

Research

Herbivory, intraspecific trait variability and back to herbivory

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Ungulate herbivory is a key driver of leaf trait syndromes and defense strategies at the ecological and macroevolutionary levels. Herbivory should also cause short-term evolution within plant populations, but few studies have experimentally tested this prediction. We set out to experimentally assess the plastic and heritable effects of contemporary history of ungulate herbivory on 1) leaf trait syndromes at the intraspecific level, and on the defense traits, 2) leaf size and 3) size of spines.

We measured leaf traits (specific leaf area, leaf dry matter content, leaf nitrogen concentration and leaf mechanical resistance) in populations of six grass and four woody legume species that had grown under contrasting pressures of domestic ungulate herbivory for at least 40 years. We then performed a common garden experiment, measuring the same traits. Next, we performed cutting experiments on the plants grown in the common garden to measure leaf trait plasticity. In the woody species, we also measured the length of spines and the leaf size (average area per leaf), in the field and in the common garden experiment.

We found that in grasses field herbivory over decades produced a displacement toward more acquisitive leaf trait syndromes. Most of these changes appeared largely due to plasticity. In woody legume species, in contrast, herbivory produced a displacement towards more conservative leaves, longer spines and smaller leaves, with the trait differences between histories of herbivory being mostly hereditary. These results point out that vertebrate herbivory can lead to evolutionary processes at contemporary time scales even in long-lived perennial plant species, but the direction of such change depends on the life form of the species. These divergent effects could depend on the predominant anti-herbivore strategies in the different life forms (tolerance versus resistance).

Keywords: acquisitive syndrome, Argentina, conservative syndrome, evolutionary ecology, Fabaceae, herbivory, leaf traits, Poaceae, spines

Introduction

Since herbivory has shaped plant evolution over millions of years (Agrawal 2007, Charles-Dominique et al. 2016), it is logical to assume it must also drive their evolution over contemporary timescales (one to hundreds of generations). However, few studies have explicitly linked the presence of herbivores to actual evolutionary



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responses within plant populations; these studies are typically observational (but see Agrawal et al. 2012, Turley et al. 2013, Fitzpatrick et al. 2015) and focus on short-lived herbaceous plants. Our understanding of which plant traits evolve in the face of herbivory at contemporary timescales is thus limited.

Herbivory is the key pressure in the evolution of plant defenses, both resistance (the ability to reduce or avoid damage) and tolerance (the ability to reduce the fitness cost due to damage) (Núñez-Farfán et al. 2007). In addition, herbivory selects for plant functional attributes (Díaz et al. 2001, Moreno García et al. 2014) related to different rates of resource uptake, retention and release, which in turn affect different ecosystem processes (Díaz et al. 2004). A set of leaf traits (e.g. specific leaf area, SLA; leaf dry matter content, LDMC; leaf nitrogen content per mass unit, N_{mass}; force to tear, F,) is known to be informative of these differences (Lambers and Poorter 1992, Reich et al. 1997, Díaz et al. 2004, Wright et al. 2004, Onoda et al. 2011, Kitajima et al. 2012). Most of the variation in such traits can be summarized in a single acquisitive-conservative axis. The position of any plant species on this axis is related to its resource use strategy (Wright et al. 2004), with a gradient from 'fast' or 'acquisitive' species, species with a fast recovery of foliar investment and fast turnover of matter and energy, associated with high SLA and N_{mass} and low LDMC and F_t, to 'slow' or 'conservative' species with opposite attributes, associated with slow return on investment. SLA and N_{mass} are intimately (and positively) related to the net assimilation rate (Wright et al. 2004, Shipley et al. 2006, Onoda et al. 2017). On the other hand, leaf structural resistance (here measured as F_r) and LDMC (as a proxy of the cell wall volume to cytoplasm volume ratio) are positively related to leaf lifespan (Wright et al. 2004, Onoda et al. 2011, Kitajima et al. 2012).

Vertebrate herbivory has been shown to favor the dominance of the acquisitive over the conservative syndrome in many situations, and in particular at high generalist herbivore densities (Westoby 1989, Díaz et al. 1992, 2001, Cingolani et al. 2007, Moreno García et al. 2014, Firn et al. 2017). Plant communities dominated by these more acquisitive syndromes, in turn, tend to be less susceptible to change due to grazing or browsing because their plant species are less susceptible to damage (McNaughton 1979, Díaz et al. 2001, Cingolani et al. 2005). At the intraspecific level, higher chronic levels of vertebrate herbivory have been shown to select for plant populations with more acquisitive leaf syndromes (Oesterheld and McNaughton 1988, Westoby 1989, Rotundo and Aguiar 2008, Lind et al. 2012). On the other hand, more conservative leaves are less palatable, and therefore more resistant to herbivory (Grime et al. 1996, Pérez-Harguindeguy et al. 2003, Kurokawa et al. 2010). Additionally, spines, which can vary in form and size fairly independently from leaves (Grubb 1992) are known to be effective against mammalian herbivores by reducing the bite-size of the browsing animal, thereby reducing food intake and driving the animal to move away to seek more rewarding targets (Cooper and Owen-Smith 1986, Cooper and Ginnett 1998, Shipley 2007). In spiny species, the leaf size acts jointly with spines in reducing the effectiveness of browsing animals, i.e. the smaller the leaves and the larger the spines, the less rewarding will be the target (Cooper and Owen-Smith 1986). Therefore, spinescence and leaf size are considered resistance traits. Depending upon the browsing activity, spine production may have a fitness cost (Gómez and Zamora 2002) and their size and amount might be induced or relaxed (Milewski et al. 1991, Gadd et al. 2001, Gómez and Zamora 2002). Overall, spines evolution seems to be driven by the browsing pressure from ungulates (Milton 1991, Charles-Dominique et al. 2016).

