidence interval bar that deviates from zero shows a significant effect of treatment (positive or negative effect of non-native growing elevation) (Nakagawa and Cuthill, 2007), while a deviation of one of the interval bars from zero, but not the other, indicates G×E effects.

Results

Plant growth related traits

For both species, we observed phenotypic plasticity and ecotypic differentiation in aboveground (AG) biomass through significant effects of both ecotype and elevation (high or low elevation growing sites) (Fig. 2, 3, 4; Table 1). We observed that AG biomass of high elevation ecotypes increased by 49% (SMD = 1.17) for *C. pratensis* and by 45% (SMD = 1.48) for *P. major* at the non-native elevation (low elevation site), while low elevation ecotypes' AG biomass decreased by 61% (SMD = - 0.96) for *C. pratensis* and by 51% (SMD = - 1.93) for *P. major* at the non-native elevation (high elevation site) (Fig. 2, 3, 4; Table 1). Furthermore, our results indicated that high elevation ecotypes produced 38.5 % and 12% more AG biomass than low elevation ecotypes in *C. pratensis* and *P. major*, respectively. In addition, in *P. major* leaf chlorophyll content and SLA showed plasticity through growing elevation effect, with the latter also showing marginal G×E effect. Specifically, we observed that chlorophyll content of high elevation ecotypes increased by 4.1% (SMD = 1.55) at the non-native site (low elevation site) and low elevation ecotypes had 3.4% (SMD = -1.36) less chlorophyll content at the non-native site (high elevation) (Fig. 2B, 4; Table 1). Moreover, SLA of low elevation ecotypes significantly increased by 6.6% (SMD = 0.96) at their non-native growing site (Fig. 2B, 4; Table 1).

Plant chemical defences and resistance

The GLS profiles of *C. pratensis* leaves consisted of six GLS compounds (two aliphatic, three indoles and one aromatic), and the secondary metabolites profile of the *P. major* leaves consisted of 13 IGs and 3 CPG compounds (Supplementary Fig. S2). In *C. pratensis*, we observed phenotypic plasticity in total indole GLS, specifically through significant ecotype by elevation interaction (G×E effect), where the total indole GLS concentration in high elevation ecotypes significantly increased at the low elevation site (non-native) by 28% (SMD = 0.77) (Fig. 2A, 3; Table 1). Moreover, we found ecotypic effect for *S. littoralis* larval weight gain; larvae on low elevation ecotypes grew 81% more compared to high elevation ecotypes. Low elevation ecotypes produced 37% more aliphatic GLS than high elevation ecotypes, and high elevation ecotypes showed 25% more chemical diversity than low elevation ecotypes (Fig. 3, Table 1). Furthermore, the PERMANOVA showed that the abundance and chemical diversity of GLS were globally affected by plant ecotypes ($P = 0.001$, Fig. 5A-B). In *P. major*, we also found ecotypic differentiation for *S. littoralis* larval weight gain; larvae on low elevation ecotypes grew 8% more than on high elevation ecotypes. Low elevation ecotypes produced 17%, 17% and 22% more total chemistry; total IGs and total CPG than high elevation ecotypes, respectively (Fig. 4, Table 1). The PERMANOVA revealed plant ecotypic effect ($P = 0.001$) and growing elevation effect ($P = 0.005$) (Fig. 5C-D) in the abundance and diversity of secondary metabolites in *P. major*. Additionally, we found that abundance of the total chemistry and diversity of the compounds were significantly affected by the AG biomass of *P. major* ($P = 0.0002$).

Overall, we also found significant population-level effects in trait expression. For instance, we found a significant effect of plant population for *C. pratensis* total GLS and aliphatic GLS (Supplementary Fig. S3 and Table 1). In *P. major*, we observed significant effects of plant population on all the measured traits (marginal for SLA and chlorophyll content) (Supplementary Fig. S4 and Table 1).

