

# Leaf CO<sub>2</sub> efflux is attenuated by acclimation of respiration to heat and drought in a Mediterranean tree

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## Summary

1. Understanding the effect of water deficit on plant respiration and its temperature sensitivity at long time-scales is important for anticipating the shift in ecosystem dynamics that is expected to occur as a result of climate change.

2. We investigated seasonal variations of leaf dark respiration and sensitivity to temperature in the upper and lower canopy of *Quercus ilex* trees in a forest stand that had been exposed to either 33% throughfall reduction or normal rainfall for the preceding 6 years.

3. For upper and lower canopy leaves, light-saturated photosynthesis and respiration at a reference temperature of 15 °C ( $R_{15}$ ) had the lowest values in late summer, when both traits were lower in trees subjected to throughfall reduction than in those subject to control conditions. Respiration acclimated to seasonal shifts in temperature, more intensively through summer than winter and in throughfall-exclusion than control trees. Foliar concentration of soluble sugars was positively correlated with  $R_{15}$  across seasons.

4. As leaf respiration decreased with increasing water deficit, and the relationship between water status and respiration did not differ between trees that had been exposed to 7 years of enhanced drought when compared with controls, we suggest that drier conditions projected for the Mediterranean may attenuate the stimulation of leaf respiratory CO<sub>2</sub> release by global warming in *Q. ilex* forests.

**Key-words:** abiotic factor, canopy depth, carbon, drought acclimation, mediterranean habitat, nonstructural carbohydrates, thermal acclimation, throughfall exclusion

## Introduction

Mitochondrial respiration produces much of the metabolic energy and carbon skeletons necessary for the growth and maintenance of plant tissues. In this process, between 25 and 75% of the carbon gained daily through photosynthesis is released into the atmosphere in the form of carbon dioxide (CO<sub>2</sub>) (Amthor 2000). Therefore, the way photosynthesis and respiration respond to variations in environmental factors – such as temperature, light and water – has important consequences for plant carbon balance, growth and survival (Maseyk *et al.* 2008; Adams *et al.* 2009). The modulation of respiration in response to environmental changes is also an important aspect for predicting future global

atmospheric CO<sub>2</sub> concentrations. It has been estimated that the emission of CO<sub>2</sub> into the atmosphere because of plant respiration approximately amounts to 60 gigatonnes of carbon per year, which is near nine times the amount released by anthropogenic activities (Schimel 1995). We are, however, far from understanding how environmental factors interact and impact on respiration.

Leaf dark respiration ( $R$ ) increases exponentially over short-term rises in temperature (e.g.  $Q_{10}$ , the proportional increase in  $R$  per 10 °C degree rise in temperature, ranges typically from 2 to 2.6 across species; Tjoelker, Oleksyn & Reich 2001; Atkin, Bruhn & Tjoelker 2005). However,  $R$  may increase less markedly, remain unchanged or even decline in response to long-term rises in temperature (i.e.  $R$  thermally acclimate, Atkin, Bruhn & Tjoelker 2005). The acclimation of  $R$  to long-term shifts in temperature is often underpinned by a change in the respiratory capacity (e.g. Tjoelker, Reich &

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Oleksyn 1999) and/or by changes in substrate supply and energy demand (i.e. in the  $Q_{10}$  of  $R$ ; e.g. Rook 1969; Ow *et al.* 2010). In spite of being a widespread phenomenon well recognized for over 50 years, only recently has thermal acclimation of plant respiration been incorporated into global climate-vegetation models (Atkin *et al.* 2008). Much research is needed before relationships between environmental factors and plant functional traits become reliably predictable and can be incorporated into models. Including the effect of water deficit on  $R$  and the temperature sensitivity of  $R$  is a further step towards improving the accuracy of models in predicting future atmospheric  $CO_2$  concentrations, but the relationship between water deficit and  $R$  remains poorly understood.

In leaves, in general, respiration declines with increasing water deficit (Mooney 1969; Flexas *et al.* 2005; Galmés *et al.* 2007; Atkin & Macherel 2009), but sometimes  $R$  increases (Slot, Zaragoza-Castells & Atkin 2008) or remains unchanged (Galmés *et al.* 2007). The decline in  $R$  could be in part because of the decline in photosynthetic  $CO_2$  assimilation ( $A$ ) caused by water deficit, as  $A$  provides soluble sugars used as substrates for  $R$ . But the interdependence of  $A$  and  $R$  does not generally lead to a homeostasis of  $R/A$ ;  $R$  proceeds in the absence of  $A$ , which abruptly increases the ratio of  $R/A$  over certain thresholds of water deficit (Slot, Zaragoza-Castells & Atkin 2008; Gimeno *et al.* 2010). A reduction in the availability of soluble sugars may also cause a reduction in the temperature sensitivity of  $R$  (Covey-Crump, Attwood & Atkin 2002 in roots), as low substrate availability limits the increase in the activity of respiratory enzymes that takes place with increasing temperature (Atkin, Zhang & Wiskich 2002). So far, however, there is no evidence that water deficit reduces the  $Q_{10}$  of respiration in leaves (Crous *et al.* 2010; Rodríguez-Calcerrada *et al.* 2010). In fact, Slot, Zaragoza-Castells & Atkin (2008) observed repeated increases in the  $Q_{10}$  of  $R$  in an herbaceous species following several short drought cycles. Over long periods (e.g. seasons), the sensitivity of  $R$  to temperature can be affected by drought-induced changes in leaf morphology, the availability of soluble sugars and the respiratory capacity (e.g. Atkin & Macherel 2009). However, the impact of drought on thermal acclimation has received little attention (Crous *et al.* 2010; Rodríguez-Calcerrada *et al.* 2010).

Plant species will probably have to face more intense droughts in the near future in some areas of the world. For example, a reduction in rainfall by more than 30% could occur over the next century in the Mediterranean basin according to global and regional climate models (Giorgi 2006; Somot *et al.* 2008). Understanding the response of  $R$  to drought may help in anticipating species susceptibility to climate change and eventually developing mitigation practices. However, most of our understanding on the mechanisms underpinning  $R$  responses to water deficit comes from short-term experiments, often performed with small plants under laboratory conditions. The response of  $R$  may change over the course of years of enhanced water deficit. Acclimation of trees to experimentally imposed drought (Metcalfe *et al.* 2010) or to naturally low soil water content (Turnbull *et al.* 2001; Xu & Griffin 2006) has resulted in an increase in  $R$  with

respect to control trees or nearby sites of higher soil water content, respectively. The acclimatory response to long-term water deficit is consistent with the evolutionary trend that has led to higher respiratory rates in species from lower- than from higher-rainfall sites (Wright *et al.* 2006) but seems to run opposite to the general trend of declining  $R$  following increasing water deficit over short terms (Atkin & Macherel 2009). Moreover, whole-plant acclimation to an accentuation of drought intensity can affect the relationship of functional traits (e.g. leaf water potential and photosynthesis) with  $R$  and alter the effects of other abiotic factors on  $R$ , such as light and temperature (Xu & Griffin 2006). To better understand the long-term consequences of a potential increase in drought severity on leaf photosynthetic and respiratory parameters, rainfall and throughfall exclusion systems are being increasingly set up in different types of forests (e.g. Hanson *et al.* 2003; Limousin *et al.* 2010; Metcalfe *et al.* 2010).

