



Intensity, frequency and rate of insect herbivory for an alpine *Rhododendron* shrub: elevational patterns and leaf-age effects

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Abstract

Uncertainty still exists on the directions and intensity of changes in leaf herbivory under scenarios of global warming. We, therefore, conducted an investigation on insect herbivory along an elevational gradient to explore how leaf herbivory may respond to future climate warming using a space-for-time substitution approach. We hypothesize that the leaf herbivory for alpine woody species should decline with elevation. We also guess the leaf herbivory may not differ between different leaf-age groups, for the old leaves are less attractive to insect due to their lower nutrients. To approve these assertions, we measured different aspects of leaf herbivory, i.e., the intensity (leaf area consumed per damaged leaf), frequency (percentage of leaves damaged), and rate (percentage of consumed leaf area over the total number of leaves), across different leaf-age groups for *Rhododendron aganniphum* var. *schizocephalum* along an elevational gradient (4280–4640 m) in the Sergymla Mountains, southeast Tibet. Related leaf traits of leaf mass per area (LMA) and nitrogen (N_{mass}), as well as total phenolics for 1-year-old leaves, were also investigated. In contrast with our expectation, the rate of herbivory did not vary with elevation, while the frequency and intensity reflected increasing and declining patterns, respectively. LMA and total phenolics tended to increase with elevation, while N_{mass} significantly declined. Further analysis indicated that N_{mass} and total phenolics mainly explained the variation of frequency of herbivory, while N_{mass} reflected a significant effect on the variation of intensity. No differences in herbivory were found between the leaf-age groups. Our results suggest that the lower intensity of leaf herbivory at higher elevations is mainly associated with the declined nutritional levels, while the higher frequency might be related to the higher costly anti-herbivore defenses like phenolics and the lower nutritional levels. Although the older leaves are exposed to herbivore attacks for a longer time, they possessed the same herbivory levels as current-year leaves partly due to their lower nitrogen concentrations. Both supporting the nutrient limitation hypothesis, i.e., plants with lower leaf nutrient levels possess less leaf herbivory. In all, the herbivory on the alpine *Rhododendron* is small in magnitude, but given the higher herbivory (for intensity at least) under persistent warming conditions and its potential impacts on mediating plant–insect interactions, insect herbivory should be included in predictions of climate change impacts on alpine ecosystems.

Keywords Alpine timberline · Leaf mass per area · Leaf nitrogen concentration · *Rhododendron aganniphum* var. *schizocephalum* · Southeast Tibet · Total phenolics

Introduction

Insect herbivory exerts substantial effects on community structure and ecological functions and represents a main pathway for energy flow from autotrophic plants to higher trophic levels (Coley and Barone 1996; Agrawal 2007; Kozlov and Zvereva 2018). The amount of plant biomass consumed by insects per year, which is assumed to range from 1 to 10% in arctic tundra to around 20% in tropical forests (Agrawal 2011), forms an important part of annual production for the terrestrial ecosystem and seems to increase from the cold high latitudes toward the equator in

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the northern hemisphere (Kozlov et al. 2015b; Wang et al. 2016). Therefore, taking into account the amount of plant biomass removed by insect herbivores is of great importance to accurately assess ecosystem productivity and predict the responses of plant–insect interaction to future climate change (Kozlov et al. 2015a; Barrio et al. 2016a; Wang et al. 2016).

The changing climate, especially the rising temperature, has profound impacts on the life history, population size, and geographical distribution of insects, which in turn mediate plant physiology and influence anti-herbivore defenses, and eventually alter plant–insect interactions (Ayres and Lombardero 2000; Bale et al. 2002; Wolf et al. 2008; DeLucia et al. 2012). Some studies even predicted that the increasing rate of insect herbivory due to the warming climate may surpass the velocity of changes in plant productivity (Ayres and Lombardero 2000; DeLucia et al. 2012). However, global data analysis on foliar losses of woody plants to insects in natural ecosystems during 1952–2013 provided no temporal trend in temperate areas and even a declining trend in the tropics (Kozlov and Zvereva 2015). Therefore, uncertainty still exists on the directions and intensity of changes in leaf herbivory under scenarios of persistent global warming, probably due to the complex and multifaceted responses of insect pests to climate warming (Lehmann et al. 2020).

Elevational gradients provide a platform to predict how ecosystems may respond to future climate change using a space-for-time substitution approach. The most dominant profile of elevational gradient is the striking variation of temperature within a short distance, while other confounding factors such as day length, species distribution, and differences in biogeography are relatively small and can usually be safely ignored (Körner 2007; Moreira et al. 2018a). It is generally assumed that the decreases in temperature and less stable climate conditions at higher elevations are not fit for insect survival and breeding (Bale et al. 2002; Rasmann et al. 2014b). Accordingly, a number of studies have reported negative relationships between leaf herbivory and elevation (Galen 1990; Garibaldi et al. 2011; Rasmann et al. 2014a; Pellissier et al. 2014, 2016; Galman et al. 2018; Buckley et al. 2019; Benevenuto et al. 2020). However, some researchers also found more leaf herbivory at higher elevations (Altmann and Claros 2015; Zhang et al. 2015; Abdala-Roberts et al. 2016) or nonlinear relationships between herbivory and elevation (Njovu et al. 2019; Sam et al. 2020). Among these studies, the extent of herbivory is usually associated with leaf nutrition status (Altmann and Claros 2015; Abdala-Roberts et al. 2016; Njovu et al. 2019), anti-herbivory defenses like trichome density (Sam et al. 2020), and insect abundance (Altmann and Claros 2015; Zhang et al. 2015). Regardless of the controversial patterns of leaf herbivory along the elevation gradients, almost all existing data are collected at relatively lower elevations, and those from