Discussion

Using reciprocal transplant experiments of ecotypes growing at different elevation, we observed ecotypic differentiation accompanied by plasticity in growth related traits, while we mainly observed ecotypic differentiation for defence and resistance traits for both *P. major*

and *C. pratensis*. Below, we outline the potential causes for such divergence along elevation gradients.

Plant biomass accumulation

We found high levels of phenotypic plasticity in the observed AG production pattern. Plasticity can be visualized as a change in the slope of the reaction norm between the ancestral and derived population or species (Doughty, 1995; Gotthard *et al.*, 1995). In this regard, for both species plant growth related traits (plant biomass, leaf chlorophyll content and SLA) showed plasticity. Our results compliment other findings where the combination of ecotypic differentiation and phenotypic plasticity in growth-related traits such as biomass and flower size was shown for invasive species at their invasive range (Martín-Forés *et al.*, 2017). More specifically, we observed that in both species, the AG biomass across both ecotypes increased at low elevation growing site and decreased at high elevation growing site. Increase in AG biomass of both ecotypes at low elevation growing site comes as no surprise, given the growing condition at low elevation are warmer and more favourable than at high elevation. Two reasons have been put forward for plants to reduce growth at high elevation. First, a decrease in the general metabolic activity as a function of colder temperature inhibits photosynthetic rate and biomass production (Boyer, 1982). Second, it has been proposed that because plants growing at higher elevations typically receive direct sunlight and higher ultraviolet radiation, and ultraviolet radiation destroys the auxins content at the apical shoots, they tend to grow much slower than lowland plants (Keller *et al.*, 2004). Furthermore, both *C. pratensis* and *P. major* are perennial species and it can be argued that high elevation ecotypes accumulated higher AG biomass than low elevation ecotypes once placed in more favourable conditions of low elevation to compensate for the next year's growing season when they would have to allocate more resource to flower and seed production. Such a scenario should be less likely for low elevation plants growing at their native site.

Interestingly, we also observed that high elevation ecotypes produced more biomass than low elevation ecotypes, and this was true for both species. This is somewhat surprising since we expected alpine plants to grow smaller in harsher and colder environments (Atkin and Day, 1990; Körner, 2003). While plant size is negatively correlated to extremely cold temperatures (Squeo *et al.*, 1991) and, as a consequence, generally decreases with elevation (Körner, 2003), it appears that high-elevation ecotypes favour fast biomass accumulation (Körner, 2016).

Plants adapted to growing in cold conditions, such as in high altitude climates, where growing season is short, pass through seasonal development taking advantage of the warmest period of the growing season. In addition, plants growing at cold condition typically exhibit greater photosynthetic and respiratory capacities than their warm-grown counterparts (Atkin *et al.*, 2006). Therefore, high-elevation ecotypes could highly benefit from faster development and high rates of metabolism (Körner, 2016), and, at equal growing conditions (same soil) and during the same growing period, can accumulate more biomass than their low-elevation counterparts.

Plant chemical defences and resistance

Concerning plant defences and resistance, we observed ecotypic differentiation across most defence and resistance measures, including total GLS, aliphatic GLS, chemical diversity, total IGs, total CPG, total chemistry and larval weight. Generally, regardless of the growing elevation, low-elevation ecotypes produced more chemical defences. The strong effect of temperature on plant primary and secondary metabolism is known and our results are in line with other findings showing the temperature-driven suppression of plant secondary metabolites at high elevation (Pellissier *et al.*, 2014) and general decrease in secondary metabolite production from low to high elevation (Kergunteuil *et al.*, 2018). However, a decrease in secondary metabolite production at high elevation could also be attributed to a relaxation of herbivory pressure at high elevation (Pellissier *et al.*, 2014). To date we have no data that allows disentangling biotic and abiotic effects of defence decline at high elevation. Interestingly, however, indole GLS showed no ecotypic differentiation, in which, high elevation ecotypes produced more of these compounds when placed at low elevation (see G x E effect in Table 1).

