


Genetic determination of tannins and herbivore resistance in *Quercus ilex*

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Abstract Genetic variability of trees influences the chemical composition of tissues. This determines herbivore impact and, consequently, herbivore performance. We evaluated the independent effects of plant genotype and provenance on the tannin content of holm oak (*Quercus ilex*) and their consequences for herbivory and performance of gypsy moth (*Lymantria dispar*) larvae. Oak seedlings of 48 open-pollinated families from six populations were grown in a common garden in central Spain. Half the plants were subjected to defoliation by gypsy moth larvae and the other half were destructively

sampled for chemical analysis. Tannin content of leaves did not differ significantly among populations but differed significantly among families. Estimates of heritability (h^2) and quantitative genetic differentiation among populations for tannin content (Q_{ST}) were 0.83 and 0.12, respectively. Defoliation was not related to the tannin content of plants nor to spine and trichome densities of leaves, although positive family–mean associations were observed between defoliation and both seed weight and plant height ($P < 0.003$). Among the oak populations, differential increase in larval weight gain with defoliation was observed. Leaf tannin content in *Q. ilex* is genetically controlled but does not influence defoliation or predict performance of the larvae. Different efficiencies of food utilisation depending on the oak genotypes indicate that other plant traits are influencing the feeding patterns and fitness of *L. dispar* and consequent population dynamics.

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Introduction

Quantitative variation in secondary plant metabolites is largely responsible for patterns of herbivore performance and distribution across available hosts (Berenbaum 1995). Numerous studies have evaluated the independent effects of plant species (e.g. Mrdaković et al. 2011; Pearse 2011; Milanović et al. 2014) and environmental factors (e.g. Milanović et al. 2015; Rubert-Nason et al. 2015; Pollastrini et al. 2016) on herbivore damage to trees and performance of insects. The relative importance of plant genetic factors in tree defoliation and insect development has been studied (Osier and Lindroth 2001; Busby et al. 2015; Rubert-Nason et al. 2015) but in some tree

systems remains poorly understood. We used an environmentally controlled experiment of holm oak (*Quercus ilex* L.) and the European gypsy moth (*Lymantria dispar* L.) to explore the effects of tree populations and families within populations on tannin content, plant damage and herbivore performance.

Tannins are widely occurring plant polyphenols of varying structure and molecular weight. Tannins from various botanical sources have extensive applications in the wine industry and are used worldwide as clarifying agents in alcoholic drinks and as aroma ingredients in alcoholic beverages, soft drinks and juices. Plant tannins and inorganic tanning agents are key compounds for the world's leather production (Kite and Thomson 2006). In recent years, the effects of plant tannins on human health have gained attention, becoming topical in food science and disease prevention. Plant tannins are divided into two major groups: hydrolysable tannins and condensed tannins (also known as proanthocyanidins), which are both important for various biological processes, such as plant defence against herbivores (Barbehenn and Constabel 2011) and pathogens (Stong et al. 2013). Total tannins was chosen as a trait to study because tannins are among the most abundant secondary metabolites made by plants, commonly ranging from 5 to 10% dry weight of tree leaves (Barbehenn and Constabel 2011).

There are diverse perspectives on tannins in the context of their roles in plant defence to herbivores (Constabel et al. 2014). Tannins are known to bind proteins, and experiments performed by Feeny (1976) led to the widespread idea that tannins can act as general defences against insect pests by reducing the digestibility of proteins. Lepidopterans have a very basic gut pH, and tannins do not bind proteins under these conditions (reviewed in Barbehenn and Constabel 2011). While tannins can have negative effects on insects, according to recent literature (Barbehenn et al. 2009a, b), these appear not to be related to their protein-binding potential. Condensed tannins act as toxins and feeding deterrents causing oxidative damage within the epithelial cells of the midgut of insects (Barbehenn and Constabel 2011). Such effects depend in particular on the interaction between the plant-specific tannin and specific pH conditions in different parts of the digestive tract of the herbivore species (Barbehenn and Constabel 2011; Salminen and Karonen 2011; Büchel et al. 2016). However, in vertebrates, tannins can bind proteins in the acidic environment of the gut, and this reduction of digestibility can have significant impact on animal health and reproduction (Wallis et al. 2012).

Quercus ilex is one of the dominant tree species of Mediterranean forests and the most abundant forest tree species in Spain. The wide ecological amplitude of holm oak and its ecophysiological adaptability to hydric and thermal stress (Gimeno et al. 2009) accord with the high heterozygosity and allelic richness reported in this species (Michaud et al. 1995; Soto et al. 2007; Ortego et al. 2010; Vernesi et al. 2012;

Guzmán et al. 2015). Intraspecific variation of morphological traits, reproductive biology and resistance to pathogens has also been reported (Michaud et al. 1992; Díaz et al. 2003; Tapias et al. 2008; Bonal et al. 2012; Galván et al. 2012; Caliskan 2014; Niinemets 2015; Corcobado et al. 2016). According to Baldantoni et al. (2013), chemical constituents of *Q. ilex* leaves (d.w.) are cellulose (330 mg g⁻¹), lignin (310 mg g⁻¹), starch (17 mg g⁻¹) and soluble sugars (173 mg g⁻¹), and main elements (d.w.) are C (480 mg g⁻¹), N (16 mg g⁻¹), Ca (49 mg g⁻¹), Mg (17 mg g⁻¹) and K (68 mg g⁻¹). Leaves of *Q. ilex* have a dense layer of stellate, tufted non-glandular trichomes on the abaxial surface (Karioti et al. 2011b). Juvenile leaves in this species also have a spiny margin with very short spines 0.6 to 0.9 mm long. Although *Q. ilex* is one of the most studied tree species in southern Europe, information about genetic variability of structural (morphological and anatomical) traits and secondary metabolites related to plant defence is lacking.

