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Temporal and spatial patterns of internal and external stem CO_2 fluxes in a sub-Mediterranean oak

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Key words

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INTERNAL AND EXTERNAL STEM CO_2 FLUXES

Summary

To accurately estimate stem respiration (R_S), measurements of both CO_2 efflux to the atmosphere (E_A) and internal CO_2 flux through xylem (F_T) are needed because xylem sap transports respired CO_2 upward. However, studies of seasonal dynamics of F_T and E_A are scarce and are non-existent in Mediterranean species under drought stress conditions. Internal and external CO_2 fluxes at three stem heights, together with stem growth, temperature, sap flow, and water potential were measured in *Quercus pyrenaica* Willd. in four measurement campaigns during one growing season. Low internal [CO_2] (<0.5%) resulted in low contributions of F_T to R_S throughout the growing season. R_S was mainly explained by E_A (>90%), which varied seasonally, mirroring trends in sap flow and stem growth. Nevertheless, at midday, when sap flow was high, F_T accounted for up to 25% of R_S. Internal [CO_2] was also found to vary vertically along the stems and in relation to root biomass among stems. Seasonality in xylem [CO_2] and resistance to radial CO_2 diffusion was related to drought stress. Enhanced radial diffusion of CO_2 through cambium, phloem, and bark tissues due to water deficit, together with down-regulation of xylem respiration ascribed to historical coppicing in Mediterranean stands, may underpin low internal [CO_2] and F_T. Long term studies analyzing temporal and spatial variation in internal and external CO_2 fluxes and their interactions are needed to comprehensively understand and model respiration of woody tissues.

Introduction

Ecosystem respiration consumes 30-70% of carbon assimilated by photosynthesis (Waring et al. 1998, Amthor 2000, Acosta et al. 2008, Ryan et al. 2009). Among terrestrial ecosystems, CO_2 released by respiration of forests is a large component of the global carbon budget, yet knowledge of respiratory processes, particularly woody tissue respiration, is limited, especially in comparison to our understanding of photosynthesis (Ryan et al. 2009, Guidolotti et al. 2013, Rambal et al. 2014). Stem respiration (R_S) has been estimated to contribute 5 to 40% of total forest ecosystem respiration, depending on forest type and season (Rodríguez-Calcerrada et al. 2014). Yet the abiotic and biotic factors controlling R_S and contributing to its temporal and spatial variability remain unclear.

To study variability in R_S, stem CO_2 efflux to the atmosphere (E_A) has been commonly assumed to equal R_S (Stockfors and Linder 1998, Damesin et al. 2002, Lavigne et al. 2004, Acosta et al. 2008, Brito et al. 2010, Yang et al. 2012, Tarvainen et al. 2014, Rodríguez-Calcerrada et al. 2015). However, in the last decade it has been demonstrated that internal fluxes of CO_2 in stems play an important role in estimating woody tissue respiration, since a portion of the CO_2 released by respiration dissolves in sap and is transported upward in the xylem stream. Measurements of E_A have generally underestimated respiration (Teskey et al. 2008, Hölttä and Kolari 2009, Angert et al. 2012, Trumbore et al. 2013, Bloemen et al. 2015), although CO_2 movement from the roots upward can also result in overestimation (Zach et al. 2010, Bužková et al. 2015). Using a mass balance approach, McGuire and Teskey (2004) revealed significant mis-estimation of R_S by measurements of E_A and the absence of a consistent relationship between R_S and E_A (Teskey and McGuire 2007). Briefly, the mass balance approach considers the sum of three respiration components in a stem segment to estimate total respiration on a volume basis. R_S components are: (i) E_A; (ii) internal flux of dissolved CO_2 through the xylem (F_T); and (iii) the change in CO_2 concentration ($[CO_2]$) (i.e. CO_2 stored in the stem segment) across two consecutive measurements (ΔS). The mass balance approach has demonstrated that relative contributions of F_T to total R_S can be highly variable, ranging from 3% to 55% for poplar and sycamore trees, respectively (McGuire and Teskey 2004, Saveyn et al. 2008). From measurements of the ratio of CO_2 efflux to O_2 influx, Angert *et al.* (2012) estimated that 35% of locally respired CO_2 was transported upward. The relevance of F_T to forest carbon budgets was noted by

Aubrey and Teskey (2009), who reported that the amount of CO_2 transported from roots into the stem was of the same magnitude as soil CO_2 efflux in a poplar plantation. On the contrary, internal CO_2 transport had no apparent effect on CO_2 efflux in large conifer trees (Ubierna et al. 2009), or young pine trees during springtime (Maier and Clinton 2006). It is becoming apparent that internal [CO_2] measurements are necessary to better integrate physiological processes at the cellular level with CO_2 fluxes from stem surfaces (Trumbore et al. 2013), and to more accurately estimate R_S (McGuire and Teskey 2004).

Previous research on R_S has revealed interesting interactions among F_T, E_A and internal [CO_2]. For instance, increased F_T during daytime is associated with lower E_A. Daytime depressions in E_A compared to nighttime values at the same temperature have been attributed to transport of locally respired CO_2 (Bowman et al. 2005, Gansert and Burgdorf 2005, Hölttä and Kolari 2009), even though direct assessments of upward transport of CO_2 are typically missing in these studies. Moreover, [CO_2] has been shown to be directly related to E_A (Teskey and McGuire 2002, 2007, Cerasoli et al. 2009), with the slope of this relationship determining the resistance to radial diffusion of CO_2 (Steppe et al. 2007, Teskey et al. 2008). Resistance to radial CO_2 diffusion is dependent on stem water status, tissue properties, and temperature (Nobel 1999). Diffusion resistance influences the quantity of internal CO_2 that diffuses to the atmosphere through cambium, phloem and bark tissues, and it could help explain the substantial variation in CO_2 efflux observed among and within tress and across stands (Steppe et al. 2007).

Spatial and temporal changes in physical and biological factors underpin changes in R_S and its components. For instance, vertical position on the stem is a significant factor affecting CO_2 concentration and fluxes. A positive relationship between E_A and height has been observed, presumably due to higher growth rates in the upper stem and tree crown (Damesin et al. 2002, Araki et al. 2010, Tarvainen et al. 2014). In parallel, a slightly increasing vertical gradient in [CO_2] was also observed from stem base to 3 m height (Teskey and McGuire 2007), and Hölttä and Kolari (2009) modeled higher ratios of CO_2 efflux to CO_2 production in the upper stem as CO_2 is transported upward. Internal [CO_2] and CO_2 fluxes, especially at the stem base, could also be affected by the influence of the extent and structure of the supporting root system on internal CO_2

107 transport from below ground (Teskey and McGuire 2007), which has never been
108 examined in natural conditions.

109 E_A varies seasonally, driven by abiotic factors such as temperature and water
110 availability, and biotic processes such as photosynthesis, phloem transport, and cambial
111 activity. Previous work based on E_A measurements has suggested that summer drought
112 reduces respiration (Maseyk et al. 2008, Brito et al. 2010, Rodríguez-Calcerrada et al.
113 2014). [CO_2] and F_T might also vary seasonally, however, data on E_A, and more so
114 internal [CO_2] and F_T, are scarce in drought-prone regions (but see Cerasoli *et al.*,
115 2009). Concomitant studies of E_A and [CO_2] have rarely lasted more than two weeks
116 (see review by Teskey *et al.*, 2008). To date, the only published data in which both
117 internal and external CO_2 fluxes have been simultaneously monitored in a long term
118 experiment consists of measurements on two Norway spruce trees instrumented for 19
119 months (Etzold et al. 2013). This study revealed a close link between internal [CO_2] and
120 both temperature and cambial activity.

