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

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Running head: plant ecotypic variation and phenotypic plasticity along elevation

Abstract

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

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

Introduction

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

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

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

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

With this study, we aimed at measuring the magnitude of ecotypic differentiation and plasticity in growth and defence traits of two unrelated plant species with similar 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 × 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 (Aeschimann *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

168 *Cardamine pratensis* seeds were collected from four different natural populations: two low
169 elevation and two high elevation populations along elevation gradients of Jura Mountains in
170 Switzerland in 2016. *Plantago major* seeds where collected from six different natural
171 populations along three elevation gradients in the Swiss Alps during summer 2016
172 (Supplementary Table S1). Seeds were collected on randomly selected plants (*C. pratensis*,
173 n= 6 plants /population; *P. major*, n= 10 plants / population) within a 100 m radius for each
174 population. We here did not track the maternal genetic background as is classically done in
175 selection experiment studies, because we were principally interests at ecotypic variation and
176 not at genotypic variation. Therefore seeds within one population were pooled to obtain
177 elevation-specific ecotypes. Seeds were germinated in Petri dishes lined with humid filter
178 paper. One week after germination, 25 seedlings of *C. pratensis* per population (total of 100
179 plants) and 24 seedlings of *P. major* per population (total of 144 plants) were transplanted
180 independently into plastic potting pots (13 cm width × 10 cm height) filled with 500 ml of
181 sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. Plants were immediately
182 transferred to a climate-controlled chamber and kept at 16h/22°C - 8h/16°C day-night, and
183 50% relative humidity conditions for two weeks. Plants received nutrients twice a week until
184 the beginning of reciprocal transplant experiment.

185 After two weeks of growth in the climate chamber, 25 *C. pratensis* plants per population and
186 24 *P. major* plants per population were equally distributed in two common gardens placed
187 along the same mountain slope: La Neuveville (N: 47°06'84.28", E: 7°10'43.9", elevation: 450
188 m), and Chasseral (N: 47°07'03.36", E: 7°01'45", elevation: 1600 m). The plants were left
189 growing for a period of two months during summer 2017.

190

191 *Plant growth-related traits*

192 For both plant species, the aboveground plant parts were separated from roots at the end of the
193 experiment, oven-dried at 40°C for 48h and weighted to determine their dry biomass.
194 Furthermore, in *P. major* plants, two additional growth-related traits were measured. The
195 chlorophyll content of the plant was measured as the average of three fully expanded leaves
196 per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta (China) Investment Ltd).
197 Specific leaf area (SLA) was measured as the area (calculated using ImageJ software) of one
198 fully expanded leaf per plant divided by their oven-dried (40°C for 48h) biomass (mm^2 mg^{-1}
199 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: H_2O: 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

232 calibration using five standard solutions of aucubin at 0.2, 0.5, 2, 5 and 10 µg/land
233 verbascoside at 0.2, 0.5, 2, 5 and 20 µg/ml. Concentrations were normalized to plant weight
234 and expressed as µg/mg. Other IGs and CPG were putatively identified based on their
235 retention time and chemical formula by comparing them to previous detection in *P. major* or
236 in species of *Plantago* genus (Rønsted *et al.*, 2000) and database (Dictionary of Natural
237 Products, CRC Press, USA, version 6.1. on DVD) containing information on known IGs and
238 CPGs and quantified as aucubin or verbascoside equivalents. IGs named with the code IGs
239 followed by numbers represent molecular formula corresponding to potential IGs for which
240 several isomers exist in the literature and thus cannot be unequivocally annotated.
241

242 *Herbivore bioassay*

243 To measure plant resistance against insect herbivores (resistance is defined as the effect of
244 plant defence traits on herbivore performance (Karban and Baldwin, 1997)); we used a
245 generalist herbivore, *Spodoptera littoralis* (Lepidoptera: Noctuidae; obtained from Syngenta,
246 Stein AG, Switzerland). *Spodoptera littoralis* is known to feed on species belonging to more
247 than 80 families of plants (Brown and Dewhurst, 1975), and is widely used for performing
248 plant resistance bioassays. Newly hatched larvae were reared on corn-based artificial diet for
249 7 days before the beginning of the bioassay. Immediately after removal of plants from the
250 field, both plant species were placed in a climate-controlled chamber (24 / 18 °C, 16/8 hr,
251 day/night regime, and 55 % R.h.) to homogenize the condition for herbivores feeding on both
252 species during bioassay performance. For *C. pratensis*, one fully expanded new leaf from 12
253 plant per ecotype and per population that were growing at the two elevation common gardens
254 (n = 48) was cut and separately placed in a Petri dish on a filter paper moisten with one drop
255 of distilled water. One 7-days old *S. littoralis* larva was added to each petri dish. For *P. major*
256 instead, we performed a whole plant bioassay. We placed two 7-day old *S. littoralis* larvae on
257 24 plant per ecotype population that were growing at the two elevation common gardens (n =
258 96). Plants were covered with nylon nets to avoid escaping of caterpillars. After five days of
259 herbivory for *C. pratensis* and three days for *P. major*, the insects were retrieved from
260 individual Petri dishes and plants, respectively and their weights were measured and recorded.
261 We consider the larval gain weight using the formula $\ln(final\ weight - initial\ weight)$. For *P. major* the larval gain weight represent the average of the two
263 caterpillar placed on each plant. Lower weight gains indicate that plants are more resistant
264 (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 × 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