Across its range, *Q. ilex* is attacked by more than 100 species of insects, many of which are prone to population outbreaks. One of these is the European gypsy moth, which causes significant but patchy defoliation of *Q. ilex* (Ibáñez-Justicia et al. 2007). Taking advantage of an environmentally controlled experiment with plant material from six differentiated *Q. ilex* populations in Spain, in this study, we explored whether there is intraspecific among- and within-population variation of the tannin content of *Q. ilex* trees, whether herbivory of *Q. ilex* by *L. dispar* varies within and among tree populations and whether the performance of *L. dispar* larvae could be influenced by the genetic structure of populations. Through an ecological genetics approach, we addressed the following questions: (1) are tannins controlled genetically in *Q. ilex* trees? (2) Can herbivory and herbivore performance be influenced by tree origin? Because structural characteristics of leaves can act as mechanical plant defences against herbivory (Hanley et al. 2007; Pearse 2011), spines and trichomes of leaves in *Q. ilex* were assessed.

Materials and methods

Genetic material

The genetic material studied comprised six *Q. ilex* ssp. *ballota* natural populations (Fig. 1), and seedlings grown from field-collected acorns were used. Sampling was performed in November and December 2013 and included trees from well-preserved forests. The sampling procedure comprised selecting randomly 10–12 fruit-bearing trees per population at minimum distances of 70 m apart to minimise the chances of intercrossing. About 100 acorns per tree were hand collected directly from the crown and stored in a cold chamber at 4 °C. In January 2014, the

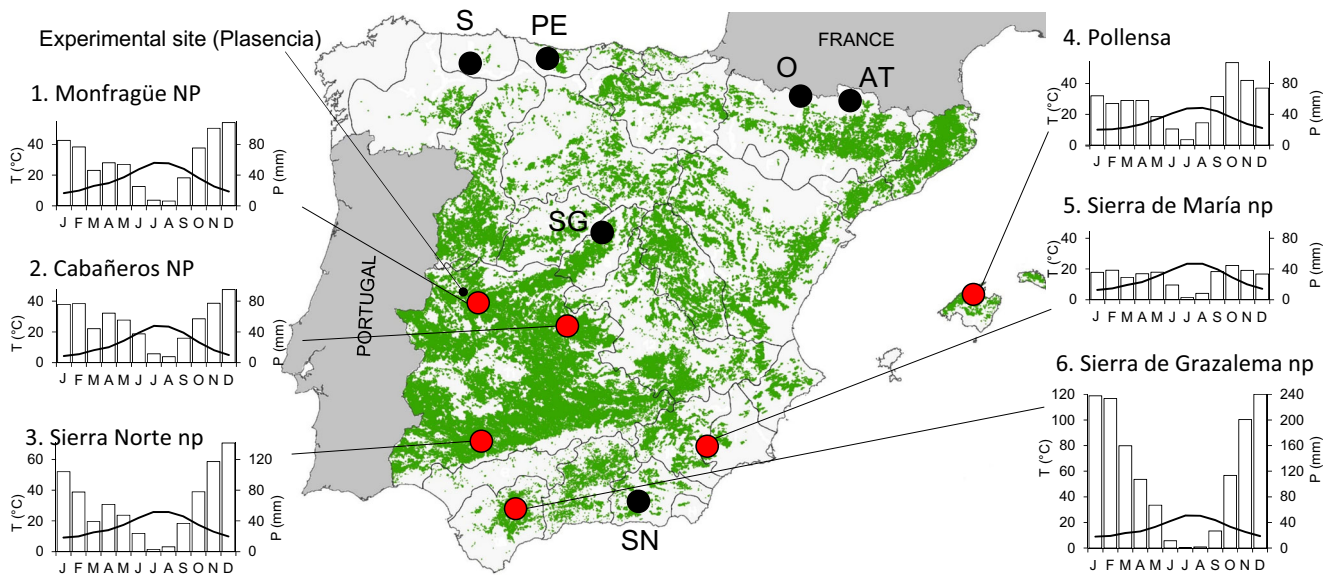


Fig. 1 Distribution of *Quercus ilex* in Spain (green) and location (red dots) and climographs of the six populations used in this study. Black dots indicate additional populations sampled but with few or no viable acorns (S Somiedo Natural Park, PE Picos de Europa National Park, O Ordesa y

Monte Perdido National Park, AT Aigües-Tortes y Lago de San Mauricio National Park, SG Sierra de Guadarrama National Park, SN Sierra Nevada National Park). Within the distribution of *Q. ilex*, grey lines delimit provenance regions according to Jiménez-Sancho et al. (1996)

acorns were placed on trays with sterilised sand, moistened close to field capacity with deionised water, covered with transparent plastic film and left to germinate under laboratory conditions.

Acorns with emerging radicles were individually weighed and planted in 48-cell rigid plastic root trainers (300 mL volume; 18 cm high, 5.3×5.3 cm upper surface) containing sand and peat (5:1, pH 5.5). Earlier research showed that this pot size would provide seedlings with unrestricted root growth during the experiment (Corcobado et al. unpublished results). The plants were kept in natural daylight under greenhouse shade that reduced solar radiation by 50% and were hand watered every 3–4 days to field capacity until they were well established.