121 Here, we aim to assess the main drivers of diel, seasonal, and vertical variability of E_A,
122 internal [CO_2], R_S, and their interactions in a sub-Mediterranean tree species (*Quercus*
123 *pyrenaica* Willd.) traditionally coppiced and currently abandoned. Variables E_A, F_T, and
124 internal [CO_2] were examined at three different stem heights during the 2013 growing
125 season on four dates with contrasting sap flow rates, drought stress, and growth rates.
126 First, we examined variation in E_A across the growing season, and the effect of sap flow
127 and F_T on the widely reported daytime depression in E_A. We predicted that annual peak
128 E_A would occur at the beginning of the growing season, when water availability and
129 early-wood formation are highest, and that E_A would decrease over the course of the
130 growing season in response to drought and reduced growth. We also predicted
131 increasing daytime depression in E_A with increasing sap flow rate driven by the dilution
132 of [CO_2^*] in the stem with water coming from the soil at relatively low [CO_2^*], or with
133 drought severity as water limitation causes loss in cell turgor reducing respiration rates.
134 Second, we examined seasonal variation in internal [CO_2] and how its relationship with
135 E_A (i.e. the resistance to radial CO_2 diffusion) changes as drought stress increases. We
136 expected that [CO_2] would follow a similar pattern to E_A in response to cambial activity,
137 and that resistance to radial CO_2 diffusion would decline as water deficit increased.
138 Third, we calculated the contribution of F_T to R_S across the growing season and at
139 different stem heights. Using the mass balance approach proposed by McGuire and

Teskey (2004), we hypothesized that the contribution of F_T to R_S would be greater in the middle of the growing season, coinciding with high sap flow rates, as well as at higher stem positions, due to the buildup of CO_2 along the stem. Fourth, we examined the relationship between internal [CO_2] and root properties (living biomass and root connections), with the expectation of higher [CO_2] in stems supported by larger root systems.

Materials and Methods

Experimental plot and set-up

The study was performed in a pure stand of *Q. pyrenaica* at the core of the species distribution in the Central Mountain Range of the Iberian Peninsula. *Q. pyrenaica* is a marcescent oak found in siliceous sub-Mediterranean mountains. Due to its high root-resprouting capability, the species has been historically coppiced, leading to anthropogenic ecosystems which physiognomy, functionality and ecology have been altered (Valbuena-Carabaña and Gil 2013). The study plot was located in the buffer zone of the national Park of "Sierra de Guadarrama", in the "Monte Matas de Valsaín", at an altitude of 1140 m.a.s.l. Average annual rainfall and temperature were 885 mm and 10ºC, respectively. Soil type is humic cambisol. The stand has been traditionally coppiced but was abandoned after 1970, when the last cutting was performed. Stem density was 781 stems ha^{-1}, with most of the stems ranging from 15 to 25 cm diameter at breast height.

Eight stems were instrumented to measure internal and external CO_2 fluxes at their base. All stems were part to the same clone. Only one clone was used because we planned to excavate the root system of the entire multi-stemmed clone to link stem [CO_2] with root properties. For detailed methodology on clonal assignment see Valbuena-Carabaña et al. (2008). Diameter at breast height (dbh) of the eight stems ranged from 12.4 to 23.9 cm, and total height from 10.5 to 13.8 m. Four measurement campaigns were made during the growing season of 2013. On each measurement date we completed 24 hours of measurements, starting and finishing at approximately 12:00 h. The first measurement date, (D1), was on DOY 143-144 (end of May), during bud-burst, when sap flow was negligible. The second and third measurement dates (D2 and D3) were on DOYs 183-184 (beginning of July) and 218-219 (beginning of August), respectively, with warmer temperatures and higher sap flow rates. Finally, D4 was on

DOY 266-267 (end of September) when transpiration was reduced, stem growth had ceased, and drought stress, indicated by shoot water potential, was maximum (Figure 1).

Stem harvest and root excavation started on DOY 280. Stems were felled to quantify sapwood volume per stem segment to calculate R_S by the mass balance approach (see below). The root system was hydraulically excavated with a high-pressure water pump down to 1m depth. Root connections were then quantified and categorized as parent roots, from which stems sprout, and root grafts, formed after stem sprouting (DesRochers and Lieffers 2001). The cross section of each root connection (cm^2) was measured at the midpoint between connected stems (in the case of parental roots), or estimated from two perpendicular diameters at each graft side (considering the smallest root within the graft). After these measurements were complete, the root system was lifted with a backhoe, coarse and taproots were separated and all roots were weighed on a balance (BMM-BR80, Baxtran, Spain) *in situ* to the nearest 20 g.

Climatic conditions, diameter increment, and shoot water potential

Air temperature (T_{air}, ºC) and precipitation were monitored during the study every 30 minutes by a weather station located 2.1 km away from the plot. Diameter increment of every stem was measured by dendrometer bands (DS20, GIS Iberica, Spain) placed at 1.3 m height and inspected ca. every two weeks. As a surrogate for tree water status, shoot water potential was measured on every measurement date with a pressure chamber (PMS Instrument Co., OR, USA) at predawn (between 04:00 and 06:00 h) and midday (between 13:00 and 15:00 h).

CO_2 efflux to the atmosphere (E_A)

CO_2 efflux to the atmosphere was measured every six hours at the base (20 cm above ground level) of each stem (at 12:00, 18:00, 00:00 and 06:00 h). Additionally, two stems were intensively measured (18 times per day) at the base and two additional heights (1.5 m and 3 m). These two stems (S1 and S2) had dbh of 22.9 and 21.0 cm, respectively. E_A was measured with a portable infrared gas analyzer (LI-6400, Li-Cor Inc., Lincoln, NE, USA) and a soil chamber (LI-6400-09) as described in Xu *et al.*, (2000). Measurements were made at the same stem location each time by fixing permanent PVC collars to the stem. Loose bark was removed and the PVC collars were sanded to better fit stem curvature. Silicone sealant and liquid rubber (Plasti-Dip, TRP, Spain) were applied to

prevent leaks. Measurements were made by reducing CO_2 concentration inside the chamber and then letting it increase to an upper concentration limit (i.e., gas analyzer operated in closed system configuration). These limits changed depending on the respiration rate of the measured stem and the atmospheric [CO_2] concentration, which averaged 370 ppm during the day and 420 ppm at night. The rate of change of [CO_2] inside the chamber was calculated at ambient [CO_2] and humidity in three consecutive measurement cycles. E_A values were corrected to account for stem collar volume, which was measured at the end of the experiment by sealing the open end of the collar and filling it with water through a hole in the top from a graduated cylinder. A correction was applied to account for stem curvature using the equations developed by Xu *et al.* (2000), so that E_A was calculated on stem surface area basis ($E_{A(S)}$ [μmol m^{-2} s^{-1}]).

Stem temperature (T_{stem}, ºC) was continuously measured with type-T thermocouples (copper-constantan) inserted at 2 cm depth and 5 cm away from each stem collar. Temperature sensitivity of E_A (Q_{10}) was assessed by the equations:

$$\ln (E_A) = a + b\, T_{stem} \quad (1)$$

$$Q_{10} = e^{(b10)} \quad (2)$$

For comparison among measurement campaigns, $E_{A(S)}$ was standardized to 15ºC ($E_{A(S)15}$) – a temperature registered in all four campaigns and widely reported in literature – using Q_{10} values obtained by pooling nighttime and daytime data.

To assess daytime depressions in efflux, residuals ($E_{A(S)\_RES}$) were calculated in the intensively measured stems (S1 and S2) as the difference between $E_{A(S)}$ predicted from the nighttime relationship between T_{stem} and $E_{A(S)}$ (from 22:00 to 06:00 h) and measured daytime $E_{A(S)}$.

Internal [CO_2] and the mass balance approach

Internal [CO_2] was measured at the base (10 cm above ground level) of the stems. Additionally, in the intensively measured stems (S1 and S2), internal [CO_2] was measured 5 cm above and below the stem collars (at 1.5 and 3 m height) to calculate respiration by mass balance as proposed by McGuire and Teskey (2004). Internal [CO_2] was measured with solid state non-dispersive infrared (NDIR) CO_2 sensors (model GMM221, Vaisala, Finland) inserted into the stem. Holes of 40 mm length and 25 mm

width were drilled to place the sensor. NDIR sensors were isolated from the external atmosphere with rubber sealant. To minimize temperature effects associated with stem position stem collars, NDIR sensors and thermocouples were all oriented facing north to minimize effects of solar radiation (Stockfors and Linder 1998, Acosta et al. 2008).

