

## The induction of spinescence in European holly leaves by browsing ungulates

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### Abstract

Evidence is presented which suggests that the spinescence of leaves of European holly, *Ilex aquifolium*, deters feeding by ungulates and is induced by browsing. Spinescence decreased as leaf size increased; hence, spinescence may be achieved by reducing adult leaf size. Holly shrubs with very spiny leaves were browsed less often than less spiny shrubs. In the absence of browsing ungulates during a one year period, the spinescence of leaves of holly shrubs significantly decreased. Browsed shrubs exhibited reduced annual shoot growth, increased branching, and produced smaller leaves with high spinescence. The regrowth on browsed branches of holly trees was characterized by increased leaf spinescence relative to unbrowsed branches. Hence, the induced response was localized, thereby reducing the ability of browsing ungulates to exert selective pressures on holly trees.

### Introduction

Thorns, prickles, spines and trichomes have often been considered a defence against herbivory (Ehrlich & Raven 1967; Grubb 1992; Myers & Bazely 1991; Pollard 1992). Studies of natural and experimental herbivory have shown: increased thorn length in *Acacia senegal* (Seif el Din & Obeid 1971), *A. depreanlobium* (Young 1987) and *A. seyal* (Milewski et al. 1991); stimulation of spine production in *Opuntia stricta* (Myers 1987); an increased number of prickles in *Rubus trivialis* (Abrahamson 1975), *R. vestitus* (Bazely et al. 1991; Gibson et al. 1993) and *Aralia spinosa* (White 1988); and increased trichome density in *Urtica dioica* (Pullin & Gilbert 1989) and *Cnidioscolus texanus* (Pollard 1986).

Despite the scarcity of experimental evidence, spiny leaves are widely believed to function as a deterrent against browsing (e.g. Crawley 1983). Supnick (1983) reported that the major function of leaf spines in American holly, *Ilex opaca*, is to deter herbivory rather than to assist in the convective cooling of leaves. However, this hypothesis was not substantiated by Potter & Kimmerer (1988) who found no relationship between the degree of spinescence and feeding damage: the thick glabrous cuticle and tough leaf mar-

gin were more important than spines in deterring the edge-feeding caterpillar *Hyphantria cunea*.

While the spines of holly leaves are set too far apart to deter insect herbivores (Givnish 1979; Potter & Kimmerer 1988), they have been found to interfere with browsing by ungulates (Cooper & Owen-Smith 1986). Spines would be more effective against vertebrates than invertebrates because of their size relative to the herbivore, and hence vertebrate herbivores should exert a stronger and more consistent selection on leaf spines than invertebrate herbivores (Grubb 1992). However, one unresolved issue is whether the induction of spinescence is a systemic response or a localized response. A localized response does not prevent browsing ungulates from feeding elsewhere on the same tree and it reduces their ability to exert selection pressure at the whole plant level (Karban & Myers 1989).

European holly, *Ilex aquifolium*, like American holly, exhibits higher spinescence on lower branches, and leaves with almost smooth edges occur in the upper canopy (Crawley 1983; Supnick 1983). European holly is browsed by mammals (Peterken 1966; Peterken & Lloyd 1967), but Potter & Kimmerer (1988) found little evidence of browsing on American holly. In *Ilex aquifolium*, heavy browsing maintains low mound-forming

shrubs and it appears to be accepted that after browsing or pruning the leaves become more spiny (Peterken & Lloyd 1967).

In this study I have used a combination of observations and field experiments to determine changes in holly leaf spinescence following herbivory by browsing ungulates. Supnick (1983) and Potter & Kimmerer (1988) used spine number per leaf as their measure of spinescence. However, this variable may not give an entirely reliable description of spinescence. Therefore I have extended the measure of leaf spinescence to include both spine number and mean spine length per leaf relative to leaf size. I have examined variation in leaf characteristics in relation to spinescence in browsed and unbrowsed holly shrubs and branches of holly trees. Specifically, I addressed the following questions: 1) Are markedly spiny holly shrubs less affected by browsing ungulates than shrubs showing less leaf spinescence? 2) Do leaf characteristics change after exclusion of ungulates? 3) Within a tree, do leaves from browsed branches differ from those of unbrowsed branches?

