



## Original article

# Population variation and natural selection on leaf traits in cork oak throughout its distribution range



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## ABSTRACT

A central issue in evolutionary biology is the exploration of functional trait variation among populations and the extent to which this variation has adaptive value. It was recently proposed that specific leaf area (SLA), leaf nitrogen concentration per mass ( $N_{\text{mass}}$ ) and water use efficiency in cork oak play an important role in adaptation to water availability in the environment. In order to investigate this hypothesis, we explored, first, whether there was population-level variation in cork oak (*Quercus suber*) for these functional traits throughout its distribution range; if this were the case, it would be consistent with the hypothesis that different rainfall patterns have led to ecotypic differentiation in this species. Second, we studied whether the population-level variation matched short-term selection on these traits under different water availability conditions using two fitness components: survival and growth. We found high population-level differentiation in SLA and  $N_{\text{mass}}$ , with populations from dry places exhibiting the lowest values for SLA and  $N_{\text{mass}}$ . Likewise, reduced SLA had fitness benefits in terms of growth for plants under dry conditions. However, contrary to our expectations, we did not find any pattern of association between functional traits and survival in nine-year-old saplings despite considerable drought during one year of the study period. These results together with findings from the literature suggest that early stages of development are the most critical period for this species. Most importantly, these findings suggest that cork oak saplings have a considerable potential to cope with dry conditions. This capacity to withstand aridity has important implications for conservation of cork oak woodlands under the ongoing climate change.

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## 1. Introduction

To understand the evolution of adaptive traits, it is necessary both to document functional trait variation within populations and assess the adaptive value of this variation (Ackerly, 2004; Poorter et al., 2008). Spatially structured selection usually leads to the development of adaptations to the local environment, generating population differentiation within species in important functional traits (Kawecki and Ebert, 2004; Savolainen et al., 2007).

Additionally, neutral evolutionary processes and demographic history can also cause population differentiation (Merilä and Crnokrak, 2001; Leinonen et al., 2006). The adaptive significance and fitness consequences of trait variation can be examined through analysis of variances and covariances between traits and fitness (Lande and Arnold, 1983; Arnold and Wade, 1984a,b). In this framework, a given trait is considered adaptive for a particular species in a given environment only if that trait is related to fitness in that environment, and the association is weak or absent in other environments where the trait is not expected to be adaptive (e.g. Dudley, 1996; Donovan et al., 2007).

An accurate estimation of the fitness of an organism requires the consideration of multiple components of overall fitness (Campbell, 1991; Charlesworth, 1994; van Tienderen, 1995, 2000; Kozłowski,

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1999; Gomez, 2004). For example, plants move through different vital stages during their life cycles, and these are connected by transitional processes such as germination, survival, growth and reproduction. Each process constitutes a fitness component and the resulting fitness will be a function of all of them (van Tienderen, 2000; Crone, 2001; Gomez, 2004). Surprisingly, a single component, such as survival, is often considered to estimate the phenotypic selection on functional traits (Bell, 1997; Fairbairn and Reeve, 2001; Donovan et al., 2007).

Specific leaf area (SLA) and leaf nitrogen concentration per mass ( $N_{\text{mass}}$ ) are leaf traits that play central roles in plant growth rate and survival (Lambers and Poorter, 1992; Grime et al., 1997; Wright et al., 2004). In fact, these traits have been proposed to be universally important for plant fitness (Poorter et al., 2008). Species having high SLA and  $N_{\text{mass}}$  usually have high relative growth rate (Reich et al., 1997; Cunningham et al., 1999; Schulze et al., 2006; Poorter and Bongers, 2006; Poorter et al., 2009). SLA and  $N_{\text{mass}}$  are important for growth because high SLA leads to a higher leaf area displayed per a given unit leaf mass, and high leaf N invested in carbon uptake increases the photosynthetic capacity of the leaf (Antunez et al., 2001; Ruiz-Robledo and Villar, 2005). Conversely, leaves having high SLA and  $N_{\text{mass}}$  may be more vulnerable to desiccation, leading to reduced survival in arid and nutrient-poor environments (Reich, 2001). But these observations are based on inter-specific studies across biomes. Recent studies have demonstrated that when intraspecific variation is considered for a single biome, the relationships between trait values and fitness may be considerably modified (Cianciaruso et al., 2009; Hulshof and Swenson, 2010; Messier et al., 2010). Particularly, since carbon gain is accomplished at the cost of water loss, transpiring more water than the plant can replace causes water stress. As a consequence, the plant may be unable to acquire the carbon necessary for growth, or under extreme conditions, survival. Thus, although an increased net carbon acquisition strategy gives plants more resources and higher growth rates in most environments, traits controlling water losses may be more critical to survive, grow, and reproduce in dry habitats.