Leaf canopy position can play a role in the response of  $R$  to drought and temperature in large trees. The variability of  $R$  and its sensitivity to temperature within the tree canopy has been studied in few species (Bolstad, Mitchell & Vose 1999; Griffin, Turnbull & Murthy 2002; Zha *et al.* 2002; Turnbull *et al.* 2003; O'Grady *et al.* 2010).  $R$  is lower in the base than the top of the canopy, which is caused by shade-induced declines in leaf mass per area (Griffin, Turnbull & Murthy 2002; Tissue *et al.* 2002; Zha *et al.* 2002) and, less frequently, in the availability of nitrogen and nonstructural carbohydrates (O'Grady *et al.* 2010) and the respiratory capacity (Noguchi *et al.* 2005). On the contrary, the sensitivity of  $R$  to temperature does not show a consistent pattern of variation with canopy depth, being either higher (Turnbull *et al.* 2003), lower (Griffin, Turnbull & Murthy 2002) or similar (Bolstad, Mitchell & Vose 1999) in the upper than in the lower canopy. The variability is generally related to the foliar concentration of nitrogen and the concentration of nonstructural carbohydrates, sometimes in an unexpected manner (e.g. higher nonstructural carbohydrates and lower  $Q_{10}$  in the canopy top; Griffin, Turnbull & Murthy 2002). Differences in chemical traits can also mediate a different response of  $R$  to seasonal shifts in temperature among canopy positions, but few studies have addressed this point (O'Grady *et al.* 2010).

*Quercus ilex* L. is an evergreen Mediterranean tree species tolerant of drought and extreme temperatures. In this study, we assessed the impact of a long-term 33% reduction of throughfall on  $R$  and other related traits at two canopy levels of *Q. ilex* trees. We compared trees that were constantly subjected to partial throughfall exclusion since 2003 with others subjected to normal rainfall conditions. 6 years after the onset of throughfall exclusion, we measured leaf photosynthesis and dark respiration and the response of dark respiration to diurnal changes in temperature in leaves in the upper and lower crown of the trees over approximately 1 year. Our main objective was to assess the influence of increased drought severity on (i) the seasonal variation of respiratory traits and (ii) the relationship of water deficit and biochemical traits with  $R$  and the diurnal temperature sensitivity of  $R$ . By comparing trees potentially prone to suffer different levels of

water deficit along the season, we also aimed at estimating the importance of water deficit on the acclimation of  $R$  to varying seasonal temperatures, if any. Finally, we aimed to examine the variability in  $R$  and temperature sensitivity within the canopy and their potential relationship with other leaf morphological and biochemical traits.

## Materials and methods

### SITE DESCRIPTION AND EXPERIMENTAL DESIGN

This experiment was carried out in an even-aged *Quercus ilex* forest stand in the south of France (Puéchabon State Forest; 3°35'E, 43°44'N; 270 m a.s.l.). The forest was used as a coppice until the first half of the 20th century and the last clear cut occurred in 1942. Today, density is 5100 stems ha<sup>-1</sup>, basal area is 27.4 m<sup>2</sup> ha<sup>-1</sup> and canopy height is 5.5 m. The soil is extremely rocky, with 75% of stones by soil volume for the top 0–50 cm and 90% below. The fine fraction of the topsoil is a homogeneous silty clay loam. The climate is Mediterranean, with hot and dry summers and cold and wet winters. Mean annual, summer and winter temperatures (averaged from the 1984–2009 period) are 13.2, 20.7 and 6.3 °C, respectively. Mean values of annual, summer and winter precipitation are 902, 110, and 243 mm, respectively, with 80% of rainfall occurring between September and April. More details about the study area can be found in the study by Rambal *et al.* (2003).

In spring 2003, a system of partial throughfall exclusion was set up on a flat plateau in three plots of 10 × 10 m size [exclusion (E) plots], with three adjacent plots being left as controls [control (C) plots]. Several lines of PVC rain gutters were suspended below the canopy at a height of 1.5–0.5 m above the soil to conduce water outside the plots (Fig. 1). On the control plots, the same area was covered by gutters turned upside down to even out understory microclimatic conditions between plots. Gutters are effectively reducing approximately 27% of net precipitation because of canopy interception. Taking into account stemflow, interception by the canopy – and by gutters in the case of E plots – yearly averaged net precipitation at the soil level is 69.1% of incident rainfall for C plots and 50.4% for E plots (see Limousin *et al.* 2008). The water stress integral (WSI) is an index used to quantify drought intensity. Monthly WSI was calculated from 2003 to 2008 as the sum of the soil water potential of all the days in a month; it was on



Fig. 1. Picture of a *Quercus ilex* stand in SE France subjected to a long-term treatment of throughfall exclusion. Photograph courtesy of Raquel Rodríguez-Cortina.

average 26.1% higher in E than in C plots during summer months (Fig. 2). Values of soil water potential are modelled from the retention curve linking soil water content and predawn leaf water potential, which is assumed to be 0.43 MPa lower than that of the soil according to Donovan, Linton & Richards (2001) (see Rambal *et al.* 2003 for more details).

For our study, we selected five healthy trees of *Q. ilex* from the centre of one C and one E plot where a scaffolding system allowed access to the canopy. We selected branches from the upper canopy and lower canopy (at approximately 5 and 2 m height, respectively) for repeated measures over approximately 1 year (every 1–2 months, from April 2009 to March 2010). Orientation varied from NE to NW, but it was paired for C and E trees. Mean light availability was 84.2 ± 2.0 and 85.8 ± 2.2% of above-canopy photosynthetic photon flux density in the upper canopy and 18.2 ± 3.5 and 20.2 ± 2.5% in the lower canopy of C and E trees, respectively. Measurements were taken using an LI-250 Light Meter (LI-COR Inc., Lincoln, NE, USA) in a completely overcast day of March 2010. At each sampling campaign, one leaf was measured from each canopy height. Leaves were fully developed and belonged to the youngest cohort at the time of sampling. Because of the ancient coppice management of the forest, sampled trees were formed by several stems clumped at the base; we chose the dominant stem for measurements.

### TEMPERATURE AND LEAF WATER POTENTIAL

Air temperature was measured at the two sampled canopy heights in both C and E plots during the study period. We also attached thermocouples to the abaxial side of leaves (one sensor per leaf in three leaves

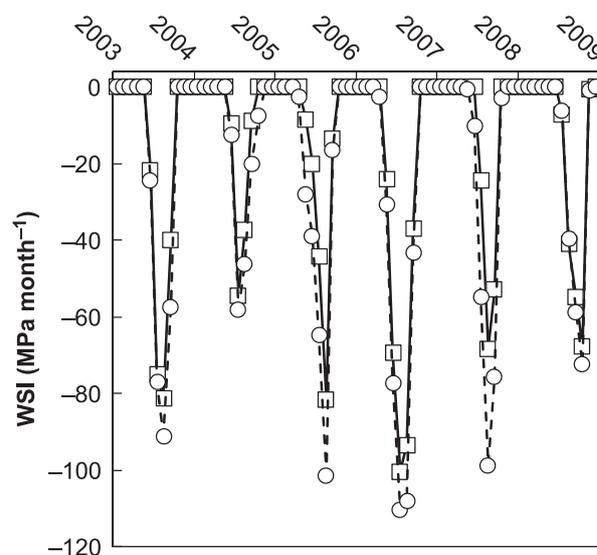


Fig. 2. Monthly water stress integral (WSI) over the years preceding this study in control (C, squares) and throughfall-exclusion (E, circles) plots. WSI was on average 30.2% higher in E than in C plots: 14.9% in 2003, 31% in 2004, 49.6% in 2005, 14.8% in 2006, 67% in 2007 and 3.7% in 2008. During winter, spring and autumn, rainfall is generally sufficient to reach soil field capacity in both plots. However, during the summer months of June through September, the partial exclusion of throughfall becomes relevant for the trees' water status. WSI was on average 26.1% higher in E than in C plots during summer months: 14.9% in 2003, 24.1% in 2004, 38.6% in 2005, 13% in 2006, 64.6% in 2007 and 1.5% in 2008. Maximum water stress occurs in August in all years except in 2008 (September).

per throughfall treatment and canopy position) with porous adhesive tape to monitor leaf temperature continuously. Measurements were taken every five minutes and averaged in intervals of 30 min.

