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Understanding the importance of intrapopulation functional variability and phenotypic plasticity in *Quercus suber*

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Abstract Climate is one of the main abiotic factors influencing natural selection patterns. Year-to-year variation in climate is postulated to elicit temporal shifts in the direction and form of selection. Here, we examine the importance of trait means and plasticities for fitness under interannual variation in rainfall and assess the shifts in selection in cork oak. We performed selection analyses using the progeny of 45 mother trees established in a common garden experiment across two consecutive years that differed in rainfall. Growth and seven functional traits (specific leaf area, leaf size, leaf shape traits, 13-carbon isotope discrimination, and leaf nitrogen) related to drought tolerance were measured. Selection analyses showed fitness benefits of reduced specific leaf area (SLA) in a dry year and increased leaf size in a mesic year, indicating that they are key traits for this evergreen oak to cope with different water availabilities. SLA and leaf size were also particularly plastic traits, but the adaptive significance of plasticity could not be confirmed. The absence of correlation between growth across years using family means and the absence of correlations between SLA and leaf size suggested that fluctuating selection over time favored different maternal families under different annual weather conditions, which could promote functional diversity within populations in this long-lived species.

Keywords Fluctuating selection · Adaptive plasticity · Genetic diversity · Canalization

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Introduction

Climate is one of the main abiotic factors influencing natural selection patterns. Since the development of phenotypic selection analyses in the 1980s (Lande and Arnold 1983; Arnold and Wade 1984a, 1984b), there has been a marked increase in the number of studies on selection (Kingsolver and Diamond 2011). Phenotypic selection analysis (sensu Lande and Arnold 1983) is a simple methodology based on regression models that allows us to estimate the strength, direction, and type of selection on individual traits by studying the relationship between traits and fitness (or, more often, proxies thereof). It can be also used to investigate adaptive hypotheses for traits by testing the correlation between trait and fitness in different environments (Dudley 1996). Differences in strength and sign of the correlation across environments are indicative of contrasting selection and provide evidence for the adaptive significance of a given trait under specific environmental

conditions (Wade and Kalisz 1990; Dudley 1996; Donovan et al. 2009). A significant number of studies have found that the magnitude and direction of selection vary depending on the levels of environmental factors such as light, nutrients, and water (Dudley 1996; Etterson 2004; Heschel and Riginos 2005; Sherrard and Maherali 2006; Saldana et al. 2007; Donovan et al. 2007; Donovan et al. 2009). In particular, temporal shifts in the direction of selection caused by year-to-year variation in climate are apparently frequent in nature (Siepielski et al. 2011). This varying selection could lead to changes in relative frequencies of different alleles in natural populations and over the long-term lead to evolutionary change of the species (Siepielski et al. 2009). In the context of the ongoing climate change, this phenomenon is particularly relevant because shifts in the selective pressures are expected to occur in many ecosystems throughout the world (Umina et al. 2005; Jump et al 2006).

In sessile species like plants, with a limited migration capacity, phenotypic plasticity (i.e., the capacity of a given genotype to produce different phenotypes in response to the environment; Reed et al. 2010) can also be crucial to an organism's response to variation in selective pressure over time. Some authors suggest that the adaptive significance of a plastic response depends on the relative length of the organism life cycle with respect to the length of the cycle of environmental variation to which it is subjected (Fusco and Minelli 2010). They suggested that long-lived species, whose life cycle is longer than the temporal variation, would preferentially respond to environmental variability by means of morphophysiological plasticity. In contrast, adaptation to the environment in species with short life cycles is postulated to be driven by selection on specific allelic variants of genes encoding for functional characters that produce specific (and relatively invariant) trait means. Other authors postulate that the adaptive significance of phenotypic plasticity primarily depends on the nature of the environmental variation. In particular, it has been suggested that when the environment is fluctuating and unpredictable in time, phenotypic plasticity would be maladaptive due to the costs of maintaining induced plastic responses (Valladares, et al. 2000, 2002, 2006). In such variable habitats, including Mediterranean ecosystems, a conservative resource-use strategy together with a phenotypic canalization would be favored (see Matesanz and Valladares 2014 for a review).

Cork oak (*Quercus suber* L.) is an evergreen, wind-pollinated tree with a wide distribution in the western Mediterranean basin (Alía et al. 2009; Aronson et al. 2009). As with other sympatric Mediterranean oak species, cork oak defines the ecosystem and plays a keystone resource role for wildlife (Aronson et al. 2009). The species is found in a high variety of climatic conditions: Annual precipitation ranges from 400 to 2000 mm and annual temperatures from 12 to 20 °C (Catalan 2003; Alía et al. 2009; Aronson et al. 2009). Previous studies in common gardens

have shown that cork oak populations present high variation in survival, growth, and leaf traits (Gandour et al. 2007; Ramirez-Valiente et al. 2009a, b; 2010; 2011; 2014a, b, c). Adaptation to contrasting environmental conditions has been suggested as the main mechanism responsible for the differences observed in functional traits among populations (Ramirez-Valiente et al. 2009b, 2010, 2014b). Cork oak also exhibits high phenotypic plasticity in leaf traits (Ramirez-Valiente et al. 2010, 2014b). In addition, the detection of a significant population-by-environment interaction for growth indicates that different plastic responses are promoted in different environments (Ramirez-Valiente et al. 2010).