In this regard, natural selection is expected to maximize water use efficiency (i.e. the ratio of total carbon gained to total water lost over the growing season) in dry environments (Nicolson and Davidson, 2010). WUE can be measured as the instantaneous rates of photosynthesis and transpiration, but also by means of the integrative Carbon-13 isotope discrimination ( $\Delta^{13}\text{C}$ , Farquhar et al., 1982; Farquhar and Richards, 1984; Brugnoli and Farquhar, 2000).  $\Delta^{13}\text{C}$  is negatively related to water use efficiency (WUE) during periods of photosynthetic activity. The negative linear relationship between  $\Delta^{13}\text{C}$  and WUE is based on the ratio of intercellular ( $c_i$ ) to atmospheric  $\text{CO}_2$  partial pressures. A low  $c_i$  reflects either a high photosynthetic efficiency, or more commonly, a low stomatal conductance. Thus, when plants increase WUE (via both photosynthetic capacity and stomatal conductance), carboxylating enzymes diminish their discrimination against  $^{13}\text{C}$  due to the limited  $c_i$ . Genetic variation in carbon isotope discrimination have been found among and within species, including differences among conspecific populations (Correia et al., 2008; Aranda et al., 2010), open-pollinated families (Brendel et al., 2002; Ramírez-Valiente et al., 2011) and clones (Prasolova et al., 2003). Many of these studies have also documented clear evidence of local adaptation, with ecotypes from arid environments displaying higher  $\Delta^{13}\text{C}$  than ecotypes from mesic environments (Marchin et al., 2008).

In a recent study on cork oak (*Quercus suber*), population-level differences were observed in SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$  (Ramírez-Valiente et al., 2010). Populations originating from humid places presented the highest values of SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$ . Associations between SLA,  $\Delta^{13}\text{C}$  and growth (used as a fitness surrogate) were

also observed across two climatically contrasting years in a common garden. Results showed that individuals with reduced SLA in a dry year grew better, whereas individuals showing increased  $\Delta^{13}\text{C}$  (reduced WUE) grew more in a wet year. In that study, it was proposed that SLA,  $N_{\text{mass}}$  and WUE play an important adaptive role for cork oak to adapt to hydrological conditions. In order to investigate this hypothesis, we explored, first, whether there was population-level variation in cork oak for these functional traits throughout its range in a way that was consistent with the hypothesis that different rainfall patterns have led to ecotypic differentiation in this species. Second, we studied whether the population-level variation was associated with short-term selection on these traits under different water availability conditions. For this purpose, we performed phenotypic selection analyses under contrasted water conditions using two fitness components: survival and growth. We estimated phenotypic selection on traits separately for each fitness component considered in this study. If selection differentials for SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$  were negative under dry conditions and they were weaker, null or positive under mesic conditions, we could conclude that low SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$  (higher water use efficiency) play an adaptive role in dry environments.

## 2. Material and methods

### 2.1. Study system: cork oak

Cork oak (*Quercus suber*) is an evergreen forest tree species with a wide distribution in the western Mediterranean basin (Aronson et al., 2009). Like other sympatric Mediterranean oak species, cork oak defines local ecosystems and plays a keystone role for wildlife (Aronson et al., 2009). It is a useful species for the examination of the mechanisms underlying the ecotypic differentiation because its range spans a large variety of climatic conditions: mean annual rainfall of 400–1500 mm and a mean annual temperature of 13–20 °C (Aronson et al., 2009; Alía et al., 2009).

### 2.2. Study area, common-garden design, and description of study population

The study was carried out in a common garden trial established in western Spain at Monfragüe National Park (39° 51' N, 6° 1' W, 375 m, Cáceres), which is located within the core of the cork oak natural range (Aronson et al., 2009). This trial was established next to the cork oak trial examined in our previous study, where thirteen Spanish populations were assayed (see Ramírez-Valiente et al., 2010). In this case, fifteen populations drawn from the natural range of the species in the Mediterranean basin were established (Table 1). Six Spanish populations were shared by both trials. The studied populations covered the total variety of climates where the species can be found naturally (but see Aronson et al., 2009; Alía et al., 2009). The seeds for the experiment were collected from 20 to 30 open-pollinated trees per population in the autumn of 1996. Mother trees were separated by at least 100 m, which represents enough distance to avoid familial structuring in cork oak (Soto et al., 2007). Acorns were sown in a nursery for a year and seedlings were planted in a field trial in 1998. The trial followed a randomized complete block design. Thirty blocks were established and four plants per population were planted within each block (15 populations × 30 blocks × 4 plants per block = 1800 plants).

The climate of this area is typically Mediterranean characterized by cool winters and hot and dry summers. The average annual precipitation is 755.2 mm and the average annual temperature is 17.2 °C for the period 1964–2005 (AEMET, National Meteorological Agency).

**Table 1**

Location and climatic details for the fifteen cork oak populations established in the common garden experiment. P: Annual precipitation (mm), Ps: summer precipitation (mm), T (°C): annual mean temperature (°C), MMH: mean maximum temperature of the hottest month (°C), MMC: mean minimum temperature of the coldest month (°C).

