

Light and sex interplay: differential herbivore damage in sun and shade in dioecious *Mercurialis perennis*

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Abstract: Interactions between plants and herbivorous insects can be shaped by light environment, resulting in differential herbivory in sun and shade. In dioecious species, the combination of plant sex and light-induced changes in defense traits and nutritive value can alter the patterns of foliar damage. We explored the combined effects of light environment and plant sex on natural herbivore damage and plant traits in the dioecious understory forb *Mercurialis perennis* on Mt. Kopaonik (Serbia). The role of plant traits in predicting the extent of damage was examined as well. Male and female plants from contrasting light environments (shade vs. sun) were analyzed with respect to leaf damage, as well as plant morphological and biochemical traits (size, specific leaf area, carbon-based defensive compounds and nutritional quality). We found prominent differences in herbivore damage between sun and shade conditions, but not between the sexes. Plants from the sun-exposed site had a significantly larger leaf area removed. The specific leaf area co-varied with herbivore damage in an inverse fashion, while leaf nutritional value had a moderate effect. Contrasting light conditions influenced the patterns of intersexual differences in the contents of condensed tannins and soluble proteins, with females exhibiting higher trait values. We found that factors defining risk of consumption were related to plant morphological traits and nutritive value rather than to chemical defenses.

Keywords: folivory; plant defense; plant sexual dimorphism; secondary metabolites; specific leaf area

INTRODUCTION

Interplay between plants and herbivorous insects is an omnipresent ecological force in nature [1]. The outcome of these complex interactions may be altered by various abiotic factors, including the light regime [2-4]. Natural populations are faced with immense spatiotemporal variations in light availability, exhibiting adaptive responses aimed at maximizing performance [3]. These responses include alterations in leaf structural characteristics, physical resistance, chemical defense and nutritive value, all of which may have implications for plant interactions with herbivores [5,6].

Studies exploring differential herbivory in sun and shade have grown in number over the past decades. For various plant species, increased rates of herbivory

in the shade have been reported [7,8]. Contrary to this are the findings of increased damage by insects in sunlit sites [3,9,10] or similar herbivore loads under contrasting light conditions [11].

The patterns of folivory have been explained in the light of various plant structural and chemical traits [3,8,12]. Requirements for efficient carbon gain in deep shade result in thinner, softer leaves, with higher water and protein contents and lower fiber content [5,6,8]. Leaves produced in the shade are more palatable and attractive to herbivores, consequently sustaining greater damage [13], while leaves exposed to full sunlight possess less soluble proteins and water [8] and higher amounts of carbon-based secondary metabolites such as phenolics and tannins that are traditionally expected to deter insects feeding on them [5,14]. However, it

has been recorded that understory plants growing in canopy gaps and sun-exposed sites can sustain higher levels of damage as a consequence of lower contents of other types of defensive compounds, such as glucosinolates [9,10 and references therein]. During the past decade, much effort has been put into discriminating between reasons for irradiance-dependent herbivore loads on saplings of light-demanding tree species [15-18] and shade-tolerant shrubs [8,12,19,20]. However, there is a paucity of data addressing this matter for key understory species, such as annual and perennial herbs.

In dioecious species, apart from vegetative traits and ecological context, plant sex is another major factor contributing to intrapopulation variations in herbivore loads [21]. The sexes differ in investment in mutually competing functions: growth, reproduction and maintenance [22]. Due to trade-offs in these traits, herbivory rates are expected to differ between the sexes because of alterations in plant nutritional value [21]. It has been proposed that in dioecious plants in general, herbivore pressure influenced the evolution of separate sexes [23].

The morphs bearing female function generally sustain higher costs of reproduction (although the reverse could also be the case in some anemophilous species [24]), which are then traded-off against reduced growth rates and higher investment in chemical defense against herbivores [25]. Males maintain low chemical defenses and high concentrations of nutrients in vegetative tissues, along with the ability to compensate for increased consumption by insects [25,26]. In fact, male-biased herbivory has been suggested as a rule in nature [21]. However, a number of studies have presented different results, showing female-biased herbivory [27,28] or lack of bias [29,30].

In this study, we explored the presence of differential herbivory in sun and shade in the dioecious species, dog's mercury (*Mercurialis perennis* L. (Euphorbiaceae)), an anemophilous, perennial forb with a wide latitudinal and altitudinal distribution throughout Europe. This is a typical member of ground-level communities of beech and oak woods with different canopy closures, and it is sporadic in sun exposed sites [31]. Being a shade-tolerant herb, for *M. perennis* light is a key resource, dictating distribution, performance and sex ratios [32]. Experimental

data showed that the main factor affecting biomass and production is light availability, with light-exposed plants being overall smaller in size [33]. This species has proved to be a suitable model system for exploring patterns of natural variation in sexually dimorphic traits and sex ratios across environmental gradients [32-35]. In *M. perennis*, females were often found to be more frequent under low light conditions [31,35,36]. One of the possible explanations, suggested previously for other species [37], is that different abundances of males and females could result from sex-biased herbivore pressure. Various invertebrates were recorded on dog's mercury [31], but the main leaf damage originates from specialist folivores such as *Hermaeophaga mercurialis* (Coleoptera). Given that Sánchez-Vilas and Pannell [38], when investigating sex-differential herbivory, found evidence of male bias in congeneric *M. annua*, we considered it important to explore this possibility under natural conditions in *M. perennis*. Therefore, the questions raised herein are as follows: (i) Does the damage inflicted by herbivorous insects differ significantly between contrasting light environments? (ii) Is herbivory sex-biased under contrasting light conditions? (iii) Are the analyzed plant traits sexually dimorphic? (iv) Is herbivore damage related to specific aspects of plant morphological and biochemical traits?