Little is known about the simultaneous effect of vertebrate herbivory on the contemporary evolution of leaf syndromes and defenses in long-lived species. For grasses, under chronically intense grazing, where biomass loss is unavoidable, increasing tolerance should be the only viable strategy (Rosenthal and Kotanen 1994, Garrido et al. 2016). Additionally, tolerance to herbivory is often associated with high growth rates and high photosynthetic capacity (Rosenthal and Kotanen 1994, Agrawal 2000, Tiffin 2000), i.e. a more acquisitive syndrome. As a consequence we hypothesized that, for grasses, chronic intense grazing should lead to 1) more acquisitive phenotypes (through genetic change and/or phenotypic plasticity). For woody legumes, whose spines are effective against ungulates, we expected that chronic intense browsing should 2) have no effect on leaf syndrome but lead to 3) larger spines and 4) smaller leaves.

To test our hypotheses we worked with ten species from two groups with documented ecological and evolutionary links with ungulates: C4 Poaceae (Bouchenak-Khelladi et al. 2009) and woody Fabaceae (Charles-Dominique et al. 2016, Tomlinson et al. 2016). First, we measured leaf traits (SLA, LDMC, F_t and N_{mass}), as well as the length of spines and the average area per leaf (LA), in woody legumes, in populations growing under contrasting long-term densities of domestic ungulates in the field (residents). We then grew individuals from the seeds of such populations in a common garden experiment (descendants), and measured the same traits in them in order to assess the transgenerational effects of herbivory. Finally, we simulated herbivory in cutting experiments on the descendants in order to evaluate leaf trait plasticity.

Material and methods

Study system and sampling

Our study site was located in central Argentina, at the southern extreme of the South American Gran Chaco (ca 31°18′–31°32′S, 65°23′–65°32′W). The area sampled is approximately 25 km N to S, and 10 km E to W. For further description of the study site see Gorné et al. (2020). Land use, mostly logging and livestock grazing at different intensities over centuries, has produced a gradient from this primary forest to communities with less tree coverage and more bare soil (Cabido et al. 1994).

We focused on six C4 perennial grasses (Aristida mendocina, Gouinia paraguayensis, Neobouteloua lophostachya, Sporobolus pyramidatus, Leptochloa crinita, Leptochloa pluriflora) and four woody legumes (Prosopis flexuosa, Prosopis torquata, Senegalia gilliesii and Vachellia aroma), all common in the Argentine Chaco (Cabido et al. 1994). These species are consumed by livestock, with some of them even preferred and thus traditionally used for fodder (Catan and Degano 2007, Karlin et al. 2013, Silvetti 2015, Martínez et al. 2016). The four woody legumes are typically 3-10 m tall, and have compound bipinnated leaves and woody (stipular or caulinar) spines. Vachellia aroma and P. torquata develop straight stipular spines (according to the classification from Charles-Dominique et al. 2016); P. flexuosa has similar spines but developing from axillary buds, and Senegalia gilliesii develops paired hooked epidermal spines at the top of a single-node short specialized branch forming a bifurcated thorn.

We selected between 39 and 49 sampling points per species. Sampling points of each species were separated from each other by at least 100 m to minimise cross-pollination both for grasses (Bateman 1947, Griffiths 1950) and woody species (Vilardi et al. 1988, Saidman and Vilardi 1993, Butcher et al. 1998, Casiva et al. 2004). Three individuals of a given species with < 20 m distance from each other correspond to a single sampling point for grasses. We collected a leaf sample from each one of these three individuals at each sampling point. For woody species, a single mature individual constituted a sampling point. We collected two leaf samples of the outer canopy, from each one of these individuals (i.e. in each sampling point), one sample from the lowest three terminal branches and one sample from the three terminal highest reachable branches (higher than 1.5 m). On these branches, having discarded the apical 3 cm, we also measured (at mm precision) the length (SL) of each one of the spines at the 3rd and 5th nodes, starting from apical to proximal. Each leaf sample, both for grasses and woody legumes, consisted of at least three leaves.

For each species, we placed half of the sampling points at sites with high local livestock density (hereafter heavy herbivory) for at least four decades, and the other half at sites with no or very low livestock density during the same period (hereafter light herbivory). The main livestock are goats and cattle, which make highly heterogeneous use of the landscape. Even when goats prefer to browse and cattle prefer to graze, both species consume both resources. Goats make a more even, diverse and stable use of fodder along the year, but cattle also browse when grass is scarce (Dicko and Sikena 1992, Papachristou et al. 2005, Ouédraogo-Koné et al. 2006). Many properties are not fenced and the fences are often not well maintained; as a consequence, the home ranges of different flocks overlap. Cattle are more constrained by fencing, and owners tend to use the land in a less communal way than goat herders (Silvetti 2012), but paddocks are often very large. As a result, different goat flocks and cattle herds overlap and their grazing does not necessarily correspond to property limits, and the stocking rates are not homogeneous within paddocks. Because of these reasons, the density of animals per unit surface tends to be a poor indicator of the actual herbivory pressure experienced by different sectors within the paddock. In practice, the intensity of grazing and/ or browsing (hereafter grazing) is inversely proportional to the distance from water points, defining a highly disturbed zone concentric to the water point (Lange 1969, Senft et al. 1983, Andrew 1988), becoming increasingly less grazed as the circles become wider. In the semiarid Chaco, realized livestock density decreases exponentially with distance to the water point; most of the activity concentrates within 500 m from the water point (Macchi and Grau 2012). In order to identify areas subjected to heavy grazing for several decades, we identified water points in the field that, according to aerial photographs, have been in existence at least since 1970. Heavy herbivory sampling points were located in these zones, from 25 to 400 m from the water points, and always in areas with more than 50% bare soil or on livestock tracks. Light herbivory sampling points were located in two zones: The first was the conservation area 'Parque Natural Chancani' which is fenced and under effective protection since 1966 (Cabido and Pacha 2002). The second light herbivory area was a wide strip of land between the Sierra de Pocho mountain range and the road RP51, 12 km south from Chancaní. This strip is in a good state of conservation at least since 1979 (Hoyos et al. 2013) and has contained no water point since at least 1970.