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

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

309

310 **Results**

311 *Plant growth related traits*

312 For both species, we observed phenotypic plasticity and ecotypic differentiation in
313 aboveground (AG) biomass through significant effects of both ecotype and elevation (high or
314 low elevation growing sites) (Fig. 2, 3, 4; Table 1). We observed that AG biomass of high
315 elevation ecotypes increased by 49% (SMD = 1.17) for *C. pratensis* and by 45% (SMD =
316 1.48) for *P. major* at the non-native elevation (low elevation site), while low elevation
317 ecotypes' AG biomass decreased by 61% (SMD = - 0.96) for *C. pratensis* and by 51% (SMD
318 = - 1.93) for *P. major* at the non-native elevation (high elevation site) (Fig. 2, 3, 4; Table 1).
319 Furthermore, our results indicated that high elevation ecotypes produced 38.5 % and 12%
320 more AG biomass than low elevation ecotypes in *C. pratensis* and *P. major*, respectively. In
321 addition, in *P. major* leaf chlorophyll content and SLA showed plasticity through growing
322 elevation effect, with the latter also showing marginal G×E effect. Specifically, we observed
323 that chlorophyll content of high elevation ecotypes increased by 4.1% (SMD = 1.55) at the
324 non-native site (low elevation site) and low elevation ecotypes had 3.4% (SMD = -1.36) less
325 chlorophyll content at the non-native site (high elevation) (Fig. 2B, 4; Table 1). Moreover,
326 SLA of low elevation ecotypes significantly increased by 6.6% (SMD = 0.96) at their non-
327 native growing site (Fig. 2B, 4; Table 1).

328 *Plant chemical defences and resistance*

329 The GLS profiles of *C. pratensis* leaves consisted of six GLS compounds (two aliphatic, three
330 indoles and one aromatic), and the secondary metabolites profile of the *P. major* leaves
331 consisted of 13 IGs and 3 CPG compounds (Supplementary Fig. S2). In *C. pratensis*, we
332 observed phenotypic plasticity in total indole GLS, specifically through significant ecotype by
333 elevation interaction (G×E effect), where the total indole GLS concentration in high elevation
334 ecotypes significantly increased at the low elevation site (non-native) by 28% (SMD = 0.77)
335 (Fig. 2A, 3; Table 1). Moreover, we found ecotypic effect for *S. littoralis* larval weight gain;
336 larvae on low elevation ecotypes grew 81% more compared to high elevation ecotypes. Low
337 elevation ecotypes produced 37% more aliphatic GLS than high elevation ecotypes, and high
338 elevation ecotypes showed 25% more chemical diversity than low elevation ecotypes (Fig. 3,
339 Table 1). Furthermore, the PERMANOVA showed that the abundance and chemical diversity
340 of GLS were globally affected by plant ecotypes (P= 0.001, Fig. 5A-B). In *P. major,* we also
341 found ecotypic differentiation for *S. littoralis* larval weight gain; larvae on low elevation
342 ecotypes grew 8% more than on high elevation ecotypes. Low elevation ecotypes produced
343 17%, 17% and 22% more total chemistry; total IGs and total CPG than high elevation
344 ecotypes, respectively (Fig. 4, Table 1). The PERMANOVA revealed plant ecotypic effect
345 (P= 0.001) and growing elevation effect (P= 0.005) (Fig. 5C-D) in the abundance and
346 diversity of secondary metabolites in *P. major*. Additionally, we found that abundance of the
347 total chemistry and diversity of the compounds were significantly affected by the AG biomass
348 of *P. major* (P= 0.0002).

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

354

355 **Discussion**

356 Using reciprocal transplant experiments of ecotypes growing at different elevation, we
357 observed ecotypic differentiation accompanied by plasticity in growth related traits, while we
358 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 wildly 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

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

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

456 time-dependency of the growing season. For instance, phenotypic plasticity in flowering time
457 in response to seasonal variations has been shown both in controlled environment (Anderson
458 *et al.*, 2011) as well as from long-term field survey in *Boechera stricta* (Anderson *et al.*,
459 2012). In addition, two studies, one on *C. cordifolia* and the second on *P. lanceolata*, showed
460 phenological variation in GLS and IGs plant tissue content, respectively (Darrow and Deane
461 Bowers, 1997; Rodman and Louda, 1984). Therefore, ontogeny should also be addressed
462 when measuring plasticity because plants have been shown to express different levels of
463 plasticity in defence traits as they grow.

464

465 ## Conclusions

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

474

475 ## Supplementary Data

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

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

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

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

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

482

483 ## Acknowledgments

484 We thank Adrienne Godschalx for her valuable comments on the manuscript. This work was
485 financed by a Swiss National Science Foundation grant 159869 to SR.