Leaf water potential was measured using a pressure chamber (PMS Instrument Company, Albany, OR, USA) before dawn and around midday in two leaves per tree, with a third leaf measured if the two-first values were more than 0.4 MPa different.

#### GAS EXCHANGE

We used a portable gas exchange system (LI-6400; LI-COR Inc.) to measure photosynthesis and dark respiration. Measurements were taken on intact leaves attached to trees in a 2 × 3 cm chamber (6400–02B LED Light Source), at 400 ppm CO<sub>2</sub> (using the CO<sub>2</sub> mixer), 300 μmol s<sup>-1</sup> air flow rate and ambient relative humidity and temperature. Light was set to 1500 μmol m<sup>-2</sup> s<sup>-1</sup> for the measurements of photosynthesis (*A*<sub>sat</sub>) and stomatal conductance (*g*<sub>s</sub>) at saturating light and 0 μmol m<sup>-2</sup> s<sup>-1</sup> for those of respiration (*R*), making sure that actual values did not deviate from target values.

*A*<sub>sat</sub> was measured once at each sampling campaign. We obtained the maximum daily value of *A*<sub>sat</sub> by measuring in early morning in spring/summer (i.e. when air temperature was near 25 °C – actual mean of 25.8 °C) and at midday in autumn/winter (i.e. when air temperature was the highest – actual mean of 15.6 °C). *R* was measured several times during the night and day at each campaign, with the aim of obtaining a response curve of *R* to temperature. Measuring *R* at the prevailing ambient temperature during the day has proven adequate for constructing temperature response curves of *R* (Atkin, Holly & Ball 2000; Zaragoza-Castells *et al.* 2008). One leaf (occasionally more if it resulted damaged) was marked and used for repeated measures at each campaign and finally collected for the determination of its mass and area. During the day, leaves were darkened for approximately 30 min before being placed into the chamber; then, CO<sub>2</sub> efflux rates were allowed to equilibrate for several minutes. There were at least 2-h intervals between measurements to avoid the depletion of substrates of *R* (Azcón-Bieto 1992). We recorded a minimum of 10 values per leaf at each temperature.

#### CHEMICAL TRAITS

We measured the concentration of nitrogen (N), soluble sugars (SS, i.e. fructose, sucrose and glucose) and starch in 5–10 leaves that were near the leaf used for gas exchange and belonged to the same cohort. Leaves were collected 3–4 h after sunrise and stored in liquid nitrogen for transport to the laboratory, where they were finally stored at –80 °C. Before analyses, all leaf material was lyophilized and milled to a fine powder using a ball mill.

Nitrogen (N) was measured using an elemental analyser (Flash EA1112 Series, Thermo Finnigan, Milan, Italy). Soluble sugars (SS) were extracted from leaf material as described by Göttlicher *et al.* (2006). From the extract, 25 μL supernatant was dried *in vacuo* (Concentrator plus, Eppendorf, Hamburg, Germany) and derivatized for gas chromatography-mass spectrometry (GC-MS) analysis according to Fiehn (2006). Quantitation was performed on a GC-MS system (7890A/5975C; Agilent Technologies, Waldbronn, Germany), using internal and external sugar standards (Sigma, Munich, Germany). For starch analysis, aliquots of 25 mg were extracted as detailed in Göttlicher *et al.* (2006) and hydrolysed for 60 min at 60 °C with 10 U amyloglucosidase from *Aspergillus niger* (Sigma) in 20 mM sodium acetate buffer (pH 4.8). Released glucose equivalents were quantitated by GC-MS as described previously.

#### DATA ANALYSES

Values of *A*<sub>sat</sub> and *R* were corrected by the leaf area enclosed into the chamber (measured using a MK2 Area Meter; Delta-T devices Ltd., Cambridge, UK) and divided by leaf dry mass per unit area (LMA) for calculating dry mass–based rates of *R*. LMA was also used to express the concentration of chemical components on a leaf area basis.

The short-term temperature sensitivity of *R* was first described using *Q*<sub>10</sub> values of temperature response curves from individual plants. Log-transformed values of *R* obtained across the diurnal range of measurement temperatures were used to calculate the base rate of respiration at 0 °C (i.e. Y-intercept, *R*<sub>0</sub>), the slope of the regression line (*k*) and then the *Q*<sub>10</sub> for each tree and canopy position according to:

$$Q_{10} = e^{10k}.$$

We also estimated the value of *R* at a temperature of 15 °C (*R*<sub>15</sub>), common to all dates except August (minimum temperature of 18 °C) and January (maximum temperature of 12 °C), and at the mean temperature of the week preceding measurements (*R*<sub>amb</sub>) according to:

$$R_x = R_0 Q_{10}^{\left(\frac{T_x}{10}\right)},$$

where *R*<sub>x</sub> is either *R*<sub>15</sub> or *R*<sub>amb</sub> and *T*<sub>x</sub> is either 15 °C or ambient temperature, respectively.

The *Q*<sub>10</sub> is dependent on the range of temperatures across which it is calculated (Atkin, Zhang & Wiskich 2002). Therefore, because our measurements were taken *in situ* and thus at different temperatures through the year, we used the equation given in Turnbull *et al.* (2001) to estimate the short-term temperature sensitivity of *R*:

$$R = R_x e^{\left(\frac{E_0}{R_g} \left(\frac{1}{T_x} - \frac{1}{T}\right)\right)}$$

This is a modified Arrhenius equation, where *E*<sub>0</sub> is a variable related to activation energy and *R*<sub>g</sub> is the ideal gas constant (8.314 J mol<sup>-1</sup> K<sup>-1</sup>). To estimate *E*<sub>0</sub> and *R*<sub>x</sub> at a given reference temperature (*T*<sub>x</sub>), nonlinear models were fitted to the relationship between temperature (*T*) and *R* using the least-squares Levenberg–Marquardt method incorporated in STATISTICA 7.1 (Statsoft Inc., Tulsa, OK, USA).

We estimated foliar respiration at the stand scale in C and E plots following a simple ‘up-scaling’ approach (e.g. Griffin, Turnbull & Murthy 2002; Metcalfe *et al.* 2010). Instantaneous rates of *R* per unit leaf area were multiplied by the leaf area index (LAI) to estimate rates per ground area. Base respiration was verified to decline with increasing canopy depth in a similar way as to LMA does (Rambal *et al.* 1996):

$$R_0(z) = R_{0top} e^{-kz},$$

with *z* varying from 0 in top canopy leaves to LAI values corresponding to lower canopy leaves. The *k* parameter is equal to the rate constant of the LMA profile (*k*<sub>LMA</sub>) and is related to LAI (Rambal *et al.* 1996). Respiration for the canopy profile was then calculated as:

$$\overline{R_0} = \frac{1}{\text{LAI}} \int_0^{\text{LAI}} R_0(z) dz.$$

Stand-scale CO<sub>2</sub> release was estimated at the average night-time temperature of each field campaign over a night period of 8 h:

$$R_d = \text{LAI} \int_0^8 \overline{R_0} Q_{10}^{T_{\text{leaf}}(t)/10} dt.$$

We considered the  $Q_{10}$  was constant through the canopy and equal to the mean of upper and lower canopy leaves. Values of LAI of *Q. ilex* for C and E plots have recently been estimated by Limousin *et al.* (2009); LAI in the C plot was 1.89 m<sup>2</sup> leaf m<sup>-2</sup> soil and 1.55 m<sup>2</sup> leaf m<sup>-2</sup> soil in the E plot, typical to a forest with a fairly open canopy.

