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**Research paper**

# Leaf traits and insect herbivory levels in two Mediterranean oaks and their hybrids through contrasting environmental gradients

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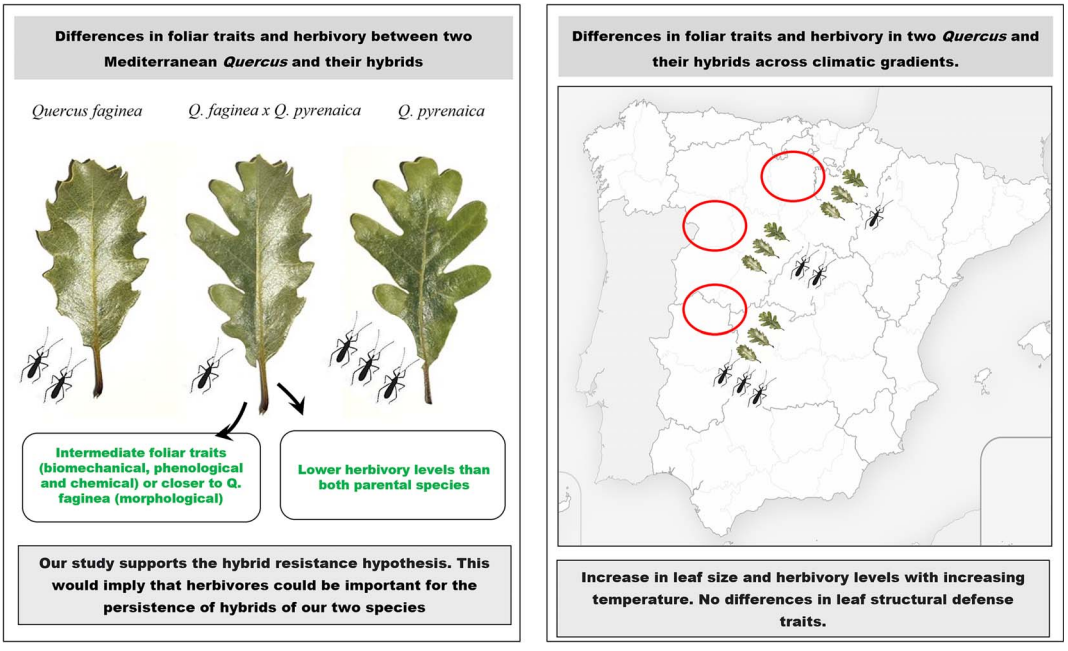
Insect herbivory has attracted enormous attention from researchers due to its effects on plant fitness. However, there remain questions such as what are the most important leaf traits that determine consumption levels, whether there are latitudinal gradients in herbivore pressure, or whether there are differences in susceptibility between hybrids and their parental species. In this work, we address all these issues in two species of Mediterranean *Quercus* (*Quercus faginea subsp. faginea* Lam. and *Quercus pyrenaica* Wild.) and their hybrids. Over 2 years, we analyzed leaf emergence and 11 leaf traits (biomechanical, chemical and morphological), as well as the levels of herbivory by insects in leaves of the three genetic groups in different locations distributed along a climatic gradient. The hybrids showed intermediate values between both species in leaf emergence, chemical traits and structural reinforcement. By contrast, they were more similar to *Q. faginea* in leaf size and shape. Despite their intermediate leaf traits, hybrids always showed lower losses by consumption than both parental species, which suggests that they possess inherent higher resistance to herbivores, which cannot be explained by their dissimilarities in leaf traits. Within each genetic group, differences in leaf size were the most important determinant of differences in herbivory losses, which increased with leaf size. In turn, leaf size increased significantly with the increase in mean annual temperatures across the climatic gradient, in parallel with herbivory losses. In conclusion, contrary to our expectations, hybrids maintained lower levels of herbivory than their parent species. Given the potential negative effect of leaf herbivory on carbon fixation, this advantage of the hybrids would imply a threat to the persistence of both pure species. More research is needed to elucidate possible alternative mechanisms that allow for maintaining species integrity in the absence of reproductive barriers.

## Graphical Abstract

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## Introduction

Insect herbivory has attracted enormous attention from researchers for decades due to its effects on plant fitness and, therefore, on the competitive relationships and species composition of plant communities. The losses of photosynthetic potential associated with the lost leaf area (LA) and the costs of investment in defenses entail negative effects on plant performance that can severely affect competitive capacity (Lehndal and Agren 2015, Wise 2023). However, the truth is that it is a still elusive topic that continues to raise significant questions about its ecological implications.

There is no unanimity, for example, on the foliar traits that explain the differences in herbivory levels. Different studies have revealed the existence of a wide range of foliar characteristics that can affect consumption levels, which can be assigned to three categories: phenological, chemical and biomechanical. It is generally accepted that aspects such as the timing of leaf emergence or the number of leaf flushes can lead to important differences in the final levels of herbivory (Boege and Marquis 2005, Pearse and Karban 2013). Regarding leaf chemical traits, secondary metabolites (particularly phenols) have traditionally been given a predominant role in defense against herbivores (Mithöfer and Boland 2012, Moreira et al. 2018). However, findings from other studies suggest the opposite (Carmona et al. 2011, Salgado-Luarte et al. 2023). Many studies have reported strong effects of leaf nitrogen (N) contents on herbivores preferences (Cronin et al. 2010, Loranger et al. 2012), while others have found only slight relationships between N and herbivory levels (Moreira et al. 2018, Ruíz-Carbayo et al. 2020). It is also a common finding that leaf traits that enhance leaf toughness (leaf mass per unit area, leaf thickness (LT), leaf tissue density (LD) and fiber content) confer resistance against herbivores (Kozlov et al. 2015, Muiruri et al. 2019). Finally, leaf size and shape have also been considered important determinants of herbivore activity (Novais et al. 2015, Higuchi and Kawakita 2019, Li et al. 2021), with, in most cases, a preference of herbivores for larger leaves with long petioles and a small number of lobes (NL) (Brown et al. 1991, Novais et al. 2015, Muiruri et al. 2019, Li et al. 2021).

There is also no consensus on the existence of trade-offs between different anti-herbivore defenses. Some authors have suggested negative associations between physical and chemical defenses (Eichenberg et al. 2015, Wang et al. 2016), while others have proposed that plants simultaneously allocate resources to several defensive traits, thus exhibiting a range of different combinations against their herbivores (Moles et al. 2013, Gianoli and Salgado-Luarte 2017).

Whatever the importance of each of these traits and the possible trade-offs, they are all known to be affected by environmental conditions (Pearse and Hipp 2012, Jamieson et al. 2017). Therefore, a fundamental objective in relation to the study of herbivory is to understand how climate (especially temperature and precipitation) can affect consumption levels in different environments (Lemoine et al. 2013, Hamann et al. 2021). Site differences in air temperature may affect timing of bud break, speed of leaf maturation and leaf chemical composition, which, combined with direct effects of temperatures on insect physiology, may determine the intensity of herbivory damages (Jamieson et al. 2012). Given the complexity of interactions that determine herbivory, there is no unanimity about the trends in herbivory associated with the temperature changes that accompany changes in latitude and altitude. Thus, while numerous studies confirm the latitudinal Herbivory-Defense Hypothesis, which proposes a decrease in herbivory with increasing latitude (Moreira et al. 2018, Nakamura et al. 2022), others have found higher herbivory at higher latitudes (Adams and Zhang 2009, Anstett et al. 2015), or no latitudinal gradients in herbivory (Moles et al. 2011, Moles and Ollerton 2016). Given the variety of responses, the effects of temperature on herbivory are difficult to predict, but previous research has revealed that, for evergreen Mediterranean species, herbivory damages tend to decrease with decreasing temperature across climatic gradients (González-Zurdo et al. 2016*a*), thus supporting the idea that higher temperatures contribute to increasing the strength of species interactions (Moreira et al. 2018) and that these temperature effects also occur under the relatively high temperatures typical of the Mediterranean climate.