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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
C. pratensis	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
P. major	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.

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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

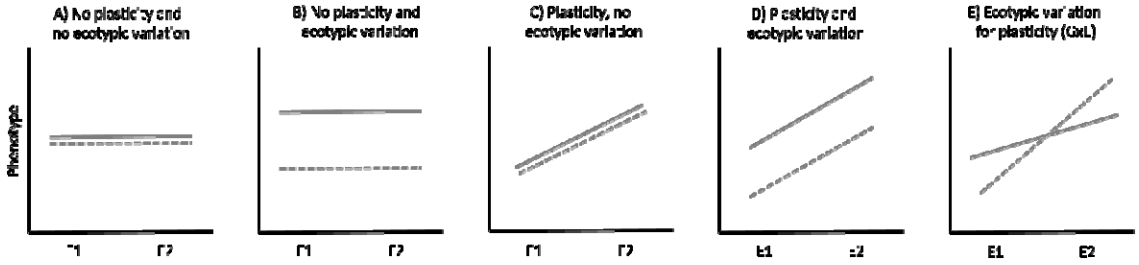


Fig. 1.

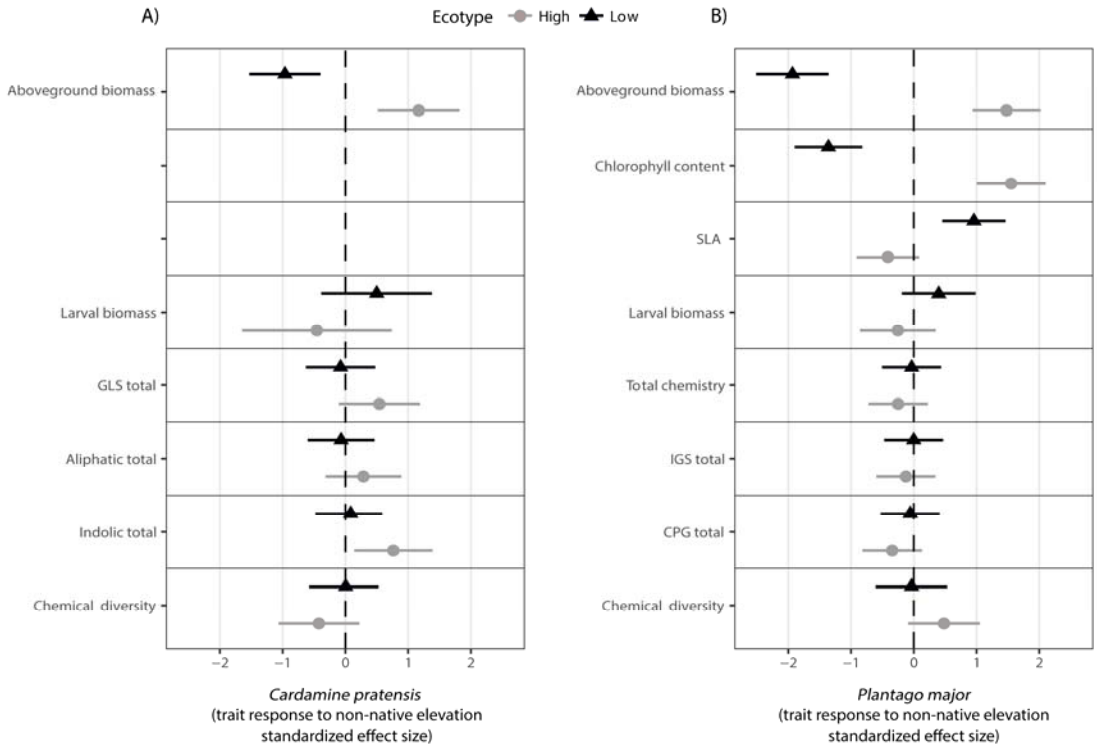


Fig. 2.

