Received: 7 July 2019 | Accepted: 5 September 2019

DOI: 10.1111/1365-2745.13286

RESEARCH ARTICLE

Journal of Ecology | BRITISH ECOLOGICAL SOCIETY

Plant physical and chemical traits associated with herbivory in situ and under a warming treatment

Patrice Descombes[1,2] | Alan Kergunteuil[3] | Gaëtan Glauser[4] | Sergio Rasmann[3] | Loïc Pellissier[1,2]

[1]Landscape Ecology, Institute of Terrestrial Ecosystems, Department of Environmental Systems Science, ETH Zürich, Zürich, Switzerland

[2]Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland

[3]Laboratory of Functional Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

[4]Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland

Correspondence
Patrice Descombes
Email: patrice.descombes@wsl.ch

Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 162604 and 179481

Handling Editor: Ayub Oduor

Abstract

1. Plants protect themselves against herbivore attacks with physical traits and toxic secondary metabolites. Levels of plant defences and herbivore performance might shift under climate warming, particularly in alpine habitats, where herbivore pressure is currently low. Plant responses to warming should be driven by species-specific shifts in physical and chemical defence traits.
2. We investigated the association between plant leaf physical and chemical traits and herbivory under current and warmer climates in three grasslands along a subalpine to alpine gradient. Specifically, we measured the rate of in situ natural herbivory, and performed bioassays to measure overall plant species-level resistance using the extreme generalist non-native caterpillar *Spodoptera littoralis*. We simulated warmer conditions by using open-top chambers and assessed the effect of warming on leaf physical and chemical traits, and how trait changes affect caterpillar performance.
3. Natural herbivory and caterpillar performance were associated with plant physical traits, including specific leaf area, and with ordination axes representing dimensions of the plant chemical profile. We found that the warming treatment independently decreased the number of distinct chemical compounds per species, and marginally increased specific leaf area. Changes in leaf functional traits were not systematically associated with changes in caterpillar performance.
4. *Synthesis*. Plant physical traits and chemical profiles are both related to natural herbivory and plant resistance against *Spodoptera littoralis*. While leaf physical and chemical traits of high elevation plants were modified by the warming treatment, these changes did not result in predictable effects on plant resistance against herbivores.

KEYWORDS

climate change, functional traits, herbivore, liquid chromatography, metabolomics, open-top chamber, phylogenetic signal, plant–herbivore interactions

1 | INTRODUCTION

Plants have evolved a wide array of defence traits that provide protection against herbivore attacks, including physical structures and toxic secondary metabolites (Agrawal & Fishbein, 2006; Farmer, 2014; Rhoades, 1979; Schoonhoven, van Loon, & Dicke, 2005). Physical defences, such as leaf toughness, trichomes or silica content, affect herbivore performance by decreasing leaf palatability and digestibility (Awmack & Leather, 2002; Brizuela, Detling, & Cid, 1986; Hanley, Lamont, Fairbanks, & Rafferty, 2007; Massey, Ennos, & Hartley, 2006; Massey & Hartley, 2009). Chemical defences, such as alkaloids, terpenoids and phenolic compounds, act as toxins or digestibility reducers (Mithöfer & Boland, 2012). The current paradigm indicates that chemical and physical defences act together, in the form of syndromes, to counteract a wide variety of herbivores (Agrawal & Fishbein, 2006; Callis-Duehl, Vittoz, Defossez, & Rasmann, 2017; Kursar et al., 2009). In addition to the selective effect of herbivores on plant defence traits (Agrawal, 1998; Kessler & Baldwin, 2001), abiotic factors, especially temperature, can change the expression of plant phenotypes (Gutbrodt, Mody, & Dorn, 2011; Pellissier, Roger, Bilat, & Rasmann, 2014; Totland, 1999). For instance, leaf functional traits have been shown to shift under warmer conditions (Hudson, Henry, & Cornwell, 2011), which may in turn influence plant interactions with herbivores (Lemoine, Drews, Burkepile, & Parker, 2013). Hence, to understand how climate change may reshape plant–herbivore interactions, the relationship between the multivariate plant defence phenotype and herbivory should be documented under both current temperatures and warmer conditions (DeLucia, Nabity, Zavala, & Berenbaum, 2012).

The amount and diversity of physical and chemical defences can vary among species, implying different susceptibility to herbivores (Agrawal & Fishbein, 2006). Physical defences result from a combination of leaf toughness, abrasive compounds such as silica, and other structures such as trichomes (Awmack & Leather, 2002; Brizuela et al., 1986; Hanley et al., 2007; Massey et al., 2006; Massey & Hartley, 2009). Beyond physical traits, the elemental composition of leaves has a large influence on their palatability. Loranger et al. (2012) identified that leaf nitrogen and lignin concentration determined the level of natural herbivory in a local pool of plant species. Because nitrogen is generally scarce in plants and a limiting nutrient for many herbivores (Mattson, 1980), herbivores display a preference for tender leaves with higher nitrogen content (Pérez-Harguindeguy et al., 2003). In addition to containing primary metabolites, plants produce a myriad of secondary metabolites to counteract herbivory (Mithöfer & Boland, 2012; Rhoades, 1979). Because quantifying and documenting complex chemical profiles of plant extracts is technically challenging, researchers have often measured the effect of plant chemistry on herbivory using controlled feeding bioassays, in which the performance of highly polyphagous insect herbivores is used as a proxy for general plant toxicity (Pellissier et al., 2012; Pérez-Harguindeguy et al., 2003). Therefore, the current challenge for research on plant–herbivore interactions is to combine recent developments in analytical chemistry with the direct assessment of herbivory and herbivore performance for a large range of plant species growing under natural conditions (Coley, Endara, & Kursar, 2018; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018; Richards et al., 2015; Salazar et al., 2018).

The levels and diversity of physical and chemical defences varies across plant lineages. In particular, plant phylogenetic position has traditionally been used as a proxy for the effect of phytochemical diversity on plant–herbivore interactions (Futuyma & Agrawal, 2009). The level of herbivory has been shown to vary among lineages, and these differences might be associated with phylogenetically conserved traits such as plant chemical compounds (Ehrlich & Raven, 1964). For instance, Wink (2003) used molecular phylogenies of Fabaceae, Solanaceae and Lamiaceae to map the distribution of defence compounds that are typical for each of these plant families and showed that classes of secondary metabolites are generally conserved. Moreover, phylogenetically conserved interactions within plant–herbivore networks suggest that plant phylogenies can be used as proxies both for plant physical and chemical profiles (Farrell & Mitter, 1998; Janz & Nylin, 1998; Pellissier et al., 2013; Rasmann & Agrawal, 2011; Rønsted et al., 2012). For example, Rasmann and Agrawal (2011) showed that the expression of secondary metabolites in *Asclepias* is associated with both the ecology and the phylogenetic position of the species. However, only a weak phylogenetic signal of leaf secondary chemicals was found for the genus *Piper* (Salazar, Jaramillo, & Marquis, 2016) and for the tropical species in the genus *Inga* (Kursar et al., 2009), both cases where co-occurring species tend to diverge in chemical composition. Hence, whether plant chemical profiles have a strong phylogenetic signal and whether the latter is stronger than the signal from physical traits remain to be evaluated across systems. A phylogenetic signal in defence traits would possibly imply different levels of herbivory, but also potentially different responses of plant traits to climate change (Pellissier et al., 2018).

Climate change might modify plant phenotypes and reshape plant–herbivore interactions (Gutbrodt et al., 2011; Pellissier et al., 2018; Pellissier & Rasmann, 2018), and such changes could depend on a species' functional group or phylogenetic position. Higher temperatures can alter the efficiency of plant defences against herbivores, making plants either more or less susceptible (Lemoine et al., 2013; Pellissier et al., 2014; Stamp & Yang, 1996). In particular, plants might be better defended under warming as a result of either a more rapid metabolism with increased metabolite production or an increase in tolerance. First, experimental warming has been shown to enhance plant biomass (Dawes et al., 2015; Doiron, Gauthier, & Lévesque, 2014) and modify leaf traits (Hudson et al., 2011; Baruah, Molau, Bai, & Alatalo, 2017), which might also increase the rate of herbivory. Increased temperature has further been shown to modulate the nutritional content and the concentrations of defence compounds in plants, thereby affecting herbivore feeding preferences (Coley, Bryant, & Chapin, 1985; Evans & Burke, 2013; Gutbrodt et al., 2011). Moreover, a meta-analysis by Zvereva and Kozlov (2006) showed that warming can decrease leaf nitrogen or sugar content, thereby influencing plant palatability to herbivores. Warming further induces plant stress (Melillo et al., 2002),