Code	Nearest locality	Latitude	Longitude	Altitude	P	Ps	T	MMH	MMC
COL	Santa Coloma de Farnés, SP	41° 54' N	2° 30' E	175	805	160	15.6	30.5	0.0
PAR	El Pardo, SP	40° 31' N	3° 45' W	750	474	56	13.5	31.4	-0.1
LV	Cañamero, SP	39° 22' N	5° 21' W	600	1008	55	15.4	34.8	2.2
FUE	Fuencaliente, SP	38° 24' N	4° 16' W	670	432	39	14.8	33.3	0.9
JC	Jerez de los Caballeros, SP	38° 13' N	6° 42' W	492	627	39	16.1	33.5	4.1
HZ	Haza de Lino, SP	36° 47' N	3° 18' W	1300	607	24	13.0	29.3	1.3
ALM	Castellar de la Frontera, SP	36° 16' N	5° 22' W	118	813	20	17.4	29.0	7.5
SOR	Ponte do Sor, PO	39° 04' N	8° 10' W	119	681	37	16.7	30.4	7.2
SAL	Alcaçer do Sal, PO	38° 22' N	8° 32' W	30	709	31	16.3	27.8	7.8
BRAS	San Bras de Alportel, PO	37° 09' N	7° 53' W	245	558	18	16.1	28.7	6.3
TUS	Tuscania, IT	42° 25' N	11° 58' E	176	728	140	13.6	29.3	1.9
CAT	Catania, IT	37° 06' N	14° 30' E	289	448	9	16.1	27.6	6.6
AIN	Ain Rami, MO	35° 04' N	5° 12' W	425	865 <sup>a</sup>	10	17.4 <sup>a</sup>	32.7	4.8
OUL	Oulmes, MO	33° 26' N	6° 01' W	1115	725	25	14.0 <sup>a</sup>	31.6	-0.2
SOUS	Soustons, FR	43° 46' N	1° 20' W	70	1289	244	13.6	24.8	3.5

<sup>a</sup> Data obtained from a climatic model (Hijmans et al., 2005). SP: Spain, PO: Portugal; IT: Italia, MO: Morocco, FR: France.

### 2.3. Phenotypic measurements: survival, growth and leaf traits

The ecophysiological study extended over two climatically contrasting years: one mesic and one extremely dry. The differences in amount of annual and summer rainfall were particularly remarkable between years. Annual rainfall was 468.7 mm in 2005 versus 644.5 mm in 2006. Rainfall during June–September was 27.5 and 79.6 mm for 2005 and 2006, respectively. Temperatures were very similar in both years. Annual mean temperature was 17.1 °C in 2005 and 17.4 °C in 2006, mainly due to differences in autumn mean temperatures (October and November), which were 17.0 and 10.8 °C in 2005 and 18.6 and 13.5 °C in 2006. (AEMET, Spanish National Meteorological Agency).

Aboveground growth and a set of functional leaf traits were measured and survival was recorded in one plant per population in each block (15 populations × 30 plants per population = 450 plants) in both years. We recorded “annual survival” for each year and “long-term survival” in the last year of the study. “Annual survival” was defined as survival over a single year, ignoring those seedlings that had died at the time of the previous census. “Long-term survival” was defined as the survival from the moment that plants were established in field until the end of the study. Accumulated aboveground growth was measured by estimating basal diameter and total height. Diameter was measured at the base of the plants with calipers, and total height was measured with a pole ruler by considering the length from the bottom to the top of the main axis of each tree. Aboveground annual growth was measured as the elongation of six shoots of the spring flush, each in one of the six main compass orientations, located in the top third of the crown. This metric is highly correlated with other growth metrics such as increase in diameter (data not shown). In this study, we measured only aboveground growth because of the logistical limitations of analyzing belowground growth in nine-year old trees. Nevertheless, we consider the limitations of this approach in the Discussion.

Three functional leaf traits highly related to the control of water loss and water use efficiency were measured (SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$ ). For this purpose, twelve sun leaves were sampled from three of the six shoots selected for annual growth measurements (N, SE, SW oriented branches). Leaves were sampled in the spring and the leaf area for each leaf was measured after scanning. Image analysis was carried out with the image analyzer software WINFOLIA v. 2002 (Régent, Quebec, Canada). Individual leaf area (LA) for each plant was estimated as the average of the twelve measured leaves. Leaves were oven dried at 65 °C to a constant weight after scanning.

Specific leaf area (SLA) was estimated in one leaf per orientation using the ratio of dry weight to leaf area of one leaf. Specific leaf area can be decomposed as the product of leaf density and leaf lamina thickness (Poorter et al., 2009). In order to investigate the influence of these traits on SLA values and analyze their evolutionary role for cork oak, we measured leaf thickness in each leaf and calculated leaf density in the dry year (Vile et al., 2005; Poorter et al., 2009).

Some dry material was ground in a ball mill and used to determine the isotopic composition of  $\text{C}^{13}$  ( $\delta^{13}\text{C}$ ) with a Carlo Erba NC1500 (Milan, Italy) elemental analyser and a Delta Plus XL (ThermoQuest, Bremen, Germany) mass spectrometer (EA-IRMS).  $\delta^{13}\text{C}$  values were used to estimate the isotopic discrimination ( $\Delta^{13}\text{C}$ ) where  $\delta^{13}\text{C}$  of the air was assumed to be  $-7.9\text{‰}$  (Farquhar et al., 1982). Nitrogen content was analyzed on the same pool of leaves by the Kjeldahl method.

