Bottom-up effects of woodland eutrophication: Interacting limiting nutrients determine herbivory frequency in northwestern Patagonia

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HIGHLIGHTS

- Effects of soil eutrophication on plant–herbivore interactions remains understudied.
- Interactive effects of N, P, K addition on herbivory patterns were studied.
- Nitrogen soil enrichment reduced herbivory frequency by 41%.
- Potassium addition buffered the effect of N-enrichment.
- Effects of nutrient enrichment were mediated by changes in leaf density and leaf size.

GRAPHICAL ABSTRACT
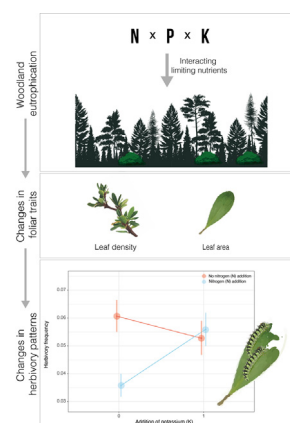
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ABSTRACT

Nutrient enrichment disrupts plant–animal interactions and ecosystem functioning globally. In woodland systems, the mechanisms of bottom-up turnover on plant–herbivore interactions remain understudied. Here, we performed a full-factorial field experiment to evaluate the interactive effects of nutrient addition (nitrogen, phosphorus, and/or potassium) on the assemblage of foliar herbivores and the interaction frequency with *Berberis microphylla*, a dominant shrub species in Patagonian woodlands. Additionally, we assessed whether these effects could be mediated by changes in vegetative traits and microhabitat characteristics (i.e., canopy cover) that may ultimately influence the foraging behavior of herbivores. The addition of nitrogen reduced the herbivory frequency by 41%, yet this effect was diluted in the presence of potassium. We found no effects of phosphorus addition. Our results suggest that the impact of multiple nutrient additions (N and K) on herbivory patterns could be mediated by changes in two important foliar traits, leaf size and leaf density. This study shows how multiple nutrient addition can change the magnitude of antagonistic plant–animal interactions in woodlands. Since herbivory by arthropods has a relevant role in net primary productivity, our results highlight the importance of buffering human-driven woodland eutrophication to maintain important ecological functions (e.g., herbivory) associated with antagonistic plant–animal interactions and avoiding ecosystem dysfunction.

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1. Introduction

Human activity in the Anthropocene is driving multiple changes at a global scale, including woodland eutrophication (i.e., excessive nutrient enrichment) (Smith et al., 1999). The deposition of nitrogen (N), phosphorus (P), or potassium (K) derived from industrial and agricultural activities (Sardans and Peñuelas, 2015; Penuelas et al., 2020) causes changes to biological and physicochemical soil conditions that regulate plant populations at both the community and species levels (Smith et al., 1999; Elser et al., 2007; Verheyen et al., 2012; Harpole et al., 2016; Stevens et al., 2018). Recent experimental evidence shows that an increase in the number of different limiting nutrients added to soils depauperates diversity and promotes changes in the community structure of grasslands (Harpole et al., 2016). At the species level, nutrient enrichment has been shown to change the response of vegetative and reproductive traits of plants which in turn influence interacting patterns with both mutualistic (e.g., mycorrhizal fungi, pollinators or seed dispersers) (Carvalheiro et al., 2020, 2021) and antagonistic partners (herbivores or parasites) (Lind et al., 2017; Pöyry et al., 2017). However, the evidence is scarce and scattered, and there is little mechanistic knowledge about how the effects of eutrophication propagate toward higher trophic levels and ultimately shape plant–animal interaction patterns and ecosystem functioning.