sites above 4000 m are scarce (but see Galen 1990), which limits our understanding of how leaf herbivory may respond to changing environments, especially at extreme elevations. Evergreen plants at extremely high elevations may suffer from low temperature-induced nutrient deficits, and usually reflect lower leaf nutrient concentrations (Zhang et al. 2020; Kong et al. 2012). According to the nutrient limitation hypothesis, plants possessing lower leaf nutrient levels generally correspond to lower leaf herbivory (Abdala-Roberts et al. 2016; Njovu et al. 2019). In this study, we hypothesize that leaf herbivory by insects on evergreen woody species should decline with elevation (due to the lower temperature), and vary with the changes of leaf nutritional levels. Also, the above assertion may lead to another hypothesis that leaf herbivory for evergreen woody species will increase under persistent warming.

Besides elevational variation, leaf herbivory may differ along leaf ontogeny. Many studies have compared leaf damage between expanding new leaves and mature ones, and find more damage in the former (e.g., Coley and Barone 1996; Gherlenda et al. 2016; Baskett and Schemske 2018), probably because the new leaves possess greater concentrations of nutrients and reduced toughness (Coley and Barone 1996; Barton et al. 2019) though sometimes higher secondary chemicals (Barton et al. 2019). Till now, however, very few studies have focused on leaf-age effects for species with relatively longer leaf lifespan (but see Gherlenda et al. 2016). As the leaves age, a certain amount of nutrients such as nitrogen and phosphorus are translocated from old to new leaves (Aerts 1996; Güsewell 2005), reflecting a gradual decline of nutrients along leaf-age sequence. Also, leaf mass per area (LMA), which correlates well with leaf toughness (Onoda et al. 2011), tends to increase with leaf age (Poorter et al. 2009). Therefore, older leaves are less attractive to insect herbivores than younger leaves, though they are exposed to herbivore attacks for a longer time (Fernández Honaine et al. 2019). We may thus expect little difference in leaf herbivory between different-aged mature leaves. However, this assertion still needs to be tested.

In the Sergyemla Mountains, southeast Tibet, the evergreen shrub *Rhododendron aganniphum* var. *schizopeplum* is widely distributed in the alpine vegetation belt above the conifer timberline, forming a unique alpine vegetation landscape. Long-term climatic data from both meteorological observations and tree-ring chronologies of smith fir *Abies georgei* var. *smithii* and *R. aganniphum* indicate the climate in southeast Tibet tended to be warmer since the 1960s (Liang et al. 2009; Kong et al. 2012). Since alpine ecosystems on the Tibetan Plateau are mainly characteristic of low temperature, and the warming trend during 1960–2012 for the plateau is about twice of the global mean value (IPCC 2013), plants in these regions are assumed to be extremely sensitive to the changing climate, and their related traits may

reflect significantly variation even during a short elevation gradient. We therefore focused on low-temperature effects here and concentrated on how leaf herbivory and leaf traits might respond to warming along an elevational gradient that provides a space-for-time substitution approach. In this study, based on simultaneous measurements of intensity, frequency and rate of leaf herbivory, and leaf traits of LMA, N_{mass} and total phenolics (representative of leaf toughness, palatability and chemical defenses, respectively) for *R. aganniphum* along an elevational gradient, we aim to: (1) test if the within-species variation of leaf herbivory at extremely high elevations reflects declining pattern with elevation; (2) disclose the relationships between leaf herbivory and associated leaf functional traits, which would provide possible mechanisms underlying the variations of leaf herbivory. Specifically, leaf herbivory for both current-year and 1-year-old leaves were investigated to (3) test if there exist significant leaf-age effects on leaf herbivory, for variations in leaf structure and/or nutrients with leaf age are usually assumed to mediate leaf herbivory (Kozlov et al. 2015c; Abdala-Roberts et al. 2016; Njovu et al. 2019).

