

1 **Foliar photochemical processes and carbon metabolism**
2 **under favourable and adverse winter conditions in a**
3 **Mediterranean mixed forest, Catalonia (Spain)**

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15 **Abstract:**

16 Evergreen trees in the Mediterranean region must cope with a wide range of environmental
17 stresses from summer drought to winter cold. The mildness of Mediterranean winters can
18 periodically lead to favourable environmental conditions above the threshold for a positive
19 carbon balance, benefitting evergreen woody species more than deciduous ones. The
20 comparatively lower solar energy input in winter decreases the foliar light saturation point.
21 This leads to a higher susceptibility to photoinhibitory stress especially when chilly (<12 °C)
22 or freezing temperatures (<0°C) coincide with clear skies and relatively high solar irradiances.
23 Nonetheless, the advantage of evergreen species that are able to photosynthesis all year round
24 where a significant fraction can be attributed to winter months, compensates for the lower
25 carbon uptake during spring and summer in comparison to deciduous species. We
26 investigated the ecophysiological behaviour of three co-occurring mature evergreen tree
27 species (*Quercus ilex* L., *Pinus halepensis* Mill., and *Arbutus unedo* L.). Therefore, we
28 collected twigs from the field during a period of mild winter conditions and after a sudden

1 cold period. After both periods, the state of the photosynthetic machinery was tested in the
2 laboratory by estimating the foliar photosynthetic potential with CO₂ response curves in
3 parallel with chlorophyll fluorescence measurements. The studied evergreen tree species
4 benefited strongly from mild winter conditions by exhibiting extraordinarily high
5 photosynthetic potentials. A sudden period of frost, however, negatively affected the
6 photosynthetic apparatus, leading to significant decreases in key physiological parameters
7 such as the maximum carboxylation velocity ($V_{c,max}$), the maximum photosynthetic electron
8 transport rate (J_{max}), and the optimal fluorometric quantum yield of photosystem II (F_v/F_m).
9 The responses of $V_{c,max}$ and J_{max} were highly species-specific, where *Q. ilex* exhibited the
10 highest and *P. halepensis* the lowest reductions. In contrast, the optimal fluorometric quantum
11 yield of photosystem II (F_v/F_m) was significantly lower in *A. unedo* after the cold period. The
12 leaf position played an important role in *Q. ilex* showing a stronger winter effect on sunlit
13 leaves in comparison to shaded leaves. Our results generally agreed with the previous
14 classifications of photoinhibition-tolerant (*P. halepensis*) and photoinhibition-avoiding (*Q.*
15 *ilex*) species on the basis of their susceptibility to dynamic photoinhibition, whereas *A. unedo*
16 was the least tolerant to photoinhibition, which was chronic in this species. *Q. ilex* and *P.*
17 *halepensis* seem to follow contrasting photoprotective strategies. However, they seemed
18 equally successful under the prevailing conditions exhibiting an adaptive advantage over *A.*
19 *unedo*. These results show that our understanding of the dynamics of interspecific competition
20 in Mediterranean ecosystems requires consideration of the physiological behaviour during
21 winter which may have important implications for long-term carbon budgets and growth
22 trends.

23 **1 Introduction**

24 Mediterranean-type ecosystems are widely associated with broadleaved evergreen
25 sclerophyllous shrubs and trees, the classic vegetation types in climates where hot and dry
26 summers alternate with cool and wet winters (Aschmann, 1973; Blumler, 1991; Orshan, 1983;
27 Specht, 1969). In summer, water is undoubtedly the most important factor limiting growth
28 and survival in the Mediterranean region, whereas spring and autumn provide better growing
29 conditions (Gracia et al., 1999; Orshan, 1983; Sabaté and Gracia, 2011). In winter, the low
30 temperatures and solar radiation limit the amount of energy available for the vegetation,
31 although soil-water contents and water-pressure deficits are favourable. This highly dynamic
32 seasonality of favourable and unfavourable conditions produces a rich diversity of plants in

1 these regions (Cowling et al., 1996). In turn, this features a highly diverse range of traits and
2 taxa that has produced multiple survival strategies that help to explain the abundance and
3 distribution of species (Matesanz and Valladares, 2013). Nonetheless, the predicted
4 reductions in annual precipitation, increases in mean temperature, and increases in the
5 variability and occurrence of extreme droughts and heat waves in arid and semi-arid regions
6 are likely to affect species abundance and distribution (Friend, 2010; IPCC, 2013; Somot et
7 al., 2008). The battle for survival and dominance in plant communities facing these novel
8 changes in their environments evokes great uncertainties and worries in the scientific
9 community concerning the adaptive ability, distribution shifts, or, at worst local extinction of
10 species especially in Mediterranean type ecosystems (Matesanz and Valladares, 2013;
11 Peñuelas et al., 2013).

12 In this context, a pivotal role devolves on the winter period in Mediterranean type- climates as
13 mild winter temperatures can suddenly provide potential periods of growth and recovery from
14 stressful summer drought periods, above all for evergreen trees. Thus, the success in the
15 future dynamics of competition and novel environmental conditions will not only depend
16 upon the tolerance to withstand abiotic stresses, but also on their effectiveness to benefit
17 rapidly from periods when environmental conditions may be favourable such as in winter.
18 The effective acclimation of the photosynthetic apparatus during winter was hereby in the
19 focus of interest for this study. This acclimation is particularly essential for evergreen tree
20 species in order to compensate for their lower photosynthetic rates during the growth period,
21 relative to deciduous species. Plants have evolved diverse adaptive mechanisms to cope with
22 the consequences of stress and to acclimate to low temperatures (Blumler, 1991; Öquist and
23 Huner, 2003).

24 Hereby, mixed forests provide us with an ideal test-bed for investigating the different
25 ecophysiological strategies and their sensitivities to abiotic stresses, because all tree species
26 have to contend equally with the yearly variability of environmental conditions. Nevertheless,
27 most ecophysiological studies have been conducted in spring and summer, and winter has
28 been surprisingly overlooked despite its importance for our understanding of the dominance
29 of certain vegetation-types and of the responses of vegetation to stress, seasonality, and
30 species composition (Oliveira and Peñuelas, 2004; Orshan, 1983; Tretiach et al., 1997). Even
31 though efforts have recently been made to elucidate the behaviour of sclerophyllous
32 ecosystems under variable winter conditions (e.g. García-Plazaola et al., 1999, 1997;

1 Kyparissis et al., 2000; Levizou et al., 2004; Martínez-Ferri et al., 2004; Oliveira and
2 Peñuelas, 2004, 2000), the physiological behaviour of co-occurring species of evergreen trees
3 in the Mediterranean region, including leaf gas exchange (GE) and chlorophyll fluorescence
4 (CF) methods, have been insufficiently studied for understanding the dynamics of
5 photoinhibitory stress and interspecific competition. Therefore, in our study we used an ample
6 set of parameters from GE & CF measurements in order to provide a snapshot in the plant's
7 physiology and in order to characterize in detail the effects on the photosynthetic light and
8 carbon reactions during winter (Flexas et al., 2008; Guidi and Calatayud, 2014). This study
9 was conducted on three species of evergreen trees (*Quercus ilex* L., *Pinus halepensis* Mill.,
10 *Arbutus unedo* L.) in northern Catalonia near Barcelona, Spain.

11 Our aims were to i) investigate the foliar physiology of these three species under mild winter
12 conditions, ii) analyse the effect of sudden changes from favourable to unfavourable
13 conditions on photochemical and non-photochemical processes associated with electron
14 transport, CO₂ fixation, and heat dissipation, iii) determine if leaves exhibit distinct locational
15 (sunlit or shaded) responses to winter stress, and iv) identify the species-specific strategies
16 when coping with stress, induced by low temperatures and frost. These topics are of particular
17 interest due to the recent report of an increased dominance of angiosperm trees and the
18 negative impacts on pines over extensive areas of the Iberian Peninsula (Carnicer et al.,
19 2013). Therefore, we must improve our understanding of the interactions among co-occurring
20 tree species competing for scarce resources and trying to survive and tolerate novel
21 environmental conditions to be able to predict ecosystem responses to global climate change.

22

23 **2 Material and Methods**

24 **2.1 Field site**

25 Our experiment was conducted at the field station of Can Balasc in Collserola Natural Park, a
26 coastal massif (8500 ha) in the hinterlands of Barcelona, northeastern Spain (41° 25' N, 2°
27 04' E, 270 m a.s.l.). The forest stand at the study site has an area of 0.7 ha and is on a
28 northeast-facing slope. The climate is characterised by typical Mediterranean seasonal
29 summer droughts and warm temperatures, with a mean August temperature of 22.8 °C. The
30 proximity to the Mediterranean Sea provides mild winters where frosts and snow are rare, as
31 reflected in the mean January temperature of 7.9 °C. Mean annual precipitation and

1 temperature are 723 mm and 15.1 °C (1951-2010), respectively (Ninyerola et al., 2000). The
2 soils have predominantly developed above lithological strata of shales and granite (Sanchez-
3 Humanes and Espelta, 2011). Sensors for measuring air temperature (HMP45C, Vaisala Oyj,
4 Finland) and solar radiation (SP1110 Skye Instruments Ltd., Powys, UK) were installed at a
5 height of 3 m, in a clearing *ca.* 1 km from the plot.