Foliar herbivory by insects drives plant–animal coevolution and modulates key ecological processes such as primary productivity, plant community composition or nutrient cycling (Schmitz, 2008). Soil nutrient enrichment has been shown to modify growth rates and a wide range of vegetative traits of plants, such as leaf density, leaf size, specific leaf area or nutrient content in leaves. Consequently, soil eutrophication may shape microhabitat characteristics (e.g., light availability) by modifying the canopy cover of coexisting plant species (Borer et al., 2014). Changes in microhabitat and vegetative traits could influence foraging behavior and plant selection patterns by herbivorous insects with different nutritional needs, thus potentially entailing changes in the composition of herbivore assemblages and interaction frequency with plants (Maiorana, 1981; Throop and Lerdau, 2004; Pöyry et al., 2017). However, most of this evidence derives from studies evaluating the impact of N or NPK enrichment, but it remains unclear how multiple additions of different limiting nutrients (i.e., N, P, and K) can impact herbivory patterns. Understanding the mechanisms underlying the bottom-up effects of soil nutrient enrichment on herbivore assemblages and herbivory patterns is relevant to better anticipate their potential consequences in ecosystem (mis)functioning (Schmitz, 2008). While most research to address this problem was developed in grasslands (Borer et al., 2014; Harpole et al., 2016; Lind et al., 2017; Anderson et al., 2018), woodland ecosystems where shrub species dominate the community have rarely been evaluated (Campo and Dirzo, 2003; Andersen et al., 2010).

Patagonian temperate woodlands harbor a diversity of shrub species that grow in nutrient-limited soils (Diehl et al., 2008), making them a good study system to test a nutrient-related hypothesis experimentally. In this study, we performed a manipulative field experiment to assess the bottom-up effects of soil nutrient enrichment on the interaction patterns between *Berberis microphylla*, a dominant shrub species in the area, and the assemblage of its foliar herbivores. We also evaluated whether bottom-up effects (if present) could be related to changes in canopy cover and vegetative traits that can change the foraging decisions of herbivorous insects. We used a full-factorial design encompassing eight fertilization treatments (i.e., no nutrient addition, N, P, K, NP, NK, PK, and NPK) that represent a gradient of progressive addition of the three most important limiting nutrients in the Patagonian woodlands. We evaluated the effects of nutrient addition on *i*) the guild diversity of foliar herbivores and *ii*) the interaction frequency with foliar herbivores. We then explored whether these effects might be mediated by changes in canopy cover, plant volume, and foliar traits (leaf density, leaf size, and specific leaf area). Although

multiple responses may arise from the addition of a different combination of nutrients, we overall hypothesize that if herbivores have different nutritional needs we should find a parallel increase in both the diversity of functional guilds and the interaction frequency with *B. microphylla* as the number of limiting resources added increases.

2. Material and methods

2.1. Study system

The study was carried out during spring 2018 (October–December) at a 4.5-ha site embedded within a temperate woodland in northwestern Patagonia (El Foyel, 41°38′37″S, 71°26′54″W, Rio Negro Province, Argentina). The study site is located at an altitude between 790 m and 880 m a.s.l. with annual precipitation of 920–1300 mm, mainly concentrated during the fall and winter seasons. The average annual temperature is 3 °C in winter (range: −2–9 °C) and 15 °C in summer (range: 6–24 °C), and frosts are present approximately 80 days per year, mainly during June–August. Soils are Hapludands (Diehl et al., 2008), usually limited both by N and P with minimal atmospheric pollution (Perakis and Hedin, 2002). Vegetation is characterized by a high-density mixed woodland where species are typical of the secondary succession stage. Dominant species include *Nothofagus antarctica* (Nothofagaceae), *Lomatia hirsuta*, *Embothrium coccineum* (Proteaceae), *Diostea juncea* (Verbenaceae), *Schinus patagonica* (Anacardiaceae), *Discaria chacaye* (Rhamnaceae) and *Berberis microphylla* (Berberidaceae), which are all widely distributed throughout the study site. The community of foliar herbivores in the study site is broadly diverse and includes at least nine different functional guilds: leaf chewers, bud feeders, hole feeders, skeletonizers, sticky feeders, surface abrasion feeders, miners, sap-sucking, and gallers (Garibaldi et al., 2011; Nacif et al., 2021).