Materials and methods

Study area and plant species

The study site sits at the peak of the Sergyemla Mountains (29° 36' N, 94° 36' E), southeastern Tibetan Plateau. We conducted an investigation along a north-facing slope of the timberline observation, which belongs to the Southeast Tibet Observation and Research Station for the Alpine Environment, Chinese Academy of Sciences. Based on the long-term (2006–2018) meteorological observations at 4390 masl on the north-facing slope, the mean annual, January, and July air temperatures and annual rainfall were 0.8 °C, – 7.7 °C, 8.6 °C and 863.4 mm, respectively. Along the slope (4170–4640 m), subalpine and timberline evergreen needle-leaved forests of *Abies georgei* var. *smithii* were dominated below about 4320 m, while alpine *Rhododendron*

shrublands were mainly distributed above 4320 m. The meteorological data at 4390 m and 4640 m along the slope indicate that the lapse rates of mean air temperatures for the year, January and July were calculated as – 0.87 °C/100 m, – 0.88 °C/100 m and – 0.98 °C/100 m, respectively (Zhang et al. 2020). The monthly rainfall and soil moisture for the topsoil (5–10 cm) at 4390 m was significantly higher than at 4640 m across different months (Appendix 1a). However, both the mean wind speed and solar radiation were almost twice higher at 4390 m than these at 4640 m (Appendix 1b), which are assumed to exert strong impacts on the differences in rainfall (Yang et al. 1999) and soil moisture (Liu and Luo 2011) between the two elevations.

Rhododendron aganniphum var. *schizopeplum* is a widely distributed Ericaceae shrub species generally spanning 4100 m to 4700 masl in the Sergyemla mountains. This species forms typical dense shrublands above timberline, with a relative coverage of more than 60% and a height of 1–2 m (Table 1). As an important understory species, it is also distributed in subalpine and timberline forests below 4300–4400 m, where it reaches a height of 3–4 m. Flowering generally begins in late May or early June, and can last for about 2–3 weeks. There are also some companion shrubs like *R. aganniphum* var. *flavorufum*, *Sorbus rehderiana*, *Ribes glaciale*, *Salix oritrepha* and *Salix pilosomicrophyua* generally occurring with *R. aganniphum* at lower elevations (<4400 m) (Zhang et al. 2020). The understory herb species mainly include *Bergenia purpurascens*, *Cassiope fastigiata*, *Anaphalis xylorhiza*, *Rhodiola fastigiata* and *Potentilla* spp. etc. Soils contain less organic carbon when the aboveground vegetation change from forests to shrublands.

Leaf herbivory and traits measurement

During the middle of August 2009, when current-year leaves of *R. aganniphum* were fully spread, we set eight sites along the elevational gradient from 4280 to 4640 m, with an average elevational interval of 50 m (Table 1). At each site, we randomly selected five or six *R. aganniphum* individuals (five individuals for the four lower elevations and six for

Table 1 Coordinates and related information of the sampling sites along the transect

Site no.	Elevation (m)	Longitude (°)	Latitude (°)	Mean height of canopies (m)	Habitats
1	4285	94.609	29.599	2.42 ± 0.60	Subalpine forest
2	4335	94.609	29.598	2.60 ± 0.39	Treeline ecotone
3	4385	94.611	29.597	2.05 ± 0.37	Shrubland
4	4435	94.611	29.596	1.31 ± 0.36	Shrubland
5	4485	94.610	29.595	0.62 ± 0.18	Shrubland
6	4535	94.610	29.594	0.47 ± 0.05	Shrubland
7	4585	94.610	29.593	0.41 ± 0.07	Shrubland
8	4635	94.610	29.591	0.55 ± 0.04	Shrubland

higher ones due to declining shrub size along the slope) as sampling targets with horizontal spacing more than 10 m between each two. The heights of the target individuals are comparable with the mean height for each site (Table 1). For each target individual, 10 twigs with all leaf-age classes were randomly sampled. Current-year and 1-year-old leaves were separated and collected according to leaf cohort characteristics (Ewers and Schmid 1981). A total of 3142 leaves from 44 individuals were collected for leaf herbivory and related traits measurements.

All the sampled leaves were scanned within 24 h after excision from the twigs. Consumed leaves and non-consumed leaves were distinguished according to the sign of insects bitten, and it seems that most of the consumed leaves were eaten by chewing insects since only two kinds of consumed signs (edge defoliation and perforation) were detected. Then, the potential intact leaves were obtained by filling the gaps in the scanned images for consumed leaves in Adobe Photoshop 7.0 according to the expected shape (Zhang et al. 2015), and the consumed leaf area (CA), potential intact leaves area (PA) and non-consumed leaf area (NCA) were calculated by Image-Pro 6.0 (Media Cybernetics, Inc. USA). The intensity, frequency, and rate of herbivory were calculated in the following formulas:

$$\text{Intensity} = \bar{X} \left(\frac{\text{PA} - \text{CA}}{\text{PA}} \right) \times 100\%, \quad (1)$$

$$\text{Frequency} = \frac{\text{NO}_{\text{cl}}}{\text{NO}_{\text{sl}}} \times 100\%, \quad (2)$$

$$\text{Rate} = \frac{\Sigma(\text{PA} - \text{CA})}{\Sigma(\text{PA} + \text{NCA})} \times 100\%, \quad (3)$$

where, NO_{sl} and NO_{cl} are the numbers of sampled and consumed leaves, respectively.