NDIR sensors measure internal [CO_2] in gaseous phase (%) in equilibrium with CO_2 dissolved in sap solution ([CO_2^*], mmol L^{-1}). Sap [CO_2^*] is estimated by Henry's law from gas phase [CO_2], sap temperature, and sap pH. Sap pH was measured at midday, at maximum transpiration rates, using a portable pH meter (model 25+, Crison, Barcelona) and a pH electrode (model 52 07, Crison, Barcelona) on sap expressed from twigs of each stem. To measure sap flux density (L cm^{-2} h^{-1}), two thermal dissipation probes (Granier 1985) were inserted to a depth of 2 cm into the sapwood on opposite sides of each stem (west and east oriented) at 1 m height. The paired needles of each probe were separated 10 cm vertically. Daily zero flow was calculated for each probe from the temporal mean of the temperature difference of the thermocouple pair between 04:00 and 06:00 h. Data from the two probes per stem were averaged to account for circumferential variability in sap flux. Calibration parameters developed by Sun *et al.* (2011) were applied to sap flux density to improve accuracy according to the ring porous xylem anatomy of *Q. pyrenaica* stems. Sap flux density was multiplied by sapwood area to determine sap flow (F_{H2O}, L h^{-1}). Stem temperature, sap flux density, and internal [CO_2] were measured every minute and averaged every 15 minutes with a data logger (model CR23X, Campbell Scientific, Spain).

The mass balance approach was applied to calculate the relative contributions of E_A, F_T, and ΔS to R_S following equations in McGuire and Teskey (2004):

$$R_S = E_A + F_T + \Delta S \quad (3)$$

$$F_T = (F_{H2O}/V)\ \Delta[CO_2^*] \quad (4)$$

$$\Delta S = ([CO_2^*]_{T1} - [CO_2^*]_{T0})\ L/T \quad (5)$$

where R_S, E_A, F_T and ΔS are the stem respiration components on a volume basis (μmol CO_2 m^{-3} s^{-1}), F_{H2O} is sap flux (L s^{-1}), V is sapwood volume of stem segment (m^3), $\Delta[CO_2^*]$ is the difference in sap [CO_2^*] above and below the PVC collar, $[CO_2^*]_{Ti}$ is the mean of sap [CO_2^*] above and below the collar at *i*-th time period, L is the stem water content (L m^{-3}), and T is the elapsed time (s) between consecutive measurements.

E_A was initially estimated on a stem surface area basis ($E_{A(S)}$). To express E_A on a volume basis ($E_{A(V)}$), we assumed uniform efflux around the stem circumference and used this equation:

$$E_{A(V)} = E_{A(S)} \frac{S}{V} = E_{A(S)} \frac{2\pi r_{h+s} height}{\pi({r_{h+s}}^2 - {r_h}^2)\, height} = E_{A(S)} \frac{2r_{h+s}}{({r_{h+s}}^2 - {r_h}^2)} \quad (6)$$

where S and V are the axial surface area and volume of sapwood of the stem segment, respectively; r_h and r_{h+s} denote the radius of heartwood and heartwood plus sapwood, respectively; and height is the vertical length of the stem segment. The mass balance approach, on a sapwood volume basis, was applied on the four dates and at two heights (1.5 and 3 m) of the intensively measured stems (S1 and S2). However, in one stem segment (S2 at 3 meters) a NDIR probe failed on two sampling dates and these values were removed from analyses.

Data analysis

To study the influence of T_{stem} on diel E_A and internal [CO_2], linear regressions were performed (Eq. 1). Seasonal differences in E_A and internal [CO_2] at the stem base were assessed with hierarchical mixed models performed in R using the *lme* function in the nlme library (Pinheiro et al. 2015) considering measurement date as a fixed factor (n=4) and stem as a random factor (n=8). To evaluate the influence of stem height on E_A and [CO_2], mixed models were performed considering date (n=4) and height (n=3) as fixed factors and stem (n=2, intensively measured stems S1 and S2) as a random factor. Backward selection was used to determine the most appropriate model until all variables included in the model were significant ($P < 0.05$). Multiple comparisons of means were performed using *glht* function in the multcomp library (Hothorn et al. 2008).

To evaluate the influence of sap flow on daytime depression of efflux, mixed models were performed. At a diel scale (dates separately examined), $E_{A(S)\_RES}$ (µmol CO_2 m^{-2} s^{-1}) was regressed against F_{H2O} (L h^{-1}) which was treated as a fixed factor, while stem segment nested within its corresponding stem (n = 2) was treated as a random factor. To test differences in slopes between F_{H2O} and $E_{A(S)\_RES}$ among dates, ANCOVA was performed. At a seasonal scale, and to further distinguish between water status and internal CO_2 transport as potential drivers of daytime depression in efflux (Saveyn et al. 2007a), daily accumulated $E_{A(S)\_RES}$ (mmol CO_2 m^{-2} day^{-1}) was regressed against

accumulated F_{H20} (L day^{-1}) and predawn and midday shoot water potential (MPa) considering the four measurement dates. Additionally, to estimate the portion of efflux depression attributed to the internal transport of CO_2, the ratio between F_T and $E_{A(V)\_RES}$ (on a volume basis, mmol CO_2 m^{-3} day^{-1}) was calculated for the four dates. According to Fick's first law, the resistance to radial CO_2 diffusion (s m^{-1}) was calculated by the ratio $\Delta[CO_2]/E_{A(S)}$, where $\Delta[CO_2]$ is the difference in $[CO_2]$ (µmol m^{-3}) between the xylem and the atmosphere (Steppe et al. 2007, Teskey et al. 2008). Resistance to radial CO_2 diffusion across dates was assessed with mixed models and differences among dates were tested by ANCOVA. To evaluate the effect of root size and root connections on internal $[CO_2]$ at the stem base, average $[CO_2]$ per stem was linearly regressed against root variables. All values presented in the text are means ± (SE).

Results

Climatic conditions, water potential, and stem growth

Over the growing season (from DOY 100 to 304), average T_{air} was 15.7 ºC (daily means ranged from 0.6 to 26.6 ºC) and total precipitation was 214 mm, 60% of which occurred before or during budburst (from DOY 140 to150, Figure 1a). Summer was hot and dry: from DOY 160 to 270, mean T_{air} was 19.7 ºC (daily means ranged from 8.7 to 26.6 ºC) and total precipitation was 37.6 mm. Mean predawn and midday shoot water potential at the end of the growing season (DOY 267) reached minimum values of -1.4 and -2.7 MPa, respectively. Stem growth started by DOY 110 and finished by DOY 260. Mean accumulated diameter growth was 0.8 mm, of which 25% took place before and during budburst (Figure 1b).

Stem CO_2 efflux (E_A) and daytime depression in E_A

$E_{A(S)}$ was strongly related to T_{stem}. On a diel basis, T_{stem} explained 82-97% of the variability in $E_{A(S)}$ during the night (Table 1). The Q_{10} values were 1.85, 1.60, 1.76 and 1.99 for the four sampling periods, D1 to D4. Pooling nighttime and daytime data reduced the number of significant relationships between T_{stem} and $E_{A(S)}$, as well as the variability in $E_{A(S)}$ explained by T_{stem} (33-64%); the Q_{10} values decreased to 1.33, 1.30, 1.30 and 1.39 for the four sample periods, respectively.

Stem CO_2 efflux was standardized to 15°C ($E_{A(S)15}$) to examine the effect of season on $E_{A(S)}$. For the four sampling periods $E_{A(S)15}$ varied significantly from 1.28 (0.14), 2.00

(0.27), 2.39 (0.36) and 1.65 (0.28) µmol CO_2 m^{-2} s^{-1}, for D1, D2, D3 and D4, respectively (n = 8, $P < 0.001$), and every contrast examined among dates was significant ($P < 0.05$). $E_{A(S)}$ at actual mean temperature on each date showed greater differences: 1.09 (0.13), 2.18 (0.28), 2.76 (0.37) and 1.86 (0.27) µmol CO_2 m^{-2} s^{-1}, measured at T_{stem} of 11.1 (0.2), 17.6 (0.2), 18.4 (0.1) and 18.9 (0.1) ^oC, on the four dates, respectively. In the stems measured at three heights (S1 and S2), $E_{A(S)15}$ was marginally influenced by stem height ($P = 0.085$). Stem efflux ($E_{A(S)15}$) was similar at the base of the stem and at 1.5 m height ($P = 0.744$), but at 3 m it was significantly lower than at 1.5 m ($P = 0.027$) and tended to be lower than at the stem base ($P = 0.059$).