6 **2.2 Stand history and composition of tree species**

7 The history of Collserola Natural Park is typical for the area, being characterised by intensive
8 exploitation for charcoal in *Quercus*- coppice forests and for agricultural purposes such as
9 olive production until the 20th century. The abandonment of these practices at the beginning
10 of the 20th century led to forest succession and restoration with the early successional and fast
11 growing Aleppo Pine (*P. halepensis* Mill.). As in wide parts of the Mediterranean basin, this
12 tree species was favoured by forest management for its rapid growth rates and timber yields
13 (Maestre and Cortina, 2004). The cessation of forest practices in the early 1950s led to a
14 second wave of succession characterised by extensive regeneration of the evergreen Holm
15 Oak (*Q. ilex* L.) and the deciduous Pubescent Oak (*Q. pubescens* Willd.). As a result, many
16 mixed forest stands in Collserola are currently characterised by two-layered canopies
17 consisting of a dense layer from *Quercus* species surmounted by shelter trees of *P. halepensis*.
18 The forest stand at our experimental site has reached the next and final stage of forest
19 succession, where the dense *Quercus* canopy is out-competing the early successional *P.*
20 *halepensis*, simply by suppressing the growth of the light demanding pine seedlings and
21 saplings. This final stage of succession is typical of many pine-oak forest-type sites in the
22 Iberia Peninsula. *P. halepensis*. is dependent mainly on fire disturbances for natural
23 regeneration (Zavala et al., 2000). Interestingly, the diversity of tree species is enriched by the
24 scattered occurrence of Strawberry trees (*A. unedo*) in the forest canopy being usually more
25 characterised as a shrubby species widely abundant in the macchia ecosystems of the Iberian
26 peninsula (Beyschlag et al., 1986; Reichstein et al., 2002). Its existence adds an ecological
27 value to the forest due to its flowering and fruiting behaviour attracting insects and birds. It
28 raises questions about its performance as a mature tree within the interspecific competition of
29 this mixed forest. The forest diversity also encompasses a dense understory mainly consisting
30 of *Pistacia lentiscus* L., *Erica arborea* L., *Phillyrea latifolia* L., *Rhamnus alaternus* L., *Cistus*
31 spp, *Crataegus monogyna* Jacq., *Bupleurum fruticosum* L., and other less abundant species.
32 The stand at our study site has reached a highly diverse stage of forest succession and has

1 provided us with a rare set of some of the most important Mediterranean tree species growing
2 together naturally.

3 **2.3 Sampling**

4 The sampling of the mild winter period took place between 09.01.-19.01.12 (DOY 9-19). The
5 frosty/chilly period lasted from 19.01.-04.02.12 (DOY 21-35). The sampling period after the
6 frosty/chilly period took place between 14.02.-24.02.12 (DOY 45-55). We obtained sunlit
7 leaves for GE-analyses by sampling five twigs with a pruning pull from the outer part of the
8 upper third of the crown, and shaded leaves by sampling five twigs from the inner part of the
9 crown, optimally at similar heights. In the second field campaign after the frost occurrence,
10 however, we were constrained to sample shaded leaves only from *Q. ilex* due to limitation in
11 labour and equipment. The shaded leaves of *P. halepensis* and *A. unedo* could only be
12 sampled in the first, but not in the second field campaign. The twigs were immediately re-cut
13 under water in buckets in the field and transported to the laboratory retained in plastic bags to
14 minimise transpiration. Five replicates of each species were collected for the analysis of GE.
15 The twigs were pre-conditioned in the laboratory at a room temperature of 24-28 °C in dim
16 light for 1-3 d and freshly cut the following morning before the measurement of GE
17 (Niinemets et al., 1999, 2005). We intended to avoid the problems we had faced in the field,
18 such as the limited ability of the instruments to reach the standard operating temperature of 25
19 °C, which was hampered by low ambient temperatures or unpredictable plant responses such
20 as closed stomata or patchy stomatal conductance (Mott and Buckley, 1998, 2000). The pre-
21 conditioned twigs instead had a stable C_i and sufficiently high g_s , which are required for
22 conducting a noise-free CO₂-response curve. The method of cutting twigs rehydrated stressed
23 leaves at optimum conditions and allowed us to analyse their long-term acclimation to the
24 environmental conditions from which they were derived. This method has been used in other
25 studies (Epron and Dreyer, 1992; Haldimann and Feller, 2004; Laisk et al., 2002; Niinemets
26 et al., 1999, 2005), and we confirmed that the leaves remained fresh and functional for several
27 days controlled by g_s and fluorescent signals (data not shown). Our ambient values of the GE-
28 and CF-derived parameters accordingly represented the “ambient capacity” of pre-conditioned
29 leaves under near-optimal ambient environmental conditions of CO₂ concentrations and
30 saturating light and at a room temperature of 20-25 °C (Reich et al., 1998).

31

1 **2.4 GE and CF analyses**

2 GE and CF were measured with a Li-Cor LI-6400XT Portable Photosynthesis System
3 equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA).
4 Response curves for foliar net assimilation versus CO₂ concentration were recorded from five
5 apparently healthy leaves per tree species and leaf position. CF was measured in parallel. *A.*
6 *unedo* leaves were sufficiently large to cover the leaf cuvette (2 cm²), whereas sunlit leaves of
7 *Q. ilex* were in some cases too small, and the area of the leaves had to be adjusted after the
8 measurements. For the leaves of *P. halepensis*, we positioned a layer of needles (appr. 10-15)
9 on the leaf cuvette, avoiding gaps and overlays. The putty-like adhesive 'Blu-tack' (Bostik
10 SA, La Plaine St Denis, France) was also used to seal the gaskets and to keep the needles in
11 position.

12 **2.4.1 Preparation and acclimation**

13 Prior to recording the response curves, the temperature of the clamped leaves (T_{Leaf}) was
14 adjusted to 25 °C, and the flow of ambient CO₂ in the leaf chamber (C_a) was set to 400 μmol
15 CO₂ m⁻² s⁻¹ (controlled with a CO₂ mixer). The leaves were dark-adapted for 15-20 min before
16 the measurements, and the data were logged when the GE-derived parameters such as
17 stomatal conductance (g_s), stomatal internal CO₂ concentration (C_i) and mitochondrial
18 respiration in darkness (R_n) had stabilised. For our purposes, dark-adaptation did not necessarily
19 mean strict prolonged darkness but referred to a sufficiently low level of ambient background
20 light that did not cause an accumulation of reduced photosystem II (PSII) acceptors, which
21 could be detected as an increase in fluorescence. The leaves were also pre-darkened with
22 special leaf clips or a dark cloth to save time. The chamber light was then turned on at a
23 saturating quantum flux density of 1000 μmol photons m⁻² s⁻¹ (20% blue LED, 80% red
24 LED). The relatively high percentage of blue light stimulated the stomata to open (Farquhar
25 and Sharkey, 1982; Kang et al., 2009; Niinemets et al., 2005). The relative humidity was
26 maintained at 50% (±10%), and the air flow was maintained at 500 μmol s⁻¹. The above
27 conditions were maintained for approximately 20-30 min until the net rate of carbon
28 assimilation (A_{net}), g_s , and C_i of the leaf stabilised.

29 The GE-derived parameters A_{net} , g_s , and C_i likely require less time to stabilize, especially in
30 healthy and unstressed leaves, but this minimum time range was necessary for the CF-derived
31 parameters to ensure accurate measurement of the efficiency of harvesting light energy by

1 maximal oxidation and therefore open PSII reaction centres under ambient conditions of CO₂
2 and saturating light, which can be monitored by observing the stability of steady-state
3 fluorescence (F_s). If this stability is not achieved, the effective quantum yield of PSII (Φ_{PSII})
4 and subsequent calculations of important parameters such as the rate of electron transport
5 based on the CF measurement (J_{CF}) could be underestimated. After all parameters had
6 stabilised, the steady-state GE-derived parameters and several CF-derived parameters in the
7 light-adapted state were recorded simultaneously. F_s followed shortly afterwards by the
8 maximum fluorescence yield in the light-adapted state (F_m') were logged by the emission of a
9 pulse of white light at 10000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to close all PSII reaction centres, followed by a so-
10 called 'dark pulse' for measuring the minimal fluorescence (F_o') of a light-adapted leaf that
11 has been momentarily darkened. The measurement of CO₂ began after the completion of the
12 preparation and acclimation, which required approximately 30 min in unstressed leaves and
13 up to 2 h in stressed leaves.

14 **2.4.2 CO₂ experiments**

15 The CO₂-response curves were recorded at a T_{Leaf} of 25 °C and a quantum flux density of
16 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. The values of C_a used to generate the response curves were 400
17 \rightarrow 300 \rightarrow 200 \rightarrow 150 \rightarrow 100 \rightarrow 50 \rightarrow 400 \rightarrow 400 \rightarrow 600 \rightarrow 800 \rightarrow 1200 \rightarrow 2000 (in μmol
18 $\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). The minimum and maximum times for stabilising A_{net} , g_s , and C_i for each log
19 were set to 4 and 6 min, respectively.

20 **2.4.3 Light experiments**

21 Light-response curves (A/PPFD) were generated at a C_a of 400 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ by
22 automatically applying changes in the photosynthetically active radiation with the LI-6400XT
23 light source. To obtain precise responses at the low range of the light gradient for estimating
24 the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following
25 PPFD sequence: 2500 \rightarrow 2000 \rightarrow 1500 \rightarrow 1000 \rightarrow 800 \rightarrow 600 \rightarrow 500 \rightarrow 400 \rightarrow 300 \rightarrow 200
26 \rightarrow 150 \rightarrow 125 \rightarrow 100 \rightarrow 75 \rightarrow 50 \rightarrow 40 \rightarrow 30 \rightarrow 20 \rightarrow 10 \rightarrow 5 \rightarrow 0 (in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$).
27 The minimum and maximum times between each light level for the generation of the
28 A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light
29 during an A/PPFD curve led to a drop in T_{Leaf} as the light decreased. The rapid changes in the
30 light levels prevented the adjustment of T_{Leaf} while guaranteeing stable air and water fluxes
31 and avoiding noisy measurements of C_i and g_s . We thus decided to maintain a stable Peltier-

1 block temperature (T_{block}) in the leaf cuvette. Hence, T_{block} was first adjusted so that T_{leaf} was
2 25 °C at the beginning of the A/PPFD curve and then kept stable throughout the experiment.
3 T_{leaf} had dropped by approximately 1-3 °C by the completion of the A/PPFD curve.