Insect material

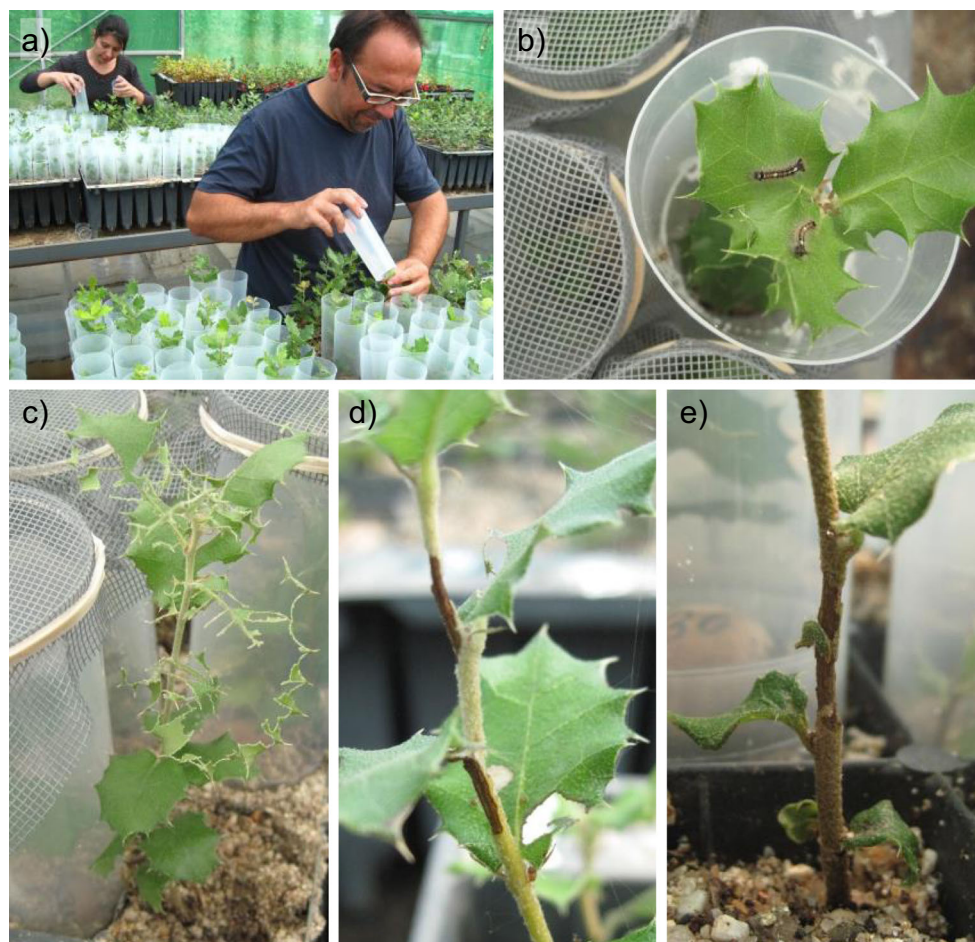
In October 2013, egg masses of *L. dispar* were collected from *Quercus cerris* L. trees in a forest ($44^{\circ} 20' 37''$ N, $22^{\circ} 22' 22''$ E; 231 m a.s.l.) near Jabukovac, about 160 km from Belgrade, Serbia. Insect material was collected at considerable distances from the plant material to prevent herbivory variation among populations being biased by any interaction between the insect and the host (e.g. by coevolutionary relationships). Eggs were processed and hatched, and larvae were grown on an artificial medium ('Gypsy moth diet', MP Biomedicals, LLC) as described in Milanović et al. (2014). Freshly moulted third-instar larvae were used to cause defoliation and study the effect of *Q. ilex* provenance on *L. dispar* performance.

Experimental design and defoliation treatment

The study was conducted in a greenhouse at the Faculty of Forestry of Plasencia, University of Extremadura, Spain ($40^{\circ} 02' 06''$ N, $6^{\circ} 04' 59''$ W; 374 m a.s.l.) under natural light at 17 to 26 °C. The plants were arranged following a split-plot design replicated in five blocks, with the two defoliation treatments acting as the main factor (two categories: defoliated and control; whole plots) and the six populations as the split factor (six categories: MO, CA, SN, PO, SM and GR as shown in Table S1 and Fig. 1; split plots). In all five blocks, each population was represented in each whole plot by two individuals from each of the eight open-pollinated families. Individuals were randomly positioned within each block. In total, there were 960 plants corresponding to 5 blocks \times 2 defoliation treatments \times 6 populations \times 8 families \times 2 individuals. The experiment therefore included 160 plants per population and 20 plants per family.

Half the seedlings were exposed to defoliation by larvae on 22 May 2014, coinciding with the expected period of attack in the field by outbreaking herbivores such as gypsy moth. On this date, about 1200 third-instar *L. dispar* larvae were kept under 21 °C in 12-cm-diameter Petri dishes. Two or three larvae per plant were used, depending on plant size. All plants were large enough to preclude larval starvation. The larvae were carefully placed with a soft brush on the upper leaves of plants. To stop the larvae from escaping, transparent rigid plastic tubes (one per plant), 5 cm in diameter, 15 cm high and 1 mm thick, were used (Fig. 2a). The tubes were individually covered with a plastic mosquito net attached by a rubber band (Fig. 2b). The average

Fig. 2 Placement of individual plastic tubes around *Quercus ilex* plants before defoliation by *Lymantria dispar* (a); plant and *L. dispar* larvae in their L3 instar, before being covered with a mosquito net (b); plant with ca. 5 cm² defoliation 4 days after exposure to larvae (c); stem heavily damaged but not girdled (d); main stem girdled (e)



temperature of the plants covered with the plastic cages did not differ significantly from the average temperature of uncovered plants. Defoliation lasted 4 days.