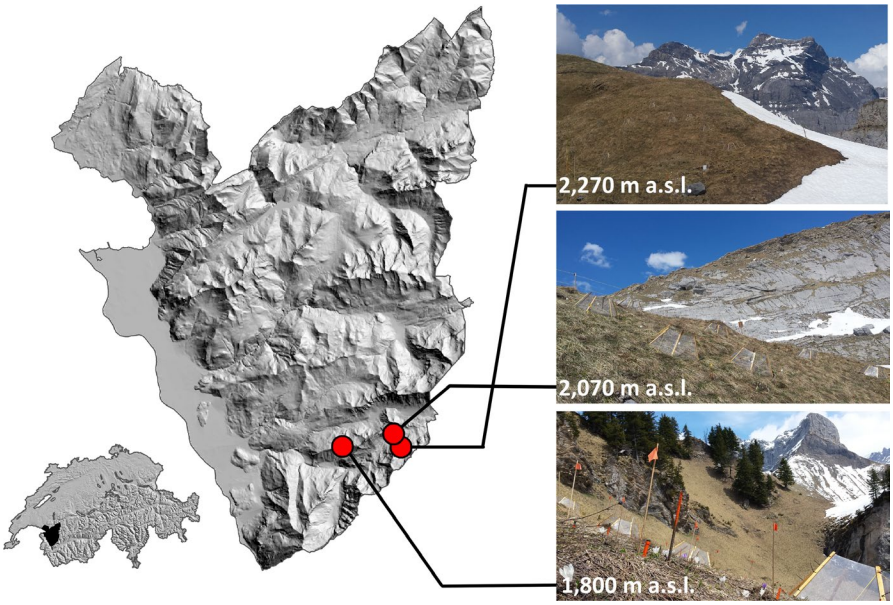

FIGURE 1 The study sites, indicated by the red points, are located in the western Swiss Alps at 1,800, 2,070 and 2,270 m a. s. l. On each site, open-top chambers (OTCs) were placed for warming vegetation plots directly after snowmelt and until the first snowfall in autumn. Terrain map: www.swisstopo.admin.ch. Picture credit: P. Descombes [Colour figure can be viewed at wileyonlinelibrary.com]

and thus plant susceptibility to herbivory (Evans & Burke, 2013; Gutbrodt et al., 2011). Second, temperature might increase the rate of plant metabolism and the amount of leaf chemicals (Pellissier et al., 2014). Most studies quantifying the impact of warming on plant–herbivore interactions have focused on how warming affects foliar damage caused by herbivores (Lemoine, Burkepile, & Parker, 2014; Lemoine et al., 2013), plant growth rates (O'Connor, 22009; Richardson, Press, Parsons, & Hartley, 2002) or plant nutrient contents (Zvereva & Kozlov, 2006). However, few studies have been conducted to investigate how altered physical and chemical plant traits under warmer climate conditions might affect herbivore performance (DeLucia et al., 2012).

Here, we measured physical defence traits, nutritional composition, and chemical traits of *c*. 135 subalpine and alpine plant species, and investigated their association with natural herbivory and with plant palatability assessed with a generalist caterpillar. Moreover, we quantified the effect of temperature change on leaf traits and plant palatability to herbivores by warming alpine plant communities using open-top chambers (OTC). We asked the following questions:

1. Are natural herbivory and plant palatability to a generalist caterpillar associated with physical and chemical traits?
2. Are chemical traits more phylogenetically-conserved than physical traits?
3. How does an increase in temperature reshape physical and chemical traits and plant palatability to a generalist caterpillar?

Our study provides a large-scale multi-species analysis of the association between physical and chemical traits and herbivory. Based on present associations between traits and herbivory and the change in these traits under a warming treatment, we discuss the predictability of plant–herbivore interactions under a warmer climate. We expected both natural herbivory and plant palatability to be associated with leaf physical traits and chemical properties, owing to biomechanical and chemical feeding constraints for the herbivores. We also expected a stronger phylogenetic signal in chemical traits than in physical traits, owing to higher conservatism of plant chemical profiles. Finally, we expected that warming would speed up plant metabolism, thus modifying plant leaf traits and chemical diversity and decreasing plant palatability for herbivores.

2 | MATERIALS AND METHODS

2.1 | Study sites

The study sites are located in the western Swiss Alps in the Chablais region (Figure 1). We selected three calcareous grasslands with similar floristic compositions (Seslerion vegetation type community; Delarze, Gonseth, & Galland, 1998), where pasture grazing is very limited or absent. The three grasslands are distributed along a straight elevation gradient from the subalpine to the alpine belt at 1,800 (46°16′04″N, 7°06′27″E), 2070 (46°16′35″N, 7°09′19″E) and 2,270 (46°16′04″N, 7°09′45″E) m a.s.l. (Figure 1). We used three sites because it enables better generalization of results and inclusion of a larger pool of plant species. On each grassland, we established 16 vegetation plots of 50 cm × 50 cm that were as similar as possible with regard to their floristic composition, canopy structure and available plant biomass. We randomly allocated a warming treatment or an ambient control to the plots, leading to eight replicated plots per site and treatment.

2.2 | Measurement of natural herbivory

We counted the number of leaves with and without herbivory marks for 101 plant species occurring in the ambient control

vegetation plots of the three selected grasslands in September 2016. For the dominant plant species, we counted the number of leaves over a reference surface (10 cm × 10 cm) and extrapolated it over the entire plot. When herbivory marks were present, the percentage of leaf eaten was visually estimated according to a seven-level scale: 1 = <1%, 2 = [1–5]%, 3 = (5–13]%, 4 = (13–25]%, 5 = (25–50]%, 6 = (50–75]%, 7 = (75–100]% of each leaf (e.g. an herbivory of 1% belongs to category 2, 5% to category 2, 13% to category 3, and so on). We estimated herbivory only for chewing damage because sap sucking, leaf mining and rasping marks were rarely observed. We quantified the total dry leaf mass of each species in each plot (g), as a measure of biomass availability for herbivores, by multiplying the total number of leaves by the average dry mass of ten undamaged leaves of the plant species collected on the field site. We estimated a standardized dry leaf mass of each species removed by herbivores in each plot (mg) by multiplying the proportion of the leaf eaten by the average dry mass of 10 undamaged leaves of the plant species.

2.3 | Measurement of plant palatability in a bioassay

We assessed species-level herbivore performance using a bioassay experiment by quantifying the weight gain of non-native chewing insect herbivore across 135 plant species. We used caterpillars of the African cotton leafworm *Spodoptera littoralis* (Lepidoptera, Noctuidae) (Brown & Dewhurst, 1975) obtained from Syngenta (Switzerland). This species is known for being extremely polyphagous (Brown & Dewhurst, 1975) and is commonly used in bioassays to provide integrated information about the combined nutritional content, as well as the physical and chemical defences of plants (Bossdorf, Schröder, Prati, & Auge, 2004; Descombes, Marchon, et al., 2017; Edwards, Wratten, & Cox, 1985; Pellissier et al., 2012; Ruhnke, Schädler, Klotz, Matthies, & Brandl, 2009; Schädler, Roeder, Brandl, & Matthies, 2007). The use of a non-native herbivore ensures that there are no pre-adaptations between the herbivore and the plants.

Well-developed and undamaged leaves were collected in August 2016 from at least five undamaged individuals randomly sampled in the grasslands outside of the experimental plots. When both basal and cauline leaves were available, we collected cauline leaves. Leaves were collected in the morning, placed in moist bags and stored in a cool box (~8°C) until the start of the bioassay in the laboratory. Each plant species was tested separately in the bioassay so that *S. littoralis* caterpillars could only feed on one plant species at a time (no-choice experiment). Leaf material was placed in a Petri dish together with 10 randomly selected freshly hatched first-instar larvae that hatched on wet paper at 20°C without food maximum 12 hr prior to the onset of the bioassay. We offered the caterpillars a non-limiting amount of plant leaves (two to 10 leaves) collected in the grasslands, representing approximately the same total fresh biomass in each Petri dish. We randomized the position of the Petri dishes in the climate chamber every 2 days. The bioassay lasted for 5 days in a climate chamber at 24°C (light; L) and 18°C (dark; D), 55 ± 5% RH and a 14:10 L:D photoperiod. During the experiment, leaves were replaced by leaves stored at 4°C in moist bags, every 2 days or at a higher frequency when leaves were eaten. After 5 days of feeding, all the larvae were dried for 72 hr at 50°C and weighed. While all larvae replicates were in a unique Petri dish, we repeated the experiment for plant species showing high larvae mortality to confirm a direct effect of the plant leaf structure and compounds on larvae survival and not mortality induced by other possibly uncontrolled factors. We calculated the mean dry larval weight (mg) in each Petri dish and averaged these values across replicates. The final dry weight of the caterpillars was considered a reflection of their ability to process the fresh plant tissue.