MATERIALS AND METHODS

Study sites

For this study, we chose neighboring sites on Mt. Kopaonik (Serbia) at 1700 m above sea level (N 43° 18' 42" E 20° 50' 32"); of the many we have studied previously, this location provided a rare opportunity to analyze *M. perennis* growing in abundance in adjacent sites, but under sharply contrasting light environments. The first (sun-exposed) site is in an open field (*Festuco-Seslerietea* class), exposed to full daylight throughout the growing season. The other site is in a spruce stand (*Vaccinio-Piceetea* association), where *M. perennis* grows in larger stands in constant shade.

Light environment evaluation

The light environment was characterized by measuring canopy openness, defined as the percentage of the

open sky seen from beneath the canopy, including the effect of additional topographic shading [39]. Since the plants were collected from an evergreen forest and a field fully exposed to sun, we assumed there would be no significant effect of changes in canopy structure and openness during the growing season. Hence, hemispherical photographs were taken once during the season, along two transects at each site, approximately 10 m apart, every 5 m, at dusk on a clear day. We used a 180° fisheye lens (F2.8 EX DC circular fisheye, Sigma) mounted on a digital camera (Rebel T3i/600 D, Cannon), leveled 30 cm above the ground and oriented toward the magnetic north with a compass. Pictures were converted into black-and-white using an automated threshold algorithm of the Side Look program [40]. Canopy openness was calculated with the Gap Light Analyzer software version 2.0 [39]. In the spruce stand, canopy openness was $43.60 \pm 9.28\%$, and in the sun-exposed site it was $66.89 \pm 3.87\%$ (t-test, $t = -7.32$, $df = 18$, $P < 0.0001$).

Sample collection

A total of 120 fully grown plants, 30 males and females each, from sun-exposed and shaded sites, were analyzed. All plants were collected on the same day in early June 2015. Sampling was performed at each site taking care to collect shoots from distinct individual clones [35], as far apart as possible. Immediately upon collection, the cut ends of the shoots were submerged in water and the plants were transported to the laboratory, where they were kept in a dark and cool room at least for 6 h before processing [41]. Fully rehydrated leaves were then carefully separated from the shoots and placed on a flatbed scanner (Hewlett-Packard ScanJet G4010) (600dpi resolution, JPEG format) in the same order they were on the plant, with the abaxial side facing the glass.

Plant height from ground level up was measured on fresh plants. All the leaves produced by each individual were dried at 65°C for 48 h, weighed, and then ground with a mortar and pestle in liquid nitrogen in preparation for chemical analysis. The first pair of fully expanded leaves with no or as little as possible visible damage was weighed fresh after scanning, reweighed after drying and used to calculate the specific leaf area (SLA) expressed as $\text{mm}^2 \text{mg}^{-1}$.

Leaf damage quantification

To assess damage from folivores, leaf scans were processed through an automated threshold binary conversion algorithm and pixel counting, followed by extrapolation to an area in cm^2 in ImageJ software [42]. In this manner, the total leaf area (TLA) for each individual and the leaf area consumed by herbivores (LAC) were determined. The percentage of leaf damage on each leaf was calculated as follows: $(\text{LAC}/\text{TLA}) \times 100$, averaged out per plant (LAR – leaf area removed).

Leaf chemical analysis

Leaf water content (LWC) was determined and expressed as the percentage of fresh leaf mass. Approximately 20-mg samples were thoroughly extracted using 80% acetone and 1% acetic acid for 12 h at room temperature. The total soluble protein content (TSPC) was determined based on the method of Bradford [43], using bovine serum albumin as standard. Proteins were extracted from the plant material using ice-cold 50 mM sodium-phosphate buffer, pH 7.4. The assay is based on the absorbance shift of the dye Coomassie Brilliant Blue G-250, which occurs under acidic conditions. The dye forms a strong, non-covalent complex with the protein's carboxyl group by Van der Waals force, and with the amino group through electrostatic interactions. Absorbance of the samples at ($\lambda = 595 \text{ nm}$) was measured on a spectrophotometer (Ultrospec 3200 pro GE, Healthcare). The amount of proteins is presented as mg g^{-1} dry matter content.

For determination of the total phenolic content and total condensed tannin content, approximately 20-mg samples were thoroughly extracted using 80% acetone and 1% acetic acid for 12h at room temperature. The total phenolic content (TPhC) was measured as gallic acid equivalent according to Singleton and Rossi [44] using Folin-Ciocalteu (F-C) reagent and 20% Na_2CO_3 . The F-C assay has been used as a measure of total phenolics in natural products, and the basic mechanism is an oxidation/reduction reaction. Although the chemical nature of the F-C reagent is not fully understood, it is believed to be based on the reduction of heteropolyphosphotungstates-molybdates in the presence of phenolic compounds. The reaction was initiated by mixing the diluted plant extract (1.60

mL) with 0.1 mL of F-C reagent, followed by 15-min incubation at room temperature. Then, 0.3 mL of 20% Na_2CO_3 was added and incubation was extended for an additional 2 h. The absorbance of the samples was measured at $\lambda=765$ nm and the results are expressed as gallic acid equivalents mg GAE g^{-1} dry matter content.

Total condensed tannins (TCT) were determined by the colorimetric method of Broadhurst and Jones [45], modified by Xu and Chang [46]. Plant extracts were incubated with 4% vanillin solution and concentrated HCl for 2 h at room temperature. The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the meta-substituted ring of flavanols to yield a red adduct that can be detected at 500 nm, and it has been widely used to estimate the condensed tannin content. The general problem associated with tannin estimation methods is the lack of proper quantification standards due to their vast structural diversity. This may cause significant overestimation of condensed tannins in analyzed samples [47], and the absolute values are impossible to obtain, so we expressed relative TCT values in absorbance units ($A_{500\text{nm}}$) per g dry matter content, which allowed us to compare the individual samples.