Many different theories have also been proposed regarding the intensity of herbivory in hybrids and parental species and the possible role of herbivory in the maintenance of different plant species or in the selection of hybrid populations. Different studies have obtained inconsistent results, with higher herbivory in hybrids than in parental species ('Hybrid susceptibility hypothesis') (Mattson et al. 1996, Cuevas-Reyes et al. 2018), lower herbivory in hybrids ('Hybrid resistance hypothesis') (Boecklen and Spellenberg 1990), intermediate herbivory levels in hybrid plants ('Additive hypothesis') (Tovar-Sanchez and Oyama 2006; Pearse and Baty 2012) or no differences from both parents ('Hybrid no difference hypothesis') (Fritz et al. 1996, Cheng et al. 2011). This wide divergence in results for different species suggests that investigating the implications of herbivory on the fitness of hybrids probably requires case-by-case studies.

In the present work, we intend to address all these issues in two species of deciduous Mediterranean *Quercus* and their hybrids located in different enclaves distributed across a climatic gradient. The two species (*Quercus faginea* subsp. *faginea* Lam. and *Quercus pyrenaica* Willd) show clearly different morphological and ecophysiological traits (Mediavilla et al. 2018, San-Eufrasio et al. 2020). Although both species tend to present different distribution patterns, they coexist in numerous places and show frequent hybridization (Amaral-Franco 1997). There is a general idea that hybridization can pose a serious threat to species maintenance (Rhymer and Simberloff 1996). However, despite the apparent absence of reproductive barriers between our two species, they still appear as different pure species across wide areas. Traditionally, it has been considered that the persistence of parental species in the absence of reproductive barriers would be explained by the lower fitness of the hybrids, but the mechanisms by which this integrity is maintained are poorly known (Lepais and Gerber 2011, Leroy et al. 2020).

Herbivory is one of the stress factors that contribute to determining leaf-level $CO_2$ fixation, and the tree responses to herbivore pressure could be one important factor determining fitness. Accordingly, we first hypothesize that parental species should be less susceptible to herbivory than their hybrids. Secondly, we try to identify the foliar traits that could help explain the possible differences in herbivory between the three study groups (the two species and their hybrids). Third, we also postulate that herbivory rates should decrease as mean temperature decreases across gradients in latitude and/or

**Table 1.** Characteristics of the study sites. Climatic data were obtained for an average of 5 years (2019–23).

| | Warm zone | | | Intermediate zone | | | Cold zone | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sites** | **S** | **T** | **R** | **B** | **V** | **H** | **A** | **M** | **VP** |
| Geographic characteristics | | | | | | | | | |
| Longitude (W) | 5°57′ | 5°36′ | 5°42′ | 6°05′ | 5°54′ | 5°50′ | 3°46′ | 3°76′ | 4°21′ |
| Latitude (N) | 40°13′ | 39°59′ | 39°44′ | 41°36′ | 41°33′ | 41°33′ | 42°31′ | 42°57′ | 42°10′ |
| Altitude (m above sea level) | 636 | 287 | 422 | 776 | 690 | 691 | 999 | 947 | 770 |
| Climatic characteristics | | | | | | | | | |
| Mean annual temperature (MAT, °C) | 17.5 | 17.3 | 17.8 | 13.8 | 13.9 | 14.5 | 11.9 | 12.1 | 11.9 |
| Absolute maximum temperature (°C) | 38.0 | 41.5 | 41.7 | 38.1 | 38.9 | 40.1 | 37.9 | 36.5 | 38.8 |
| Absolute minimum temperature (°C) | −2.15 | −5.64 | −2.20 | −6.21 | −6.81 | −5.95 | −8.89 | −8.27 | −8.45 |
| Number of frost days per year | 6 | 15 | 6 | 42 | 51 | 47 | 77 | 66 | 78 |
| Mean annual precipitation (MAP, mm) | 939 | 726 | 650 | 535 | 463 | 482 | 482 | 520 | 390 |
| Summer precipitation (mm) | 98 | 69 | 62 | 49 | 63 | 74 | 38 | 37 | 54 |
| Dominant genetic group | Qp | Qp × Qf | Qf | Qp | Qp × Qf | Qf | Qp | Qp × Qf | Qf |

Qp, *Quercus pyrenaica*; Qf, *Quercus faginea*; Qp × Qf, hybrids.

altitude, as seen before in other *Quercus* species under similar environmental conditions (González-Zurdo et al. 2016*a*). To achieve this, we have analyzed the relationships between levels of consumption by insects and leaf traits in each genetic group through the climatic gradient. Since it has been shown that the environmental conditions affect phenology, chemical composition and leaf morphology (Pearse and Hipp 2012, Jamieson et al. 2017), changes in these traits are expected to occur across the climate gradient, which in turn should also determine changes in consumption levels.

## Materials and methods

### Specimens and study areas

The study was carried out in three zones located in the regions of Castilla-León and Extremadura (central-western Spain). In each zone, three sites were selected within a range of <50 km: one with apparent dominance of *Q. faginea* (in the absence of *Q. pyrenaica*), another with *Q. pyrenaica* (in the absence of *Q. faginea*) and intermediate sites with presence of both species. The characteristics of each of the nine sites are shown in Table 1. The sites consisted of sparse populations of isolated mature trees (between 50 and 100 specimens $ha^{-1}$) over 100 years old, with areas covered mainly by small shrubs among them. Most trees had a hemispheric shape with a clustered distribution of leaves and an open crown structure (Mediavilla and Escudero 2023).

Air temperature data were obtained for each site by means of two data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA) programmed to obtain temperature data every 10 min. The loggers were placed in the shade within the crown of some of the selected specimens in each site. Annual rainfall data from the station nearest to each stand were provided by the Spanish National Meteorological Institute. All climatic data reflected in Table 1 correspond to the average of five study years (2019–23) after verifying that the between-site differences in temperature and rainfall were consistent in all study years.

Among the three zones, there is a temperature gradient, with maximum, minimum and average values always lower in zone 3 (cold zone) and higher in zone 1 (warm zone), with intermediate values in zone 2 (intermediate zone). The differences among the three zones are especially marked in absolute minimum temperatures and the number of frosts per year (Table 1). The highest values of precipitation occurred in the warm zone (which contributes to compensating for the effects of their higher temperatures on drought stress), with fewer differences between colder and intermediate zones. In most cases, summer precipitation is below 10% of the annual total. Within each zone, the climatic differences between sites are much less accentuated, so the selected enclaves will allow us to study between-species differences in foliar traits and herbivory levels for similar climatic conditions within each area, as well as the changes for each genetic group across the climatic gradient.