Our previous phenotypic selection analyses have shown that interannual variation in annual rainfall alters the patterns of selection on traits over time (Ramirez-Valiente et al. 2010, 2014b). Differences in magnitude and sign of selection on specific leaf area and water use efficiency were particularly remarkable (Ramirez-Valiente et al 2010, 2014b). However, the adaptive significance of phenotypic plasticity for different traits in temporally fluctuating environments has not yet been explored. In fact, only a few studies in long-lived species have included formal tests to explore the adaptive significance of trait plasticities; most studies assess this question through indirect inferences based on theoretical predictions (but see Matesanz and Valladares 2014).

Another issue that has not traditionally been taken into account in selection studies is the effect of micro-environmental variation on selection estimates (Schluter et al. 1991; Rausher 1992; van Tienderen 1991; Mauricio and Mojonnier 1997). The logic behind this is that if trait means and fitness are affected by common environmental factors, then significant correlations between trait and fitness can arise because of this covariation. This bias can be considerably high (Stinchcombe et al. 2002). For this reason, Rausher (1992) and Stinchcombe et al. (2002) claimed that selection studies should use some kind of genetic structure within populations (i.e., clone, full-sib family, and half-sib family) as mean genotypic values as opposed to individual values take into account the potential microenvironmental variation (Rausher 1992). However, obtaining real genotypic values requires control for maternal effects, which is not always possible in species with long life cycles.

In this study, we measured growth and seven phenotypic traits related to drought tolerance (specific leaf area, leaf size, leaf shape traits, 13-carbon isotope discrimination, and leaf nitrogen) in 45 open-pollinated families of cork oak in two climatically contrasting years. Our goals were (1) to assess the extent to which fluctuations in annual rainfall cause selection to vary in sign, magnitude, and direction, and (2) to explore the importance of trait means and plasticities for explaining growth as a proxy of fitness in this period. Differences in magnitude and/or direction of selection across years would be indicative that fluctuating climate is causing selection to

vary (Grant and Grant 2002). More specifically, we tested whether selection favored cork oak families with traits conferring increased water use efficiency and reduced water loss (e.g., lower specific leaf area, and smaller and more elongated leaves) in dry conditions. To explore our second goal, we analyzed the relationship between trait plasticities and growth. If selection analyses show a positive relationship between phenotypic plasticity of a given trait and growth, then it would support the hypotheses that plasticity is adaptive in a fluctuating environment.

Material and methods

Common garden

The study was conducted in a common garden experiment established in the "Sierra de Andújar" Natural Park (38° 21′ 54″ N, 3° 51′ 40″ W, 560 m a.s.l.), which is located in southern Spain, within the core of the cork oak natural range. The trial followed a design of randomized complete blocks. Sixty-six open-pollinated maternal families of three populations were assayed. Two plants per family were set in each block. Populations originated from sites in Morocco, Spain, and Portugal that have similar annual rainfall, but contrasting severity of the dry season (Ramirez-Valiente et al. 2011). Briefly, there is a pattern of increasing drought severity and length toward southern sites. The Portuguese population exhibits the highest amount of rainfall in summer (31 mm) and 3 months of dry season (where $2TM \leq PM$, TM being monthly mean of the maximum and minimum temperatures and PM the monthly precipitation; Grossmann et al. 2002). In contrast, Moroccan population presents the lowest rainfall in summer (10 mm) and the longest dry season (6 months). The Spanish population has intermediate values, 20 mm of summer rainfall and 4 months of dry season.

Seeds for the trial were collected from randomly selected mother trees within each population. They were separated at least 150 m from one other, which is an enough distance to avoid familial structures in cork oak (Soto et al. 2007). The seeds were collected from the mother trees during winter of 1996. They were sown in the beginning of 1997 and grown in a nursery for 1 year under standard conditions of water and nutrient availability before being planted in the field during the spring of 1998.

Climate in the trial site is Mediterranean, characterized by dry warm summers and moderately cold winters. The average annual precipitation is 617 mm and the average annual temperature is 14.7 °C. The average temperatures for the most contrasting months are 5.9 °C in January and 25.3 °C in July. Most precipitation falls between October and January (329.3 mm), and very little during the summer months (34.3 mm on average from June to September) (data for the

interval 1987–2005, taken from the closest climatic station to the plot, located about 4 km from the common garden).

For the present study, 45 maternal families (15 families per population) were followed over two climatically and consecutive contrasting years, one dry and one mesic. Specifically, we randomly selected one plant per family in eight blocks of the common garden (i.e., 15 families×3 populations×8 blocks×1 plant per family/block=360 plants). The between-year differences in amount of annual, spring, and summer rainfall were remarkable (Fig. 1). Annual rainfall was 306.0 mm in 2005 versus 600.6 mm in 2006. Precipitation during the period of April–September was 36.3 and 168.7 mm for 2005 and 2006, respectively. Annual mean temperature was similar in the 2 years, 15.3 °C in 2005 and 15.6 °C in 2006, mainly due to differences in autumn mean temperatures.