All the three parameters for each site were calculated as the arithmetic mean values for the five or six individuals. After scanning, different leaf-age samples for each twig were dried in the oven at 65 °C to constant weight used for leaf trait measurements. LMA was calculated as the ratio of dry weight to corresponding leaf area. Mass-based leaf nitrogen concentration (N_{mass}) was analyzed with a micro-Kjeldahl assay (Campbell and Hanna 1937).

Phenolic compounds were chosen as a proxy of leaf defenses since they are widely recognized as herbivore feeding deterrents across many plant taxa (Mithöfer and Boland 2012; Abdala-Roberts et al. 2016). In this study, total phenolic content was measured for all samples from 1-year-old leaves and only 4 samples from current-year leaves because most current-year leaf samples were used for other measurements. Leaf total phenolic content was determined using the Folin–Ciocalteu procedure (Ainsworth and Gillespie

2007). The Folin–Ciocalteu assay relies on the transfer of electrons from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in an alkaline environment to form blue complexes that are measured optically at 760 nm. 1 g dry mass for each sample was extracted with 50 mL 50% ethanol in an ultrasonic bath for 15 min and the supernatants were collected after filtration. The process was repeated three times for each sample, and then the combined extracts were concentrated to 50 mL. One milliliter of these concentrated extracts was diluted to 10 mL using deionized water. The modified Folin–Ciocalteu procedure consisted of transferring 1 mL solution into a 25 mL volumetric flask, followed by additions of 6 mL deionized water and 0.5 mL Folin–Ciocalteu reagent. After mixing the samples, 1.5 mL 20% Na_2CO_3 and 16 mL deionized water were added. The reaction solutions were incubated at room temperature for 30 min and then measured using a UV/Vis spectrophotometer (722N, INESA, CN). The detected wavelength was set to 760 nm. The standard curve was calculated from the blank-corrected A760 of the Gallic acid standards. Total phenolic content was calculated as Gallic acid equivalents using the regression equation between Gallic acid standards and A760.

Statistical analysis

The elevational patterns in leaf herbivory and leaf functional traits (LMA, N_{mass} , and phenolics) were explored by linear regression. The age differences in herbivory (i.e., intensity, frequency, and rate) and functional traits (LMA and N_{mass}) at the individual level were tested using one-way analysis of variance (One-way ANOVA) followed by post hoc Tukey tests. The correlations among the intensity, frequency, and rate of herbivory at the individual level were explored by linear regression analysis. Generalized linear mixed model (GLMM) was used for quantifying the relative effects of N_{mass} , LMA, and total phenolics on the variation of intensity and frequency of herbivory, in which N_{mass} , LMA, and total phenolics were taken as fixed factors, while elevation was included as a random effect. All statistical analysis were performed with SPSS 22 for Windows (SPSS Inc., Chicago, USA).

Results

Elevational changes in leaf herbivory and their relationships

The mean rate, intensity, and frequency of leaf herbivory were $0.24 \pm 0.09\%$, $2.00 \pm 0.70\%$, $20.7 \pm 6.64\%$ for current-year leaves, and $0.25 \pm 0.12\%$, $1.97 \pm 0.63\%$, $25.3 \pm 6.76\%$ for 1-year-old leaves, respectively. No significant difference existed between current-year and 1-year-old leaves in

herbivory (Table 2). Contrary to our expectation, the rate of herbivory for both leaf-age classes did not vary with rising elevation (Fig. 1a, $R^2 = 0.06\text{--}0.25$, $P > 0.05$). However, its components of intensity and frequency showed significantly decreasing (Fig. 1b, $R^2 = 0.62\text{--}0.94$, $P < 0.05$) and increasing patterns (Fig. 1c, $R^2 = 0.70\text{--}0.71$, $P < 0.05$), respectively.

At the individual level, the rate of herbivory was positively associated with intensity across different leaf-age groups and with frequency for 1-year-old leaves. No significant correlations were detected between intensity and frequency (Appendix 2).

Variations in leaf traits and their effects on herbivory

Potential intact leaf area for both age classes tended to decline along the elevation gradient (Appendix 3a, $R^2 = 0.88$, $P < 0.05$). Also, the leaf area consumed by insects at a leaf level also tended to decline with elevation (Appendix 3b, $R^2 = 0.89\text{--}0.94$, $P < 0.05$). From the lowest to the highest site, the values of potential intact leaf area and consumed leaf area declined by 60.75% and 74.70%, respectively, indicating that the elevational variations of intensity mainly resulted from the variations of consumed leaf area rather than those of leaf sizes.

Leaf functional traits of leaf mass per area (LMA) and mass-based leaf nitrogen (N_{mass}) showed significant differences between current-year and 1-year-old leaves (Table 2). With increasing elevation, LMA for both age classes tended to increase (Fig. 2a, $R^2 = 0.60\text{--}0.68$, $P < 0.05$), while N_{mass} showed opposite trends (Fig. 2b, $R^2 = 0.57\text{--}0.77$, $P < 0.05$). Total phenolics for 1-year-old leaves showed a significantly increasing trend with rising elevation (Fig. 2c, $R^2 = 0.97$, $P < 0.05$).