Cardamine pratensis

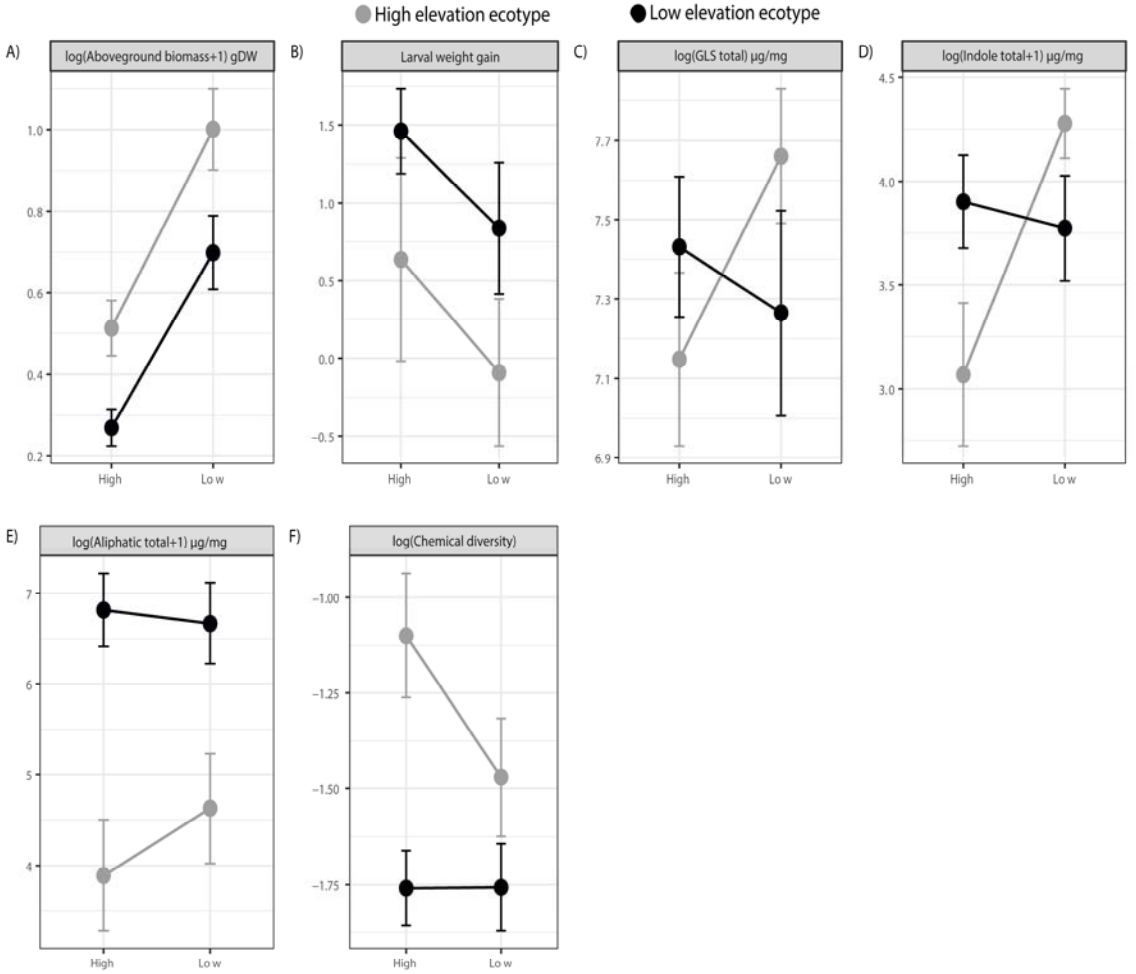
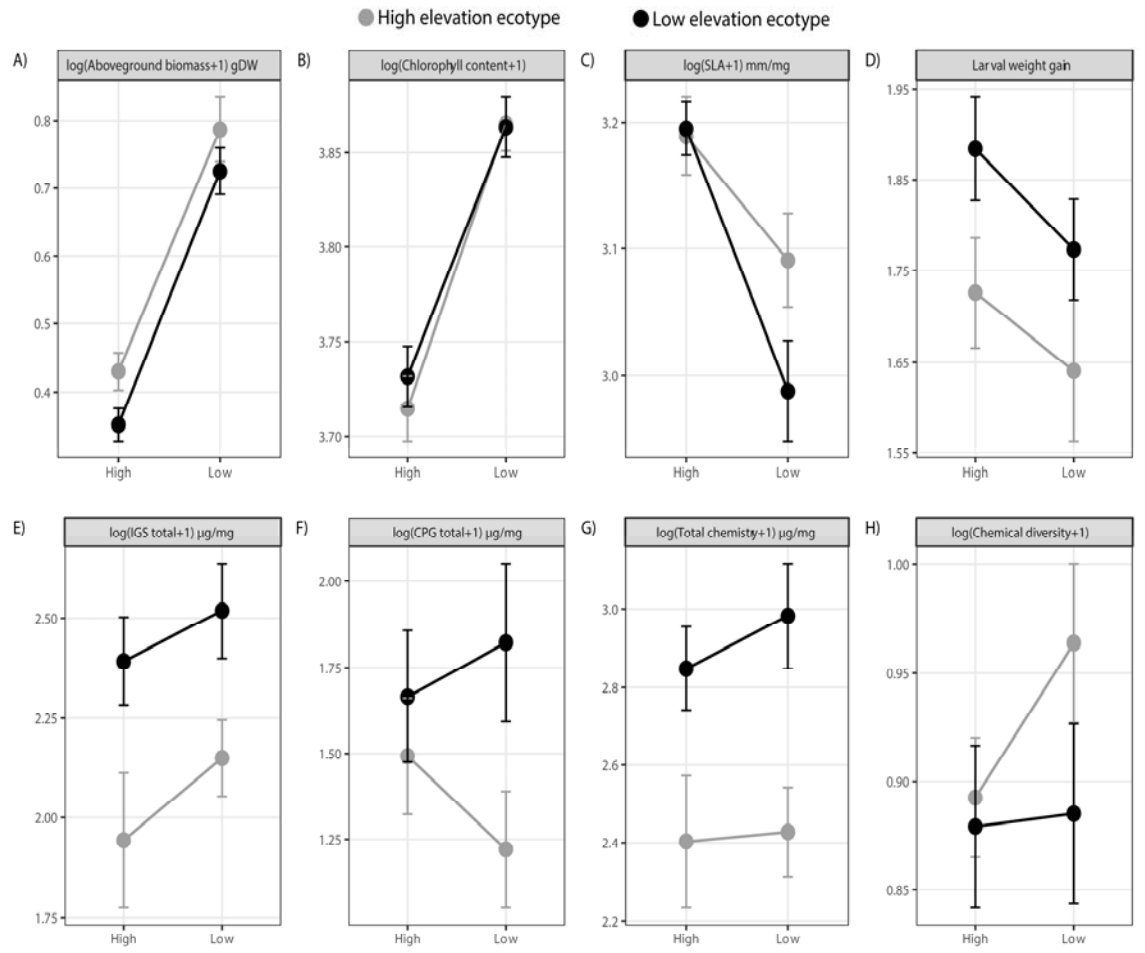