## Materials and methods

### Study sites

The study was conducted at two localities in the Cantabrian Range, Asturias province, northern Spain, where holly is a common understorey tree. Pome Forest is located in Picos de Europa National Park (950 m a.s.l., 43° 16' N, 4° 59' W). It is a beech forest with *Ilex aquifolium* and *Crataegus monogyna* present as undergrowth trees. Grandiella (Sierra del Aramo, 1050 m a.s.l., 43° 14' N, 5° 55' W) is a pasture zone with second-growth forest dominated by *Ilex aquifolium*. *Crataegus monogyna*, *Sorbus aria* and *S. aucuparia* are also present.

Cattle (*Bos taurus*), goats (*Capra hircus*) and roe deer (*Capreolus capreolus*) feed on holly in Pome forest and cattle and horses (*Equus caballus*) in Grandiella. The incidence of browsing is higher in the Pome forest, and many holly bushes at this site are small, and heavily-browsed, and the density of browsers is higher than at Grandiella. At Pome Forest the holly trees are scarcely browsed because of the height of the canopy. In contrast, at the Grandiella site, small shrubs are rare but most of the trees have a dense mass of branches in the lower sections which are heavily browsed. In order to examine forest regeneration a 1-ha enclosure

was built in July 1993 in an open-canopy area of Pome Forest. The growth of the shrubs during 1994 in the presence (outside the enclosure) and in the absence (inside) of browsing ungulates was determined.

### Browsing pressure and leaf spinescence

I measured holly leaf characteristics during November and December 1993 in Grandiella and during the same months in 1994 in Pome Forest. Several different sampling designs were used.

In order to examine the relationship between spinescence and leaf size and the effect of spinescence on the incidence of consumption by browsing ungulates, ten browsed shrubs were sampled outside the enclosure at the Pome Forest site. These shrubs were selected to span the range of variation in leaf size and spinescence. Two leaves from each of five current-year twigs were randomly sampled per shrub and they were used to examine the relationship between leaf area and spinescence. These shrubs were also examined for vertebrate herbivory, which usually consists of the removal of the current year's growth. Approximately 100 twigs were examined per shrub and were divided into browsed and unbrowsed categories. The frequency of branching in the current year's growth shoots was also recorded. The frequency of branching may depend on herbivory because regrowth on browsed branches is only possible by axillary buds. These shrubs were then divided into two groups of four shrubs differing markedly in their spinescence: shrubs with a high density of spines and shrubs with a low density of spines. The two other shrubs showing intermediate values for spinescence were discarded from the analyses.

To determine if the absence of browsing ungulates had an effect on leaf spinescence, small shrubs (<70 cm tall) were selected inside and outside the enclosure at Pome Forest from homogeneous open-canopy beech forest. Seven shrubs were randomly selected inside the enclosure and another seven shrubs were selected outside the enclosure. To limit errors from pseudo-replication, shrubs outside the enclosure were located far from each other and selected to span all the range of variability in density of leaf spines. There is no reason to suspect that before enclosure shrubs differed in size or spinescence (i.e. there were no differences in shading). Five branches were randomly selected from each shrub and the twigs of the current year's growth were sampled. The dry weights of the shoot and leaves were determined; three leaves

per twig were randomly sampled and the characteristics of these leaves were determined.