Statistical analyses

Data were transformed where necessary, to meet the assumption of normality: LAR, TPhC and TSPC were square root (sqrt) transformed, while the TCT content was inversely transformed. Nested analyses of variance (ANOVAs) were performed to explore the effects of site (sun-exposed vs. shaded) and sex (male vs. female) nested in site as fixed factors on dependent variables: LAR, size traits (height and TLA), leaf morphological and chemical traits (SLA, TPhC and TCT), and nutritional quality (LWC and TSPC). A least-squares means (LSMeans) test was used for *a posteriori* comparison. To explore the relationships between the amount of herbivore damage in shade and sun with the analyzed plant traits, we conducted regression analyses for each site. The leaf area removed was the response variable, while the height, TLA, SLA, LWC, TSPC, TPhC and TCT were predictor variables. All graphs and analyses were performed using packages of the R program version 3.2.0 (<http://www.R-project.org>).

RESULTS

The percentages of leaf areas removed by herbivores showed highly significant differences between the sites with different light conditions (Table 1). Plants from the sun-exposed site sustained greater damage than plants from the shaded site (Fig. 1A). However, the effect of sex nested in-site was not significant (Fig. 1A).

Plants from the shade were significantly taller (Table 1, Fig. 1B), with higher TLA (Table 1, Fig. 1C) and SLA values (Table 1, Fig. 1D). Plant sex nested in-site had no effect on height, TLA and SLA (Table 1). *A posteriori* tests showed that although female plants were on average shorter than male plants, with lower SLA, these differences were not significant. A similar result was found for TLA.

The examined biochemical plant traits varied significantly between the sites, apart from TPhC (Table 1). Plants in the shade had significantly higher water (Fig. 1E), protein (Fig. 1F) and TCT contents (Fig. 1H) compared to their sun-exposed counterparts. Plant sex had no significant effect on LWC or TPhC (Table 1), while TSPC and TCT exhibited significant intersexual differences. According to the *a posteriori* test, female plants in the sun-exposed site had significantly higher TSPC compared to males, while in the shade TSPC was higher in males, but not significantly. The intersexual difference in TCT content was significant only in the shade, with higher trait values in female plants.

The effect of height on the degree of foliar damage was significant in the shade, but not in the sun-

Table 1. Results of nested ANOVA with the effects of site and sex nested in-site on the leaf area removed (LAR), height, total leaf area (TLA), specific leaf area (SLA), leaf water content (LWC), total soluble protein content (TSPC), total phenolic content (TPhC) and total condensed tannins (TCT) of *M. perennis*.

Source	Site			Site: Sex		
	df	F	p	df	F	p
LAR (%)	1, 116	84.83	<0.0001	2, 116	0.78	0.4600
Height (cm)	1, 116	83.02	<0.0001	2, 116	0.08	0.9240
TLA (cm^2)	1, 116	12.91	<0.0001	2, 116	0.01	0.9878
SLA ($\text{mm}^2 \text{g}^{-1}$)	1, 116	100.81	<0.0001	2, 116	0.23	0.7970
LWC (%)	1, 116	79.69	<0.0001	2, 116	0.19	0.8200
TSPC (mg g^{-1})	1, 116	17.15	<0.0001	2, 116	13.52	<0.0001
TPhC (mg g^{-1})	1, 116	0.07	0.7890	2, 116	0.33	0.7230
TCT ($A_{500} \text{g}^{-1}$)	1, 116	115.06	<0.0001	2, 116	11.96	<0.0001

LAR, TPhC and TSPC – sqrt transformed, TCT – inversely transformed. Statistically significant differences are indicated by bolded p-values.