Tissue sampling and chemical analyses

On 26 May 2014, leaves and roots from the other half of the plants (control) were collected manually and flash-frozen in liquid nitrogen. Fine roots were easily collected by immersion of the root ball in water then merged with the main root into one sample. Samples were immediately transported to the laboratory, lyophilised and ground into powder in a ball mill (Mixer Mill MM 400, Retsch GmbH, Haan, Germany). The milled samples were stored at -80°C until analysis.

The concentration of total tannins present in the lyophilised leaves and roots was determined by radial diffusion in agarose gels, as described by Hagerman (1987). This method is based on the observation that tannins form a stable complex with bovine serum albumin (BSA), forming a clearly visible ring whose area is linearly proportional to the tannin level in samples. Petri dishes containing 1% agarose (Sigma-Aldrich) and a solution of 50 mM acetic acid, 60 μM ascorbic acid and 0.1% BSA were prepared. Fifty milligrams of milled tissue

was extracted with 250 μL 50% (v/v) aqueous methanol for 60 min in an ultrasonic bath. The crude extracts were centrifuged at 5000g for 5 min, and the supernatant was collected and stored at -80°C . Four uniform wells per Petri dish, 4 mm in diameter, had been made in the solidified agarose medium beforehand using a punch. Sixteen microlitres of each extract was inserted into each perforation via a micropipette. A calibration curve for tannic acid (0.1 to 1 mg) and a negative control (16 μL of 50% aqueous methanol) were used. Petri dishes were covered, sealed with parafilm and incubated at 30°C for 120 h. The Petri dishes were then scanned, and ImageJ 1.44p software (NIH, Bethesda, MD, USA) was used to obtain the area of the rings. Tannin content was expressed as milligrams of tannic acid equivalent per 100 mg lyophilised sample.

Defoliation and larval assessment

Four days after plant exposure to larvae (Fig. 2c), plant defoliation was individually assessed by quantifying the defoliated areas of all leaves. Defoliated leaves were placed above a 0.25×0.25 cm grid, and their defoliated areas were estimated. The defoliated area per plant was obtained by the sum of

defoliating values per leaf. Defoliation was expressed in square centimetres per number of larvae used. Stem damage was assessed as follows: 0 = no damage, 1 = some damage present, 2 = heavily damaged but not girdled (Fig. 2d) and 3 = main stem girdled (Fig. 2e). Caterpillars used to defoliate each plant were weighed together before and after plant exposure. Larval weight gain (growth rate) (mg day^{-1}) was obtained using the following formula (modified from Milanović et al. 2015):

$$WG_{\text{larval}} = (WG_{t_2} - WG_{t_1}) / n (t_2 - t_1)$$

where WG_{t_1} is the total weight of larvae at the beginning of the experiment, WG_{t_2} the total weight of larvae at the end of the experiment, n the number of larvae used and $t_2 - t_1$ the number of days plants were exposed to defoliation (i.e. 4 days).

Assessment of structural traits of leaves

In 20 control seedlings per population, the spines of the leaf margins were counted. Trichome density was measured in three leaves per seedling using a pre-weighed self-adhesive tape (Karioti et al. 2011b). Trichomes were removed from the abaxial leaf surface. Trichome density was expressed as milligrams per square centimetre and as trichome dry weight per leaf dry weight (%) (Karioti et al. 2011b). Water content (WC) of leaves was expressed in grams per gram and determined as $WC = (FW - DW) / DW$, where FW is the fresh weight of leaves and DW is the dry weight of leaves after drying in an oven for 48 h at 60 °C. Succulence of leaves was expressed in grams per square centimetre and determined per control plant as $(FW - DW) / LA$ where LA is the total leaf area. Leaf area was measured by the Image-Pro Plus v.4.5 software (Media Cybernetics, Inc., Silver Spring, MD, USA). Softness or specific leaf area (SLA) was calculated as LA / DW and expressed in square centimetres per gram dry weight (Hanley et al. 2007).

Data analysis

To ensure that plant development variation among populations was not biased by the environmental conditions of the experimental site, we compared the germination and growth rates of seedlings with the environmental distances between the experimental site and the conditions of origin of each population (for a similar procedure, see Hernández-Serrano et al. 2014). Climate data were obtained from www.opengis.uab.es/wms/iberia/mms/index.htm and www.climate-data.org (see Supplementary Data Table S1). We first calculated the Gower distance between the climate variables of each population and those of the experimental site, and then correlated the germination and growth rates of each provenance with its environmental (Gower) distance. Correlations were not

significant (Pearson $r < 0.17$, $P > 0.21$), indicating that the particular environment of the experimental site did not differentially affect development of the seedlings evaluated.

To estimate the variation in tannin content among and within populations of *Q. ilex*, a general linear mixed (GLM) model was used. The tannin content (expressed in mg TA eq/100 mg) was used as the dependent variable; block was used as a fixed factor; population and mother tree (nested within the population) were used as random factors; and individual seed weight, time to germinate and plant height were used as covariates. The block \times mother tree-within-population interaction was not significant ($P > 0.05$) and therefore was not included in the model. To estimate the variation in defoliation by *L. dispar* among and within populations, a similar GLM model was applied, this time using defoliated area (expressed in cm^2) as the dependent variable, block as a fixed factor and population and mother tree (nested within the population) as random factors. The block \times population interaction, and individual seed weight, time to germinate, plant height and initial larval weight covariates, were included in the model. With these models, we estimated variance components, and narrow-sense heritability across populations (h^2) was estimated as the additive genetic variance (V_A) divided by the phenotypic variance (V_P). To correct estimates of h^2 for a generalised selfing rate of about 1 to 3% in native *Q. ilex* stands (Ortego et al. 2014), an r coefficient of relatedness of 0.27 rather than 0.25 for true half-sibs was used (because full-sibs share half their genes and half-sibs share one quarter). In the numerator, the additive genetic variance was therefore calculated as 3.704 times the variance component among families ($\sigma^2_{f(\text{pop})}$). Phenotypic variance was deemed to be the sum of $\sigma^2_{f(\text{pop})}$, the among-populations variance (σ^2_p) and the error variance (σ^2_e). Pooled within-populations narrow-sense heritability was then estimated as

$$\hat{h}^2 = \hat{V}_A / \hat{V}_P = (\hat{\sigma}^2_{f(\text{pop})} \cdot 1/r) / (\hat{\sigma}^2_{f(\text{pop})} + \hat{\sigma}^2_p + \hat{\sigma}^2_e)$$