Hysteresis between diel T_{stem} and $E_{A(S)}$ was observed on all measurement dates, stems, and heights; i.e., for a given temperature $E_{A(S)}$ was higher at nighttime than at daytime (Figure 2). At a diel scale (dates separately examined), hysteresis ($E_{A(S)\_RES}$, i.e. predicted $E_{A(S)}$ - measured $E_{A(S)}$, µmol CO_2 m^{-2} s^{-1}) was related to F_{H20} from D2 to D4, which was the period in which transpiration occurred ($P < 0.001$, Figure 3). ANCOVA showed that the slopes of these relationships were similar on D2 and D3 ($P = 0.378$), whereas slopes on D2 and D3 were different than the slope on D4 ($P < 0.001$). Interestingly, on D1, during budburst, hysteresis occurred in the absence of sap flow, and $E_{A(S)\_RES}$ was as large as on the dates with fully developed leaves (Figure 3). Therefore, at a seasonal scale, daily accumulated $E_{A(S)\_RES}$ (µmol CO_2 m^{-2} day^{-1}) per measurement date was not influenced by daily accumulated sap flux (L m^{-2} day^{-1}) ($P = 0.525$) but tended to increase with decreasing shoot water potential at predawn ($P = 0.066$) and midday ($P = 0.054$). Considering daily accumulated F_T (calculated by the mass balance approach) and $E_{A(V)\_RES}$ (both on a sapwood volume basis, mmol CO_2 m^{-3} day^{-1}), the mean ratio F_T/ $E_{A(V)\_RES}$ was -0.01 (0.01), 0.70 (0.23), 0.80 (0.26) and 0.49 (0.28) for dates D1 to D4, indicating that hysteresis in E_A was not fully explained by F_T, and not at all on D1 when transpiration was negligible.

Internal [CO_2] and resistance to radial CO_2 diffusion

Relationships between internal [CO_2] and T_{stem} at the base of the stem were predominantly positive at night throughout the growing season, but pooling nighttime and daytime data substantially reduced the number of significant positive relationships between internal [CO_2] and T_{stem} (Table 1). The shifting pattern of this relationship

prevented us from estimating [CO_2] at a reference temperature as we had done for E_A using $E_{A(S)15}$. Internal [CO_2] decreased over the growing season from 0.26 (0.06), 0.14 (0.03), 0.13 (0.02) to 0.11 (0.02)%, on D1, D2, D3, and D4, respectively ($P < 0.001$).

The ratio between Δ[CO_2] and $E_{A(S)}$, i.e. the resistance to radial CO_2 diffusion, changed across the growing season (Figure 5). On D1, this resistance was the highest (1.08×10^5 (3.63×10^3) s m^{-1}), and a strong positive relationship between Δ[CO_2] and $E_{A(S)}$ was found (average $R^2 = 0.96$, $P < 0.001$). On the remaining measurement dates, the relationships between Δ[CO_2] and $E_{A(S)}$ were weaker: average R^2 values were 0.72 , 0.19 and 0.66 for D2, D3 and D4 respectively. From D2 to D4, resistance to radial CO_2 diffusion was lower, decreasing by up to 1/4 compared to D1: 3.68×10^4 (2.07×10^3), 2.61×10^4 (6.98×10^3) and 3.85×10^4 (3.12×10^3) s m^{-1}, respectively ($P < 0.001$) (Figure 5). The contrasts comparing slopes (i.e., resistance to radial CO_2 diffusion) by ANCOVA were significant between D1 and all other dates ($P < 0.001$), but not among D2, D3, and D4 ($P > 0.1$). Resistance to diffusion tended to decrease with decreasing midday shoot water potential ($R^2 = 0.893$, $P = 0.055$, n = 4) but not with predawn shoot water potential ($P = 0.924$, n = 4), suggesting that the resistance to diffusion was influenced by midday water status.

Among the eight stems, the seasonal mean of internal [CO_2] at the base of the stem was positively related to taproot biomass ($P = 0.049$; $R^2 = 0.50$), and the accumulated cross section of its root connections ($P = 0.027$; $R^2 = 0.58$). In the stems measured at three heights (S1 and S2), internal [CO_2] was influenced by date, height, and their interaction ($P < 0.001$), varying from 0.52% (at 3 m height on D1) to 0.12% (at the stem base on D2; Figure 4). Pooling dates, internal [CO_2] increased from 0.17 (0.02)% at stem base, to 0.26 (0.02)% at 1.5 m, and finally 0.40 (0.02)% at 3 m height ($P < 0.001$).

Components of stem respiration by a mass balance approach

Daily values of R_S and its components ($E_{A(V)}$, F_T, and ΔS), as well as their proportional contributions to R_S, varied with date, stem, and stem height (Table 2; Figure 6). The lowest R_S, 4.69 (0.37) mol m^{-3} day^{-1}, was found on D1; and Rs increased to 13.68 (0.86), 13.94 (0.63) and 13.75 (2.30) mol m^{-3} day^{-1} on consecutive subsequent dates. The contribution of ΔS to R_S was always negligible, with daily contributions ranging from -0.4 to 0.1%. However, a linear model testing the contribution of F_T to R_S by date and height showed that F_T contributions changed significantly across the growing

season (P = 0.046) and also changed marginally with stem height (P = 0.071). The average contribution of F_T to R_S was nil at D1 due to negligible transpiration and increased to 5 - 6.5% on the remaining dates (P = 0.046), among which no significant differences were found (P > 0.1). The contribution of F_T to R_S tended to be 3.3% larger at 3 m compared to 1.5 m height (P = 0.071). $E_{A(V)}$ was the main component of daily R_S, with contributions above 90% in most cases (Table 2). At a diel scale, the contribution of $E_{A(V)}$ to R_S was close to 100% at night (Figure 6). In the daytime, the contribution of $E_{A(V)}$ to R_S declined (in some cases to 75%) at high sap flow rates, when the contribution of F_T to R_S reached maximum values close to 25% (Figure 6a).

Discussion

Stem CO_2 efflux (E_A) and daytime depression in E_A across the season

Stem CO_2 efflux varied from 1.28 to 2.39 μmol CO_2 m^{-2} s^{-1} at a reference temperature of 15ºC, which is within the range of values reported for different *Quercus* species (Edwards and Hanson 1996, Saveyn et al. 2007a, 2007b, Yang et al. 2012, Rodríguez-Calcerrada et al. 2014, 2015). In agreement with data from Barbaroux and Bréda (2002), Corcuera *et al.* (2006) and Courty *et al.* (2007), accumulated stem diameter increment before and during budburst accounted for one fourth of total annual stem diameter increment. Thus, we expected high $E_{A(S)15}$ at the beginning of the growing season, driven by the absence of drought stress and high rates of wood production (Lavigne et al. 2004). However, $E_{A(S)15}$ was lowest on D1. Negligible respiration of phloem tissues (Amthor 2000) and no supply of new assimilates (Wertin and Teskey 2008, Maier et al. 2010) may result in low $E_{A(S)15}$ before leaf expansion. $E_{A(S)15}$ showed maximum values in mid-summer (D2 and D3), coinciding with maximum transpiration and high stem growth (e.g. Damesin *et al.*, 2002; Acosta *et al.*, 2008; Yang et al. 2012; Guidolotti *et al.*, 2013; Tarvainen *et al.*, 2014). The decrease in $E_{A(S)15}$ at the end of the growing season (D4) could be explained by stem growth cessation and reduced water availability (Figure 1b). Yet, contrary to other studies in drier Mediterranean regions (Maseyk et al. 2008, Brito et al. 2010, Rodríguez-Calcerrada et al. 2014), mild drought stress (with minimum predawn shoot water potential of -1.4 MPa) did not result in large reductions of $E_{A(S)15}$ in our study.