4 The calculation of the parameters F_v/F_m , NPQ, qp, and temperature functions, in
5 supplementary material.

6 **2.5 Calculation of CF-derived parameters**

7 The maximum efficiency of PSII was calculated by:

$$8 \quad \frac{F_v}{F_m} = \frac{(F_m - F_o)}{F_m} \quad (1)$$

9 where F_v is the variable fluorescence of a dark-adapted sample, F_m is the maximal
10 fluorescence measured after a saturating light pulse, and F_o is the minimal fluorescence
11 measured under darkness. These parameters were obtained from dark-adapted leaves with
12 closed PSII reaction centres as described in the previous sections. The F_v/F_m ratio describes
13 the fraction of photochemically absorbed photons under dark conditions. Typical values range
14 between 0.75 and 0.85, depending on age, health, and preconditioning (Maxwell and Johnson,
15 2000). The F_v/F_m ratio provides information about the maximum or optimum quantum yield
16 and serves as indicator of stress in the photosystems. Ratios below 0.80 are indicative of
17 induced photoprotection and sustained energy dissipation (Maxwell and Johnson, 2000;
18 Verhoeven, 2014), whereas leaves with ratios below 0.74 are considered to be below the
19 recovery threshold (Björkman and Demmig, 1987). The effective quantum yield of PSII was
20 estimated by:

$$21 \quad \Phi_{\text{PSII}} = \frac{(F_m' - F_s)}{F_m'} \quad (2)$$

22 where F_s is the steady-state fluorescence in a fully light-adapted sample, and F_m' is the
23 maximal fluorescence yield after a pulse of high light. The Φ_{PSII} is the counterpart of the
24 optimum quantum yield and represents the fraction of photochemically absorbed photons in a
25 light-adapted leaf (Maxwell and Johnson, 2000).

$$26 \quad J_{CF} = \varepsilon * \Phi_{\text{PSII}} * \alpha_L \quad (3)$$

27 where ε is a scaling factor for the partitioning of intercepted light between photosystems I and
28 II. We assumed that light was equally distributed between both photosystems ($\varepsilon = 0.5$)

1 (Bernacchi et al., 2002; Niinemets et al., 2005). α_L is the foliar absorbance determined in
2 separate measurements of foliar reflectance and transmittance. The following values of α_L
3 were determined: 0.932 for *Q. ilex* and 0.912 for *P. halepensis*, with no differences between
4 sunlit and shaded leaves of these two species, and 0.935 for sunlit leaves of *A. unedo*, and
5 0.917 for shaded leaves of *A. unedo*. For the determination of these leaf absorptances (α_L),
6 foliar reflectance and transmittance were measured at midday in August 2012 using a
7 spectroradiometer UniSpec Spectral Analysis System (PP Systems, Haverhill, MA, USA).
8 The value of J_{CF} at a CO_2 concentration of $400 \mu mol CO_2 m^{-2} s^{-1}$ and a PPFD of $1000 \mu mol$
9 $photons m^{-2} s^{-1}$ was termed ambient photosynthetic electron transport (J_{amb}). Its relationship
10 with the net assimilation rate (J_{amb}/A_{net}) was used for the analyses of alternative electron sinks
11 beside carbon metabolism.

12 **2.6 Estimation of light respiration and calculation of the effective quantum** 13 **yield of CO_2 (Φ_{CO_2})**

14 In the literature, the term R_d was sometimes used for dark respiration (Farquhar et al., 1980;
15 Turnbull et al., 2003), but also for day respiration (Flexas et al., 2012; Yin et al., 2011). We
16 will use R_d to represent mitochondrial respiration during the day or under lighted conditions
17 and R_n to represent mitochondrial respiration at night or under dark-adapted conditions. We
18 estimated R_n during the day after darkening the leaf for at least 30 min. R_d was estimated from
19 the light-response curves with the combined GE and CF measurements proposed by Yin et al.
20 (2009), named the CF method. This method amended the Kok method (Kok 1948) by
21 substituting the A/PPFD relationship with A/PPFD * Φ_{PSII} (Yin et al., 2009). See reference for
22 details.

23 The effective quantum yield of CO_2 (Φ_{CO_2} , unitless) can be calculated using the estimated α_L ,
24 R_d , together with A_{net} and PPFD as follows

$$25 \quad \Phi_{CO_2} = \frac{(A_{net} - R_d)}{PPFD * \alpha_L} \quad (4)$$

26 **2.7 The Farquhar, von Caemmerer, and Berry (1980) photosynthesis model** 27 **(FvCB)**

28 The FvCB photosynthesis model was employed on the assumption that foliar carbon
29 assimilation was limited either by Rubisco activity (A_c) or by ribulose-1,5-bisphosphate

1 (RuBP) regeneration (A_j) and was driven by light, temperature, and CO_2 . The model was
 2 further complemented with a third limitation: the photosynthetic rate limited by triose-
 3 phosphate use (A_p) (Sharkey, 1985). A_{net} can then be determined by the minimum of these
 4 three potential rates from an A/C_c curve:

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

6 where

$$7 \quad A_c = V_{c\text{max}} * \left[\frac{C_c - \Gamma^*}{C_c + K_c \left(1 + \frac{O}{K_o}\right)} \right] - R_d \quad (6)$$

8 where $V_{c,\text{max}}$ represents the maximum rate of Rubisco carboxylation, K_c is the Michaelis-
 9 Menten constant of Rubisco for CO_2 , O is the partial pressure of O_2 at Rubisco, and K_o is the
 10 Michaelis-Menten constant of Rubisco for O_2 (Table C1, see Appendix C) and C_c determined
 11 with the variable J method (Equ. A7 and A8, see Appendix A). The equation representing
 12 photosynthesis limited by RuBP regeneration is:

$$13 \quad A_j = J * \left[\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} \right] - R_d \quad (7)$$

14 where J is the rate of electron transport. The denominator of the above equation
 15 represents the stoichiometry of the number of electrons required to regenerate ATP and
 16 NADP; we have used four for C_c and eight for Γ^* (Flexas et al., 2012). J becomes J_{max}
 17 under light and CO_2 saturation when the maximum possible rate of electron transport is
 18 theoretically achieved.

19 The photosynthetic rate limited by triose-phosphate use is estimated by:

$$20 \quad A_p = \frac{3TPU * C_c}{\Gamma^* \left[C_c - \left(\frac{1 + 3\alpha TPU}{2} \right) \right]} - R_d \quad (8)$$

21 where TPU is the rate of triose-phosphate use at saturating CO_2 concentrations, and α_{TPU} is
 22 the proportion of glycerate not returned to the chloroplasts. This equation fits the A/C_c curve
 23 plateau at high concentrations of CO_2 when a further increase in C_c no longer increases A_{net}
 24 or, in some cases, decreases A_{net} .

25 These three estimated parameters ($V_{c,\text{max}}$, J_{max} , and TPU) define the biochemical capacity to
 26 drive the photosynthetic assimilation of CO_2 but are defined here as the photosynthetic

1 potential (Niinemets et al., 2006). The term photosynthetic capacity is here dismissed, despite
2 its frequent use in the literature, to avoid confusion with studies that have used this term for
3 the maximum rate of assimilation under saturating light conditions (e.g. Bertolli and Souza,
4 2013).

5 **2.8 Curve fitting**

6 The procedure for fitting the curves to estimate the photosynthetic parameters $V_{c,max}$, J_{max} , and
7 TPU applied the least square fit method using the SOLVER estimator tool in Excel. In this
8 procedure, the squared errors of the observed points on the A/C_c curve and the modelled
9 points of Eq.(s) 6, 7, and 8 were calculated and summed. Prior to the fitting procedure, the
10 user must assess the limiting factors, i.e. which points are allocated to which Eq. (6 or 7 or 8).
11 The initial slope of the A/C_c curve is attributed to non-saturating CO_2 conditions when
12 Rubisco activity limits A_{net} (Eq. 6), while the slope of the curve is smoothed at higher CO_2
13 conditions (usually > 35 Pa), representing the limitation of the regeneration of ribulose-1,5-
14 biphosphate (RuPb) (and hence light is a limiting factor) (Eq. 7). The transition zone
15 (approximately at 25-35 Pa of C_i), however, is a grey zone where one point can be attributed
16 to either one or another limitation. These points can also introduce noise in the estimations in
17 cases of doubt and are best discarded. Moreover, unusual points with evidence of an error
18 during the measurements were not included in the curve-fitting procedure. At very high CO_2
19 concentrations, the A/C_c curve plateaus or even decreases slightly. In this case, these points
20 can be attributed to the limitation of triose-phosphate use (Eq. 8). The CO_2 response curves,
21 however, rarely exhibit such a plateau or decrease at high CO_2 concentrations when working
22 on a C_c rather than a C_i basis, so TPU could seldom be estimated in our study. Finally, when
23 attributing all observed points to one or another limitation, we could then estimate the values
24 of $V_{c,max}$ and J_{max} (and possibly TPU) with the SOLVER Excel tool, which iteratively changes
25 the three parameters to minimise the sum of squares of deviation from the observation.

26 **2.9 Correction for diffusion leakage**

27 Large gradients between the ambient air and the CO_2 concentrations inside the chamber are
28 created during the generation of a carbon-response curve. This leakage is particularly
29 important at the high and low ends of the carbon-response curve when a large CO_2 -
30 concentration gradient exists between the leaf chamber and the surrounding ambient
31 concentration. Based on the findings by Flexas et al. (2007a), we corrected A_{net} by

1 subtracting the diffusion leakage for each step of the A/Cc curve obtained from separate
2 response curves with leaves thermally killed in hot water.

3 **2.10 Statistical Analyses**

4 All statistical analyses were performed using the R software package, version 3.0.2
5 (<http://www.r-project.org/>). Differences in the parameters between the mild and cold winters
6 were determined with Student's *t*-tests ($P \leq 0.05$). Shapiro-Wilk tests of normality tested for
7 normality of the data. Data were normalised at $P \leq 0.1$. One-factorial analyses of variance
8 (ANOVAs) with tree species as the main factor tested for differences between tree species of
9 the parameters in the sampling periods. Significant differences were determined at $P \leq 0.05$
10 with Tukey's HSD tests. Regression analyses were conducted to study the relationship
11 between J_{\max} and $V_{c,\max}$ and between J_{amb} and A_{net} . Analyses of covariance (ANCOVAs)
12 tested for differences in slopes and intercepts.