Among all local plant species, we selected *B. microphylla* as model species because *i*) it grows in all experimental plots, *ii*) it is one of the most abundant shrub species at the study site and *iii*) it is an important plant species that provides feeding resources to a wide broad range of organisms (e.g., herbivores, pollinators or frugivores). Furthermore, *B. microphylla* is an evergreen shrub that can grow up to 2 m tall and can be easily recognized by its thorns located in the stems in groups of three. It presents simple oval leaves with dimensions up to 40 mm × 14 mm and an area up to 2.8 cm^2.

2.2. Experimental design

To evaluate the bottom-up effects of soil nutrient enrichment (i.e., a progressive addition of different limiting nutrients to woodland soils) on the interaction patterns of *B. microphylla* with foliar herbivores, we performed a fertilization field experiment with a full factorial design. We applied eight different fertilization treatments resulting from a factorial combination of N, P, and K (Fig. 1). Each of the eight treatments (N, P, K, NP, NK, PK, NPK, Control) was replicated with four blocks, resulting in a total of thirty-two 31.5 × 45 m experimental plots (Fig. 1). After consulting local soil experts and following recommendations of previous research, we fertilized at a nutrient concentration of 100 kg/ha of time-release urea for nitrogen (Lindberg and Persson, 2004), 75 kg/ha of triple-superphosphate for phosphorus, and 56 kg/ha of potassium sulfate for potassium (Kim, 2008). Experimental plots were fertilized three times during the three years prior to the first sampling date (fertilizer application dates: November 2016, September 2017 and 2018). Nutrients were applied by hand, resulting in an increment of 5%, 180% and 75% of nitrogen, phosphorus and potassium, respectively, when compared to soils in nonfertilized plots. Although some variation in nutrient content among pre-treatment plots may exist, we expect that our randomized block design capture and account for that variation.
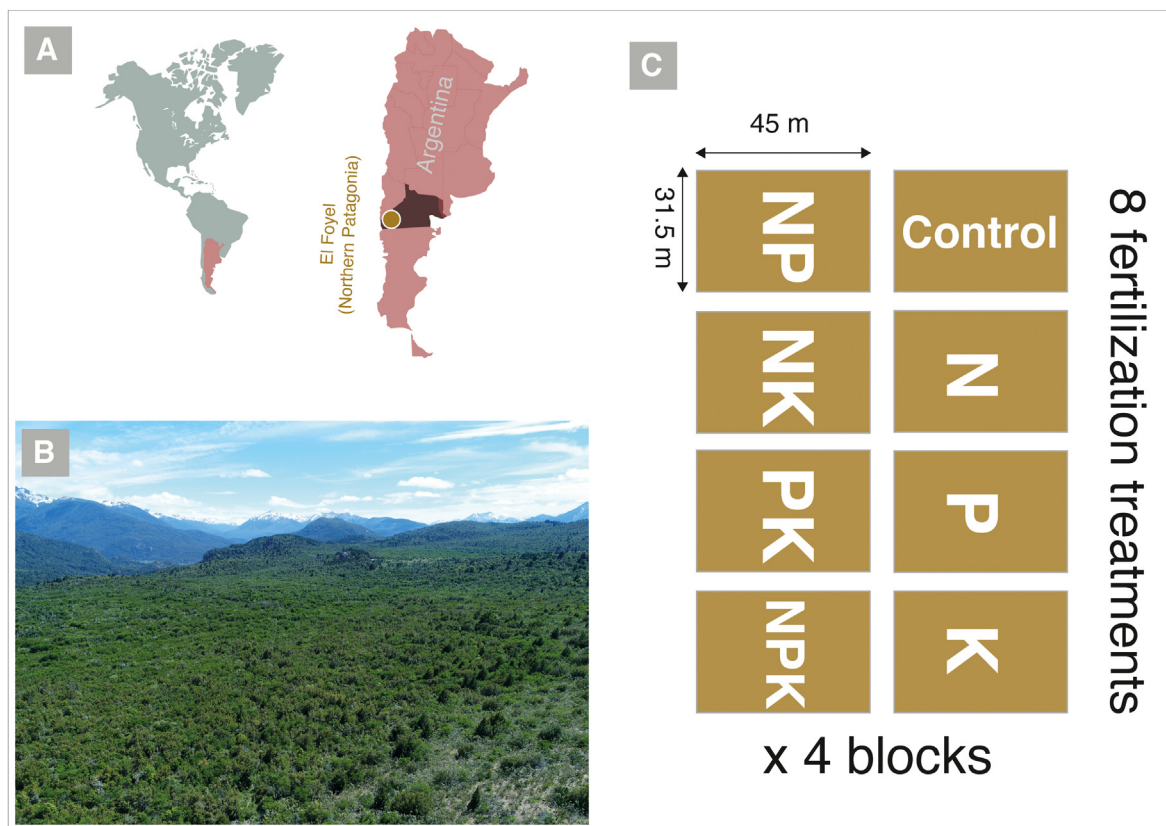
Fig. 1. Study site and experimental design: A) Map of geographical location of "El Foyel" temperate woodland; B) Illustrative picture of the physiognomy of "El Foyel" temperate woodland C) Experimental setup consisted of 32 experimental plots distributed across 8 fertilization treatments (factorial combination of N, P and K addition and a control non-fertilization treatment) and 4 replication blocks.