Approximate standard errors of heritability estimates were obtained following Jayaraman (1999). In accordance with Leinonen et al. (2013), Q_{ST} was calculated as

$$Q_{ST} = \hat{\sigma}^2_p / (\hat{\sigma}^2_p + 2\hat{\sigma}^2_{f(\text{pop})})$$

To check whether different populations and mother trees influence larval performance, the same GLM model was applied, this time with larval weight gain (expressed in mg day^{-1}) as the dependent variable. During the 4 days of larval growth, larval weight gain was assumed to follow a linear function. Initial larval weight was included as a covariate. The residuals of the models were checked for normality and means were compared using the Tukey HSD test.

To estimate the variation of structural traits of leaves among populations, a GLM model was used. Spines per leaf, spines per leaf area, trichome density, trichome dry weight per leaf dry weight, water content, succulence and specific leaf area were used as the dependent variables, population was used as the random factor and individual seed weight, time to germinate and plant height were used as covariates.

Genetic (r_g) and phenotypic (r_p) Pearson correlations among seed weight, time to germinate, plant height, leaf tannin content, defoliation and larval weight gain variables were obtained using family-mean and individual values, respectively. The Bonferroni correction was used, and significances were divided by the number of statistics involved ($\alpha/15$). At the population level, relationships between defoliation (and larval weight gain) and structural traits of leaves were examined by means of pairwise Pearson correlations. To assess whether relationships between performance of *L. dispar* larvae and defoliation vary as a function of populations and mother trees, tests for homogeneity of slopes on covariates were performed. A GLM analysis included larval weight gain (mg day^{-1}) as the dependent variable and the interaction between defoliation (cm^2 ; continuous predictor) and population (categorical predictor) as variables. The same GLM was then performed using mother tree as the categorical predictor. All analyses were performed with STATISTICA v10 (Stat Software Inc., Tulsa, OK, USA).

Results

Tannin content variation

Leaf tannin content did not differ significantly among populations, but it differed significantly among families and covaried positively with plant height (Table 1). In some populations, mean values of tannins were significantly different

Table 1 Results of the general linear mixed model for analysis of tannin content of leaves of *Q. ilex* seedlings

Effect	DF	<i>F</i>	<i>P</i> value
Random factors			
Population	5	1.6	0.160
Mother tree (population) ^a	7	1.8	<i>0.024</i>
Fixed factor			
Block	4	6.6	<i>0.002</i>
Covariates			
Seed weight	1	3.6	0.061
Plant height	1	11.5	<i>0.001</i>

Degrees of freedom (DF) and *F*-ratios are shown. Significant *P* values are shown in italics

^a The mother tree was nested within the population

among their mother trees (e.g. SN and GR) whereas in others not (Fig. 3). Within the SN population, for example, seedlings from tree 1 had 70% higher leaf tannin content than seedlings from tree 2 ($P = 0.008$; Fig. 3). Tannin content of leaves of 458 individuals ranged from 16.31 to 2.87 mg TAEq/100 mg and showed a mean value of 7.5 mg TAEq/100 mg (SD = 2.1). Leaf tannin content showed significant narrow-sense heritability across populations (h^2) of 0.83 ± 0.23 and genetic differentiation (Q_{ST}) of 0.12 (Table 2).

Root tannin content was not significantly variable at either the population or within-population level. Tannin content of roots of 460 individuals ranged from 7.65 to 0.09 mg TAEq/100 mg and showed a mean value of 1.9 mg TAEq/100 mg (SD = 0.9). Because of the lack of significance, h^2 and Q_{ST} of root tannin content are not shown.

Leaf tannin content of trees in the common garden was not related to root tannin content or the climate parameters of the provenance sites of trees. At the population level, despite the failure to detect statistical population differences, root tannin content was significantly related to summer rainfall of sites ($r = 0.88$; $P = 0.018$). A positive relation between leaf tannin content and time to germinate ($r = 0.92$; $P = 0.010$) was observed, indicating a higher tannin level in provenances germinating later. At the family level, time to germinate was also predictive of leaf tannin content (Table 3, above the diagonal). At the individual level, however, time to germinate was not related to leaf tannin content (Table 3, below the diagonal).

Herbivory variation

Defoliation was strongly dependent on population ($P = 0.011$) and family ($P = 0.012$) and was affected by plant height (Table 3). Narrow-sense heritability across populations and Q_{ST} estimates of defoliation were 0.13 ± 0.17 and 0.36, respectively (Table 2). Defoliation was not related to leaf or root tannin content, or stem damage. Positive family-mean relations were observed between defoliation and seed weight and between defoliation and plant height (Table 3).

Herbivore performance variation

Larval weight gain differed significantly depending on which populations (Fig. 4b) and families of *Q. ilex* seedlings were defoliated. Interestingly, larval weight gain covaried positively with seed weight ($P = 0.017$). At the population level, larval weight gain was not related to plant tannin content, defoliation or stem damage. Other significant relations at the family and individual levels are shown in Table 3.