We hypothesized that E_A would increase with height due to increased metabolic activity (Damesin et al. 2002, Araki et al. 2010, Tarvainen et al. 2014) and internal upward

movement of CO_2 (Hölttä and Kolari 2009). However, stem height barely affected $E_{A(S)15}$. The short vertical gradient (3 m) and the fact that the highest sampling point was below the tree crown may have contributed to the limited spatial variability observed in $E_{A(S)15}$.

Sap flow was a reliable predictor of depression in daytime CO_2 efflux (Bowman et al. 2005, Gansert and Burgdorf 2005, Hölttä and Kolari 2009), but only after leaf expansion (from D2 to D4, Figure 3). The ratio between F_T and $E_{A(V)\_RES}$ ranging from 0.49 to 0.80 from D2 to D4 suggests that 50% or more of the hysteresis in $E_{A(V)}$ could be attributed to the internal transport of CO_2 after leaf expansion. However, measurements on D1 during budburst, when hysteresis in $E_{A(S)}$ could not be ascribed to transpiration, showed $E_{A(S)\_RES}$ as large as after leaf expansion (Figure 3), suggesting that transpiration is not the only process determining daytime depression in $E_{A(S)}$ (Saveyn et al. 2007a). Similarly, Etzold *et al.* (2013) found that relationships between depressions in $E_{A(S)}$ and sap flow in Norway spruce trees were significant only during the growing period. The inverse relationship ($P < 0.1$) between daily accumulated $E_{A(S)\_RES}$ and shoot water potential observed in this study reinforces the suggestion of Saveyn *et al.*, (2007*a*) that low water availability and low turgor pressure limit respiration and elicit a depression in $E_{A(S)}$ during daytime.

Internal [CO_2] and its relationship to E_A (resistance to radial CO_2 diffusion)

Internal [CO_2] varied between 0.11 and 0.52%. These values were an order of magnitude lower than those generally observed using this methodology. However, internal [CO_2] lower than 0.5% was also observed for several species using different methodologies (see Table 1 in Teskey *et al.*, 2008), and at this site during the 2012 growing season (Salomón et al. 2015). Comparatively low xylem [CO_2] and $E_{A(S)}$ similar to commonly reported rates might be explained by the combination of low respiration of xylem living cells and high radial diffusion of CO_2. First, low xylem respiration in roots and stems can be attributed to the over-aged status of trees in this historically coppiced stand (Manuel Valdés and Rojo y Alboreca 1993). Limited growth (Figure 1b, Corcuera et al. 2006, Salomón et al. 2013) and low proportion of sapwood (mean sapwood thickness was 2.5 cm) in over-aged stems relative to trees in other experiments using this methodology might result in comparatively low respiration rates of xylem tissues. The reduction in carbon expenditures by an acclimation of respiration

may enhance carbohydrate storage to support resprouting (Zeppel et al. 2015), the reproductive strategy of this species. Second, limited water availability in drought-prone regions may have a key role explaining the amount of respired CO_2 that built up in roots and stems or diffused to the soil and atmosphere. Gas diffusion is much higher (~10^4) in gas than in water (Nobel 1999). Thus, progressive soil drying across the growing season might have resulted in a progressively larger proportion of root-respired CO_2 diffusing through soil to the atmosphere relative to the amount dissolving in sap solution and moving upward in the transpiration stream. Consistently, F_T as low as 2% of the root-respired CO_2 that diffused through soil to the atmosphere has been observed at this experimental site (Salomón et al. 2015), which likely decreased the [CO_2] at the base of the stem relative to that measured in other studies (e.g. Aubrey and Teskey 2009). This is the first study in which [CO_2] was measured in a sub-Mediterranean species during the dry season. To date, [CO_2] has been monitored in temperate and boreal species where water availability is not a limiting factor for stem respiration. In the only manipulative drought experiment using this methodology, values as low as 0.3 mmol L^{-1} (≈ 0.3% in the gaseous phase assuming T_{stem} = 15ºC and sap pH = 6.5) were observed in *Q. robur* trees at higher midday water potential than in this study (Saveyn et al. 2007b). In support of this idea, it is worth noting that in the same experimental site, [CO_2] exhibited a 20-fold increase after a heavy rain at the end of the dry season (manuscript in prep.), similar to observations by Saveyn et al. (2007b) after rewatering. Furthermore, due to the steep decline in latewood formation in over-aged stems of *Q. pyrenaica* (Corcuera et al. 2006), the high proportion of large early-wood vessels may facilitate the radial diffusion of respired CO_2 (Sorz and Hietz 2006).

Our observation that [CO_2] was highest on D1 compared with the other dates was most likely due to the absence of [CO_2] dilution by transpiration on D1 (Teskey and McGuire 2002), but increasing drought and healing of wounds caused by CO_2 sensor installation (see Etzold et al. 2013) could have also contributed to the decline in [CO_2] over the growing season. Additionally, enhanced cambial activity due to primary root elongation (Courty et al. 2007) and early-wood formation in *Q. pyrenaica* branches (Corcuera et al. 2006) during spring may have also increased [CO_2] at the stem base and at 3 m, respectively, compared with measurements later in the season (Figure 4). This pattern of [CO_2] did not hold at 1.5 m, which was similar to the date-by-height interaction observed in a pioneering study of stem [CO_2] (Chase 1934). In any case, pooled data

showed an increasing vertical gradient in [CO_2], suggesting that respired CO_2 was transported upward (Teskey and McGuire 2007, Hölttä and Kolari 2009).

Recent work has provided interesting data on the amount of CO_2 that moves from the roots upward (Bloemen et al. 2015), but there are still challenging issues regarding internal CO_2 transport, such as the effect of root structure on internal [CO_2]. Multi-stemmed trees offer a good opportunity to study how root-respired CO_2 moves horizontally through roots and upward into stems. Here we found that stems supplied by larger taproots connected to greater amounts of belowground biomass had higher [CO_2] at the base than those connected to fewer and smaller roots. This possibility was suggested by Teskey & McGuire (2007) and has found empirical support here, in natural conditions. It provides a new evidence of the difficulty in ascribing stem CO_2 efflux to the respiration rate of directly adjacent cells.

Seasonal variability in the resistance to radial CO_2 diffusion is another overlooked factor that could help to comprehensively understand and model stem respiration. Resistance to radial CO_2 diffusion determines the fate of locally respired CO_2 by changing the potential for CO_2 to diffuse outward and contribute to $E_{A(S)}$ or alternatively, to accumulate and dissolve in xylem sap and contribute to F_T. Resistance to radial diffusion of CO_2 was estimated from the relationship between internal [CO_2] and $E_{A(S)}$ (Steppe et al. 2007, Teskey et al. 2008). Although no relationship between [CO_2] and $E_{A(S)}$ has been found in some studies (see Maier & Clinton, 2006 and Saveyn *et al.*, 2007b), here, a strong direct relationship between internal [CO_2] and $E_{A(S)}$ was found on D1 during budburst ($R^2 = 0.96$, $P < 0.001$) and, similar to findings of Saveyn *et al.* (2007b), the strength of the relationship diminished during the dry season. In addition to affecting the rate of respiration, seasonal changes in temperature and water availability also alter the relative volume of gas and water in stems, and thus influence stem permeability to CO_2 (Nobel 1999). In our study, resistance to radial CO_2 diffusion was 3-4 times higher on D1 than D2, D3 or D4. Similarly, Fick's radial diffusion coefficient increased 5- fold in *Q. robur* when gas volume increased (to the detriment of water volume) from 15 to 40% (Sorz and Hietz 2006). The direct relationship between the resistance to radial CO_2 diffusion and midday water potential ($R^2 = 0.89$, $P = 0.06$) suggests that greater drought stress concomitantly reduced stem water content and the resistance to radial CO_2 diffusion. Low resistance to radial diffusion after leaf expansion

519 (from D2 to D4) might be an underlying cause of the low contribution of F_T to R_S
520 observed during dates of substantial transpiration (see below).