We used the dry weight as a proxy for herbivore performance instead of fresh weight because it is unbiased by the water status of the plant leaves, which may affect caterpillar fresh weight independently from physical or chemical traits. While calculating the difference between the final and initial dry weight of every single larvae would provide a better estimate of herbivore performance, we opted to use only the final average dry weight because (a) measuring larvae initial dry weight is not possible without killing the larvae and (b) the initial weight of *S. littoralis* larvae (<0.01 mg) is very close to the detectability threshold of high-precision balance. While this might lead to uncertainty in the final comparisons, we expect that not considering the initial dry weight (<0.01 mg) for estimating herbivore performance is not critical given that the average caterpillar final dry weight is 0.2 mg (up to 0.94 mg for the best performing caterpillars), much larger than the initial dry weight (weight increase: 40× on average, 188× for the best performing caterpillars). In addition, while genetic variation in the egg quality might affect the performance of some caterpillar individuals, we do not expect much genetic variability among individuals in this population raised for industrial purposes. Furthermore, we randomly selected 10 larvae from the received batch, which allow accounting for genetic variability in caterpillar performance, something that could not be detected visually on freshly hatched larvae.

2.4 | Measurements of plant physical traits

On different individuals of the same 135 species used in the palatability bioassay, we next measured a set of physical and chemical traits. Individuals from plant species occurring on several sites were measured at each site. For each species, plant height was assessed at each site in 2014 using a vegetation height survey performed with the point-intersect method (Jonasson, 1988; Mueller-Dombois & Ellenberg, 1974) in 32 plots 50 cm × 50 cm in area (see Note S1 for further details).

Leaf area (LA), specific leaf area (SLA) and leaf dry matter content (LDMC) were measured following standard protocols (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013) in August 2015 on at least 10 well-developed and undamaged leaves collected from different plant individuals randomly sampled in the grasslands outside of the experimental plots. When both basal and cauline leaves were available, we collected cauline leaves. Leaves were collected in the morning, placed in moist bags and stored in a cool box (~8°C) until

performing the measurements in the laboratory in the afternoon. Leaves were scanned and weighed, then dried for 4 days at 50°C and weighed. LA (mm^2) was estimated from the scanned leaves in R version 3.4.1 (R Development Core Team, 2014). SLA (mm^2/mg) was measured as LA divided by leaf dry mass, and LDMC (mg/g) was calculated as the ratio of the leaf dry mass to its fresh mass.

We mixed all dry leaf samples into one single sample per species and site for leaf silica and chemical measurements. We mixed and ground the leaves using a mixer mill (RETSCH MM 400 Mixer Mill from Retsch), which provides a good homogenization of the dry plant tissue. Pooled samples provide an indication of average differences among species, as we were not interested in the variation within species. Leaf silica content (% dry mass) was measured using an alkaline extraction of biogenic silica with a sodium carbonate solution (Callis-Duehl et al., 2017; Hallmark, Wilding, & Smeck, 1982).

Leaf toughness was measured on 10 individuals per species in August 2016, using a punching test machine (Imada Inc.), as the force required to pierce a hole through the lamina of the leaf (Aranwela, Sanson, & Read, 1999; Sanson, Read, Aranwela, Clissold, & Peeters, 2001). The device consists of a flat-ended cylindrical steel rod (2 mm in diameter) mounted onto a moving head, which can be passed through a sharp-edged hole with a 0.15 mm clearance located on a stationary base (Aranwela et al., 1999; Sanson et al., 2001). We collected 10 well-developed and undamaged leaves from different plant individuals randomly sampled in the grasslands outside of the experimental plots. Leaves were collected in the morning, placed in moist bags and stored in a cool box (~8°C) until measurement in the laboratory in the afternoon. One punch test measurement was performed near the centre of the leaf (on the left or right side), avoiding primary and secondary veins whenever possible. Before conducting the punch test, we measured leaf thickness with a digital calliper gauge (0.01 mm precision). From these measurements, we calculated the specific punch strength representing the strength per unit leaf thickness at the testing point and expressed in GN m^{-2} m^{-1}. A few species had a leaf width smaller than the diameter of the cylindrical steel rod (<2 mm). In those cases, because the punch strength is applied over a smaller contact area, we estimated the contact area (see Note S2 for further details) and calculated the force required to pierce a hole through the lamina of the leaf based on this area. We used the average trait value for each species and site among all sampled individuals for further analyses.

2.5 | Measurements of plant chemical traits

We measured the elemental content and chemical profile of the 135 plant species recorded in the three grasslands by using the same mix sample as for leaf silica content analysis, as described above. Leaf nitrogen (%) and carbon (%) concentrations were measured using an elemental analyser (NC-2500 from CE Instruments). We calculated the carbon to nitrogen ratio (C:N), which indicates plant nitrogen availability to herbivores (i.e. plants with low C:N are more nitrogen rich).

We performed untargeted metabolomics analyses for estimating chemical richness, chemical diversity, and total chemical abundance. We extracted 20 mg of dry ground tissue with 0.5 ml extraction solution (MeOH: MilliQ water: formic acid; 80:19.5:0.5) and analysed the sample via ultra-high-pressure liquid chromatography—quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) using an Acquity UPLC™ coupled to a Synapt G2 MS (Waters). A volume of 2.5 µl of extract was injected into an Acquity UPLC™ C18 column (50 mm × 2.1 mm, 1.7 µm). We used a binary solvent system consisting of H_2O and acetonitrile, both supplemented with 0.05% formic acid. The chromatographic separation was carried out at a flow rate of 0.6 ml/min under a temperature of 40°C using a linear gradient of 2%–100% acetonitrile in 6.0 min. MS detection was done in positive electrospray ionization over a mass range of 85–1,200 Da. The MS source was cleaned before each of the three batches running over three consecutive days, and peak picking was performed in Markerlynx XS (Waters; Gaillard, Glauser, Robert, & Turlings, 2018). We used the full chemical profile to assess the number of individual chemical compounds per species (chemical richness), the summed abundance of chemical compounds per species (chemical abundance), and the inverse Simpson chemical diversity index (chemical diversity), based on the abundance of individual chemical compounds per species, using the package *vegan* in R (Oksanen et al., 2007). To detect family patterns in plant chemical profiles, we applied the binarized chemical profile to a correspondence analysis (CA) using the *ade4* package in R (Dray & Dufour, 2007). We used a CA to reduce the chemical profile into orthogonal components, as recommended by Bagnères and Hossaert-McKey (2016). We binarized the chemical profile to give more weight to compounds that are produced in small amounts in plants and which might be specific to some plant families. Note that this CA also included a few additional plant species (n = 19) that occurred at the field sites but not in the plots that were not used in further analyses. Because of the nature of the metabolomics approach, with 15,668 peaks of chemical compounds recovered in 154 plant species (248 samples when considering plant species occurring on several sites), each axis of the CA explains only a small percentage of the total variation in chemical compounds. To explore a sufficient fraction of the variation, we retained the first nine axes (CA Axes 1–9), representing 10.4% of the total variability (see Figure S1), as descriptors of the plants' chemical profiles. We calculated the number of chemical compounds associated with the nine axes with explained deviance (D^2) higher than 0.5 and 0.2 by using a binomial generalized linear model and the 'ecospat.adj.D2.glm' function from the *ecospat* package in R (Di Cola et al., 2017).

2.6 | Phylogenetic signal of plant physical and chemical traits and herbivory

The phylogenetic signal of plant physical and chemical traits, as well as natural herbivory and plant palatability, was assessed by Blomberg's K statistic with the *phylosignal* package in R (Blomberg, Garland, Ives, & Crespi, 2003; Kembel et al., 2010). Phylogenetic relationships between plants were retrieved from a well-resolved and dated phylogeny of European plant species (Durka & Michalski, 2012). We estimated K for the average plant trait values

calculated across sites on the pruned tree that included only the plant species occurring in the grasslands (n = 132). K-values close to zero indicate that trait values are randomly distributed within the phylogeny, while K-values close to one indicate that closely related species share more similar trait values than random as expected under Brownian Motion, and K-values greater than 1 indicate stronger similarities among closely related species than expected under Brownian Motion (Blomberg et al., 2003). The statistical significance of the observed K-values was tested with a null distribution (Kembel et al., 2010).

2.7 | Statistical analyses of natural herbivory and plant palatability

We tested the association between natural herbivory and plant palatability with a spearman rank correlation test. We tested family differences in plant natural herbivory and plant palatability with an analysis of variance by averaging palatability and natural herbivory values for each species across sites.