### Genetic analysis

In each site, around 30 specimens of each of the dominant groups (*Q. faginea*, *Q. pyrenaica* or hybrids) were selected according to their leaf morphology. All selected trees (272 in total) were fully sun-exposed mature specimens with heights between 8 and 10 m and diameters (at 1.3 m) between 40 and 60 cm. Leaf samples were collected from each specimen throughout 2021, and the genetic structure and categorization were performed using AFLPs through the specific procedure detailed in Table and text S1 (available as Supplementary data at *Tree Physiology* Online). Following the method implemented in Structure Harvester (Evanno et al. 2005), Bayesian clustering analysis supported an optimal partition of the genetic structure in two clusters. Optimal value of K was $K = 2$ within all three studied contact zones. In accordance with the obtained Q values, three genetic categories (i.e. genetic clusters) were established, based also on the a priori morphological identification of the individuals according to standard keys. Based on the admixture coefficient ($Q \geq 0.9$), a total of 76 individuals were assigned to the genetic cluster identified as *Quercus pyrenaica*, 70 to that corresponding to *Q. faginea* and 126 were identified as hybrids. The final assignment of individuals to genetic clusters within the three contact zones is shown in Tables S2–S4 (available as Supplementary data at *Tree Physiology* Online). As we expected, no specimen of *Q. faginea* was located in the plots dominated by *Q. pyrenaica*, nor vice versa.

### Insect herbivory assessment

In each site, 10 specimens (although only nine hybrids were identified in the intermediate zone) corresponding to the

dominant genetic category in the site (*Q. pyrenaica*, *Q. faginea* or hybrids) were selected for sampling. Five sun-exposed branches were sampled from different positions in the upper crown of each tree. All branches contained between 100 and 400 leaves and were of similar length (between ∼50 and 70 cm). Sampling was done in mid-June 2022–23, once all the leaves had been fully formed and leaf expansion had been completed (Mediavilla and Escudero 2003). A previous study with the same species revealed that the percentage of LA lost to herbivores was the same for the leaves sampled in mid-June and those sampled after summer (Mediavilla et al. 2022). Visual inspection revealed that, also at the sites studied in the present paper, leaf damage did not increase after the sampling period. This suggests that all insect attacks occur before mid-June, which permitted us to estimate the total losses suffered during the whole leaf life span. In each year, 25 leaves were taken at random from the branches sampled on each tree (250 leaves per genetic group, site and year), which were visually analyzed and classified depending on whether they presented visible symptoms of herbivory or not and the number of healthy and consumed leaves was counted. In the leaves where damage was observed, the percentage of the damaged LA was determined using five randomly selected leaves per tree (50 leaves per genetic group and per site and year). First, the leaves were subjected to high-resolution scanning (2300 × 3300 pixels) and the ImageJ program (http://rsb.info.nih.gov/ij/; Abramoff et al. 2004) was then used to measure both the actual area of each leaf and the area lost to herbivores, which was estimated by reconstructing leaf outlines and calculating the difference between the reconstructed leaf and the actual area (Mediavilla et al. 2018). Some examples of the procedure used to reconstruct the area lost appear in Figure S1 (available as Supplementary data at *Tree Physiology* Online). From the data obtained, the percentage of the total LA affected by insect damage at the branch level was calculated by multiplying the percentage of leaves damaged by the fraction of area lost per leaf.

## Leaf traits measurements

The dates of leaf emergence were determined in each site by analyzing the images taken by two time-lapse cameras (Time Lapse Camera TL2300), which were maintained in the field over the two years of study (2022–23). Morphological traits were measured in undamaged leaves taken from the same specimens used for the herbivory measurements in each site. From each specimen, in each year, five leaves were taken from the branches sampled, and LT was measured with a digital micrometer (Digimatic Micrometer, Mitutoyo, Japan) as a mean of three measurements taken at random positions on each leaf, avoiding the main ribs. Leaf area and petiole length (PL) were calculated by photographing the leaves against a scale and using ImageJ software (Abramoff et al. 2004). In each case, the total NL was also counted. The samples were then oven-dried for 24 h at 60 °C to constant mass and the total dry mass was determined with an analytical balance (Sauter AR70, Sauter, Ebingen, Germany). From the data thus obtained, we calculated the leaf dry mass per area (LMA) as the ratio of dry mass to LA and LD as dry mass/volume. All biomechanical and leaf shape traits were also measured in consumed leaves of the same specimens to verify possible differences in the preferences of herbivores at the level of the same individuals.

The individual undamaged leaves taken from each specimen were later ground together to obtain a large enough sample for the chemical analyses. Leaf N concentrations were determined with a CE-instruments NA-2100 auto analyzer (ThermoQuest, Milan, Italy). After the N analyses, the remaining material was used to analyze the fiber content (hemicellulose, cellulose and lignin) with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). The N and fiber concentrations of leaves were expressed as milligrams of nutrient or fiber per gram of leaf dry mass. The total polyphenolic content in the different *Quercus* samples was determined by a modification of the Folin–Ciocalteu method (Singleton and Rossi 1965). In the present study, 100 $\mu$L of Folin–Ciocalteu reagent (VWR International, Rosny-sous-Bois, France) was added to 100 $\mu$L of each *Quercus* aqueous extract, previously filtered (0.45 $\mu$m Clarinert™ syringe filters, Bonna-Agela Technologies, USA). Then, 2 mL of an aqueous solution of $Na_2CO_3$ (7.5%, Fisher Chemical, Loughborough, UK) and 2.8 mL of ultrapure water (Autwomatic, Wasserlab) were added to reach a final volume of 5 mL. Blank sample was prepared similarly but replacing 100 $\mu$L of the *Quercus* extract with 100 $\mu$L of ultrapure water. The solutions were kept in darkness for 50 min to allow the completion of the reaction. The absorbance was measured at 750 nm in quartz cuvettes (1 cm path length). Calibration curve (y = 0.021x + 0.0316; R2: 0.9966) was built with gallic acid (Merck, Darmstadt, Germany) following the same procedure as for samples, but adding 100 $\mu$L of the gallic acid solutions of known concentrations instead of 100 $\mu$L of the sample. The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g of dry sample.

## Data analysis

In each tree (89 specimens in total), a value of each variable was obtained as an average of the five leaves analyzed each year, and finally, for each specimen, an average value of the 2 years of study was obtained after verifying that there were no significant differences between years in any case. The analysis of variance (ANOVA) was carried out to search differences between genetic groups (two parental species and hybrids) and between the three study areas for the different parameters considered, followed by post-hoc Tukey–Kramer HSD test (at a significance level of $P = 0.05$). Prior to any analysis, data were tested for normality (using the Kolmogorov–Smirnov statistic test) and variance homogeneity (Levene's test) to proceed with data transformation when necessary. Mean values and standard error were obtained for each variable, genetic group and zone across the gradient. The variability of the biomechanical, morphological and chemical characters within each species and between zones was measured by calculating the coefficient of variation (CV). To assess the most useful traits for discrimination and to determine how leaf biomechanical, morphological and chemical traits separate individuals into groups or zones, the leaf characters analyzed were used for principal components analysis (PCA), considering the individual trees as the units in this analysis. Pearson correlation was used to determine associations between variables, with log transformation used when necessary. Regression analyses were used to explore the relationships between herbivory rates vs climate and leaf traits. All tests were performed with the SPSS v. 28 program (SPSS Inc., Chicago, IL, USA).