2.3. Characterization of vegetative plant traits, canopy cover, guild diversity and herbivory frequency

We considered four vegetative traits relevant for interactions with foliar herbivores that might be sensitive to nutrient availability: plant volume, leaf density, leaf size and specific leaf area (SLA hereafter). We characterized these four traits at the plot level as follows. Plant volume (m^3) was estimated from 4 to 8 individuals per experimental plot (mean $= 5.5$, $n = 178$) as $Height \cdot D_{max} \cdot D_{per}$, where D_{max} is the largest diameter of the plant and D_{per} is its perpendicular diameter. To characterize the rest of the vegetative traits, we additionally sampled four random branches from each plant individual ($n = 538$ branches). Leaf density was estimated as the $number\ of\ leaves/branch\ length, m$. We selected 16 green leaves per individual (4 random leaves per branch) for estimating leaf size and SLA. Individual leaf size (cm^2, $n = 2084$ leaves) was estimated by using ImageJ software (Rueden et al., 2017). Then, selected leaves were oven dried at 60 °C for two days and weighed by using a digital balance ($\pm$ 0.1 mg). SLA was defined as $leaf\ area, cm^2/leaf\ dry\ mass, g$ for each leaf. We also characterized the canopy cover (%) of the tree layer for each sampled plant as a surrogate for light availability by using the *Canopeo* app for Android (Patrignani and Ochsner, 2015). Light availability was estimated as it is known to influence the vegetative traits of plants and the foraging behavior of herbivorous animals (Maiorana, 1981).

We followed the approach proposed by Nacif et al. (2021) for characterizing herbivore guild diversity, e.g., leaf chewers, bud feeders, hole feeders, miners (Nacif et al., 2021), yet we only observed foliar damage mediated by leaf chewers. Therefore, we finally did not include the diversity of herbivore guilds as an interesting variable in our analyses as no variation was found. Finally, the interaction of *B. microphylla* with leaf chewers was estimated as the frequency of damaged leaves (i.e., $damaged\ leaves/total\ leaves$) for each sampled branch ($n = 538$). The leaf area damage (i.e., the damaged surface of each leaf) was not considered as we found little variation in this variable.

