



## RESEARCH PAPER

# Balance between carbon gain and loss under long-term drought: impacts on foliar respiration and photosynthesis in *Quercus ilex* L.

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## Abstract

Terrestrial carbon exchange is a key process of the global carbon cycle consisting of a delicate balance between photosynthetic carbon uptake and respiratory release. We have, however, a limited understanding how long-term decreases in precipitation induced by climate change affect the boundaries and mechanisms of photosynthesis and respiration. We examined the seasonality of photosynthetic and respiratory traits and evaluated the adaptive mechanism of the foliar carbon balance of *Quercus ilex* L. experiencing a long-term rainfall-exclusion experiment. Day respiration ( $R_d$ ) but not night respiration ( $R_n$ ) was generally higher in the drought treatment leading to an increased  $R_d/R_n$  ratio. The limitation of mesophyll conductance ( $g_m$ ) on photosynthesis was generally stronger than stomatal limitation ( $g_s$ ) in the drought treatment, reflected in a lower  $g_m/g_s$  ratio. The peak photosynthetic activity in the drought treatment occurred in an atypical favourable summer in parallel with lower  $R_d/R_n$  and higher  $g_m/g_s$  ratios. The plant carbon balance was thus strongly improved through: (i) higher photosynthetic rates induced by  $g_m$ ; and (ii) decreased carbon losses mediated by  $R_d$ . Interestingly, photosynthetic potentials ( $V_{c,max}$ ,  $J_{max}$ , and  $TPU$ ) were not affected by the drought treatment, suggesting a dampening effect on the biochemical level in the long term. In summary, the trees experiencing a 14-year-long drought treatment adapted through higher plasticity in photosynthetic and respiratory traits, so that eventually the atypical favourable growth period was exploited more efficiently.

**Key words:** Carbon-use efficiency, day respiration,  $J_{max}$ , mesophyll conductance, rainfall exclusion,  $V_{c,max}$ .

## Introduction

Warmer and drier conditions are expected globally under current climate change scenarios and particularly in the Mediterranean region (Somot *et al.*, 2008; Friend, 2010; IPCC, 2013). Seasonal reoccurring drought is the main natural environmental factor in the Mediterranean region

limiting plant growth and yield (Specht, 1969; Di Castri, 1973). Projected water shortages are thus likely to intensify the limitations on plant productivity and forest growth. Several studies have already reported drought-induced forest impacts and diebacks in the Mediterranean region (Peñuelas

*et al.*, 2001; Martínez-Vilalta and Piñol, 2002; Raftoyannis *et al.*, 2008; Allen *et al.*, 2010; Carnicer *et al.*, 2011; Matusick *et al.*, 2013), as well as shifts in vegetation composition (Jump and Peñuelas, 2005; Anderegg *et al.*, 2013). Seasonal summer drought limits plant growth and productivity most strongly through reductions in the plant carbon budget, which depends on the balance between photosynthesis and respiration (Flexas *et al.*, 2006). Winter has been somehow overlooked, despite the importance of potential recovery and growth periods for the annual carbon budget, especially for evergreen vegetation (Sperlich *et al.*, 2014, 2015). The Mediterranean region is characterized by a high variability in temperature and precipitation regimes, especially in mountainous areas such as the Prades Mountains in north-eastern Spain (Barbeta *et al.*, 2013). Climate extremes combined with high interannual variability complicate the scaling of carbon dynamics from one year to another (Reynolds *et al.*, 1996; Morales *et al.*, 2005; Gullías *et al.*, 2009). In fact, the modelling performance in Mediterranean-type ecosystems is particularly poor (Morales *et al.*, 2005; Vargas *et al.*, 2013) owing to under-represented soil–water patterns and our limited understanding of the effects of water stress on both carbon uptake and release (Hickler *et al.*, 2009; Niinemets and Keenan, 2014).

The non-photorespiratory carbon release in leaves is called mitochondrial respiration—a central metabolic process that produces energy (ATP, NADPH) and carbon skeletons for cellular maintenance and growth. It also contributes to significant carbon losses—especially under stress conditions—altering the net carbon gain (van Oijen *et al.*, 2010). Although the drought responses of Mediterranean vegetation have been investigated extensively, most studies concern *photosynthetic* responses (reviewed by Flexas *et al.*, 2014), whereas *respiratory* responses in leaves have largely been neglected (Niinemets, 2014). Also, it is not clear how seasonality and other abiotic stressors affect the balance of night respiration ( $R_n$ ) and day respiration ( $R_d$ ) in the leaves. This is partly owing to measurement difficulties;  $R_n$  can easily be measured by darkening the leaf, but  $R_d$  is harder to obtain and is traditionally estimated from carbon-response curves with the Laik method, from light-response curves with the Kok method, or with an amended version of the Kok method with chlorophyll fluorescence developed by Yin *et al.* (2009) (reviewed by Yin *et al.*, 2011). Measurement constraints and lacking research priorities can account for the dearth of data on respiratory responses to abiotic stress, particularly drought (Atkin and Macherel, 2009; Heskell *et al.*, 2014). Wright *et al.* (2006) provided evidence that irradiance, temperature, and precipitation affect respiration in a wide range of woody species around the world; Mediterranean species, however, were not covered. Catoni *et al.* (2013) recently provided evidence that temperature, and monthly rainfall to a lesser extent, could explain the seasonal variation of  $R_d$  in several Mediterranean maquis species. Galmés *et al.* (2007) noted that the number of studies on plant respiration responses to drought is generally limited, but particularly so for Mediterranean species. This is surprising considering the obvious importance of water stress in the Mediterranean region. Seasonal acclimation of respiration

is believed to be more important in sclerophyllic perennial leaves (Galmés *et al.*, 2007; Zaragoza-Castells *et al.*, 2007, 2008) than in plants with short-lived leaves (review by Atkin and Macherel, 2009). A better characterization of the respiratory responses to drought relative to carbon gain is vital for elucidating the overall effects on carbon exchange dynamics in water-limited environments. Rainfall-exclusion experiments in natural ecosystems are laborious and expensive but are highly valuable to simulate more realistically long-term drought. Some studies have addressed the photosynthetic limitations under long-term drought in natural ecosystems comprising stomatal, mesophyll, and biochemical components (Limousin *et al.*, 2010; Martin-StPaul *et al.*, 2012). To the best of our knowledge, the effects of long-term experimental drought on photosynthesis in parallel with night and day respiration has not been investigated so far on mature species in natural ecosystems.

*Quercus ilex* L. is one of the ‘flagship’ species for the Mediterranean Basin because it is a typical evergreen sclerophyllic tree extending over a large geographical range and forms the terminal point of secondary succession over vast areas in the Iberian Peninsula, including low and higher altitudes, and near-coastal sites with an oceanic climate, as well as inland sites with a semi-arid climate (Lookingbill and Zavala, 2000; Niinemets, 2015). However, reduced stem growth and higher mortality rates found for *Q. ilex* in response to drought (Barbeta *et al.*, 2013) could decrease the distribution under predicted future drier conditions. Hence, *Q. ilex* is the ideal candidate to evaluate the seasonal acclimation of the foliar carbon balance in the long-term drought experiment of Prades (north-eastern Spain) where partial rainfall exclusion has been applied for the last 14 years, reducing soil moisture by an average of 13% (Ogaya *et al.*, 2014; Barbeta *et al.*, 2015).

We investigated the variations of foliar respiratory and photosynthetic traits of *Q. ilex* affected by seasonal changes in growth temperature and precipitation from winter to spring and summer. Furthermore, we studied the impact of long-term experimental drought on key limitations of photosynthesis comprising stomatal, mesophyll, and biochemical components, as well as mitochondrial respiration during the day and night. Based on these parameters, we evaluated the response pattern of the foliar intrinsic water- and carbon-use efficiency ( $WUE_i$  and  $CUE_i$ , respectively) with respect to the simulated drought. Our aim was to improve our understanding of the boundaries and mechanisms of foliar respiration and photosynthesis in terms of seasonal acclimation and adaptation to drought. We provide here a set of needed parameters that potentially help to improve model simulation of ecosystem carbon fluxes.