## Results

### Herbivory levels: differences between genetic groups and zones

In all zones, hybrids experienced lower levels of damage by insects than both parental species (around 30% less than *Q. pyrenaica* and 20% less than *Q. faginea*), mainly due to a significantly lower proportion of area missing per leaf (Figure 1b), with less differences in the percentage of attacked leaves between the three groups (Figure 1a). Between parental species, there were also differences in the percentage of area lost per leaf and total area consumed per branch, with higher values always in *Q. pyrenaica*. These between-group differences were consistent in all areas throughout the gradient (Figure 1c). There were between-site differences in the area consumed per leaf and total herbivory per branch, with no general changes in the percentage of leaves attacked (Figure 1). In the three genetic groups, total LA lost per branch increased with mean annual temperature (MAT) (Figure 2a), although for a given MAT, leaf losses tended to be larger for *Q. pyrenaica* and lowest for the hybrids. Because of the trend of warmer sites to have higher rainfall, there was also a significant relationship between losses and mean annual precipitation (MAP) (Figure 2b).

### Leaf traits: differences between genetic groups and zones

In the three study areas, *Q. faginea* showed the earliest emergence (around 2 or 3 weeks earlier than *Q. pyrenaica*), with emergence in the hybrids on intermediate dates between both (Figure 3). Across the gradient, leaf unfolding was earlier in the three groups in the warmer than in the colder zone (up to 7 weeks earlier in *Q. pyrenaica* and hybrids and around 5 weeks in *Q. faginea*), showing the intermediate zone also intermediate values in emergency (Figure 3). The results were similar for both years of study.

We found consistent differences among the three genetic groups for all leaf traits analyzed, except for LT (Table S5 available as Supplementary data at *Tree Physiology* Online). Across the temperature gradient, only LA, PL, NL, total phenolics (TP) and N concentration differed significantly among the three climatic zones, with non-significant interaction term (genetic group × area), except for TP (Table S5 available as Supplementary data at *Tree Physiology* Online).

There were significant correlations between most of the traits studied (Table S6 available as Supplementary data at *Tree Physiology* Online), especially strong between LMA and LD and between LA and PL (Pearson coefficient >0.8). Since the differences in LT were non-significant for all comparisons, the variation in LMA was almost exclusively due to differences in leaf LD. Interestingly, LMA and LD correlated negatively with the concentration of fibers and N but positively with that of phenols (Table S6 available as Supplementary data at *Tree Physiology* Online).

The results of Principal Component Analysis based on the leaf traits were consistent with the ANOVAs. The first and second extracted factors accounted for 60% of the total variances (43.2% for PC1 and 16.5% for PC2) (Table 2). LMA, together with cellulose, lignin and phenol content and LA, showed the highest (either positive or negative) correlations with PC1. Morphological traits, such as LA and NL, together with TP, exhibited the highest correlations with PC2. The first component clearly discriminates *Q. pyrenaica* (distributed on

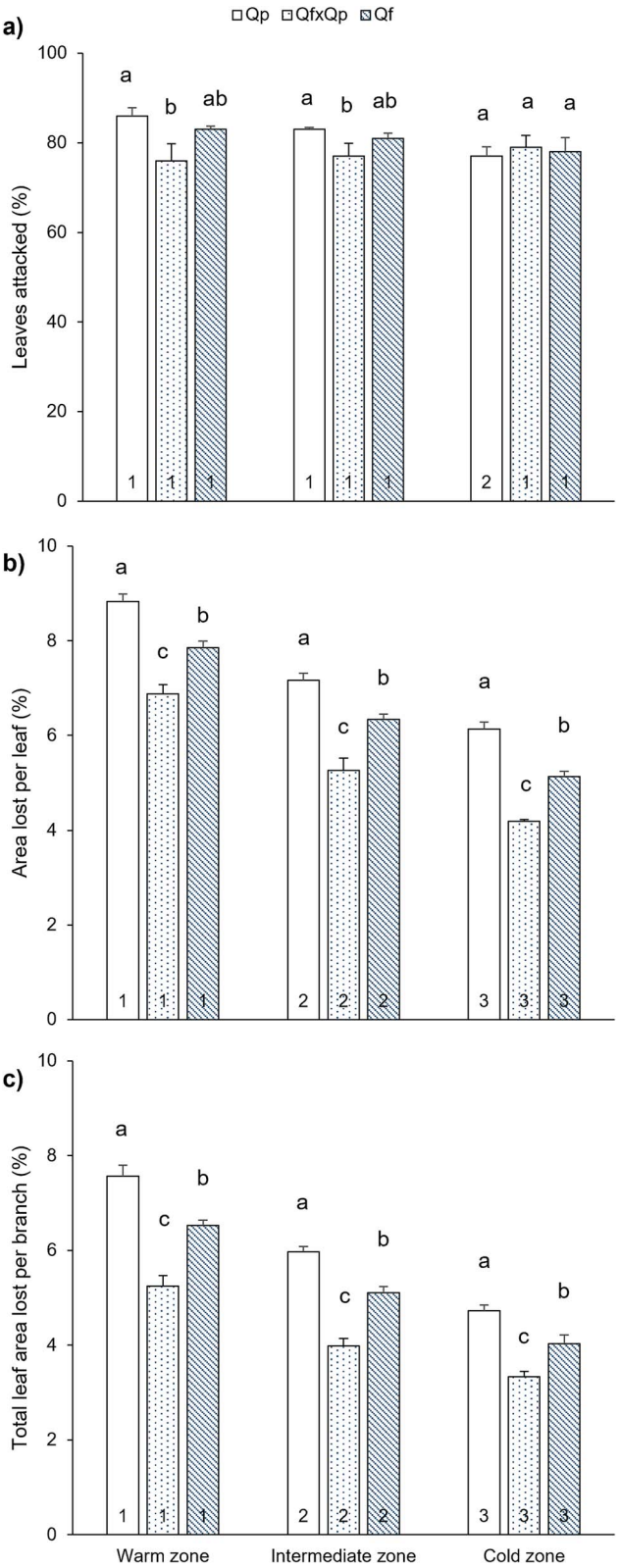


**Figure 1.** Average values + SE ($n$ = 10 trees for each leaf type and climatic zone across the gradient) of (a) the number of leaves attacked by herbivores (% of total leaf number per branch), (b) percentage of area lost per leaf and (c) the fraction of the total LA per branch lost to herbivores (%). For each leaf type (Qp = *Q. pyrenaica* open bars, Qf = *Q. faginea* striped bars, and Qp × Qf = hybrids dotted bars), different numbers within bars indicate significant differences ($P < 0.05$) among zones. For each climatic zone, different letters above bars indicate significant differences ($P < 0.05$) among leaf types.