521 *Seasonal dynamics of stem respiration and its contributors*

522 Stem respiration (R_s) was calculated by the mass balance approach proposed by
523 McGuire and Teskey (2004), which elucidated the contributions of $E_{A(V)}$, F_T, and ΔS to
524 R_S. Average R_S on D1 was 4.7 mol m^{-3} day^{-1}, and increased to 13.7, 13.9, and 13.8 mol
525 m^{-3} day^{-1} on D2, D3, and D4, respectively. Values of R_S were similar to the range of
526 reported values applying this methodology: 3.3 mol m^{-3} day^{-1} for *Dacrydium*
527 *cupressinum* trees (Bowman et al. 2005); 5.1 mol m^{-3} day^{-1} for *P. occidentalis* trees
528 (calculated from Teskey & McGuire, 2007); 7.4, 5.8 and 4.2 mol m^{-3} day^{-1} for *Fagus*
529 *grandifolia*, *Platanus occidentalis* and *Liquidambar styraciflua*, respectively (McGuire
530 and Teskey 2004); and from 21.3 to 49.5 mol m^{-3} day^{-1} in young *Populus deltoides* trees
531 (Saveyn et al. 2008). The contribution of ΔS to *R*s was negligible, ranging from -0.4 to
532 0%, which was consistent with previous studies. Thus F_T, and particularly E_A, were the
533 main contributors to *R*s. The contribution of F_T during budburst was nil and then
534 increased to an average of only 5 - 6.5% of R_S after leaf expansion. This contribution is
535 in the lower range of reported values: 3 - 18 % for *P. deltoides* trees (Saveyn et al.
536 2008); 10.6% for *D. cupressinum* trees (Bowman et al. 2005); 14% for *F. grandifolia*
537 and 15% for *L. styraciflua* (McGuire and Teskey 2004); and 34 % and 55% for *P.*
538 *occidentalis* at different times (McGuire and Teskey 2004, Teskey and McGuire 2007).
539 Although [CO_2] in *Q. pyrenaica* stems was an order of magnitude lower than in
540 previous studies, the contribution of F_T to *R*s was within the same order of magnitude.
541 This apparently counterintuitive observation is explained by the fact that F_T is not
542 proportional to the absolute internal [CO_2], since F_T is directly calculated from the
543 difference in dissolved [CO_2^*] between two stem points (here separated by 25 cm; Eq.
544 4).

545 The only significant variation in the contribution of F_T to R_S across the growing season
546 was explained by the resumption of transpiration that occurred between D1 and D2.
547 After leaf expansion (from D2 to D4) the contribution of F_T did not show substantial
548 changes (< 2%) despite temporal variation in water availability and cambial activity.
549 This consistency indicates that the contribution of E_A to R_S was constant and high
550 during the vegetative period, once leaves had expanded, and suggests that E_A may be a

551 good estimator of R_S at large temporal scales in *Q. pyrenaica* and perhaps other
552 drought-adapted species (Rodríguez-Calcerrada et al. 2014). Low transpiration rates
553 characteristic of sub-Mediterranean species, together with low resistance to radial CO_2
554 diffusion and low internal [CO_2], may explain the strong coupling between E_A and R_S in
555 this study. Nonetheless, despite the small contribution of F_T to R_S on a daily basis, we
556 emphasize that the influence of internal transport of CO_2 across interconnected roots
557 and stems of *Q. pyrenaica* on R_S (with F_T accounting up to 25% of R_S in daytime) is
558 important to our understanding of the fate of respired CO_2.

559 *Conclusions*

560 Relative to previous reports, xylem [CO_2] was low in *Q. pyrenaica* stems. Due to low
561 [CO_2], F_T did not account for more than 10% of total R_S on a daily basis, suggesting that
562 E_A may be a suitable estimator of R_S for this species, and perhaps others, under drought
563 conditions. Low stem [CO_2] and F_T was related to high permeability of phloem and bark
564 tissues to diffusion of CO_2 that appeared to be driven by water deficit, as the resistance
565 to radial CO_2 diffusion decreased across the growing season along with decreasing
566 midday water potential. Variation in the resistance to radial CO_2 diffusion in relation to
567 environmental conditions might be a currently overlooked factor that could be important
568 to our understanding of stem respiration.

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574 **Conflict of interest**

575 None declared.

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Figure 1. Daily air temperature (T_{air}), precipitation, and average sap flow per tree (F_{H20}) (a); and accumulated diameter increment and predawn and midday shoot water potential (b) across the growing season in an experimental plot of *Quercus pyrenaica*. Stem growth began before budburst and leaf expansion and ceased at the end of the dry season before first autumn rains (DOY 249). Four measurement campaigns are depicted by grey vertical bars. The gap in the F_{H20} series from DOY 236-253 corresponds to the theft of the power supply on DOY 236.