In order to examine the effect of browsing ungulates on leaf characteristics of holly trees, six trees were randomly selected at the Grandiella site. The samples were collected from the bottom branches, where they were within reach of browsing ungulates. I selected three browsed and three unbrowsed branches per tree at the same level (about 1 to 1.5 m height). After selection of one browsed branch showing the current year's regrowth, the nearest undamaged branch was selected as an unbrowsed branch. Within the branches (both browsed and unbrowsed), I considered leaf position on branches. The terminal portion of the branches corresponded to the current year's growth (upper position) and the part of the branches nearest the centre of the tree represented growth of the previous year (lower position). Four leaves were sampled per position (i.e. from upper and lower end of branches) and their characteristics were determined. Leaves on upper positions were 6-month old and leaves on lower position were 1.5-yr old. However, in some browsed branches the leaves on the lower position might have been older than 1.5-yr, since an ungulate may have browsed more than the current year's growth (if the growth of two years was removed, leaf age at lower position was 2.5-yr old), or regrowth after browsing may have been delayed more than one year.

To compare the regrowth on browsed branches with annual growth on control branches, seven trees were randomly chosen at the Grandiella site and the current year's growth was collected for three control and three browsed branches per tree.

#### *Measurements and rationale for the development of an estimate of spinescence*

To determine the leaf water content, both the fresh and dry weight after oven drying (one week at 50 °C) were determined for each individual leaf to the nearest 0.1 mg. The number of spines was counted and three spines (2nd, 3rd and 4th) of the right margin were measured with a caliper from the base to the tip to the nearest 0.1 mm and a mean spine length per leaf was calculated. To estimate the specific weight of the leaves, two to three leaf discs (7 mm diameter) were cut from each leaf and their dry weight determined. Leaf area was measured by digitizing the leaf contours with an image analysis program for leaves from the ten shrubs sampled outside the fence. This generated an

estimate of leaf size that was statistically independent of leaf weight.

Leaf water content was obtained by subtracting the dry weight from the fresh weight of individual leaves. The specific weight was estimated as mg per mm<sup>2</sup> based on the mean weight of dried leaf discs. Leaf spinescence was calculated as the number of spines multiplied by mean spine length and divided into dry leaf weight. Spine lengths varied from 2 to 15 mm and leaves showing larger spine number (20–25) were big and frequently exhibited short spines. Despite their high spine number, browsing ungulates ate them (pers. obs.). However, spiny leaves frequently exhibited 8–12 spines 10–15 mm long which usually grew at a right angle to the leaf surface. Thus, spine length together with spine number was considered a better estimate of leaf defence against browsing ungulates than spine number alone. Finally, the relative importance of spine number and length depends on leaf size. If spine length and number are kept constant it is predicted that they should defend a small leaf better than a large one.

#### *Statistical analyses*

I used contingency table analyses and performed G-tests in order to determine the effect of spinescence on the incidence of browsing and the effect of browsing on branching. To avoid possible lack of statistical independence by pooling all twigs across the shrubs, the correlation between the proportion of twigs browsed per shrub and mean spinescence per shrubs was also calculated.

Nested ANOVAs were employed to test differences in leaf characteristics for shrubs growing inside and outside the fence, and differences in annual growth between control and previously browsed branches. Since there were several twigs per shrub and several leaves per twig, shrub factor was nested within treatment (inside vs outside the enclosure). This design reduces the d.f. for testing the effect of herbivory (Tables 1 and 2) but permits the use of several leaves per tree.

The sampling structure for leaf characteristics of tree branches corresponded to a mixed multifactorial ANOVA. Tree and branch were random factors and browsing and position were fixed factors. Branch factor was nested within browsing × position. The estimates of the *F*-ratios were calculated according to the rules summarized by Underwood (1981). ANOVAs were performed using SPSS statistical package. Means are given ± 1 SD.