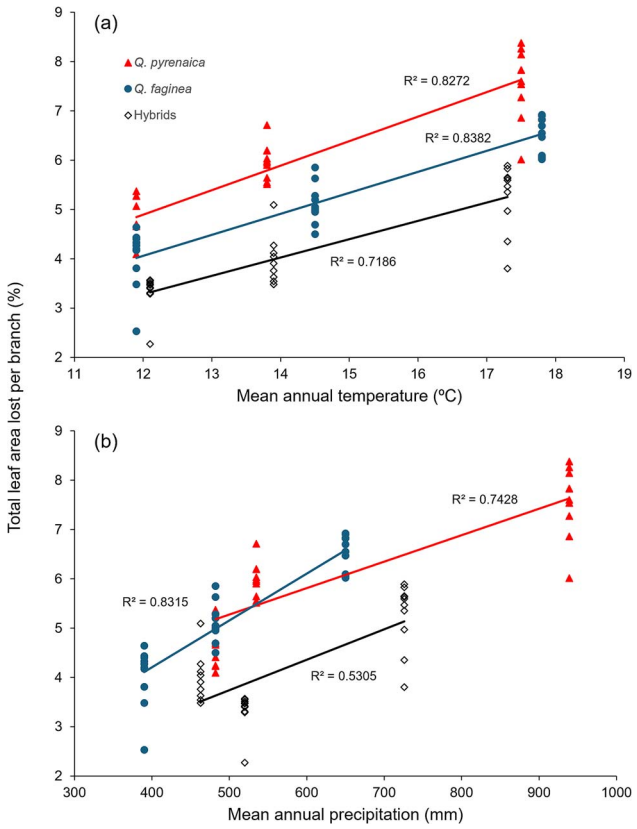


**Figure 2.** Relationships between the fractions of the total LA per branch lost to herbivores (%) and (a) MAT and (b) MAP in each site. $R^2$ values are shown.

**Table 2.** Eigenvalues, percentage of the variance explained by the first two principal components and factor scores of leaf traits.

| | PC1 | PC2 |
|---|---|---|
| Eigenvalue | 4.76 | 1.81 |
| Percentage variance (%) | 43.3 | 16.5 |
| Leaf traits | | |
| LT | −0.306 | 0.036 |
| LMA | −0.860 | 0.035 |
| LA | 0.688 | 0.575 |
| NL | −0.472 | 0.567 |
| L | 0.766 | −0.319 |
| H | 0.554 | −0.241 |
| C | 0.716 | 0.078 |
| N | 0.646 | 0.324 |
| TP | −0.647 | 0.570 |

LT, leaf thickness ($\mu$m); LMA, leaf mass per area ($gm^{-2}$); LA, leaf area ($cm^2$); NL, number of lobes; L, lignin concentration (mg $g^{-1}$); H, hemicellulose concentration (mg $g^{-1}$); C, cellulose concentration (mg $g^{-1}$); N, N concentration (mg $g^{-1}$); TP, total phenolics (mg $g^{-1}$ dry weight).

the right side of the diagram) from *Q. faginea* and most hybrid individuals, which occupied both the central and left parts of the diagram (Figure 4). Traits of hybrids are, therefore, closer to one of the two parents (*Q. faginea*). The second component discriminates between zones, with a clear separation between the warmest zone at the top of the axis and the coldest zone at the negative side, with individuals from the intermediate climatic zone distributed between both areas, although with more overlap with that of the negative side of the axis (Figure 4).

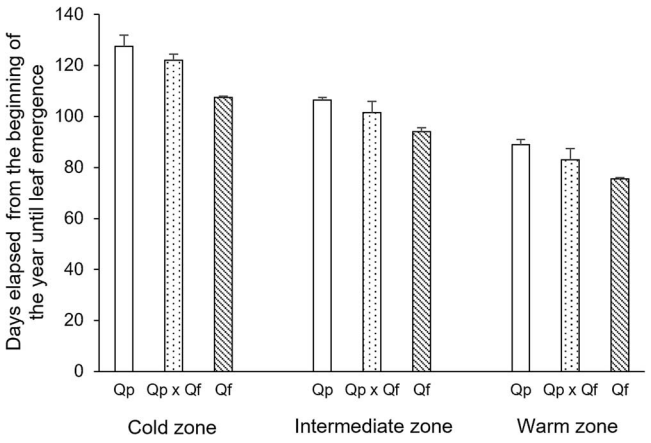


**Figure 3.** Days elapsed from the beginning of the year until leaf emergence in the three genetic groups and in the three zones across the climatic gradient (Qp = *Q. Pyrenaica* open bars, Qf = *Q. Faginea* striped bars, and *Qp* × *Qf* = hybrids dotted bars). The data correspond to the average + SE of the 2 years of study (2022–2023).

In the three genetic groups, LA, NL and TP tended to increase with MAT (Figure 5). Leaf N concentration also exhibited a positive relationship with MAT, except for *Q. faginea*. The remaining leaf traits, except LP, did not show any trend related to climate. In all study areas, the leaves of *Q. pyrenaica* showed significantly lower LD and greater LA than the other two groups (Figure 5a and b). Between *Q. faginea* and the hybrids, there were differences in LD, with lower values in the hybrids (Figure 5a). With respect to chemical composition, *Q. pyrenaica* showed the lowest TP content and highest N and fiber content, with *Q. faginea* at the opposite extreme and the hybrids showing intermediate values (Figure 5d–f). Both chemical and biomechanical traits tended to show moderate variability within each population (CV < 10%). The variability was highest for LA, followed by NL and, contrary to expectations, it was not generally greater in the hybrids than in the parental species (Figure 5).

## Relationships between herbivory levels and leaf traits

Among the different leaf traits, only LA, NL and LD exhibited significant effects on total leaf losses per branch when controlling for MAT, according to the results of multiple regression analysis (Table 3). As expected, LA had a strong positive effect on leaf losses, whereas NL and LD had negative effects. The larger herbivory losses experienced by larger leaves were confirmed by the fact that, within each genetic group and site, the reconstructed LA of attacked leaves was significantly larger than that of intact leaves (Figure 6), which suggests that, other things being equal, herbivores show a preference for larger leaves. Losses to herbivores tended to increase with leaf size in each climatic zone (Figure 7), which also suggests that this effect is independent of climatic differences, although the trends were much less intense for the coldest sites (Figure 7a). However, hybrids maintained lower herbivory levels for leaf sizes similar to those of *Q. faginea*, and the relationship of herbivory levels with LA was also less apparent.