To ensure the selected individuals had grown under herbivory pressures similar to those experienced at present, we selected the woody individuals as small as possible but following two restrictions: first, they had to produce seeds to perform the common garden experiment; second, they had to have branches currently exposed to browsing (below 1.5 m) and branches above browsing height (above 1.5 m). The diameter at the base was measured for all individuals and no size difference between heavy herbivory and light herbivory groups was found. Additionally, on the basis of previous studies, we were able to estimate an age for the selected individuals of V. aroma and P. flexuosa, based on stem diameter. The selected individuals of V. aroma were approximately between 13 and 35 years old (Bravo et al. 2006), and those of *P. flexuosa* were approximately between 16 and 40 years old (Perpiñal et al. 1995).

Soil analyses

Soil properties can affect leaf traits (Ordoñez et al. 2009), so at each sampling point we collected a soil sample (four sub-samples 0–10 cm deep). Soil samples were pooled by the closeness of the sampling points, grouping all samples into 22, ten from light herbivory and twelve from heavy herbivory. The soil properties measured were: organic matter content (Walkley and Black technique, Sparks et al. 1996), total nitrogen content (elemental analyser), extractable phosphates (Olsen technique, Sparks et al. 1996), silicates (Wei-min et al. 2005), pH, conductivity, cation concentration (Ca²⁺, Mg²⁺, Na⁺ and K⁺) (atomic absorption spectroscopy), compaction at the soil surface (pocket penetrometer), at 0–5 and 5–10 cm (dynamic cone penetrometer) and volumetric soil water

content at 3 inches. Then, we compared sites with different histories of herbivory. A T-test was performed for each variable by comparing the mean for each history of herbivory. p-values were corrected according to Hommel (1988).

Common garden

We collected seeds from all sampling points (2013–2014) and produced seedlings in pots in a common garden at the National University of Córdoba, Argentina. For woody species seeds from a given sampling point were all from a single mother plant, the same whose leaves and spines were measured. In the case of grasses, seeds from a sampling point were collected from several random plants and pooled. Seeds from grass species were sown from October to December 2014 and woody species from January to February 2015. For all pots we used commercial potting soil. Pots were 0.79 l (10 cm diameter, 10 cm height) for grasses and 2.4 1 (10 cm diameter, 30 cm height) for woody legumes. Pots containing a given species were placed together in the same block. Within each block, the origin of the seeds (sampling points) was completely randomized, so the history of herbivory (heavy versus light) was randomized too. During the growing season, all pots were weeded periodically and kept free from insect herbivores by applying insecticide. All plants were watered with sprinklers, making sure that they received enough water but did not become waterlogged. We obtained seedlings from 28 to 40 sampling points per species. A year after germination, we measured leaf traits of two to four plant individuals from each sampling point for each species and performed the cutting experiments with two grass (N. lophostachya and L. pluriflora) and two woody species (P. flexuosa and P. torquata). At that time grasses had reached maturity and reproduced sexually, but woody species were still juvenile.

Cutting experiment

The cutting experiment was performed between March and May 2016, in the common garden, on two grass species (N. lophostachya and L. pluriflora) and two woody species (P. flexuosa and P. torquata). For the two grass species, from the six individuals produced from each sampling point, we selected the four most similar in size. From these four, the two closest in size were assigned one to control and the other to moderate cutting treatment. The third was assigned to severe cutting and the last one was only used for leaf trait measurement. The cutting treatments (both moderate and severe) consisted of cutting each tiller at half of its total length. At that moment, a leaf sample was obtained from each plant to measure leaf traits. Plants under the severe cutting treatment were re-cut 2-3 weeks after the first time. In this second cutting, all biomass growth above the first-cut marks was removed. Plants being measured and treated were kept away from the common garden and from the control group for two days to avoid any response induced by volatile compounds released as a result of cutting (Turlings and Tumlinson 1992).

Forty-two to forty-nine days after the initial measurement all plants were re-measured. The leaves measured at the end of the experiment were leaves grown after the treatments and initial measurement.

For the woody species, from the six individuals produced from each sampling point, we selected the four most similar in size. We assigned the three treatments randomly to the individuals and the remaining one was only used for leaf trait measurement. First, leaf samples were taken from each individual to measure leaf traits. We then applied the cutting treatment, which consisted of cutting the main branch and up to three branches if the individual had ramified, below the third node from apical to proximal (7 cm approx.). This treatment simulates the type of damage produced by livestock browsing (Gowda 1996). Plants under the severe cutting treatment were re-cut 15-18 days after first-cut, with the same intensity but in different branches, if any. Final measurements were made 71-77 days after the onset of the experiment. At that moment, only P. torquata had produced new leaves in enough individuals to allow the analysis of trait response to treatment. Specifically, 40 individuals (12 control, 13 moderate cutting and 15 severe cutting) from the heavy herbivory and 36 (13 control, 11 moderate cutting and 12 severe cutting) from the light herbivory sites had produced new leaves. Prosopis flexuosa had produced new leaves in only three individuals.