Table 1. Characteristics of current year growth (mean  $\pm$  SD) for unbrowsed twigs from shrubs growing inside the enclosure and unbrowsed twigs from browsed shrubs outside the enclosure. F values correspond to a nested design nesting shrubs (d.f. = 12) within treatment (inside vs outside), and error term (d.f. = 57) was twigs within shrub

	Outside (N = 35)	Inside (N = 35)	$F_{(1,12)}$	P
Number of leaves	4.8 $\pm$ 1.1	6.0 $\pm$ 1.2	9.75	0.009
Fresh weight of leaves (g)	1.48 $\pm$ 0.49	2.66 $\pm$ 0.84	28.75	<0.001
Dry weight of shoot (g)	0.081 $\pm$ 0.037	0.172 $\pm$ 0.10	11.59	0.005

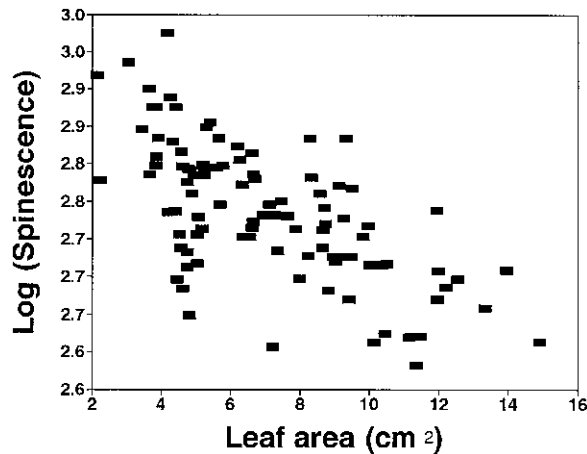


Figure 1. Relationship between log-transformed spinescence (number of spines by mean spine length per mg of leaf dry weight) and leaf size (leaf area).  $r^2 = 0.440$ ,  $N = 100$ ,  $P < 0.0001$ .

## Results

### Spinescence and leaf size

The spinescence of *Ilex aquifolium* leaves decreased significantly with leaf size (leaf area);  $r^2 = 0.440$ ,  $N = 100$ ,  $P < 0.0001$ , for the log-transformed spinescence (Figure 1).

### Spinescence and incidence of browsing on shrubs

Shrubs with high spinescence (mean values per shrubs  $> 0.670$ ) and low spinescence ( $< 0.570$ ) differed significantly in the incidence of twig consumption by browsing ungulates,  $G_{(1)} = 29.1$ ,  $N = 719$ ,  $P < 0.001$ . While 43.3% ( $N = 335$ ) of the twigs from low spiny shrubs were browsed, only 17.4% ( $N = 384$ ) of the twigs from highly spiny shrubs were affected. Furthermore, the relationship between the proportion of twigs browsed per shrub and mean leaf spinescence

per shrub, approached significance ( $r = -0.612$ ,  $N = 10$ ,  $P = 0.059$ ).

### Influence of browsing on growth and spinescence of shrubs

Current year growth was greater for unbrowsed shrubs within the enclosure than those outside, after one year of protection from herbivory (Table 1). Unbrowsed shrubs produced more leaves and larger shoots. Branching during current year growth was greater in browsed shrubs growing outside the enclosure than in unbrowsed shrubs (35% of branches for browsed and 4% for unbrowsed shrubs;  $G_{(1)} = 20.89$ ,  $N = 200$ ,  $P < 0.001$ ). The characteristics of the leaves from unbrowsed shrubs differed significantly from those of shrubs growing outside the enclosure (Table 2). Leaves produced during 1994 in the absence of browsing ungulates were larger, exhibited lower spinescence and had a higher water content.

### Spinescence in regrowth branches from browsed trees

The annual growth of branches (shoot and leaves) from trees at Grandiella was greater for control (unbrowsed) branches ( $1.238 \pm 0.338$  g dry weight,  $N = 21$ ) than the regrowth of previously browsed branches ( $0.759 \pm 0.394$  g dry weight,  $N = 21$ ;  $F_{(1,41)} = 17.874$ ,  $P = 0.0001$ ). However, the annual growth of the shoots did not differ among treatments ( $0.218 \pm 0.096$  g dwt for unbrowsed and  $0.208 \pm 0.107$  g dwt for browsed branches,  $F_{(1,41)} = 0.117$ ,  $P = 0.734$ ).