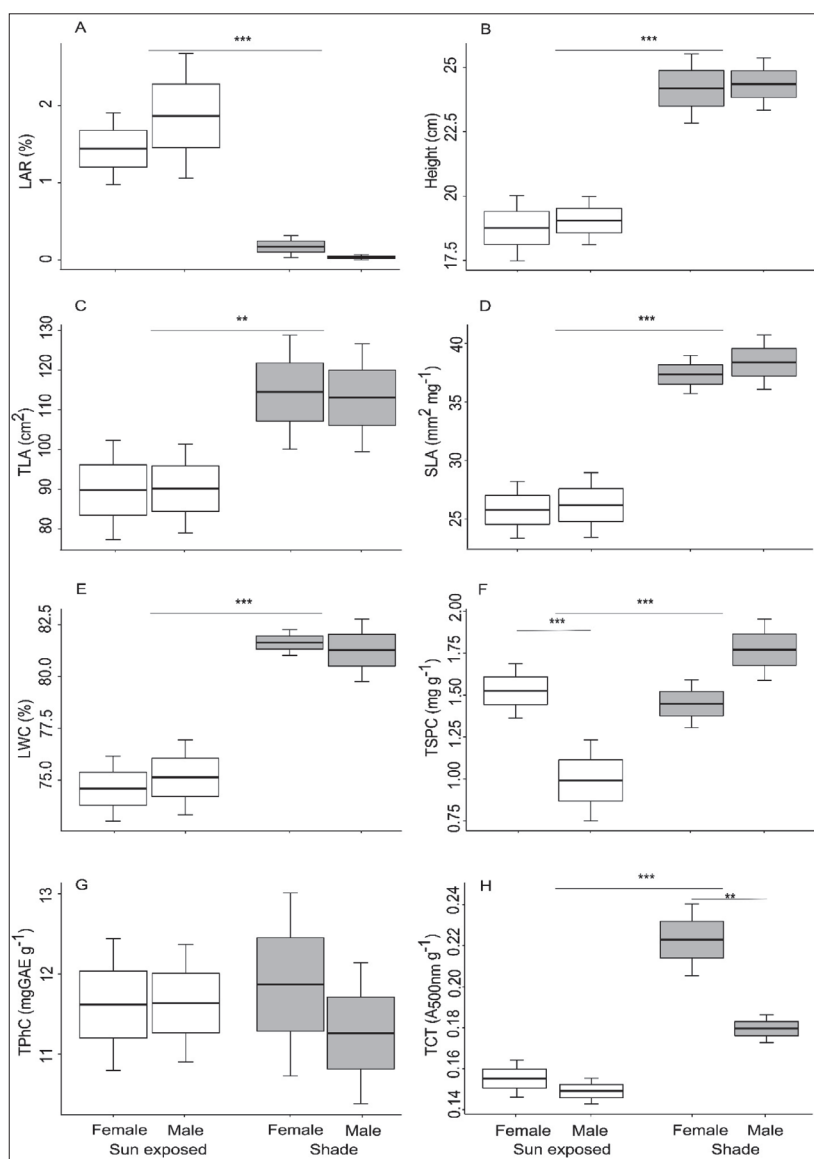


Fig. 1. Differences between male and female *M. perennis* from sunny and shaded sites. **A** – leaf area removed (LAR); **B** – height; **C** – total leaf area (TLA); **D** – specific leaf area (SLA); **E** – leaf water content (LWC); **F** – total soluble protein content (TSPC); **G** – total phenolic content (TPhC); **H** – total condensed tannins (TCT); white boxes – sun exposed site; gray boxes – shaded site; bold lines in the middle of the boxes indicate the mean value; the boxes indicate mean \pm SE; the error bars show mean \pm SD; p-values according to nested ANOVA and LSMeans test, **p<0.01, ***p<0.001.

exposed site. Taller plants in the shade were less damaged (Fig. 2A, $R^2_{\text{shade}}=0.08$, $P_{\text{shade}}=0.0178$). The specific leaf area affected the degree of foliar damage negatively, as indicated by linear regression analysis: plants with lower SLA were more damaged at both sites (Fig. 2C; $R^2_{\text{shade}}=0.05$, $P_{\text{shade}}=0.0441$, $R^2_{\text{sun exposed}}=0.24$, $P_{\text{sun exposed}}<0.0001$). In the shade, the water content did not af-

fect leaf damage significantly; on the other hand, the relationship between the percentage of water and LAR at the sun-exposed site was statistically significant and negative (Fig. 2D, $R^2_{\text{sun exposed}}=0.07$, $P_{\text{sun exposed}}=0.0206$). The same was found for the relationship of LAR with protein content in the shade (Fig. 2E; $R^2_{\text{shade}}=0.06$, $P_{\text{shade}}=0.0386$). No significant effect of TLA, TPhC or TCT on leaf damage was found at either site (Fig. 2B, F and G, respectively).

DISCUSSION

Folivory patterns

Environmental heterogeneity dictates dynamics of plant-herbivore interactions, maintaining variability and selecting for defense strategies of hosts [2]. Light exposure directly shapes a plant's structural and functional traits and, therefore, mediates resistance and tolerance to folivory. To date, studies of light-differential herbivory that provided insight into the observed patterns of leaf damage were mostly focused on woody species [3,15,16,18,48]. Forest understory, however, is carpeted with herbaceous species that are strong competitors, capable of excluding less shade-tolerant species. When faced with an altered light regime, the physiological constraints of shade-adapted species are considered the main factor explaining their absence from the sun-exposed sites

[6]. However, an alternative hypothesis has been that the main selection agent could in fact be differential herbivory rates [9,49].

Light limitations can make the leaves of forest understory species vulnerable to insects; increased consumption of leaves in the shade may further lower

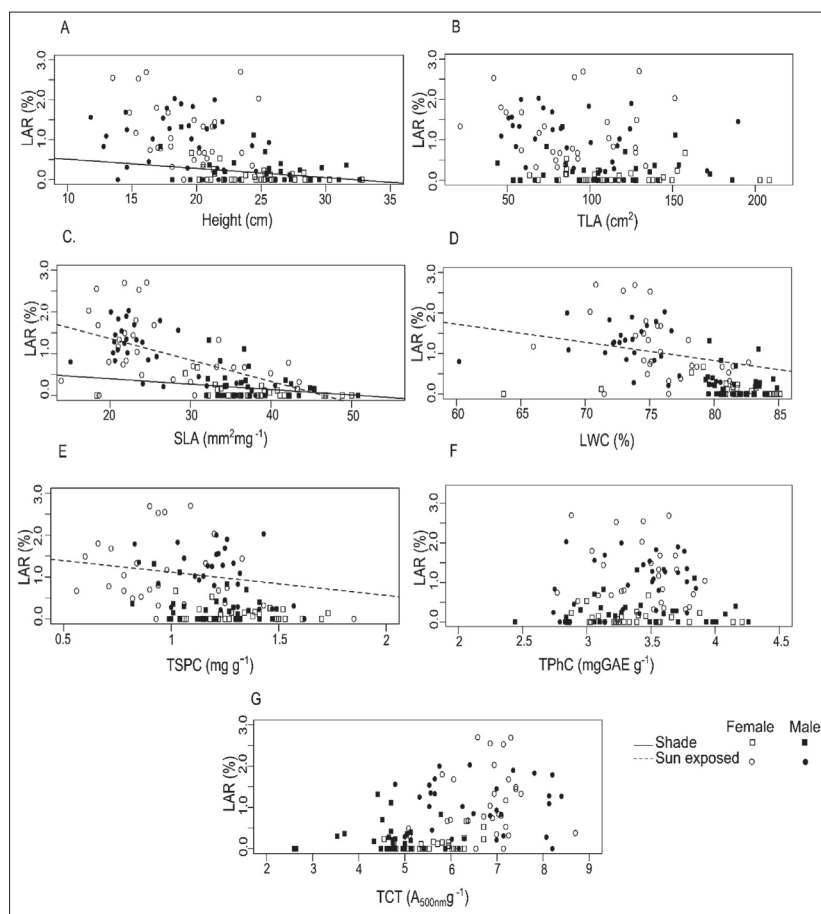


Fig. 2. Regression analysis of the relationships between the leaf area removed (LAR) for *M. perennis* plants from sunny and shaded sites. **A** – height; **B** – total leaf area (TLA); **C** – specific leaf area (SLA); **D** – leaf water content or (LWC); **E** – total soluble protein content (TSPC); **F** – total phenolic content (TPhC); **G** – total condensed tannins (TCT). LAR, TPhC and TSPC – sqrt transformed; TCT – inversely transformed