Repeated-measures ANOVA were conducted to test for the main effects and interactions between throughfall treatment (1 d.f.), canopy position (1 d.f.) and date (within-subject factor, 6 d.f.). In addition, we explored the main and interactive effects of throughfall treatment and canopy position for some dates. To compare the relationships between leaf traits in C and E trees, we used ANCOVA and tested for the interaction between throughfall treatment and leaf traits (covariates). Before analyses were made, variables leaf water potential,  $Q_{10}$ ,  $R_n/A_{\text{sat}}$  and starch content were logarithmically transformed to attain normality and reduce heteroscedasticity. Observations subject to technical failure and statistical outliers were eliminated for a final number of replicates of 4–5. *F* values (with degrees of freedom for factor and error) and *P* values from ANOVAs were given in the text. All statistical analyses were carried out using STATISTICA 7.1.

## Results

### ENVIRONMENTAL CONDITIONS AND LEAF WATER DEFICIT

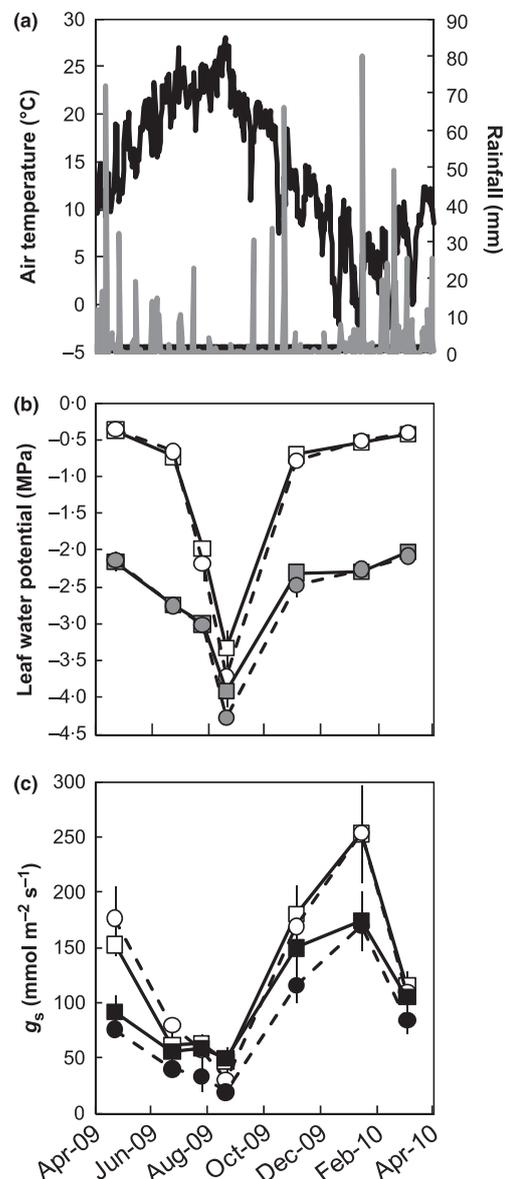
Rainfall and air temperature varied greatly through the study period (Fig. 3a). Rainfall was high in spring and autumn but low in summer, with 47.1 mm during July and August. Air temperature increased progressively from the beginning of the study in April 2009 until the end of August and then declined until January. Mean daily air temperatures were similar between throughfall treatments and canopy positions and were similar to mean daily leaf temperatures. Differences were only evident between canopy positions when considering daily maximum values of temperature (see Figure S1, Supporting Information). With increasing air temperatures, top canopy leaves tended to become hotter than the air to a higher extent than lower canopy leaves, without difference for C and E trees.

Leaf water potential was similar between C and E trees during most of the study period (Fig. 3b). Only in August, E trees exhibited water potentials 0.4 MPa lower than C trees before dawn and at midday (both  $P > 0.10$ ). At this date, E trees exhibited significantly lower values of stomatal conductance ( $g_s$ ), which is suggested to be a more integrative trait of water deficit than leaf water status (Flexas *et al.* 2004);  $g_s$  in E trees was almost half that of C trees: 24.7 vs. 49.3 mmol m<sup>-2</sup> s<sup>-1</sup>,

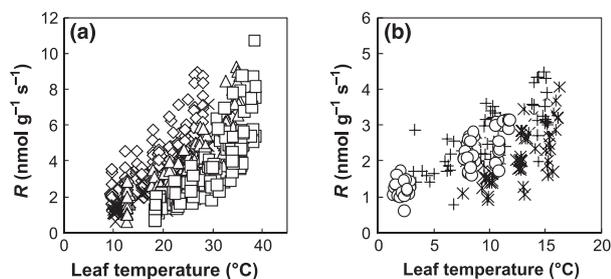
respectively ( $F_{1,15} = 6.109$ ,  $P = 0.026$ ; Fig. 3c). Leaf pre-dawn water potential and log-transformed  $g_s$  were positively correlated across dates (C trees:  $r^2 = 0.43$ ,  $P = 0.01$ , E trees:  $r^2 = 0.68$ ,  $P < 0.001$ ).

### GAS EXCHANGE AND SHORT-TERM TEMPERATURE RESPONSE OF RESPIRATION

*R* increased exponentially with increasing diurnal temperature (Fig. 4). The shape of the curves did not change significantly between treatments or canopy positions.  $Q_{10}$  and the



**Fig. 3.** (a) Mean daily air temperature and rainfall over the study period; (b) mean ( $\pm$  ISE) leaf water potential before dawn (white symbols) and at midday (grey symbols) in control (squares) and throughfall-exclusion (circles) trees; (c) mean ( $\pm$  ISE) stomatal conductance to water vapour at saturating light in upper (open symbols) and lower (closed symbols) canopy leaves of control (squares) and throughfall-exclusion (circles) trees.



**Fig. 4.** Response of leaf dark respiration ( $R$ ) to diurnal changes in temperature in (a) spring/summer and (b) autumn/winter. Each point represents one measure of respiration at the prevailing ambient temperature of the night/day. Different symbols are for different dates: April 2009 (diamonds), June 2009 (saltires), July 2009 (triangles), August 2009 (squares), November 2009 (asterisks), January 2010 (circles) and March 2010 (crosses). Throughfall treatments, canopy positions and trees are pooled. Notice the different scale of axes between panels.

analogous variable  $E_o$  obtained for each plant from a modified Arrhenius equation were positively correlated ( $r^2 = 0.63$ ,  $P < 0.001$ ), so similar results were found irrespective of the model fitted to diurnal temperature response curves (compare Fig. 5a and Fig. S2, Supporting Information). The temperature sensitivity of  $R$  varied among dates ( $F_{6,78} = 5.99$ ,  $P < 0.001$  for  $Q_{10}$  and  $F_{6,78} = 5.45$ ,  $P < 0.001$  for  $E_o$ ). There was a trend for higher  $Q_{10}$  values in autumn/winter than in spring/summer, but the trend was less evident from  $E_o$  values, which suggests differences in  $Q_{10}$  were partly because of the variable measuring temperatures among dates. No significant relationship of  $Q_{10}$  (or  $E_o$ ) with leaf water potential,  $A_{\text{sat}}$ , or chemical traits appeared across dates (not shown).

We used the daily temperature response curves of  $R$  to estimate  $R$  at 15 °C ( $R_{15}$ ) and at the mean temperature of the week preceding each date of measurements ( $R_{\text{amb}}$ ). It is important to note that estimations were rather independent of the model fitted and that there was a strong correlation between the estimations given by each model ( $r^2 = 0.97$ ,  $P < 0.001$  for both  $R_{15}$  and  $R_{\text{amb}}$ ; compare Fig. 5 with Fig. S2, Supporting information). Differences between seasons and treatments were independent of the method of prediction. Thus hereafter, we only present values obtained from the  $Q_{10}$  model.

