RESEARCH ARTICLE

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Plant geographic distribution influences chemical defences in native and introduced *Plantago lanceolata* populations

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Abstract

1. Plants growing outside their native range may be confronted by new regimes of herbivory, but how this affects plant chemical defence profiles has rarely been studied.

2. Using *Plantago lanceolata* as a model species, we investigated whether introduced populations show significant differences from native populations in several growth and chemical defence traits. *Plantago lanceolata* (ribwort plantain) is an herbaceous plant species native to Europe and Western Asia that has been introduced to numerous countries worldwide.

3. We sampled seeds from nine native and 10 introduced populations that covered a broad geographic and environmental range and performed a greenhouse experiment, in which we infested half of the plants in each population with caterpillars of the generalist herbivore *Spodoptera littoralis*. We then measured size-related and resource-allocation traits as well as the levels of constitutive and induced chemical defence compounds in roots and shoots of *P. lanceolata*.

4. When we considered the environmental characteristics of the site of origin, our results revealed that populations from introduced ranges were characterized by an increase in chemical defence compounds without compromising plant biomass. The concentrations of iridoid glycosides and verbascoside, the major anti-herbivore defence compounds of *P. lanceolata*, were higher in introduced populations than in native populations. In addition, introduced populations exhibited greater rates of herbivore-induced volatile organic compound emission

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For Affiliation refer page on 11

and diversity, and similar chemical diversity based on untargeted analyses of leaf methanol extracts.

5. In conclusion, the geographic origin of the populations had a significant influence on morphological and chemical plant traits, suggesting that *P. lanceolata* populations are not only adapted to different environments in their native range, but also in their introduced range.

1 | INTRODUCTION

The range of many plant species has been extended into new regions by accidental or deliberate anthropogenic introduction (Mack & Lonsdale, 2001). Plants may exhibit new phenotypes in response to changing biotic and abiotic environmental factors in their new range (van Kleunen et al., 2018). Studies comparing native and introduced plant populations often emphasize the importance of chemical defences against herbivores because they are considered critical in controlling plant establishment, development and survival (Cipollini et al., 2005; Huang et al., 2010). However, most of these studies have examined only a few plant populations and a small fraction of defence metabolites. Therefore, it remains unclear whether the establishment of a plant species in a new range leads to a rapid evolutionary change in the composition of its defences.

Various non-mutually exclusive hypotheses have been proposed to explain the mechanisms underlying the establishment and dominance of some introduced species. Introduced plants may experience reduced biotic selection pressures due to partial or complete release from pathogens and herbivores found in their native range (ERH: enemy release hypothesis; Keane & Crawley, 2002). They can thus reallocate resources from defence to growth or reproduction, leading to enhanced competitiveness (EICA: evolution of increased competitive ability hypothesis; Blossey & Nötzold, 1995; Bossdorf et al., 2005), or shift defence strategies towards less-costly chemical compounds (SDH: shifting defence hypothesis). Moreover, under some conditions, they might also benefit from the synthesis of new defence compounds (NWH: novel weapon hypothesis; Callaway & Ridenour, 2004). Numerous studies have tested these hypotheses with varying degrees of support (Yannelli et al., 2020) suggesting that the response and successful establishment of a plant species in a new range might vary depending on genetic diversity, spatial–temporal scales and environmental conditions (Bock et al., 2015; Catford et al., 2021; Smith et al., 2020; Theoharides & Dukes, 2007).

The introduction of plants into new ranges often occurs over a large area, spanning different countries and continents, such that new populations are exposed to a wide variety of different climatic conditions, soil properties and biotic factors. As a result, introduced populations may show local adaptations (Hunter, 2016). Differing intensities of biotic and abiotic factors might lead to clinal patterns

in plant chemical profiles (Moreira et al., 2018). It has been hypothesized that investment in plant defence decreases at higher latitudes and altitudes due to lower herbivory pressure and diversity than in tropical environments or at lower elevations (latitudinal/elevational herbivory defence hypothesis, Coley & Aide, 1991; Pellissier et al., 2014). Although many studies provide explicit support for this hypothesis (e.g. Rasmann & Agrawal, 2011), others do not and suggest that many plant-herbivore interactions are too variable to result in such a uniform pattern (Anstett et al., 2015; Cremieux et al., 2008; Moles et al., 2011).

Recent studies have found evidence that the chemical profiles of introduced populations differ from those of populations in their native range (Bhattarai et al., 2017; van Boheemen et al., 2019; Wolf et al., 2011). For example, introduced individuals of *Tanacetum vulgare* L. showed increased concentrations of terpenes and other volatile compounds (Wolf et al., 2011). A study of native and introduced populations of *Ambrosia artemisiifolia* L. showed that phenolic compound composition did not only vary between ranges but also with latitude (van Boheemen et al., 2019). Similarly, the defence chemistry of *Phragmites australis* (Cav.) Trin. ex Steud. was strongly influenced by biogeographic range and latitude. Native genotypes had higher total phenolic contents than introduced genotypes, and concentrations of phenolics increased with latitude in native populations but not in introduced populations (Bhattarai et al., 2017).

In this study, we used *Plantago lanceolata* L. (ribwort plantain) to investigate how the colonization of a new range may lead to changes in chemical defence compounds. *Plantago lanceolata* is a common forb native to Europe and Western Asia that has been introduced and successfully established in numerous countries worldwide (Alexander et al., 2012; Penczykowkski & Sieg, 2021), with records in North America, New Zealand, Australia and Japan dating back 200 years and Chile and South Africa for around 150 years (Cavers et al., 1980; Philippi, 1881). Aside from a high degree of neutral and adaptive genetic diversity in both introduced and native ranges (Smith et al., 2020), *P. lanceolata* displays plastic and adaptive responses to environmental variation (Bischoff et al., 2006; Skinner & Stewart, 2014). Different populations of *P. lanceolata* exhibit different metabolic profiles (Iwanycki Ahlstrand et al., 2018), but there is relatively little knowledge on how herbivore-induced metabolite patterns vary in this plant species across a geographic gradient. There

are also numerous studies on the chemical defences of *Plantago lanceolata*. For example, the iridoid glycosides (e.g. aucubin and catalpol) and phenylethanoid glycosides (e.g. verbascoside) are characteristic defence compounds against insect herbivores and pathogens. Their concentrations can be increased by insect herbivory (Darrow & Bowers, 1999; Marak et al., 2003), but also change in response to light, nutrient availability, mycorrhizal status and neighbouring plant species composition (Fontana et al., 2009; Miehe-Steier et al., 2015; Mraja et al., 2011).

To examine whether *P. lanceolata* populations change after their establishment in new geographic ranges, we quantified levels of chemical defence compounds in nine native and 10 introduced populations that covered a broad range of geographic and environmental conditions. We asked the following questions: (1) how do native and introduced populations differ in size-related and resource-acquisition traits? (2) What are the differences in chemical traits both prior to and in response to insect herbivory? (3) Does the local climate of native and introduced populations influence their levels of chemical defence?