the potential for light exploitation, making plant fitness losses even more severe [3]. In our study, plants growing in the shade were significantly taller, with larger canopies, higher SLA values, higher amounts of water and total soluble protein contents. These traits, consistent with a shade-tolerant strategy [6], theoretically should implicate higher herbivory rates in the shade. We found that the leaves from the sun-exposed site sustained on average six-fold greater damage than leaves from the shade. However, this result should be taken with caution, since the overall percentage of foliar damage in this study was rather low, ranging from 0 to 7.3%. Our results showing increased herbivore pressure on a shade-tolerant forb in sun-exposed sites underpin the claim of Louda and Rodman [49]. It should be noted that, apart from light conditions, other factors (e.g. temperature, soil characteristics, diversity

and size of herbivore communities) may have also affected the observed patterns, but the magnitude of differences in light flux between the sites allows for the assumption that their contribution is not as significant. In line with this, Niesenbaum and Kluger [7] have shown that the effects of temperature on herbivory rates under natural conditions were “muted” by the differences in leaf traits in sunny and shady environments. The differences in diversity and size of herbivore communities between the environments with contrasting light conditions might have influenced, to a certain extent, the patterns of herbivore damage. However, light-differential herbivory in natural conditions was recorded in cases where no significant difference in abundance or herbivore species composition between sun and shade was observed [50]. Therefore, to fully elucidate this matter in *M. perennis*, further research is needed.

There is evidence of sex-biased herbivory in numerous plant model systems, but less attention has been paid to how it can be affected by the interplay between resource availability and sexual dimorphism [21]. Previous field and experimental research in *M. perennis* often revealed a decrease in the proportion of females as light fluxes increased [31,35,36]. Since females do not appear to be physiologically intolerant to high solar irradiation, one of the possible explanations could be a bias in herbivore loads (male-bias in shade or female-bias in sun-exposed sites). However, in the present study, we found no significant overall effect of sex on the extent of foliar damage (although in the shade females suffered more damage – 0.24% vs. 0.09%), thus other factors could have produced the observed patterns in distribution of females and males of *M. perennis* in relation to light regime. Sexual dimorphism (SD) was recorded in biochemical traits TSPC and TCT; these patterns differed between sites but were not reflected in sex differential herbivore pressure.

Differences between sexes in contrasting light environments

In dioecious plants, sexual size dimorphism (SSD) is one of the manifestations of divergence in resource allocation [51]. In species where males exhibit greater total aboveground biomass, longer shoots and bigger leaves, these traits can make them more apparent, conferring greater susceptibility to herbivores, especially to folivores [21]. Previous research found prominent male-biased SSD in height in *M. perennis* populations at lower altitudes [34,52]; however, this difference tended to decrease with increasing altitude [34]. In the present study, at the upper limit of altitudinal distribution, no significant effect of sex on plant height or total leaf area was found.

In terms of intersexual differences in vegetative traits, a trade-off between intrinsic growth rates, leaf thickness and herbivore loads has been suggested. Jing and Coley [53] found males of *Acer negundo* to be larger than females, with thinner leaves that sustained greater levels of damage. In contrast, Li et al. [54] reported SD in leaf thickness in *Hippophae rhamnoides*, with females having higher SLA, even in populations at the highest altitudes. We found no such pattern in *M. perennis*. It is possible that under environmental stress, the evolution of mechanisms of resource garner and translocation (i.e. canopy size and shoot height) includes minimizing intersexual differences in vegetative allocation [55,56]. If females are the sex with higher energy demands for reproduction, they would increase the capacity for resource acquisition, especially under harsh conditions.

Due to their higher reproductive investment, females are often regarded as the sex that is better protected from herbivores [25]. Our results are in line with this claim, since female *M. perennis* plants had higher overall levels of total condensed tannins, though the difference in this particular defensive trait was significant only in the shade. Although the TCT content had no significant effect on the extent of foliar damage, the lack of intersexual differences in TCT at the sun-exposed site can be attributed to more stressful environmental conditions, i.e. a tendency of SD to decrease under such conditions [55,56]. In high altitude open habitats, plants are subjected to stressful conditions

[55], and specifically high light fluxes are known to have a detrimental effect on *M. perennis* [31].

Intersexual differences can also be found in the redistribution of metabolic exchanges. Seed and fruit production require greater amounts of carbon-based compounds, whereas males of wind-pollinated herbs allocate more nitrogen to the production of large quantities of pollen, described in the closely related *Mercurialis annua* [24]. The overall amount of leaf nitrogen correlates with the soluble protein content, which represents another factor that can contribute to the increased risk of consumption by folivores [57]. This trait was found to exhibit sexual dimorphism in *M. perennis* at the sun-exposed site, with females having a higher protein content. Similar intersexual differences in protein and nitrogen contents were recorded in *Salix repens* [58] and *Rhamnus alpinus* [59] as well.

The explanation for the observed pattern of intersexual differences in TSPC under contrasting light conditions might lie in the dynamics of sexual reproduction and within plant source-sink translocations [60]. Males finish pollen production prior to fruit development [24]; during that time nitrogen is allocated from leaves to pollen production. Following the end of pollen dispersal, females are expected to divert the assimilated carbon from the leaves to the developing fruits [38]. The sampling took place after pollen dispersal in the shade was finished, while in the sun-exposed site, under higher herbivore pressure, it was not fully completed. Therefore, TSPC of males from the shaded site could be increasing, since the sink demands for nitrogen have been fulfilled, which explains the absence of SD. Sánchez-Vilas and Pannell [38] found a significant difference in nitrogen content between the sexes of *M. annua* only in young leaves. Since plants from the sun-exposed site were slightly younger compared to those from the shade, our findings are in accordance with the results on *M. annua* [24,38].

Herbivore damage in the sun and shade related to plant morphological and biochemical traits

Plant defenses against herbivores consist of traits such as nutritive value, morphological characteristics, compensatory growth potential, production of secondary metabolites and toxins. A certain combination of

these traits acting simultaneously may have stronger effects on insect performance than any one of them individually. This is known as the defense syndrome that may vary across environments, conferring adaptive advantage [61].