## Material and methods

### Experimental site

The experimental site was situated in the Prades Mountains in southern Catalonia (north-eastern Spain; 41°21'N, 1°2'E) at 950 m above sea level on a 25% south-facing slope. Temperature, photosynthetically active radiation, air humidity, and precipitation have been monitored continuously with a meteorological station installed at

the site. The climate is Mediterranean, with a mean annual rainfall of 609 mm and a mean annual temperature of 12.2 °C (climate data from the meteorological station for 1999–2012). The soil is a Dystric Cambisol over Paleozoic schist with a depth of 35–90 cm. The forest is characterized by a dense, multi-stemmed crown dominated by *Q. ilex* and *Phillyrea latifolia* L. with a maximum height of 6–10 m. The understorey is composed of *Arbutus unedo* L., *Erica arborea* L., *Juniperus oxycedrus* L., and *Cistus albidus* L. A long-term rainfall-exclusion experiment has been established and maintained in this forest since 1999 to simulate *in situ* projected decreases in precipitation in the Mediterranean region (Peñuelas *et al.*, 2007). Four control and four treatment plots of 15 × 10 m were installed at the same altitude along the mountain slope. In the treatment plots, rain was partially excluded by PVC strips suspended 0.5–0.8 m above the soil (covering 30% of the soil surface). A ditch of 0.8 m in depth around the plots intercepted the runoff water from above the plots and conducted the water around to the bottom. The control plots received no treatment.

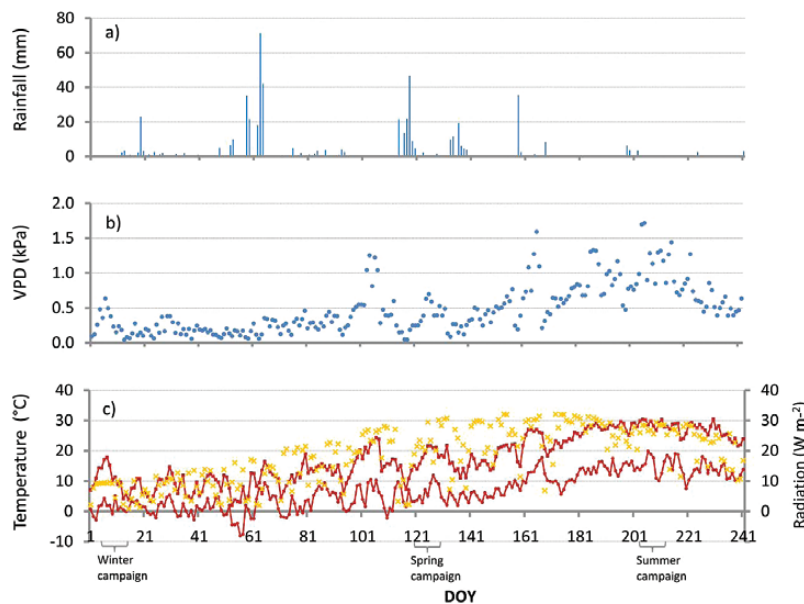
#### Sampling method

We conducted three seasonal field campaigns: winter (5–11 January 2013), spring (30 April–4 May 2013) and summer (24–29 July 2013) (Fig. 1). Eight twigs for each drought and control plot (two replicates for each plot) were cut with a pruning pull from the sun-exposed crowns of *Q. ilex* trees. We recut the twigs under water in the field, wrapped them in plastic bags to minimize transpiration, and transported them in water buckets to a nearby laboratory. The twigs were pre-conditioned overnight in the laboratory at room temperature (22–26 °C), freshly recut in the morning, and then kept in dim light [50–100 photosynthetic photon-flux density (PPFD) in  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ]. Once the leaves showed open stomata [stomatal limitation ( $g_s$ ) > 0.03 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>] and a stable stomatal internal CO<sub>2</sub> concentration ( $C_i$ ,  $\mu\text{mol CO}_2 \text{ mol air}^{-1}$ ), we started the response curves. In a few cases, the twigs were kept for one or two additional nights until gas exchange was sufficiently stable to conduct a light-response curve. We have adopted this method to overcome limitations that we often faced in the field such as: (i) accessibility of the branches of mature trees (canopy height between 6 and 10 m); (ii) limited ability of the instruments to reach the standard leaf temperature ( $T_{\text{leaf}}$ ) of 25 °C; and (iii) unpredictable plant responses

such as closed stomata or patchy stomatal conductance (Mott and Buckley, 1998, 2000). With the pre-conditioned twigs, in contrast, we reached stable gas-exchange values that are required for conducting a noise-free light- or CO<sub>2</sub>-response curve and to estimate reliably the photosynthetic potentials (see Supplementary Fig. S1 at *JXB* online). The leaves remained fresh and functional for several days controlled by stomatal conductance and fluorescent signals. The cut twigs showed stable values of night respiration for several days (see Supplementary Fig. S2 at *JXB* online). This method works well on Mediterranean oak species including *Q. ilex* as shown in other studies (Haldimann and Feller, 2004; Niinemets *et al.*, 2005; Sperlich *et al.*, 2015). This method provided us with the opportunity to look at the potential physiological properties under standardized conditions that are representative for each season of a control group and a drought treatment and that are independent of short-term meteorological variability (e.g. cloud cover, extreme temperatures, chilly periods, rain events) and unpredictable plant responses.

#### Analyses of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Lincoln, USA). Response curves of net assimilation versus PPFD were recorded in parallel with chlorophyll fluorescence measurements on mature, fully expanded leaves. In the summer campaign, we additionally conducted response curves of net assimilation versus CO<sub>2</sub>. Some of the *Q. ilex* leaves were too small to fill the leaf cuvette (2 cm<sup>2</sup>), so the measured parameters were adjusted after the measurements. The leaves were prepared and acclimated prior to recording the response curves as described by Sperlich *et al.* (2014). First, we measured the maximum quantum yield of photosystem II (PSII; unitless) [ $F_v/F_m = (F_m - F_o)/F_m$ ] of a dark-adapted leaf (>30 min) at ambient CO<sub>2</sub> ( $C_a$  of 400  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and  $T_{\text{leaf}}$  of 25 °C.  $F_o$  is the minimal fluorescence measured under darkness, and  $F_m$  is the maximal fluorescence measured after a saturating light pulse.  $F_v/F_m$  describes the fraction of absorbed photons used in photochemistry under dark conditions and serves as the primary stress indicator of the photosystems. Typical values range between 0.74 and 0.85. Ratios of <0.80 are indicative of induced photoprotection (sustained energy dissipation), and ratios <0.74 are indicative



**Fig. 1.** Environmental variables for the days of the year (DOY) from January to August 2013: rainfall (a), atmospheric vapour-pressure deficit (VPD) (b), and maximum and minimum temperatures (°C) (c) on the primary y-axes (red circles) and radiation (yellow crosses) on the secondary y-axes. The field campaigns are indicated.

of chronic photoinhibition (Björkman and Demmig, 1987; Maxwell and Johnson, 2000; Verhoeven, 2014). We then acclimated the leaf to saturating light conditions (PPFD of 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and simultaneously recorded gas exchange and chlorophyll fluorescence parameters at ambient  $\text{CO}_2$  and  $T_{\text{leaf}}$  as above: net assimilation rate ( $A_{\text{net}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ),  $g_s$ ,  $C_i$ , non-photochemical quenching (unitless)  $[\text{NPQ}=(F_m - F_m')/F_m']$ , where  $F_m'$  is the maximal fluorescence of a light-adapted leaf], and the effective quantum yield of PSII (unitless)  $[\Phi_{\text{PSII}}=(F_m' - F_s)/F_m']$ , where  $F_s$  is the steady-state fluorescence of a light-adapted leaf].