Taking into account the leaf traits in browsed and unbrowsed branches, browsing significantly decreased leaf dry weight, especially on regrowth branches (Table 3, Figure 2). Leaf dry weight also showed significant differences among branches within tree and browsing treatment. Leaf specific weight and water content varied significantly among branches and

Table 2. Leaf characteristics (mean  $\pm$  SD) of shrubs growing inside and outside the enclosure. Dry weight in g and water content is the proportion of fresh weight. F values correspond to a nested design nesting shrubs (d.f. = 12) within treatment (inside vs outside), and error term (d.f. = 197) was leaves within shrub

	Outside (N = 105)	Inside (N = 105)	$F_{(1,12)}$	P
Leaf dry weight	0.127 $\pm$ 0.035	0.162 $\pm$ 0.029	24.13	<0.001
Leaf spinescence	683.3 $\pm$ 153.8	605.0 $\pm$ 95.8	9.04	0.011
Leaf water content	0.548 $\pm$ 0.022	0.570 $\pm$ 0.024	9.84	0.009

among trees. The specific weight of leaves at the lower position, which corresponded to older leaves, were significantly higher, especially on browsed branches (Table 3, Figure 2). Leaves from the upper position of browsed branches (regrowth) were significantly smaller and showed higher spinescence, while those from the lower position exhibited higher specific weight. However, the effect of browsing depended on branch position and showed variation among branches within tree and among trees for specific weight, water content and spinescence (other significant interaction terms in Table 3).

## Discussion

This study supports the widely stated view that the leaf spines of hollies function to deter vertebrate herbivory and is in contrast to previous results of a study in which holly spines did not affect invertebrate herbivory (Potter & Kimmerer 1988). The spinescence of holly leaves was increased by reduced size. The allometric relationship between spine growth and leaf growth subsequently resulted in increased spinescence of smaller leaves. Spines are relatively longer in small leaves (they approach their maximum size in leaves 50% of mean adult size) and remain closer to each other than spines on larger leaves. Leaf size reduction in holly after browsing was observed but not quantified by Peterken & Lloyd (1967). Leaf size reduction appears to be a common response to herbivory or experimental defoliation (Gibson et al. 1993 in *Rubus vestitus*; Cornelissen 1993 in *Castanopsis fargesii*; Honkanen & Haukioja 1994, in *Pinus sylvestris*).

One explanation for leaves on regrowth shoots of holly being smaller is that they were younger, since they were produced after browsing and had less time to grow. However, most of the regrowth shoots were produced the year after browsing and showed the same

phenology as control shoots (pers. obs.). Furthermore, at sampling dates (winter), leaves from regrowth shoots had characteristics of adult leaves other than leaf size: leaf water content and specific weight did not differ from corresponding values for current year leaves of control branches. Leaf toughness increases and water content declines with leaf age in American holly (Potter & Kimmerer 1986). Therefore, the observed reduction in leaf size may not be considered an effect of leaf age.

It appeared that basal leaves of browsed branches were sometimes older than leaves at the same position in control branches because regrowth from axillary buds was not initiated immediately after branch browsing. This may explain the characteristics of old leaves (lower) on browsed branches, since they exhibited the highest dry weight and specific leaf weight and the lowest water content (Figure 2, Table 3).