To address these questions, we performed a greenhouse experiment in which we infested half of the plants in each experimental population with generalist *Spodoptera littoralis* caterpillars. Using GC–MS and HPLC–MS, we compared constitutive and induced chemical defence compounds, both volatile and non-volatile from native and introduced populations of *P. lanceolata* and related them to geographic and climatic variables in the area of plant origin. We expected that plants from introduced ranges would have lower concentrations of defence compounds and higher biomass, consistent with the ERH, EICA and SDH hypotheses. Our results, however, show that introduced populations were better defended chemically in terms of both volatile and non-volatile defences when we considered the geographical distribution of the populations.

2 | MATERIALS AND METHODS

2.1 | Seed collection and plant cultivation

Plantago lanceolata seeds were collected from nine native and 10 introduced populations (following the Nagoya protocol requirements from each population sampled) across a wide latitudinal and longitudinal gradient (Figure 1a, Table S1) throughout the world. In each population, seeds were collected as bulk samples from at least five different plants growing at least 3 m apart. Seeds were cleaned and stored at −20°C until the start of the experiment. To sterilize the seeds, we shook them for 2 min in a sterilization solution (100 mg sodium dichloro-isocyanurate [DCCA 2% w/v, Sigma, St. Louis, MO, USA] in 5 mL deionized H_2O + 50 μL Tween 20 [1% v/v, Merck, Darmstadt, Germany] and afterwards rinsed for 30 s with tap water. The seeds were then germinated in 0.44 L pots (Ø 10 cm) filled with a mixture of sand and nutrient-poor soil (50:50) under greenhouse conditions in Jena, Germany (20 ± 2°C during the day [16 h]) and

18 ± 2°C at night [8 h], 30%–55% relative humidity). After germination, 10 seedlings per population were planted individually in pots of the same size and filled with the same substrate as germination pots. Plants were automatically irrigated once a day for 5 min and fertilized (0.025% Ferty 3, Planta Düngemittel, Regenstauf, Germany) after 35 days. The arrangement of plants on the greenhouse table was re-randomized once a week to account for variability in light exposure.

2.2 | Environmental variables

Given the wide geographic distribution of the studied populations, we characterized the environment of each sampled population using 19 bio-climatic variables from WorldClim (http://www.worldclim.org). These variables are a set of climatic variables that capture temperature and precipitation annual and seasonal conditions. Variables were extracted on a 2.5-arc min scale based on measurements from 1970 to 2000 (Fick & Hijmans, 2017). We conducted a principal components analysis to reduce the axes variation and included the first principal component (hereafter PC1) as an explanatory variable in our model to estimate trait differences between native and introduced populations (see statistical analysis below). PC1 explains 33.3% of the variance among population sites and represents a gradient from sites with warmer temperatures (mean annual temperature and mean temperature of the coldest quarter) and smaller seasonal and day-to-night temperature fluctuations (isothermality), to sites with colder temperatures and larger seasonal and day–night temperature fluctuations (Figure 1b, Tables S2 and S3).

2.3 | Herbivory treatment

We selected *Spodoptera littoralis* Boisd (Lepidoptera: Noctuidae) as an insect herbivore because caterpillars of this species are broad generalists that will feed on *P. lanceolata*. Caterpillars of *S. littoralis* were hatched from eggs (Syngenta, Basel, Switzerland) and reared in a climate chamber (23 ± 2°C, with 16 h light per day) on an agar-based artificial diet until they reached the third larval instar (this study did not require ethical approval). Half of the 8-week-old *P. lanceolata* plants were infested with five third-instar *S. littoralis* caterpillars for 48 h, and the other half of the plants functioned as undamaged controls (*n* = 5 plants per herbivore treatment, per population). All plants (190 plants) were enclosed in mesh bags that were tightened at the bottom (at the outer rim of the pots) and at the top with cable ties (Figure 1c).
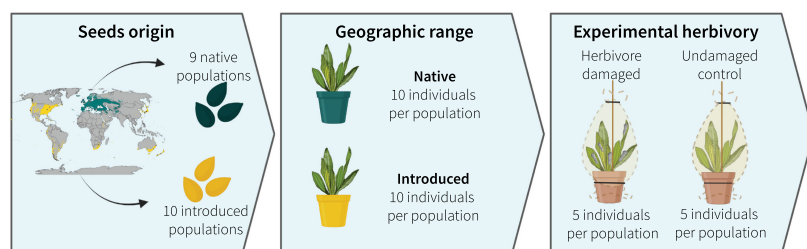
2.4 | Experimental design

The experiment was staggered over 5 days, and each day a block of 38 plants (with two plants per population) was processed. To determine chemical defence traits, we first collected volatile organic
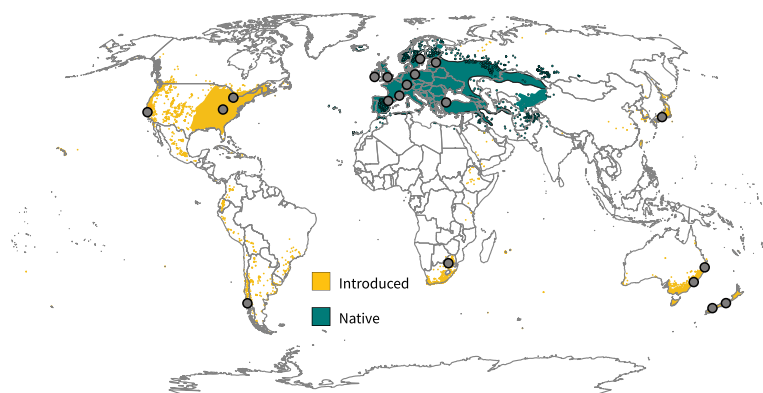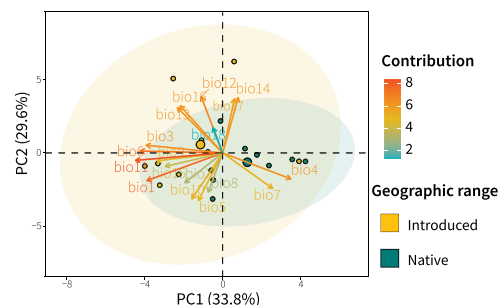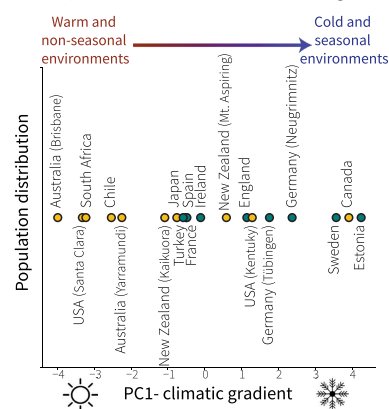
FIGURE 1 Native and introduced populations of *Plantago lanceolata* selected for this study and experimental design. (a) Distribution map of native and introduced ranges of *P. lanceolata*. Dots represent the areas where populations were sampled. (b) Principal component analysis results from the first two principal coordinates on the 19 bio-climatic variables extracted from the WORLCLIM data set (Table S2). (c) Experimental design. Seeds were collected from nine native and 10 introduced populations across all five populated continents. Ten seedlings from each population were germinated and planted in pots under greenhouse conditions. Half of the 8-week-old plants were exposed to five third-instar *Spodoptera littoralis* caterpillars for 48h, and the other half of the plants functioned as undamaged controls. $N=190$ ($n=5$ plants per herbivore treatment, per population). (d) Populations included in the study and their position along the first principal coordinates of the PCA climatic gradient. Figure created with QGIS, R software and Biorender.com.