We investigated the relationship between the plant susceptibility to natural herbivory and plant physical and chemical traits using a Monte Carlo Markov Chain generalized linear mixed model (MCMCglmm) implemented in the *MCMCglmm* package (Hadfield, 2010) in R version 3.4.1 (R Development Core Team, 2014). We used a Bayesian approach, which makes it possible to account for phylogenetic relatedness between plant species as a random factor in the model, and because more traditional models such as linear mixed effect models (Pinheiro, Bates, DebRoy, & Sarkar, 2014) were not converging. We related natural herbivory to the physical and chemical traits, dry plant biomass and the elevation of the site. Vegetation plot and site identity were also included as random effects in the model with uninformative priors (V = 1 and nu = 0.002). We used the 'vifstep' function in the R package *usdm* (Naimi, 2015) to calculate a variance inflation factor (VIF; Quinn & Keough, 2002), to identify highly correlated predictors. A VIF value that exceeds 10 is indicative of multicollinearity of a predictor variable (Montgomery & Peck, 1992; Quinn & Keough, 2002), while VIF values less than 6 are considered acceptable (Zuur, Ieno, & Elphick, 2010). We removed chemical abundance from the predictors because it had a high level of collinearity (VIF = 8.02). All remaining predictors had VIF < 6.2. The natural herbivory response variable was square-root transformed and all variables were rescaled around their mean using the 'scale' function in the *base* package in R. Because the response variable of the natural herbivory model was zero-inflated, we rounded it to the nearest integer and used a MCMCglmm with a zero-inflated hurdle Poisson distribution, following the recommendation of Hadfield (2010, 2015). The model was run with 500,000 iterations with a thinning factor set to 200 to reduce autocorrelation of consecutive samples. We visually checked for convergence of posteriors and autocorrelation of consecutive samples in the models with the 'xyplot' and 'autocorr.plot' functions in the R packages *lattice* and *coda*, respectively.

We related plant palatability to *S. littoralis* to the physical and chemical traits and the elevation of the site with a MCMCglmm (Hadfield, 2010). We removed chemical abundance from the predictors because it had a high level of collinearity (VIF = 7.12). All remaining predictors had VIF < 4.3. Plant palatability was square-root transformed to reach a normal distribution and all variables were rescaled around their mean. The plant palatability model was run with a Gaussian distribution, with 500,000 iterations and a thinning factor of 200, and phylogenetic relatedness between the plant species and site identity was accounted for by including them as random effects with uninformative priors. We visually checked for convergence of posteriors and autocorrelation of consecutive samples in the models.

2.8 | Plant traits and plant palatability under the warming treatment

The warming treatment consisted of hexagonal OTC following the International Tundra Experiment standards (Henry & Molau, 1997; Marion et al., 1997). OTCs provide an effective and simple method of climate change simulation and consist of a hexagonal enclosure built of clear transparent 2-mm-thick polymethylmethacrylate material (PMMA-XT transparent clear, Angst + Pfister SA). The walls of the OTC have a 60° inclination relative to the ground, a ground diameter of 111 cm, a top opening of 60 cm in diameter and a height of 38 cm. The experiment lasted from spring 2014 to autumn 2017. OTCs were set up over the plots as soon as possible after spring snowmelt and removed before the first snowfall in autumn. We characterized the effect of the OTCs on air temperature by using high-resolution temperature loggers (DS1922L-F5; Homechip Ltd) placed in the middle and just outside of each OTC from the beginning of July to the end of August in 2017. Loggers were placed 20 cm above the ground and fixed under a small white cup to avoid direct solar radiation heating effects, and they were parameterized at high resolution (0.0625°C) with a sampling rate of 30 min. We averaged daily (00:00–24:00 hr), diurnal (11.00–17.00 hr) and nocturnal (23.00–05.00 hr) temperature over the sampling period for each logger. We assessed the effect of the OTCs on temperature with a linear mixed-effects model fitted by maximum likelihood, including site identity and plot as random factors, with the 'lme' function (Pinheiro et al., 2014) in the *nlme* package in R. The warming treatment increased the mean daily air temperature (20 cm above ground) by 1.1°C (p < .001) and strengthened mean diurnal (+3.8°C) and nocturnal temperature (−0.6°C) during the summer season. The OTC greenhouses decrease mixing with ambient air, which in turn favours heat accumulation in the chamber during daytime warming and enhances cooling at night by trapping cold, dense air during night-time inversions (Dabros, Fyles, & Strachan, 2010; Marion et al., 1997).

We focused the trait analyses on a subset of 16 plant species out of the 135 species collected in August 2016 (Table S1). The 16 species were a subset of the most frequent species observed in each site and were selected to represent a good phylogenetic coverage and physical trait variability. We collected each species from only one site, with the exception of *Sesleria cearulea*, which we collected from more than

one site. For each species, we randomly selected four to five warmed plots inside the OTCs and four to five ambient control plots where more than five individuals were present. In these plots, we collected three to five well-developed leaves from different undamaged individuals, with a preference for cauline leaves. Leaves were collected in the morning, placed in moist bags and stored in a cool box (~8°C) until measurement in the laboratory in the afternoon. Leaf toughness was measured individually for each collected leaf (one measurement per leaf), and values were averaged per species and per plot. We measured LA, SLA and LDMC on all leaves at the same time to obtain an average value per species per plot. We mixed the leaf samples per species and per plot, dried (4 days at 50°C) and ground them, and performed nutrient content (C:N) and chemical content analyses (chemical richness, chemical diversity and total chemical abundance). All trait measurements followed the protocols described above.

On a subset of seven plant species (Table S1), we evaluated palatability under the warming treatment and ambient control using *S. littoralis* caterpillars. For each of these species, we collected three to five well-developed leaves from different undamaged individuals, whenever possible, on the same four to five warmed plots inside the OTCs and four to five ambient control plots as described above. The leaf sampling procedure and the bioassay experiment followed the same protocol as described above.

2.9 | Statistical analyses of the warming treatment

We analysed the change in chemical profile between the ambient control and the warming treatment for the 16 plant species collected in warmed OTCs plots and in ambient control plots using the 'adonis' function in the R package *vegan* (Oksanen et al., 2007). This function performs an analysis of variance based on Euclidean distance matrices and a permutation test by setting treatment, site identity and species (and their interactions) as fixed factors and setting species and site identity as strata for within-species randomizations (1,000 permutations). For each species, we calculated the number of chemical compounds with significantly increased or decreased expression by comparing expression profiles between the ambient control and warming treatment with a two-sample *t*-test. We selected the four chemical compounds that showed the greatest increase or decrease in their expression. Compounds of interest were tentatively identified on the basis of their molecular formulae (determined from measurements of both mass-to-charge ratios and isotopic abundances), fragmentation patterns, and comparison with available databases such as the Dictionary of Natural Products (CRC Press), ReSpect for Phytochemicals and Massbank.

We investigated the effect of the warming treatment on single traits (LA, SLA, LDMC, toughness, chemical richness, chemical diversity and chemical abundance) with a linear mixed-effects model fitted by maximum likelihood, including site and species identity as random factors, with the 'lme' function (Pinheiro et al., 2014). For each species, we calculated the average trait change (%) across plots, and we calculated the average and standard deviation of trait changes (%) across species. We used a binomial model as a discriminant analysis (Kuhn & Johnson, 2013) to discriminate between the two treatments regarding the physical and chemical traits expressed, with a MCMCglmm (Hadfield, 2010). This analysis detects associations between trait changes and the warming treatment. We removed chemical abundance, as in the abovementioned analyses. All remaining predictors had VIF < 6. We rescaled all selected variables around their mean. Traits were set as fixed effects and the phylogenetic relatedness of the plant species and site identity were set as random effects with uninformative priors ($V = 1$ and $nu = 0.002$) and strong priors for the residuals ($V = 1$, $fix = 1$; Hadfield, 2015). We ran the model with 1,500,000 iterations and a thinning factor of 200. We visually checked for convergence of posteriors and autocorrelation of consecutive samples in the models.

We related plant palatability, measured as dry larval weight, to the ambient control and warming treatment, additionally considering an interaction with the species leaf traits, using a MCMCglmm with a Gaussian distribution (Hadfield, 2010). The treatment and traits were set as fixed effects. The phylogenetic relatedness between plant species and site identity were set as random effects with uninformative priors. We removed chemical abundance as in the abovementioned analyses. All remaining predictors had VIF < 4. We rescaled all selected variables around their mean. We ran the model with 500,000 iterations and a thinning factor of 200, and we visually checked for convergence of posteriors and autocorrelation of consecutive samples in the models. A significant interaction between the warming treatment and the traits would indicate that the treatment changed the traits, with consequences for plant palatability.