GLMM analysis indicated that both the total phenolics ($F = 10.976$, $P = 0.002$, Table 3) and N_{mass} ($F = 5.866$, $P = 0.021$, Table 3) reflected significant effects on frequency of herbivory for 1-year-old leaves, whereas only N_{mass} influenced the intensity of herbivory ($F = 5.589$, $P = 0.025$,

Table 3). For current-year leaves, a significant effect of N_{mass} on the frequency of herbivory was also detected ($F = 8.021$, $P = 0.007$, Table 3). However, LMA did not exert significant effects on the intensity and frequency of herbivory for both leaf-age classes (Table 3).

Discussion

According to our knowledge, this is the first attempt to quantify leaf area losses by insects at such high elevations [Galen (1990) only reported percent of plants infested by aphids at 4025 m]. In this study, the rate of herbivory for the alpine *Rhododendron* species ranged from 0.1 to 0.5%, which is far less than those in tropical or temperate regions (Kozlov et al. 2015b; Kozlov and Zvereva 2015), and is comparable to those in arctic tundra (Barrio et al. 2016a, 2017; Rønbjerg et al. 2019) or at high elevations of Swiss (Pellissier et al. 2014). However, only a few studies have paid attention to the frequency of herbivory, and even fewer to intensity, probably because the two parameters of frequency and rate are tightly correlated with each other (Garibaldi et al. 2011; Liu et al. 2013; Kozlov et al. 2015b), or in some cases almost every observed target (usually leaves or flowers) is attacked by insects and some can be completely eaten leading to very high values of intensity and frequency (even close to 100%, English-Loeb and Karban 1992; Altmann and Claros 2015; Wang et al. 2016; Baskett et al. 2018). In this study, the mean value of frequency at site level is around 20%, which is significantly lower than those of deciduous oaks in temperate (Abdala-Roberts et al. 2016; Wang et al. 2016; Moreira et al. 2018b) or evergreen woody species in subtropical forests (Liu et al. 2013), but is comparable with those in alpine (2–22%, Galen 1990; 10–22%, Birkemoe et al. 2016) or subarctic tundra ecosystems (11.2%, Barrio et al. 2017). Therefore, it seems that the low temperature mainly regulates the lower levels of leaf herbivory in alpine and arctic ecosystems.

Table 2 Differences in leaf herbivory (intensity, frequency and rate of herbivory) and leaf functional traits of specific leaf area (LMA) and N_{mass} (mass-based leaf nitrogen concentration) between current-year

and 1-year-old leaves for *Rhododendron aganniphum* var. *schizopeplum* in the Serjymla Mountains

Category		Current-year leaves	1-year-old leaves
Leaf herbivory	Intensity (%)	2.00 ± 0.70a	1.97 ± 0.63a
	Frequency (%)	20.70 ± 6.64a	25.30 ± 6.76a
	Rate (%)	0.24 ± 0.09a	0.25 ± 0.12a
Leaf functional traits	LMA (mg cm ⁻²)	20.76 ± 1.18a	24.26 ± 1.22b
	N_{mass} (mg g ⁻¹)	13.12 ± 0.71a	11.25 ± 0.55b
	Total phenolics (mg g ⁻¹)	NA	133.72 ± 4.96

Different letters in the same line represent significant difference of $P < 0.05$, $n = 44$