### 2.4. Data analyses

Generalized Linear Models were conducted to determine population-level differentiation in “annual survival” and “long-term survival”. Analogously, General Linear Models were constructed to test for differences in growth and leaf traits among populations. To investigate whether climate predicts population divergence in leaf traits and fitness, we performed linear regression of average population trait values on climatic variables, including one moisture index (which takes into account potential evapotranspiration in its estimates). The moisture index was calculated as  $I_m = 100 \times (P - \text{PET})/\text{PET}$ , where  $P$  is the annual precipitation and PET the potential evapotranspiration (following Edwards, 2006). Our analyses showed that the amount of rainfall and the moisture index were highly correlated (e.g.  $r = 0.98$ ,  $P < 0.0001$ ) for annual rainfall. As a consequence, only rainfall will be shown in further results. SAS 9.2 and R 2.15.2 were used to implement General and Generalized Linear Models.

We also estimated phenotypic selection on SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$  using two fitness components: (a) growth and (b) survival (see Adriaenssens and Johnsson, 2013 for a similar approach). Phenotypic selection analyses were implemented for each year: mesic and dry. However, no plants were dead in the mesic year (2006), so the phenotypic selection analysis using survival as the fitness component was performed only during the dry year.

Phenotypic gradients ( $\beta$ ), which estimate direct selection, were calculated performing a multiple linear regression where standardized trait values [(value – mean)/SD] were related to relative

**Table 2**  
Mean  $\pm$  standard error of survival, growth, 13-carbon isotope discrimination ( $\Delta^{13}\text{C}$ ), specific leaf area leaf (SLA), leaf thickness, leaf density and mass-based nitrogen concentration ( $N_{\text{mass}}$ ) for nine-year old cork oak saplings originating from fifteen populations. Values for the dry and mesic years are indicated. Survival was 100% for all populations in the mesic year. The significance level ( $P$ ) of differences among populations for each variable is indicated in the last row (see Table S1 for more details).

Population	Survival (%)	Growth (cm)		$\Delta^{13}\text{C}$ (‰)	SLA ( $\text{m}^2 \text{kg}^{-1}$ )		Leaf thickness (mm)	Leaf density ( $\text{kg m}^{-3}$ )	$N_{\text{mass}}$ ( $\text{mg g}^{-1}$ )	
	Dry	Dry	Mesic	Dry	Dry	Mesic	Dry	Dry	Dry	Mesic
Santa Coloma de Farnés, SP	96.7	4.67 $\pm$ 0.65	12.92 $\pm$ 1.61	20.38 (0.30)	5.51 $\pm$ 0.12	5.78 $\pm$ 0.11	0.21 $\pm$ 0.01	886 $\pm$ 24	18.51 $\pm$ 0.31	17.04 $\pm$ 0.47
El Pardo, SP	100.0	3.36 $\pm$ 0.39	10.95 $\pm$ 1.76	20.35 $\pm$ 0.42	5.00 $\pm$ 0.14	5.40 $\pm$ 0.15	0.23 $\pm$ 0.01	904 $\pm$ 21	17.77 $\pm$ 0.51	16.55 $\pm$ 0.58
Cañamero, SP	100.0	4.13 $\pm$ 0.44	13.96 $\pm$ 1.84	20.49 $\pm$ 0.28	5.44 $\pm$ 0.11	5.52 $\pm$ 0.12	0.21 $\pm$ 0.01	886 $\pm$ 20	18.94 $\pm$ 0.53	17.75 $\pm$ 0.56
Fuencaliente, SP	96.7	3.35 $\pm$ 0.64	11.45 $\pm$ 1.60	20.28 $\pm$ 0.26	5.36 $\pm$ 0.12	5.46 $\pm$ 0.13	0.22 $\pm$ 0.01	879 $\pm$ 21	18.20 $\pm$ 0.41	16.63 $\pm$ 0.54
Jerez de los Caballeros, SP	100.0	4.58 $\pm$ 0.66	9.91 $\pm$ 1.12	20.51 $\pm$ 0.22	5.21 $\pm$ 0.12	5.41 $\pm$ 0.12	0.22 $\pm$ 0.01	905 $\pm$ 22	18.25 $\pm$ 0.51	17.16 $\pm$ 0.65
Haza de Lino, SP	96.7	5.14 $\pm$ 0.87	11.68 $\pm$ 2.64	20.43 $\pm$ 0.23	5.01 $\pm$ 0.14	4.90 $\pm$ 0.10	0.24 $\pm$ 0.01	875 $\pm$ 30	16.85 $\pm$ 0.41	15.44 $\pm$ 0.44
Castellar de la Frontera, SP	100.0	5.02 $\pm$ 0.64	15.69 $\pm$ 2.02	20.07 $\pm$ 0.23	5.57 $\pm$ 0.12	5.58 $\pm$ 0.11	0.21 $\pm$ 0.01	871 $\pm$ 17	18.32 $\pm$ 0.40	17.86 $\pm$ 0.66
Ponte do Sor, PO	100.0	5.61 $\pm$ 0.87	11.79 $\pm$ 1.36	20.73 $\pm$ 0.18	5.53 $\pm$ 0.16	5.70 $\pm$ 0.12	0.21 $\pm$ 0.01	891 $\pm$ 24	17.90 $\pm$ 0.37	16.56 $\pm$ 0.42
Alcaçer do Sal, PO	100.0	3.31 $\pm$ 0.36	10.50 $\pm$ 1.32	20.74 $\pm$ 0.35	5.69 $\pm$ 0.12	5.83 $\pm$ 0.13	0.20 $\pm$ 0.01	889 $\pm$ 25	19.28 $\pm$ 0.47	17.71 $\pm$ 0.54
San Bras de Alportel, PO	93.3	4.26 $\pm$ 0.58	13.39 $\pm$ 2.05	19.86 $\pm$ 0.42	5.25 $\pm$ 0.10	5.46 $\pm$ 0.12	0.22 $\pm$ 0.01	889 $\pm$ 26	18.86 $\pm$ 0.42	18.45 $\pm$ 0.57
Tuscania, IT	93.3	4.61 $\pm$ 0.54	13.6 $\pm$ 1.69	20.72 $\pm$ 0.25	5.64 $\pm$ 0.12	5.87 $\pm$ 0.10	0.20 $\pm$ 0.01	887 $\pm$ 22	19.72 $\pm$ 0.47	18.36 $\pm$ 0.69
Catania, IT	100.0	5.14 $\pm$ 0.81	14.76 $\pm$ 1.69	20.42 $\pm$ 0.30	5.46 $\pm$ 0.16	5.52 $\pm$ 0.13	0.22 $\pm$ 0.01	873 $\pm$ 24	19.95 $\pm$ 0.48	19.12 $\pm$ 0.50
Ain Rami, MO	90.0	6.52 $\pm$ 0.93	12.77 $\pm$ 1.16	20.16 $\pm$ 0.17	5.80 $\pm$ 0.11	5.66 $\pm$ 0.16	0.20 $\pm$ 0.01	871 $\pm$ 26	20.02 $\pm$ 0.55	19.15 $\pm$ 0.69
Oulmes, MO	100.0	9.09 $\pm$ 1.13	14.46 $\pm$ 1.41	20.56 $\pm$ 0.33	5.33 $\pm$ 0.15	5.51 $\pm$ 0.16	0.23 $\pm$ 0.01	837 $\pm$ 23	18.21 $\pm$ 0.57	17.64 $\pm$ 0.71
Soustons, FR	96.7	3.75 $\pm$ 0.44	11.56 $\pm$ 1.26	19.87 $\pm$ 0.42	5.82 $\pm$ 0.13	5.82 $\pm$ 0.13	0.19 $\pm$ 0.01	956 $\pm$ 26	20.17 $\pm$ 0.53	18.26 $\pm$ 0.52
Population variation significance	$P = 0.149$	$P = 0.080$		$P = 0.577$	$P < 0.001$		$P < 0.001$	$P = 0.454$	$P < 0.001$	