3 | RESULTS

3.1 | Metabolomics ordination

The UHPLC-QTOFMS profiling detected 15,668 peaks of chemical compounds across the 154 plant species and 248 samples. When applying a CA on the plant chemical profiles, we could observe that species were grouped by families along these axes (Figure S1). In particular, the first axis (CA Axis 1; 1.81% of total variance) discriminated Gentianaceae, Caprifoliaceae, Orobanchaceae and Plantaginaceae (high scores) from other plant families (low scores; Figure S1). The second axis (CA Axis 2; 1.66% of total variance) discriminated Gentianaceae, Brassicaceae, Euphorbiaceae, Primulaceae and Violaceae species (low scores) from Geraniaceae, Asteraceae, Ericaceae and Plantaginaceae species (high scores; Figure S1). The third axis (CA Axis 3; 1.22% of total variance) discriminated Orobanchaceae and Plantaginaceae (high scores) from other plant families (low scores; Figure S1). The fourth axis (CA Axis 4; 1.14% of total variance) set the taxonomic group of Poales (high scores; i.e. Poaceae, Cyperaceae and Juncaceae) apart from Rosaceae, Cistaceae, Ericaceae and Fabaceae species (low scores; Figure S1). The fifth axis (CA Axis 5; 1.03% of total variance) discriminated Poales, Ericaceae, Cistaceae and Rosaceae (low scores) from other plant families (high scores; Figure S1). The sixth axis (CA Axis 6; 0.97% of total variance) strongly discriminated Orchidaceae (low scores) from other plant families (high scores; Figure S1). The seventh axis (CA Axis 7; 0.88% of total variance) discriminated Fabaceae (low scores) from other plant families (high scores; Figure

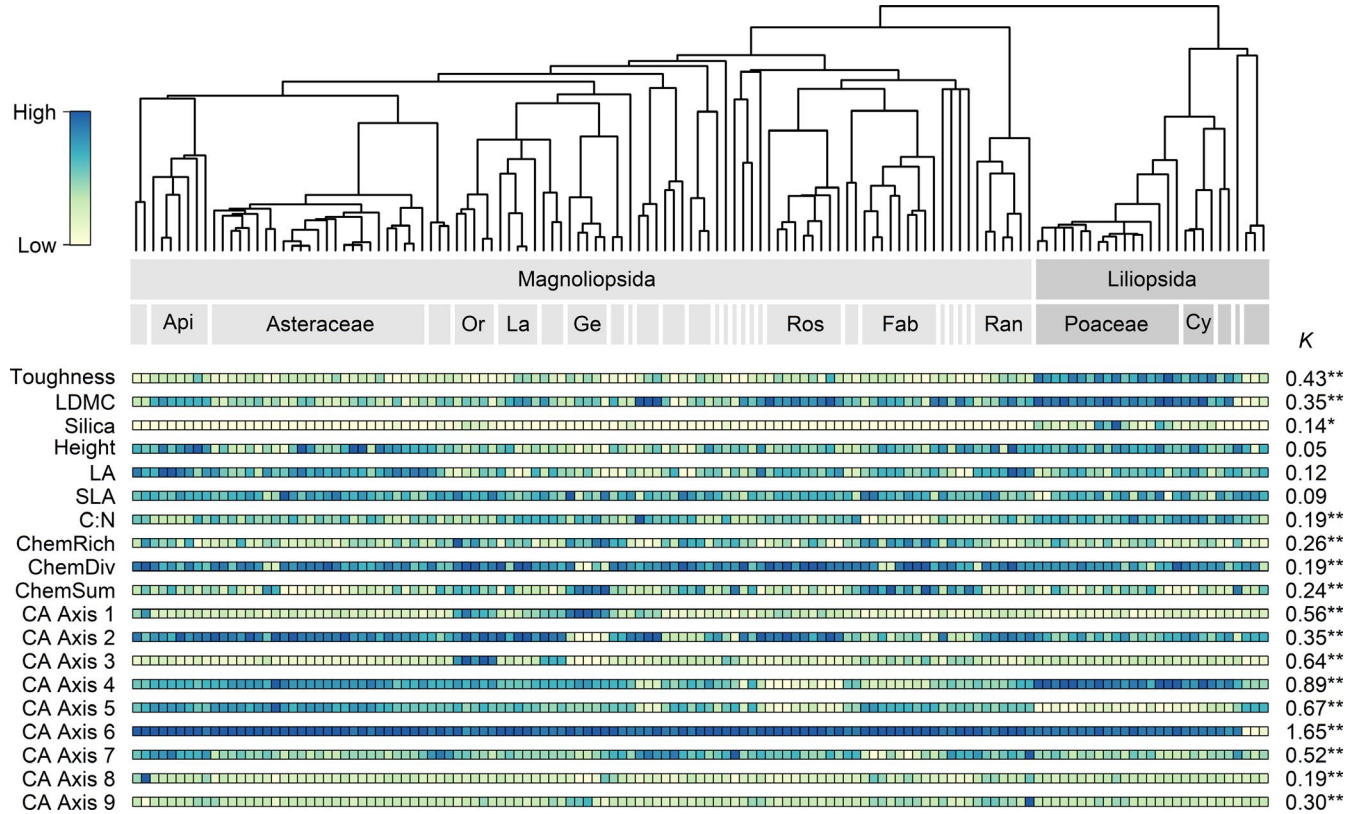

FIGURE 2 Phylogenetic signal in plant functional traits assessed with Blomberg's *K* statistic for 132 plant species. Trait values were averaged across the three sites. The colour scale represents the strength of the trait value. For visual ease, traits represented in the figure were log + 1 transformed (except CA Axes 1–9) and normalized. LA = leaf area, SLA = leaf mass per area, LDMC = leaf dry matter content, Toughness = leaf penetration force, Silica = amount of silica per mg dry leaf tissue, C:N = leaf carbon to nitrogen ratio, Height = plant height, ChemRich = chemical richness representing number of individual chemical compounds obtained from the untargeted metabolomics analyses, ChemDiv = chemical diversity based on the abundance of individual chemical compounds per species, ChemSum = total chemical abundance based on the abundance of individual chemical compounds per species, CA Axes 1–9 = first nine axes of the metabolomics ordination, Api = Apiaceae, Or = Orobanchaceae, La = Lamiaceae, Ge = Gentianaceae, Ros = Rosaceae, Fab = Fabaceae, Ran = Ranunculaceae, Cy = Cyperaceae. $^{**}p < .01$, $^{*}p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

S1). The eighth axis (CA Axis 8; 0.84% of total variance) discriminated Caprifoliaceae (high scores) from other plant families (low scores; Figure S1). The ninth axis (CA Axis 8; 0.82% of total variance) showed no clear pattern of family discrimination (Figure S1). Together, retaining the first nine axes of the ordination meant 10.4% of the variation in secondary metabolites was explained. The selected nine axes were associated with 86 peaks of chemical compounds with an explained deviance (D^2) > 0.5 (34.7% of the total number of peaks correlated to the 247 axes with this explained deviance) and 1,217 chemical peaks with an explained deviance (D^2) > 0.2 (43.2% of the total number of peaks correlated with the 247 axes with this explained deviance) among the 15,668 peaks (Table S2).

3.2 | Phylogenetic signal in leaf traits and herbivory

We found a weak but significant phylogenetic signal for most of the plant traits ($K < 0.700$, $n = 132$, $p < .050$; Figure 2 and Table S3), indicating that they are relatively labile across the plant phylogeny. In contrast, the fourth axis of the metabolomics ordination showed a strong phylogenetic signal (CA Axis 4: $K = 0.893$, $n = 132$, Z-score = −5.381, p =.001), while the sixth axis showed a K-value higher than 1 (CA Axis 6: $K = 1.651$, $n = 132$, Z-score = −1.874, p =.001; Figure 2 and Table S3). The phylogenetic signal was on average 2.75 times higher for chemical traits (mean $K = 0.54$) than for traits relating to competition, leaf structure and nutritional value (mean $K = 0.20$). Magnoliopsida and Liliopsida plant species showed distinct patterns in both physical and chemical traits (e.g. tougher leaves, higher silica content and high scores on the fourth axis of the metabolomics ordination for Liliospida; Figure 2). We found a weak and significant phylogenetic signal in the performance of *S. littoralis* ($K = 0.156$, $n = 132$, Z-score = −2.425, $p = .001$), and a weak and marginally significant phylogenetic signal in natural herbivory ($K = 0.159$, $n = 101$, Z-score = −1.101, $p = .067$; Table S3), justifying the need to consider phylogenetic relationships in further analysis.