We used the relationship of  $A_{\text{net}}$  versus  $g_s$  to estimate the foliar water-use efficiency ( $WUE_i$ ) which is defined as the amount of carbon gained per unit water used (Flexas *et al.*, 2013). The electron-transport rate based on the effective quantum yield of PSII ( $J_{\text{CF}}$  in  $\mu\text{mol electron m}^{-2} \text{s}^{-1}$ ) was calculated as

$$J_{\text{CF}} = \epsilon * \Phi_{\text{PSII}} * \alpha_L * \text{PPFD} \quad (1)$$

where  $\epsilon$  is a scaling factor accounting for the partitioning of intercepted light between PSI and PSII. We assumed that light was equally distributed between the two photosystems ( $\epsilon=0.5$ ) (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2005). The foliar absorbance ( $\alpha_L$ , unitless) was 0.932 for *Q. ilex* (Sperlich *et al.*, 2014).  $J_{\text{CF}}$  at ambient  $\text{CO}_2$  and saturating light was termed  $J_{\text{amb}}$ .

#### Light experiments

Light-response curves ( $A/\text{PPFD}$ ) were generated by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source at a leaf chamber internal concentration ( $C_a$ ) of 400  $\mu\text{mol CO}_2 \text{ mol air}^{-1}$ . To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence (in  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ): 2500→2000→1500→1000→800→600→500→400→300→200→150→125→100→75→50→40→30→20→10→5→0. The minimum and maximum times between each light level for the generation of the  $A/\text{PPFD}$  curves were set to 1 and 2 min, respectively. The rapid changes in light levels prevented the correct adjustment of  $T_{\text{leaf}}$ . We fixed the Peltier-block temperature ( $T_{\text{block}}$ ) in the leaf cuvette, so that  $T_{\text{leaf}}$  was 25 °C at the beginning of the  $A/\text{PPFD}$  curve. In the lower light levels where day respiration was estimated,  $T_{\text{leaf}}$  had dropped on average by 0.8 °C (standard error  $\pm 0.0004$ ). Day respiration ( $R_d$  in  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ) was estimated from the light-response curves with the method proposed by Yin *et al.* (2009) combining measurements of gas exchange and chlorophyll fluorescence. This method amended the Kok method (Kok, 1948) by substituting the  $A/\text{PPFD}$  relationship with  $A/(\text{PPFD} \times \Phi_{\text{PSII}}/4)$  (see Yin *et al.*, 2009, for details on the protocol). We estimated night respiration ( $R_n$  in  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ) after darkening the leaf for 20–30 min, ensuring that all reaction centres had been closed (controlled with  $F_o$ ).  $R_d$  and  $R_n$  were then normalized to unity at 25 °C with an Arrhenius function [parameter= $\exp(c - \Delta H_a / RT_k)$ ].  $T_k$  is the leaf temperature (in Kelvin) and  $R$  is the molar gas constant (0.008314  $\text{kJ K}^{-1} \text{mol}^{-1}$ ). The values of the scaling constant  $c$  (18.72, dimensionless) and energy of activation  $\Delta H_a$  (46.39  $\text{kJ mol}^{-1}$ ) for leaf respiration were taken from Bernacchi *et al.* (2001).

Thereafter, we applied a correction of  $R_d$  to account for the increase of  $C_i$  with decreases of PPFD described by Kirschbaum and Farquhar (1987). In this method, the intercept of plots of photosynthetic electron transport to PPFD is minimized through the adjustment of  $R_d$  via iteration (see Weerasinghe *et al.*, 2014, for details on the protocol).

With  $R_d$  from the light-response curves, we calculated the intrinsic carbon-use efficiency ( $CUE_i$ ) as proportion of carbon assimilated per carbon respired (Gifford, 2003), which served as a rough indicator for the foliar carbon balance (Pattison *et al.*, 1998; Galmés *et al.*, 2007):

$$CUE_i = 1 - \frac{R_d}{A_{\text{net}}} \quad (2)$$

#### $\text{CO}_2$ experiments

The  $C_a$  concentrations used to generate the  $\text{CO}_2$ -response curves were 400→300→200→150→100→50→400→400→600→800→1200→2000  $\mu\text{mol CO}_2 \text{ mol air}^{-1}$ .  $T_{\text{leaf}}$  was set to 25 °C. The saturating PPFD used was 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  based on light-response curves conducted prior to the measurements campaigns. The results of all light-response curves after the measurement campaign, however, indicated a saturating PPFD of 1500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The minimum and maximum times for stabilizing  $A_{\text{net}}$ ,  $g_s$ , and  $C_i$  for each log were set to 4 and 6 min, respectively. Diffusion leakage was corrected as described by Flexas *et al.* (2007).

#### Estimation of mesophyll conductance

We estimated  $g_m$  ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ ) using the variable- $J$  method of Harley *et al.* (1992):

$$g_m = \frac{A_{\text{net}}}{C_i - \frac{\Gamma^* [J_{\text{CF}} + 8(A_{\text{net}} + R_d)]}{J_{\text{CF}} - 4(A_{\text{net}} + R_d)}} \quad (3)$$

where  $\Gamma^*$  is the  $\text{CO}_2$  concentration at which the photorespiratory efflux of  $\text{CO}_2$  equals the rate of photosynthetic  $\text{CO}_2$  (37.43 ppm at 25 °C).  $\Gamma^*$  and its temperature response were taken from Bernacchi *et al.* (2002). The chloroplastic  $\text{CO}_2$  concentration ( $C_c$  in  $\mu\text{mol CO}_2 \text{ mol air}^{-1}$ ) was determined as:

$$C_c = C_i - \frac{A_{\text{net}}}{g_m} \quad (4)$$

#### Photosynthesis model

The photosynthesis model of Farquhar *et al.* (1980) considers photosynthesis as the minimum of the potential rates of Rubisco activity ( $A_c$ ) and ribulose-1,5-bisphosphate (RuBP) regeneration ( $A_j$ ). A third limitation ( $A_p$ ) was implemented that considers the limitation by triose-phosphate use at high  $\text{CO}_2$  concentrations when the  $\text{CO}_2$  response shows a plateau or decrease (Sharkey, 1985). The model was further modified by replacing  $C_i$  with  $C_c$  for the chloroplast where the actual carboxylation takes place (reviewed by Flexas *et al.*, 2008). As outlined above, we used the variable- $J$  method for the  $C_c$  calculation to create  $A/C_c$  curves. The modelled assimilation rate  $A_{\text{mod}}$  was then calculated by the minimum of these three potential rates from the  $A/C_c$  curves:

$$A_{\text{mod}} = \min\{A_c, A_j, A_p\} \quad (5)$$

where:

$$A_c = V_{c,\text{max}} * \left[ \frac{C_c - \Gamma^*}{C_c + K_c \left(1 + \frac{O}{K_o}\right)} \right] - R_d \quad (6)$$

where  $V_{c,\text{max}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ) is the maximum rate of Rubisco carboxylation,  $K_c$  is the Michaelis–Menten constant of Rubisco for  $\text{CO}_2$ ,  $O$  is the partial pressure of  $\text{O}_2$  at Rubisco, and  $K_o$  is the Michaelis–Menten constant of Rubisco for  $\text{O}_2$ , taken from Bernacchi *et al.* (2002). The equation representing photosynthesis limited by RuBP regeneration is:

$$A_j = J_{1200} * \left[ \frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} \right] - R_d \quad (7)$$

where  $J_{1200}$  (in  $\mu\text{mol electron m}^{-2} \text{s}^{-1}$ ) is the rate of electron transport at a PPFD of 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and saturating  $\text{CO}_2$ .