Depending on the tree population, differential increase in larval weight gain with defoliation was observed, as indicated by the significant population \times defoliation interaction revealed in the homogeneity of slopes test ($P = 0.001$). Leaves of SM trees, for example, resulted in higher larval weight gain with

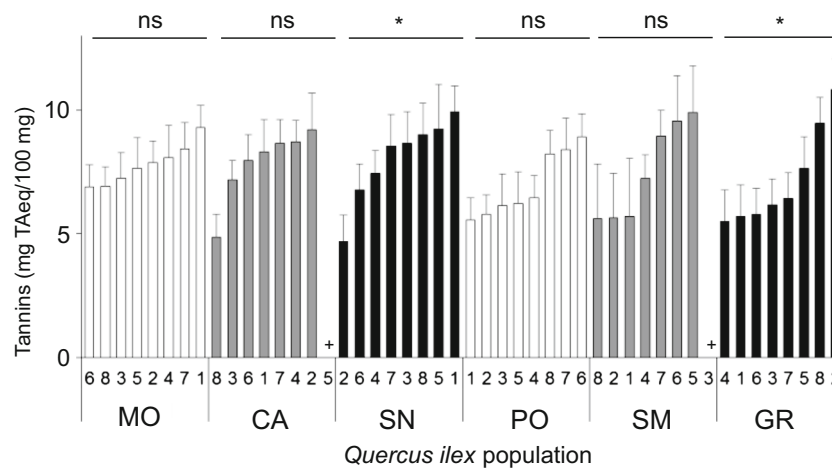


Fig. 3 Tannin content of leaves of *Quercus ilex* families originating from Monfragüe National Park (MO), Cabañeros National Park (CA), Sierra Norte de Sevilla Natural Park (SN), Pollensa (PO), Sierra de María-Los Vélez Natural Park (SM) and Grazalema Natural Park (GR) populations. Eight

families per population were analysed except for CA and SM, in which one family had less than three seedlings available (+). Vertical bars are standard errors and asterisks indicate significant differences of mean values within populations (asterisk $P < 0.05$) according to the Tukey HSD test

defoliation than leaves of MO trees (Fig. 5). Differential increase in larval weight gain with defoliation was not dependent on the mother tree ($P = 0.577$). Differential increase in larval weight gain with seed weight was not dependent on the population, but was dependent on the mother tree ($P = 0.236$ and $P = 0.029$, respectively).

Variation of structural traits of leaves among populations

The number of spines per leaf did not differ significantly among populations (Table 4). However, trichome dry weight per leaf dry weight, water content and succulence of leaves varied among populations (Table 4). At the population level, defoliation and larval weight gain were not related to spine and trichome densities but larval weight gain was significantly related to water content of leaves ($r = 0.92$, $P = 0.010$).

Discussion

Tannins are genetically controlled in *Q. ilex*

Estimated within- and among-population genetic variances of leaf tannin content in *Q. ilex* were in the ratio 78:22%. The presence of substantial additive genetic variation within the species ($\hat{h}^2 = 0.83$) enables short-term evolutionary change in response to natural selection and makes future breeding initiatives designed to obtain tannin-rich plant material feasible. Although earlier studies explored the genetic basis of several adaptive traits in oak species (Ramírez-Valiente et al. 2014; Nisar et al. 2016), as far as we know, this study is the first to provide a quantitative estimation of heritability and genetic differentiation for a chemical trait in a Mediterranean oak species. Genetic control of condensed tannins in trees was reported only for *Populus* sp., with broad-sense heritability

Table 2 Estimates of variance components (σ^2), narrow-sense heritabilities (\hat{h}^2) and quantitative genetic differentiation among populations (Q_{ST}) of *Q. ilex* seedlings for tannin content of leaves, tannin content of roots, defoliation by *L. dispar*, time to germinate and plant growth

Statistic	Tannin content of leaves ^a	Defoliation ^b	Time to germinate ^c	Plant growth ^d
$\hat{\sigma}^2_{f(pop)}$	0.000947	0.193	48.7	0.96
$\hat{\sigma}^2_p$	0.000259	0.222	106.0	0.14
$\hat{\sigma}^2_e$	0.002999	5.127	41.2	7.60
\hat{h}^2	0.83 ± 0.23	0.13 ± 0.17	0.92 ± 0.18	0.41 ± 0.13
Q_{ST}	0.12	0.36	0.52	0.07

^a Variance components were adjusted for seed weight, time to germinate and plant height covariates

^b Variance components were adjusted for seed weight, time to germinate, plant height and initial larval weight covariates

^c Variance components were adjusted for the seed weight covariate

^d Variance components were adjusted for seed weight and time to germinate covariates

Table 3 Family-mean (above the diagonal) and individual-seedling phenotypic (below the diagonal) correlations between traits assessed in *Quercus ilex* seedlings exposed to defoliation by *Lymantria dispar*

	Seed weight	Time to germinate	Plant height	Leaf tannin content	Defoliation	Larval weight gain
Seed weight	1	ns	0.68**	ns	0.38*	0.48**
Time to germinate	0.17*	1	ns	0.41*	ns	ns
Plant height	0.41**	-0.29**	1	ns	0.41*	0.42*
Leaf tannin content	ns	ns	ns	1	ns	ns
Defoliation	ns	ns	0.18*	–	1	0.45*
Larval weight gain	0.30**	ns	ns	–	0.22*	1

Asterisks indicate significance after applying the Bonferroni correction ($\alpha/15$) at * $P < 0.05/15$ and ** $P < 0.01/15$ ns not significant since $P \geq 0.05/15$

values ranging from 0.52 ± 0.42 to 0.90 ± 0.01 (Stevens and Lindroth 2005; Bailey et al. 2006; McKown et al. 2014). Genetic control of time to germinate (since sowing) and plant growth (\approx increase of plant height since sowing) in *Q. ilex* is also reported here for the first time ($h^2 = 0.92 \pm 0.18$ and $h^2 = 0.41 \pm 0.13$, respectively; Table 2). Despite the small standard errors, values of h^2 should be taken with caution given the relatively small number of families and individuals per family used.