3.3 | Natural herbivory and plant palatability in relation to traits

We found no correlation between natural herbivory and plant palatability assessed with *S. littoralis* (spearman rank correlation test:

rho = −0.047, p = .267), suggesting different feeding preferences for the two herbivory measurements. We found family differences in plant palatability (ANOVA: p < .001) and no significant differences for natural herbivory (ANOVA: p = .968; Figure S2).

We found that herbivory in grasslands is greater for tall plants (MCMCglmm 95% credible interval [CrI]: 0.123, 0.427), with a large amount of available biomass in the vegetation plots (CrI: 0.139, 0.299), low C:N ratio (CrI: −0.609, −0.179), low silica content (CrI: −0.338, −0.059), low SLA (CrI: −0.359, −0.035), high scores on the third axis of the metabolomics ordination (CrI: 0.056, 0.415), high scores on the sixth axis (CrI: 0.126, 0.455) and decreasing overall with increasing elevation (CrI: −0.581, −0.070; Table 1a). Based on the metabolomics ordination axes, herbivory was generally lower for Ophioglossaceae (CA Axis 6) and Gentianaceae (CA Axis 3; Table 1a, Figures S2 and S3). In contrast, herbivory was generally higher for Cistaceae, Carpifoliaceae, Asteraceae and Fabaceae species (CA Axis 6) and for Orobanchaceae and Plantaginaceae species (CA Axis 3; Table 1a, Figures S2 and S3).

We found that *S. littoralis* caterpillars had a higher weight when fed with plants with a high SLA (MCMCglmm 95% credible interval (CrI): 0.011, 0.061), high scores on the second axis of the metabolomics ordination (CrI: 0.003, 0.093) and low scores on the fourth axis of the metabolomics ordination (CrI: −0.130, −0.019; Table 1b). *Spodoptera littoralis* caterpillars performed marginally better on plants with a higher chemical richness (CrI: −0.001, 0.074; Table 1b). Based on the metabolomics ordination axes, the performance of *S. littoralis* was generally lower when they were fed Cyperaceae and Poaceae species (CA Axis 4) and on Gentianaceae species (CA Axis 2; Table 1b, Figures S2 and S4). In contrast, performance was generally higher on Ericaceae, Cistaceae, Geraniaceae, Plantaginaceae, Rosaceae and Fabaceae species (CA Axes 2 and 4, Figures S2 and S4).

3.4 | Effect of warming on plant functional traits and plant palatability

The leaf chemistry of the 16 plant species was significantly affected by the warming treatment (Adonis test: R^2 = .004, p = .001; Table S4). On average, 97.6 ± 1.2% (mean ± 1 *SD*) of the chemical compounds remained unchanged under the warming treatment, while 1.4 ± 0.9% decreased and 1.0 ± 0.4% increased their expression significantly (number of chemical compounds per species: mean ± 1 *SD* = 4,588 ± 127; Table S5 and Figure S5). Changes were driven by a limited number of chemical compounds, including flavonoids, terpenoids and phospholipid diesters (Table S6).

Analyses of changes in individual traits revealed that the warming treatment significantly increased LA (linear mixed-effects model: estimate = 0.34, p < .001) and SLA (estimate = 0.33, p < .001), but significantly decreased LDMC (estimate = −0.12, p < .001) and chemical abundance (estimate = −0.17, p = .029) and marginally significantly decreased chemical richness (estimate = −0.11, p = .089). Leaf toughness, C:N and chemical diversity did not change under warming (p > .100). The warming treatment tended to increase the LA of all species, by an average of 19.6 ± 13.3% (mean ± 1 *SD*; Figure S6). On average, SLA increased by 9.3 ± 8.0%, LDMC decreased by 3.6 ± 4.0%, leaf toughness decreased by 0.6 ± 9.9%, C:N increased by 1.9 ± 8.1%, chemical richness decreased by 1.1 ± 3.6%, chemical diversity increased by 4.5 ± 11.7%, and chemical abundance decreased by 1.9 ± 3.6% (Figure S6).

The discriminant analyses considering phylogenetic relationships between species revealed that the warming treatment decreased chemical richness (MCMCglmm 95% credible interval (CrI): −2.466, −0.038) and marginally increased SLA (CrI: −0.063, 2.648), independently from other leaf traits (Table 2). No significant changes in other leaf traits, such as LA, LDMC, toughness, C:N or chemical diversity, were observed despite a marginal increase in C:N (CrI: −0.196, 2.167; Table 2).

The warming treatment did not significantly affect larval weight and was not significantly associated with changes in leaf traits (no MCMCglmm 95% credible intervals for the interaction terms were different from 0; Table S7).

4 | DISCUSSION

Using untargeted metabolomics applied to 135 plant species in combination with measurements of leaf physical traits, we provided evidence for the relationship between the composite physical and chemical multivariate plant phenotypes and herbivory in subalpine and alpine grasslands. Our results using metabolomics expand on traditional measures of physical and chemical traits in herbivory studies and underline the important role of the plant secondary metabolites in mediating herbivore preferences and performances. In contrast, our investigation of the effect of experimental warming on plant physical and chemical traits and on palatability to a generalist herbivore did not highlight a link between changes in plant physical and chemical traits and changes in susceptibility to herbivory. While warming modified physical and chemical plant phenotypes, trait changes did not result in a consistent effect on plant resistance against *S. littoralis*. The development of metabolomics analyses promises new understanding in plant–herbivore interactions, but considering a broader range of herbivores in warming experiments could provide a more general understanding of future interactions under climate change.

4.1 | Plant traits, palatability and natural herbivory

Natural herbivory and *S. littoralis* performance responded to phylogenetically conserved plant chemical profiles. Beyond the effects of physical and nutritional traits documented in many studies (Descombes, Marchon, et al., 2017; Hanley et al., 2007; Massey et al., 2006; Peeters, Sanson, & Read, 2007; Pérez-Harguindeguy et al., 2003), we found that both natural herbivory and the performance of *S. littoralis* were associated with the plant chemical profile summarized in ordination axes. Supporting the general assumption of phylogenetic conservatism of chemical profiles (Ehrlich & Raven, 1964;

TABLE 1 (a) Relationship between natural herbivory and plant functional traits, elevation and plant available biomass, estimated with a MCMCglmm with a zero-inflated hurdle Poisson distribution. The hurdle zero-inflated model is associated with two latent variables (intercept = mean parameter of a zero-truncated Poisson distribution; hurdle = probability on the logit scale that the response variable is zero). (b) Relationship between plant palatability and plant functional traits and elevation, estimated with a MCMCglmm with a Gaussian distribution. Estimates for the random effects (site, plot and species) are provided in italics at the bottom of (a) and (b)

	Mean posterior distribution	Lower 95% CrI	Upper 95% CrI	Effective sample size	*p*
(a)					
(Intercept)	**5.603**	**2.609**	**8.306**	**2,232**	<.001***
Hurdle	**−4.841**	**−5.091**	**−4.636**	**1,590**	<.001***
Available plant biomass	**0.221**	**0.139**	**0.299**	**2,313**	<.001***
LDMC	0.241	−0.043	0.516	2,238	.098·
Toughness	0.194	−0.041	0.435	2,181	.123
SLA	**−0.192**	**−0.359**	**−0.035**	**2,485**	.021*
Height	**0.269**	**0.123**	**0.427**	**2,485**	<.001***
Silica	**−0.189**	**−0.338**	**−0.059**	**2,485**	.006**
C:N	**−0.399**	**−0.609**	**−0.179**	**2,485**	<.001***
ChemRich	0.050	−0.178	0.269	2,485	.689
ChemDiv	0.012	−0.163	0.175	2,485	.895
CA Axis 1	0.146	−0.036	0.342	2,485	.126
CA Axis 2	0.211	−0.046	0.451	2,174	.094·
CA Axis 3	**0.248**	**0.056**	**0.415**	**2,485**	.006**
CA Axis 4	−0.131	−0.385	0.109	2,485	.288
CA Axis 5	−0.239	−0.503	0.051	2,484	.076·
CA Axis 6	**0.283**	**0.126**	**0.455**	**2,485**	<.001***
CA Axis 7	−0.099	−0.290	0.067	2,210	.280
CA Axis 8	0.007	−0.163	0.167	2,485	.942
CA Axis 9	0.114	−0.071	0.323	2,187	.246
Elevation	**−0.321**	**−0.581**	**−0.070**	**1,637**	.009**
Site	*3.685*	*0.000*	*3.508*	*2,485*	
Plot	*0.016*	*0.001*	*0.038*	*2,485*	
Species	*3.446*	*1.480*	*5.734*	*1,983*	
(b)					
(Intercept)	0.454	−0.195	1.071	2,485	.103
LDMC	−0.015	−0.055	0.026	2,485	.472
Toughness	−0.006	−0.045	0.035	2,739	.785
SLA	**0.035**	**0.011**	**0.061**	**2,485**	.006**
Height	0.018	−0.005	0.039	2,558	.096·
Silica	−0.018	−0.043	0.007	2,485	.146
C:N	−0.011	−0.040	0.020	2,485	.477
ChemRich	0.037	−0.001	0.074	2,485	.061·
ChemDiv	0.011	−0.013	0.035	2,485	.365
CA Axis 1	−0.035	−0.081	0.007	2,485	.126
CA Axis 2	**0.047**	**0.003**	**0.093**	**2,485**	.043*
CA Axis 3	0.007	−0.033	0.043	2,485	.698
CA Axis 4	**−0.075**	**−0.130**	**−0.019**	**2,485**	.007**
CA Axis 5	0.025	−0.025	0.078	2,485	.357
CA Axis 6	−0.028	−0.067	0.011	2,485	.163