2.4. Data analysis

To evaluate the fertilizer effects on herbivory patterns and to assess whether they are mediated by changes in foliar traits and/or canopy cover, we followed a multi-step GLMM approach (Fig. S1). It is worth noting that we used this multistep GLMM approach instead of a structural equation model (SEM) approach because current analytical development does not allow us to properly accommodate interactions among three factors (e.g., N × P × K interactions) in SEMs (Lefcheck, 2016). To assess the impact of nutrient amendment on the herbivory frequency, we first applied a generalized linear mixed-effect model (GLMM) (Pinheiro and Bates, 2000; Gelman and Hill, 2006). The model (GLMM-1) included the interaction among the different fertilizers (N, P, K) as fixed factors that take two possible values (1 = fertilized, 0 = not fertilized), and the experimental block was included as a random effect. We fitted the model using a binomial distribution with a *logit*-link function and applied a Tukey post hoc contrast to assess pairwise differences among fertilization treatments that were involved in significant interactions. Given that we were interested in identifying those mediator variables that can potentially mediate the effects of fertilizer amendment on herbivory patterns, we then applied an additional GLMM (GLMM-2). We used herbivory frequency as a response, potential mediator variables (i.e., plant volume, leaf density, SLA and canopy cover) as predictors and the experimental block as a random factor. The independent SLA variable was standardized to allow model convergence. All mediator variables were included as model predictors except leaf size since it correlated with leaf density (Spearman's $r = -0.45$, $p <$
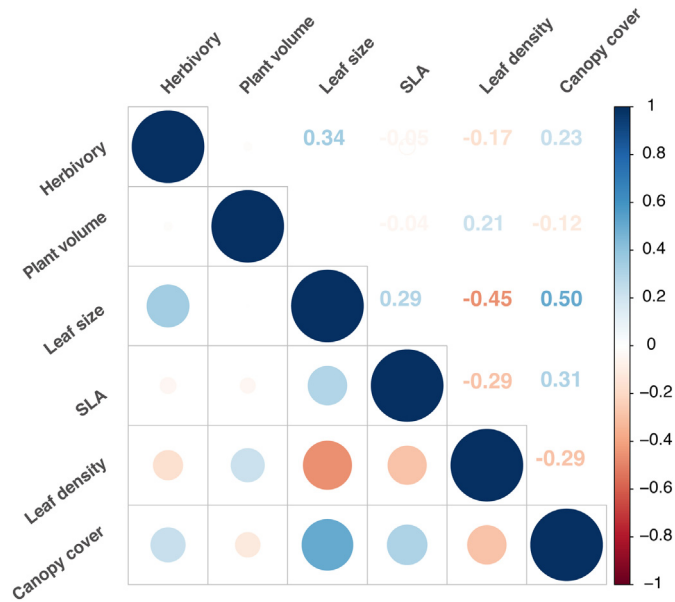
Fig. 2. Correlogram showing correlations (Spearman's correlations) among all variables characterized. Positive correlations are displayed in blue and negative correlations in red. Circle size and intensity of the color are proportional to the correlation coefficients (see the legend at the right side of the plot to associate colors and correlation coefficients). Only significant correlations are displayed ($p < 0.01$).

0.001; Fig. 2) and canopy cover (Spearman's $r = -0.50$, $p < 0.001$; Fig. 2), therefore entailing potential multicollinearity issues. Note that the N × P × K interactions were not included in this model as we were interested in assessing the direct effect of the mediator variables on the herbivory frequency independent of the influence of the fertilizer application. Finally, we applied a set of GLMMs (GLMM-3) to assess the influence of nutrient enrichment on the significant mediator variables detected in GLMM-2. All models included the significant interactions among nutrients (if any) detected in the GLMM-1 (i.e., in the herbivory frequency GLMM, Fig. S1). The experimental block was also included as a random effect across the different models. All models were fitted using a Gaussian distribution of errors with a log-link function, and the *DHARMa* R package (Hartig, 2020) was used to verify that model residuals were normally distributed. Finally, when a statistically significant interaction was detected in any of the previous GLMMs, we also applied Tukey's post hoc contrast to assess pairwise differences among fertilization treatments involved in that interaction. We used the *lme4* R package (Bates et al., 2015) to perform all the abovementioned GLMMs and the *emmeans* R package to apply the post-hoc analyses (Lenth, 2020).