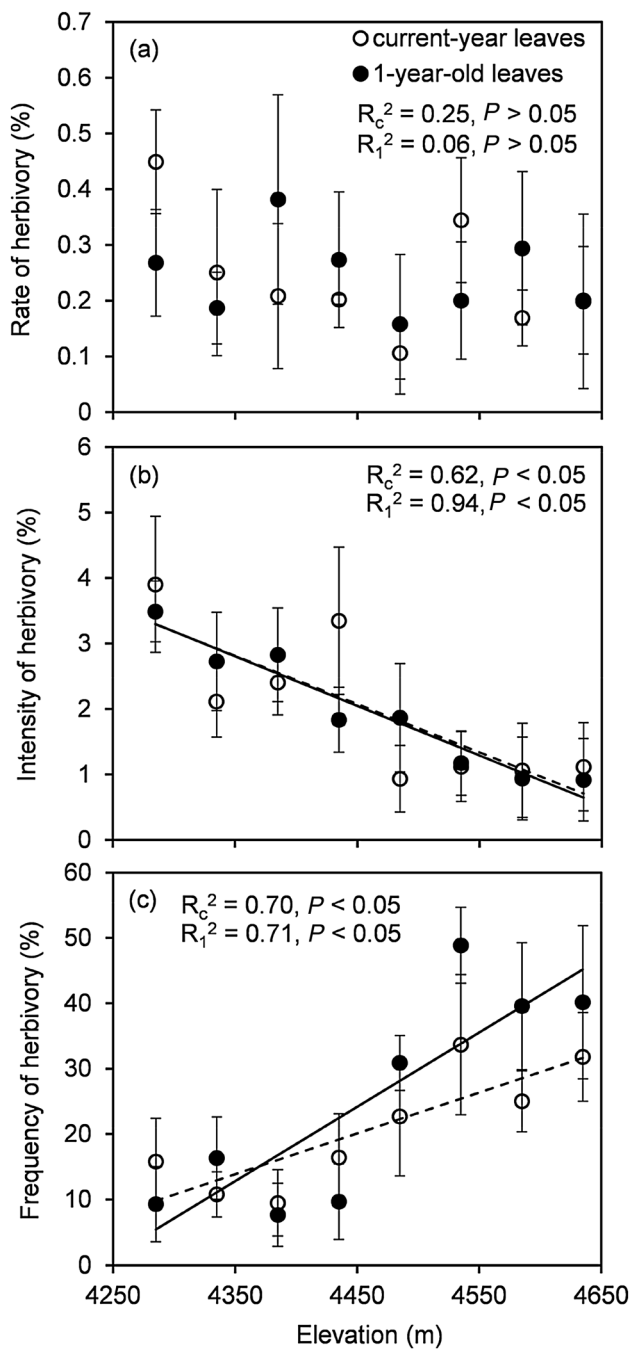


Fig. 1 Elevational variations of **a** rate, **b** intensity and **c** frequency of leaf herbivory for different leaf-age groups of *Rhododendron aganiphum* var. *schizopeplum* in the Sergymla Mountains. Solid and dashed lines are for trends line of current-year and 1-year-old leaves, respectively. R_c and R_1 represent for correlation coefficient of current-year and 1-year-old leaves, respectively

Elevational changes in leaf herbivory

Inconsistent with many studies reported that the rate of leaf herbivory decreased with increasing elevation (Pellissier et al. 2014, 2016; Birkemoe and Schemske 2016; Galman

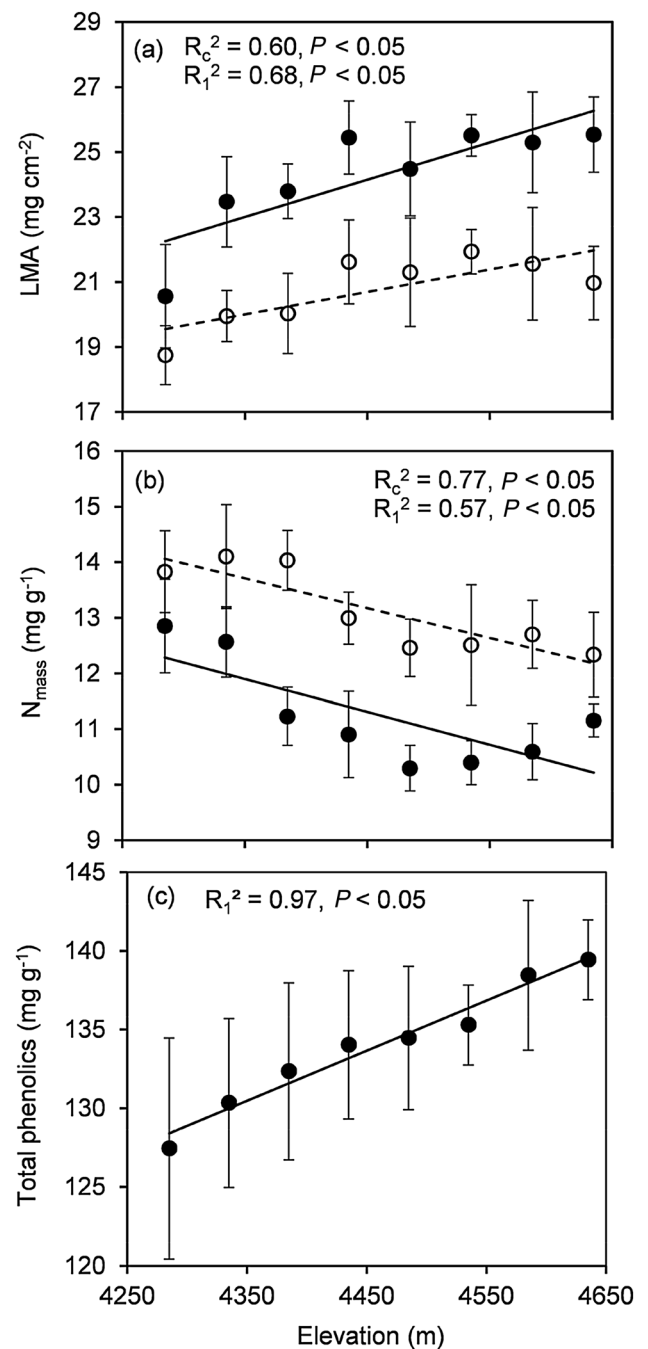


Fig. 2 Elevational variations of **a** leaf mass per area, **b** nitrogen concentration for different leaf-age groups and **c** total phenolics for 1-year-old leaves of *Rhododendron aganiphum* var. *schizopeplum* in the Sergymla Mountains. LMA leaf mass per area, N_{mass} leaf nitrogen concentration. Solid and dashed lines are for trends line of current-year and 1-year-old leaves, respectively. R_c and R_1 represent for correlation coefficient of current-year and 1-year-old leaves, respectively

et al. 2018), we only found the parameter of intensity tended to decrease, whereas the rate itself varied little. More interestingly, the frequency in this study reflected significant increasing trends with elevation, which is in contrast with