(Continues)

TABLE 1 (Continued)

	Mean posterior distribution	Lower 95% CrI	Upper 95% CrI	Effective sample size	*p*
CA Axis 7	0.001	−0.034	0.039	2,485	.965
CA Axis 8	0.006	−0.025	0.036	2,240	.719
CA Axis 9	0.019	−0.014	0.049	2,485	.231
Elevation	−0.113	−0.555	0.446	2,485	.666
Site	*19.610*	*0.000*	*1.312*	*2,485*	
Species	*0.073*	*0.036*	*0.117*	*2,179*	

Significant variables based on posterior distributions and 95% credible intervals (CrI) are highlighted in bold. *p*-values based on randomizations are also provided. Estimates for the random effects are provided in italics. Abbreviations are explained in the caption of Figure 2.
$^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$, $^{\cdot}p < .1$

TABLE 2 Effect of warming with open-top chambers (OTCs) on plant functional traits for 16 plant species collected in warmed plots and in ambient control plots, as estimated with discriminant analysis using a MCMCglmm with a binomial distribution

	Mean posterior distribution	Lower 95% CrI	Upper 95% CrI	Effective sample size	*p*-value
(Intercept)	0.232	−4.383	5.890	7,246	.892
LA	0.821	−0.546	3.004	2,408	.316
SLA	1.167	−0.063	2.648	3,576	.038*
LDMC	−1.234	−3.708	0.727	4,697	.185
Toughness	0.060	−1.290	1.668	7,776	.972
C:N	0.892	−0.196	2.167	3,593	.079·
ChemRich	**−1.148**	**−2.466**	**−0.038**	**7,136**	.049*
ChemDiv	0.230	−0.631	1.079	6,135	.586
Site	*13.370*	*0.000*	*30.920*	*7,189*	
Species	*12.18*	*0.000*	*61.92*	*2,755*	

Estimates for the random effects (site and species) are provided in italics at the bottom of the table. Significant variables based on posterior distributions and 95% credible intervals (CrI) are highlighted in bold. *p*-values based on randomizations are also provided. Abbreviations are explained in the caption of Figure 2.
$^{*}p < .05$, $^{\cdot}p < .1$

Wink, 2003), the phylogenetic signal of chemical traits was, on average, 2.75 times stronger than that of traits relating to competition, leaf structure and nutritional value. This suggests that untargeted plant metabolomics analyses enable the discrimination of plant families in their chemical profiles and that plant chemistry carries stronger phylogenetic inertia than the other plant functional traits. Nevertheless, the interpretation of the chemical profile is difficult given the high dimensionality of the data (15,668 peaks of chemical compounds from 248 samples and 154 plant species), where the nine axes used in our analysis explain only 10.4% of the variation. Presently, only approximatively 1.8% of an untargeted metabolomics spectrum analysis can be annotated (Da Silva, Dorrestein, & Quinn, 2015). Hence, a majority of the chemical signatures remains uncharacterized, which limits the understanding of the functions behind the ordination patterns (Da Silva et al., 2015). While our study suggests an association between metabolomic signature and herbivory, the development of novel machine learning approaches based on metabolomics reference databases could enable the classification of individual molecules and be more informative regarding the underlying physiological mechanisms (Blaženović, Kind, Ji, & Fiehn, 2018; Zhou, Huang, Guo, & dos-Santos, and Vivanco, 2018).

Both physical traits and chemical profiles were associated with natural herbivory and palatability in a bioassay, but relationships differed among herbivory measures (Table 1). Regarding the chemical profile, the performance of *S. littoralis* was associated with axes 2 and 4, which mainly discriminated Poales and Gentianaceae species from other plant families. Lower palatability of these plant families could be explained by their distinct chemical profiles, with the presence of toxic or repellent chemical compounds (e.g. bitterness in Gentianaceae, which are rich in both alkaloids and tannins; Callis-Duehl et al., 2017) or other family-specific leaf characteristics which were not captured by our selection of physical and chemical traits (e.g. leaf texture, presence of volatile compounds) but indirectly detected with the ordination axis. Natural herbivory in grasslands was also associated with metabolomics axes, but in this case with axes 3 and 6, which mainly discriminated Ophioglossaceae and

Gentianaceae species from other plant families. Hence, as for *S. littoralis* palatability measures, natural herbivores tended to avoid feeding on Gentianaceae species. This finding suggests that this plant family harbours specific chemical compounds which are repellent for herbivores, independently of their leaf physical traits and the availability of plant biomass in the grasslands. Despite this congruent result, the difference in the axes of the chemical ordination which better explain both herbivory measures might be the result of the different sensitivity to secondary metabolites in *S. littoralis* and the herbivores occurring naturally in the grasslands, such as orthopterans, which are among the top arthropod grazers in alpine grassland ecosystems (Blumer & Diemer, 1996).

In agreement with the literature (Descombes, Marchon, et al., 2017; Hanley et al., 2007; Massey et al., 2006; Peeters et al., 2007; Pérez-Harguindeguy et al., 2003), plant physical and nutritional traits were also associated with natural herbivory and the performance of *S. littoralis* (Table 1). We found negative relationships between foliar C:N, as well as silica content, and natural herbivory in grasslands (Table 1a), suggesting a preference for plants with a high nutritional value and low silica content in natural systems (Mattson, 1980; Pérez-Harguindeguy et al., 2003), but not in the *S. littoralis* bioassay. Hence, herbivores display a preference for tender leaves with higher nitrogen content (Loranger et al., 2012; Pérez-Harguindeguy et al., 2003), because nitrogen is generally scarce in plants and a limiting nutrient for many herbivores (Mattson, 1980). Silica content reflects the content of phytoliths (rigid, microscopic structures made of silica) stored in leaf tissue and mainly present in Poales and, in lower amounts, in Orobanchaceae (Figure 2). Silica has been shown to have an abrasive impact on the mandibula of insect herbivores and to affect herbivore performance by decreasing leaf palatability and digestibility (Awmack & Leather, 2002; Brizuela et al., 1986; Hanley et al., 2007; Massey et al., 2006; Massey & Hartley, 2009). The lack of a relationship between plant palatability for *S. littoralis* and silica content might be explained by the general low ability of *S. littoralis* young caterpillars to feed on tough, silica-rich plants in the Poales. Surprisingly, while we found that *S. littoralis* performed better on plants with high SLA values (Table 1b), natural herbivory was higher in plants with low SLA (Table 1a). Plants with a high SLA are typically fast-growing and more palatable species (Pérez-Harguindeguy et al., 2013, 2003; Villar & Merino, 2001), which are more easily processed by *S. littoralis*. On natural subalpine and alpine grasslands, herbivory is mainly dominated by orthoptera herbivore species (Blumer & Diemer, 1996), and their high mandibular strength enables them to feed on tougher plants, such as Poaceae and Cyperaceae species (Ibanez, Lavorel, Puijalon, & Moretti, 2013). Tough plants tend to have thicker leaves, a high LDMC and a low SLA (Cornelissen et al., 2003). Hence, the feeding preference for tougher leaves for the majority of naturally occurring herbivores might partly explain why we found a preference for plants with a low SLA, in contrast with the pattern for *S. littoralis*. Finally, Poales species dominate plant communities (up to 60% of relative cover by a few species) and structure vegetation communities in natural grasslands (Delarze et al., 1998; Descombes, Vittoz, Guisan, & Pellissier, 2017). Hence, dominant plants may be more attractive to herbivores because of their higher natural abundance, which is supported by the significant positive relationship between herbivory and available plant biomass in our analysis (Table 1a). While we found associations between the bioassay and natural herbivory and plant physical and chemical traits, divergent patterns suggest that predicting the responses of plants in a natural community context is complex and depends on both the plant traits in question and the type of herbivores in the community.