We assumed that  $J_{1200}$  became  $J_{\max}$  under light and  $\text{CO}_2$  saturation when the maximum possible rate of electron transport was theoretically achieved, although we may have underestimated the true  $J_{\max}$  (Buckley and Diaz-Espejo, 2015). The limitation of triose-phosphate use was estimated as:

$$A_p = \frac{3TPU * C_c}{C_c - (1 + 3\alpha_{TPU}) * \Gamma^*} - R_d \quad (8)$$

where  $TPU$  is the rate of triose-phosphate use at saturating  $\text{CO}_2$  concentrations, and  $\alpha_{TPU}$  is the proportion of glycerate not returned to the chloroplasts. Eqn 8 is from von Caemmerer (2000) after correcting a typographical error in the expression  $3\alpha_{TPU}/2$  to  $3\alpha_{TPU}$ , as described by Gu *et al.* (2010). This equation fits the  $A/C_c$  curve plateau at high  $\text{CO}_2$  when a further increase in  $C_c$  does not produce any increase of  $A_{\text{net}}$  anymore or, in some cases, even produces a decline of  $A_{\text{net}}$ .

In addition to the  $A/C_c$  curves, we replaced  $C_c$  with  $C_i$  in Eqns 6–8 and thus applied the above photosynthesis model to the traditional  $A/C_i$  curve. We used an adequate set of kinetic constants from Bernacchi *et al.* (2001). We considered  $V_{c,\max}$ ,  $J_{1200}$ , and  $TPU$  from the  $A/C_c$  curve as the ‘true’ biochemical potential to drive photosynthesis whereas the parameters from the  $A/C_i$  curve were the ‘apparent’ photosynthetic potential.

### Statistical analyses

We estimated the true and apparent values of  $V_{c,\max}$ ,  $J_{1200}$ , and  $TPU$  from Eqns 6–8 with the SOLVER Excel tool. SOLVER iteratively changes the parameters to minimize the sum of squares of the deviation of observed  $A_{\text{net}}$  versus modelled  $A_{\text{mod}}$ . We then performed further statistical analyses with R version 3.0.2 (<http://www.r-project.org/>). Differences in the parameters between control and drought plots were determined with Student’s *t*-test ( $P \leq 0.05$ ). The normality of the data was tested with the Shapiro–Wilk test, and the data was normalized if not normally distributed. One-factorial ANOVA with season as the main factor was tested for seasonal differences in the parameters. Significant differences were determined at  $P \leq 0.05$  with Tukey’s honestly significant difference test. Linear regression analyses were conducted to study the relationships among various leaf traits such as  $A_{\text{net}}/g_s$ ,  $A_{\text{net}}/g_m$ ,  $J_{1200}/V_{c,\max}$ ,  $g_m/g_s$ , and  $R_n/R_d$ . We tested for differences in regression slopes and intercepts with analyses of co-variance (ANCOVAs).

## Results

### Environmental conditions over the sampling period

Frost events were frequent in winter and snowfall was also observed. The maximum temperatures during the day were on an average 4.9 °C (Table 1). The spring was humid with a precipitation comparable to that in winter (246 and 269 mm, respectively) and was relatively cold (average of 12 °C) with

occasional night frosts (Fig. 1). Spring together with winter accounted for nearly 80% of the annual average precipitation. The summer, in contrast, was dry and warm (total precipitation of 21 mm and average temperature of 20.3 °C), with a vapour-pressure deficit (VPD) nearly twice as high as in spring (0.83 kPa) (Table 1). The partial rainfall exclusion reduced the soil water content (SWC) by a total of 13% from the beginning of the experiment in 1999 until the end of our measurement campaign in 2013. For the period of our measurement campaign, the SWC was on average 14% lower in the partial rainfall-exclusion plots compared with the control plots (Table 1). This difference was highest in spring, with a 24% lower SWC in the drought plots compared with the control plots.

### Seasonal changes in photosynthetic parameters

We analysed the seasonality of the photosynthetic parameters using the full dataset independent of treatment. Winter had a strong effect on several parameters with lower average values than in spring and summer, except for  $R_n$  and  $C_i$  (Table 2).  $A_{\text{net}}$ ,  $g_s$ ,  $g_m$ , and  $F_v/F_m$  were significantly ( $P < 0.05$ ) and  $R_d$ ,  $C_c$ ,  $CUE_i$  were marginally significantly ( $P < 0.10$ ) lower in winter than in either spring or summer (Figs 2 and 3). In summer, we found the highest mean values of  $A_{\text{net}}$ ,  $g_s$ ,  $g_m$ , and  $C_c$ , which were significantly different from those in spring and winter (Fig. 3).  $F_v/F_m$  was also highest in summer, demonstrating that the photosynthetic systems in spring had not yet fully recovered from the low winter temperatures but operated at peak efficiency in summer (Fig. 4b). NPQ is an indicator for photoinhibitory stress and dissipation of excess energy and was lowest in spring (significantly different from both winter and summer) (Fig. 4a). Neither  $\Phi_{\text{CO}_2}$  nor  $\Phi_{\text{PSII}}$  differed significantly between the seasons (Table 2 and Supplementary Fig. S3 at JXB online). The saturating PPFDs for  $A_{\text{net}}$  and  $J_{\text{cf}}$  were 1484 and 1552, respectively, and did not change seasonally.

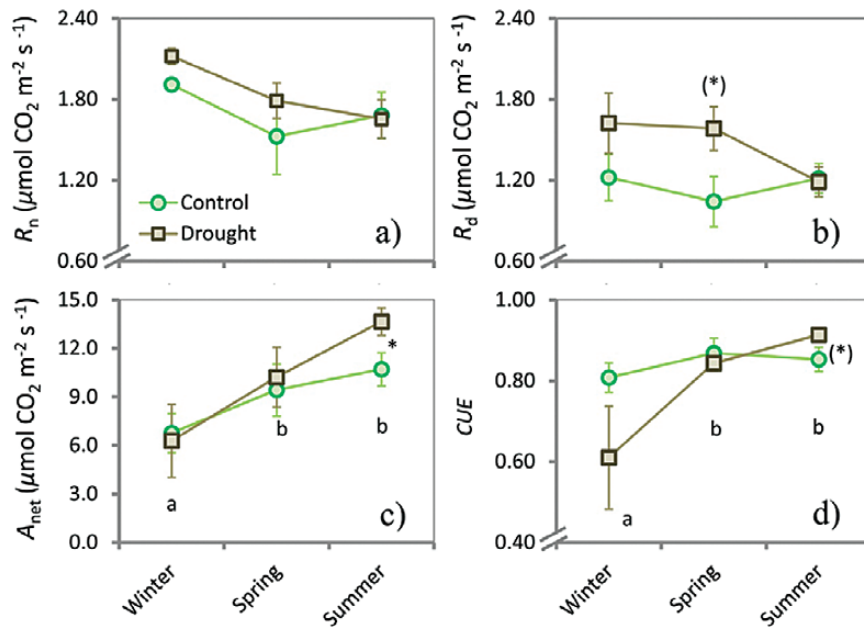
Several relationships were analysed with ANCOVAs to test whether seasonal changes in environmental conditions produced significant differences in slopes (Table 3). We analysed the relationship of  $A_{\text{net}}/g_s$  as an indicator for  $WUE_i$ . The slope of this relationship for the control group was significantly gentler in winter compared with spring and summer, indicating a lower  $WUE_i$ . For the relationship of  $A_{\text{net}}/g_m$ , we analysed the effect of the mesophyll internal  $\text{CO}_2$  diffusion on net carbon assimilation. This relationship had a significantly steeper slope in winter in comparison with summer in the drought

**Table 1.** Dates and days of the year (DOY) for each season in 2013 with mean temperature (*T*), total precipitation (*Prec.*), mean vapour-pressure deficit (VPD), mean radiation, and the percentage of the difference in the soil water content between the control and drought plots ( $\Delta\text{SWC}$ )

Season	Date	DOY	T (°C)	Prec. (mm)	VPD (kPa)	Radiation (W m <sup>-2</sup> )	$\Delta\text{SWC}$ (%)
Winter	1 January–21 March 2013	1–79	4.9	269	0.20	9.1	5.3
Spring	22 March–21 June 2013	79–171	12.0	246	0.45	21.3	23.9
Summer	22 June–31 August 2013	172–242	20.3	21.8	0.83	25.0	7.7
Total	1 January–31 August 13	1–242	12.1	537	0.48	18.3	13.5