13 **3 Results**

14 **3.1 Environmental Variables**

15 Collserola Natural Park experienced extremely mild winter conditions in November and
16 December 2011 and January 2012, when average minimum temperatures (10.4 °C in
17 November, 5 °C in December, and 3.4 °C in January) remained above 0 °C and no frosts
18 occurred. Average maximum temperatures were 16.3 °C in November, 12.2 °C in December,
19 and 11.4 °C in January. All species had considerable shoot growth of up to 15 cm during this
20 mild period. Sudden low temperatures, however, led to frost on six consecutive days and a
21 minimum average temperature of -2.3 °C (Day of the year (DOY) 21-26) followed by eight
22 days of cool temperatures averaging +2.6 °C (DOY 27-35) (Figure 1). The average radiation
23 during first field campaign (DOY 9-19) was 46 and during the period of frost 58 W m⁻².

24 **3.2 Photosynthetic potentials**

25 Of the three photosynthetic parameters describing the photosynthetic potential, $V_{c,\max}$ and
26 J_{\max} , and TPU, only the first two could be satisfactorily estimated from the A/C_c-response
27 curves. The leaves were only occasionally limited by TPU (6 out of 42), despite the excessive
28 CO₂ concentrations in the higher section of the CO₂-response curve. TPU was therefore
29 discarded from further analysis. $V_{c,\max}$ and J_{\max} were highest in *Q. ilex* but more importantly

1 also decreased most strongly after the period of frost by nearly 50% ($P \leq 0.05$; Fig. 2). The
2 photosynthetic potential of *P. halepensis* was affected the least, reflected by moderate
3 decreases in $V_{c,max}$ and J_{max} (16% and 19%), which were not significant. $V_{c,max}$ and J_{max} were
4 lowest in *A. unedo* during the mild winter period and decreased by approximately 33% after
5 the period of frost. This decrease, however, was not significant due to a large standard error.

6 **3.3 GE-derived parameters under ambient conditions**

7 The period of frost had a strong effect on several GE-derived parameters in *Q. ilex* leaves.
8 The cold temperatures decreased R_n in *Q.ilex* leaves, but the effect was much weaker than for
9 R_d and was not significant (Fig. 3). These parameters responded very weakly to the cold and
10 frost in the leaves of *A. unedo* and *P. halepensis*. A_{net} and Φ_{CO_2} were also reduced in *Q. ilex*
11 leaves by approximately 50% which was significant for the former (Fig. 4A) and low
12 significant for the latter parameter (Fig. 4B). Further differences were only significant for
13 Φ_{CO_2} in *P. halepensis* leaves being reduced by 12 % ($P \leq 0.05$). The CO_2 conductance was
14 more strongly reduced in g_m than in g_s for *Q. ilex* and *A. unedo* leaves which was only
15 significant for the former whereas these parameters seemed unaffected in *P. halepensis* leaves
16 (Fig. 5A and 5B). As a consequence, we observed a tendency of an C_i increase in parallel with
17 a C_c decrease in *Q. ilex* and *A. unedo* leaves due to a lower CO_2 uptake in carbon metabolism,
18 but not in *P. halepensis* (Fig. 6A and 6B). The differences observed were not significant ($P \leq$
19 0.05). The lower drawdown of CO_2 to the chloroplasts ($\Delta C_i - C_c$) (Fig. 7B) led to the observed
20 decreases in A_{net} (Fig. 4).

21 **3.4 CF-derived parameters under ambient conditions**

22 The GE-derived parameters enabled us to study the immediate responses, but several CF-
23 derived parameters allowed us to determine in more depth the physiological changes in parts
24 of the light-harvesting apparatus, namely PSII. F_v/F_m estimated the maximum quantum yield
25 of PSII and represented the health of a leaf (Fig. 7B). *A. unedo* leaves were most strongly
26 affected by the period of frost, followed by *Q. ilex* leaves, whereas *P. halepensis* leaves were
27 only marginally affected. The changes were not statistically significant in the latter two
28 species ($P \leq 0.05$). Φ_{PSII} tended to decrease in all species but most strongly in *Q. ilex* leaves
29 (42 %), however insignificantly (Fig. 7A). NPQ responded very differently in the three
30 species. NPQ did not change much between the two sampling periods in the leaves of *P.*

1 *halepensis* (6%) but decreased significantly by 25% ($0.05 \leq P \leq 0.1$) in *A. unedo* leaves and
2 tended to increase in *Q. ilex* leaves by 31% ($P \geq 0.05$), however insignificantly (Fig. 8).

3 **3.5 Relationships of foliar photosynthetic variables**

4 The covariance of several relationships of the foliar photosynthetic variables were analysed in
5 an ANCOVA to test for differences in the slopes and intercepts in these relationships. The
6 ANCOVA for the relationship between $V_{c,max}$ and J_{max} in *Q. ilex* leaves indicated a marginally
7 significant ($P \leq 0.1$) reduction in the slope and a highly significant ($P \leq 0.01$) reduction in the
8 intercept, revealing a comparatively stronger effect on J_{max} than on $V_{c,max}$ due to the change in
9 weather (Fig. 10 and Table 2). The relationship between the rate of electron transport at
10 ambient conditions derived from CF and the CO_2 assimilation at ambient CO_2 concentrations
11 (J_{amb}/A_{net}) was similar in all tree species (Fig. 10, 11 and Table 2). The slopes were higher in
12 response to the stress imposed by the low temperatures but were not significant. When all
13 species were combined the change of the slope was marginally significant, indicating a
14 possible increased alternative electron sink other than carbon metabolism (Table 2).

15 **3.6 Role of leaf position**

16 Under mild conditions, the leaves of *Q. ilex* showed the most strongly pronounced differences
17 in the leaf position (data of *P. halepensis* Mill. & *A. unedo* L. not shown). Leaves growing
18 under high irradiances had a more active carbon metabolism (A_{net} , R_d , R_n , and Φ_{CO_2}),
19 photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) in all tree
20 species (data only shown for *Q.ilex*). As described in *Material and Methods* the effect of the
21 leaf position after the sudden cold period was only studied for *Q. ilex*. After the sudden frost
22 period, the photosynthetic potential was much higher in sunlit than in shaded leaves of *Q. ilex*,
23 with both J_{max} and $V_{c,max}$ being highly significant (Fig. 2 and Table 1). These differences
24 disappeared after the cold period, because J_{max} and $V_{c,max}$ in the shaded leaves remained
25 unaffected by the frost. F_v/F_m was generally higher in the shaded leaves, but not significantly
26 ($P \leq 0.05$) (Fig. 8 and Table 1). The photosynthetic parameters under ambient conditions,
27 such as A_{net} , g_s , C_i , C_c , and g_m , were not affected much by the leaf position (Fig. 4, 5, 6 and
28 Table 1). Although not significant, the effects of the cold period on these parameters were
29 stronger in the sunlit leaves. The leaf position had more pronounced effects on R_n and R_d (Fig.
30 3 and Table 1). The response of respiration to winter stress, however, differed depending on
31 the location of the leaves. R_n maintained the same balance between sunlit and shaded leaves

1 before and after the cold period, but R_d decreased comparatively more in sunlit leaves due to
2 the period of frost. This pattern was also reflected in Φ_{CO_2} (Fig. 4B and Table 1) and in the
3 CF-derived parameters Φ_{PSII} and NPQ, (Fig. 7A, 8 and Table 1) indicating a stronger effect on
4 the photochemical machinery of sunlit leaves than on shaded leaves. Shaded leaves also
5 exhibited a lower J_{amb}/A_{net} ratio, but the ratio increased equally in both leaf positions after the
6 cold period, indicating a similar behaviour of dissipating energy by alternative electron sinks
7 (Fig. 10A and Table 1).

8 **4 Discussion**

9 **4.1 Winter in the Mediterranean region**

10 Mediterranean-type ecosystems are exposed to stress from summer droughts but also from
11 low temperatures in winter (Mitrakos, 1980). Less attention, however, has been paid to the
12 degree and extent of these stress periods and the wide variation among years and regions, in
13 response to which Mediterranean evergreen species have developed a dynamic
14 photoprotective ability to withstand these stressors (Kyparissis et al., 2000; Martínez-Ferri et
15 al., 2004). Despite the occurrence of lower temperatures than in spring conditions, the
16 photosynthetic potential recovered in winter once the leaves became acclimated to the new
17 conditions (Dolman et al., 2002; Hurry et al., 2000). This is important for the plants overall
18 performance because the photosynthetic exploitation of favourable conditions in winter is
19 crucial for achieving a positive carbon balance in Mediterranean evergreen tree species
20 (García-Plazaola et al., 1999b; Martínez-Ferri et al., 2004). We showed how a long lasting
21 comfortable winter period without frost lead to notably high photosynthetic potentials and
22 carbon assimilation in winter being equal to or partly even exceeding spring values (Sperlich
23 et al, unpublished data). As a result, increased winter temperatures influenced phenological
24 responses, advanced winter cambium activation, spring bud burst and leaf unfolding which
25 has been reported in an increasing number of studies (Peñuelas and Filella, 2001). These
26 observations were also reflected in the high sap flow per tree (J_t), ranging for all tree species
27 on average between 5 and 10 kg d⁻¹ during the mild winter period (Sánchez et al., unpublished
28 results). Whereas sudden frosts have often been attributed to higher altitudes of the
29 Mediterranean region (Blumler, 1991; Tretiach et al., 1997), we showed that it can also be an
30 important factor for plant growth and distribution in other areas such as the sub-humid
31 Mediterranean climate of our study site (Garcia-Plazaola et al., 2003a). At night when frosts
32 are more likely to occur, we observed the lowest temperatures whereas at daytime the

1 temperatures were often above zero degrees. However, as we showed, not only cool daytime
2 but also nighttime temperatures or frosts can affect subsequent daytime photosynthesis and
3 induce photoprotective processes (see also Flexas et al., 1999). In our study, the sudden
4 occurring low temperatures affected strongly the photosynthetic apparatus, although the
5 responses were highly species specific. We will elucidate the physiological mechanism in the
6 following.