Table 3 Summary tests of generalized linear mixed model for fixed effects of LMA, N_{mass} and total phenolics on the variation of frequency and intensity of herbivory along the elevational gradient

Leaf age	Source	Frequency		Intensity	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
1-year-old leaves	LMA	0.743	0.395	2.135	0.154
	N_{mass}	5.866	0.021	5.589	0.025
	Total phenolics	10.976	0.002	2.551	0.121
Current-year leaves	LMA	0.374	0.544	2.158	0.150
	N_{mass}	8.021	0.007	1.448	0.236

Bold denotes a significant level at 0.05

F and *P* denote the statistical value of *F*-test and significance level, respectively

most existing studies reporting declining trends (Galen 1990; Garibaldi et al. 2011; Buckley et al. 2019; Benevenuto et al. 2020). Since the parameters of frequency and intensity showed the inverse patterns along the elevation gradient, it is not surprising that the rate, i.e., the product of frequency and intensity, did not vary with elevation. Declining intensity might indicate relatively lower amounts of insects feeding on *Rhododendron* leaves or less palatable leaves (see below) towards higher elevations. And all these are assumed to be associated with low-temperature conditions at higher elevations (Garibaldi et al. 2011; Rasmann et al. 2014a; Abdala-Roberts et al. 2016). In our study, the lapse rate of air temperature along the gradient exceeds more than 0.8 °C per 100 m which is around 1.5 folds of the mean value in the world and would exert strong impacts on herbivores. Further, a significant effect of leaf nitrogen concentration on intensity was found in 1-year-old leaves, which means that the lower leaf nitrogen status at higher elevations may influence the intensity of herbivory through mediating the palatability of leaves since leaf nutrient concentration is closely associated with foliage palatability (e.g., Abdala-Roberts et al. 2016; Njovu et al. 2019).

However, it is more complex when concerning the pattern of increasing frequency. The analysis of the generalized mixed model indicated that the leaf total phenolic and N_{mass} simultaneously mediate the variations of frequency. The first point to explain these might be associated with the pattern of increasing leaf total phenolics which is assumed to be the most important means of defense against herbivores (Moreira et al. 2018b). This is consistent with Abdala-Roberts et al. (2016) who found the increase in leaf total phenolics with elevation plays a role in mediating herbivory frequency for *Quercus robur* in northwestern Spain. A similar relationship between total phenolics and frequency was also found by Wang et al. (2016). Second, since chewing insects at higher elevations are strongly limited by the lower nitrogen content of their diets, they must consume more

foliage to meet their nutritional needs resulting in frequent feeding on different targets. Although it might be beneficial for the insects to eat as much as possible on a single leaf and spend less energy on moving to different leaves, it is more dangerous for the herbivores to spend more time on a same leaf because of bird predation (Kozlov et al. 2017). Also, the postponed leaf phenology at higher elevations may coincide with the pattern of frequency, i.e., the onset of shoot growth may delay for about 2 weeks along the gradient (Wang et al. 2013), and the relatively young leaves are thus providing better foliage for insects.

Besides, elevational variation of plant growth, as well as the changing environment at the same time, may influence plant allocation to defense like the shift of direct (physical and chemical traits that resist herbivores, reduce consumption or decrease their survival, Agrawal 2007) to indirect (traits that provide food and shelter, information on herbivore presence for the natural enemies of herbivores, Kessler and Heil 2011) anti-herbivory defenses. At higher elevations, plants generally invest more direct defenses (phenolics), while those at lower ones deploy both direct and indirect defenses due to higher growth rate and warmer climate (Pellissier et al. 2014, 2016; Rasmann et al. 2014b; Moreira et al. 2018a). It is possible that the relatively simplified anti-herbivory defenses at higher elevations probably induced more frequent attacks by insects. There still exist other possible reasons like the changes in the herbivore community with varying elevations (e.g., Zhang et al. 2015; Sam et al. 2020).

The unchanged rate of herbivory with elevation (i.e., temperature) found in this study might infer that the warming climate will not exert significant impact on the magnitude of herbivory for the alpine *Rhododendron* species in the future. However, under warming climate, the evergreen *Rhododendron* leaves will be more *N*-enriched (from Fig. 2b), and the number and the activity of feeding herbivores are assumed to increase (Pöyry et al. 2011), leading to positive feedback. Furthermore, concerning about the maximum speed at which species can move under scenarios of global warming, IPCC (2013) predicted that the woody species would possess the lowest speed, while the plant-feeding insects are among the highest. Therefore, higher herbivory (for intensity at least) and stronger *Rhododendron*-herbivore interactions would be inferred under persistent warming conditions.