Fig. 3.

Plantago major



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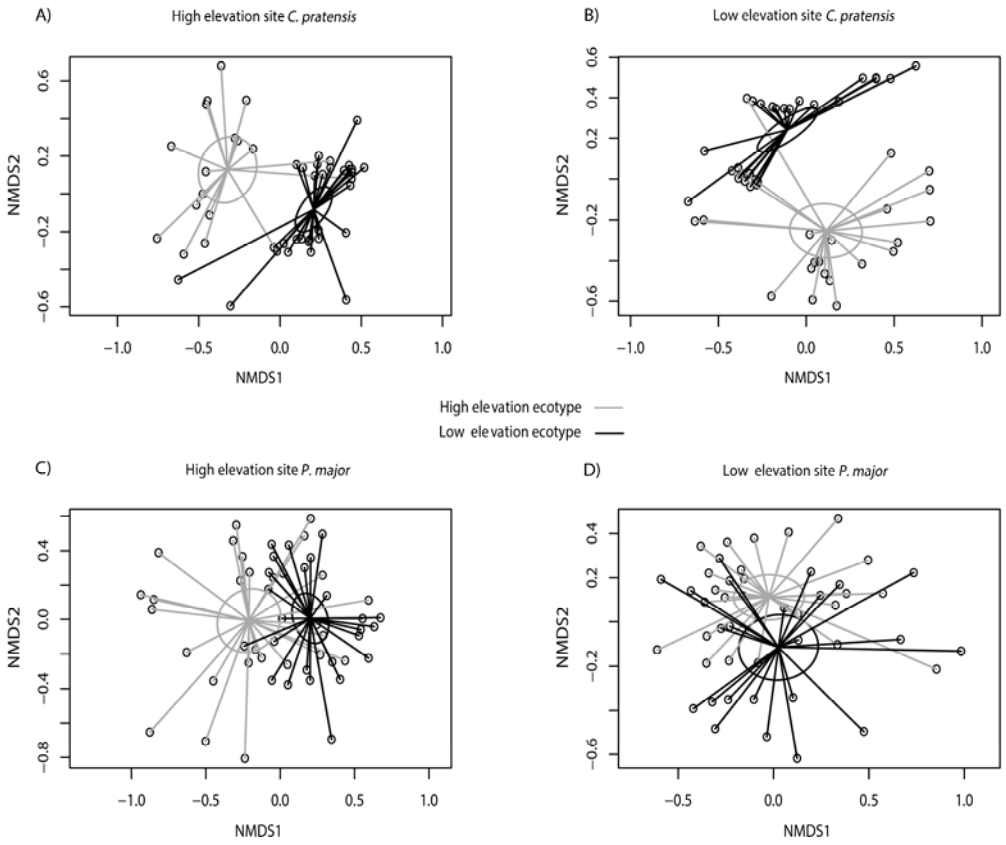

Fig. 5.

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
C. pratensis	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
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		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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		Population	4	2.34	6.97	**<0.001*****
		Elevation	1	0.7	2.07	0.2
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	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*** **
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	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****
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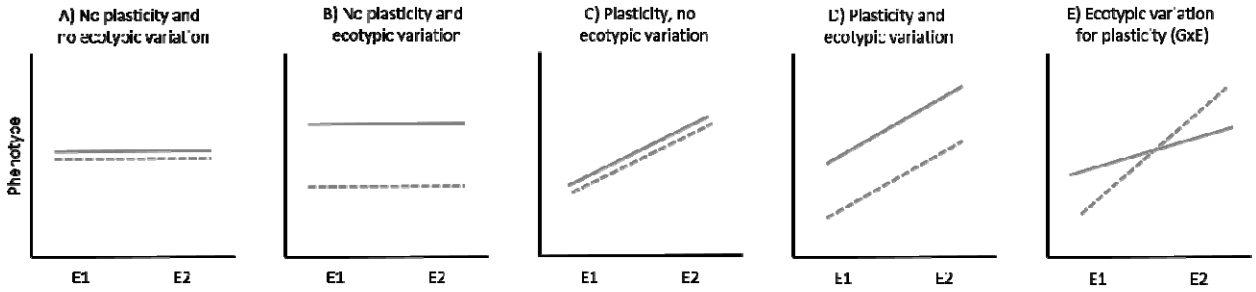
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Figures