We found that traits as predictors of the degree of foliar damage in *M. perennis* were SLA at both sites, height, leaf water and total soluble protein contents in shade. Taller plants were less damaged in the shade. Total leaf area had no significant effect on the amount of damage. Thus, our results did not confirm the predictions of the Plant Vigor Hypothesis, according to which the most apparent hosts would be attacked more frequently [62]. However, the results obtained for the plant traits mediating herbivore loads should be taken with caution since the overall regression coefficients were low. Low levels of damage sustained by plants might have skewed the correlations between LAR and the examined traits.

It is well known that leaves with higher SLA values are thinner [13] and softer, which would lead to higher damage [2]. Although not as often, the opposite pattern of leaves with lower SLA sustaining greater damage has been recorded in various plant-herbivore systems [63,64]. Leaves of *M. perennis* that were thicker also had a larger proportion of leaf lamina that was consumed both in the shade and in the sun-exposed sites. One possible explanation for the negative relationship between SLA and the degree of damage might lie not in palatability, but in the nutritional value of the leaf tissue. Lusk et al. [63] found that leaves with high SLA values presented a poor energy source compared to the effort invested in chewing and digesting such leaves. Still, in the shade, leaves with lower TSPC suffered more damage. This finding is consistent with the hypothesis of increased consumption of lower quality food to compensate for poor nutritive gain [21,63], although TSPC explained a small part of the overall variation in leaf damage.

At the sun-exposed site, SLA accounted for the great part of variability in the leaf area missing/removed, while nutritional quality contributed moderately. Well hydrated leaves sustained less damage. It was suggested that the inverse relationship between the extent of foliar damage and water content is due to the fact that leaves of water-stressed plants had higher con-

centrations of osmolytes, sugars and amino acids when compared to the well-hydrated ones [65]. Bisigato et al. [64] posited that food choice represents a hierarchical process with different traits influencing consumption rates. They demonstrated that the main factors determining the rates of consumption at the intraspecific level were SLA and LWC – thicker and drier leaves that were more prone to consumption by folivores.

It was reported that leaf physical traits such as toughness, thickness and specific area represent the strongest predictors of the degree of foliar damage in the field across light gradients [16]. Spatial patterns of leaf chemical defensive traits and nutritive value could be more strongly affected by the environmental context and may not contribute to the risk of consumption to the same extent [2]. Though secondary metabolites such as condensed tannins and phenolics are traditionally considered as defensive compounds against a broad spectrum of herbivores [66], they showed little overall effect on the degree of foliar damage in *M. perennis*. Increased levels of these compounds should theoretically deter herbivores and ultimately result in lower foliar damage. Although *M. perennis* plants growing in the shade did exhibit higher levels of tannins and sustained less foliar damage, this trait, along with the total phenolics content, showed no significant effect on the rates of consumption either in the shade or in the sun. There are reports that these compounds do not affect the extent of foliar damage [64] and that leaves with higher phenolic contents do not sustain greater overall damage [20]. It is possible that other compounds with a broad antiherbivore activity, or toxic metabolites such as alkaloids, which are present in leaves of dog's mercury [67], confer increased resistance to specialist feeders. Further research is needed to elucidate the main contributors to the chemical defense in *M. perennis*.

In this study, one of the examined plant morphological traits (SLA) explained the largest portion of variability in herbivore loads in different natural light environments. Our results are in agreement with the recent meta-analysis of functional and chemical traits in relation to plant resistance [68]. They suggested that novel insight into the microevolutionary pathways of plant resistance show that relative contributions of biochemical and physical traits linked to resistance may be more complex than previously believed. Our

findings contribute to the idea that plant morphology and leaf morphological traits can affect insect preference and performance more strongly as compared to chemical defenses.

CONCLUSIONS

The question of how light environments and sex influence natural herbivore loads in dioecious species remains particularly interesting. The present study revealed differential herbivore damage under contrasting light conditions in dioecious shade-tolerant *Mercurialis perennis*. Plants suffered significantly greater leaf damage at the sun-exposed site than in the shaded site. Overall, factors defining the risk of consumption were related to plant morphological traits and nutritive value rather than to chemical defenses. Nonetheless, we have to take into account that differential herbivory is not mediated solely by plant traits. Further research into other factors and on more populations is needed to elucidate the observed variation patterns and to fully understand the factors that shape one of the most important interactions in nature.

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Author contributions: SS. and VV. carried out data collection in the field, analysis and interpretation of the results, and wrote the manuscript. LR carried out data collection in the field and obtained hemispherical photography of the sites. NS and ŽJ conducted the chemical analyses and revised the manuscript. DC and SR contributed substantially to revising the manuscript.