Unlike aliphatic GLS, for which induction has been rarely observed (Koritsas *et al.*, 1991; Li *et al.*, 1999), induction of indolic GLS has been widely documented in several systems (Agrawal *et al.*, 1999; Doughty *et al.*, 1995; Griffiths *et al.*, 1994; Moyes *et al.*, 2000; Raybould and Moyes, 2001; Siemens and Mitchell-Olds, 1998), including the closely related *Cardamine hirsuta* (Bakhtiari *et al.*, unpublished data). Additionally, in contrast to the aliphatic GLS that are under strong genetic control (Raybould and Moyes, 2001), indole GLS have been shown to be strongly influenced by environmental factors with some heritable variation in production (Rücker and Röbbelen, 1994). Altogether, this could indicate that the

production of indole GLS might be more costly than the production of other GLS (Bidart-Bouzat *et al.*, 2005; Traw, 2002) in *C. pratensis*. Several studies detected a relatively high cost of plasticity for chemical defences and emphasized on the fact that the limit of plasticity in expression of chemical traits may be attributed to such cost (Agrawal *et al.*, 2002; Agrawal *et al.*, 2010; Züst and Agrawal, 2017). On the other hand, plasticity in defence-related traits is the reflection of both biotic and abiotic environmental conditions that affect the expression of defences, and plasticity of defence-related traits in response to biotic pressures, such as herbivory, is well-documented (Agrawal *et al.*, 2002; Humphrey *et al.*, 2018; Wagner and Mitchell-Olds, 2018). Such results may suggest that plants show higher degree of plastic response to biotic stimuli compared to abiotic stress such as environmental fluctuations. Thus, the lack of plasticity in the majority of the measured defence-related traits in our study could be due to the fact that the benefits of plasticity in expression of defence cannot outweigh the costs of biotic pressure occurred early in the season or other potential costs of defence plasticity. For example, indolic GLS were not plastic, in contrast to plastic non-indolic GLS, in *Cardamine cordifolia* plants growing in shaded-common gardens, that are characterized by low herbivory (Humphrey *et al.*, 2018). In contrast to our results, in the same study, Humphrey *et al.* found plasticity in larval weight gain of specialist herbivore (*Scaptomyza nigrita*).

On a broader perspective, detailed analysis of the effect sizes (ESs) between growth and defence related traits in *C. pratensis* indicates that the magnitude of plastic responses displayed by high elevation ecotypes is higher for AG biomass (very large ES) compared to indolic GLS production (large ES). In *P. major* the magnitude of plastic responses in all the growth-related traits were also very large, compared to the non-significant plastic responses for all the defence-related traits (except for some the individual compounds, Supplementary Fig. S2B). Nevertheless, the lack of plastic response to elevation in defence-related traits does not rule out the potential for plasticity in chemical defences. Given the fact that the environmental effect of the growing elevation could affect the plant chemistry at any time throughout the growing season and the chemistry was measured only at the end of the field season, a potential plasticity in expression of such traits could have disappeared by the end of the season. Moreover, detecting plastic response in defence traits upon biotic stress such as herbivory is simpler. Upon herbivory, the phytohormone activation machinery behind expression of chemical defences is an immediate process, whereas the detection of the potential plastic responsiveness of plant defence to abiotic stimuli might be masked by the

time-dependency of the growing season. For instance, phenotypic plasticity in flowering time in response to seasonal variations has been shown both in controlled environment (Anderson *et al.*, 2011) as well as from long-term field survey in *Boechera stricta* (Anderson *et al.*, 2012). In addition, two studies, one on *C. cordifolia* and the second on *P. lanceolata*, showed phenological variation in GLS and IGs plant tissue content, respectively (Darrow and Deane Bowers, 1997; Rodman and Louda, 1984). Therefore, ontogeny should also be addressed when measuring plasticity because plants have been shown to express different levels of plasticity in defence traits as they grow.

Conclusions

Few studies have assessed phenotypic variation of plant growth versus defence traits in response to contrasting environments. Here, we document that plant growth traits displayed strong ecotypic differentiation accompanied by plasticity, but, in contrast, we find little support of phenotypically plastic defence and resistance traits in response to different growing habitat across step elevation gradient. Future research on similar systems would require coupling the observed effects on plant phenotypes with fitness measurements and selection gradient analyses in order to disentangle the fitness benefits of phenotypic plasticity versus fixed ecotypic differentiation for individual plant traits.