compounds (VOCs) right after the herbivory treatment. After VOC collection, plant tissues (leaves, inflorescences, root crown and roots) were harvested separately, and immediately flash-frozen in liquid nitrogen and then stored at −80°C until further chemical analyses. Leaf and root samples were then lyophilized (Alpha 1–4 LD-plus, Martin Christ, Osterode am Harz, Germany) and weighed. Leaf samples were divided in two batches, one for metabolome analysis and another for carbon and nitrogen concentrations. Both batches include representative leaves of an individual plant (damaged and undamaged leaves). Samples were homogenized to a fine powder using a ball mill (MM200, Retsch, Haan, Germany). For metabolome analysis, leaf and root powder (10mg) was extracted with 1mL methanol for 30min shaking at 240rpm on a horizontal shaker (IKA® Labortechnik, Steifen im Breisgau, Germany) at room temperature and then centrifuged (2000g for 5min; Method S1).

2.5 | Leaf area and leaf damage measurements

To estimate leaf area and experimental leaf damage by *S. littoralis* caterpillars, we took pictures of all leaves of each *P.*

lanceolata individual right after harvesting. Damage by herbivory was determined by reconstructing the original leaf area with Adobe Photoshop CS5 (Adobe, California, USA; see Unsicker & Mody, 2005 for details) to calculate the proportion of total leaf area (cm^2) and grams consumed (leaf area lost divided by specific leaf area (SLA)).

2.6 | Size-related traits

Number of leaves, leaf area (cm^2) and plant dry mass (g_{dw}), including leaves, roots, flower stem and flower biomass, were analysed as plant size-related traits taking into account only non-infested plants. Leaf area was calculated as described above.

2.7 | Resource-acquisition traits

Specific leaf area (cm$^2_{leaf}$ $g_{dw}^{-1}$$_{leaf}$), leaf carbon concentrations (mg C g_{dw}^{-1}) and nitrogen concentrations (mg N g_{dw}^{-1}) were analysed as resource-acquisition traits. For analyses of carbon and nitrogen

TABLE 1 Replication statement of the experimental design.

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriated scale
Plant (*Plantago lanceolata*)	Geographic range	1 (native) 1 (introduced)
Plant (*Plantago lanceolata*)	Population	9 (native populations) 10 (introduced populations)
Plant (*Plantago lanceolata*)	Herbivory	5 (undamaged controls) 5 (herbivore-damaged plants)
Plant (*Plantago lanceolata*)	Plant	190 individuals

concentrations, approximately 10 mg homogenized leaf material was weighed into tin capsules and measured with an Elemental Analyzer (Vario EL Cube, Elementar, Hanau, Germany).

2.8 | Untargeted analysis

Untargeted metabolic profiles for leaves of *P. lanceolata* populations were obtained by ultra-high performance liquid chromatography coupled via electrospray ionization (ESI) to a qTOF mass spectrometer (UHPLC-ESI-HRMS), using both positive and negative ionization modes separately, with automatic MS2 scans ('autoMS', data-dependent acquisition) enabled. The mobile phases consisted of 0.1% v/v formic acid in water and acetonitrile. Raw data files from UHPLC-HRMS were transferred to MetaboScape (Bruker, Germany) to perform bucketing based on MS1 spectra (Method S2). In order to have additional information about the classes of compounds detected in *P. lanceolata*, we used SIRIUS (version 5.6.3) classification tool for systematic compound class annotation (Dührkop et al., 2021). The chemical taxonomy of the predicted metabolite structures was obtained by CANOPUS (class and subclass; Dührkop et al., 2021).

2.9 | Targeted chemical analysis

Targeted analyses for leaf and root tissues of iridoid glycosides, verbascoside, flavonoids and phytohormones were conducted using an HPLC-MS/MS system (HPLC 1260 Infinity II [Agilent Technologies, Santa Clara, USA]—QTrap® 6500+ [AB Sciex, Waltham, Massachusetts, USA]) in multiple reaction monitoring (MRM) mode. For the iridoid glycosides and verbascoside, identification was based on comparison to authentic standards (aucubin: Carl Roth, Germany; catalpol: Wako, Japan; verbascoside: Extrasynthese, France) and quantitative data for these compounds and the phytohormones were calculated by means of internal standards applying experimentally determined response factors when necessary (Method S3, Table S4). For flavonoids (apigenin 7-O-glucoside, luteolin, luteolin-7-glucoside, rutin, quercitrin), relative concentrations were calculated by dividing the peak area by the weight of the sample and the peak area of the internal standard D6-JA.

2.10 | VOC analysis

Volatile organic compound emissions of *P. lanceolata* were measured using a closed push-pull system for 3 h. Single plants were enclosed with PET bags that were tightened on top with a cable binder and with a rubber band right at the upper rim of the pots (Figure S1). Compressed air entered the system after passing through an activated charcoal filter (0.7 L/min) and it was pumped out (0.4 L/min) at the top through a Poropak-Q absorbent filter (Volatile Collection Trap [VCT] LLC, USA; Figure S1). After VOC collection, the traps were eluted with 200 μL dichloromethane containing nonyl acetate (Sigma-Aldrich, 10 ng μL^{-1}), as an internal standard. Subsequently, VOCs were analysed using a GC–MS chromatograph with helium as the carrier gas for identification and GC-FID for the quantification (Method S4).

2.11 | Statistical analyses

To test whether size-related traits, resource-acquisition traits and herbivore damage differ between native and introduced ranges of *P. lanceolata* populations, we performed mixed-effects models. For size-related traits, only undamaged control plants were taken into account, whereas for herbivore damage, we performed generalized mixed models only for herbivore-damaged plants. 'PC1-climatic gradient' (continuous variable, Figure 1d) and 'range' (factor with two levels) and their interactions were treated as fixed effects and 'population' (population nested within range) and 'harvest day' as crossed random effects. Investment in reproduction may influence the chemical defence and response to herbivory. Since several individuals of the introduced populations flowered during the experiment, we included flowering of plants (as a factor with two levels for non-flowering vs. flowering) as a covariate that we entered before the other explanatory variables in the model to account for variation attributable to the reproductive stage ($y \sim$ Flowering + PC1* Range + [1|Range/Population] + [1|Harvest date]). We tested the significance of the fixed effects using Type I Wald tests with a chi-square statistic. When needed, data was transformed to meet the assumptions of normality and homogeneity of variances (Tables S5–S8; Table 1).