Leaf morphology

Four sun-leaves formed in spring were collected from each of three orientations (N, SE, SW) for each sampled plant (12 leaves per plant in total) in each studied year to characterize
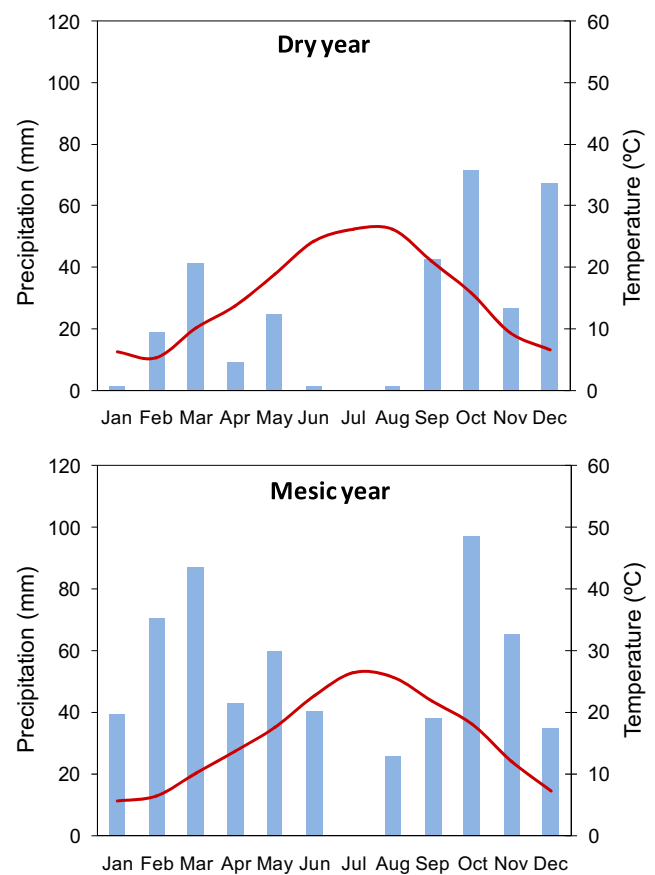

Fig. 1 Average monthly rainfall (*blue bars*) and temperature (*red curve*) for the two study years in the common garden: dry (2005) and mesic (2006). Data were obtained from the nearest meteorological station located 4 km from the common garden (Color figure online)

leaf morphology (N=360 plants×12 leaves/plant×2 years= 8640 leaves in all). Leaf collection took place in autumn to be sure that the vegetative period, which can extend over summer, had ended. Leaves were digitalized and analyzed using the software WINFOLIA v. 2002 and Image J. Average leaf size of the plant was estimated by means of several morphological leaf traits: individual leaf area, maximum length, maximum width, and perimeter. All these parameters were strongly correlated among each other (all r>0.80, P<0.0001), so we finally used only leaf area (leaf size from now on) for further analyses. Leaf shape was measured by (1) circularity index ($4\pi A/P^2$), where A is the leaf area and P is the perimeter (this index grades the leaf shape between circular and filliform); (2) aspect ratio or elongation factor, which is the ratio of maximum horizontal width to vertical length (W/L); and (3) perimeter square/area ratio (P/A), which is usually called the shape factor. High P/A indicates more complex leaf shape (e.g., lobing and dissection). Leaves were oven-dried at 65 °C to a constant weight after scanning. Specific leaf area (SLA) was calculated for one leaf per orientation (three leaves per plant) using the ratio of leaf area to dry weight of each leaf.

Biochemical traits

Two biochemical leaf traits highly related to control of water losses and water use efficiency were measured: 13-carbon isotope discrimation ($\Delta^{13}C$) and leaf nitrogen content per unit mass (N_{mass}). The dry leaf material above was ground in a ball mill (following Ramírez-Valiente et al. 2011). Carbon-13 isotopic composition ($\delta^{13}C$) was determined with a Micromass Isochrom mass spectrometer and leaf nitrogen content by the Kjeldahl method (Vapodest 50, Gerhardt) for each plant. The $\delta^{13}C$ values were used to estimate the isotopic discrimination ($\Delta^{13}C$) as

$$\Delta^{13}C(‰) = \delta_a - \delta_p / (1 + \delta_p/1000)$$

where δ_p (‰) refers to $\delta^{13}C$ of bulk leaf material, and δ_a is the $\delta^{13}C$ of the air acting as carbon source (it was assumed to be −7.9‰).

Fitness

Plant size has been documented to be related to both age of first reproduction and total acorn yield, in terms of mass and number in oaks, at both interspecific (Guyette et al. 2004) and intraspecific levels (Greenberg 2000). Our unpublished results from other common garden trials of a close-related taxon (*Quercus oleoides*) also suggest that time for the first reproduction is highly associated to tree height ($F_{1,585}$=61.35, P<0.001; Ramirez-Valiente & Cavender-Bares, unpublished).