Trait measurements

For each species of woody legume growing in the field, all leaf samples were collected and processed, and spines were measured, in a single trip (S. gilliesii and P. flexuosa in December 2013 and *V. aroma* and *P. torquata* in February 2014). For the six grass species, leaf samples were collected and processed during four field trips, two in March 2013, one in April 2013 and one more in May 2014; this information was included in the analysis as a random factor. In the common garden experiment, leaf samples were taken from 23 February to 29 April 2016 but with less than a week difference within each species (this is without considering the second measurement in the cutting experiment). For the species not used in the cutting experiment, two to four (depending on availability) randomly selected individuals per sampling point origin were measured for leaf traits. Each leaf sample consists of at least three healthy, fully expanded, nonsenescent leaves, weighing at least 50 mg in total. These leaf samples were collected in plastic bags, water-sprayed and kept in the fridge until processing. At the same time, we measured the length of the spines (SL) in two to four individual plants per sibling family (of the woody legumes), in one spine of each 4th and 5th subapical node (from distal to proximal) in all the terminal branches.

From each leaf sample, processed independently, we measured LA (mm²), SLA (mm² mg⁻¹), LDMC (proportion), F_t (N mm⁻¹) and N_{mass} (%) (Pérez-Harguindeguy et al. 2013), with the following exceptions: 1) in woody species all measures were performed on the leaflets (excluding rachis),

 F_{τ} could not be measured because they are too small to be handled into our measuring device, LA was recorded only for woody species, 2) N_{mass} was measured (elemental analysis) for a subset of samples ranging all sampling points both in residents (field) and descendants (common garden) for N. lophostachya, L. pluriflora, P. flexuosa and P. torquata.

Because SLA, LDMC, N_{mass} and F_{t} are not independent from each other (Hu et al. 2015, Gorné et al. 2020) we computed the position of each individual (within each species in each growing condition) on a major axis of covariation among the leaf traits measured in all species of the same growth form (i.e. SLA, LDMC and F, for grasses; SLA and LDMC for woody species). We ran a PCA for each species in each condition (field or common garden) on the basis of their (log-transformed and standardized) trait values; we then considered the position of each individual on the first principal component (PC1), and thus a 'synthetic' indicator of its leaf syndrome. PC1 correlated positively with SLA and negatively with LDMC (and F, when applicable). Thus the higher the PC1 value, the more acquisitive the leaf trait syndrome. N_{mass} was not included because we were able to measure this trait only on a subset of the species and samples.

Data analysis

For all species, in both conditions (field and common garden), we compared means of leaf traits (SLA, LDMC, F, N_{mass}) as well as PC1, the spine length (SL), and the average area per leaf (LA) as a function of the history of herbivory (light versus heavy). PCA to compute PC1 was performed with the *prcomp* function from the R-package stats. To assess the effect of herbivory history on these variables we applied linear mixed-effects models by using the *lme* function from the R-package nlme (Pinheiro et al. 2020). For grasses, species and herbivory history (and their interaction) were included as fixed-effect factors. In the analysis of resident plants, the field trip and sampling point were included as nested random-effect factors. In the analysis of descendant plants, the sampling point was included as a random-effect factor. For woody species, the sampling point was included as a random-effect factor. In resident plants, species, history of herbivory and position in the canopy (low or high), as well as interactions, were included as fixed-effect factors. In descendant plants, species, history of herbivory and interactions were included as fixed-effect factors. The variables SL and LA were transformed to $\log_{10}(SL+1)$ and log₁₀(LA) respectively to meet normality and homogeneity of residuals. In the analysis of N_{mass} for descendants, only one plant per sampling point was measured. As a consequence the random-effect factor 'sampling point' is no longer needed (their effect would be the same as the error), so we performed a generalized least squares linear model (gls function, nlme package) instead of a mixed-effects model.

In the cutting experiments, we analyzed the relative change of each leaf trait ($\Delta'X = (X_{t1} - X_{t0}) \times X_{t0}^{-1}$) but LA, and the change in PC1 (Δ PC1 = PC1_{t1} – PC1_{t0}) as a function of species, history of herbivory, treatment and interactions.

The sample point was included as a random-effect factor in all these analyses. As in previous analyses, we analysed grasses and woody species separately.

In all cases, a backward and forward model selection procedure was performed to select the best fixed-effect structure according to the Akaike information criterion (AIC). For all analyses, assumptions were tested and logtransformation or variance modeling were included when necessary. When a posteriori contrasts from a given model were necessary to test a specific hypothesis, these contrasts were computed with the function *glht* from the multcomp R-package (Hothorn et al. 2008) and the p-value was corrected by Bonferroni (Bonferroni 1936). While no exact equivalent to the R² of linear regression exists for mixedeffects and generalized least squares linear models, a pseudo-R² for the selected models was computed to summarize the amount of variance explained (Nakagawa and Schielzeth 2013). We report the marginal pseudo- R^2 ($R^2_{GLMM(m)}$), which includes the variance explained by fixed factors (Nakagawa and Schielzeth 2013, Johnson 2014) by using r2glmm R-package (Jaeger 2017).

Finally, we tested the correlation between LA, SL and the other leaf traits at the sibling family level in the common garden experiment. To do that, we computed the average value of the $\log_{10}(LA)$, $\log_{10}(SL+1)$, PC1, SLA and LDMC for each sibling family. Then, we computed the Pearson correlation coefficient at the sibling family level. All analyses were performed in R ver. 4.1.2 (<www.r-project.org>).