3. Results and discussion

Northwestern Patagonian woodlands host a high diversity of generalist herbivorous insects, including different functional guilds such as leaf chewers, miners, skeletonizers or sticky feeders (Garibaldi et al., 2011; Nacif et al., 2021). However, we found that leaf damage in *B. microphylla* was mediated exclusively by leaf chewers, which include several lepidopteran larvae from the Geometridae, Noctuidae and Saturniidae families in our study system (Garibaldi et al., 2011). Similarly, other coexisting evergreen shrub species with similar ecological requirements and leaf traits such as *Maytenus chubutensis* (Celastraceae), showed a low diversity assemblage of herbivores dominated by leaf chewers (Nacif et al., 2021). Generalist herbivore species (e.g., leaf chewers) tend to select plants with high N content (Whitfeld et al., 2012), which is higher in deciduous than evergreen species in our study system (Diehl et al., 2008). Therefore, the low

Table 1

Analysis of deviance (χ^2) for generalized linear mixed effects models assessing changes in herbivory frequency, leaf density and canopy cover under the factorial nutrient addition experiment. Note that for leaf density and canopy cover we only included the N × K interaction in the models as P was not found to influence the herbivory frequency in GLMM-1. Intercepts were allowed to vary by block (random effect). Bold values represent statistically significant effects (*** p-value <0.001; ** < 0.01; * < 0.05).

	Analysis of deviance (χ^2)		
	Herbivory frequency	Leaf density	Canopy cover
Control	–	–	–
N	**12.91*****	0.1149	1.108
P	0.50	–	–
K	3.23	**4.9364***	2.582
NP	3.15	–	–
NK	**22.50*****	**2.9697***	0.135
PK	2.25	–	–
NPK	0.05	–	–

diversity of herbivores in *B. microphylla* is most likely explained by the low palatability of leaves (i.e., low N content) when compared to deciduous plant species that dominate the plant community (e.g., *Nothofagus* sp.) (Diehl et al., 2008; Nacif et al., 2021).

Overall, herbivory frequency was low (0.05 ± 0.006; mean $\pm$ SE) but consistent with values reported for other evergreen shrub species of the community (Nacif et al., 2021). As stated above, generalist herbivores tend to avoid low palatable leaves in favor of more nutritional leaves produced by alternative plant species (Nacif et al., 2021). The addition of N fertilizer further accentuated this pattern by decreasing the herbivory frequency by 41% when compared to the control plots ($\chi^2 = 12.9$, $p < 0.01$; Table 1, Fig. 3). However, as shown by the significant N × K interaction detected ($\chi^2 = 22.5$, $p < 0.01$; Table 1), herbivory reduction mediated by nitrogen addition was only evident when potassium was not added to the experimental plots (Table 1; Fig. 3). Finally, we found no effect of phosphorus addition on herbivory patterns (Table 1), most likely because this nutrient was not limited in our study site (Diehl et al., 2008).

Previous studies suggest that the effects of nutrient enrichment on plant–animal interactions, rather than direct interactions, might be explained by changes in foliar traits and microhabitat characteristics (Borer et al., 2014; Lind et al., 2017). Accordingly, our GLMM analyses showed that leaf density had a marked negative effect on herbivory frequency ($Z = -6.1$, $p < 0.01$; Table S1, Fig. 4a), which in turn was highly influenced by nitrogen and potassium addition in opposite directions
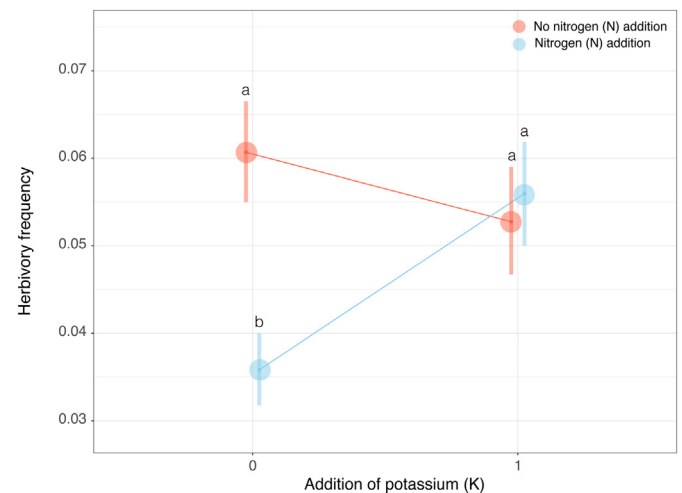