There are several lines of evidence which suggest that spinescence in *Ilex aquifolium* leaves could evolve under selective pressures exerted by browsing ungulates: (1) Spinescent species make up a small proportion of the genus *Ilex* (13 out of 400 to 800 species). They are predominantly evergreen and occur in forests dominated by deciduous trees. In these forests there is a scarcity of available foliage other than holly leaves during a good part of the year (Grubb 1992). (2) Higher spinescence in *Ilex* species is maintained on leaves accessible to browsing ungulates. This includes leaves of seedlings, sucker shoots, lower branches and also young leaves (Grubb 1992; Peterken & Lloyd 1967; Potter & Kimmerer 1988; Supnick 1983; Trippi 1963, and present study). (3) Spinescent branches of holly shrubs were browsed less than less-spiny branches. Browsing ungulates consume holly leaves mainly during spring when leaves are still developing, their toughness is low and they have marginal teeth but not spines. At this phase of growth leaves have a high nutritional quality, as occurs in American holly (Potter & Kimmerer 1986). Nevertheless, it seems that the spinescence of

Table 3. ANOVA results from leaf characteristics in different browsing treatments and positions within branch. Significance is given at the Bonferroni-adjusted level. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$

Source of Variation	d.f.	Dry weight		Specific weight		Water content		Spinescence	
		SS	F	SS	F	SS	F	SS	F
Browsing <sup>a</sup>	1	372906.07	225.03 ***	11.94	8.51	11.22	12.52	1.18	9.92
Tree <sup>b</sup>	5	75967.84	0.51	73.26	4.43 **	79.19	11.59 ***	1.08	1.16
Position <sup>c</sup>	1	295680.97	10.91	34.43	468.52 ***	1.79	0.89	1.60	6.39
Browsing × tree <sup>b</sup>	5	8285.83	0.06	7.02	0.42	4.48	0.66	0.59	0.64
Browsing × position <sup>d</sup>	1	309347.30	29.58 *	10.14	39.36 *	0.63	1.01	0.83	14.34 *
Tree × position <sup>e</sup>	5	135463.98	2.22	0.37	0.14	10.00	5.04 *	1.25	3.46
Branch (browsing × tree)	24	712280.97	9.52 ***	79.37	26.99 ***	32.81	6.54 ***	4.46	7.14 ***
Pos. × bra. (brow. × tree)	24	293047.66	3.87 ***	12.40	4.22 ***	9.52	1.90 *	1.73	2.77 ***
Browsing × tree × position <sup>e</sup>	5	52297.23	0.86	1.29	0.50	3.13	1.58	0.29	0.80
Error	216	681578.60		26.46		45.15		5.85	
Total	287								

<sup>a</sup>Browsing × tree as error term.

<sup>b</sup>Branch (browsing × tree) as error term.

<sup>c</sup>Tree × position as error term.

<sup>d</sup>Browsing × tree × position as error term.

<sup>e</sup>Position × branch (browsing × tree) as error term.

older leaves reduces browsing in leaves representative of the current year's growth. Browsed shrubs and the branches in the lower sections of trees reduce annual shoot growth and increase branching. This results in a spiny mound-forming shrub in which there is a dense mass of spiny foliage which inhibits browsing by ungulates. (4) Holly shrubs subject to browsing by ungulates developed larger leaves with reduced spinescence one year after ungulate exclusion; in contrast, shrubs growing outside the enclosure maintained high spinescence. Hence, leaf characteristics depended not only on plant age or size but also on the incidence of browsing. However, this conclusion cannot be generalized due to absence of true replication in the experiment of ungulate exclusion.

The above responses provide the basis for a mechanism resulting in browsing ungulates exerting selection pressure on holly spinescence. However, there is evidence for an alternative hypothesis: spinescence in holly leaves could evolve under selection pressures other than that of browsing ungulates. (1) Branches accessible to browsing ungulates are also proximate to roots, the place of cytokinin synthesis, which mediates the rejuvenation response (Trippi 1990). Spinescence is a characteristic of juvenile phases of some *Ilex* species. Hence the spinescence of adult phases may have developed through neoteny (Grubb 1992). Heterophylly is a characteristic of some woody plants, in which the leaves from juvenile trees are more spines-