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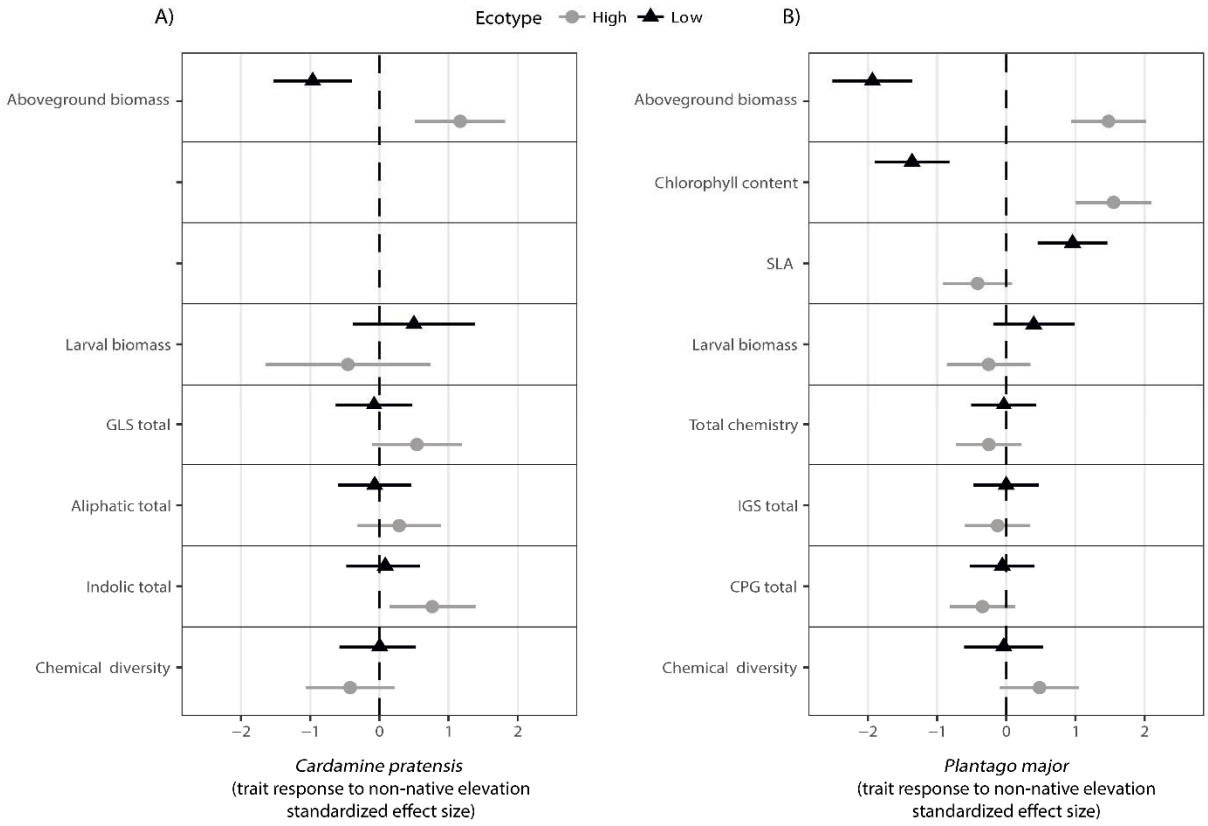