Supplementary Data

Table S1. Coordinates of the populations of *C. pratensis* and *P. major*.

Fig. S1. Natural distribution map of *C. pratensis* and *P. major* along elevation.

Fig. S2. Effect sizes of individual chemical compounds of *C. pratensis* and *P. major* growing at their non-native elevation.

Fig. S3. Reaction norms of growth and defense traits for populations of *C. pratensis*.

Fig. S4. Reaction norms of growth and defense traits for populations of *P. major*.

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Tables

Table 1. Two-way ANOVA Table for measuring the interaction between the effects of high and low elevation ecotypes and the elevation of growth in two common garden sites on growth and defence traits.

Plant species	Response variable	Factor	Df	Mean SQ	F value	P value
<i>C. pratensis</i>	AG biomass	Ecotypes	1	2.15	14.59	<0.001***
		Population	2	0.09	0.64	0.53
		Elevation	1	5.22	35.41	<0.001***
		Ecot *Elev	1	0.02	0.14	0.7
	Total GLS	Ecotypes	1	0.16	0.17	0.7
		Population	2	4.71	5	0.009**
		Elevation	1	0.38	0.40	0.5
		Ecot *Elev	1	3.21	4	0.07
	Total indole	Ecotypes	1	0.6	0.38	0.5
		Population	2	2.59	1.63	0.2
		Elevation	1	5.46	3.44	0.07
		Ecot *Elev	1	11.45	7.22	0.009**
	Total aliphatic	Ecotypes	1	154.86	23.40	<0.001***
		Population	2	56.78	10.41	<0.001***
		Elevation	1	1.52	0.28	0.6
		Ecot *Elev	1	4.72	0.87	0.4
	Chemical diversity	Ecotypes	1	4.69	12.33	<0.001***
		Population	2	0.72	1.89	0.2
		Elevation	1	0.59	1.55	0.22
		Ecot *Elev	1	0.91	2.4	0.12

	Resistance	Ecotypes	1	7.73	4.38	0.04*
		Population	2	0.06	0.04	1
		Elevation	1	4.03	2.28	0.1
		Ecot *Elev	1	0.02	0.01	0.9
<i>P. major</i>	AG biomass	Ecotypes	1	0.18	4.75	0.03*
		Population	4	0.1	2.47	0.047*
		Elevation	1	4.63	118.88	<0.001***
		Ecot *Elev	1	0.004	0.09	0.8
	Chlorophyll content	Ecotypes	1	0.0008	0.1	0.8
		Population	4	0.02	2.28	0.06
		Elevation	1	0.68	81.79	<0.001***
		Ecot *Elev	1	0.003	0.32	0.6
	SLA	Ecotypes	1	0.07	1.89	0.2
		Population	4	0.08	2.38	0.05
		Elevation	1	0.81	23.14	<0.001***
		Ecot *Elev	1	0.1	2.78	0.09
	Total IGs	Ecotypes	1	4.26	12.65	<0.001***
		Population	4	2.34	6.97	<0.001***
		Elevation	1	0.7	2.07	0.2
		Ecot *Elev	1	0.04	0.1	0.7
	Total CPG	Ecotypes	1	3.51	4.1	0.04*
		Population	4	2.14	2.49	0.04*
		Elevation	1	0.09	0.11	0.7
		Ecot *Elev	1	1.1	1.28	0.3
	Total chemistry	Ecotypes	1	6.2	14.78	<0.001***

		Population	4	1.4	3.33	0.01*
		Elevation	1	0.0.16	0.37	0.5
		Ecot *Elev	1	0.08	0.18	0.7
	Chemical diversity	Ecotypes	1	0.05	1.66	0.2
		Population	4	0.09	3.11	0.02*
		Elevation	1	0.04	1.28	0.3
		Ecot *Elev	1	0.02	0.76	0.4
	Resistance	Ecotypes	1	0.2	8.66	0.004**
		Population	4	0.36	14.78	<0.001***
		Elevation	1	0.1	4.07	0.047*
		Ecot *Elev	1	0.0003	0.01	0.9

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure legends

Fig. 1. Theoretical framework for measuring ecotypic differentiation and phenotypic plasticity using reciprocal transplant experiments and reaction norms. The different panels represent all likely scenarios.