For this reason, annual growth, which is a component of total growth, is considered an important response variable for juvenile oaks. In fact, aboveground growth has been considered as proxy of fitness in many other selection studies (e.g., Donovan et al. 2007, 2009, Nicotra et al. 2008).

In our experiment, the annual growth per plant was obtained by averaging the growth of six shoots of the spring flush located in the top-third of the crown and covering six different orientations of the plants. Our preliminary analyses on 1-year-old saplings of five climatically contrasting populations suggest that aboveground and belowground growth is highly correlated for cork oak. In this study, we measured only aboveground growth because of the logistic limitations of analyzing belowground growth in 8-year-old trees.

Statistical analyses

Linear mixed models were implemented for growth and the seven measured traits (specific leaf area, leaf size, width/ length, perimeter/area, circularity index, 13-carbon isotope discrimination, and leaf nitrogen) to analyze the maternal family and population differentiation in trait means and plasticity. The size of the plants estimated by total height was included in the model as a covariate because of its potential influence on leaf function and annual growth (Ramirez-Valiente et al. 2010). These analyses were carried out using the procedure PROC MIXED of the statistical package SAS 9.1 (SAS/ STAT® Software; SAS Institute), with Type III sum of squares, and a restricted maximum likelihood method (REML; Littell et al. 1998) that renders unbiased estimates.

We implemented linear and non-linear (quadratic) selection analyses on functional trait means measured in two climatically contrasting years, dry (2005) and mesic (2006), using two approaches: multiple regression (Lande and Arnold 1983) and structural equation modeling (Scheiner et al. 2000). Annual aboveground growth was considered the fitness proxy. This metric is assumed to be appropriate for selection studies in long-lived species when plants are not reproductively mature (Nicotra and Davidson 2010). Selection analysis estimates direct and total selection on traits by calculating what are called "selection gradients" and "selection differentials." Following Lande and Arnold (1983), linear selection gradients (β) are estimated performing a multiple regression where standardized trait values are regressed against relative fitness. Analogously, non-linear selection gradients (γ) are the partial regression coefficients for quadratic terms in multiple polynomial regressions. Linear selection differentials (S) are calculated using simple regression between each standardized trait and relative fitness. Non-linear selection differentials (C) are the partial regression coefficients for quadratic terms in quadratic regressions (for further explanation, see Lande and Arnold 1983). Maternal-family trait means were used instead of individual trait values to test for selection. Differences between

years in selection coefficients were tested through a test of heterogeneity of slopes in an analysis of the covariance (ANCOVA). The analyses were performed using the average maternal family values. "Population," "year," and their inter-action were included as fixed factors, and plant size and traits were included as covariates. ANCOVAs were only performed for those traits presenting significant selection differentials between years. The relationships between traits and growth were also represented by plotting the least squares means of significant traits and growth in each year.

Structural equation modeling (SEM) provides an aprioristic-statistical approach that can be used to unravel the linking structure of traits and is normally implemented to explore the biological mechanisms underlying the observed covariance structure among traits. In addition, SEM can be used to make predictions about patterns of selection (Scheiner et al. 2000). In selection analyses using SEM, direct selection is represented by β' for linear terms and γ' for quadratic terms, while direct+indirect (total) selection is represented by S' for linear terms and C' for quadratic terms. In our study, several tentative specific models relating traits and fitness were generated according to the previous knowledge of the species. For this approach, we assessed whether our aprioristic SEM (Supplementary Fig. S1) fitted the data by a series of goodness-of-fit tests. Covariance structure patterns among traits and between traits and fitness were calculated for each year. In addition, because the metrics of leaf shape (perimeter square/area and circularity index) were highly correlated with leaf size ($r=0.90$, $P<0.0001$) and width/length ($r=0.49$, $P<0.0001$), respectively, they were excluded from the analyses to reduce multicollinearity. For similar approaches, see Scheiner et al. (2000), Shefferson and Roach (2010), and Gimenez-Benavides et al. (2011). Population and plant size were also included in the models to control for possible effects on trait relationships with fitness. See Milla et al. (2009) for a similar procedure.

Additionally, we examined whether different families were favored in different years due to changes in selection. For this purpose, we investigated (1) the relationship between traits within years and (2) the relationship between growths across years using family means. Non-positive relationships between traits within years and values of growth across years would be evidence of absence of trait integration for the studied traits. Non-positive association between growths across years would denote that different environmental conditions favor families with different leaf traits.

Trait and growth values over two successive years might not be statistically and biologically independent. In fact, it has been demonstrated in some species that growth in year n is dependent on the number of growth units that were present in the bud and formed during year $n-1$ (Stanturf et al. 1989; Bisang et al. 2008). For this reason, we tested whether there was autocorrelation across years in growth and traits under selection by

assessing the correlation between the residuals of the growth/ traits over successive years. Correlations were performed using residuals obtained from mixed models for family values.