Results

Herbivory and leaf trait syndromes

In the case of grass species growing in the field, chronic heavy herbivory was associated with a more acquisitive syndrome (Fig. 1A–B, Supporting information). This effect was found across all species and traits, with the exception of N_{mass} in *N. lophostachya* (Fig. 1B) and F_{t} in *G. paraguayensis* (Supporting information), in which no difference was found between histories of herbivory.

Among the grass descendants growing in the common garden, this general pattern remained for most species but *G. paraguayensis* and *L. crinita*. However, the differences were statistically significant only for *A. mendocina* and *S. pyramidatus* (Fig. 1C, Supporting information). In *N. lophostachya* and *L. pluriflora*, descendants from populations with different herbivory histories showed no significant difference (Fig. 1C–D, Supporting information). These two species showed a plastic response towards a more acquisitive syndrome when subjected to the cutting treatments (Fig. 2A, Supporting information), but this plasticity was not related to herbivory history.

For woody species in the field, we found mixed effects of the history of herbivory on leaf traits. PC1 values for plants from the heavy history of herbivory sites were lower (more conservative syndrome) than those from plants from the light

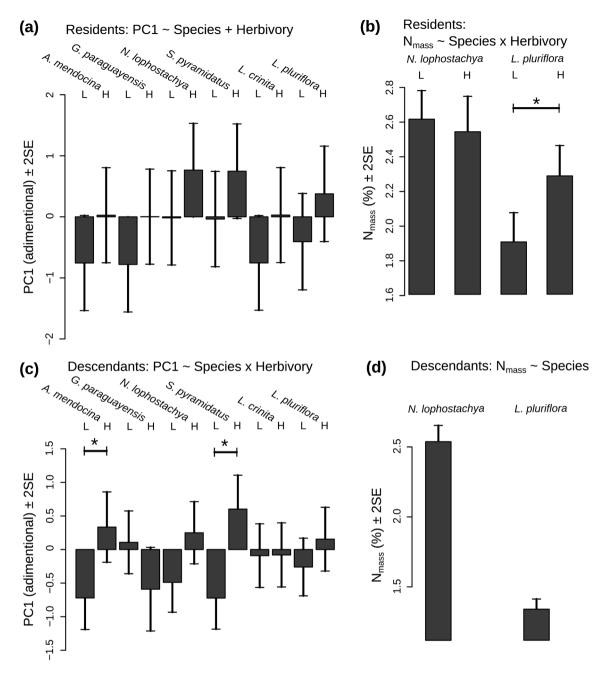


Figure 1. Relationship between leaf traits and history of ungulate herbivory among grass species. Bars in each panel show results from the best model according to AIC; the fixed-effect part of each model is also shown at the top of each panel. Asterisks show significant differences between groups when interaction among factors was retained in the final model. PC1 is the score on the first principal component of PCA analysis including SLA, LDMC and F_t . L: light history of herbivory; H: heavy history of herbivory. (A) PC1 of residents (plants growing in the field) as a function of species and history of herbivory. (B) N_{mass} of residents as a function of species and history of herbivory. (C) PC1 of descendants growing in the common garden, as a function of species and history of herbivory. (D) N_{mass} of descendants as a function of species; history of herbivory was not retained in the model selection procedure.

history of herbivory sites (Fig. 3A, Supporting information). This pattern was mostly driven by the effect of the history of herbivory on SLA (Supporting information). For N_{mass} we found the opposite pattern in relation to herbivory (Fig. 3B, Supporting information). We also found an effect of the position in the canopy, where leaves located lower in the canopy were more acquisitive than those located higher

(Fig. 3A, Supporting information). While not statistically significant, *V. aroma* showed a different trend in heavy herbivory history, with apparently no difference in leaf traits between positions in the canopy.

For woody descendants in the common garden, the pattern was more consistent among all traits (SLA, LDMC and N_{mass}). In general, descendants from the heavy history of

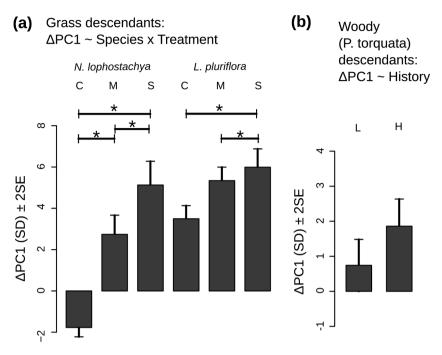


Figure 2. Effect of simulated herbivory (cutting experiment) on leaf traits of descendants growing in a common garden. PC1 is the score on the first principal component in PCA including SLA, LDMC and F_t for grasses, but only SLA and LDMC for woody species. Bars in each panel show result from the best model according to AIC; the fixed-effect part of each model is also shown at the top of each panel. Asterisks show significant differences between groups when interaction among factors was retained in the final model. C: cutting treatment control; M: cutting treatment moderate; S: cutting treatment severe. L: light history of herbivory; H: heavy history of herbivory. (A) Change in PC1 in grasses the cutting experiment (Δ PC1) in grasses as a function of species and treatment; history of herbivory was not retained in the best model according to AIC. (B) Change in PC1 after the cutting experiment (Δ PC1) in a woody legume as a function of the history of herbivory, treatment was not held in the best model according to AIC. There were not enough individuals of *P. flexuosa* with new leaves at the end of the experiment to be included in the analysis.

herbivory sites had lower values of PC1 (more conservative) than woody descendants from the light herbivory sites (Fig. 3C, Supporting information). This was mostly driven by the effect of the history of herbivory on the LDMC (Supporting information), but also a significant effect of the history of herbivory was found on the SLA for P. flexuosa (Supporting information). For N_{mass}, we also found a general effect of the history of herbivory: descendants from sites with a heavy history of herbivory had less N_{mass} (Fig. 3D, Supporting information). In the cutting experiment, only P. torquata produced new leaves in enough individuals to analyze plasticity. In the analysis of ΔPC1, herbivory history, but not cutting treatment, was retained in the model selection (Fig. 2B, Supporting information). In the analyses of the Δ 'SLA and Δ 'LDMC, cutting treatment was retained in the final models but a posteriori comparisons did not show differences among treatment levels (Supporting information).