**Table 2.** Means ( $\pm$ standard errors) of a set of photosynthetic parameters and foliar traits for *Q. ilex* for the control group and the drought treatment in three seasonal campaigns ( $n=5-9$ )

Variable	Control			Drought		
	Winter	Spring	Summer	Winter	Spring	Summer
$R_n$	1.91 $\pm$ 0.03	1.53 $\pm$ 0.28	1.68 $\pm$ 0.17	2.12 $\pm$ 0.06	1.79 $\pm$ 0.13	1.65 $\pm$ 0.14
$R_d$	1.22 $\pm$ 0.17	1.04 $\pm$ 0.19	1.21 $\pm$ 0.11	1.62 $\pm$ 0.22	1.58 $\pm$ 0.16	1.19 $\pm$ 0.11
$R_d/R_n$	0.64 $\pm$ 0.09	0.69 $\pm$ 0.03	0.74 $\pm$ 0.05	0.77 $\pm$ 0.11	0.88 $\pm$ 0.03	0.73 $\pm$ 0.06
$A_{net}$	6.76 $\pm$ 1.2	9.43 $\pm$ 1.0	10.71 $\pm$ 1.0	5.52 $\pm$ 2.0	10.17 $\pm$ 0.7	13.66 $\pm$ 0.9
$g_s$	0.077 $\pm$ 0.032	0.090 $\pm$ 0.016	0.116 $\pm$ 0.012	0.054 $\pm$ 0.021	0.113 $\pm$ 0.009	0.161 $\pm$ 0.013
$g_m$	0.054 $\pm$ 0.009	0.085 $\pm$ 0.014	0.097 $\pm$ 0.011	0.047 $\pm$ 0.017	0.074 $\pm$ 0.017	0.137 $\pm$ 0.014
$C_i$	206 $\pm$ 30	198 $\pm$ 21	234 $\pm$ 8	210 $\pm$ 20	227 $\pm$ 8	243 $\pm$ 6
$C_c$	74 $\pm$ 9	77 $\pm$ 3	119 $\pm$ 7	61 $\pm$ 10	81 $\pm$ 5	139 $\pm$ 23
NPQ	2.70 $\pm$ 0.29	0.82 $\pm$ 0.02	2.97 $\pm$ 0.26	2.61 $\pm$ 0.14	0.80 $\pm$ 0.02	2.74 $\pm$ 0.31
$F_v/F_m$	0.80 $\pm$ 0.011	0.81 $\pm$ 0.007	0.83 $\pm$ 0.005	0.78 $\pm$ 0.022	0.80 $\pm$ 0.007	0.82 $\pm$ 0.005
$\Phi_{CO_2}$	0.0074 $\pm$ 0.0020	0.0092 $\pm$ 0.0009	0.0102 $\pm$ 0.0014	0.0054 $\pm$ 0.0014	0.0097 $\pm$ 0.0008	0.0119 $\pm$ 0.0018
$\Phi_{PS2}$	0.215 $\pm$ 0.045	0.250 $\pm$ 0.024	0.206 $\pm$ 0.029	0.220 $\pm$ 0.009	0.273 $\pm$ 0.021	0.218 $\pm$ 0.030
$V_{c,max}$			107 $\pm$ 9			120 $\pm$ 11
$J_{max}$			132 $\pm$ 11			148 $\pm$ 12
TPU			9.4 $\pm$ 1.2			7.6 $\pm$ 1.3

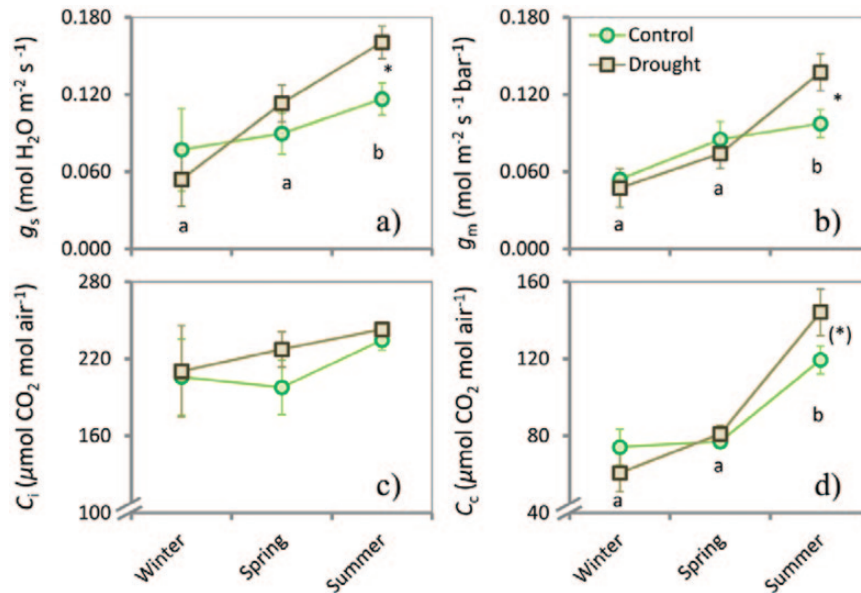
**Fig. 2.** Line graphs depicting seasonal changes of night respiration ( $R_n$ ) (a), day respiration ( $R_d$ ) (b), net assimilation rate ( $A_{net}$ ) (c), and (d) carbon-use efficiency ( $CUE$ ) (d) for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Asterisks and asterisks in brackets indicate significant ( $P<0.05$ ) and marginally significant ( $P<0.1$ ) differences between the control and drought plots for each season, respectively. Different lower-case letters indicate differences between seasons. Vertical bars indicate standard errors of the mean ( $n=59$ ).

group. The relationship of  $g_m/g_s$  unveils the relative contribution of stomatal and mesophyll diffusion limitation on the net carbon assimilation. The relationship of  $g_m/g_s$  was significantly steeper in the control plot in spring in comparison with summer. We analysed the relative importance of day and night mitochondrial respiration with the relationship of  $R_d/R_n$ . The slope was significantly steeper in winter compared with spring and summer in both the control and drought plots.

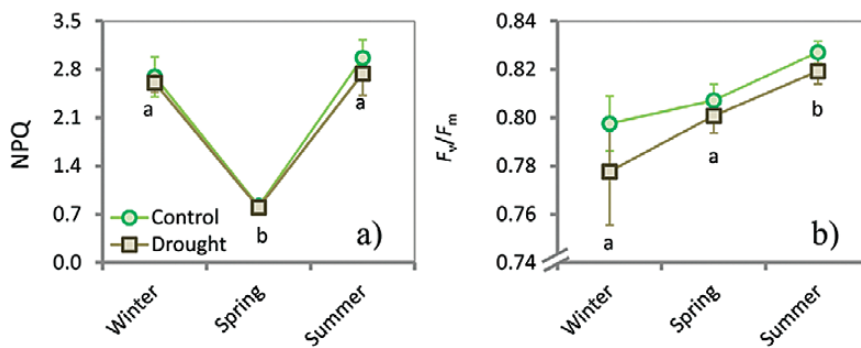
#### Effect of experimental drought

$R_d/R_n$  for all seasons combined was significantly higher in the drought treatment ( $0.79 \pm 0.06$ ) compared with the control

plots ( $0.71 \pm 0.03$ ). No other general trends were detected. In the respective seasons, however, we found significant effects of the drought treatment, with several parameters showing higher average values compared with the control group (Figs 2 and 3):  $A_{net}$ ,  $g_s$ , and  $g_m$  were significantly higher, and  $CUE_i$  and  $C_c$  were marginally significantly higher in summer, and  $R_d$  was marginally significantly higher in spring. We conducted carbon-response curves in summer only (see Material and methods).  $J_{1200}$ ,  $V_{c,max}$ , and  $TPU$  were thus only available for the summer campaign. The drought treatment had no significant effect on these photosynthetic potentials when estimated from an  $A/C_c$  curve (Fig. 5). Additionally, we estimated the apparent photosynthetic potential from  $A/C_i$  curves. The



**Fig. 3.** Line graphs depicting seasonal changes of stomatal conductance ( $g_s$ ) (a), mesophyll conductance ( $g_m$ ) (b), stomatal internal  $\text{CO}_2$  concentration ( $C_i$ ) (c), and chloroplastic  $\text{CO}_2$  concentration ( $C_c$ ) (d) in sunlit leaves of *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Asterisks and asterisks in brackets indicate significant ( $P < 0.05$ ) and marginally significant ( $P < 0.1$ ) differences between the control and drought plots for each season, respectively. Different lower-case letters indicate differences between seasons. Vertical bars indicate standard errors of the means ( $n=59$ ).