Q_{ST} is a standardised measure of genetic differentiation among populations displayed by quantitative traits. Comparative studies of the divergence of quantitative traits and neutral molecular markers, known as Q_{ST} - F_{ST} comparisons, provide a means for researchers to distinguish between natural selection and genetic drift as causes of population differentiation in traits (Leinonen et al. 2013). The use of Q_{ST} - F_{ST} comparisons has increased rapidly in the last few years (Hernández-Serrano et al. 2014; Santiso et al. 2015),

enabling a wide range of questions relevant to evolutionary and ecological genetics to be addressed. However, estimates of Q_{ST} and other traits, especially those related to plant defence, are lacking (Solla et al. 2015). The genetic differentiation of tannin content among *Q. ilex* populations observed ($Q_{ST} = 0.12$) is a first step in providing some insight into the variation of a secondary plant metabolite throughout the vast heterogeneous area occupied by this oak species in Spain.

Variation of herbivory within and among *Q. ilex* populations

Herbivory is not distributed uniformly across available hosts among and within tree populations. Comparison of responses to defoliation in conspecific populations with different origins is a common experimental strategy for studying plant resistance to defoliation. The variation in the defoliation observed in seedlings from different mother trees and populations explains the non-uniform defoliation observed in *Q. ilex* forests when *L. dispar* outbreaks occur (Ibáñez-Justicia et al. 2007). Environmental factors and genetic differences among caterpillars

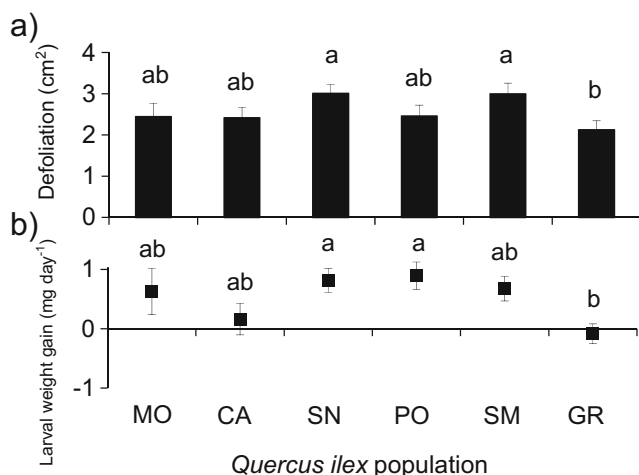


Fig. 4 Defoliation rates (a) and larval weight gain (b) following herbivory by *L. dispar* of *Q. ilex* seedlings originating from Monfragüe National Park (MO), Cabañeros National Park (CA), Sierra Norte de Sevilla Natural Park (SN), Pollensa (PO), Sierra de María-Los Vélez Natural Park (SM) and Grazalema Natural Park (GR) populations. Values were not adjusted for covariation. Vertical bars are standard errors and different letters indicate significant differences of mean values between populations ($P < 0.05$) according to the Tukey HSD test

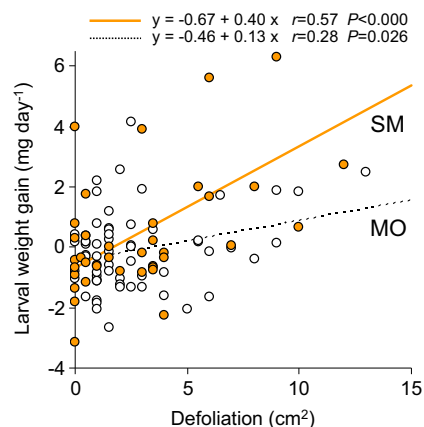


Fig. 5 Larval weight gain following defoliation by *L. dispar* of individual *Q. ilex* seedlings originating from Sierra de María-Los Vélez Natural Park (SM, orange circles, continuous line) and Monfragüe National Park (MO, empty circles, dashed line) populations as a function of defoliation ($n > 30$)

Table 4 Average (\pm standard deviation) and range of individual values of structural leaf traits in *Q. ilex* seedlings, and *F*-ratios resulting from a general linear mixed model for analysis of these traits among tree populations

	Average \pm SD	Range	Effect: population ^a	
			<i>F</i>	<i>P</i> value
Spines per leaf (<i>N</i>)	12.7 \pm 2.7	5.0–20.1	1.6	0.146
Spines per leaf area (<i>N</i> cm ⁻²)	4.0 \pm 1.3	1.9–9.2	1.7	0.131
Trichome density (mg cm ⁻²)	0.9 \pm 0.2	0.1–1.5	0.5	0.708
Trichome dry weight per leaf dry weight (%)	9.5 \pm 4.2	2.1–25.4	2.0	<i>0.010</i>
Water content (g g ⁻¹)	1.0 \pm 0.2	0.1–1.9	6.2	<i><0.001</i>
Succulence (g cm ⁻²)	0.013 \pm 0.003	0.001–0.026	4.3	<i>0.001</i>
Specific leaf area (cm ² g ⁻¹ dry weight)	53 \pm 11	6.7–86.5	1.3	0.260

Significant *P* values are shown in italics

^a Seed weight, time to germinate and plant height were included in the model as covariates

(Mrdaković et al. 2013) may also explain non-uniform defoliation of *Q. ilex* forests.