Respiratory rates were higher in the upper canopy leaves than in the lower canopy leaves. Differences in  $R_{15}$  were statistically significant regardless of whether the rates were expressed per unit of leaf area or dry mass ( $F_{1,14} = 135.10$ ,  $P < 0.001$  and  $F_{1,14} = 30.52$ ,  $P < 0.001$ , respectively); however, higher LMA in the upper canopy ( $F_{1,16} = 212.61$ ,  $P < 0.001$ ; Fig. 5b) made that differences were larger on an area basis (Fig. 5e,f). The same results were found for  $R_{\text{amb}}$  ( $F_{1,14} = 149.83$ ,  $P < 0.001$  and  $F_{1,14} = 48.59$ ,  $P < 0.001$ , for area- and mass-based values, respectively). The effect of leaf canopy position did not greatly change among dates, despite a strong seasonality in  $R_{15}$  and  $R_{\text{amb}}$ .  $R_{15}$  declined from spring to the end of summer (in parallel to increasing

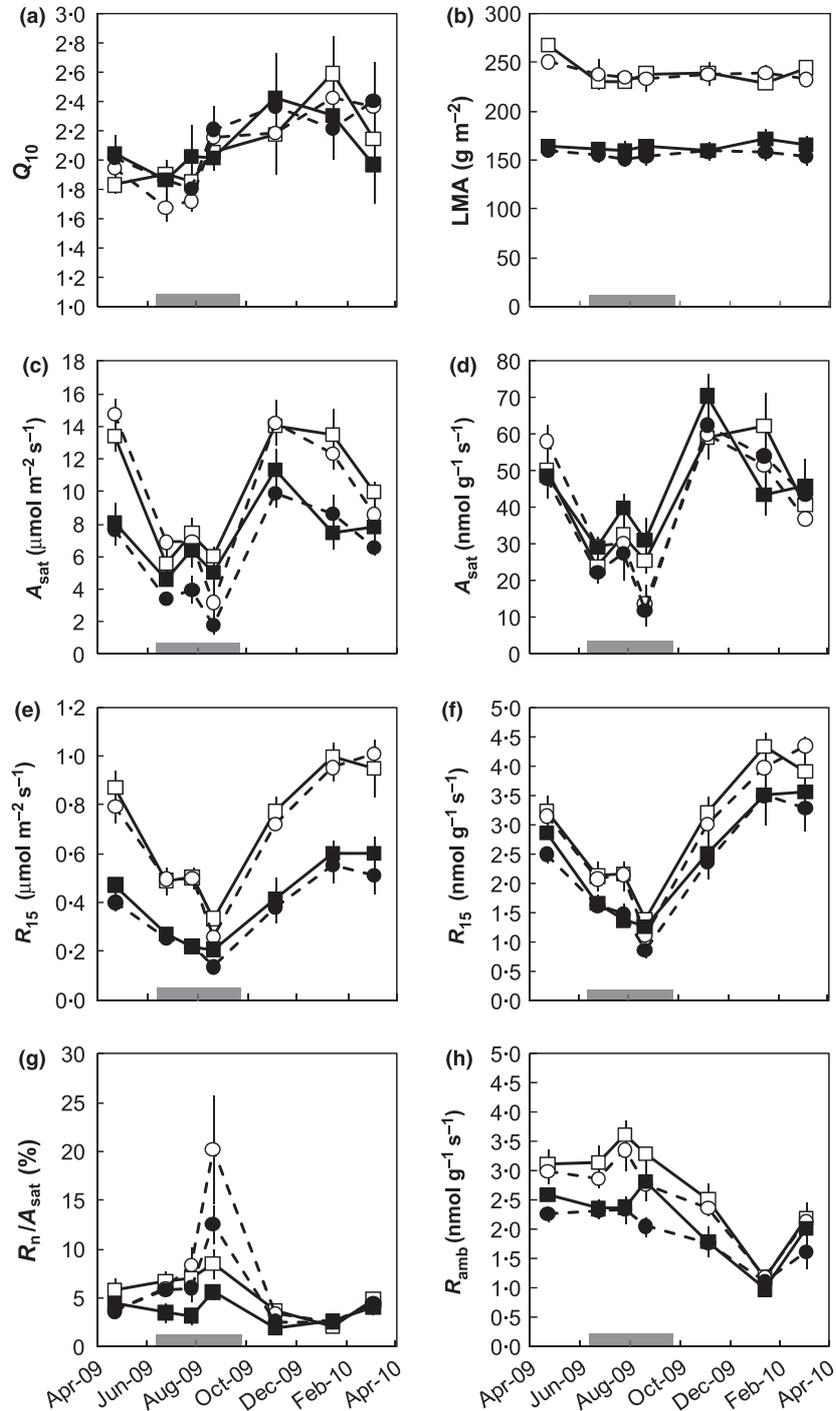
temperatures and decreasing soil water content) and recovered in autumn ( $F_{6,84} = 71.96$ ,  $P < 0.001$ , for mass-based values).  $R_{\text{amb}}$ , on the contrary, was higher in summer and lowest in January ( $F_{6,84} = 39.32$ ,  $P < 0.001$ ; Fig. 5h), although the seasonality was less accentuated than for  $R_{15}$ .

Values of light-saturated net photosynthesis ( $A_{\text{sat}}$ ) per unit leaf area were also higher in the upper canopy than in the lower canopy ( $F_{1,15} = 32.36$ ,  $P < 0.001$ ; Fig. 5c,d), but differences disappeared when this variable was expressed per unit dry mass. Consequently, the ratio of  $R_n$  to  $A_{\text{sat}}$  was slightly higher in the upper canopy layer ( $F_{1,15} = 7.02$ ,  $P = 0.018$ ; Fig. 5g). Seasonal changes in  $A_{\text{sat}}$  tended to parallel those in  $R_{15}$ , with  $A_{\text{sat}}$  declining significantly from spring to summer and recovering afterwards ( $F_{6,90} = 35.87$ ,  $P < 0.001$ , for mass-based  $A_{\text{sat}}$ ). The relatively constant  $R_n/A_{\text{sat}}$  ratio over time was abruptly broken in August, when  $R_n/A_{\text{sat}}$  was higher than in the other dates ( $F_{6,90} = 24.44$ ,  $P < 0.001$ ), especially for E trees ( $F_{6,90} = 3.94$ ,  $P = 0.002$  for the interaction between date and throughfall treatment). The seasonality in the rates of CO<sub>2</sub> exchange was important when comparing C and E trees:  $A_{\text{sat}}$  and  $R_{15}$  (as well as  $R_{\text{amb}}$ , Fig. 5h) were lower in E trees than in C trees in late summer (i.e. in August, when all  $P < 0.006$ ), but differences were absent for the other dates. Taken together, the results suggest that there is a threshold of water deficit above which the decline in  $R$  is proportionally lower than that in  $A_{\text{sat}}$ .  $R_{15}$  was positively correlated with  $\Psi_{\text{pd}}$  and  $A_{\text{sat}}$  across dates, with the slopes being similar in both throughfall treatments (Fig. 6a,b).

Modelled stand-scale night-time foliar CO<sub>2</sub> emission was on average 24% lower in E plots than in C plots (Table 1). This was mainly attributed to the decline in LAI that has occurred since the onset of throughfall exclusion (Limousin *et al.* 2009). However, the difference between both treatments was greater in August, when the instantaneous rates of  $R$  were lower in E than in C trees.

#### LEAF NITROGEN AND TOTAL NONSTRUCTURAL CARBOHYDRATES

As for gas exchange traits, higher values of LMA in the top canopy layer resulted in higher values of N, SS and starch per unit leaf area than in the lower canopy ( $F_{1,16} = 73.00$ ,  $P < 0.001$ ;  $F_{1,14} = 67.74$ ,  $P < 0.001$ ; and  $F_{1,13} = 7.46$ ,  $P = 0.017$ , respectively; Fig. 7a,c,e). Although there was no difference in these traits when they were expressed per unit dry mass (all  $P > 0.10$ ; Fig. 7b,d,f), a marginally significant canopy effect existed for SS ( $F_{1,14} = 4.17$ ,  $P = 0.060$ ). Irrespective of the unit of expression, foliar content of N, SS and starch varied clearly through the study period (all  $P < 0.001$ ; Fig. 7), while there was no significant effect of throughfall treatment for any variable (all  $P > 0.10$ ). Seasonal changes were fairly similar for both canopy positions and throughfall treatments. The significant interaction between factors date and canopy position for SS and N, and factors date and throughfall treatment for starch reflected a slight departure from a common seasonal pattern in some dates (Fig. 7).