7 **4.2 PSII – primary target of stress induced by low temperatures**

8 Typically in winter there is an imbalance between light energy absorbed in photochemistry
9 and light energy used in metabolism. This is shown in our data by increased thermal energy
10 dissipation (NPQ) and reduced PSII efficiency (Φ_{PSII}) in order to reduce the harmful effects of
11 excess energy reflecting an inactivation and damage of PSII reaction centres, more precisely,
12 the reaction-centre protein D1 (Aro et al., 1993; Demmig-Adams and Adams, 1992; Mulo et
13 al., 2012). More precise information about the underlying processes that have altered this
14 efficiency is provided by the F_v/F_m ratio. Chronic changes occurring in the F_v/F_m ratio can be
15 related to a cascade of processes which are induced to protect the photosynthetic apparatus
16 including i) re-organisation of the thylakoid membrane, ii) closure of reaction centres, iii)
17 and/or reduced antennal size (Ensminger et al., 2012; Huner et al., 1998; Maxwell and
18 Johnson, 2000; Verhoeven, 2014). The small changes in the F_v/F_m ratio observed in the leaves
19 of *Q. ilex* and *P. halepensis* reflected photoprotective responses without any photodamage.
20 The significantly decline of F_v/F_m in *A. unedo*, however, indicated strong chronic
21 photoinhibition and is an indication of severe photodamage (Martínez-Ferri et al., 2004). We
22 conclude that *A. unedo* suffered most notably from the low temperatures. whereas *Q. ilex* and
23 *P. halepensis* were equipped with a good photoprotective capacity able to keep the
24 photosynthetic apparatus intact (Öquist and Huner, 2003). *Q. ilex* showed the most dynamic
25 responses, negating the harmful excitation stress by lowering the photochemical operating
26 efficiency (Φ_{PSII}) and increasing the use of alternative thermal-energy pathways (NPQ). This
27 photoprotective capability represented by a higher NPQ is usually linked to the xanthophyll
28 cycle that responds to environmental factors such as temperature, water deficit, and nutrient
29 availability (Demmig-Adams and Adams, 1996; García-Plazaola et al., 1997). Inter-
30 conversions of the cycle and pool sizes occur following the need to dissipate excess excitation
31 energy in response to summer drought (García-Plazaola et al., 1997; Munné-Bosch and
32 Peñuelas, 2004), but also to winter stress (Corcuera et al., 2004; Garcia-Plazaola et al., 2003a;

1 Kyparissis et al., 2000; Oliveira and Penuelas, 2001). The implicit interpretation of being
2 equipped with a high capacity of photoprotection when NPQ increases was recently
3 questioned by Lambrev et al. (2012). This study reported that quenching and photoprotection
4 were not necessarily linearly related and stated that several possibilities of photoprotective
5 responses other than NPQ of CF existed, such as antennal detachment that could possibly
6 vary with species and growth conditions. The highly dynamic and photoprotective capability
7 of *Q. ilex* leaves, however, was also demonstrated by several other photosynthetic parameters
8 such as $V_{c,max}$, J_{max} , A_{net} , Φ_{CO_2} , and R_d , which confirmed this trend and were in accord with the
9 findings by Corcuera *et al.* (2004). Despite reports of several mechanisms of resistance to
10 drought stress in *A. unedo*, including increased levels of zeaxanthin that indicates an enhanced
11 thermal dissipation of excess excitation energy in periods of summer stress (Munné-Bosch
12 and Peñuelas, 2004), we found that *A. unedo* leaves had a lower capacity of photoprotection
13 in response to induced over-excitation of the photosystems by winter stress.

14 **4.3 High photosynthetic potentials and strong effects of low temperatures**

15 $V_{c,max}$ and J_{max} were strongly correlated, being regulated in a coordinated manner
16 (Wullschlegler, 1993). Interestingly, the ANCOVAs indicated that J_{max} decreased more
17 strongly than did $V_{c,max}$ in *Q. ilex* and *P. halepensis* leaves. This is because the above
18 described photoprotective adjustments lead to a lower energy-use efficiency in the reaction
19 centres and consequently also to a downregulation of the photosynthetic electron transport
20 J_{max} . The larger decrease of J_{max} relative to $V_{c,max}$ indicated that low temperature stress
21 became manifest first in a hampered pathway of photochemical energy, because PSII
22 complexes are primarily affected by light-induced damage (Maxwell and Johnson, 2000; Taz
23 and Zeiger, 2010; Vass, 2012). Hence, the limitations of the photosynthetic rate by RuBP
24 regeneration are stronger affected by frost and cold induced stress than those by RuBP
25 carboxylation. The relative amounts of photosynthetic proteins can probably explain the
26 differences observed in the $J_{max}/V_{c,max}$ ratio (Hikosaka et al., 1999; Onoda et al., 2005).

27 The physiological responses were highly species-specific. *Q. ilex* leaves responded with
28 significant decreases (approximately 50%) in their photosynthetic potentials (both $V_{c,max}$ and
29 J_{max}). In contrast, $V_{c,max}$ and J_{max} decreased in *P. halepensis* leaves by only 16 and 19%,
30 respectively, and in *A. unedo* leaves by approximately 30% (for both parameters).

1 **4.4 Inhibition of carbohydrate metabolism**

2 As demonstrated above, adjustments to the frost event took place via the energy flow in the
3 antennal systems and a downregulation of photosynthetic electron transport as well as
4 regulatory mechanisms including the inhibition of Rubisco activity, but also via stomatal and
5 mesophyllic diffusion behaviour (Ensminger et al., 2012; Gratani et al., 2000; Taz and Zeiger,
6 2010). Interestingly, the mesophyllic diffusion resistance was stronger pronounced as a
7 response to low temperatures, especially in *Q. ilex* reducing the CO₂ available for fixation in
8 the chloroplasts. This underlines the recently growing awareness in the scientific community
9 about the important role of g_m as an additional regulating parameter as response to stress,
10 above all in sclerophyllic species (Flexas et al., 2008; Niinemets et al., 2011). In general, our
11 results demonstrated that the efficiency of carbon use in the photosynthetic metabolism and
12 foliar respiratory responses were highly species dependant (Zaragoza-Castells et al. 2007,
13 2008). For instance, *P. halepensis* and *Q. ilex* leaves depicted extraordinarily high values of
14 A_{net} , R_d , R_n , and Φ_{CO_2} in in the mild winter period, but only *Q. ilex* exhibited a significant
15 downregulation after the frost event. Downregulating photosynthesis, the most efficient
16 process to get rid of excess energy, suggests alternative energy pathways such as
17 photorespiration. We did not measure photorespiration directly, but we could infer some of its
18 characteristics by studying the relationship between J_{amb} and A_{net} . All tree species had a
19 relatively higher proportion of electron flux during the period that can be explained by
20 utilization in the carbon metabolism. This has been mainly attributed to photorespiration, but
21 also to the Mehler reaction that protects plants from photodamage in bright light (Allen and
22 Ort, 2001; D'Ambrosio et al., 2006; Flexas et al., 1998, 1999; Fryer et al., 1998; Huner et al.,
23 1998).

24 **4.5 Leaf position specific responses to abiotic stress in winter**

25 It is well know that leaves growing under high irradiances have a more active carbon
26 metabolism (A_{net} , R_d , R_n , and Φ_{CO_2}), photochemical efficiency (Φ_{PSII}), and photosynthetic
27 potential (high J_{max} and $V_{c,max}$) (Taz and Zeiger, 2010). Hereby, *Q. ilex* showed the most
28 strongly pronounced differences between sunlit and shaded leaves. Plants develop leaves with
29 a highly specialised anatomy and morphology for the absorption of the prevailing light in
30 their local environments resulting generally in smaller but also thicker sunlit leaves (Kull and
31 Niinemets, 1993; Terashima and Hikosaka, 1995). Nevertheless, the higher carbon
32 metabolism and photochemical activity of sunlit leaves decreased strongly, partly below the

1 level of shaded leaves, whereas shaded leaves showed little sign of any downregulation but
2 maintained a relatively stable effective quantum yield of CO₂ assimilation in both periods.
3 Furthermore, the photosystems showed no sign of photodamage and generally maintained a
4 higher maximum efficiency than did sunlit leaves. We concluded that foliar-level physiology
5 during winter was better protected in the shaded crown of *Q. ilex* unexposed to the dramatic
6 changes in radiation in the outer canopy, confirming the results by Valladares et al. (2008).
7 We also concluded that *Q. ilex* is a highly dynamic species able to rapidly change its
8 metabolism on the antioxidant and photoprotective level in dependence to its leaf position
9 (García-Plazaola et al., 1997, 1999a; Martínez-Ferri et al., 2004). We show that the foliar
10 plasticity in morphology and anatomy of *Q. ilex* (Bussotti et al., 2002; Valladares et al., 2000)
11 can also be attributed to its biochemical metabolism. We stress that the solar environment of
12 the leaves is a crucial factor when assessing tree performance, especially when comparing tree
13 species in a competitive context.