To test for differences in chemical traits between the native and introduced populations, we performed mixed-effect models using the same mixed models as described before but adding 'herbivory' (factor with two levels) and their interactions as fixed factors ($y \sim$ Flowering $+$ PC1* Range * Herbivory $+$ [1|Range/Population] $+$ [1|Harvest date]). To test for differences in untargeted metabolite composition between the native and introduced populations, we performed a partial least squares-discrimination analysis (PLS-DA) and a heatmap of relative abundances of metabolome features and VOC emission using the same mixed models. We calculated the metabolome diversity via Hill number measures (Chao et al., 2014). In analogy to Hill number measures for species, we used metabolite features and VOC identities, and abundance quantified as peak intensity derived from UHPLC-ESI-HRMS traces and concentration of the VOCs derived from GC-FID analyses (Morris et al., 2014). All analyses were run in R version 4.3 (R Development Core Team, 2023) using the packages, *raster* and *sp* for worldclim data extraction and map visualization (Hijmans, 2021; Pebesma & Bivand, 2005); *lme4*, *lmerTest*, *performance*, *factoextra*, *mixOmics* and *hillR* for statistical and diversity analysis (Bates et al., 2015; Kassambara & Mundt, 2020; Kuznetsova et al., 2017; Li, 2018; Lüdecke et al., 2021; Rohart et al., 2017); *notame* for filtering false positive signal of untargeted metabolites (Klåvus et al., 2020); and *ggplot2*, *pheatmap* and *circlize* for graphical visualization (Gu et al., 2014; Wickham, 2016).

3 | RESULTS

3.1 | Plant morphological traits and herbivory damage

Several morphological traits (biomass, SLA, proportion of flowering plants) and herbivory damage showed clinal patterns in relation to the original geographical distribution of *P. lanceolata* populations, and the patterns varied between native and introduced populations (Figure 2). Both leaf and inflorescence biomass were significantly lower in populations from cooler and more seasonal environments (Figure 2a, Range: $x^2 = 6.25$, $p = 0.012$). Introduced populations had more leaves, started flowering earlier and had a higher proportion of flowering plants (33%) at harvest date, compared with native populations (3%) (Table S5). Specific leaf area was higher in populations from cooler and more seasonal environments for native but not introduced populations (Figure 2c, PC1×Range: $x^2 = 4.09$, $p = 0.042$). Plants from introduced populations had lower SLA due to higher leaf mass, rather than smaller leaf area. While foliar nitrogen concentration did not differ among populations, foliar carbon concentration was higher in populations from introduced ranges, and overall, carbon concentrations decreased in populations from cooler and more seasonal environments (Figure 2d, PC1 $= x^2 = 4.52$, $p = 0.034$, Range: $x^2 = 7.35$, $p = 0.006$).

Experimental herbivory by *S. littoralis* caterpillars for 2 days caused on average 5% leaf area loss, with introduced populations
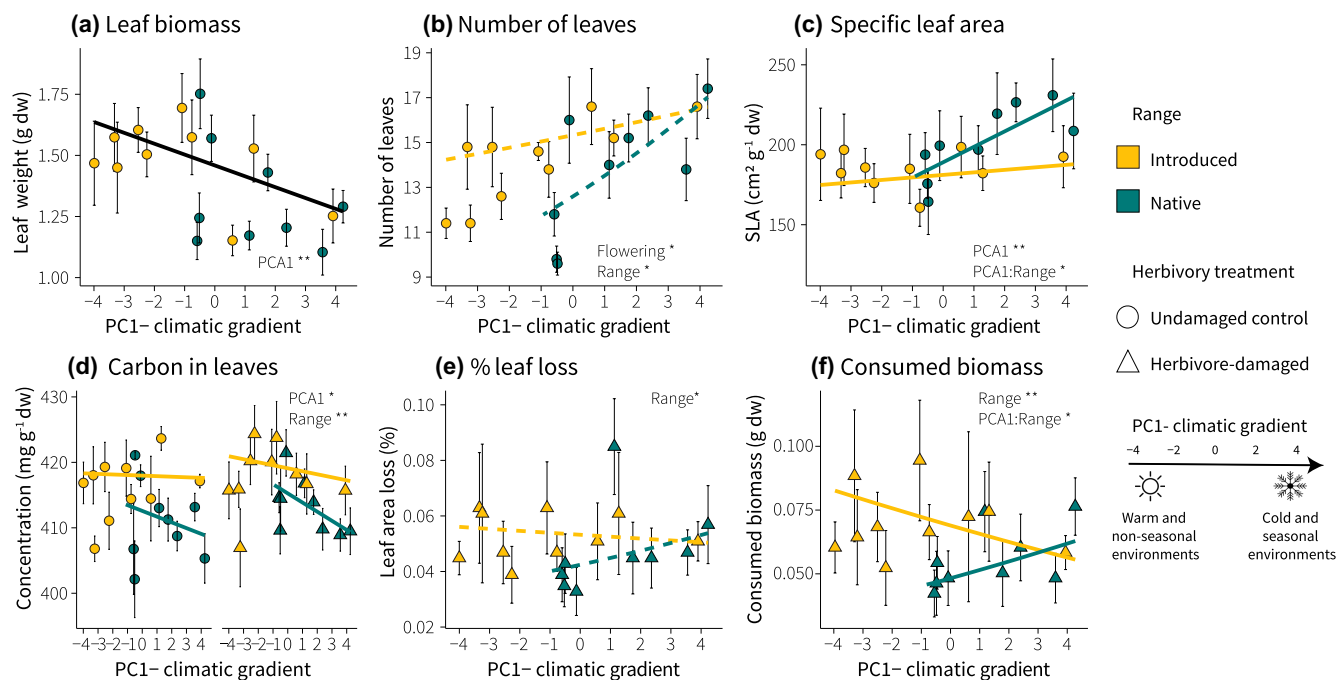

FIGURE 2 Effect of climatic conditions at population sites (native and introduced populations) on plant morphological traits and herbivore damage in *Plantago lanceolata*. (a) leaf biomass, (b) number of leaves, (c) specific leaf area (SLA), (d) foliar carbon concentration, (e) % of leaf loss and (f) consumed biomass by *Spodoptera littoralis* caterpillars. Each point (mean ± 1 SE) within a panel represents a single population. Size-related traits were based on undamaged control plants only. For carbon concentration, herbivore-damaged plants were included in the model. Broken lines denote insignificant relationships with PC1 of the climactic gradient. Asterisks indicate significant effects ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

losing slightly more leaf area than native populations (Figure 2e, 5.2% vs. 4.6% respectively). Consistent with this, caterpillars consumed more biomass from plants of introduced populations as compared to native populations (Figure 2f, Range: $x^2=6.78$, $p=0.009$). Similar to plant resource-acquisition traits, we found climate effects on consumed biomass only in native populations, with leaf damage being higher in populations from cooler and more seasonal environments (Figure 2f, PC1×Range: $x^2=5.23$, $p=0.02$).