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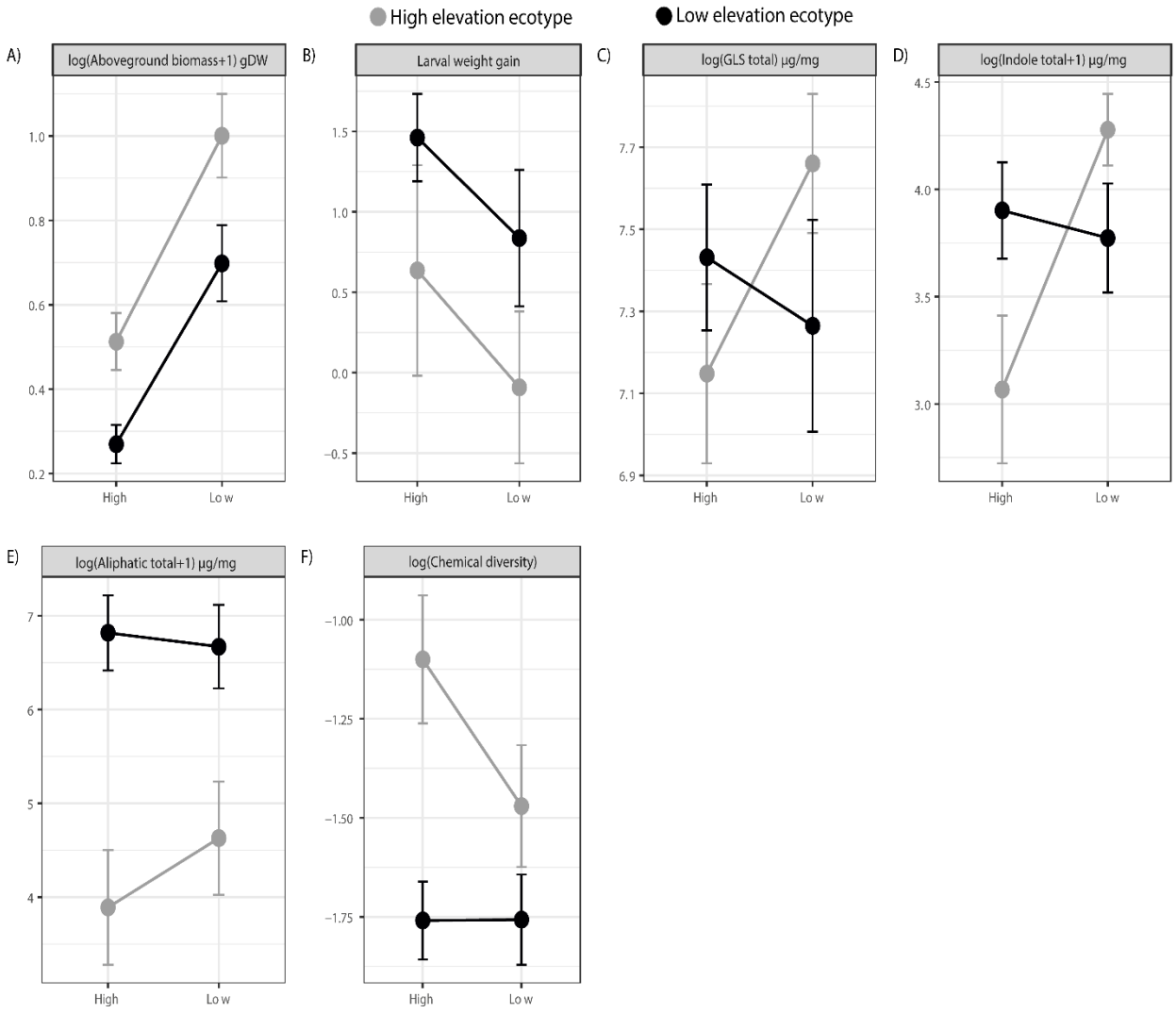

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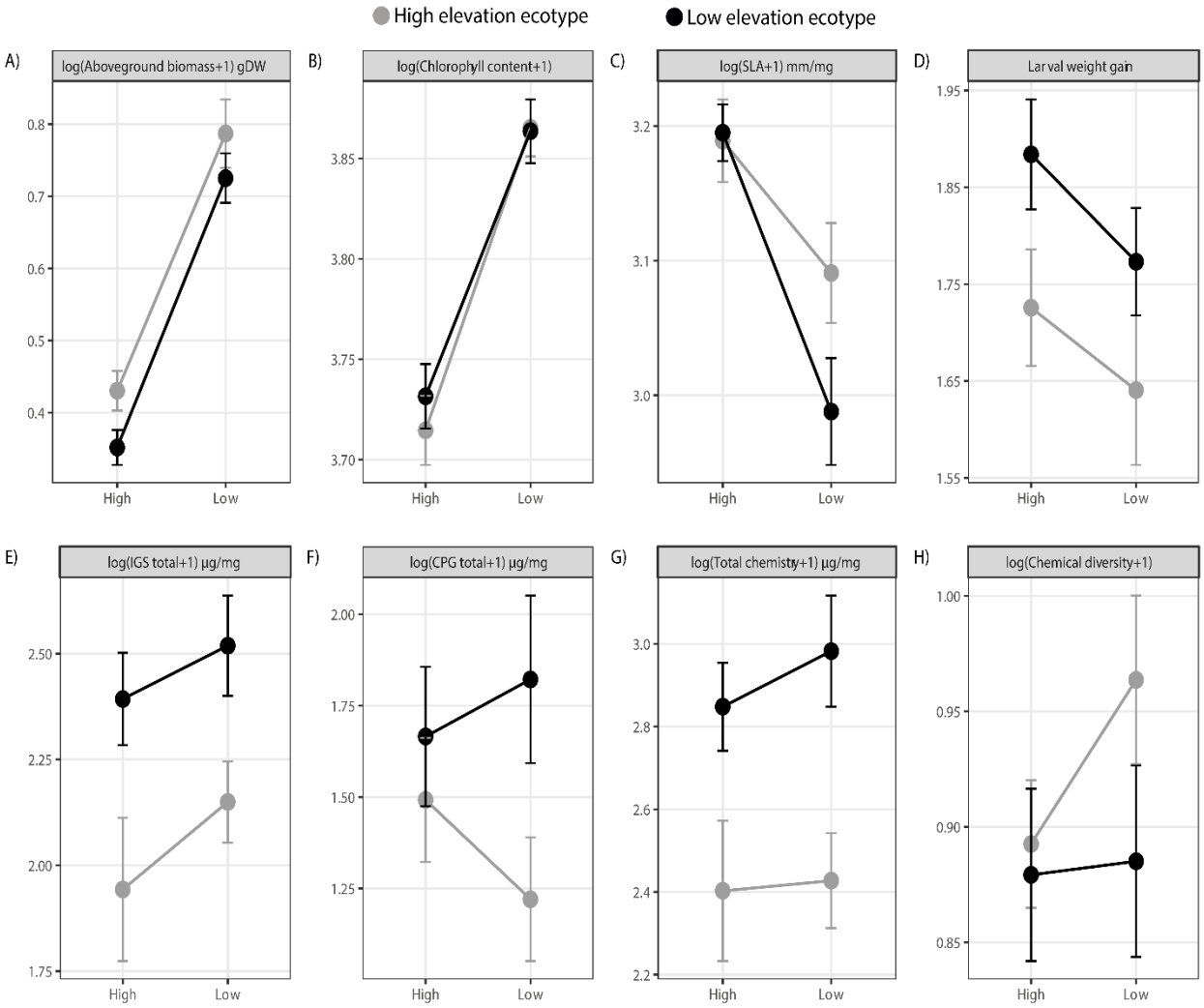