Finally, to explore the importance of plasticity for explaining fitness in the 2-year period, we used regression analyses and SEM (see previous paragraphs for details). For this purpose, we regressed each individual plant's relativized fitness across years (i.e., relativized increment in growth) on its standardized functional trait plasticities. Plasticity for traits was estimated by subtracting the maternal-family mean of the dry year from the maternal-family mean in the mesic year (Valladares et al. 2006; Caruso et al. 2006). Thus, we calculated S, β, C, and γ for trait plasticities by using linear regressions and SEM as made for trait means. These analyses were performed only for those traits that showed significant plasticity in response to annual conditions of water, i.e., significant year effect in mixed models (Caruso *et al.* 2006).

Results

Interpopulation and intrapopulation variation in functional traits and plasticity

The results from the mixed models showed significant differences among populations in leaf size and specific leaf area (SLA) and a marginally non-significant difference for N_{mass} (Table 1). The Moroccan population had the largest leaves as well as the highest SLA and N_{mass} (Supplementary Table S1). In contrast, cork oaks originating from Portugal exhibited on average the smallest leaves and lowest SLA values (Supplementary Table S1). Spanish population had the lowest N_{mass}. In addition, we found a high among-maternal family variation in most studied traits (Table 1). Maternal families within populations differed in all morphological traits and water use efficiency (measured by $\Delta^{13}C$) (Table 1 and Supplementary Table S1).

The mixed models also revealed significant effect of the year on all variables including growth, leaf morphology, and biochemistry (Table 1). On average, plants had larger leaf sizes, higher W/L, $\Delta^{13}C$, and N_{mass}, and lower P/A in the mesic year than in the dry year indicating high phenotypic plasticity for these traits (Supplementary Table S1). The population by year and maternal-family by year interactions were not significant for any trait, indicating absence of differences in phenotypic plasticity across populations and maternal families within populations (Table 1).

Selection analyses

The results of the selection analyses using multiple regression (Table 2) and SEM (Supplementary Table S2 and Fig. S2) were quite similar, as expected. They both showed linear

Table 1 General linear mixed model results for fitness and seven morphological and biochemical traits

		Population	Maternal family (Population)	Year	Block	Population×year	Maternal family×year	Plant size
Fitness traits								
Annual growth	Test	2.33	1.07	780.70	4.30	0.06	–	81.29
	P	0.110	0.286	**<0.001**	**<0.001**	0.942	–	**<0.001**
	% variance	0.07	0.19	26.81	0.45	0	0	62.89
Morphological traits								
Leaf size	Test	39.62	2.71	321.12	3.67	0.75	–	24.82
	P	**<0.001**	0.007	**<0.001**	**<0.001**	0.471	–	**<0.001**
	% variance	8.47	2.08	24.73	0.85	0	0	43.37
Circularity index	Test	0.57	3.57	18.33	3.50	1.81	–	13.11
	P	0.569	**<0.001**	**<0.001**	**<0.001**	0.165	–	**<0.001**
	% variance	0	8.85	2.41	1.57	0.20	0	47.13
Width/length	Test	2.22	3.45	90.50	4.13	2.51	–	71.70
	P	0.121	**<0.001**	**<0.001**	**<0.001**	0.081	–	**<0.001**
	% variance	0.19	2.38	4	0.57	0.16	0	80.72
Perimeter/area	Test	1.07	3.45	1.61	5.00	0.03	–	16.88
	P	0.352	**<0.001**	0.204	**<0.001**	0.966	–	**<0.001**
	% variance	0.03	6.46	0.09	1.86	0	0	59.87
SLA	Test	4.89	2.30	174.42	3.71	0.60	–	12.13
	P	**0.012**	**0.022**	**<0.001**	**<0.001**	0.552	–	**<0.001**
	% variance	1.28	2.51	24.3	1.59	0	0	33.76
Biochemical traits								
$\Delta^{13}C$	Test	2.20	3.53	8.73	31.02	0.08	–	3.41
	P	0.123	**<0.001**	**0.003**	**<0.001**	0.922	–	*0.065*
	% variance	0.85	7.81	0.93	47.43	0	0	7.4
N_{mass}	Test	2.91	0.96	4.38	10.50	0.81	0.22	9.45
	P	*0.065*	0.355	**0.04**	**<0.001**	0.452	0.826	**0.002**
	% variance	0.73	1.39	0.7	7.2	0	0.32	38.27

Fixed factors: Population, Year, Block, Population × year. Random factors: Maternal family nested within Population [represented as Maternal family (Population)], and Maternal family (Population) × year. Covariates: Plant size. F-test and Z wald were implemented for fixed and random factors, respectively

SLA specific leaf area, $\Delta^{13}C$ 13-carbon isotope discrimination, N_{mass} leaf nitrogen content

Significant *P* values (*P* < 0.05) are given in bold

direct and total selection (i.e., directional selection) on reduced SLA and *W/L* means in a dry year, but this selection differential was lacking in a mesic year (Table 2 and Supplementary Table S2). That is, maternal families that had lower SLA also had higher growth under dry conditions, but not under mesic conditions (Fig. 2a, b). In addition, both analyses found linear selection on increased leaf size mean in the mesic year, but not in the dry year (Table 2 and Supplementary Table S2), indicating that maternal families with increased leaf size had higher growth in mesic conditions but not in dry conditions (Fig. 2c, d).