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REFERENCES

- Johnson MT. Evolutionary ecology of plant defenses against herbivores. *Funct Ecol.* 2011;25(2):305-11.
- Hakes AS, Cronin JT. Environmental heterogeneity and spatiotemporal variability in plant defense traits. *Oikos.* 2011;120(3):452-62.
- Salgado-Luarte C, Gianoli E. Herbivores modify selection on plant functional traits in a temperate rainforest understory. *Am Nat.* 2012;180(2):42-53.
- Valladares F, Laanisto L, Niinemets Ü, Zavala M. Shedding light on shade: ecological perspectives of understorey plant life. *Plant Ecol Divers.* 2016;9(3):237-51.
- Roberts MR, Paul ND. Seduced by the dark side: integrating molecular and ecological perspectives on the influence of light on plant defence against pests and pathogens. *New Phytol.* 2006;170(4):677-99.
- Valladares F, Niinemets Ü. Shade tolerance, a key plant feature of complex nature and consequences. *Annu Rev EcolEvol Syst.* 2008;39:237-57.
- Niesenbaum RA, Kluger EC. When studying the effects of light on herbivory, should one consider temperature? The case of *Epimecishortaria* F. (Lepidoptera: Geometridae) feeding on *Lindera benzoin* L. (Lauraceae). *Environ Entomol.* 2006;35(3):600-6.
- Muth NZ, Kluger EC, Levy JH, Edwards MJ, Niesenbaum RA. Increased per capita herbivory in the shade: necessity, feedback, or luxury consumption. *Ecoscience.* 2008;15:182-8.
- Salgado-Luarte C, Gianoli E. Herbivory on temperate rainforest seedlings in sun and shade: resistance, tolerance and habitat distribution. *PLoS One.* 2010;5(7):e11460.
- Takafumi H, Kawase S, Nakamura M, Hiura T. Herbivory in canopy gaps created by a typhoon varies by understory plant leaf phenology. *EcolEntomol.* 2010;35(5):576-85.
- Coley PD. Intraspecific variation in herbivory on two tropical tree species. *Ecology.* 1983;64(3):426-33.
- Karolewski P, Giertych MJ, Żmuda M, Jagodziński AM, Oleksyn J. Season and light affect constitutive defenses of understory shrub species against folivorous insects. *ActaOecol.* 2013;53:19-32.
- Reich PB, Wright IJ, Cavender-Bares J, Craine JM, Oleksyn J, Westoby M, Walters MB. The evolution of plant functional variation: traits, spectra, and strategies. *Int J Plant Sci.* 2003;164(S3):S143-64.
- Barbehenn RV, Constabel CP. Tannins in plant-herbivore interactions. *Phytochemistry.* 2011;72(13):1551-65.
- Baraza E, Gómez JM, Hódar JA, Zamora R. Herbivory has a greater impact in shade than in sun: response of *Quercuspyrenaica* seedlings to multifactorial environmental variation. *Can J Botany.* 2004;82(3):357-64.
- Guerra PC, Becerra J, Gianoli E. Explaining differential herbivory in sun and shade: the case of *Aristoteliachilensis* saplings. *Arthropod-Plant Inte.* 2010;4(4):229-35.
- Sinimbu G, Coley PD, Lemes MR, Lokvam J, Kursar TA. Do the antiherbivore traits of expanding leaves in the Neotropical tree *Inga paraensis* (Fabaceae) vary with light availability? *Oecologia.* 2012;170(3):669-76.
- Stoepler TM, Rehill B. Forest habitat, not leaf phenotype, predicts late-season folivory of *Quercusalba* saplings. *Funct Ecol.* 2012;26(5):1205-13.
- Mooney EH, Tiedeken EJ, Muth NZ, Niesenbaum RA. Differential induced response to generalist and specialist herbivores by *Lindera benzoin* (Lauraceae) in sun and shade. *Oikos.* 2009;118(8):1181-9.
- Mooney EH, Niesenbaum RA. Population-specific responses to light influence herbivory in the understory shrub *Lindera benzoin*. *Ecology.* 2012;93(12):2683-92.
- Cornelissen T, Stiling P. Sex-biased herbivory: a meta-analysis of the effects of gender on plant-herbivore interactions. *Oikos.* 2005;111(3):488-500.
- Obeso JR. The costs of reproduction in plants. *New Phytol.* 2002;155(3):321-48.