Herbivory and defenses

The heavy herbivory leads to longer spines both, in the field and in the common garden experiment (Fig. 4). In the field, this difference was more marked in the lower branches (Fig. 4A, Supporting information). This effect of a heavy herbivory history on the length of spines was maintained

in the descendants grown in the common garden free from herbivory (Fig. 4C, Supporting information). However, the difference between histories of herbivory was larger in the field-grown individuals than in their garden-grown descendants (1.42 mm versus 1.12 mm).

The average area per leaf (LA) showed a similar pattern to spine length, but in the opposite direction, both in the field and in the common garden experiment. Leaves of individuals from heavy herbivory sites were smaller than leaves of individuals from light herbivory sites (Fig. 4B, D, Supporting information). Additionally, the leaf size was mostly independent of the other leaf traits. In the case of plants growing in the common garden experiment, free from livestock browsing, and at the maternal family level, only *V. aroma* showed a significant (positive) correlation between LA and PC1 (Table 1).

Discussion

Herbivory and leaf trait syndromes

Depending on growth form, chronic heavy herbivory selects for different leaf trait syndromes in plant populations. In accordance with our expectations, heavy herbivory during decades in the field consistently led to a more acquisitive

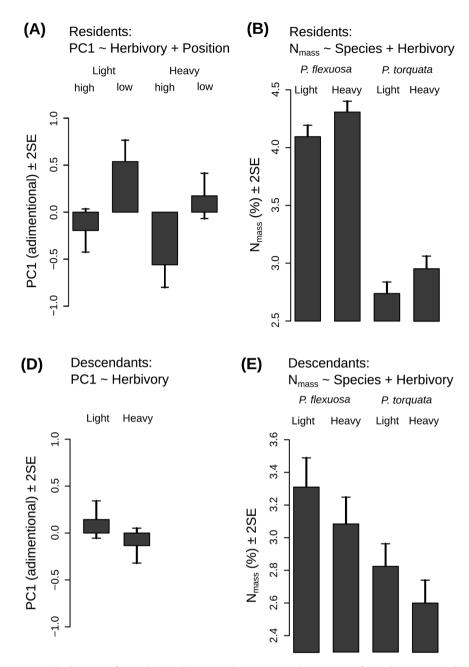


Figure 3. Relationship among the history of ungulate herbivory and position in the canopy of woody species with leaf traits. Bars in each panel show results from the best model according to AIC; the fixed-effect part of each model is also shown at the top of each panel. PC1 is the score on the first principal component of PCA analysis including SLA and LDMC. (A) PC1 of residents as a function of the history of herbivory and position; species was not retained in the best model according to AIC. (B) N_{mass} of residents as a function of species and the history of herbivory; the position was not retained in the best model according to AIC. (C) PC1 of descendants growing in the common garden, as a function of the history of herbivory; species were not retained in the best model according to AIC; there were no different positions in descendants. (D) N_{mass} of descendants as a function of species and the history of herbivory; there were no different positions in descendants.

syndrome in the grasses analyzed (Fig. 1A–B). Similar findings have been reported by ecological field studies of other systems (Oesterheld and McNaughton 1988, Rotundo and Aguiar 2008). Here, by performing a common garden experiment with plants grown from seeds, we found that these differences are partially heritable. Many of the differences

between histories of herbivory attenuate or disappear under common garden conditions (Fig. 1C–D). These differences between resident and descendant patterns suggest an important role of phenotypic plasticity of leaf traits in response to current herbivory in the field. The plastic change toward a more acquisitive syndrome in the face of heavy herbivory

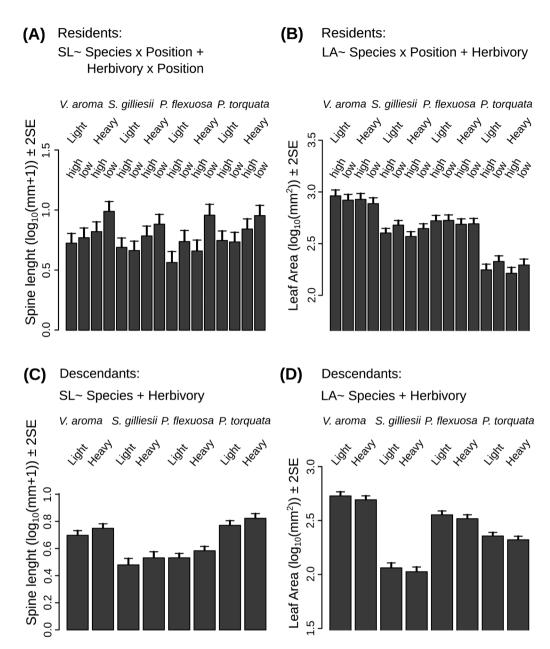


Figure 4. Relationship among the length of spines (SL) and the average area per leaf (LA) with history of ungulate herbivory and position in the canopy in woody species. Bars in each panel show results from the best model according to AIC; the fixed-effect part of each model is also shown at the top of each panel. (A) Length of the spines ($\log_{10}(mm+1)$) of residents as a function of species, history of herbivory and position in the canopy according to the best model (AIC criteria). (B) Average area per leaf ($\log_{10}(mm^2)$) of residents as a function of species, history of herbivory and position in the canopy according to the best model (AIC criteria). (C) Length of the spines ($\log_{10}(mm+1)$) of descendants as a function of species and history of herbivory according to the best model (AIC criteria). (D) Average area per leaf ($\log_{10}(mm^2)$) of descendants as a function of species and history of herbivory according to the best model (AIC criteria).