annual growth. To calculate the non-linear gradients ( $\gamma$ ), quadratic terms and two-way interactions between traits were included as independent variables in a polynomial multiple regression, where growth was the dependent variable. The  $\gamma$  values for quadratic terms were obtained by multiplying by two the coefficients from the regression analysis (see Lande and Arnold, 1983; Stinchcombe et al., 2008). To determine the total selection on traits, phenotypic differentials ( $S$ ) were calculated using simple regression of each standardized trait on relative annual growth. Phenotypic differentials estimate the strength of total selection, including both direct and indirect effects. The quadratic differentials ( $C$ ) were estimated as the partial regression coefficient of the quadratic term in a quadratic regression.

Phenotypic selection was also estimated using survival as a fitness component. Survival in this study is a dichotomous variable. Standard linear regressions are not appropriate to analyze phenotypic selection using these types of data (Brodie et al., 1995). In this case, multiple logistic regressions were conducted to estimate the phenotypic selection gradients ( $\beta$ ) of SLA,  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$ , where survival was the dependent variable (see Janzen and Stern, 1998). To calculate the non-linear gradients ( $\gamma$ ), quadratic terms and two-way interactions between traits were included as independent variables in a polynomial multiple logistic regression. To determine the total selection, phenotypic differentials ( $S$ ) were calculated using simple logistic regressions. The quadratic differentials ( $C$ ) were estimated using logistic regressions with quadratic terms. The logistic regression coefficients were transformed using the technique proposed by Janzen and Stern (1998) to calculate the phenotypic selection gradients and differentials. R 2.15.2 was employed to perform the phenotypic selection analyses.

Environmental dissimilarities in standardized gradients were assessed by performing a test of homogeneity of slopes in analyses of covariance. When growth was used as a fitness component, we examined for differences between years in standardized differentials by implementing a test of homogeneity of slopes in an analysis of covariance. Phenotypic relationships between traits were assessed using Pearson correlations between all pair traits.

### 3. Results

#### 3.1. Population-level differentiation and phenotypic plasticity

The results from the generalized linear models showed no significant differences among populations in survival in the dry year ( $P = 0.149$ , Tables 2 and S1). In contrast, population did differ significantly in long-term survival ( $P < 0.001$ , Tables 3 and S2). The hierarchical ANOVAs showed marginal differences among populations in annual growth ( $P = 0.080$ , Table 2 and S1). There were also large population-level differences in height and diameter ( $P < 0.001$  for both traits, Table 3 and S2). In addition, significant population variation was observed for SLA,  $N_{\text{mass}}$  and leaf thickness but not in leaf density and  $\Delta^{13}\text{C}$  (Table 2 and S1). Population factors

**Table 3**

Mean  $\pm$  standard error of long-term survival, height and diameter for nine-year old cork oak saplings originating from fifteen populations. The significance level ( $P$ ) of differences among populations for each variable is indicated in the last row (see Table S2 for more details).