14 **4.6 Ecological context**

15 *Q. ilex* had the most drastic photoprotective response to frost and cool temperatures, whereas
16 *P. halepensis* exhibited a homeostatic behaviour with a very active carbon assimilatory and
17 respiratory metabolism in both periods. *A. unedo* was intermediate, with large decreases in the
18 parameters of carbon metabolism but also a high variability in its response to frost. *A. unedo*
19 also had the lowest photoprotective capability, which might be explained by previous
20 characterisations to be semi-deciduous to drought being at the borderline to evergreen
21 sclerophyllous species (Gratani and Ghia, 2002a, 2002b). Moreover, *A. unedo* occurs
22 naturally most commonly as a shrub and is less frequently found in the forest canopy of
23 mixed forests growing up to 8-10 m tall as in our study site (Beyschlag et al., 1986;
24 Reichstein et al., 2002). Investments in leaves are thus lower and their life cycles shorter.
25 Leaves of *A. unedo* are more rapidly replaced relative to more sclerophyllic leaves such as
26 those of *Q. ilex*. We postulated that *A. unedo*, considered a relict of the humid-subtropical
27 Tertiary tree flora, was more sensitive to winter stress, which is consistent with its presence
28 mostly in the western Mediterranean basin and its frequent occurrence in coastal zones where
29 humidity and temperature are the main factors determining its geographical distribution
30 (Gratani and Ghia, 2002a and references therein). Our results suggested that *Q. ilex* could
31 greatly benefit from favourable winter conditions exhibiting a high photosynthetic potential
32 and carbon metabolism. Angiosperms are known to make efficiently use of favourable winter

1 periods to recover depleted carbon reserves and embolism induced loss of hydraulic capacity
2 (Carnicer et al., 2013 and references therein). When these relatively favourable conditions
3 changed, *Q. ilex* quickly re-adjusted the photosynthetic machinery to the prevailing
4 conditions, as indicated by the largest decreases in photosynthetic potential and carbon
5 metabolism. Some researchers have proposed the lutein-epoxy cycle in photoprotection of
6 *Quercus* as a mechanism to maintain sustained energy dissipation (Garcia-Plazaola et al.,
7 2003b), which could help to account for the higher tolerance to low temperatures in *Q. ilex*
8 relative to other co-occurring Mediterranean trees or shrubs (Ogaya and Peñuelas, 2003,
9 2007). *P. halepensis* did not suffer a pronounced chronic photoinhibition, confirming the
10 results by Martínez-Ferri *et al.* (2004). Despite a pronounced downregulation of
11 photosynthetic electron transport and an increase in alternative electron sinks, the light-
12 saturated ambient photosynthesis and stomatal conductance remained surprisingly high and
13 constant. *P. halepensis* thus exhibited a successful refinement of photosynthetic electron flow
14 and possibly a successful repair of protein D1 in the PSII reaction centre. The strong
15 downregulation in *Q. ilex* and the homogenous response of *P. halepensis* were possibly due to
16 distinct, previously described strategies. *Q. ilex* has been characterised as a photoinhibition-
17 avoiding species and *P. halepensis* as a photoinhibition-tolerant species (Martinez-Ferri et al.,
18 2000). We have extended this categorisation for *A. unedo*, a less photoinhibition-tolerant tree
19 species, which favoured carbon metabolic processes at the cost of chronic photoinhibition and
20 photodamage. This strategy is similar to those in other semi-deciduous shrubs (Oliveira and
21 Penuelas, 2001; Oliveira and Peñuelas, 2004). The physiological responses of *Q. ilex*, a
22 slowly growing late-successional species, to environmental stressors are highly plastic
23 (Zavala *et al.*, 2000) due to its vegetative activity in a wide range of temperatures and high
24 stomatal control in stressful conditions (Gratani et al., 2000; Savé et al., 1999), high plasticity
25 index and resprouting dynamics (Espelta et al., 1999; Gratani et al., 2000), deep rooting
26 system and large carbohydrate pools (Canadell and Lopez-Soria, 1998; Canadell et al., 1999),
27 and high adaptive variability in foliar phenomorphology (Sabaté et al., 1999). Our findings
28 showed the intra-crown variability in *Q. ilex*, where shaded leaves were widely unaffected by
29 the inhibitory cold stress (Oliveira and Penuelas, 2001). The ability of *Q. ilex* to perform rapid
30 metabolic changes in the antioxidant and photoprotective mechanisms could be of adaptive
31 importance (García-Plazaola et al., 1999a). In contrast, *P. halepensis* is a fast growing conifer
32 that quickly occupies open spaces after disturbances such as fires (Zavala *et al.*, 2000). *P.*
33 *halepensis*, as do all pines, has a low ability to store carbohydrates and therefore follows a

1 strategy of water conservation and embolism avoidance (Meinzer et al., 2009). High rates of
2 photosynthesis and growth require high concentrations of carboxylation enzymes in the
3 carbon cycle that have high maintenance costs (Valladares and Niinemets, 2008), perhaps
4 accounting for the high respiration rates found in *P. halepensis* leaves. Moreover, differences
5 among the species are also likely to be the result of distinct foliar morphologies and crown
6 architectures. Pine trees are characterised by a relatively low exposure of foliar surface area to
7 direct sunlight due to the cylindrical shape and steep angles of their needles but at the same
8 time are able to exploit a wider range of incident light angles than broadleaved trees. Despite
9 reported flexible adjustments in the orientation of the leaves in several Mediterranean
10 broadleaved sclerophyllic species (Oliveira and Peñuelas, 2000; Vaz et al., 2011; Werner et
11 al., 2002), needle leaves probably still confer some benefits to attain near-saturated
12 photosynthetic rates over a wider range of diurnal and seasonal variation in sun angles (Jordan
13 and Smith, 1993; Lusk et al., 2003), while at the same time showing a high tolerance to
14 photoinhibition. This might account for the good performance of *P. halepensis* under mild
15 winter conditions with moderate abiotic stresses such as in our study. However, under more
16 severe and re-occurring frost events, *P. halepensis* might reach the threshold of its tolerance
17 and severe frost damage can occur. This explains also its absence in mountain regions with
18 more severe winters where *Q. ilex* becomes more competitive. Despite following distinct
19 physiological strategies, both *Q. ilex* and *P. halepensis* seem to cope equally well with the
20 winter conditions they were exposed to whereas the foliar photosynthetic systems of *A. unedo*
21 were more sensitive to sudden frost impacts. Thus, *A. unedo* might have been in a competitive
22 disadvantage for the following growing season.

23 Overall, we conclude that the photosynthetic exploitation of relatively favorable winter
24 conditions might be crucial for evergreen Mediterranean tree species for achieving a positive
25 annual carbon balance. The winter period might give important insights helping to explain the
26 dynamics of Mediterranean forest communities when withstanding increased novel
27 environmental conditions projected in multiple climate change scenarios and benefitting from
28 periods of potential recovery and growth in winter.

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- 3

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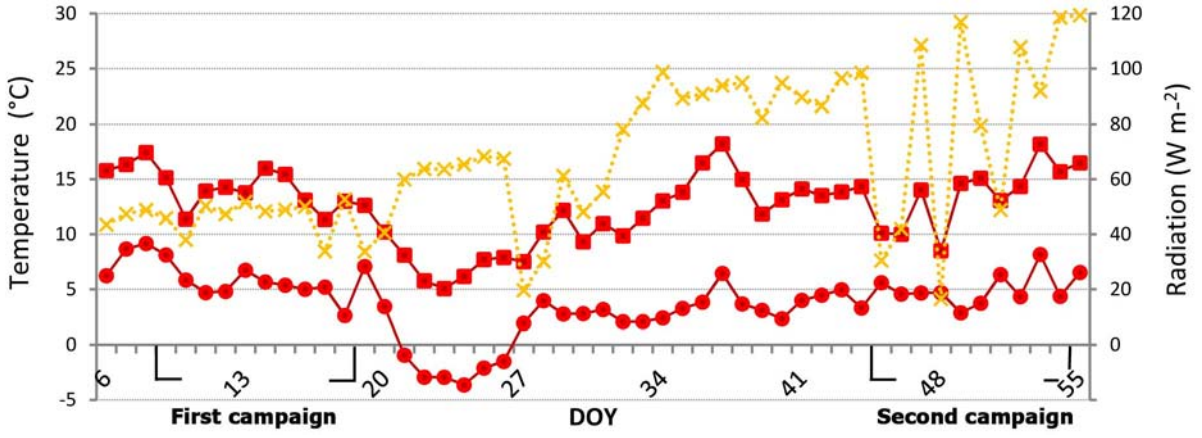
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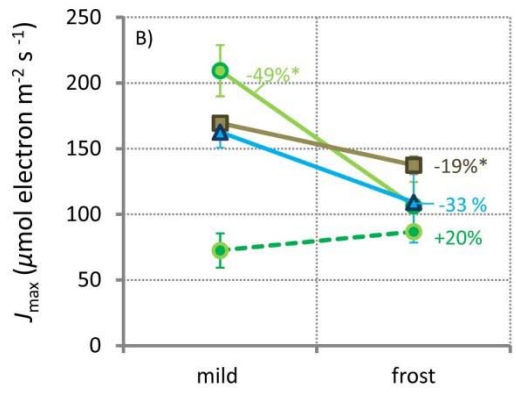
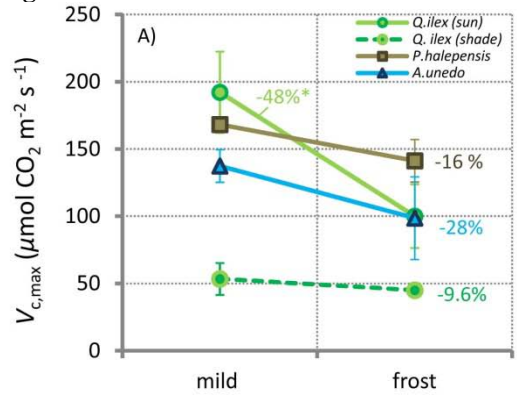
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1 **Fig.1**
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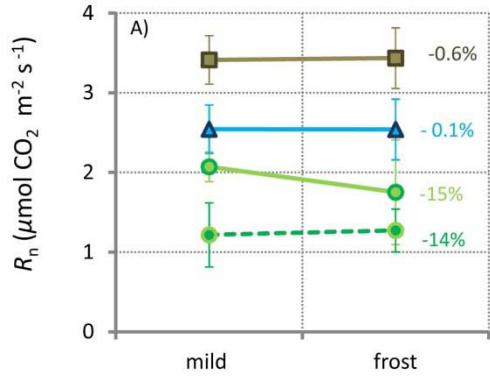
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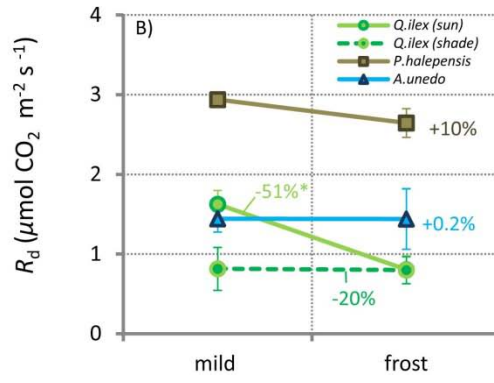
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Fig.3