ANCOVAs implemented to test for differences between years in selection coefficients showed significant year-by-trait interactions for SLA and leaf size, which indicates

significant differences in the selection coefficients for the 2 years (Table 3). There was not a significant inter-action between year and trait for *W/L*. ANCOVAs for traits showed no relationship between leaf size and SLA in dry (*P*=0.842) and mesic years (*P*=0.540), indicating that maternal families that produce thick leaves in dry years are not the ones that produce large leaves in wet years. Likewise, there was no significant relationship between growth in dry and mesic years, indicating that different maternal families were favored depending on the annual climatic conditions (*P*=0.249, Fig. 3).

Correlation analyses of residuals showed that there was autocorrelation across years in both SLA (*r*=0.33, *P*=0.025) and leaf size (*r*=0.80, *P*<0.001). Interestingly, there was no autocorrelation for growth residuals across years (*r*=0.00, *P*=

Table 2 Selection analyses showing linear selection gradients (β) and differentials (S) and non-linear selection gradients (γ) and differentials (C) for trait means in dry and mesic years and plasticity across years using multiple regression models

Trait	Dry year		Mesic year		Plasticity	
	Direct selection (β)	Total selection (S)	Direct selection (β)	Total selection (S)	Direct selection (β)	Total selection (S)
SLA	**−0.16**	**−0.15**	−0.04	−0.03	−0.04	−0.05
Leaf size	0.00	0.08	0.04	**0.09**	0.07	0.03
W/L	**−0.11**	**−0.14**	−0.01	−0.05	0.00	0.01
$\Delta^{13}C$	0.00	−0.06	0.00	−0.05	−0.04	−0.04
N_{mass}	−0.03	−0.02	−0.02	0.02	−0.03	−0.03
Trait	Dry year		Mesic year		Plasticity	
	Direct selection (γ)	Total selection (C)	Direct selection (γ)	Total selection (C)	Direct selection (γ)	Total selection (C)
SLA^2	0.02	−0.02	0.02	−0.02	−0.02	−0.02
Leaf size^2	−0.14	−0.04	−0.07	−0.08	−0.02	−0.01
W/L^2	−0.07	0.02	0.01	0.09	−0.02	−0.03
$\Delta^{13}C^2$	−0.05	−0.11	0.04	0.04	−0.02	−0.02
$N_{mass}{}^2$	−0.10	0.01	−0.03	−0.02	−0.02	−0.04

Coefficients in bold type are statistically significant

0.990) consistent with the absence of correlation for growth means (see results above).

No significant linear selection differentials or gradients were observed for plasticity of any trait and nor were quadratic selection differentials and gradients significant for trait plasticity (Table 2). In general terms, this indicates that plasticity on the studied traits did not have a clear effect on growth across two climatically contrasting years in the common garden.

Fig. 2 Relationships between growth and leaf traits in dry and mesic years. Relationships are shown for **a** growth and SLA in a dry year, **b** growth and SLA in a mesic year, **c** growth and leaf size in a dry year, and **d** growth and leaf size in a mesic year. *Points* represent maternal family means
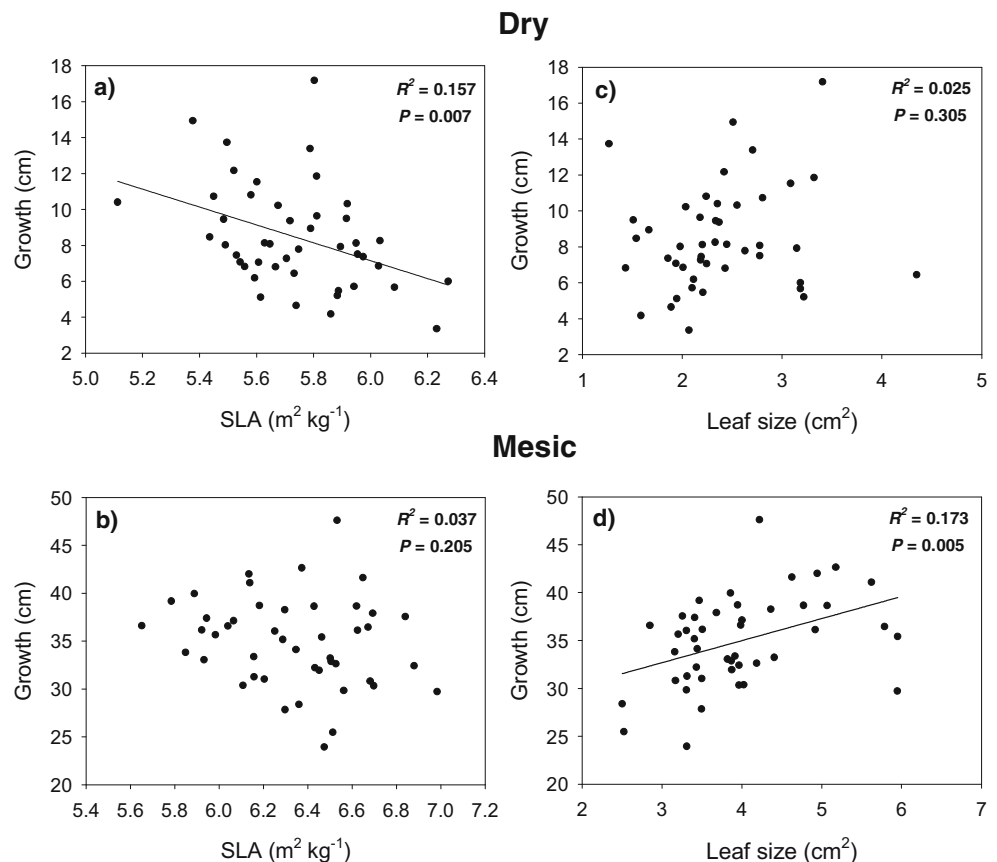
Table 3 ANCOVA results indicating the effects of leaf size, specific leaf area, and leaf width/length ratio on annual growth across 2 years with contrasting rainfall