3.2 | Plant chemical traits

The metabolic fingerprinting of leaf methanol extracts with UHPLC-ESI-HRMS in negative ionization mode resulted in a total of 1216 metabolic features. The in silico classifications of these metabolic features showed that the metabolic profiles of *P. lanceolata* are dominated by iridoid glycosides, secoiridoid glycosides, megastigmanes, phenylethanoid and phenylpropanoid glycosides, flavonoids, simple phenolics, cinnamic acids derivatives and shikimic acid derivatives (Figure 3a). The PLS-DA model revealed that the metabolomic profiles of *P. lanceolata* plants clustered together based on population range and herbivore damage. Undamaged plants and herbivore-damaged plants showed a strong differentiation, explained by the first component (Figure 3c). In total, 315 features significantly differed between range and herbivory treatment. The heatmap (169 metabolic features, $p<0.01$) showed four broad metabolite clusters (Figure 3b). Cluster I contained metabolic features that are relatively high in native populations in contrast to introduced populations. Cluster II included features that are relatively low in herbivore-damaged plants compared with undamaged control plants. In cluster III, we found that metabolic features that were relatively high in herbivore-damaged plants compared with undamaged control plants. Cluster IV included features that were relatively high in introduced populations compared with native populations. When we included climate variables (PC1) in the model, metabolite feature diversity showed different clinal patterns between native and introduced populations. Hill Shannon diversity of metabolites of native populations increased towards cooler environments with greater seasonality, while in introduced populations, climate had no effect on diversity (Figure 3d; PC1×Range: $x^2=9.39$, $p=0.002$). In both population ranges, metabolite diversity increased after herbivory damage (Figure 3d; Herbivory: $x^2=14.58$, $p<0.001$). The patterns of metabolic features from UHPLC-ESI-HRMS analysis in positive ionization mode (Table S8, 4654 metabolic features detected) were similar to those in negative ionization mode.

We conducted targeted analysis of leaf and root extracts for iridoid glycosides, verbascoside, flavonoids and phytohormones using an HPLC-MS/MS system. When we compared native and introduced populations, we did not find differences among plant traits (Table S6). However, when the climate at the site of origin was taken into account, the foliar concentrations of verbascoside and aucubin were significantly higher in introduced populations, whereas catalpol concentrations did not differ between populations from native

and introduced ranges (Figure 4a–c; aucubin: $x^2=7.10$, $p=0.008$; catalpol: $x^2=0.52$, $p=0.476$; verbascoside: $x^2=7.67$, $p=0.006$). In response to herbivory, verbascoside and iridoid glycoside concentrations in roots and leaves did not increase (Figure 4a–c, Table S6). Phytohormones in leaves increased after herbivory regardless of population range, with jasmonate concentrations being on average 2.6 times higher and ABA concentrations 1.7 times higher (Figure 4c,d). In general, the relative abundance of flavonoids decreased in populations from cooler, more seasonal environments. After *S. littoralis* exposure, the relative abundance of flavonoids increased in the leaves, primarily due to the increase in luteolin and luteoloside (Figure 4f, Table S6). Investment in reproduction was associated with changes in the chemical defence of *P. lanceolata*. In general, flowering plants had significantly lower leaf concentrations of verbascoside, aucubin and the flavonoid quercetin than non-flowering plants (Table S6).

Across the 19 sampled populations, we identified 28 VOCs released from *P. lanceolata* leaves that belonged to five major groups, namely green leaf volatiles, aromatic compounds, monoterpenes, sesquiterpenes, the homoterpene DMNT and a few other compounds that did not belong to any of these groups (Figure 5b, Table S7). Terpenes were the largest group of VOCs, but green leaf volatiles were the most abundant. PLS-DA revealed that whereas herbivore-induced VOCs from native and introduced populations of *P. lanceolata* tended to differ, constitutive VOCs emitted from both populations were very similar in their composition. (Figure 5a,b). Herbivore-induced VOC emission increased in populations from cooler and more seasonal environments, primarily due to the increase in monoterpenes, sesquiterpenes and green leaf volatiles, with introduced populations showing higher emissions (Figure 5c, Table S7). After herbivore damage, VOC diversity increased due to the increase in emission and number of compounds. In general, we found differences in VOC profiles of native and introduced populations along climatic gradients. In native populations, VOC diversity decreased towards cooler and more seasonal environments, whereas introduced populations did not show any clinal pattern (Figure 5d, Table S7).

4 | DISCUSSION

Introduced species that spread from points of the initial introduction, often occur on a global scale, encompassing large latitudinal and longitudinal gradients. *Plantago lanceolata* is a good example of this phenomenon, because despite its large native range, its current geographical distribution extends across all inhabited continents. Using a greenhouse experiment, we compared traits related to plant size, resource acquisition and chemical defence of introduced and native populations of *P. lanceolata* to investigate whether the establishment of a plant species in a new range leads to shifts in its defence metabolites. Our results showed that when we considered the environmental characteristics of the site of origin, populations from introduced ranges were characterized by an increase in chemical
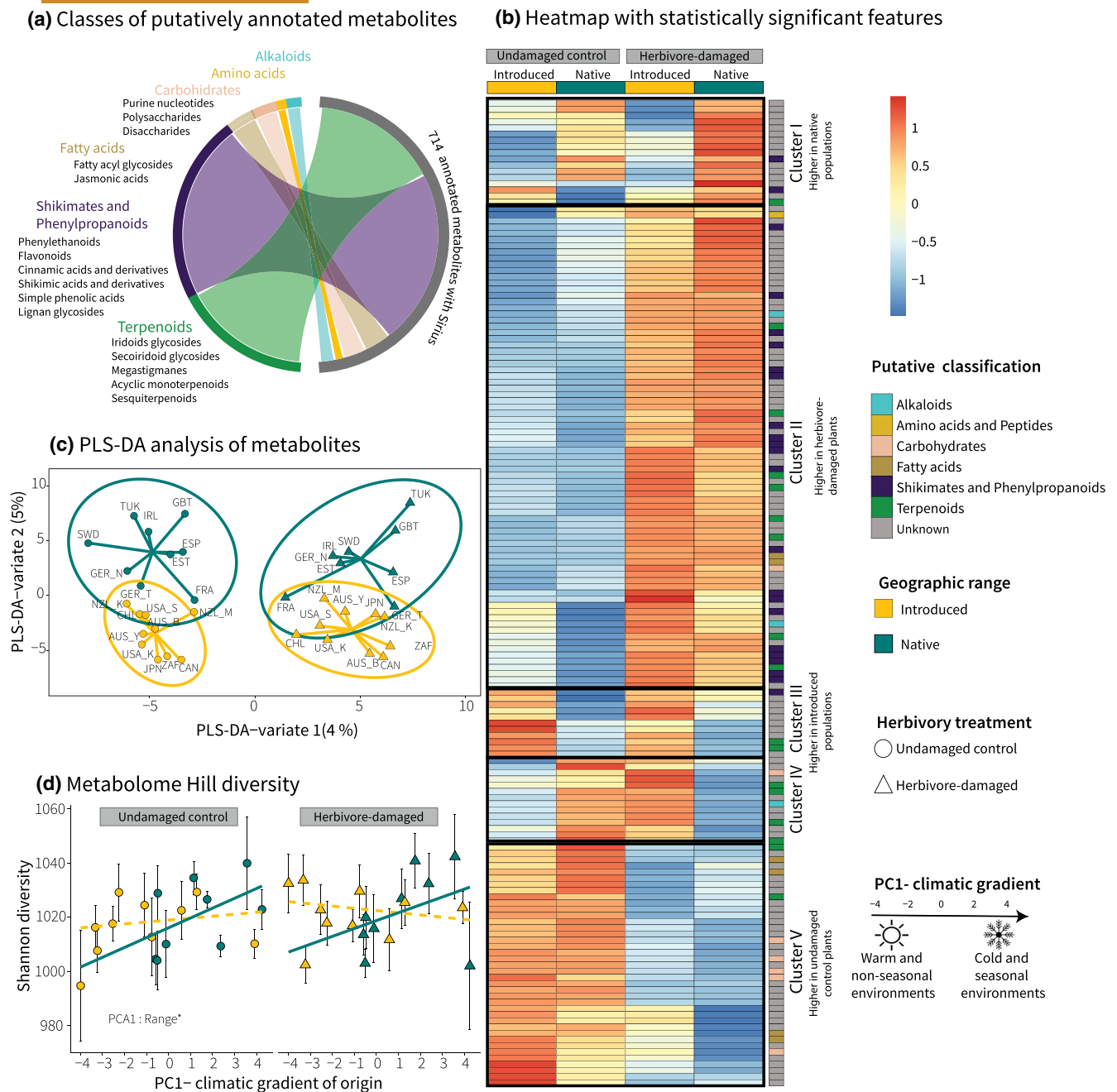
(a) Classes of putatively annotated metabolites