Fig. 3. Impact of fertilizers (N–K) on the herbivory frequency of *Berberis microphylla*. Note that phosphorus addition was not included as it did not influence the frequency of herbivory. Points indicate the estimates of GLMMs while error bars show the estimated Standard Errors (SE). Different letters indicate statistical differences (*p-value* < 0.05) among treatments after applying a Tukey post-hoc contrast.
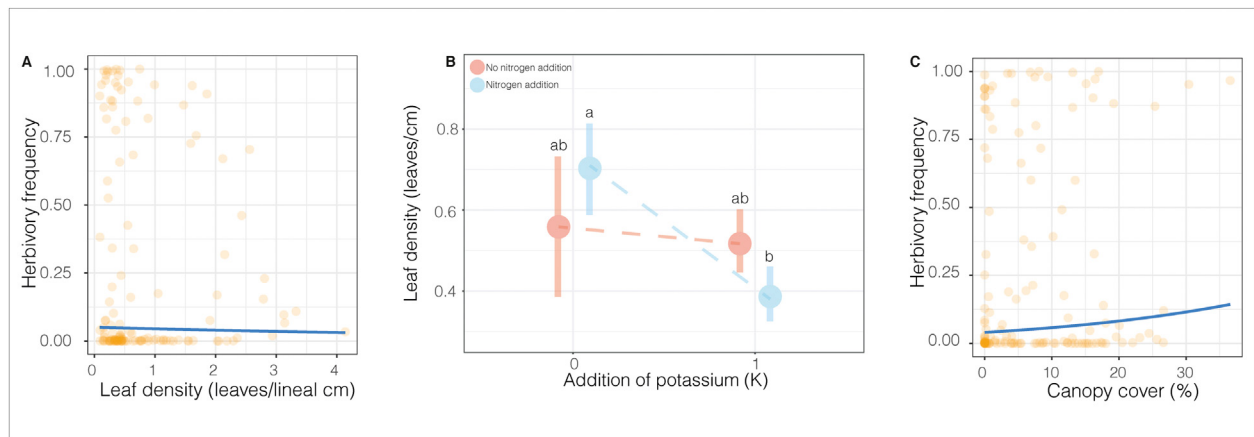
Fig. 4. *Panel A*: Relationships between leaf density and the herbivory frequency (i.e., the proportion of damaged leaves) resulting from the fitted GLMM. *Panel B*: Impact of nitrogen and potassium amendment (N:K) on leaf density. Points indicate the estimates of GLMMs while error bars show the estimated Standard Errors (SE). Different letters indicate statistical differences (*p-value* < 0.05) among treatments after applying a Tukey post-hoc contrast. Note that phosphorus addition was not included as it did not influence the frequency of herbivory. *Panel C*: Relationship between the canopy cover and the herbivory frequency resulting from the fitted GLMM.

(Table 1, Fig. 4b). On the one hand, individuals located in N-fertilized plots exhibited small leaves growing at high densities (N-fertilized plots: 0.70 ± 0.11 leaves/cm vs. nonfertilized plots: 0.56 ± 0.17 leaves/cm; Table 1), as shown by the negative correlation among leaf density and leaf size (*Spearman's r = −0.45, p < 0.001*; Fig. 2). As reported in previous research, the reduced herbivory detected in N-fertilized plots suggests that herbivorous insects might be avoiding small (and high dense) leaves, in favor of larger leaves (and low dense) as they are usually more palatable (Stiling and Moon, 2005). Alternative nonexclusive explanations cannot, however, be discarded; for example, nitrogen enrichment might promote a more efficient synthesis of nitrogenous anti-herbivory compounds such as alkaloids, which would discourage herbivore foraging (Mattson, 1980). Alternatively, a high foliar N content might reduce herbivory frequency if herbivores are able to meet their nutritional requirements at low per capita consumption rates.