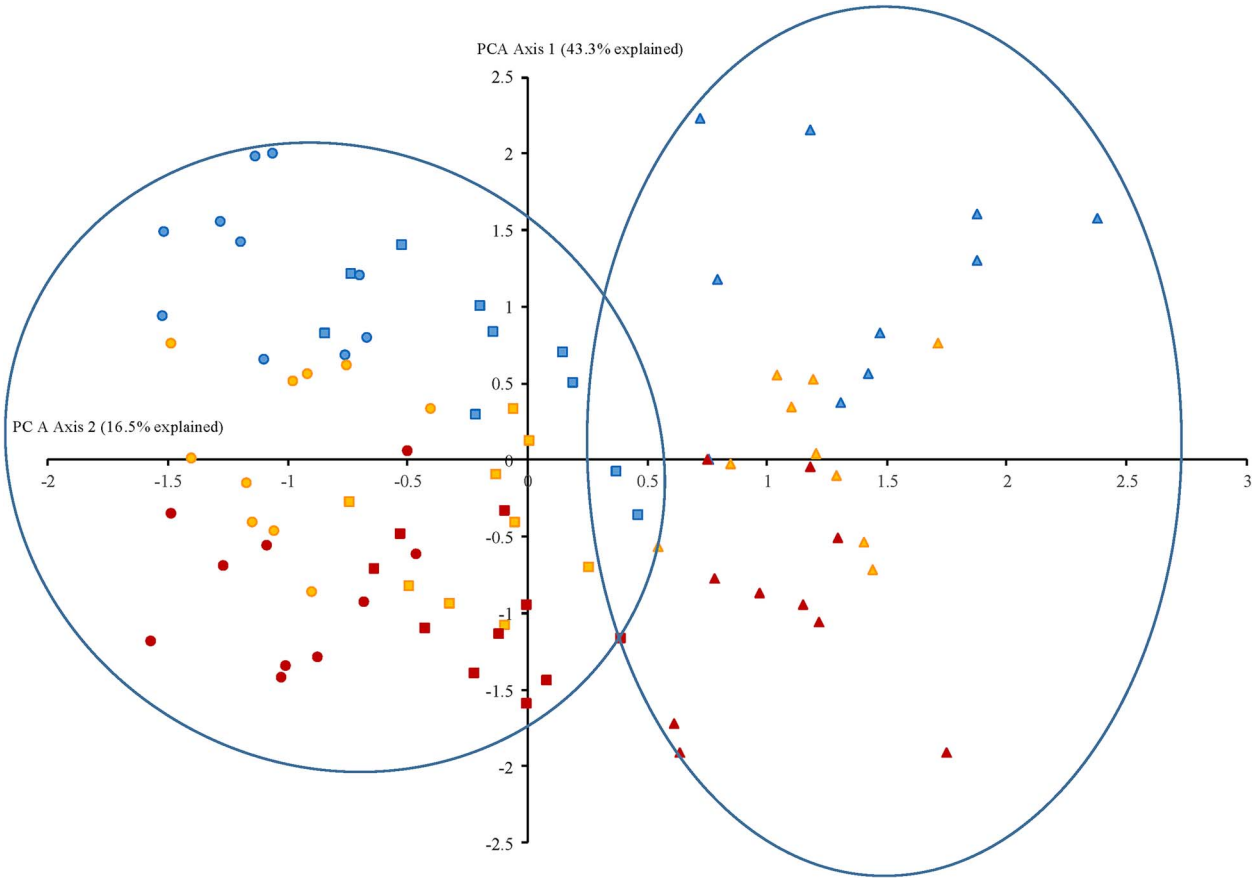


**Figure 4.** PCA based on the 11 morphological and chemical leaf traits (climatic zones were labeled with different colors: warm—blue, intermediate—yellow, cold—red; genetic groups were labeled with different symbols: *Q. pyrenaica*—triangle, *Q. faginea*—circle, hybrids—square).

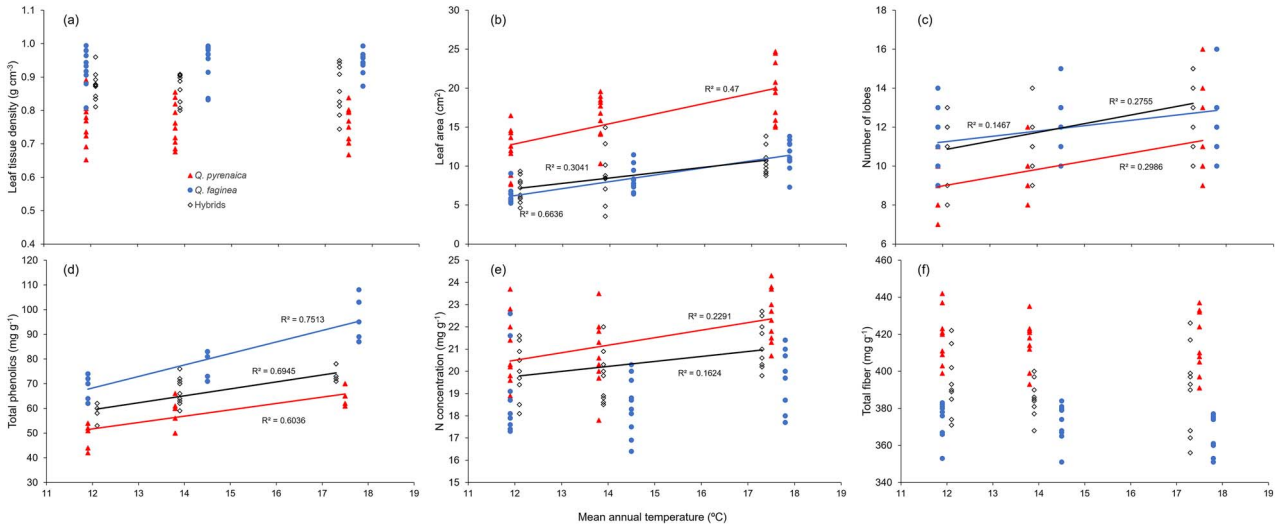


**Figure 5.** Relationships between MAT in the different sites and leaf traits. Only significant relationships ($P < 0.05$) are shown.

## Discussion

### Herbivory levels and leaf traits: differences between *Q. pyrenaica*, *Q. faginea* and their hybrids

The results of our study reveal that a combination of foliar traits acting together allows *Q. faginea* to maintain lower levels of consumption compared with *Q. pyrenaica* without any apparent trade-off between chemical and physical defenses, as other authors have also observed in different species (Kursar et al. 2009, Callis-Duehl et al. 2017). Despite their phylogenetic proximity and similar foliar longevity (Mediavilla et al. 2018), there were clear differences between the two study species in almost all the foliar characters considered. Compared with *Q. pyrenaica*, *Q. faginea* leaves showed greater biomechanical reinforcement (LMA, LD) and chemical defenses (TP), which are traits repeatedly associated with less herbivory (Kozlov et al. 2015, Moreira et al. 2018,

**Table 3.** Multiple regression of the percentage of total leaf area per branch lost to herbivores, as dependent variable, against different leaf traits.

| Independent variables | Intercept | $R^2$ | Standardized coefficients | $P$ |
|---|---|---|---|---|
| MAT | 2.125 | 0.741 | 0.485 | <0.0001 |
| LT | | | 0.028 | 0.640 |
| LD | | | −0.183 | 0.040 |
| LA | | | 0.441 | <0.0001 |
| PL | | | 0.051 | 0.640 |
| NL | | | −0.146 | 0.040 |
| TP | | | 0.193 | 0.090 |
| N | | | −0.099 | 0.180 |
| L | | | −0.055 | 0.520 |
| H | | | 0.060 | 0.390 |
| C | | | 0.002 | 0.980 |

MAT, mean annual temperature (°C); LT, leaf thickness ($\mu$m); LD, leaf density (g cm$^{-3}$); LA, leaf area (cm$^2$); PL, petiole length (mm); NL, number of lobes; TP, total phenolics (mg g$^{-1}$ dry weight); N, N concentration (mg g$^{-1}$); L, lignin concentration (mg g$^{-1}$); H, hemicellulose concentration (mg g$^{-1}$); C, cellulose concentration (mg g$^{-1}$).

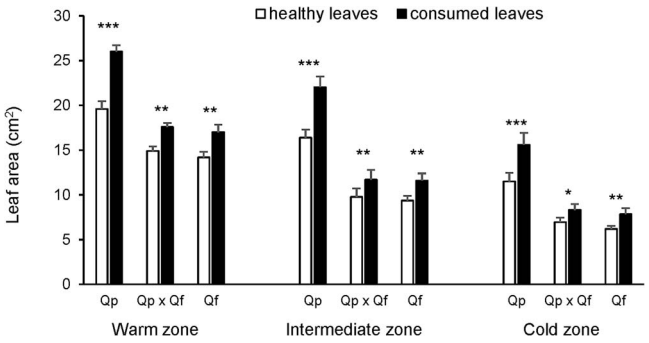


**Figure 6.** Average leaf size + SE ($n$ = 10 trees for each leaf type and climatic zone across the gradient) of the intact and attacked leaves in each genetic group (Qp = *Q. pyrenaica,* Qf = *Q. faginea* and Qp × Qf = hybrids) across the climatic gradient. Asterisks indicate significant differences between both types of leaves: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Muiruri et al. 2019). Other authors (Uzunova et al. 1997, Mediavilla et al. 2001) have also observed that *Q. faginea* leaves tend to exhibit thicker cuticles than *Q. pyrenaica*, which may also contribute to explaining the lower herbivore susceptibility of *Q. faginea*.