Table 1. Correlation Pearson coefficient between the acquisitive–conservative leaf syndrome (PC1) and the average area per leaf ($\log_{10}(LA)$) or the spines length ($\log_{10}(SL+1)$), for each woody legume species. p-value in brackets.

Species	PC1-LA	PC1-SL
V. aroma	0.387 (0.020)	-0.382 (0.022)
S. gilliesii	0.200 (0.307)	-0.343 (0.073)
P. flexuosa	0.134 (0.410)	0.009 (0.954)
P. torquata	0.108 (0.537)	-0.088 (0.617)

was confirmed by the responses of *N. lophostachia* and *L. pluriflora* in the cutting experiment: both of them showed a change toward a more acquisitive syndrome when subjected to cutting (Fig. 2A). A plastic response in leaf traits toward a more acquisitive syndrome as a consequence of biomass loss has been frequently observed in graminoids (Nowak and Caldwell 1984, Oesterheld and McNaughton 1988, McNaughton 1992, Bråthen and Odasz-Albrigtsen 2000). The plastic response remained similar between the

two herbivory histories. So, in our study system, the history of heavy grazing did not modify plasticity itself.

In the overall pattern for grasses, with the heavy herbivory leading to a more acquisitive syndrome, *L. crinita* and *G. paraguayensis* growing in the common garden were the only exceptions. The latter is peculiar in the sense that it grows in the comparatively shady understorey, almost always protected from herbivores, even in heavily grazed sites. It typically grows protected by the spiny stems of shrubs like *S. gilliesii* or among the cladodes of *Opuntia sulphurea*. This suggests that *G. paraguayensis* is particularly intolerant to herbivory and the individuals sampled in the field in heavily grazed sites were in a microsite protected from grazing.

In contrast to grasses, in woody legumes, heavy herbivory led to a more conservative syndrome and, these changes were predominately heritable rather than plastic. The observed effect of history of herbivory on leaf traits (in SLA and N_{mass}) for woody residents (i.e. in the field) are exactly the opposite to those reported by Lind et al. (2012) for the spicebush Lindera benzoin being browsed by white-tailed deer Odocoileus virginianus. Also, an effect of the position in the canopy (low versus high) was found for PC1 (as well as LDMC and SLA). This effect was additive without interaction with the history of herbivory, suggesting it is not a plastic response to current herbivory. Moreover, this pattern is consistent with the general trend of variation for SLA within (nongrazed) individuals reported for trees (Poorter et al. 2009). Additionally, no plastic response was detected in *P. torquata* in the cutting experiment. We found an effect of herbivory history on the change in leaf traits but this was unrelated to cutting treatment (Fig. 2B); this perhaps is due to differences in phenology between populations under both regimes, but not a plastic response to disturbance.

However, a plastic physiological response can explain the inconsistency between the $N_{\rm mass}$ pattern between the observations in the field and in the common garden. Du Toit et al. (1990) found that heavy browsing in the field leads to higher leaf nitrogen content in stands of *Acacia nigrescens*. They proposed that severe pruning by browsing ungulates reduces inter-shoot competition for nutrients, and as a result, it increases the concentrations of nutrients in remaining shoots. In that way, the current herbivory in the field would lead to an increase in the leaf nitrogen content. At the same time, the absence of browsing in the common garden experiment would have allowed seeing the non-plastic effect of herbivory. This last effect was consistent across all the measured leaf traits in the common garden experiment.

An important aspect to consider is the indirect effect of ungulate herbivory on plant traits via direct effects on soil processes. While effects of soil properties on leaf traits at the intraspecific level are often obscured by an overlap of soil gradients with gradients in other factors such as precipitation, temperature, disturbance, altitude or latitude, there is some evidence that leaf traits tend to show a more acquisitive syndrome under more fertile soil conditions (Walters and Gerlach 2013, Hu et al. 2015; but see Kichenin et al. 2013). In our case, ungulate herbivory did not appear to affect leaf

traits indirectly via soil changes. The differences found in soil parameters at sites with different histories of herbivory (Supporting information) indicate that the soil at sites with a light history of grazing provides better conditions for plant growth (less compaction and more organic matter content, other parameters including N–P–K content were similar) than soil at sites with a heavy history of grazing. These findings are consistent with previous studies in the same area (Cuchietti et al. 2017). Remarkably, resident plants from sites with light history of herbivory, where soils are better, have lower nitrogen content and, in the case of grasses, a more conservative leaf trait syndrome.

Herbivory and defenses

We found that chronic heavy browsing leads to heritable larger spines. Previous studies have shown that contrasting browsing activity may lead to differences in the size of spines among stands (Goheen et al. 2007) as was found in the field measurements in the present work. Here, we went further to find that sustained livestock pressure modified the expression of mechanical resistance in these populations and their descendants even at the contemporary time scale.