**Fig. 5.** Seasonal variation in (a)  $Q_{10}$ , (b) leaf mass per area, (c) area-based light-saturated photosynthesis, (d) mass-based light-saturated photosynthesis, (e) area-based dark respiration at 15 °C, (f) mass-based dark respiration at 15 °C, (g) ratio of night-time respiration to light-saturated photosynthesis and (h) mass-based dark respiration at the mean temperature of the week preceding each date. Open symbols are for upper canopy leaves and closed symbols for lower canopy leaves; control trees are symbolized with squares and continuous lines and throughfall-exclusion trees with circles and dashed lines. Horizontal grey bars indicate the summer drought period. Data are means  $\pm$  1 SE.

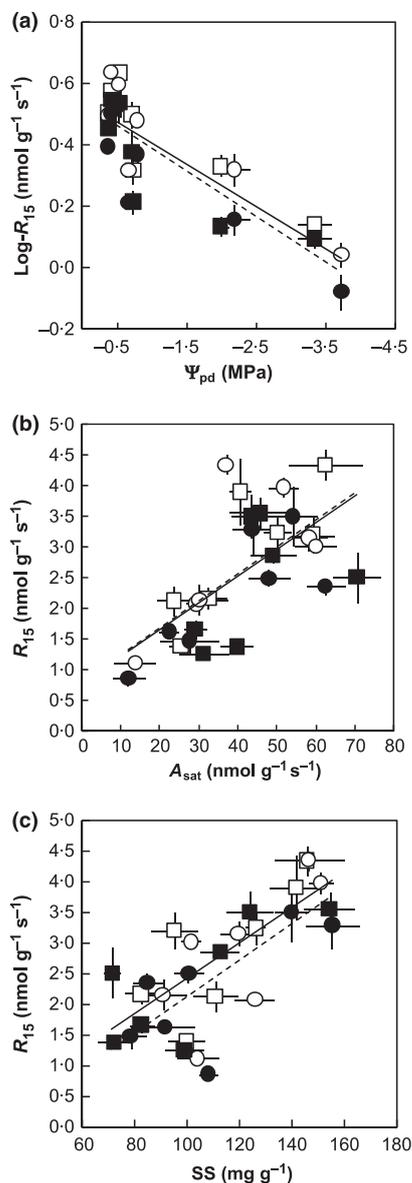
When the effect of canopy position and throughfall treatment was analysed by dates, canopy position continued to be a significant factor in explaining the variation of N, SS and starch for all dates, and throughfall exclusion continued to be an insignificant factor (except for mass-based foliar starch content in March 10:  $F_{1,15} = 6.92$ ,  $P = 0.019$ , indicating higher values in E than in C trees).

The foliar concentration of SS was the most important chemical trait involved in the variation of  $R_{15}$  (Fig. 6c), but neither starch nor N was correlated with  $R_{15}$  (not shown). Seemingly, growth demand of carbohydrates was the main

factor governing starch mobilization, as suggested by the six times higher foliar concentration of starch in April, before shoot growth in May, than in the other dates (Körner 2003).

LONG-TERM TEMPERATURE RESPONSE OF RESPIRATION

By comparing the response of  $R$  to seasonal changes in temperature in C vs. E trees and periods of variable  $\Psi_{pd}$  (summer) vs invariable  $\Psi_{pd}$  (autumn/winter/spring), we estimated the



**Fig. 6.** Relationships of different traits with leaf respiration at 15 °C ( $R_{15}$ ). Symbols as in Fig. 5. Data are date means  $\pm$  2 SE. Significant regression lines were drawn for control (C, continuous) and through-fall-exclusion (E, dashed) trees, for both canopy positions pooled. Relationship between (a) leaf predawn water potential and log-transformed values of  $R_{15}$  (C trees:  $r^2 = 0.65$ ,  $P < 0.001$ , E trees:  $r^2 = 0.73$ ,  $P < 0.001$ ); (b) mass-based light-saturated photosynthesis and  $R_{15}$  (C trees:  $r^2 = 0.38$ ,  $P = 0.020$ , E trees:  $r^2 = 0.50$ ,  $P = 0.005$ ); and (c) soluble sugar concentration and  $R_{15}$  (C trees:  $r^2 = 0.60$ ,  $P = 0.001$ , E trees:  $r^2 = 0.52$ ,  $P = 0.003$ ).

relative contribution of water deficit on the seasonal acclimation of  $R$  to temperature in *Q. ilex*. Thermal acclimation of  $R$  over the course of the study was evidenced by the linear negative relationship between  $R_{15}$  and the mean ambient temperature of the week preceding each field campaign in both upper and lower canopy leaves (Fig. 8a). Acclimation, however, did not result in the homeostasis of  $R$  across the entire range of temperatures experienced by leaves (Fig. 8b). The small difference in the base rate of  $R$  between C and E trees (and simi-

lar  $Q_{10}$ ) in late summer translated into a high difference in  $R$  at the prevailing ambient temperature (26.6 °C) in the leaves of both canopy positions. Therefore, over the warmer and drier summer dates,  $R_{amb}$  clearly deviated from the instantaneous temperature response curve of  $R$ ; acclimation was higher over summer than winter months and the extent of acclimation over this period was higher in E trees than in C trees.

## Discussion

Climate change will challenge the survival of many plant species. Where climate models predict increasing temperatures and decreasing rainfall, ecosystem dynamics are expected to change dramatically with respect to current dynamics (Breshears *et al.* 2005). Understanding how water deficit impacts on  $R$  and modifies the response of  $R$  to other abiotic factors (e.g. temperature) is important for anticipating the consequence of future reductions in rainfall for those regions. Here, we address the effect of drought on  $R$  and its sensitivity to temperature over different time-scales.

### THE EFFECT OF TEMPERATURE AND WATER AVAILABILITY ON THE SEASONALITY OF $R$

$R$  acclimated to annual changes in temperature: as temperatures increased over the year, base rates of  $R$  declined and moderated actual respiratory rates in hotter months. However, the general trend of  $R$  to decrease in response to drought (Atkin & Macherel 2009) makes possible that acclimation of  $R$  during summer was a composite effect of both heat and drought. In fact, our results suggest that water deficit enhanced thermal acclimation of  $R$  in *Q. ilex* (Fig 8b). Recent studies on different tree species point out to the same conclusion (Maseyk *et al.* 2008; Crous *et al.* 2010; Rodríguez-Calcerrada *et al.* 2010). Crous *et al.* (2010) wondered whether drought-induced increases in leaf temperature because of stomata closure, rather than water deficit *per se*, were behind the higher summer downward shift in daily temperature response curves of  $R$ . The similar values of leaf temperature in C and E trees during summer observed here suggest that the effect of drought on exacerbating thermal acclimation of  $R$  was not because of an indirect effect on increasing leaf temperatures.