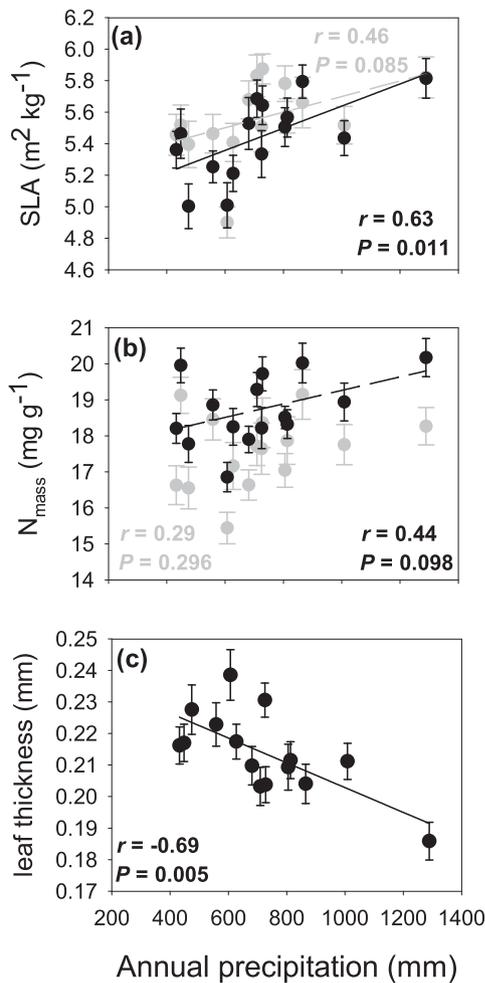
Population	Long-term survival (%)	Height (cm)	Diameter (cm)
Santa Coloma de Farnés, SP	72.3	73.8 $\pm$ 5.5	4.49 $\pm$ 0.38
El Pardo, SP	80.5	54.9 $\pm$ 3.4	3.68 $\pm$ 0.26
Cañamero, SP	76.1	79.5 $\pm$ 8.0	4.53 $\pm$ 0.35
Fuencaliente, SP	71.4	70.5 $\pm$ 7.6	3.86 $\pm$ 0.42
Jerez de los Caballeros, SP	75.6	65.1 $\pm$ 7.1	3.95 $\pm$ 0.33
Haza de Lino, SP	79.8	57.9 $\pm$ 5.1	3.90 $\pm$ 0.38
Castellar de la Frontera, SP	71.7	71.2 $\pm$ 6.0	4.47 $\pm$ 0.39
Ponte do Sor, PO	85.5	82.7 $\pm$ 6.1	4.27 $\pm$ 0.21
Alcaçer do Sal, PO	70.1	57.3 $\pm$ 4.0	3.42 $\pm$ 0.27
San Bras de Alportel, PO	68.6	66.0 $\pm$ 7.4	4.46 $\pm$ 0.34
Tuscania, IT	88.0	59.2 $\pm$ 5.1	4.63 $\pm$ 0.43
Catania, IT	78.3	62.1 $\pm$ 4.9	4.51 $\pm$ 0.30
Ain Rami, MO	77.2	105.5 $\pm$ 12.7	6.31 $\pm$ 0.38
Oulmes, MO	85.0	129.7 $\pm$ 11.1	4.73 $\pm$ 0.51
Soustons, FR	66.7	53.2 $\pm$ 3.3	3.09 $\pm$ 0.26
Population variation significance	$P < 0.001$	$P < 0.001$	$P < 0.001$

explained between <1% (for  $\Delta^{13}\text{C}$ ) and ~11% (for leaf thickness) of trait variance (Table S1). Annual precipitation was positively correlated with population values of SLA and  $N_{\text{mass}}$ , and negatively correlated with leaf thickness (Fig. 1). Similar results were found between the index of moisture and the tree leaf traits (Fig. S1).

The analyses of variance also revealed that there were significant differences among dry and wet years in growth, SLA and  $N_{\text{mass}}$  (Tables S1). The environmental factor dryness of year explained between 1.5 and 14% percentage of variance in growth (Table S1). The interaction term population  $\times$  year was not significant for any trait, indicating the absence of differences in plasticity among populations (Table S1).

### 3.2. Phenotypic selection on SLA, $N_{\text{mass}}$ and $\Delta^{13}\text{C}$

Plants with lower SLA values had significantly larger above-ground growth in the dry year ( $S = -0.29$ ,  $\beta = -0.29$ ) than in the mesic one ( $S = -0.12$ ,  $\beta = -0.11$ , Table 4). We did not find a significant relationship between SLA and survival ( $S = 0.01$ ,  $\beta = 0.01$ , Table 4). As such, reduced SLA had fitness benefits in a dry year in terms of growth but not in survival. In addition, plants with lower  $N_{\text{mass}}$  values had also larger aboveground growth in the dry year ( $S = -0.18$ ) than in the mesic one ( $S = -0.17$ , Table 4), although



**Fig. 1.** Relationship between annual precipitation with SLA,  $N_{\text{mass}}$ , leaf thickness ( $\pm$ SE) measured in dry (black points) and mesic years (gray points) in plants originating from fifteen cork oak populations established in the common garden trial. Solid lines are shown for significant ( $P < 0.05$ ) relationships. Dashed lines indicate marginally non-significant relationships ( $P < 0.10$ ).