On the other hand, our results show that potassium enrichment counteracted the negative effect of nitrogen addition on the herbivory frequency, as shown by the similar values in the NK-fertilized (0.056 ± 0.006) and the nonfertilized plots ($0.061 \pm 0.006, p > 0.05$; Fig. 3, Table 1). This counteracting effect of potassium when added simultaneously with nitrogen might be mediated by the reduced leaf density (and increased leaf size; Fig. 2) observed in these NK plots (Table 1, Fig. 4b). As discussed above, herbivores preferentially selected individuals with sparse large leaves (NK-fertilized: 0.39 ± 0.06 leaves/cm vs. N-fertilized: 0.70 ± 0.11 leaves/cm, $p < 0.001$; Fig. 4b; Table 1), most likely for optimizing their energy uptake from more palatable leaves (Mattson, 1980). In addition, if the increase in leaf size was not accompanied by an increase in photosynthetic capacity with NK addition, then the production of defensive compounds (e.g., phenolics, terpenes) could be hampered and foliar herbivory promoted (Herms, 2002; Glynn et al., 2003).

Unexpectedly, nutrient addition did not influence canopy cover (Table 1) as would be expected if canopy-dominant species of the community (e.g., *Nothofagus* sp., *Lomatia hirsuta*, *Embothrium coccineum*) increased their growth rate in response to the addition of limiting nutrients (Magnani et al., 2007; Thomas et al., 2010). However, canopy cover was still relevant to mediating herbivory patterns of *B. microphylla*, since individuals located under high canopy cover showed higher levels of herbivory ($Z = 18.8, p < 0.01$; Table S1, Fig. 4c). Despite a lack of a clear general pattern, increased herbivory in shaded microhabitats has been found in other research (Lincoln and Langenheim, 1979; Shure and Wilson, 1993; Muth et al., 2008). This preference for shaded microhabitats by herbivorous arthropods could be due to *i*) lower predation risks and *ii*) lower stressful abiotic conditions

(e.g., more stable temperatures, higher humidity). Alternative explanations may rest on the response of key plant trait responses to modified microhabitats (Lincoln and Mooney, 1984; Henriksson et al., 2003; Muth et al., 2008). Accordingly, canopy cover was positively correlated with leaf size (Spearman's r = 0.50, p < 0.001) and negatively correlated with leaf density (Spearman's r = −0.29, p < 0.001, Fig. 2), which was previously shown to also increase the herbivory frequency.

4. Conclusions

Here, we demonstrate that soil nitrogen enrichment entails marked bottom-up effects on herbivory patterns of an important shrub species of the Patagonian woodlands, yet these effects are diluted when potassium is added simultaneously. Potassium, usually disregarded as a limiting nutrient in woodlands, has an interesting role in the regulation of nitrogen impacts on ecosystem functioning (Sardans and Peñuelas, 2021). Our results further suggest that the impact of multiple nutrient additions on the interaction patterns between herbivores and *B. microphylla* might be mediated by changes in leaf density and leaf size. In conclusion, our results show that woodland eutrophication modifies antagonistic plant–animal interactions, ultimately affecting key ecosystem functions such as herbivory. We expect similar effects in other temperate woodlands hosting plant communities adapted to nutrient-limited soils (Verheyen et al., 2012; Carvalheiro et al., 2020). Buffering woodland eutrophication effects driven by human activities seems therefore essential to maintain important ecological functions associated with antagonistic plant–animal interactions and avoid ecosystem dysfunctioning.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151608.

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