The decrease in the foliar concentration of carbohydrates during summer is consistent with the strong limitation of photosynthesis caused by drought, and it could limit  $R$  (Fig. 5). However, there are two findings that are not consistent with the idea that summer drought impacts on  $R$  were mediated by a depletion of respiratory substrates. First, we observed that diurnal temperature response curves of  $R$  varied mostly in their intercept but not in their shape, as would have been the case if substrates had limited  $R$  (Atkin, Bruhn & Tjoelker 2005). Second, lower respiratory rates in E trees than in C trees in August, when predawn leaf water potential was 0.4 MPa lower in the former, did not correspond to any change in the concentration of soluble sugars. It is possible

**Table 1.** Stand-scale CO<sub>2</sub> release (mmol CO<sub>2</sub> m<sup>-2</sup>) estimated at the average night-time temperature of each date in control and throughfall exclusion treatments. Standard errors have been calculated using 300 Monte Carlo simulations

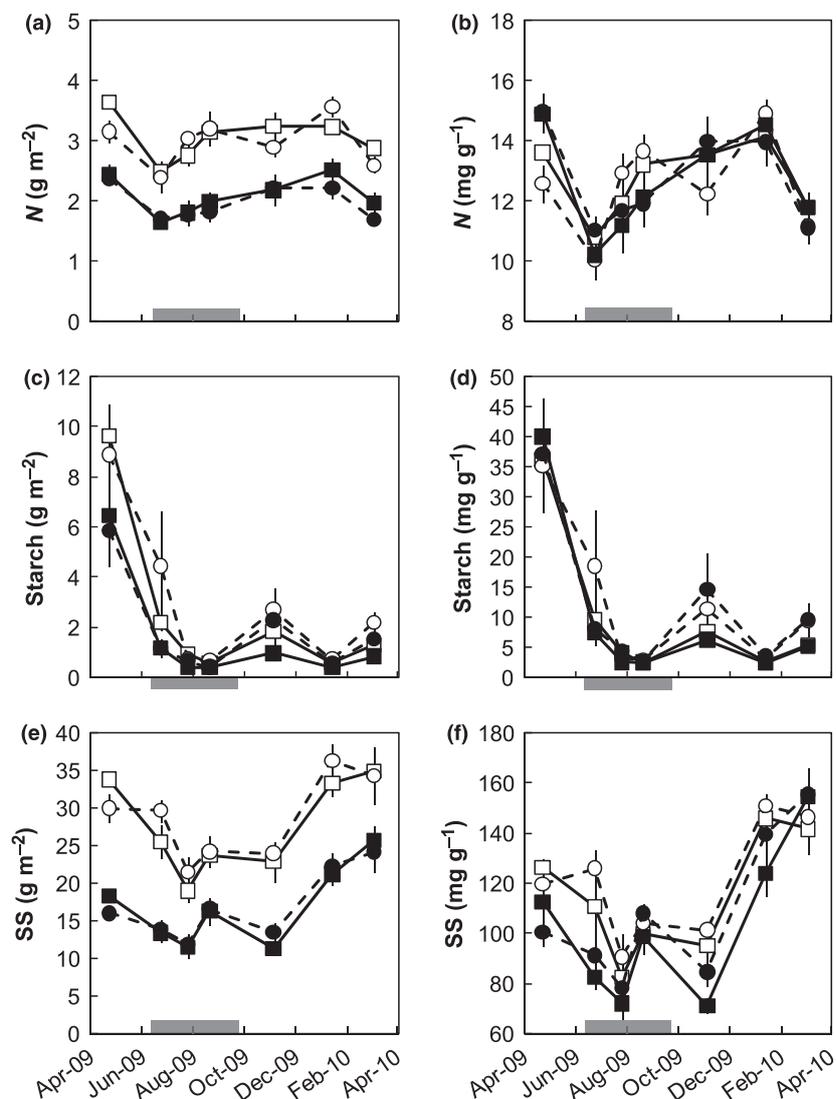
Treatment	22 April 2009	23 June 2009	25 July 2009	20 August 2009	4 November 2009	13 January 2010	4 March 2010
Temperature	16.0	13.7	15.9	20.0	8.7	3.6	2.6
Control	42.3 ± 10.6	19.5 ± 3.5	22.6 ± 4.2	21.4 ± 3.5	21.4 ± 4.1	15.2 ± 3.4	18.9 ± 4.5
Throughfall exclusion	30.2 ± 7.9	16.8 ± 4.6	17.8 ± 5.4	13.8 ± 2.3	17.1 ± 4.6	12.9 ± 2.3	12.6 ± 2.7

that diel patterns in the concentration of carbohydrates played a role in such inconsistencies. Alternatively, the homeostasis in  $R_{amb}$  over summer was because of a reduction in the overall respiratory capacity, which is a key factor controlling thermal acclimation of  $R$  in mature leaves (Tjoelker, Reich & Oleksyn 1999; Bruhn *et al.* 2007; Tjoelker *et al.* 2009; Rodríguez-Calcerrada *et al.* 2010). There is growing evidence that acclimation of  $R$  to heat and drought reflects some sort of metabolic downregulation that reduces carbon depletion and thus helps plants to grow and survive in Mediterranean-type environments (Maseyk *et al.* 2008; Crous

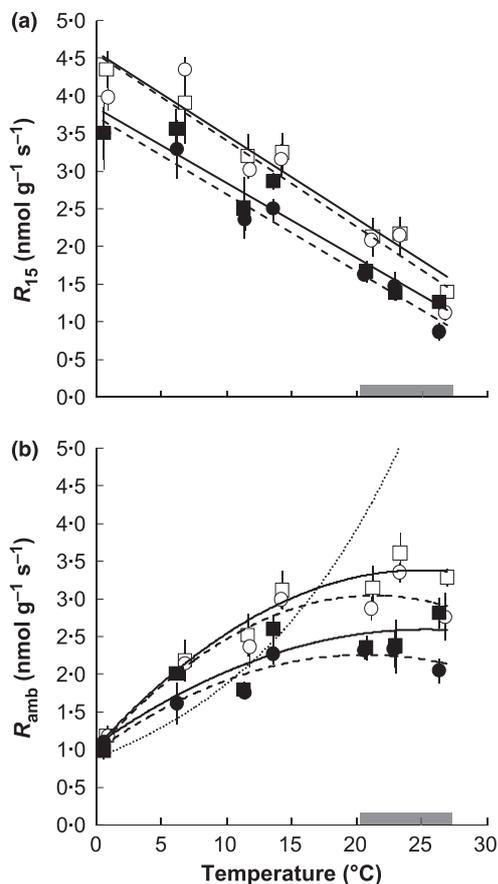
*et al.* 2010; Rodríguez-Calcerrada *et al.* 2010; this study); yet the exact physiological mechanisms through which this occurs remain unclear (Atkin & Macherel 2009; Sala, Piper & Hoch 2010).

#### DID THE LONG-TERM DECLINE IN THROUGHFALL AFFECT $R$ AND THE TEMPERATURE SENSITIVITY OF $R$ ?

E trees suffered higher water deficit than C trees during the six summers that preceded the year of study (Fig. 2). Thus, our results reflect the effect of a current-year summer drought



**Fig. 7.** Seasonal variation in (a–b) area- and mass-based foliar nitrogen content (N), (c–d) area- and mass-based foliar starch content and (e–f) area- and mass-based foliar soluble sugars content (SS). Horizontal grey bars indicate the summer drought period. Symbols as in Fig. 5. Data are means ± 1 SE.