cent, toothed or lobed than leaves from adults plants (Kozłowski 1971). Rejuvenation or reversion to juvenile forms of adult phases may be induced by pruning, which causes development of adventitious buds in woody plants (e.g. apple and pear trees, ivy; Trippi 1990). These changes may also affect leaf size and be induced by several environmental factors: light intensity, defoliation and carbon balance (Trippi 1990). However, the reversibility of these phase changes is controlled by changes in gene expression (Durzan 1990), hence the capability to produce juvenile forms on regrowth may be controlled by other types of natural selection. (2) It may be argued that shoots growing from axillary buds (which are scarce in unbrowsed hollies) develop smaller leaves. This might be simply an ontogenetic effect independent of browsing. However, shoot growth did not differ among unbrowsed and browsed (axillary growth) branches of trees. The lower annual growth of browsed branches was due to their smaller leaves. (3) The response was not systemic; browsed branches of holly trees developed leaves with smaller size and higher spinescence than leaves growing in unbrowsed branches (the spinescence of unbrowsed branches did not differ from lower position -leaves produced before consumption of browsed branches- to upper position -leaves produced after the consumption of browsed branches- as should be expected in a systemic response). This is a localized response which may not reduce whole tree damage by ungulates,

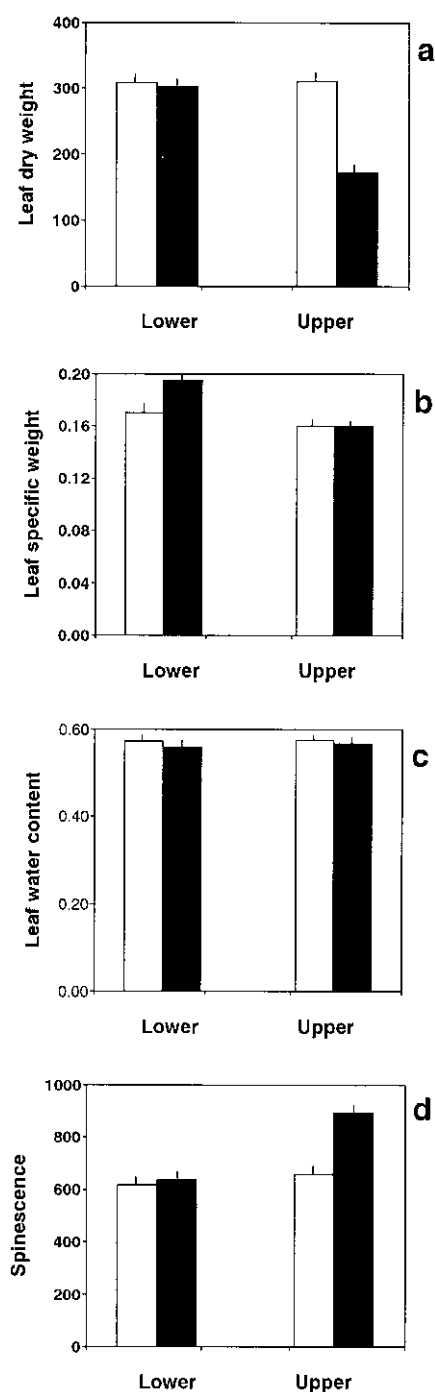


Figure 2. Effects of position on branch (upper, lower) and browsing (white bars are unbrowsed and black bars are browsed branches) on leaf traits. Leaf dry weight, mg (a), specific weight, mg mm<sup>-2</sup> (b), water content, proportion of fresh weight (c), and spinescence (d) of holly trees foliage. Mean values ± 1 S.E. and  $N = 72$  for each bar.

because they can feed elsewhere on the tree. Hence, the potential selective pressures exerted by ungulates are limited.

Differences between trees in the spinescence induced by browsing ungulates indicate phenotypic variation. However, it remains a challenge to determine whether the ability to produce spiny leaves after browsing was selected by browsing ungulates or rather is simply a physiological response depending on structural and developmental constraints which prevent browsing secondarily. It is very difficult to design an experiment to discriminate between the two theories because the leaves never regrowth from buds on the browsed shoot, they grow on axillary shoots.

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