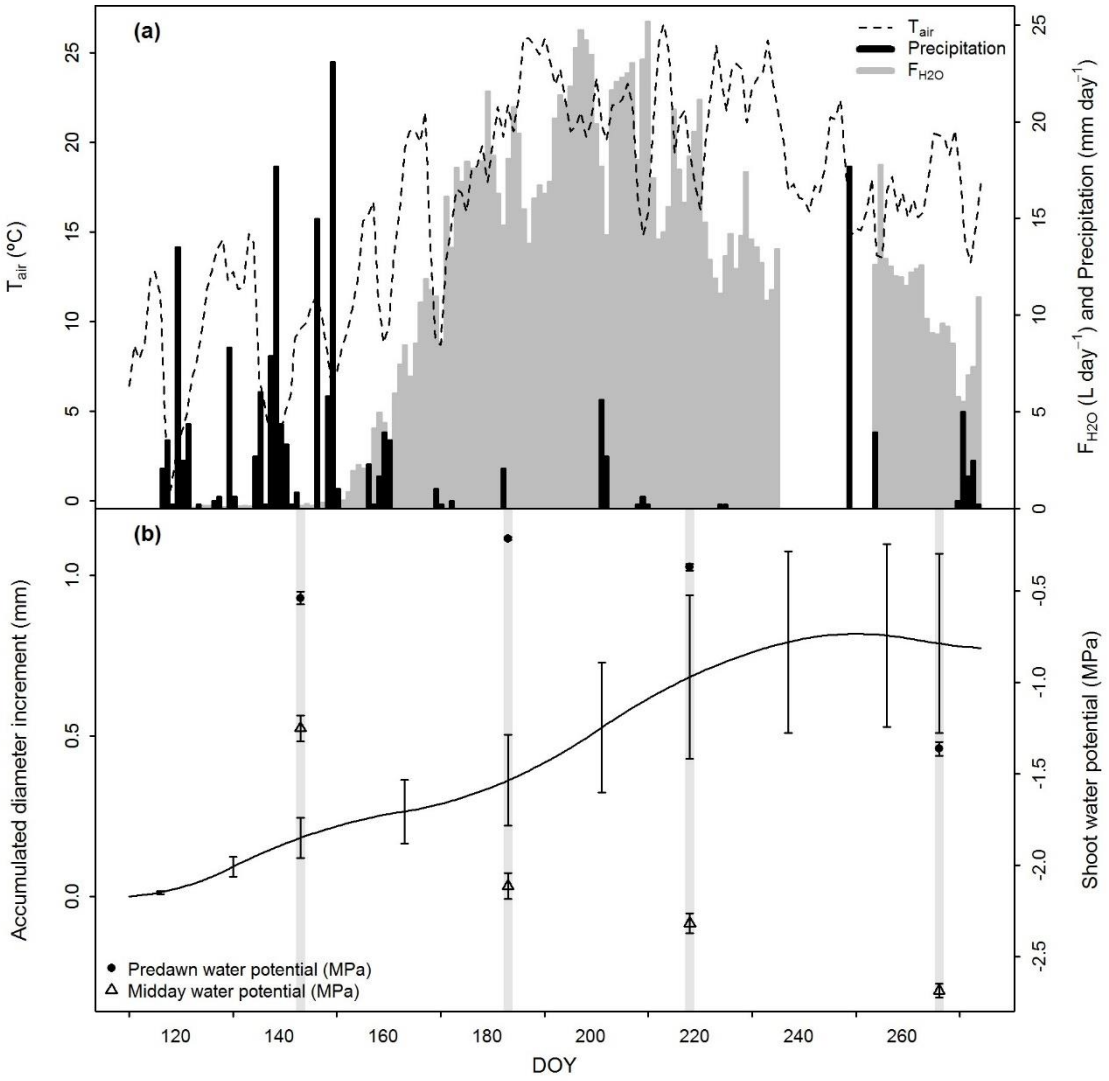

Figure 2. Example of hysteresis in the relationship between stem temperature (T_{stem}) and stem CO_2 efflux on a surface basis ($E_{A(S)}$). Measurements started at midday and follow a counterclockwise pattern. Shaded area indicates nighttime. In the example, for a given temperature, $E_{A(S)}$ was higher at nighttime than at daytime at negligible sap flow rates (DOY 143-144, during budburst). Similar hysteresis was observed throughout the course of the experiment.

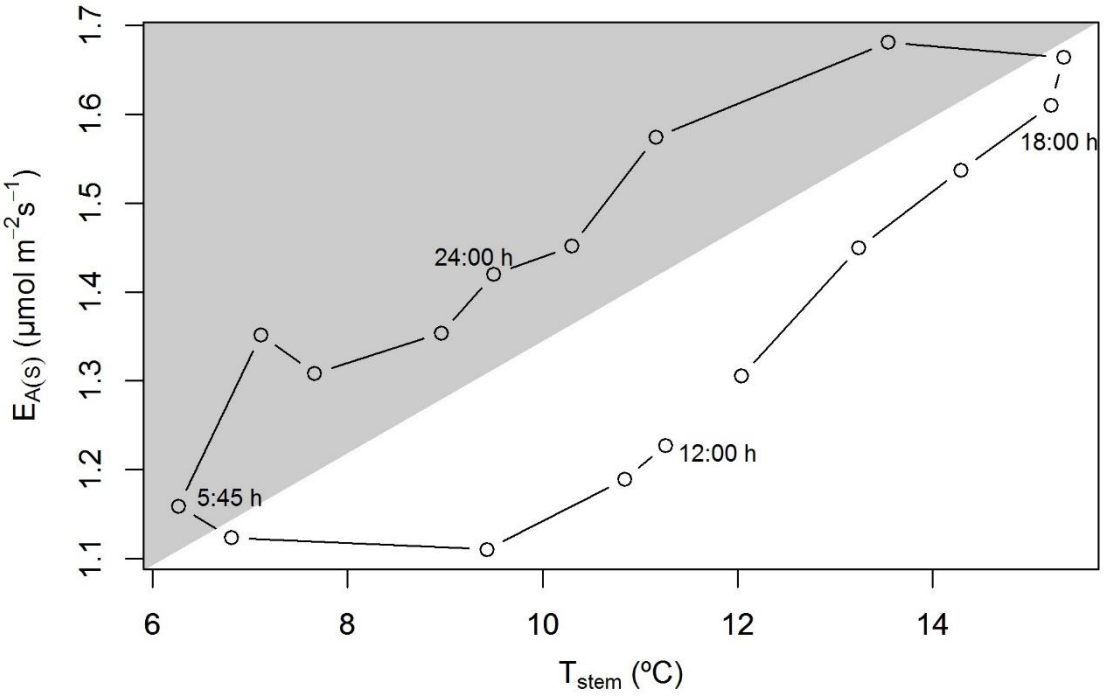

Figure 3. Residual stem CO_2 efflux ($E_{A(S)\_RES}$) regressed against sap flow (F_{H2O}). Residual stem CO_2 efflux was calculated as the difference between predicted (from the nighttime relationship between efflux and temperature, Eq. 1) and measured CO_2 efflux during the daytime. Significant regression lines ($P < 0.05$) per stem and date are depicted. On the leafless measurement date (D1), sap flow and $E_{A(S)\_RES}$ were not related ($P = 0.319$), whereas positive relationships ($P < 0.001$) were found on the remaining dates after leaf expansion ($R^2 = 0.604$, 0.699, 0.618 for D2, D3, and D4, respectively). The slopes of these relationships were similar on D2 and D3 ($P = 0.378$), whereas slopes on D2 and D3 were different than the slope on D4 ($P < 0.001$).

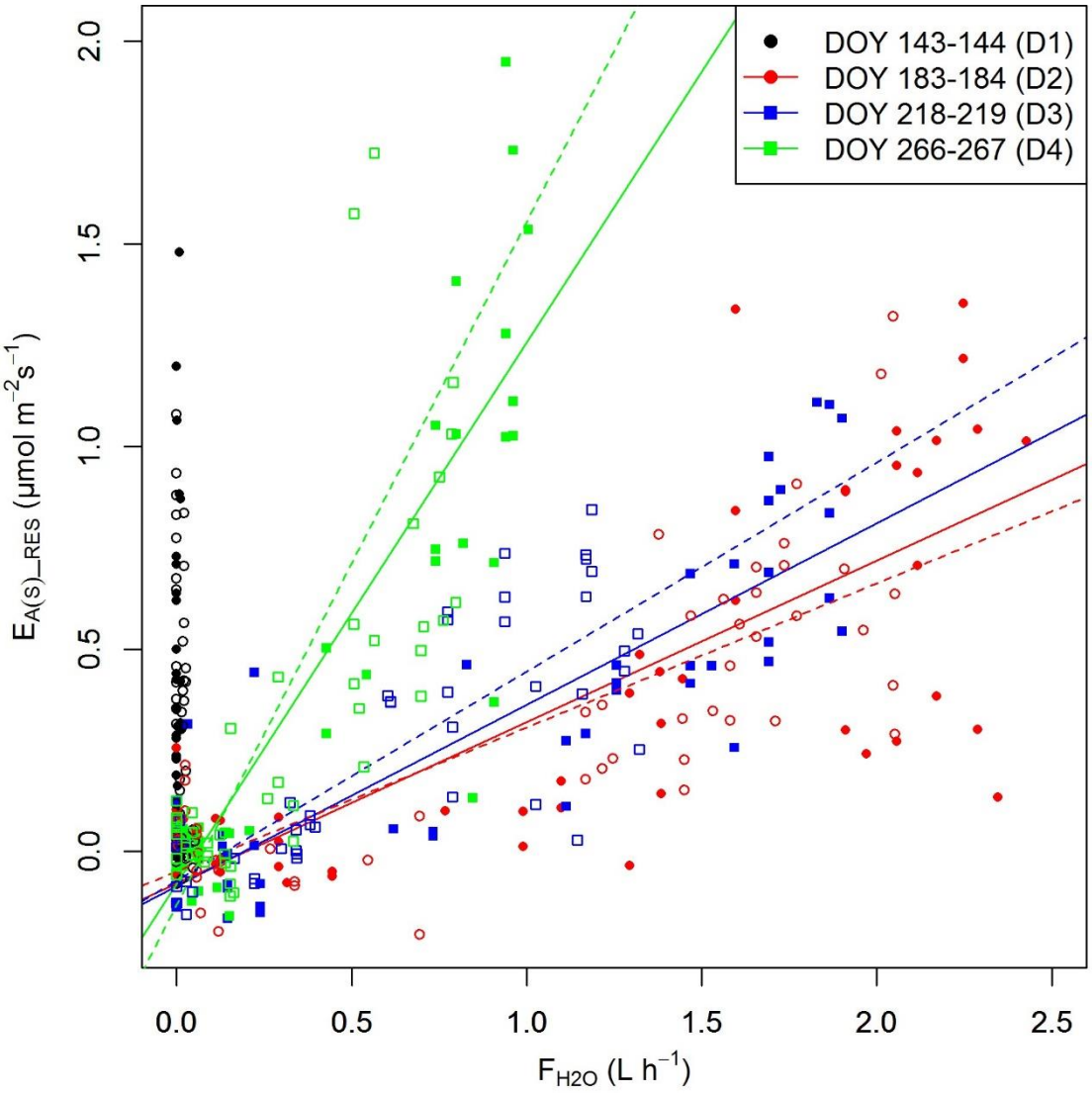

Figure 4. Mean internal [CO_2] across the growing season at three stem heights. Mean internal [CO_2] was calculated for the two stems on which measurements were made at different heights.