In addition to chemical and physical defenses, the differences in the leaf shape (smaller leaf size and PL and greater NL in *Q. faginea*) showed a clear relationship with the differences between species in herbivory in all climatic areas. In fact, LA was the trait that explained a greater percentage of the variance in herbivory levels among the different specimens (Table 3). Positive correlations between leaf size and herbivory levels have been found in numerous studies at both inter- and intraspecific levels, which suggest that larger leaves are more attractive to herbivores (Muiruri et al. 2019, Li et al. 2021). Several potential explanations for this have been presented: small leaves may reduce insect growth rates owing to the lower foraging efficiency of herbivores because a small individual LA forces insects to invest more time and energy in moving to new leaves (Brown et al. 1991). Also, shorter leaf expansion times, which is the period when the leaf is most vulnerable to herbivory (Barbehenn et al. 2017), and greater vein density, which contributes to increasing their mechanical resistance (Sack and Scoffoni 2013), could explain the lower consumption rates in smaller leaves. In some cases, longer petioles have been related to more richness and abundance of herbivores (Novais et al. 2015). However, PL and LA tended to be positively related because longer petioles provide greater mechanical support for larger leaves (Filartiga et al. 2022). In fact, the relationships between PL and herbivory disappeared for a constant LA in multiple regression (Table 3), suggesting that the effects of PL were exclusively due to its relationship with LA. Finally, lobes can interfere with the feeding of some insects, reducing the preference for herbivores (Brown et al. 1991, Higuchi and Kawakita 2019), perhaps because the presence of lobulation contributes to increased physical resistance against leaf-processing herbivores, and thus the herbivores may show a preference for less-lobed leaves (Higuchi and Kawakita 2019). This effect could also favor *Q. faginea*, helping to explain its lower consumption levels.

The hybrids showed intermediate values between both parent species in leaf chemical traits (phenolic, N and fiber content) and in structural reinforcement (LMA and LD), irrespective of the study area, but they were more similar to *Q. faginea* regarding the shape of the leaf (LA and NL). The hybrids also showed intermediate phenological patterns between the earliest emergence of *Q. faginea* and the latest of *Q. pyrenaica*. It would then be expected that the hybrids would also show intermediate levels of herbivory between the two study species. However, the hybrids showed the lowest levels of herbivory, which reveals that the differences in consumption between the hybrids and their parents are not determined by their intermediate leaf traits. Our study, therefore, provides new evidence supporting the 'hybrid resistance hypothesis' (Boecklen and Spellenberg 1990) that suggests lower herbivory in hybrid plants than in parental species. Although this same result has been obtained for other species (Boecklen and Spellenberg 1990, Boecklen and Larson 1994), until now, the hybrid resistance hypothesis has received less experimental support than the opposite 'hybrid depression hypothesis' that postulates greater herbivory in hybrids (Fritz 1999, Cuevas-Reyes et al. 2018).

This greater resistance of hybrids has been attributed to both genetic and environmental factors (Fritz 1999). Although the former are still little known and have not been explored in depth in many species, it is likely that the causes of altered resistance are related in some way to the genetic rearrangements that have occurred during the formation of the hybrid (Rieseberg and Ellstrand 1993, Fritz 1999). It could also occur that the environmental factors of the habitats where hybrids dominate lead to a lower abundance, diversity or activity of herbivores compared with those occupied by the parental species (Paige and Capman 1993, Fritz 1999). However, this explanation seems unlikely in our case because there are hardly any significant differences in most of the estimated environmental parameters between the three sites selected within each climatic zone. Also, the differences in herbivory levels among the genetic groups are consistent in the three climatic zones despite the large environmental differences among them. A third more plausible explanation could be that the parents have provided more time for their host recognition by possible herbivore species (Fritz 1999). There is evidence that herbivorous insects adapt more frequently to their host plant than vice versa (Mopper and Strauss 1997), so it could be that the number of insect species that have adapted to ingesting leaves with the characteristics of the parents is larger than in the case of the hybrids. Whatever the explanation,

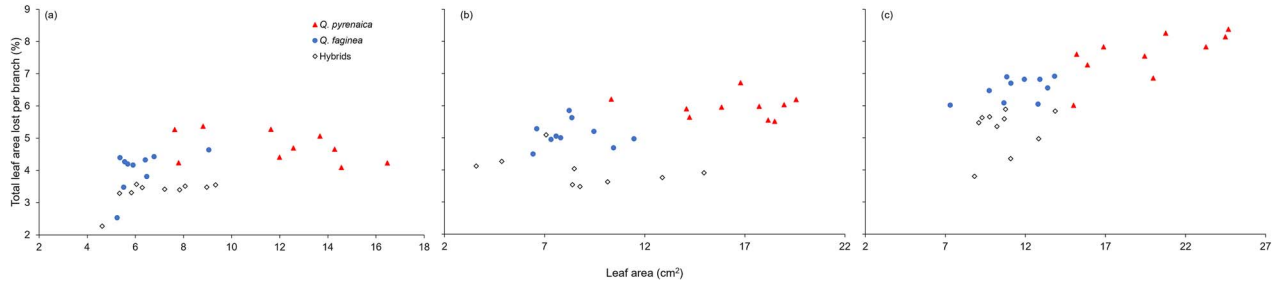


**Figure 7.** Percentage of total LA per branch lost to herbivores for the different genetic groups plotted against individual LA. (a) Cold zone. (b) Intermediate zone. (c) Warm zone.

taking our species as a study framework, herbivory pressure not only would not represent an unfavorable factor on the fitness of hybrids that would justify the persistence of the parental species but, on the contrary, differences in herbivory levels could be important in the evolution of hybrids, favoring their persistence, abundance and distribution.

## Herbivory levels and leaf traits: differences across the climatic gradient

In the three genetic groups, herbivory increased among the different populations from the lowest levels in the coldest sites to the highest in the warmest sites, thus confirming that interaction intensity between plants and their herbivores tends to increase with the increase in temperature (Johnson and Rasmann 2011, Moreira et al. 2018). Since leaf emergence in colder sites occurred later in the season, one reason for the lower levels of consumption in cold sites could be that the shorter time period elapsed between leaf emergence and sampling, leaving less time for herbivores to feed. However, it is known that insect herbivory occurs when leaves are still expanding (Barbehenn et al. 2017, Mediavilla et al. 2018). Leaf expansion in the studied species is very quick and leaves tend to achieve final size before the start of June (Mediavilla and Escudero 2003). In the coldest sites, leaf emergence occurred around early May, which implies that sampling was performed at least 1.5 months after emergence. Accordingly, all insect attacks occurred before sampling, as also seen in a previous study with the same species (Mediavilla et al. 2022). In fact, visual inspection revealed that leaf damage did not increase after the sampling period, and that leaf damages were clearly lower in the colder sites during the rest of the growth season.