**Fig. 8.** Response of (a) leaf dark respiration estimated at 15 °C ( $R_{15}$ ) and (b) leaf dark respiration estimated at the prevailing ambient temperature in each date (i.e. mean of the 7-day period preceding measurements;  $R_{amb}$ ) to seasonal changes in temperature (i.e. mean of the 7-day period preceding measurements). In (b), a dotted line represents the modelled response of  $R$  to temperature based on average values of  $R_0$  (0.89 nmol g<sup>-1</sup> s<sup>-1</sup>) and  $Q_{10}$  (2.1) observed across dates and treatments. Horizontal grey bars indicate the summer drought period. Symbols as in Fig. 5 and regression lines as in Fig. 6. Data are date means  $\pm$  1 SE. Equations of regression lines are:  $R_{15} = 4.58 - 0.11T$  ( $r^2 = 0.97$ ,  $P < 0.001$ ) and  $R_{amb} = 1.03 + 0.19T - 0.004T^2$  ( $r^2 = 0.96$ ,  $P = 0.001$ ) for upper canopy of C trees,  $R_{15} = 4.56 - 0.11T$  ( $r^2 = 0.89$ ,  $P = 0.001$ ) and  $R_{amb} = 0.99 + 0.19T - 0.004T^2$  ( $r^2 = 0.91$ ,  $P < 0.01$ ) for upper canopy of E trees,  $R_{15} = 3.85 - 0.10T$  ( $r^2 = 0.93$ ,  $P < 0.001$ ) and  $R_{amb} = 1.06 + 0.12T - 0.002T^2$  ( $r^2 = 0.80$ ,  $P < 0.05$ ) for lower canopy of C trees, and  $R_{15} = 3.73 - 0.10T$  ( $r^2 = 0.97$ ,  $P < 0.001$ ) and  $R_{amb} = 0.94 + 0.14T - 0.004T^2$  ( $r^2 = 0.91$ ,  $P < 0.01$ ) for lower canopy of E trees.

but also the potential carry-over effects of 6 years of increased drought. Is there a way to distinguish between the two time-scales? In any given year (e.g. the year of study), throughfall exclusion merely exacerbates the annual drop in water potential that takes place because of summer drought; thus, we argue that any change in the relationships between water potential and  $R$  between C and E trees would be indicative of a carry-over effect of increased drought (see Turnbull *et al.* 2001; Xu & Griffin 2006 for a similar interpretation). As the relationships between water potential and leaf traits with  $R$  did not change between C and E trees, we suggest that there was no evidence of long-term acclimation of  $R$  to enhanced

drought severity. The difference in  $R$  between both treatments was because of the extent to which water status differed between them along the season. In August, E trees had 0.4 MPa lower water potential than C trees, and rates of  $R$  were reduced by 25%. After the first autumn rains, tree water status recovered fully and similarly in C and E trees and so did  $A_{sat}$  and  $R$ .

The aforementioned results support our observation that acclimation to long-term drought occurs at the canopy level rather than at the leaf physiology level (Limousin *et al.* 2009, 2010). The clearest impact of 7 years of sustained drought accentuation is being an 18% decline in LAI. Thus, the difference in foliar respiration at the stand level between C and E plots was governed by changes in LAI and, less importantly, by a reduced rate of  $R$  at peak summer drought. Metcalfe *et al.* (2010) also found a decline in LAI (of 23%) in an Amazon rain forest after 5 years of throughfall reduction, but they reported increased rates of  $R$ . Future drought impacts on canopy CO<sub>2</sub> release will vary among ecosystems depending on species sensitivity to drought. *Q. ilex* is well adapted to drought and did not seem to suffer any metabolic damage that had exacerbated the demand of respiratory products as summer progressed, nor as the intensity of drought was experimentally augmented by throughfall exclusion (Fig. 5e,f). The tropical species studied by Metcalfe *et al.* (2010) are probably less adapted to drought than *Q. ilex* and, in addition, trees suffered a higher percentage of throughfall removal (50%) than ours' (33%). It is thus reasonable to think that trees examined by Metcalfe *et al.* (2010) experienced higher drought severity and need of respiratory products than *Q. ilex* trees in this study (Atkin & Macherel 2009).

The temperature sensitivity of  $R$  was not affected by throughfall reduction, which is consistent with the similar chemical composition of leaves of both C and E trees. To date, few studies addressing the potential long-term effects of drought on the temperature sensitivity of  $R$  have reported contrasting results. Turnbull *et al.* (2001) found a lower  $Q_{10}$  in *Quercus prinus* trees growing in drier sites (1.56) than in wetter sites (2.07), but Xu & Griffin (2006) found similar  $E_o$  values (52 kJ mol<sup>-1</sup> K<sup>-1</sup>;  $Q_{10} = 2.09$ ) in old *Quercus rubra* trees in nearby sites of contrasting soil water availability.

#### THE EFFECT OF CANOPY POSITION ON $R$ AND TEMPERATURE SENSITIVITY OF $R$

A better understanding of within-canopy variation in respiratory traits is important for improving process-based models of net ecosystem exchange. Here, we asked whether canopy position would affect  $R$  and its sensitivity to short- and long-term changes in temperature in *Q. ilex* trees with a fairly open crown.

We found that  $R$  was higher in the upper than in the lower canopy. The variation in LMA was an important driver of this change, because the difference between the two canopy positions in the area-based rates of  $R$  was higher than when  $R$  was expressed per unit dry mass. Light availability was 4.5 times higher in the upper canopy and was the abiotic factor

that mainly modulated leaf morphology, given that air temperature was almost invariable through the tree crown. Previously, LMA has been found to be the principal trait involved in light plasticity of photosynthetic (e.g. Evans & Poorter 2001) and respiratory (e.g. Tissue *et al.* 2002) rates, with thicker leaves having higher rates of CO<sub>2</sub> assimilation and efflux for a given area. Nonetheless, LMA was not the only trait involved in the variation of *R*. Mass-based rates of *R* were significantly higher in the upper canopy, which together with the similar concentration of foliar nitrogen, and the minor difference in soluble sugars between canopy positions points to a higher activity of respiratory enzymes in the upper canopy (Noguchi *et al.* 2005). The  $R_n/A_{\text{sat}}$  ratio was also higher in the upper canopy. Proportionally higher respiration than photosynthesis is probably a consequence of the higher, more stressful light intensity in the canopy top, which enhances maintenance energy demand, as in species growing at high irradiance sites (Wright *et al.* 2006).

The sensitivity of *R* to short-term changes in temperature has been found to vary within the tree canopy in some studies (Turnbull *et al.* 2003; O'Grady *et al.* 2010) but not in others (Bolstad, Mitchell & Vose 1999). We found that neither the  $Q_{10}$  nor the  $E_o$  of *R* varied with canopy position. This result is consistent with the similar concentration of soluble sugars in the upper and lower canopy, as the availability of respiratory substrates determines the effect of temperature on respiratory enzymes and thus on temperature sensitivity (Atkin, Zhang & Wiskich 2002). Over the annual range of temperatures, *R* showed a similar acclimatory response in the upper and lower canopy levels. As leaves were exposed to similar temperatures over the year and had similar chemical characteristics at both levels, they exhibited a similar degree of acclimation. Previously, Zha *et al.* (2002) observed that canopy position did not affect the response of *R* to experimentally elevated temperature in *Pinus sylvestris*. Similarly, Zaragoza-Castells *et al.* (2008) showed that irradiance did not affect the degree of thermal acclimation in *Q. ilex* saplings growing in contrasting microsites.

#### CONCLUDING STATEMENTS

In the context of modelling, these and previous results suggest that while it is important to account for different respiratory rates among different canopy levels when upscaling from leaf to tree and ecosystem levels in *Q. ilex*-dominated forests, a single temperature response of *R* through the canopy can be assumed (Zaragoza-Castells *et al.* 2007, 2008). A better characterization of the effect of drought on seasonal shifts of respiratory parameters is needed if we are to model temperature and water deficit effects on *R* and net ecosystem exchange. While it is important to consider that *Q. ilex* trees may acclimate to an increase in drought severity by reducing LAI, our results suggest that it is not necessary to include a long-term physiological acclimation factor when predicting future response of foliage *R* to climate change, as the relationship between leaf water potential and chemical traits with spe-

cific rates of *R* did not change with previous history of drought severity.

Because *R* decreased with increasing water deficit, and the relationship between water status and *R* did not change after 7 years of enhanced water deficit, it can be suggested that drier conditions projected for the Mediterranean by climate models (Giorgi 2006) may attenuate the responsiveness of leaf respiratory CO<sub>2</sub> release to global warming in *Q. ilex* forests. This conclusion is consistent with the reduction of ecosystem respiration observed over Europe during the exceptionally dry and hot summer of 2003 (Ciais *et al.* 2005).

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Figure S1.** Relationships between air and leaf temperatures.

**Figure S2.** Estimation of respiratory traits from a modified Arrhenius equation.

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