(b) Heatmap with statistically significant features

FIGURE 3 Comparison of native and introduced populations of *Plantago lanceolata* based on untargeted metabolic analysis of methanol extracts. There were 1216 metabolic features in the negative ionization mode after bucketing and filtering. (a) Chord diagram showing classes of all the putatively annotated metabolites with SIRIUS. (b) Heatmap with most significant features (*p* < 0.01) affected by population range and herbivory. (c) Partial least squares-discriminant analysis (PLS-DA) of untargeted metabolites. (d) Mean (±1 SE) of Hill Shannon diversity in relation to PC1-climate gradient. Each point (mean ± 1 SE) within panels' (c and d) represents a single population. Prior to analysis, data were normalized by sample weight, log-transformed and Pareto-scaled. Broken lines denote non-significant relationships with PC1 of the climatic gradient. Asterisks indicate significant effects (*p < 0.05; **p < 0.01; ***p < 0.001).

defence compounds without compromising plant biomass. The observed patterns did not fully meet the expectations of the ERH, the evolution of increased competitive ability hypothesis (EICA) and/or the SDH (Blossey & Nötzold, 1995; Keane & Crawley, 2002). Our findings illustrate the importance of considering environmental conditions when investigating plant traits and chemical defence divergence between introduced and native populations.

Based on the predictions made by the EICA hypothesis, we expected that introduced populations would invest more in biomass and reproduction. Similar to other studies (Blair & Wolfe, 2004; Huang et al., 2010; Villellas et al., 2021), our results were consistent with this trend, at least in the sense of more vigorous growth (higher number of leaves) and an earlier onset of flowers and thus the possibility of earlier reproductive success observed in introduced
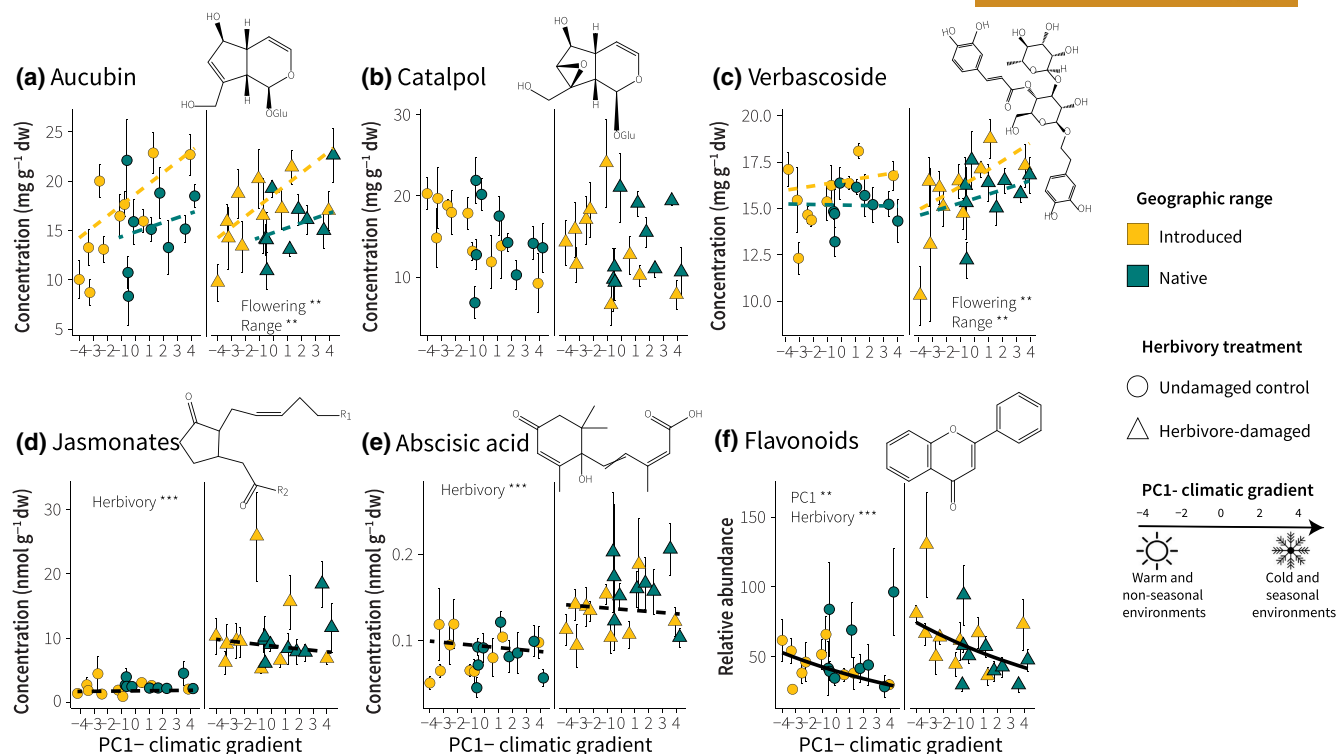
FIGURE 4 Effect of climatic conditions at population sites (native and introduced populations) and herbivory treatments on levels of characteristic defence compounds and defence hormones in *Plantago lanceolata*. Foliar concentrations of (a) aucubin, (b) catalpol, (c) verbascoside, (d) total jasmonates, (e) abscisic acid (ABA) and (f) total flavonoids of native and introduced populations in relation to PC1 derived from climate variables. Each point (mean ± 1 SE) within a panel represents a single population. Broken lines denote non-significant relationships with PC1 of the climactic gradient. Asterisks indicate significant effects (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