Contrary to what was expected, these intraspecific differences across the climatic gradient did not respond to changes in biomechanical traits since LD and fiber content did not exhibit significant changes for any of the genetic groups in response to temperature variations (Figure 5a and f). Within each genetic group, only LA, NL and TP increased with the increase in temperature, in parallel with the changes in herbivory levels. The apparent intraspecific association of large values of NL and TP with high herbivory levels across the temperature gradient is opposite to expectations based on the negative effects seen by other authors (Brown et al. 1991, Moreira et al. 2018, Higuchi and Kawakita 2019). It is possible that the intraspecific correlations found in the present study across the temperature gradient are not the result of a direct cause–effect relationship and that the tendency to develop leaves with more lobes and phenolic content in warmer sites responds to other functions. Different studies have shown a greater heat transfer capacity at low air speed in more lobed leaves, thereby contributing to the reduction in leaf temperature and, consequently, transpiration (Sisó et al. 2001, Vogel 2009). The more lobed leaves also show lower hydraulic resistance, which allows a correct supply of water to the mesophyll cells, ensuring that the leaves do not develop very negative water potentials despite living in atmospheres with a high vapor pressure deficit (Sisó et al. 2001, Vogel 2009). Regarding the phenols, the latitudinal Herbivory-Defense Hypothesis assumes that higher levels of antiherbivore defenses would be expected in plants occupying environments with higher levels of consumption (Pearse and Hipp 2012, Moreira et al. 2018). As said above, we did not find changes across the gradient in LMA, LT or LD, although these traits have been revealed to be more important than chemical defenses in deterring herbivores in several studies (Muiruri et al. 2019, Ruíz-Carbayo et al. 2020). This would suggest that investment in phenols would be the main response of our species to the increase in herbivory pressure, perhaps because phenols are cheaper to produce than the investment in greater structural reinforcement (Goodger et al. 2013). However, other authors think that, on the contrary, structural defenses are less resource-demanding than secondary chemicals (Choong 1996). Besides, it is difficult to assume that the only response to the changes in herbivory pressure across our gradient consists in the variation in phenol content, especially taking into account that phenols do not seem effective in promoting lower levels of herbivory (Ruiz-Carbayo et al. 2020, Salgado-Luarte et al. 2023). Changes in phenol content may instead serve other functions, such as protection against temperature changes, UV radiation or photodamage (Close and MacArthur 2002, Janská et al. 2010). Our results do not support, therefore, the prediction that plants should possess high antiherbivore defenses in localities with high herbivore pressure. A similar conclusion was reached in a recent meta-analysis by Zvereva et al. (2024).

Leaf area was the best predictor of interspecific as well as intraspecific differences in herbivore damage across the gradient, with higher levels of consumption associated with larger leaves that, for the three genetic groups, were found in the warmer plots. The variation in leaf size affects many ecological processes, including leaf expansion time, photosynthesis, transpiration rate, thermal regulation, self-shading and foliar support costs (Kidner and Umbreen 2010, Yates et al. 2010, Leigh et al. 2017). A smaller size has been interpreted to reduce the thickness of the boundary layer. It is known that a thick boundary layer contributes to slow sensible heat exchange with the surrounding air (Wright et al. 2017).

For this reason, we may expect that small leaves would experience larger heat losses by convection, which results in leaf temperatures closer to air temperature and lower transpiration rates (Nobel 2009, Leigh et al. 2017). Smaller leaves also tend to have a greater density of veins, which has frequently been associated with greater leaf hydraulic conductance (Scoffoni et al. 2011). Our study revealed in the three genetic groups significantly larger leaves across the temperature gradient, which in principle could be contrary to what was expected according to the implications of leaf size just discussed. Under conditions of high water availability, larger leaves that sustain high transpiration rates may operate at temperatures lower than air temperatures, thanks to a greater depth of the boundary layer (Wright et al. 2017). Reducing leaf temperature can be a priority in warmer sites, where high temperatures can cause significant foliar damage (Still et al. 2023). This may constitute an advantage for the larger leaves found in the warmer sites of the present study, where annual rainfall levels were also higher than in other sites, allowing perhaps higher transpiration rates under conditions of high water vapor pressure deficit (Gil-Pelegrín et al. 2017). By contrast, the significantly larger leaves in the intermediate sites with respect to the colder sites are difficult to explain, given that precipitation levels were not higher than in the colder sites, which would rather suggest a direct positive effect of temperature on leaf size. Smaller leaf size constitutes an advantage during cold nights because of larger sensible heat exchange with the surrounding air, which helps to compensate for long-wave radiation losses (Wright et al. 2017). Although leaf unfolding occurred later in the season at the colder sites, minimum nighttime temperatures during the leafy periods were significantly lower at the colder sites, where minimum temperatures as low as −5 °C were frequently registered during the period April–May. The predominance of nighttime constraint on leaf size found in the present study, despite the supposed prevalence of soil drought among the different stress factors in the Mediterranean environments, constitutes a confirmation of the strong effects of low temperatures on leaf traits (Niinemets 2016, González-Zurdo et al. 2016*b*).

Apart from the effects of leaf size, other factors must contribute to the between-site differences in herbivory since MAT showed strong significant effects on herbivory after fixing the effects of leaf traits in multiple regression analysis (Table 3). There are two possible explanations for the higher levels of herbivory in warmer environments regardless of foliar traits. There were differences in leaf phenology across the temperature gradient, with a much earlier unfolding of the leaves in the warmer zone. Early unfolding dates in warmer sites may coincide with the highest densities of herbivorous insects that usually occur in spring. Higher mean temperatures in spring also have positive effects on insect metabolic rates, which would increase population growth rates and reduce generation times, resulting in higher herbivory rates (Lemoine et al. 2013, Nakamura et al. 2022).

## Conclusions

Contrary to our expectations, hybrids suffered less herbivory damage than their parental species, which suggests that the persistence of the parental species in the absence of reproductive barriers must be related to other still unknown factors that compensate the differences in herbivory losses. Among the different leaf traits, individual leaf size was apparently the principal determinant of the differences in resistance to herbivory since, within a given genetic group, the level of damage tended to be significantly larger for larger leaves. In turn, leaf size was the trait that exhibited the strongest response to differences in temperature. Individual LA tended to be significantly larger in warmer sites despite the known trend of larger leaves suffering from larger temperatures and water losses in hot environments. We postulate that the most probable explanation for this trend is that the risk of frost damage during late spring selects smaller leaves in cold sites. To determine whether this supposition is correct would require more experimental work with other tree species under different environmental conditions.

## Supplementary data

Supplementary data for this article are available at *Tree Physiology Online*.

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## Authors' contributions

S.G.-C. (performed the analysis of most leaf traits, analyzed and interpreted the data, contributed to the writing of the manuscript); A.F.-F. (performed the herbivory levels measurements and analysis of some leaf traits); A.E. (conceived the study and design, analysis and statistics, contributed to the drafting and final version of the manuscript); I.G.-E. (contributed to the total phenolics content measurements and manuscript preparation); M.M.-O. (conceived the study and design and performed the genetic analysis); and S.M. (conceived the study and design, analysis and statistics, contributed to the drafting and final version of the manuscript. All authors edited, read and approved the final manuscript).

## Conflict of interest

None declared.

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## Data availability

Data can be made available upon request from the corresponding author.

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