23. Ashman TL. The role of herbivores in the evolution of separate sexes from hermaphroditism. *Ecology*. 2002;83(5):1175-84.
24. Harris MS, Pannell JR. Roots, shoots and reproduction: sexual dimorphism in size and costs of reproductive allocation in an annual herb. *P Roy Soc B-Biol Sci*. 2008;275(1651):2595-602.
25. Barrett SC, Hough J. Sexual dimorphism in flowering plants. *J Exp Bot*. 2013;64(1):67-82.
26. Ågren J, Danell K, Elmqvist T, Ericson L, Hjäältén J. Sexual dimorphism and biotic interactions. In: Geber MA, Dawson TE, Delph LF, editors. *Gender and sexual dimorphism in flowering plants*. Berlin Heidelberg: Springer;1999. p. 217-46.
27. Cibils AF, Swift DM, Hart RH. Female-biased herbivory in fourwing saltbush browsed by cattle. *J Range Manage*. 2003;56:47-51.
28. Maldonado-López Y, Cuevas-Reyes P, Sánchez-Montoya G, Oyama K, Quesada M. Growth, plant quality and leaf damage patterns in a dioecious tree species: is gender important? *Arthropod-Plant Int*. 2014;8(4):241-51.
29. Espírito-Santo MM, Neves FS, Fernandes GW, Silva JO. Plant phenology and absence of sex-biased gall attack on three species of *Baccharis*. *PloS One*. 2012;7(10):e46896.
30. Buckley NE, Avila-Sakar G. Reproduction, growth, and defense trade-offs vary with gender and reproductive allocation in *Ilex glabra* (Aquifoliaceae). *Am J Bot*. 2013;100(2):357-64.
31. Jefferson RG. Biological Flora of the British Isles: *Mercurialis perennis* L. *J Ecol*. 2008;96(2):386-412.
32. Vandepitte K, Honnay O, De Meyer T, Jacquemyn H, Roldán-Ruiz I. Patterns of sex ratio variation and genetic diversity in the dioecious forest perennial *Mercurialis perennis*. *Plant Ecol*. 2010;206(1):105-14.
33. Elemans M. Light, nutrients and the growth of herbaceous forest species. *Acta Oecol*. 2004;26(3):197-202.
34. Cvetković D, Jovanović V. Altitudinal variation of the sex ratio and segregation by gender in the dioecious plant *Mercurialis perennis* L. (Euphorbiaceae) in Serbia. *Arch Biol Sci*. 2007;59(3):193-8.
35. Vandepitte K, Roldán-Ruiz I, Leus L, Jacquemyn H, Honnay O. Canopy closure shapes clonal diversity and fine-scale genetic structure in the dioecious understorey perennial *Mercurialis perennis*. *J Ecol*. 2009;97(3):404-14.
36. Wade KM. Experimental studies on the distribution of the sexes of *Mercurialis perennis* L. II. Transplanted populations under different canopies in the field. *New Phytol*. 1981;87:439-46.
37. Dormann CF, Skarpe C. Flowering, growth and defence in the two sexes: consequences of herbivore exclusion for *Salix polaris*. *Funct Ecol*. 2002;16(5):649-56.
38. Sánchez-Vilas J, Pannell JR. Sex-differential herbivory in androdioecious *Mercurialis annua*. *PloS One*. 2011;6(7):e22083.
39. Frazer GW, Canham CD, Lertzman KP. Gap Light Analyzer (GLA), Version 2.0: Imaging software to extract canopy structure and gap light transmission indices from true-colour fisheye photographs, users manual and program documentation. Burnaby, British Columbia: Simon Fraser University; 1999. 36 p.
40. Nobis M, Hunziker U. Automatic thresholding for hemispherical canopy-photographs based on edge detection. *Agr Forest Meteorol*. 2005;128(3):243-50.
41. Cornelissen JHC, Lavorel S, Garnier E, Diaz S, Buchmann N, Gurvich DE, Reich PB, terSteege H, Morgan HD, van der Heijden MGA, Pausas JG, Poorter H. A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. *Aust J Bot*. 2003;51(4):335-80.
42. Abramoff MD, Magalhães PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int*. 2004;11(7):36-42.
43. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1-2):248-54.
44. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult*. 1965;16(3):144-58.
45. Broadhurst RB, Jones WT. Analysis of condensed tannins using acidified vanillin. *J Sci Food Agr*. 1978;29(9):788-94.
46. Xu BJ, Chang SKC. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *J Food Sci*. 2007;72(2):159-66.
47. Salminen J-P, Karonen M. Chemical ecology of tannins and other phenolics: we need a change in approach. *Funct Ecol*. 2011;25(2):325-38.
48. Salgado-Luarte C, Gianoli E. Herbivory may modify functional responses to shade in seedlings of a light-demanding tree species. *Funct Ecol*. 2011;25(3):492-9.
49. Louda SM, Rodman JE. Insect herbivory as a major factor in the shade distribution of a native crucifer (*Cardamine cordifolia* A. Gray, bittercress). *J Ecol*. 1996;84:229-37.
50. Guerra PC, Becerra J, Gianoli E. Explaining differential herbivory in sun and shade: the case of *Aristoteliachilensis* saplings. *Arthropod-Plant Int*. 4(4):229-35.
51. Fairbairn DJ, Blanckenhorn WU, Székely T. Sex, size, and gender roles: evolutionary studies of sexual size dimorphism. 1st ed. Oxford: Oxford University Press; 2007.
52. Vujić V, Rubinjoni L, Selaković S, Cvetković D. Small-scale variations in leaf shape under anthropogenic disturbance in dioecious forest forb *Mercurialis perennis*: a geometric morphometric examination. *Arch Biol Sci*. 2016;68(4):705-13.
53. Jing SW, Coley PD. Dioecy and herbivory: the effect of growth rate on plant defense in *Acer negundo*. *Oikos*. 1990;58:369-77.
54. Li C, Xu G, Zang R, Korpelainen H, Berninger F. Sex-related differences in leaf morphological and physiological responses in *Hippophae ramnoides* along an altitudinal gradient. *Tree Physiol*. 2007;27(3):399-406.
55. Sakai A, Sasa A, Sakai S. Do sexual dimorphisms in reproductive allocation and new shoot biomass increase with an increase of altitude? A case of the shrub willow *Salix reinii* (Salicaceae). *Am J Bot*. 2006;93(7):988-92.
56. Labouche AM, Pannell JR. A test of the size-constraint hypothesis for a limit to sexual dimorphism in plants. *Oecologia*. 2016;181(3):873-84.
57. Ballhorn DJ, Kautz S, Jensen M, Schmitt I, Heil M, Hegeman AD. Genetic and environmental interactions determine plant defenses against herbivores. *J Ecol*. 2011;99(1):313-26.

58. Ruuhola T, Nybakken L, Julkunen-Tiitto R. Sex-related differences of two ecologically divergent *Salix* species in the responses of enzyme activities to atmospheric CO₂ enrichment. *BiolPlantarum*. 2013;57(4):732-8.
59. Bañuelos MJ, Obeso JR. Resource allocation in the dioecious shrub *Rhamnus alpinus*: the hidden costs of reproduction. *EvolEcol Res*. 2004;6(3):397-413.
60. Wright VL, Dorken ME. Sexual dimorphism in leaf nitrogen content but not photosynthetic rates in *Sagittaria latifolia* (Alismataceae). *Botany*. 2014;92(2):109-12.
61. Agrawal A A, Fishbein M. Plant defense syndromes. *Ecology*. 2006;87(sp7):132-49.
62. Price PW. The plant vigor hypothesis and herbivore attack. *Oikos*. 1991;62:244-51.
63. Lusk CH, Onoda Y, Kooyman R, Gutiérrez-Girón A. Reconciling species-level vs plastic responses of evergreen leaf structure to light gradients: shade leaves punch above their weight. *New Phytol*. 2010;186(2):429-38.
64. Bisigato AJ, Saín CL, Campanella MV, Cheli GH. Leaf traits, water stress, and insect herbivory: Is food selection a hierarchical process? *Arthropod-Plant Inte*. 2015;9(5):477-85.
65. Meyer ST, Roces F, Wirth R. Selecting the drought stressed: effects of plant stress on intraspecific and within-plant herbivory patterns of the leaf-cutting ant *Atta colombica*. *Funct Ecol*. 2006;20(6):973-81.
66. Carmona D, Lajeunesse MJ, Johnson MT. Plant traits that predict resistance to herbivores. *Funct Ecol*. 2011;25(2):358-67.
67. Lorenz P, Hradecky M, Berger M, Bertrams J, Meyer U, Stintzing FC. Lipophilic constituents from aerial and root parts of *Mercurialis perennis* L. *Phytochem Analysis*. 2010;21(3):234-45.