Fig. 2. Effect sizes for the influence of non-native growing elevation on plant growth and defence related trait for high and low elevation ecotypes of *C. pratensis* (A) and *P. major* (B). Effects are natural log response ratios (LRRs) with 95% confidence limits.

Fig. 3. Reaction norms of *C. pratensis* ecotypes of growth (A), resistance (B) and defence (C, D, E, F) traits. Mean phenotypic values (mean $\pm$ 1 s.e. for each elevation ecotype) are represented in black (low elevation ecotypes) and in grey (high elevation ecotypes) across two contrasted growing elevations (high or low elevation).

Fig. 4. Reaction norms of *P. major* ecotypes of growth (A, B, C), resistance (D) and defence (E, F, G, H) traits. Mean phenotypic values (mean $\pm$ 1 s.e. for each elevation ecotype) are represented in black (low elevation ecotypes) and in grey (high elevation ecotypes) across two contrasted growing elevations (high or low elevation).

Fig. 5. Glucosinolates (GLS), iridoid glycosides (IGs) and caffeoyl phenylethanoid glycoside (CPG) ordinations. Representation of the non-multidimensional scaling (NMDS) indicating the GLS found in high and low *C. pratensis* ecotypes at high (A) and low (B) elevation sites and IGs and CPG found in *P. major* ecotypes at high (C) and low (D) elevation sites. The 95% confidence interval ellipses are represented based on the two elevation ecotypes (high elevation ecotype in grey and low elevation ecotype in black). Stress values: (A) and (B) = 0.12, (C) and (D) = 0.2, K = 2.

Figures

Fig. 1.

Fig. 2.

Cardamine pratensis

Fig. 3.

Plantago major

Fig. 4.

Fig. 5.

Tables

Table 1. Two-way ANOVA Table for measuring the interaction between the effects of high and low elevation ecotypes and the elevation of growth in two common garden sites on growth and defence traits.

Plant species	Response variable	Factor	Df	Mean SQ	F value	P value
<i>C. pratensis</i>	AG biomass	Ecotypes	1	2.15	14.59	<0.001***
		Population	2	0.09	0.64	0.53
		Elevation	1	5.22	35.41	<0.001***
		Ecot *Elev	1	0.02	0.14	0.7
	Total GLS	Ecotypes	1	0.16	0.17	0.7
		Population	2	4.71	5	0.009**
		Elevation	1	0.38	0.40	0.5
		Ecot *Elev	1	3.21	4	0.07
	Total indole	Ecotypes	1	0.6	0.38	0.5
		Population	2	2.59	1.63	0.2
		Elevation	1	5.46	3.44	0.07
		Ecot *Elev	1	11.45	7.22	0.009**
	Total aliphatic	Ecotypes	1	154.86	23.40	<0.001***
		Population	2	56.78	10.41	<0.001***
		Elevation	1	1.52	0.28	0.6
		Ecot *Elev	1	4.72	0.87	0.4
	Chemical diversity	Ecotypes	1	4.69	12.33	<0.001***
		Population	2	0.72	1.89	0.2
		Elevation	1	0.59	1.55	0.22

		Ecot *Elev	1	0.91	2.4	0.12
	Resistance	Ecotypes	1	7.73	4.38	0.04*
		Population	2	0.06	0.04	1
		Elevation	1	4.03	2.28	0.1
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<i>P. major</i>	AG biomass	Ecotypes	1	0.18	4.75	0.03*
		Population	4	0.1	2.47	0.047*
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		Ecot *Elev	1	0.004	0.09	0.8
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