**Table 4**

Phenotypic linear differentials ( $S$ ) and gradients ( $\beta$ ) for standardized plant ecophysiological traits and relativized annual aboveground growth and survival for dry and mesic years in a common garden experiment. Underlined text shows selection gradients and differentials varying statistically between years. <sup>(1)</sup>No plants were dead in the mesic year, so selection analyses were not implemented for survival for this year.

Trait	Survival		Survival <sup>(1)</sup>	
	Growth	Survival	Growth	Survival <sup>(1)</sup>
	Dry year $S$		Mesic year $S$	
SLA	<u>-0.29***</u>	-0.02	<u>-0.12*</u>	-
$N_{\text{mass}}$	<u>-0.18***</u>	-0.06	<u>-0.17</u>	-
$\Delta^{13}\text{C}$	-0.03	0.00	-	-
	Dry year $\beta$		Mesic year $\beta$	
SLA	<u>-0.29***</u>	0.01	<u>-0.11*</u>	-
$N_{\text{mass}}$	-0.01	0.01	-0.03	-
$\Delta^{13}\text{C}$	-0.02	0.01	-	-

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

SLA – Specific leaf area,  $N_{\text{mass}}$  – leaf nitrogen content per unit mass,  $\Delta^{13}\text{C}$  –  $^{13}\text{C}$ -Carbon isotope discrimination.

differences were not significant. In addition, when direct selection was explored, no significant gradient was observed for  $N_{\text{mass}}$  ( $\beta = -0.01$ ,  $\beta = -0.03$  for the dry and mesic year, respectively, Table 4). Carbon isotope discrimination did not show a significant relationship with any fitness component (Table 2). Non-linear terms were not significant for any trait (data not shown).

All phenotypic correlations are shown in Table 5.  $\Delta^{13}\text{C}$  was negatively correlated with  $N_{\text{mass}}$ , but not with SLA (Table 5).  $N_{\text{mass}}$  and SLA were positively correlated in both years (Table 5). SLA was negatively correlated with leaf thickness and leaf density (Table 5).

## 4. Discussion

### 4.1. Adaptive value of reduced SLA and $N_{\text{mass}}$ in dry environments

In this study, we provide evidence for the adaptive significance of reduced specific leaf area (SLA) in dry conditions. Cork oak populations from dry sites presented lower SLA and thicker leaves, consistent with the hypothesis of evolution of drought tolerance in response to low water availability. The results obtained at the population-level agree with those found from short-term selection analysis, in which we observed that individuals with reduced SLA presented increased fitness in terms of growth, similar to that observed in our previous study with Iberian populations (Ramírez-Valiente et al., 2010). Similar patterns have been documented in other plant species, where negative relationships between SLA and precipitation were observed under dry conditions (e.g. Cunningham et al., 1999; Schulze et al., 2006; Marchin et al., 2008; Milla et al., 2008; Poorter et al., 2009), including Mediterranean oaks (e.g. Castro-Díez et al., 1997; Bussotti et al., 2002; Bruschi et al., 2003; Gratani et al., 2003; Ogaya and Peñuelas, 2006; Gouveia and Freitas, 2009). However, only a reduced number of them have been able to disentangle the genetic and environmental component by

**Table 5**

Spearman's coefficient correlations among ecophysiological traits for cork oak saplings measured in a common garden trial. Pearson correlation between SLA and  $N_{\text{mass}}$  were also estimated in the mesic year ( $r = 0.35$ ,  $P < 0.001$ ).

	LT	LD	$N_{\text{mass}}$	$\Delta^{13}\text{C}$
SLA	-0.66***	-0.12*	0.53***	0.06
LT		-0.64***	-0.43***	-0.07
LD				0.05
$N_{\text{mass}}$				-0.14**

$P < 0.10$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

SLA – Specific leaf area, LT – Leaf thickness, LD – Leaf density,  $N_{\text{mass}}$  – leaf nitrogen content per unit mass,  $\Delta^{13}\text{C}$  –  $^{13}\text{C}$ -Carbon isotope discrimination.

performing experiments with specific genotypes under common conditions (e.g. Gratani et al., 2003; Marchin et al., 2008; Ramírez-Valiente et al., 2010). Leaves presenting lower SLA are usually denser and thicker, as observed in the present study. Leaf thickness and density are commonly reported to be negatively related to respiration and conductance rates (Reich et al., 1997; Poorter and Bongers, 2006). The low respiration rates observed for thick and dense leaves limit carbon depletion and enable the plant to maintain a positive carbon balance, which could explain the increased growth of plants in arid and poor-nutrient environments observed in this study relative to thinner leaves (King, 1994; Venekkaas and Poorter, 1998; Lusk, 2002).