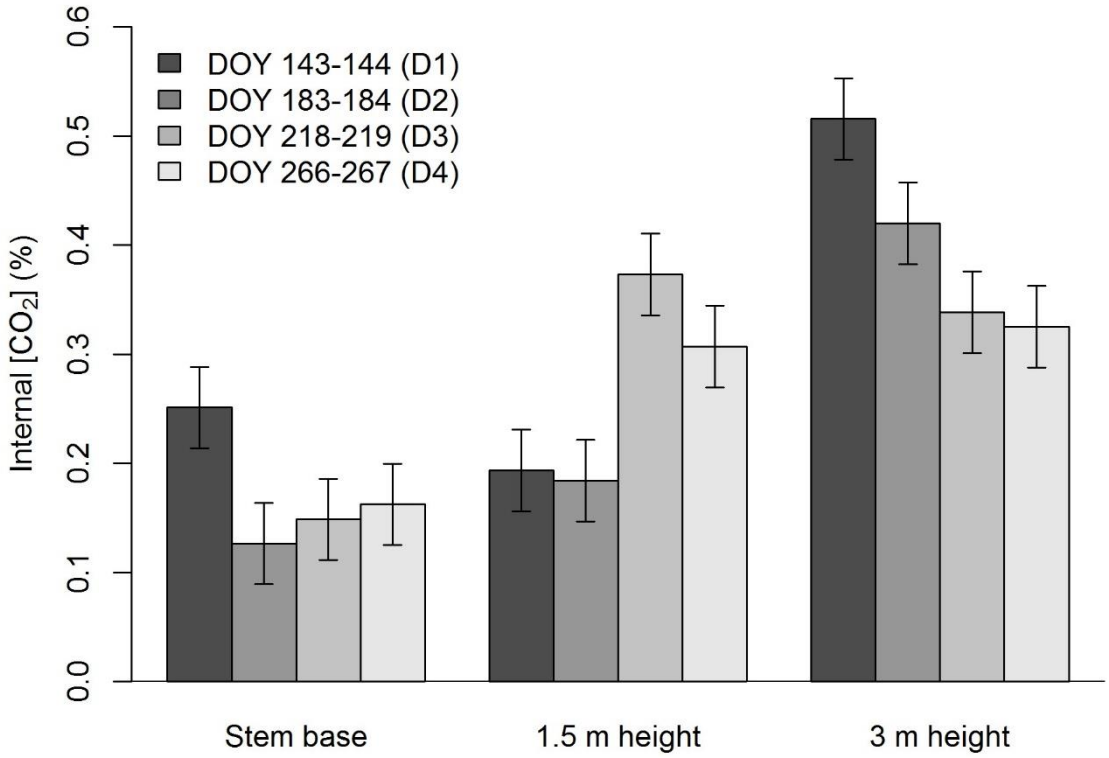

Figure 5. CO_2 concentration gradient between xylem and atmosphere ($\Delta[CO_2]$) in relation to stem CO_2 efflux to the atmosphere ($E_{A(S)}$) on four measurement dates across the growing season. The slope of this relationship represents stem resistance to radial CO_2 diffusion. Measurements at three heights on two *Quercus pyrenaica* stems are depicted by open and closed circles. Only significant regressions ($P < 0.05$) are shown. Resistance to radial CO_2 diffusion was highest on the first measurement date ($P < 0.001$).

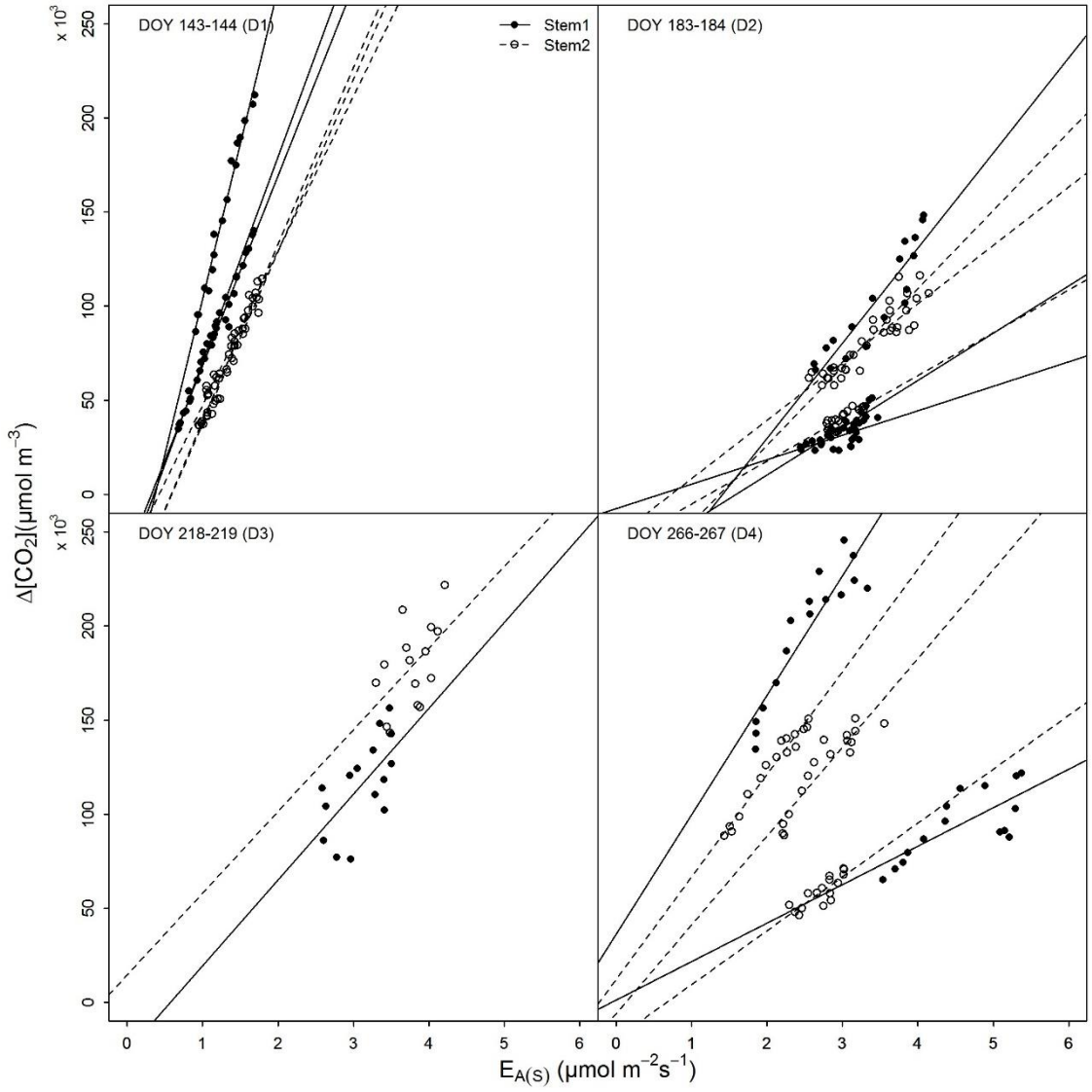

Figure 6. Diel variations in stem respiration (R_S) and its components - stem CO_2 efflux to the atmosphere ($E_{A(V)}$), internal CO_2 transport through xylem (F_T) and CO_2 storage flux (ΔS) - on four dates over the growing season. Mass balance at 1.5 m stem height calculated from average of two stems. Mass balance at 3 m stem height calculated for one stem due to NDIR probe failure in the second stem at two dates. Shaded areas indicate nighttime.