4.2 | Effect of experimental warming on plant–herbivore interactions

Among the physical traits investigated, we found that warming with OTCs marginally increased SLA, and this increase was independent from the response of other leaf traits (Table 2). While LA tended to increase across all plant species (+19.6% on average), this shift likely resulted from a modification of resource allocation driven by changes in SLA, such as an expansion of the foliar lamina associated with a decrease in leaf thickness. Hence, higher temperature likely enhances plant metabolism, favouring higher rates of cell expansion and facilitating faster production of softer tissues (Atkin, Botman, & Lambers, 1996; Körner, 1999); temperature is thus generally positively related to SLA at the intra- and interspecific level (Bjorkman et al., 2018; Woodward, 1983). Hudson et al. (2011) found that most species showed increased leaf size and plant height under a warming treatment and that traits related to growth were more affected by warming than leaf nitrogen content. Similarly, Bjorkman et al. (2018) found comparable intraspecific responses of plant traits, such as plant height, LA and SLA, to temperature gradients.

We found that the warming treatment significantly decreased plant chemical richness and significantly changed the chemical profile of the plants. Changes in the chemical profile were mainly driven by an increase or decrease in the expression of a limited number of chemical compounds, including flavonoids, terpenoids and phospholipid diesters. Phospholipid diesters are involved in membrane remodelling (i.e. glycerophosphocholine; van der Rest, Boisson, Gout, Bligny, & Douce, 2002), while terpenoids and flavonoids are involved in many basic functions in plant development and protection against stress (Tholl, 2015; Urquiaga & Leighton, 2000), including UV protection, pigmentation and freezing tolerance (Close & McArthur, 2002). Similarly, phenolic and terpenoid concentrations were previously shown to change in response to warming (Veteli, Kuokkanen, Julkunen-Tiitto, Roininen, & Tahvanainen, 2002; Zvereva & Kozlov, 2006), but it remains unclear whether this modifies plant palatability to herbivores.

Shifts in chemical richness and change in SLA might be expected to affect plant resistance to herbivory, yet we observed no change in plant palatability to *S. littoralis* as a result of changes in physical and chemical traits under the warming treatment (Table S7). However, an increase in SLA, as observed with our warming treatment (Table 2), might be associated with increased nutrient content and lower toughness (Cornelissen et al., 2003; Villar & Merino, 2001),

thereby increasing plant palatability to chewing herbivores. In contrast, a decrease in plant chemical richness (Table 2) might be either beneficial or detrimental to herbivores, depending on the nature of the compounds affected by the warming treatment. Herbivores performed better, though only marginally so, on plants with a higher chemical richness in our bioassay experiment (Table 1b). A decrease in chemical richness under warming should, by extension, negatively affect herbivore performance if beneficial chemical compounds are less expressed or altered. Nonetheless, it remains unclear to what extent interspecific trends between traits and herbivory can be generalized and extended to intraspecific variation under climate warming. Our bioassay analysis revealed no significant change in *S. littoralis* performance associated with changes in SLA or leaf chemical richness across the plant species investigated. Contrasting responses of herbivores to warming when feeding on different plant species have been reported previously (Bidart-Bouzat & Imeh-Nathaniel, 2008; Gutbrodt et al., 2011). Thus, warming might enhance or diminish insect herbivore performance by altering plant palatability, and responses to warming are likely to be plant–species and herbivore–species specific (Bidart-Bouzat & Imeh-Nathaniel, 2008; Gutbrodt et al., 2011; Lemoine et al., 2014).

4.3 | Limitations

While comprehensive, our study had limitations associated with: (a) the exploratory power of the metabolomics analyses, (b) the in-situ measurement of traits and herbivory, and (c) the quantification of palatability using *S. littoralis* in a bioassay. First, while the CA is an effective approach for reducing high-dimensional data, the first axis only explained 1.81% of total variance, and even the first nine axes only collectively explained 10.4% of the total variation. The documented association between these axes and herbivory supports the need to consider plant secondary metabolites in herbivory studies. Nevertheless, the methods used to process plant metabolomics profiles should be further developed in the future to improve the level of information gathered from such datasets, especially for gaining information on the underlying physiological mechanisms (Blaženović et al., 2018; Zhou et al., 2018). Second, because most of the studied subalpine and alpine species are perennial, measuring traits in the field made it possible to target mature individual plants under natural conditions, as done in previous herbivory association studies (Descombes, Marchon, et al., 2017; Pellissier et al., 2013). This approach does not allow to entirely tease apart individual genotypes from their local environments in producing the observed phenotypes, possibly generating noise from the experiment design (Bakhtiari, Formenti, Caggìa, Glauser, & Rasmann, 2019; Woods, Hastings, Turley, Heard, & Agrawal, 2012). In the future, physical traits and chemical analyses could be compared in more controlled settings such as climate chambers (Pellissier et al., 2014). Because plants grown under controlled climate conditions tend to grow faster and have different morphologies than plants growing in natural conditions, it may still be challenging to translate the results from controlled conditions back to natural settings (Poorter et al., 2016). Third, while Petri dish feeding-experiments using detached leaves provide a fast and holistic assessment of plant resistance, this analysis has the major limitation of not allowing plants to respond to herbivory through induction of defences, which generally modifies plant overall chemotypes, as well as physical defences to a certain degree (Karban & Baldwin, 1997). That said, while defence induction can modify individual phenotypes intraspecifically, species-level variation is generally maintained (Defossez, Pellissier, & Rasmann, 2018), which was the focus of our study. Hence, while we cannot exclude that intraspecific trait variability also impacts plant–herbivore interactions, the comparison of multiple measures of herbivory we performed enabled evaluation of the consistency of the signals of physical traits and chemical profiles across species.

5 | CONCLUSIONS

Our study provides evidence of associations between plant physical and chemical phenotypes and herbivory, either based on natural herbivory surveys, or based on *S. littoralis* larval performance. Despite altered physical and chemical properties under a warming treatment, we found no consistent directional response of plant palatability. Hence, the response of plant–herbivore interactions to climate change might be species specific and cannot be easily generalized. So far, studies investigating the relationship between plant chemical traits and herbivore performance have typically measured one or a few groups of chemical compounds, such as flavonoids, phenolics, cardenolides or glucosinolates (e.g. Callis-Duehl et al., 2017; Defossez et al., 2018; Pellissier et al., 2016; Rasmann & Agrawal, 2011). Because plants have evolved a myriad of chemical secondary metabolites to counteract herbivory (Mithöfer & Boland, 2012; Rhoades, 1979), the overall chemical arsenal in plants is unlikely to be restricted to one single compound class. Our results provide evidence that untargeted metabolomics analyses are powerful for identifying family patterns in plant defences for a large range of species growing under natural conditions. However, a major challenge of this approach is to deal with the high dimensionality of the plant chemical profile, which can contain thousands of chemical compounds (15,668 in this study), and to explain a meaningful fraction of the total variance when summarizing chemical profiles along ordination axes. Future studies should develop pipelines to better identify metabolites based on untargeted metabolomics analyses and use adequate tools to reduce the dimensionality of the data (Barker & Rayens, 2003; Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012; Salazar et al., 2018; Tibshirani, 1996) in order to decouple herbivore responses to chemical and physical phenotypes.

ACKNOWLEDGEMENTS

We are grateful to the people who helped with fieldwork and to Oliver Kindler and Roland Reist (Syngenta, Stein, Switzerland) for providing *S. littoralis* eggs. We thank Hélène Blauenstein and

Andreas Zurlinden for technical support during the palatability bioassay experiments with *S. littoralis*, and Emilien Jolidon for help in the lab. We thank Melissa Dawes for providing suggestions to improve the manuscript and Antoine Guisan for lending equipment for plant traits measurements. This study was supported by the Swiss National Science Foundation (SNSF) Project "Lif3web" (grant number 162604) to L.P. and by the University of Fribourg, and by the SNSF grant 179481 to S.R.

AUTHORS' CONTRIBUTIONS

P.D. and L.P. conceived the ideas and designed methodology; P.D. and A.K. collected the data; P.D. and G.G. analysed the data; P.D., L.P. and S.R. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data from this paper can be accessed through figshare http://doi.org/10.6084/m9.figshare.9699131 (Descombes, Kergunteuil, Glauser, Rasmann, & Pellissier, 2019).

ORCID

Patrice Descombes https://orcid.org/0000-0002-3760-9907
Sergio Rasmann https://orcid.org/0000-0002-3120-6226
Loïc Pellissier https://orcid.org/0000-0002-2289-8259

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How to cite this article: Descombes P, Kergunteuil A, Glauser G, Rasmann S, Pellissier L. Plant physical and chemical traits associated with herbivory in situ and under a warming treatment. *J Ecol*. 2020;108:733–749. https://doi.org/10.1111/1365-2745.13286