Our results also provide evidence for an adaptive role of reduced  $N_{\text{mass}}$  under dry conditions. These results support the expectation of evolution of increased drought tolerance under drought-prone habitats, through which thick, dense leaves with low  $N_{\text{mass}}$  would provide fitness benefits in dry environments.  $N_{\text{mass}}$  is usually positively correlated with SLA and negatively correlated with leaf thickness, as observed in this study and many others (Reich et al., 1997; Wright et al., 2005). The fitness benefits of reduced  $N_{\text{mass}}$  in dry environments have been commonly explained in terms of selection against the combination of high  $N_{\text{mass}}$  and thickness (low SLA), because self-shading of chloroplasts and diffusion limitations of  $\text{CO}_2$  in the thicker leaves (with lower SLA) can impair the effective photosynthetic use of high  $N_{\text{mass}}$  concentrations (Reich et al., 1997, 1999). In this study, there was no direct association between growth and  $N_{\text{mass}}$  ( $\beta$  not significant), suggesting that selection on reduced  $N_{\text{mass}}$  was instead caused by the selection on a correlated trait, SLA.

Another alternative (but non-exclusive) theory suggests that leaves with low SLA and  $N_{\text{mass}}$  may be adaptive under dry conditions because these traits are negatively correlated with water use efficiency (WUE) (Lamont et al., 2002; Sefton et al., 2002). In our study, there was a negative correlation between  $N_{\text{mass}}$  and  $\Delta^{13}\text{C}$  (a surrogate of water use efficiency, WUE). No relationship was found between SLA and  $\Delta^{13}\text{C}$ , but a negative correlation between SLA and  $\Delta^{13}\text{C}$  has been observed in cork oak in other experiments under similar dry conditions (Ramírez-Valiente et al., 2010, 2011), supporting the idea that  $N_{\text{mass}}$  and SLA are, at least in part, controlling WUE in cork oak. However,  $\Delta^{13}\text{C}$  did not exhibit any relationship with growth and survival in a dry year and it also did not show population variation, suggesting a limited role for increased WUE under dry conditions in cork oak. In dry environments, plants may increase fitness by reducing water loss, increasing water-use efficiency, or both (Givnish, 1986). Our results here suggest that cork oak has a strategy of reducing water loss in part by increasing thickness and density of leaves under dry conditions instead of increasing water use efficiency in these environments.

#### 4.2. Survival: absence of population variation and fitness-trait associations

The phenotypic selection analyses performed in this study are novel in their consideration of survival as a fitness component for cork oak. Similar approaches have been widely used in annual plants, but to our knowledge, very few studies have focused on forest tree species and morphofunctional traits (Gomez, 2004). Here, we did not find any pattern of association between functional traits and survival. In fact, there were no population differences in annual survival, contrary our expectations for the extremely arid conditions of 2005. Several factors could have produced this unexpected decoupling between the observed patterns for leaf traits and survival.

First, many studies have documented that, in Mediterranean ecosystems, early stages of plant development are particularly

vulnerable to summer drought. In fact, the first and second years after germination are considered the most critical period for plant survival in these ecosystems (Parciak, 2002a,b; Gomez, 2004). In a previous study on cork oak seedlings established in a common garden trial, we found that plants were highly vulnerable to drought during the first two years after establishment (Ramírez-Valiente et al., 2009). In that study, cork oak populations differed in their tolerance to drought and seedlings originating from dry sites had higher survival during this period. After the second year, survival rates were much higher and populations did not differ in annual survival, but the population differences were still observable in long-term survival for the whole studied period (four years). In another study, Rodríguez-Calcerrada et al. (2011) observed that seedling growth depended on seed size much more than did traits such as water use efficiency or SLA. Here, we found large population differences in long-term survival, but not in annual survival measured in nine-year-old saplings, suggesting that the impact of functional traits on survival and growth, although significant, could be less crucial in this stage than in early stages of the development. Most importantly, these findings suggest that cork oak saplings have considerable capacity to cope with arid conditions. This finding has important implications for conservation of cork oak woodlands under the increasing severity and intensity of droughts in the Mediterranean Basin during 21st century (IPCC, 2013).

In conclusion, we found significant, large population-level differentiation in SLA,  $N_{\text{mass}}$  and leaf thickness in cork oak, with populations from dry places exhibiting the lowest values for SLA and  $N_{\text{mass}}$  and the highest leaf thickness. Likewise, reduced SLA had fitness benefits in terms of growth for plants under dry conditions. Plants having low SLA values had high leaf thickness and density, which could explain the higher tolerance of drier conditions. Overall, the population-level differentiation in traits (i.e. ecotypic differentiation across climatic clines) coincided with the relationships observed between traits and fitness at individual level, suggesting an important adaptive role of reduced SLA,  $N_{\text{mass}}$  and high leaf thickness in dry environments for this Mediterranean oak. Finally, we did not find any pattern of association between functional traits and survival in nine-year-old saplings despite the severe drought in one study year. These results together with previous literature suggest that early stages of development are the most critical period for this species, and saplings are considerably tolerant to severe seasonal drought.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.actao.2014.04.004>.

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