**Fig. 4.** Line graphs depicting seasonal changes of non-photochemical quenching (NPQ) (a) and maximum quantum efficiency of PSII ( $F_v/F_m$ ) (b) for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Different lower-case letters indicate differences between seasons. Vertical bars indicate standard errors of the means ( $n=59$ ).

drought treatment had a marginally significant effect on the apparent  $J_{1200}$  and apparent  $V_{c,max}$  with lower values in the control plot, but no effect on the apparent  $TPU$  (Fig. 5). A comparison of the photosynthetic potential from  $A/C_i$  and  $A/C_c$  curves indicated that the foliar internal diffusion limitation imposed by  $g_m$  accounted on average for a 54% higher  $V_{c,max}$  and a 30% higher  $J_{1200}$  and a 29% higher  $TPU$  with regard to the apparent photosynthetic potential.

The ANCOVAs in the respective seasons identified significant differences in slopes as a result of the experimental drought. The slope of  $A_{net}/g_s$  was significantly steeper in the control compared with the treatment group in the winter campaign, indicating a higher  $WUE_i$  in the control group (Fig. 6). The slope of  $A_{net}/g_m$  was significantly steeper in the control group compared with the treatment group in the summer campaign (Fig. 6). The overall slope of  $g_m/g_s$  was significantly steeper in the control group compared with the treatment group when all seasons were combined (Fig. 6). The slope of

$R_d/R_n$  was significantly gentler in the control group compared with the treatment group in the spring campaign and when all seasons were combined. Neither season nor treatment significantly affected the slopes of  $A_{net}/R_d$ ,  $A_{net}/R_n$ ,  $J_{amb}/A_{net}$ , and  $C_i/C_c$  (Supplementary Tables S1–S4 at *JXB* online).

## Discussion

The scaling of carbon dynamics from one year to another is particularly challenging in Mediterranean environments due to climate extremes combined with a high interannual variability (Reynolds *et al.*, 1996; Morales *et al.*, 2005; Gullás *et al.*, 2009). We aimed to investigate the effect of seasonal changes in temperature and precipitation from winter to spring and summer on the photosynthetic and respiratory traits of a widely abundant Mediterranean tree species. However, abiotic stress under field conditions often hampers gas-exchange measurements due to deviations from the standard temperature

(25 °C) or unpredictable plant responses, e.g. patchy stomatal conductance (Mott and Buckley, 1998, 2000), making it impossible to conduct response curves for the estimation of photosynthetic and respiratory parameters. Our data was thus obtained on cut twigs under standardized conditions in

**Table 3.** Regression equations and coefficients of determination ( $R^2$ ) for  $A_{net}/g_s$ ,  $A_{net}/g_m$ ,  $g_m/g_s$  and  $R_d/R_n$  for *Q. ilex* in three sampling campaigns in the control and drought plots

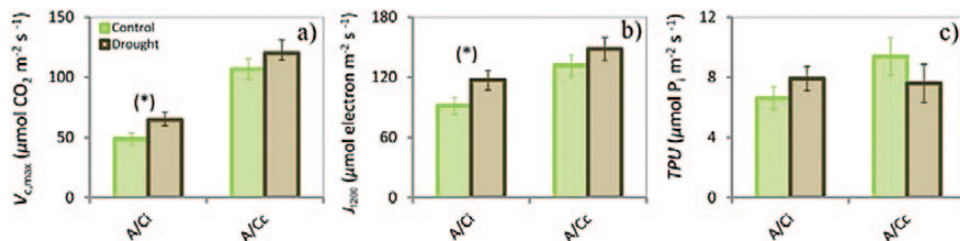
*P* values indicate the significance of the differences between the slopes for the control and drought plots. Equations for non-significant relationships are not displayed ( $n=5-9$ ).

Variable	Campaign	Plot	Equation	$R^2$	<i>P</i>
$A_{net}/g_s$	Total	Control	$y=60.7x+3.68$	0.72	0.417
		Drought	$y=74.7x+1.92$	0.88	
	Winter 2013	Control	$y=36.1x+3.98$	0.86	0.009
		Drought	$y=94.9x+0.39$	0.92	
	Spring 2013	Control	$y=104.1x+1.51$	0.98	0.380
		Drought	$y=74.0x+2.71$	0.68	
	Summer 2013	Control	$y=79.1x+1.49$	0.89	0.222
		Drought	$y=53.9x+5.01$	0.64	
$A_{net}/g_m$	Total	Control	$y=79.3x+2.61$	0.77	0.513
		Drought	$y=70.2x+4.00$	0.75	
	Winter 2013	Control			0.279
		Drought	$y=115.1x+0.08$	0.62	
	Spring 2013	Control	$y=88.5x+1.01$	0.92	0.521
		Drought	$y=63.8x+5.17$	0.80	
	Summer 2013	Control	$y=88.8x+2.07$	0.85	0.040
		Drought	$y=30.5x+9.47$	0.10	
$g_m/g_s$	Total	Control	$y=0.254x+0.059$	0.06	0.011
		Drought	$y=0.757x+0.011$	0.57	
	Winter 2013	Control			-
		Drought	$y=0.595x+0.017$	0.56	
	Spring 2013	Control	$y=1.051x+0.015$	0.86	0.337
		Drought	$y=0.637x+0.015$	0.27	
	Summer 2013	Control	$y=0.758x+0.009$	0.75	0.949
		Drought	$y=0.732x+0.020$	0.30	
$R_d/R_n$	Total	Control	$y=0.540x+0.263$	0.59	0.0035
		Drought	$y=0.980x+0.272$	0.68	
	Winter 2013	Control	$y=4.05x+6.14$	0.78	0.279
		Drought	$y=1.036x+0.343$	0.61	
	Spring 2013	Control	$y=0.639x+0.063$	0.96	0.0126
		Drought	$y=1.147x+0.427$	0.95	
	Summer 2013	Control			-
		Drought	$y=0.487x+0.373$	0.38	

order to provide insights into the photosynthetic and respiratory potential independently of meteorological variability in the field. The cutting-twig method allowed us to analyse the long-term acclimation to the environmental conditions from which the twigs were derived and has been applied by other experts in the field (e.g. Epron and Dreyer, 1992; Niinemets *et al.*, 1999, 2005; Laisk *et al.*, 2002; Haldimann and Feller, 2004; Heskell *et al.*, 2014; Dusenge *et al.*, 2015). Our study thus provides a mechanistic description of seasonal changes in photosynthetic and respiratory processes under long-term drought that contributes to improve our understanding of the impacts of future climate change in Mediterranean-type ecosystems.