Additionally, we found evidence of a contemporary and heritable effect of browsing on the leaf size. Cooper and Owen-Smith (1986) showed that interspecific differences in the leaf size in conjunction with spines affect the preference of browsing ungulates for different plant species. Here we show that even at the intraspecific level the leaf size seems to be under natural selection pressure by browsing herbivores, so that chronic heavy browsing leads to heritable smaller leaves.

Leaf trait syndrome and defenses

Previous reports show the growth of acquisitive ecotypes of Themeda triandra was less affected by clipping (Oesterheld and McNaughton 1988). Different mechanisms have been found for tolerance in grasses, including reallocation of resources from root to shoot, changes in the age composition of foliage (younger foliage usually exhibits greater photosynthetic capacity; Caldwell et al. 1981), compensatory photosynthesis and delayed senescence in remaining foliage (Nowak and Caldwell 1984). A lower carbon investment per unit of leaf area could be advantageous in frequently disturbed (grazed) sites. A higher SLA should lead to a higher net assimilation rate per unit of mass invested in leaf and should involve a cheaper way to recover the total plant leaf area. This result is consistent with the relationship between the history of herbivory and leaf traits in grass descendants, where both species showed the same trend: descendants from a heavy history of herbivory tended to be more acquisitive than descendants from a light history of herbivory. If being more acquisitive leads to a higher tolerance, this would lead to a natural selection process which will produce a more acquisitive population at sites with heavy herbivory. Previous studies have shown that tolerance can evolve at contemporary time scales among grass species. Didiano et al. (2014) found

some heritable differences in tolerance of *Festuca rubra* (Poaceae) after 21 years of rabbit exclosure. In contrast, Rotundo and Aguiar (2008) and Turley et al. (2013) found no change in the tolerance for *Poa ligularis* and *Rumex acetosa* growing under contrasting grazing regimens during < 50 years and ca 30 years, respectively.

In woody legumes, on the contrary, herbivory led to a more conservative leaf trait syndrome. Additionally, cutting did not affect leaf traits (Fig. 2B). However, tolerance could be reached by means of reallocating resources from root to shoot (Stowe et al. 2000), as previously observed for seedlings of *Prosopis glandulosa* under clipping treatment (Weltzin et al. 1998). Indeed, a compensatory response in terms of vegetative biomass is common in woody species (Du Toit et al. 1990, Dangerfield and Modukanele 1996, Haukioja and Koricheva 2000, Lehtilä et al. 2000, Fornara and du Toit 2007; but see Bergström and Danell 1987, Edenius et al. 1993).

Additionally, as observed by Gadd et al. (2001), Leimu and Koricheva (2006) and Fornara and du Toit (2007) the trade-off between resistance and tolerance does not appear to be a universal rule. Tolerance and resistance traits may coexist if browsers trigger and maintain a positive feedback loop in which trees are continually investing in regrowth (tolerance), and if the plant's physical resistance traits are not nutritionally costly and are long-lived (Herms and Mattson 1992, Fornara and du Toit 2007). Spines fit these last requirements, and the energetic cost may decouple from growing costs because of the climatic conditions (semiarid). Any factor limiting growth more than it limits photosynthesis, such as moderate drought, will increase the carbon pool available for allocation to secondary metabolism with little or no trade-off with growth (Herms and Mattson 1992). In the woody species studied here, more defended individuals displayed more conservative leaf syndrome. However, this covariation seems to be due to the simultaneous effect of herbivory on both sets of variables and not because of a tradeoff or genetic correlation between both. Indeed, in three of the four species, the leaf size and spines length were independent of the acquisitiveconservative syndrome (Table 1).

Eco-evolutionary feedback

Intraspecific phenotypic changes may be as relevant to ecosystem processes as species sorting (Des Roches et al. 2018) or abiotic factors (Fitzpatrick et al. 2015). Our findings suggest that the introduction of livestock and water points, in addition to their well-known effects on the vegetation composition and physiognomy, drive evolutionary changes which could in turn modify ecosystem processes and community dynamics. A positive eco-evolutionary feedback by which chronic heavy herbivory leads to more tolerant and palatable plant populations, which in turn should be more consumed, has been observed previously in grasses (Cargill and Jefferies 1984, Oesterheld and McNaughton 1988, Westoby 1989) and woody legumes (Du Toit et al. 1990). Our results for grass species were in accordance with this, but those on woody legumes were the opposite. This suggests that

ungulate herbivory may be driving opposite evolutionary trajectories at the same time and in the same place, depending on the plant growth form and, probably, the ability to resist herbivory through mechanisms different from fodder quality. Previous studies have shown that tolerance and resistance can be selected simultaneously (Gadd et al. 2001, Fornara and du Toit 2007).

Conclusion

We conclude that livestock herbivory at contemporary time-scales (decades) produces heritable intraspecific changes in leaf traits. The direction of such changes depends on the growth form. In perennial grasses, livestock herbivory produces a displacement toward a more acquisitive leaf trait syndrome. While some transgenerational differences in traits were found, these herbivory-induced changes seem to be largely due to phenotypic plasticity. In woody legumes, the effect was the opposite, livestock herbivory produces a displacement toward a more conservative syndrome. Also, the length of spines increases due to livestock herbivory, as well the leaf size was reduced. In spite of the longer generation time of woody species, these differences, both in leaf traits and in spines, are mostly heritable.

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Author contributions

Lucas D. Gorné: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (equal); Software (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Sandra Díaz**: Conceptualization (equal); Funding acquisition (lead); Methodology (equal); Project administration (lead); Supervision (lead); Writing – review and editing (equal).

Data availability statement

Data are available from the Zenodo Digital Repository: https://doi.org/10.5281/zenodo.5541900 (Gorné 2022).

Supporting information

The supporting information associated with this article is available from the online version.

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