Source	df	SS	F	P
Leaf size				
Population	2	0.094	0.88	0.4134
Year	1	3.935	74.09	**<0.0001**
Population × year	2	0.229	2.15	0.1171
Plant size	1	3.641	68.56	**<0.0001**
Leaf size	1	3.087	58.13	**<0.0001**
Year × leaf size	1	0.880	16.56	**0.0001**
Specific leaf area				
Population	2	0.697	6.71	**0.0013**
Year	1	0.877	16.88	**<0.0001**
Population × year	2	0.023	0.22	0.8026
Plant size	1	3.088	59.42	**<0.0001**
Specific leaf area	1	2.804	53.96	**<0.0001**
Year × specific leaf area	1	3.035	58.40	**<0.0001**
Width/length				
Population	2	0.263	2.22	0.1093
Year	1	0.330	5.56	**0.0186**
Population × year	2	0.011	0.09	0.9136
Plant size	1	4.008	67.59	**<0.0001**
W/L	1	0.110	1.86	0.1737
Year × W/L	1	0.013	0.22	0.6366

Significant year × trait interaction indicates a significant difference in the relationship of the trait and fitness for the 2 years

Significant P values (P < 0.05) are given in bold

Discussion

Fluctuating selection on SLA and leaf size

Our analyses of leaf traits showed growth benefits for reduced SLA in a dry year and for increased leaf size in a mesic year (Fig. 2). These results suggest that fluctuating rainfall was an
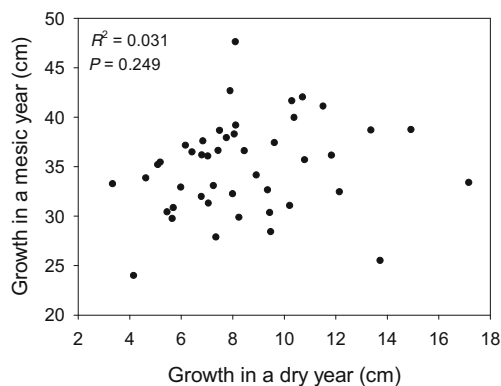

$R^2 = 0.031$
$P = 0.249$

Fig. 3 Relationship between growth in dry and mesic years. *Points* represent maternal family means

important factor causing selection to vary between years. Long-term studies of selection in some natural populations indicate that interannual variation in environmental conditions, particularly rainfall, can lead to changes in the direction or magnitude of selection over time (Siepielski et al. 2009; Bell 2010; Kingsolver and Diamond 2011; Siepielski et al. 2011). The patterns of selection found in this study were similar to those found for SLA in our previous works, which were carried out in different common gardens of cork oak without any intrapopulation genetic structure (Ramirez-Valiente et al. 2010, 2014a). Thus, our findings are consistent across trials and support the important role of reduced SLA under low water availability conditions for cork oak. Conversely, the results for leaf size contrasted with our previous observations where we found that larger leaves presented growth benefits in both dry and mesic conditions in several common gardens (Ramirez-Valiente et al. 2010, 2011). Two possibilities could have led to these differences across studies. On one hand, population was not included as a categorical factor into the phenotypic selection analysis in our previous studies, which could have led to false-positive associations between leaf size and fitness due to population differences in both variables (i.e., population structure). On the other hand, using individual trait values instead of family means could have increased the "environmental" bias in the estimates of selection gradients and differentials in our previous studies. In fact, some studies have suggested that variation in the magnitude, form, and direction of selection over time arises as a result of artifacts of sampling error and statistical biases (Kruuk et al. 2003; Kingsolver et al. 2012; Morrissey and Hadfield 2012). Environmental effects were postulated by these authors as one of the main sources of bias in selection gradients and differentials. Rausher (1992) and Scheiner et al. (2002) suggested that using a genetically structured design in selection studies, whether with clones, full-sib families, or half-sib families, would reduce the environmental biases. In the present study, we used half-sib family values instead of individual values in the selection analyses, limiting the bias from environmental origin that Morrissey and Hadfield (2012) stated as the main source of bias in selection coefficients.