populations. Yet, this was mostly because the majority of the introduced populations were from warm and non-seasonal environments, which may select for accelerated plant development (Stamp, 2004). In previous work, increases in SLA and leaf nitrogen concentration were also associated with higher growth rates in introduced populations (Leishman et al., 2007). However, introduced *P. lanceolata* populations had lower SLA but similar leaf nitrogen concentrations to those of native populations. Therefore, it remains unclear whether introduced plants invest more in growth in their non-native ranges.

There was little evidence that introduced populations possessed fewer chemical defences than native populations when we took the climatic conditions of origin into account. Compared with native populations, introduced populations had similar non-volatile metabolic diversity, higher concentrations of aucubin and verbascoside, and higher emission of VOCs. Introduced plants may escape specialist herbivores from their home ranges, but they may still suffer attacks from generalist herbivores in the introduced range (van der Putten et al., 2005). Since defence compounds such as iridoid glycosides are more effective on generalists than specialists (Bowers & Puttick, 1988; Harvey et al., 2005), the increase in aucubin levels may be considered a logical consequence of the shift from native to introduced ranges. Curiously, we found no induction of iridoid glycosides and verbascoside upon herbivory. These compounds have been reported to be inducible by herbivores in some cases (Fuchs & Bowers, 2004), but not all (Fontana et al., 2009;

Stamp & Bowers, 1996), with inducibility depending on the ontogenetic stage of the plant (Quintero & Bowers, 2012). In line with the shift in defence strategy towards generalists rather than specialists, we hypothesized that introduced populations would invest in constitutive VOC emission while native populations would invest in herbivore-induced VOCs. However, our findings revealed that introduced populations of *P. lanceolata* emitted higher concentrations of herbivore-induced volatiles compared with native populations, in contrast to earlier studies on *Jacobaea vulgaris* Gaertn. (Lin et al., 2021). This suggests that the ERH may not be applicable to all plant species and that different species may have different strategies for dealing with herbivores.

Introduced plants may come into contact with possible new specialist herbivores or reencounter with their native specialists. Zangerl et al. impressively showed with leaf material from herbaria that *Pastinaca sativa* L. reduces the concentration of phototoxic furanocoumarins in its invasive range when natural enemies are absent (Zangerl & Berenbaum, 2005). However, when these enemies are also introduced into the plant's invasive ranges, the toxin concentrations increase, suggesting that the association with co-evolved herbivores leads to fast changes in defence chemistry of introduced plant species (Zangerl et al., 2008). Caterpillars of the moth species *Junonia coenia*, which specialized in plants containing iridoid glycosides, feed on North American *P. lanceolata* populations (Bowers, 1984). Correspondingly strong effects on phytochemistry,
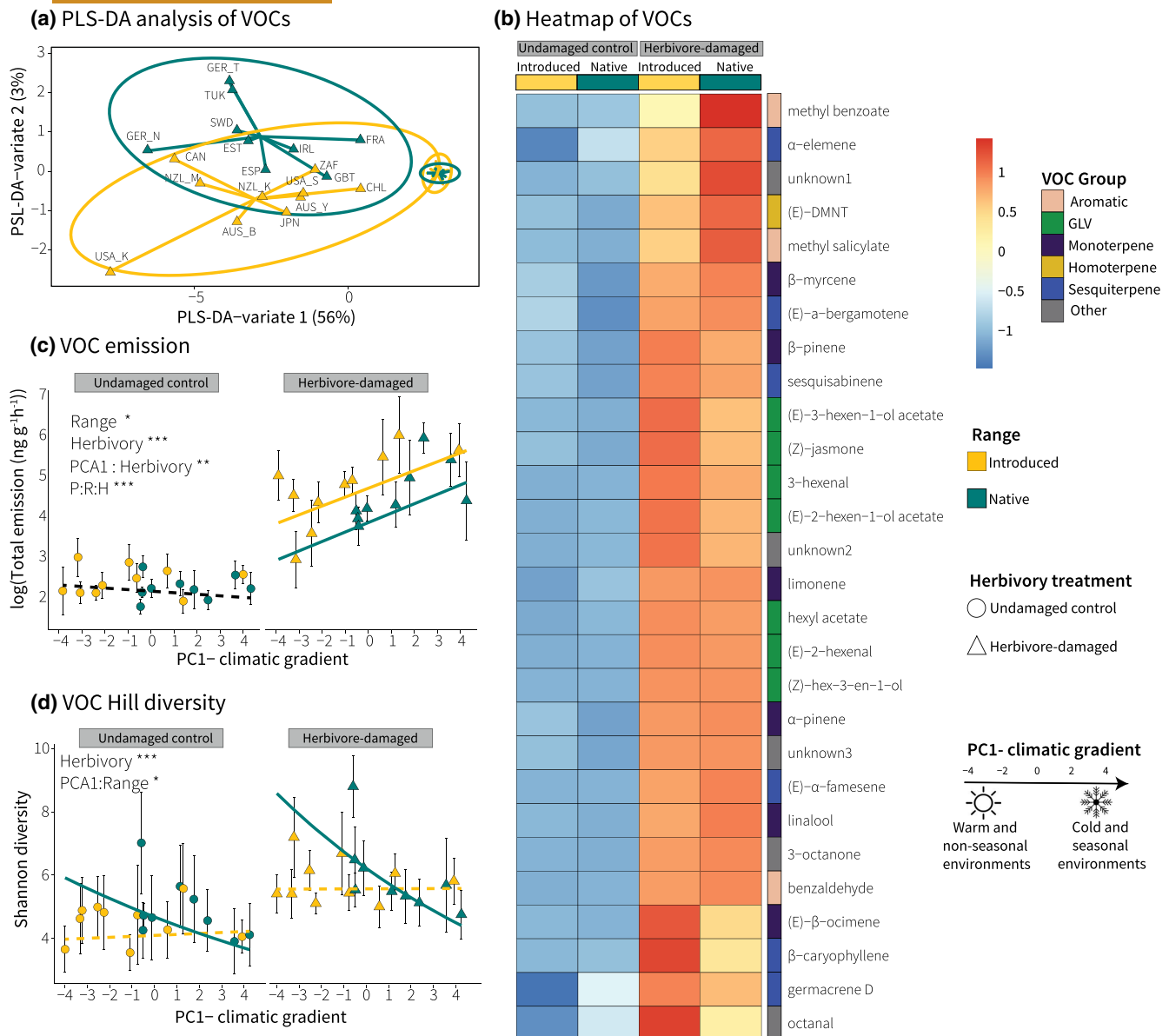
FIGURE 5 Volatile organic compound (VOC) emission from native and introduced populations of *Plantago lanceolata*. (a) Partial least squares-discriminant analysis (PLS-DA) of VOCs composition, (b) Heatmap of VOCs composition, (c) VOC richness in relation to PC1-climate variable, (d) VOC Shannon diversity in relation to PC1-climate variable. Each point (mean ± 1 SE) within panels (c and d) represents a single population. Broken lines denote non-significant relationships with PC1 of the climactic gradient. Asterisks indicate significant effects (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

such as those observed with *P. sativa*, are however not known from *P. lanceolata* in its introduced range.