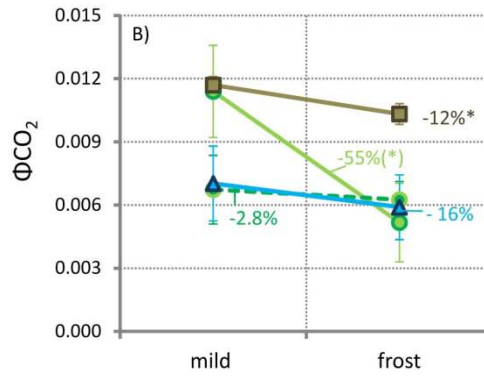
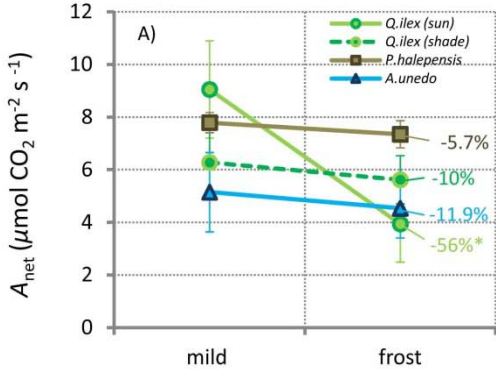


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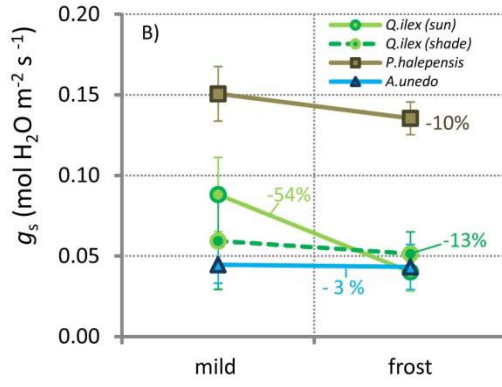
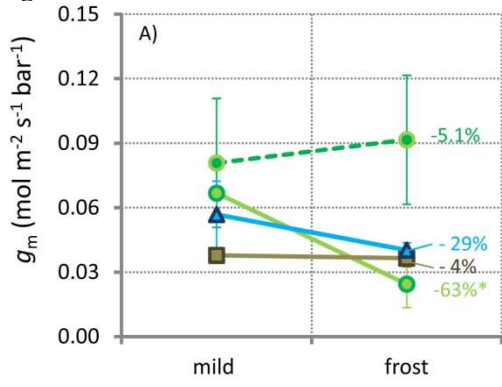
Fig.4



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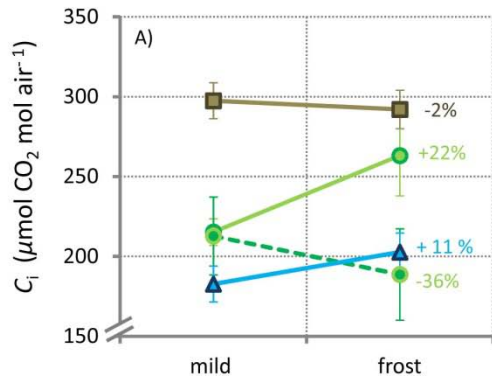
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Fig.5

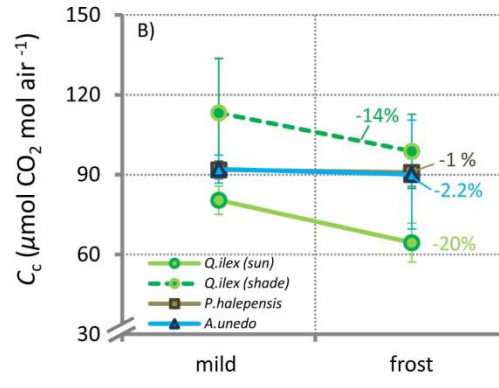


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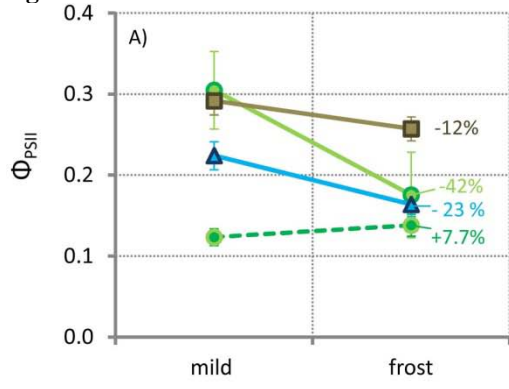


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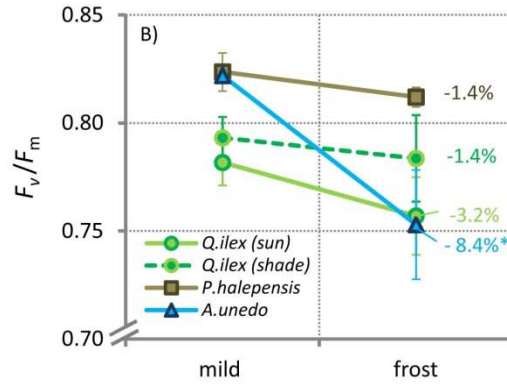


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Fig.7

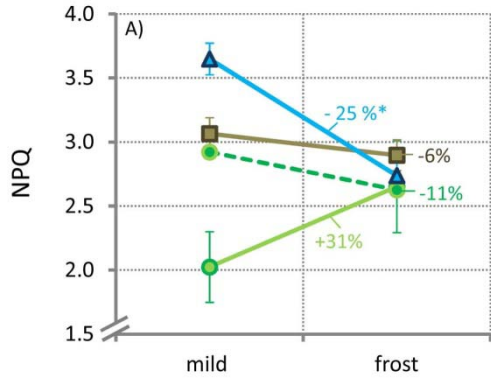


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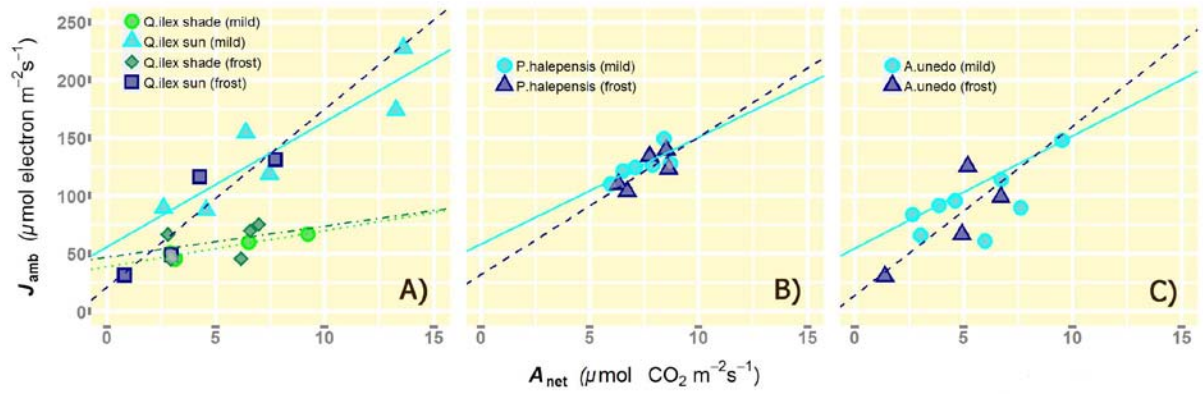
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Fig.8



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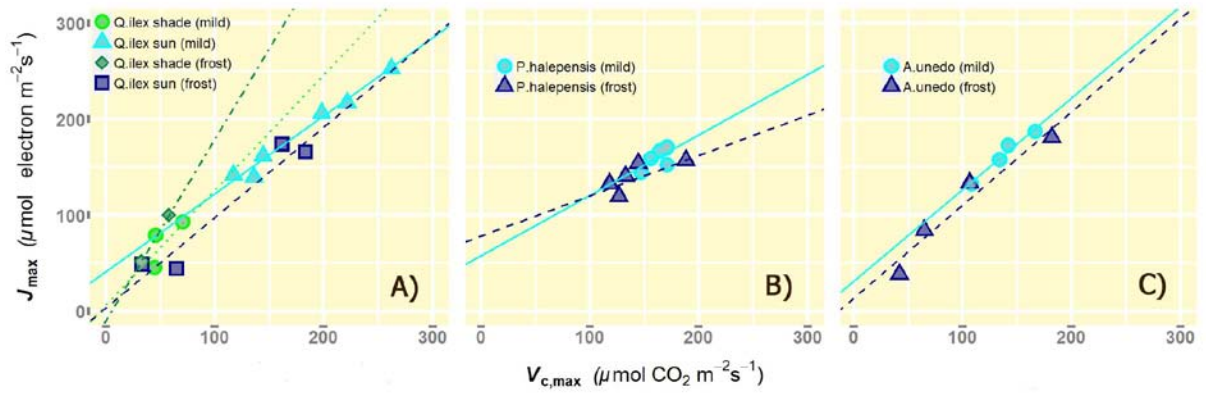
1 **Fig.10**



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Fig.11



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1 **Tables**

2 **Table 1:** *P* values of Student's *t*-tests for the differences between sunlit and shaded leaves of
 3 *Q. ilex*.

	all	mild	frost
$V_{c,max}$	0.001	0.002	0.172
J_{max}	0.006	0.002	0.553
J/V	0.279	0.797	0.249
F_v/F_m	0.611	0.533	0.535
A_{net}	0.546	0.594	0.745
g_s	0.156	0.791	0.127
C_i	0.151	0.326	0.154
g_m	0.041	0.066	0.107
C_c	0.138	0.364	0.203
CUE	0.151	0.728	0.439
R_n	0.061	0.470	0.356
R_l	0.016	0.004	0.577
J_{amb}/A_{net}	0.052	0.014	0.203
Φ_{PSII}	0.290	0.315	0.825
Φ_{CO2}	0.750	0.886	0.497
qp	0.195	0.045	0.882
NPQ	0.192	0.903	0.126
$\Delta(C_a-C_i)$	0.037	0.321	0.068
$\Delta(C_i-C_c)$	0.043	0.073	0.113
$\Delta(C_a-C_c)$	0.023	0.006	0.122

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 5

1 **Table 2.** Regression coefficients and results from ANCOVA analyses of the J_{amb}/A_{net} and $J_{max}/V_{c,max}$ relationships.