Defoliation, larval weight gain and densities of spines and trichomes did not vary significantly between different populations from the same region of provenance, e.g. among MO, CA and SN (Fig. 4), from the ‘Extremadurensis’ region (Fig. 1). However, earlier research in *Q. ilex* using enzyme polymorphism and studying acorn mass variation reported substantial genetic differentiation among nearby populations in the same region (Michaud et al. 1995; Bonal et al. 2012).

The southern populations of *Q. ilex* in Spain are currently suffering severe decline, mostly caused by episodes of drought and waterlogging and the presence of invasive *Phytophthora* species (Corcobado et al. 2014; Martín-García et al. 2015; Jung et al. 2016). Increasing temperatures have led to more abundant outbreaks of *L. dispar* (Spathelf et al. 2014), and it has recently been observed that *L. dispar* herbivory and larval growth rates are higher in declining oaks (Milanović et al. 2015). Thus, a positive feedback loop for *Q. ilex* decline is expected. This is the first study to report a heritability estimate for the level of herbivory in *Q. ilex* ($h^2 = 0.13$). The intraspecific variation observed should be taken into account in conservation and breeding initiatives to preserve *Q. ilex* forests. Moreover, the low tannin content levels in roots of *Q. ilex* populations with greater exposure to summer drought could be relevant when explaining oak decline syndrome.

Tannin content in *Q. ilex* is not predictive of defoliation by *L. dispar*

That defoliation did not reflect tannin content is not surprising; in earlier studies, tannin content either predicted defoliation (Feeny 1976; Mrdaković et al. 2011) or not at all (Osier and Lindroth 2001; Barbehenn et al. 2009a, b; Milanović et al. 2015; Caldwell et al. 2016). Holm oak contains both hydrolysable (Gharzouli et al. 1999) and condensed (Estiarte et al. 2007) tannins, which probably have very different effects on insects. Among the hydrolysable tannins, ellagitannins are especially

prone to oxidise in the midguts of several species of caterpillars (Barbehenn et al. 2009a, b; Barbehenn and Constabel 2011), forming reactive oxygen species able to cause lesions in the midgut epithelium. However, when *L. dispar* fed on *Acer saccharum* leaves coated with condensed tannins (15% d.w.), growth rates of larvae were unaffected and antioxidant effects were not observed (Barbehenn et al. 2009a). Although some classes of chemicals have been portrayed as having general effects on herbivores (e.g. condensed tannins; see Feeny 1976), other chemicals appear to be effective against specific insects (Johnson et al. 2014) or are most effective in combination with a specific mixture of other secondary metabolites. Moreover, specialist insect herbivores often evolve counter-adaptations to overcome or even benefit from specialised chemical defences (Johnson et al. 2014). Thus, correlations between herbivore susceptibility and genetic variation in the amount of a specific chemical can range anywhere from negative to positive and could therefore be non-significant, as observed here. Our results emphasise the need to measure tannin type and composition in leaves, rather than simply total tannins. Spines, trichomes, prickles and thorns on leaves and along stems can serve as defences against insects (Hanley et al. 2007; Karioti et al. 2011b; Pearse 2011). In *Q. ilex*, the number of spines and trichomes per leaf seems to have no effect on *L. dispar* attack. In consequence, other secondary metabolites (e.g. terpenoids, glucosinolates; Karioti et al. 2011a) may individually or synergistically influence *L. dispar* feeding patterns.

Performance of *L. dispar* larvae is influenced by the genetic structure and early-life traits of *Q. ilex*

Although the role of insects as a selective pressure shaping plant phenotypes has frequently been reported (e.g. Züst and Agrawal 2016), little is known about the genetic basis of hosts and early-life traits of plants influencing herbivore performance. As far as we know, variability of herbivory and insect performance has not been assessed in defoliated *Q. ilex* hosts. This lack of information limits our understanding of

defoliation outbreaks as an evolutionary process influencing herbivores and trees. Severe defoliation of oaks by *L. dispar* results in tree mortality (Morin and Liebhold 2016), acorn loss during the outbreak (unpublished results) and lower acorn production the following year (Liebhold et al. 2000).

Larval performance was positively related to defoliation and significantly conditioned by the mother tree and the tree population. That mother trees had a large effect on larval weight gain is not surprising, as differences among *Populus* genotypes were found to have similar effects in other studies (Osier et al. 2000 and references therein). In defoliated *Populus tremuloides*, performance of *L. dispar* larvae was strongly influenced by plant genotype and much less so by defoliation (Osier and Lindroth 2001). There is little information on maternal effects in oaks, but it is widely known that these effects generally appear at early ontogenetic stages, affecting early-life traits such as seed mass, germination rate, seedling growth and resistance to pathogens (Díaz et al. 2003; Solla et al. 2011; Vivas et al. 2013, 2014; Corcobado et al. 2016). Larvae feeding on some tree populations were much less efficient in converting food to body mass than larvae feeding on other populations (Fig. 5). Moreover, larvae were more efficient at converting ingested food to biomass when foliage originated from heavy acorns and freshly formed foliage, probably because leaves contained higher concentrations of compounds (e.g. nitrogen, water) favourable for caterpillar growth.

Conclusions

Three main findings can be derived from the results of this study. Firstly, constitutive leaf tannin content is a heritable trait in *Q. ilex*. Variability of constitutive tannins among and within *Q. ilex* populations may be an inherent source of stress-tolerant ecotypes in the face of current global change conditions. Secondly, tannin content in leaves and roots, and spine and trichome densities in leaves of *Q. ilex* are not predictive of *L. dispar* defoliation or larval performance. Finally, the origin and genotype of *Q. ilex* trees play a major role in determining patterns of *L. dispar* defoliation and larval performance.

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