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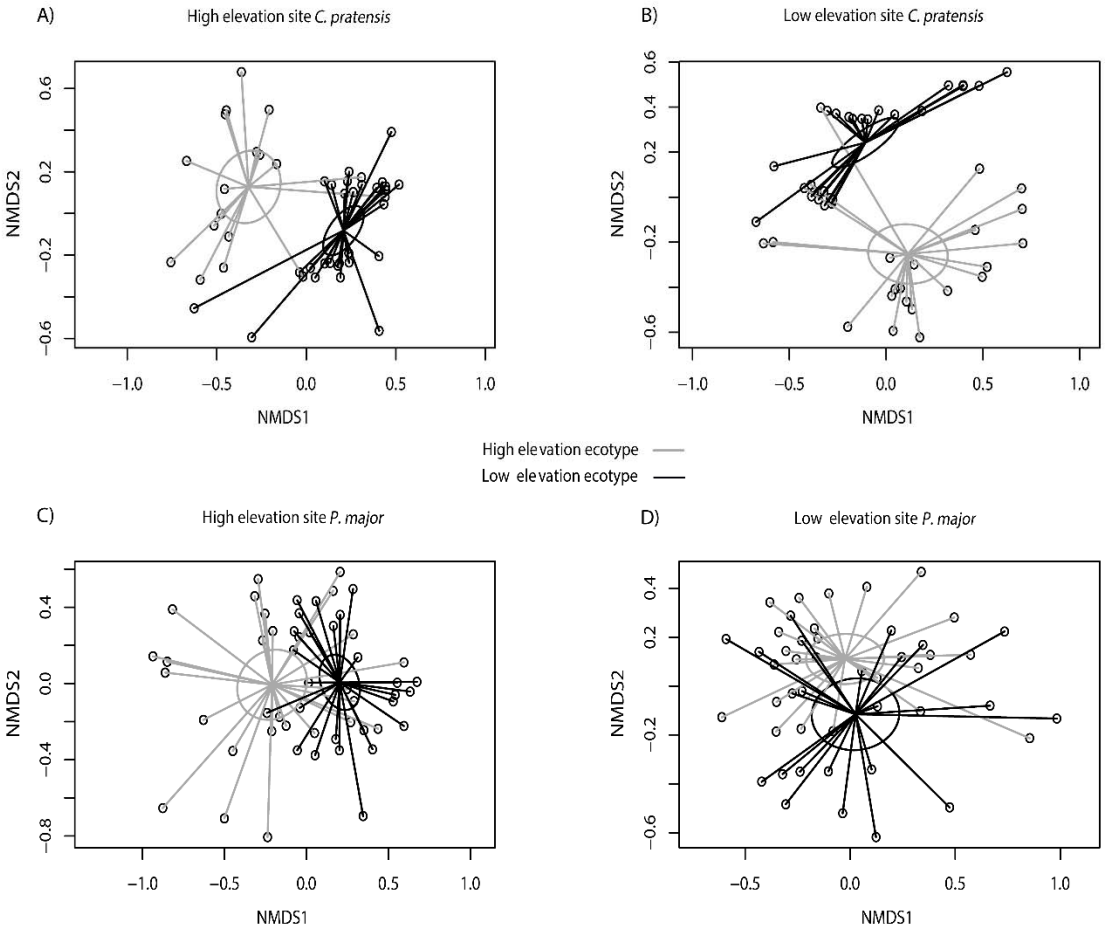

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