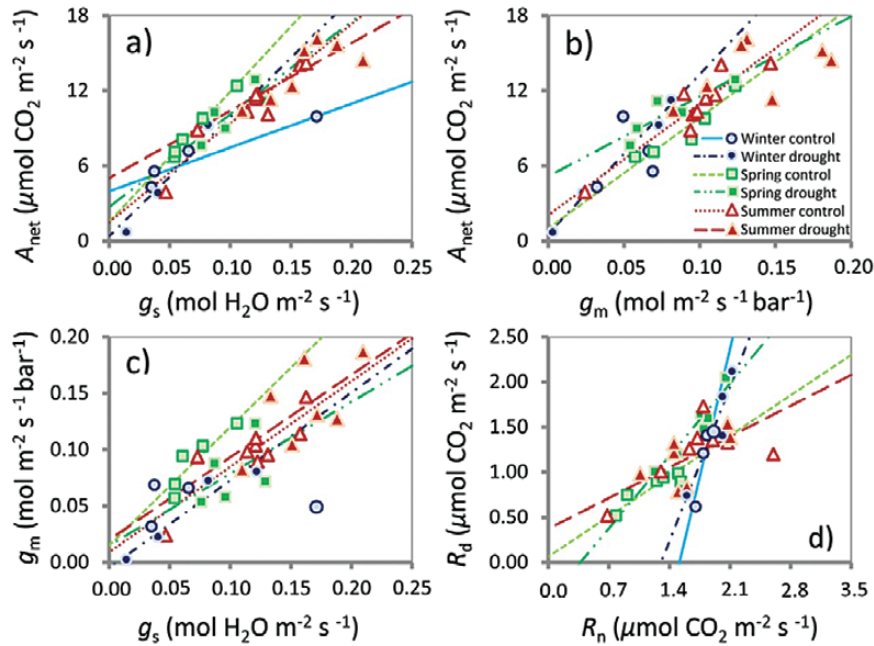
*Effect of seasonality on photosynthetic and respiratory traits*

We found that cold winter temperatures had a stronger negative impact on the leaf physiology of *Q. ilex* than summer drought. The standardized  $A_{net}$  under controlled conditions (see Material and methods) was approximately half the rate in winter compared with the peak found in summer, yet relatively high average winter values were reached ( $6.5 \pm 1.3$ ) that were comparable to those reported in other studies (Gratani, 1996; Ogaya and Peñuelas, 2003). Both  $g_m$  and  $g_s$  reduced the  $CO_2$  concentration in the chloroplasts in winter compared with spring and summer. In winter, however,  $g_m$  limited photosynthesis relatively more than  $g_s$ . High water availability and low VPDs make the reduction of transpiratory water loss through stomatal closure less urgent in the winter period. There is some evidence that  $g_m$  acts as a stronger regulator for photosynthesis in winter (Sperlich *et al.*, 2014), although very few studies have examined the behaviour of  $g_m$  under natural winter conditions. Low temperatures in winter hamper photosynthetic metabolism and enzymatic activities (e.g. Corcuera *et al.*, 2004; Aranda *et al.*, 2005), which may account for a concurrent downregulation of photosynthesis through  $g_m$ , as our results indicated. This was paralleled by a drastic decrease in the foliar carbon-use efficiency. In winter, chilly or freezing temperatures often coincide with clear skies and relatively high solar irradiances. The imbalance created between light energy absorbed in photochemistry and light energy used in metabolism increases the susceptibility to photoinhibitory stress (Demmig-Adams and Adams, 1992). This imbalance is particularly problematic



**Fig. 5.** Bar graphs of maximum carboxylation rate ( $V_{c,max}$ ) (a), electron-transport rate at saturating light and  $CO_2$  ( $J_{1200}$ ) (b), and triose-phosphate use (TPU) (c) estimated with  $CO_2$ -response curves based on  $C_i$  ( $A/C_i$ ) and  $C_c$  ( $A/C_c$ ) in the control and drought plots for the summer campaign. Marginal significant differences ( $P < 0.1$ ) between the control and drought plots are indicated by asterisks in brackets. Vertical bars indicate standard errors of the means (control,  $n=7$ ; drought,  $n=8$ ).





**Fig. 6.** Scatter plots and regression lines of stomatal conductance ( $g_s$ ) versus net assimilation rate ( $A_{\text{net}}$ ) (a), mesophyll conductance ( $g_m$ ) versus  $A_{\text{net}}$  (b),  $g_s$  versus  $g_m$  (c) and night respiration ( $R_n$ ) versus day respiration ( $R_d$ ) (d) for each season and for control and drought plots. Only the regression lines for significant relationships ( $P < 0.05$ ) are displayed.

for the evergreen vegetation, and thermal acclimation to winter conditions is essential to survive these adverse conditions (Blumler, 1991; Öquist and Huner, 2003). As a response, thylakoid membranes are reorganized, reaction centres are closed, and antennal size is reduced in order to protect the photosynthetic apparatus against overexcitation by the incoming radiation (García-Plazaola *et al.*, 1997; Huner *et al.*, 1998; Ensminger *et al.*, 2012; Verhoeven, 2014). The increased NPQ and decreased  $F_v/F_m$  found in our study are good proxies for these photoprotective processes in the thylakoid membranes, indicating an increased thermal dissipation of excess energy and a decreased photochemical efficiency (Maxwell and Johnson, 2000). Thus, we found that *Q. ilex* acclimated to the winter conditions with reoccurring night frosts, and exploited the winter period photosynthetically at the cost of lower assimilation rates and a lower carbon-use efficiency (see also Hurry *et al.*, 2000; Dolman *et al.*, 2002; Sperlich *et al.*, 2014). We underline the fact that winter acclimation and exploitation can be essential for Mediterranean evergreen tree species to recover from stressful summer periods and to achieve a positive annual carbon balance.

Notably lower NPQs in spring indicate that the photosystems experienced the least amount of photochemical stress in this period. This is because the spring in our study was particularly cool and wet and was characterized by a low VPD. The high NPQ in winter and summer, in contrast, reflects strong photoprotection against photoinhibitory stress due to the temperature extremes. However, the photoprotective mechanisms seemed to be effective: the optimal light intensity for net assimilation and the electron transport (approximately  $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for both) and the effective quantum yield of PSII ( $\Phi_{\text{PSII}}$ ) (Supplementary Fig. S3) did not change between the seasons.

The assimilation rates and the carbon-use efficiency increased from winter to spring, although it was not until summer that the peak photosynthetic activity was reached. The elevated  $F_v/F_m$  underlines the fact that the photosynthetic apparatus fully recovered its maximum photochemical efficiency in summer. This contrasts with a very low total precipitation measured during the summer (22mm). However, *Q. ilex* can benefit from water reserves in deep soil layers and also in rock fractures (Barbeta *et al.*, 2015), which also explains its water-spending behaviour during drier periods (Sánchez-Costa *et al.*, 2015). It is known that *Q. ilex* develops a profound root system with a lignotuber that can make up as much as half of the total tree biomass (Canadell *et al.*, 1999), which is vital to withstand abiotic stress periods or disturbances. The precipitation in winter and spring together nearly reached the annual mean, so that deep soil water reserves are likely to have been yet filled in summer. High water availability in combination with high summer temperatures can account for the high photosynthetic activity in a potential water stress period. The replenishment of soil water reserves early in the growing season is critical to endure seasonal summer droughts in Mediterranean trees (Sperlich *et al.*, 2015). Pinto *et al.* (2014) also found the highest sap flow rates of *Quercus suber* L. in summer because its roots had access to the groundwater.

#### Effect of rainfall exclusion on photosynthetic and respiratory traits

Drought experiments with rainfall exclusion under natural conditions can serve as valuable real-time model simulations for scenarios of future climate change. Unfortunately, long-term experiments over several years are costly and laborious

and thus are particularly scarce. The rainfall exclusion in Prades, maintained since 1999, has reduced soil moisture by 13% with respect to ambient conditions and is probably the longest continuous-drought experiment worldwide (Wu *et al.*, 2011; Ogaya *et al.*, 2014).

Plants face a trade-off under water stress: the closure of the stomata reduces transpiratory water loss but at the same time constrains CO<sub>2</sub> diffusion to the chloroplasts. Besides  $g_s$ ,  $g_m$  can act as a second leaf internal valve regulating the gas exchange through carbonic anhydrase and aquaporins and can thus help to prevent dehydration of vacuoles and cells (Terashima and Ono, 2002; Lopez *et al.*, 2013; Perez-Martin *et al.*, 2014). When chronic water stress begins to deplete stores of non-structural carbohydrates, plants are particularly reliant on photosynthetic products for refinement, repair, and protective actions (Niinemets *et al.*, 2009). We have provided novel evidence that  $g_m$  not only imposes an additional leaf internal resistance to gas exchange but can also facilitate the CO<sub>2</sub> diffusion to the chloroplasts in comparison with a stronger control by stomata under drought. This was reflected by a comparatively higher  $g_m$  that increased the  $g_m/g_s$  ratio under long-term drought (see also Galmés *et al.*, 2013). Our results are supported by recent findings obtained in *Q. ilex* and *Pinus halepensis* leaves showing a higher leaf internal CO<sub>2</sub> conductance in parallel with a stricter stomatal control under severe drought (Sperlich *et al.*, 2015).