The observed positive associations between growth and reduced SLA in a dry year and increased leaf size in a mesic year are consistent with the expectations of functional hypothesis for environments with low and high water availabilities (Etterson 2004). More sclerophyllous leaves allow plants to avoid an excess of water loss, maintaining photosynthetic activity and carbon gain over a longer time period (Reich et al. 1999, Poorter et al. 2009). For a given number of leaves, larger leaves increase the photosynthetic area, which would increase the carbon gain when water availability is high (Dudley 1996). The results from selection analyses in the two studied years did not agree with the population differentiation observed in this study, where the xeric population from

Morocco showed the largest leaves and lower SLA. However, our results did agree with the population differentiation observed in our previous studies where a higher number of populations were analyzed (Ramirez-Valiente et al. 2010, 2014b). These studies showed that populations from dry and warm places have lower SLA and leaf size and suggest that the inconsistency of that relationship in this study is probably due to the limited number of populations. Ramirez-Valiente et al. (2009a, b) investigated the relative importance of natural selection and neutral evolution on genetic differentiation among populations in leaf traits. We found that SLA (27.8 %) and leaf size (45.9 %) had the highest genetic differentiations among populations, which were much higher than that expected just by neutral evolution (3.3 %). These results indicated that population differences in these traits were mainly explained by long-term selection and adaptation to different environmental conditions. Overall, our previous and present findings indicate that specific leaf area and leaf size are key traits for cork oak response to water availability and that adaptation to local climate is the main evolutionary force leading intraspecific differences in these traits.

Phenotypic plasticity and intrapopulation variation

Cork oak saplings exhibited a plastic response to interannual variation in rainfall. The factor "year" explained over 24 % of variance in two traits, specific leaf area and leaf size, indicating high differences in trait means among years and therefore high plasticity. These results rule out the hypothesis of canalization for these traits, as has been hypothesized to occur in fluctuating and unpredictable environments such as Mediterranean climates (Valladares et al. 2002). As shown above, interannual variation in rainfall favored different trait means across years. However, selection analyses did not show fitness benefits of trait plasticities for cork oak families, contrary to our expectations (Fusco and Minelli 2010). The absence of selection on phenotypic plasticity could be due to the low family variation for plasticity, which gave us low statistical power to test for plasticity-fitness correlations. Further research is needed to conclusively rule out or support the adaptive significance of phenotypic plasticity of SLA and leaf size under interannual rainfall variations.

Mixed model results showed also high intrapopulation differentiation (maternal family differences) in SLA, leaf size, leaf shape, and $\Delta^{13}C$, which concurs with our previous report for some traits in only one year, and the high intrapopulation genetic diversity observed in neutral markers (Ramirez-Valiente et al. 2011, 2014b).

SLA and leaf size were not correlated in any year using family means, indicating that these two traits are independently expressed in the maternal families (Fig. 2). However, there was a significant autocorrelation of the residuals across years for these two traits which is indicative of values these two

traits over successive years are biologically or statistically not fully independent. In other words, maternal families exhibiting higher values of SLA or leaf size in the dry year also had higher values of these traits in the mesic year. Interestingly, our statistical analyses revealed an absence of both correlation between growth in dry and growth in mesic years using family means (Fig. 3) and autocorrelation of the residuals across years, suggesting that maternal families showing higher growths in the dry year did not have a higher growth rate in the mesic year. Overall, the results suggest that specific maternal families of cork oak (those which reduced SLA) have growth benefits in dry years, whereas other maternal families (those which increased leaf size) are favored in mesic years. These findings suggest that fluctuating selection over time could promote and maintain functional diversity within populations in this long-lived species by favoring different genotypes under different annual weather conditions (Burger and Gimelfard 2002). Nevertheless, at this point, two considerations must be taken into account: Half-sib families are not actual genotypes (they mix half-sib and full-sibs), and this study was performed in only 2 years for a long-lived species. For these reasons, further research studies including analyses with clones or controlled genotypes and also considering other fitness components over a longer period are warranted to provide a more integrated measure of selection (Arnold and Wade 1984a; Wade and Kalisz 1990; Fairbairn and Preziosi 1996; Hunt et al. 2009; Shaw and Geyer 2010; Kingsolver et al. 2012).

In conclusion, selection analyses showed growth benefits for maternal families with reduced SLA in a dry year and increased leaf size in a mesic year. These findings are consistent with studies at the population level and indicate that selection favors cork oak saplings with traits conferring increased water use efficiency and reduced water loss under dry conditions (Ramirez-Valiente et al. 2009a, b, 2014b). SLA and leaf size were also particularly plastic in response to annual variations in rainfall, but contrary to expectations, the adaptive significance of plasticity could not be confirmed. Finally, the absence of family-mean correlations across years for growth and the absence of family-mean correlation between SLA and leaf size suggested that changes in direction and magnitude of selection over time could promote functional diversity within populations in this long-lived species (Siepielski et al. 2009; Bell 2010; Kingsolver and Diamond 2011).

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