According to the leaf feeding form (edge defoliation and perforation) and our rough observation, moth larvae and beetles are assumed to be the main herbivores for the *Rhododendron* leaves. However, data about the insect species abundance and population dynamic in this region are not yet available. Also, the herbivory patterns found in this study are only applicable in extremely high elevations, and the results from a relatively shorter gradient might partly result in the inconsistencies with other studies, e.g., the transect studies

carried out in Tanzania (Njovu et al. 2019) or worldwide (Galmán et al. 2018).

Leaf age effects on herbivory

In this study, we did not find a significant difference in leaf herbivory between current-year and 1-year-old leaves, i.e., leaf-age effects on insect herbivory seemed negligible. This is consistent with the results of our phenolics measurements on different-aged leaves which showed that the total phenolics did not differ significantly between current-year and 1-year-old leaves (Appendix 4). Also, this view is in accordance with Gherlenda et al. (2016) who found similar leaf damage levels (<2% leaf area loss) for old versus fully expanded new leaves in mature *Eucalyptus tereticornis* trees in southeast Australia. However, this finding is in contrast with the assertion that the plants with long leaf lifespan may suffer from continuous attack due to the long-time exposure to the action of herbivores and eventually results in accumulated high leaf damage (Fernández Honaine et al. 2019). Till now, we have no data to quantify the variation of herbivory for young expanding leaves, which may underestimate the potential herbivory and limit our understanding of exactly when and how this damage occurs. Although many published papers have reported more herbivory damage in the tender new leaves, there yet exist totally inverse cases. For example, Fernández Honaine et al. (2019) found that herbivory damage was significantly higher in old leaves than in younger leaves for an invasive tree *Ligustrum lucidum* in Argentina. Scott-Brown et al. (2016) reported higher leaf damage in older leaves compared with younger leaves due to the lower trichome density and diterpenoid concentration in older leaves of *Rhododendron simsii*. Recently, Barton et al. (2019) pointed the generality of shifts in defense syndromes during leaf development using meta-analysis across 124 woody plant species. They found that the young leaves possessed greater secondary chemistry concentrations including phenolics but reduced toughness and indirect defense traits than mature leaves. Therefore, the effects of leaf ontogeny on herbivory are complex and might be species-specific.

Compared with the neglectable leaf-age effects on herbivory, significant leaf-age effects on functional traits do exist. In other words, the changes in N_{mass} and LMA as leaves aged did not induce reciprocal changes in leaf herbivory. For N_{mass} , this seems to be in conflict with the result of elevational transect investigations, i.e., leaf nutritional status plays an important role in regulating insect herbivory along the elevational gradient rather than across leaf-age groups. The reason may be related to the assertion that the leaf herbivory of *Rhododendron* mainly occurs in expanding new leaves rather than mature leaves (Baskett and Schemske 2018). Besides, though the older leaves may suffer from continuous attack due to the long-time exposure to

herbivores, they are less attractive (due to their lower N_{mass}) and thus possessed the same herbivory level as the current-year leaves. That is, the translocation of nitrogen from older to younger leaves in some degree reduced the possibility of being further consumed by herbivores for older leaves, partly supporting the nutrient limitation hypothesis. Also, the higher LMA in older leaves plays a role in defending against external herbivores, consistent with the existing studies (Poorter et al. 2009; Garibaldi et al. 2011; Sam et al. 2020). However, further seasonal dynamic observations are urgently needed to figure out when and how the leaf damage exactly happens, which can be fulfilled by setting cameras that can automatically take high-quality pictures.

Conclusion

In the extremely stressful environments where trees can hardly grow, the rate of leaf herbivory for the shrub *Rhododendron aganniphum* var. *schizopeplum* did not vary with increasing elevation, while its components of intensity and frequency reflected completely different patterns. The chemical traits of N_{mass} and total phenolics partially explained the variations in leaf herbivory. Compared with these, leaf-age-induced variations N_{mass} and LMA did not evoke corresponding changes in leaf herbivory, indicating the leaves are generally eaten in the early growing season when the young leaves are spreading. Since different parameters of leaf herbivory contain different ecological information, future studies should pay more attention to the parameters of intensity and frequency rather than the rate itself, which would shed new light on the interactions between plants and insects, especially in cold-limited regions.

Under scenarios of climate warming, insects are assumed to expand to higher latitudes and/or elevations leading to higher herbivory pressure (Parmesan et al. 1999; Conrad et al. 2004). Moreover, the ecosystem carbon sink can be simply converted to a carbon source if an insect burst happens (Bale et al. 2002), e.g., the plague of locusts destroyed crops and natural vegetation in vast areas of eastern Africa and southern Asia in 2020. Compared with these, the herbivory on alpine *Rhododendron* is small in magnitude, however, given its prevalence and dependence on climatic variables, as well as its potential impacts on mediating plant–insect interactions, background insect herbivory should be included in predictions of climate change impacts on alpine ecosystems (Wolf et al. 2008; Barrio et al. 2016b).

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Data availability All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

Code availability Not applicable.

Declarations

Conflict of interest This manuscript has not been published before and is not being considered for publication elsewhere. All authors have contributed to the creation of this manuscript for important intellectual content and read and approved the final manuscript. We declare there is no conflict of interest.

Ethics approval This study does not involve research on human participants or animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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