Introduced plants may come into contact with possible new specialists or reencounter with their native specialists. For instance, *P. lanceolata* populations in North America, have an iridoid glycoside specialist, *Junonia coenia* (Bowers, 1984); or in the case of *Pastinaca sativa*, which increased it defences after their association with is coevolved herbivore, *Depressaria pastinacella* in North America (Zangerl & Berenbaum, 2005). In addition, plant metabolites can be multifunctional and, as a consequence, some metabolites may play different roles in different ecosystems (i.e. volatile diversity may play a role in enhancing reproduction while in another ecosystem it

may serve as defence). Further research is needed to understand the mechanistic implications of chemical profile selection in populations as a defence against herbivores, including comparative analysis of specialist and generalist herbivores.

Environmental factors play an important role in structuring plant defence traits, both directly and indirectly, since climate can be a key selective force driving rapid trait adaptations (Colautti et al., 2009; Hahn et al., 2019; van Boheemen et al., 2019). We detected a positive correlation between herbivore-induced VOCs and decreasing temperatures in both native and introduced populations. Similar to our results, studies on *Asclepias syriaca* L. and *Vicia sepium* L. showed a greater induction of VOC emissions at higher latitude/

altitude compared with plants at lower latitude/altitude (Rasmann et al., 2014; Wason et al., 2013). Concentrations of herbivore-induced VOCs have been shown to be positively correlated with the percentage of herbivore damage. However, leaf damage was not correlated with the total emission of herbivore-induced VOCs, suggesting that the increase in VOCs in cooler environments was not explained by herbivore damage. Plant investment in herbivore-induced VOCs at cooler temperatures might reflect a shift to more investment in cost-saving inducible defences (Rasmann et al., 2014) or be a consequence of limited nutrient availability in harsh conditions (Rinnan et al., 2014). The shifting composition and concentration of plant metabolites may allow for more robust protection against a range of antagonists and unfavourable abiotic environmental conditions (Shulaev et al., 2008). Since the successful establishment of a species depends not only on defence metabolites, other compounds may also play a role in developing adaptive strategies. However, future experiments are needed to test whether these assumptions hold true under field conditions.

Given that introduced plant species are typically introduced into heterogeneous environments containing a variety of levels of biotic and abiotic factors, it is unlikely that all introduced populations will evolve similarly. While we found clinal patterns in some of the measured plant traits, we did not observe consistent responses to climatic variables in all studied traits. In fact, in some cases, responses were even different in native and introduced populations. For example, native populations showed clear clines in SLA and their metabolite diversity, with populations from cooler and more seasonal environments showing leaves with higher SLA and metabolite diversity than native plants in warmer areas, whereas introduced populations showed no variation, a pattern previously reported for SLA in *P. lanceolata* (Alexander et al., 2012; Villellas et al., 2021). Several traits studied in *P. lanceolata* exhibited clinal patterns in both native and introduced ranges, suggesting rapid adaptation to the local environments following introduction, but this may also result from the joint action of genetic drift and gene flow as a result of repeated introduction from distinct native populations or differences in introduction timing resulting in varying levels of local adaptations (Smith et al., 2020; Ward et al., 2017).

As mentioned above, environmental gradients in introduced and native ranges may influence selection for morphological and chemical traits. When comparing *P. lanceolata* populations from different environments, there is always a risk that differences will be masked by an increase in variability within their geographical origin. The patterns of most morphological and chemical traits remain consistent when comparing only native and introduced populations growing under similar climatic conditions (Tables S5–S8). However, both leaf number and leaf damage remain the same for plants in the introduced and native ranges, although there is a trend towards higher leaf aucubin concentrations in the introduced populations ($p = 0.051$). These patterns may be due to warmer environmental conditions in the introduced populations.

In this study, we integrated targeted and untargeted metabolomics to evaluate how plant growth and defence traits vary between native and introduced *Plantago lanceolata* populations. Our results now add valuable insights into changes in chemical defence traits in introduced ranges of *P. lanceolata* along environmental gradients. Due to potential maternal effects in our study, environmental-associated clines in plant phytochemical defences should be interpreted with care. Despite including reproductive status in our models to compensate for plants from warmer areas developing inflorescences earlier than those from cooler populations, such developmental changes can obscure the results (Colautti et al., 2009). It would be interesting to learn more about the underlying mechanisms of the strong correlation between climate and defence levels and why this pattern at a global scale. Follow-up experiments at the chemical and genetic levels under controlled conditions would be helpful in identifying potential drivers. Factors, such as edaphic conditions, plant–plant competition and other biotic variables may be as important as climate and herbivory in influencing chemical defence profiles.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Sally Power is an Associate Editor of Functional Ecology, but took no part in the peer review and decision-making processes for this paper.

STATEMENT ON INCLUSION

The authors from different countries contributed to this study, including scientists from the country where the seed material was collected. Each author was involved in the research from the beginning.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.5dv41nsd1 (Medina-van Berkum et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. *Plantago lanceolata* populations from which seed samples were obtained for this study.

Table S2. Explained variables and loadings of the six principal components from native and introduced populations of *Plantago lanceolata*.

Table S3. Sampled population loading in a principal component analysis (PCA) based on their climatic variables extracted from WorldClim.

Table S4. Details of analysis of phytohormones, iridoid glycosides, verbascoside, and flavonoids by LC-MS/MS [HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionization mode.

Method S1. Leaves and roots extraction.

Method S2. Untargeted metabolic fingerprinting by UHPLC–ESI–HRMS profiles.

Method S3. Quantification of targeted metabolites.

Method S4. Push-pull system for volatile organic compounds collection.

Table S5. Mean (+SE) and model comparison of native and introduced populations of *Plantago lanceolata* based on size-related traits, resource acquisition traits and herbivore damage.

Table S6. Mean (+SE) and model comparison on levels of chemical defense compounds in *Plantago lanceolata* leaves and roots.

Table S7. Mean (+SE) and model comparison of native and introduced populations of *Plantago lanceolata* based on leaf volatile organic compounds (VOCs) emission.

Table S8. Hill number diversity of volatile organic compounds (VOC_S) and untargeted metabolites of native and introduced populations of *Plantago lanceolata*.