In addition to the importance of the diffusive capacity of stomata and mesophyll for the foliar carbon balance, this balance also depends strongly on the relationship of photosynthesis with respiration. However, the extent to which  $R_n$  or  $R_d$  are affected by water scarcity is highly uncertain. We found that  $R_d$  was approximately 74% of  $R_n$  and that the long-term rainfall-exclusion experiment increased the ratio of  $R_d/R_n$  ( $0.79 \pm 0.04$ ) compared with the control plot ( $0.71 \pm 0.03$ ) due to a higher  $R_d$ . Some studies found increased foliar respiration under severe water stress (Ghashghaie *et al.*, 2001), but reductions were also reported (Flexas *et al.*, 2006). The leaf may exert an acclimation of the respiratory metabolism through  $R_d$  because the demands for energy (ATP and NADPH) for synthesis of sucrose and carbon skeletons in the cytoplasm are higher under stressful conditions (Flexas *et al.*, 2006; Zaragoza-Castells *et al.*, 2007).  $R_d$  provides the basis for building up heat-stabilizing components such as heat-shock proteins or biogenic volatile organic compounds protecting the plant against detrimental effects (Tcherkez and Ribas-Carbó, 2012). Higher photoinhibitory stress can thus increase the respiratory metabolic activity expressed as a higher protein turnover at a given overall protein content (Niinemets, 2014; Weerasinghe *et al.*, 2014). This might explain the generally higher values of  $R_d$  that we found in the drought treatment. A lower stress level would evidently lead to a lower demand for energy and carbon skeletons and hence to a lower protein turnover. We found an indication for this in the effective photoprotective mechanism and lower rates of  $R_d$  in the summer campaign characterized by favourable conditions. In contrast to the results of Zaragoza-Castells *et al.* (2007), in our study only  $R_d$  but not  $R_n$  acclimated seasonally. The higher  $R_d$  in the drought treatment decreased significantly and coincided with the lower values of the control group, which

remained unaffected throughout the seasons. This decrease of  $R_d$  in the drought treatment in summer—paralleled by higher rates of photosynthesis—significantly decreased  $R_d/R_n$  and increased the foliar carbon-use efficiency. Griffin and Turnbull (2013) showed that a decreased  $R_d/R_n$  can be explained by a suppressed light-saturated rate of oxygenation in photorespiration. Although we did not measure photorespiration directly, our data showed increased  $g_m$  and thus elevated CO<sub>2</sub> concentrations in the chloroplasts (increase of 35% from spring to summer) (Fig. 3), which would benefit carboxylation over oxygenation. Overall, we found that  $R_d$ —as the key player for the foliar carbon balance in *Q. ilex*—was the most responsive to seasons or treatment effects.

We found that the drought treatment had no significant effect on  $J_{1200}$ ,  $V_{c,max}$ , or  $TPU$  in the summer campaign. Our results emphasize that the increased photosynthetic activity in the drought treatment in summer was not attributed to a higher potential in the biochemistry of photosynthesis, but rather than to an increased diffusive capacity of both  $g_s$  and  $g_m$ . Interestingly, analysis of the apparent  $J_{1200}$  and apparent  $V_{c,max}$  (derived from  $A/C_i$  curves) identified marginally significant higher values in the rainfall-exclusion plot. This shows that the  $A/C_c$  method is more appropriate and that the traditional fitting method based on  $A/C_i$  curves may lead to confounding effects—as also shown in grapevines by Flexas *et al.* (2006). The foliar internal diffusion limitation imposed by  $g_m$  accounted on average for a 54% higher  $V_{c,max}$ , a 30% higher  $J_{max}$ , and a 29% higher  $TPU$  of the apparent photosynthetic potential. Similar values were reported for nearly 130 C<sub>3</sub> species in a recent study by Sun *et al.* (2014).

We postulate firstly, that the summer period provided counterintuitively favourable conditions (as discussed above), and secondly, that the trees in the drought treatment acclimated most efficiently the balance between energy supply versus energy consumption in this period. Recent findings underscore the high plasticity of *Q. ilex* in response to seasonal changes in temperature or soil water compared with other Mediterranean species (Sperlich *et al.*, 2015). The rainfall-exclusion experiment in Prades was shown to result in a higher stem mortality (Barbeta *et al.*, 2013) and in a reduced leaf area in *Q. ilex* (Ogaya and Peñuelas, 2006), while increasing leaf mass per area (data not shown). With fewer leaves and lower total leaf area, the concentration of biochemical resources per leaf would increase. This might contribute to explaining the partly higher photosynthetic rates and carbon-use efficiency in the drought treatment. Sperlich *et al.* (2015) also found higher photosynthetic potentials in crowns that suffered a reduced total leaf area after a severe drought.

In this study, we examined the seasonality of photosynthetic and respiratory traits and evaluated the adaptive mechanism in response to reduced soil water under partial rainfall exclusion. A high climatic variability in the Mediterranean region can lead to counterintuitive effects, with the peak photosynthetic activity in summer, which is usually characterized by a high level of abiotic stress. The trees experiencing a 14-year-long drought treatment adapted through a higher plasticity in photosynthetic traits, so that eventually an atypical favourable growth period in summer was exploited more

efficiently, with  $g_m$  and  $R_d$  as the determining parameters. Drought-induced growth declines may be attenuated in the long-term through morphological and physiological acclimation to drought (Leuzinger *et al.*, 2011; Barbeta *et al.*, 2013). Fewer leaves in the drought treatment were compensated by higher net photosynthetic rates. The similarity of photosynthetic potentials in the treatment and control plots suggests that there is also a dampening effect on the biochemical level.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Four exemplary samples of carbon-response curves conducted at leaves of (i) twigs attached to the tree (field), (ii) after cutting and pre-conditioning the twigs under dim light in water in the lab for one night (day 1) and (iii) two nights (day 2).

**Supplementary Fig. S2.** Bar chart depicting the evolution of night respiration ( $R_n$ ) of *Q. ilex* directly after cutting the twig and after darkening for 30 min (field), and at day 1, 2, and 3 after being pre-conditioned under dim light in water in the laboratory.

**Supplementary Fig. S3.** Line graphs depicting seasonal changes of (a) quantum yield of  $CO_2$  ( $\Phi_{CO_2}$ ) and (b) effective quantum yield of PSII ( $\Phi_{PSII}$ ) for *Q. ilex*.

**Supplementary Fig. S4.** Scatter plots and regression lines of maximum carboxylation rate ( $V_{c,max}$ ) versus maximum rate of electron transport ( $J_{max}$ ) derived from (a)  $A/C_c$  and (b)  $A/C_i$  response curves for control and drought plots in summer 2013.

**Supplementary Table S1.** Regression equations and coefficients of determination ( $R^2$ ) for  $A_{net}/R_d$  for *Q. ilex* in three sampling campaigns in control and drought plots.

**Supplementary Table S2.** Regression equations and coefficients of determination ( $R^2$ ) for  $A_{net}/R_n$  for *Q. ilex* in three sampling campaigns in control and drought plots.

**Supplementary Table S3.** Regression equations and coefficients of determination ( $R^2$ ) for  $J_{amb}/A_{net}$  for *Q. ilex* in three sampling campaigns in control and drought plots.

**Supplementary Table S4.** Regression equations and coefficients of determination ( $R^2$ ) for  $C_i/C_c$  for *Q. ilex* in three sampling campaigns in control and drought plots.

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