Regression analyses of J_{max} & $V_{c,max}$															
tree species	<i>Q. ilex</i>			<i>Q. ilex</i>			<i>P. halepensis</i>			<i>A. unedo</i>			<i>all species</i>		
leaf position	sunlit			shaded			sunlit			sunlit			sunlit		
	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P
mild	$y = 0.81x + 41.6$	0.97	2E-04	$y = 1.2x + 6.1$	0.48	0.193	$y = 115.9x + 148.8$	0.04	0.32	$y = 0.954x + 31.5$	0.95	0.017	$y = 50.2x + 0.77$	0.94	1.4E-07
frost	$y = 0.94x + 3.6$	0.89	0.035	$y = 1.89x - 9.19$			$y = 971x + 9.9$	0.53	0.1	$y = 0.97x + 13.7$	0.91	0.029	$y = 10.5x + 0.93$	0.90	7.2E-05
p (slope)	5.76E-02			0.83			0.022			0.69			0.072		
p (intercept)	8.91E-09			0.3			0.058			0.28			0.008		
Regression analyses of J_{amb} & A_{net}															
tree species	<i>Q. ilex</i>			<i>Q. ilex</i>			<i>P. halepensis</i>			<i>A. unedo</i>			<i>all species</i>		
leaf position	sunlit			shaded			sunlit			sunlit			sunlit		
	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P	reg. line	R^2	P
mild	$y = 10.8 + 56.1$	0.76	0.014	$y = 3.1x + 39$	0.91	0.029	$y = 9.22x + 58.3$	0.51	0.068	$y = 9.7x + 54.9$	0.96	0.005	$y = 10.9x + 51.9$	0.84	7.1E-06
frost	$y = 15.4x + 21.1$	0.73	0.093	$y = 2.7x + 46.8$	-0.13	0.52	$y = 11.9x + 31.9$	0.52	0.105	$y = 14.6x + 14.2$	0.46	0.200	$y = 13.5x + 22.3$	0.76	1.7E-04
p (slope)	0.337			0.72			0.59			0.322			0.098		
p (intercept)	0.51			0.45			0.31			0.29			0.071		

2

3

1 **Figure 1.** Maximum and minimum temperatures on the primary y-axes (in red squares and
2 circles, respectively) and radiation (in yellow crosses) on the secondary y-axes are presented
3 for the mild and frost winter period for the day of the year (DOY) in January and February
4 2012.

5 **Figure 2.** Effect of a sudden period of frost following a mild winter period in 2012 on A) the
6 maximum velocity of carboxylation ($V_{c,max}$) and B) the maximum rate of electron transport
7 (J_{max}) in sunlit leaves of *Q. ilex* (circles), *P. halepensis* (squares), and *A. unedo* (triangles) and
8 in shaded leaves of *Q. ilex* (circles and dashed lines). The error bars represent the standard
9 error, and the percentages indicate the change between periods where significance is indicated
10 with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

11 **Figure 3.** Effect of a sudden period of frost following a mild winter period on A) nighttime
12 respiration (R_n) and B) daytime respiration (R_d) in sunlit leaves of *Q. ilex* (circles), *P.*
13 *halepensis* (squares), and *A. unedo* (triangles) and in shaded leaves of *Q. ilex* (circles and
14 dashed lines). The error bars represent the standard error, and the percentages indicate the
15 change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and
16 marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

17 **Figure 4.** Effect of a sudden period of frost following a mild winter period on A) net
18 assimilation (A_{net}) and B) the effective quantum yield of net CO₂ assimilation (Φ_{CO_2}) in
19 sunlit leaves of *Q. ilex* (circles), *P. halepensis* (squares), and *A. unedo* (triangles) and in
20 shaded leaves of *Q. ilex* (circles and dashed lines). The error bars represent the standard error,
21 and the percentages indicate the change between periods where significance is indicated with
22 an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

23 **Figure 5.** Effect of a sudden period of frost following a mild winter period on A) mesophyll
24 conductance (g_m) and B) stomatal conductance (g_s) in sunlit leaves of *Q. ilex* (circles), *P.*
25 *halepensis* (squares), and *A. unedo* (triangles) and in shaded leaves of *Q. ilex* (circles and
26 dashed lines). The error bars represent the standard error, and the percentages indicate the
27 change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and
28 marginal significance with an asterisk in brackets ($0.05 \geq P \leq 0.1$).

29 **Figure 6.** Effect of a sudden period of frost following a mild winter period on A) the stomatal
30 internal CO₂ concentration (C_i) and B) the chloroplastic CO₂ concentration (C_c) in sunlit
31 leaves of *Q. ilex* (circles), *P. halepensis* (squares), and *A. unedo* (triangles) and in shaded

1 leaves of *Q. ilex* (circles and dashed lines). The error bars represent the standard error, and the
2 percentages indicate the change between periods where significance is indicated with an
3 asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \geq P \geq 0.1$).

4 **Figure 7.** Effect of a sudden period of frost following a mild winter period on A) the effective
5 quantum yield of photosystem II (Φ_{PSII}) and B) the maximum efficiency of photosystem II
6 (F_v/F_m) in sunlit leaves of *Q. ilex* (circles), *P. halepensis* (squares), and *A. unedo* (triangles)
7 and in shaded leaves of *Q. ilex* (circles and dashed lines). The error bars represent the
8 standard error, and the percentages indicate the change between periods where significance is
9 indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets
10 ($0.05 \geq P \geq 0.1$).

11 **Figure 8.** Effect of a sudden period of frost following a mild winter period on non-
12 photochemical quenching (NPQ) in sunlit leaves of *Q. ilex* (circles), *P. halepensis* (squares),
13 and *A. unedo* (triangles) and in shaded leaves of *Q. ilex* (circles and dashed lines). The error
14 bars represent the standard error, and the percentages indicate the change between periods
15 where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an
16 asterisk in brackets ($0.05 \geq P \geq 0.1$).

17 **Figure 7.** Relationship between the maximum velocity of carboxylation ($V_{c,max}$) and the
18 maximum rate of electron transport (J_{max}) in *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C),
19 leaves. Leaves measured under mild conditions are indicated by green circles and cyan
20 triangles in shaded and sunlit locations, respectively. Leaves measured after the period of
21 frost are indicated by green diamonds and blue squares in shaded and sunlit locations,
22 respectively.

23 **Figure 8.** Relationship between the rate electron transport from chlorophyllic fluorescence
24 (J_{amb}) and net assimilation (A_{net}) at ambient CO₂ concentrations and saturating light (A_{net}) in
25 *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C), leaves. Leaves measured under mild conditions
26 are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively.
27 Leaves measured after the period of frost are indicated by green diamonds and blue squares in
28 shaded and sunlit locations, respectively.

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1 Appendix A

2 Temperature functions

3 The effective Michaelis-Menten constants K_c and K_o and the photorespiratory compensation
4 point, Γ^* , were taken from (Bernacchi et al., 2002) and are summarized in Table 3. The
5 following generic temperature response functions were used to adjust these parameters to the
6 prevailing T_{Leaf} during the experiments

$$7 \quad K_c = e^{\left(c - \left(\frac{\Delta Ha}{R \times (273.15 + T_{Leaf})} \right) \right)} \quad (A1)$$

8 (13)

9 and

$$10 \quad K_o = e^{\left(c - \left(\frac{\Delta Ha}{R \times (273.15 + T_{Leaf})} \right) \right)} \quad (A2)$$

11 (14)

12 and

$$13 \quad \Gamma^* = e^{\left(c - \left(\frac{\Delta Ha}{R \times (273.15 + T_{Leaf})} \right) \right)} \times \frac{O_2}{20.9} \quad (A3)$$

14 (15)

15 where R is a unitless gas constant (0.008314), c is a scaling constant, ΔHa represents the
16 activation energy and O_2 is the oxygen concentration of the ambient air assumed to be 20.9
17 kPa.

18 CF- parameters

$$19 \quad NPQ = \frac{(F_m - F_m')}{F_m'} \quad (A4)$$

20 Photochemical quenching (qP) indicates the proportion of open PSII reaction centres and
21 tends to be highest in low light when leaves use light most efficiently (Maxwell and Johnson,
22 2000). qP was estimated by:

$$1 \quad qP = \frac{F_m' - F_s}{F_m' - F_o'} \quad (A5)$$

2 where F_o' is the minimum fluorescence in a light-adapted leaf after a pulse of darkness. The
 3 rate of photosynthetic electron transport, J_{CF} , is calculated by (Genty et al., 1989):

4 **Estimation of mesophyll conductance**

5 The CO_2 pathway leads from the atmosphere to the intercellular air spaces through the
 6 stomata and from there diffuses through the air spaces of the mesophyll, cell walls, cytosol,
 7 and chloroplastic envelopes and finally reaches the sites of CO_2 fixation in the chloroplastic
 8 stroma where it is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In
 9 this study, we call this pathway the internal mesophyll diffusion conductance (g_m) and
 10 estimate it with the variable- J method by Harley et al. (1992):

$$11 \quad g_m = \frac{A_{net}}{C_i - \frac{[\Gamma^* J_{CF} + 8(A_{net} + R_d)]}{J_{CF} - 4(A_{net} + R_d)}} \quad (A7)$$

12 where Γ^* is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate
 13 of photosynthetic uptake of CO_2 (Table 3). Similarly to g_s , g_m is defined as a unitless molar
 14 fraction, rendering the units for conductance the same as those for photosynthesis.
 15 Nonetheless, the drawdown of CO_2 from the intercellular airspaces to the sites of
 16 carboxylation is thought to be dominated by the liquid phase of the chloroplast and is hence
 17 dependent on the partial pressure of the gas according to Henry's law (Harley et al., 1992).
 18 The units for conductance ($mol\ m^{-2}\ s^{-1}\ bar^{-1}$) are thus directly comparable to g_s when the
 19 atmospheric pressure is 1 bar. We assumed normal pressure (1.01325 bar) in our experiments
 20 that were conducted in Barcelona, which is close to sea level. The variable- J method accounts
 21 for the variation in g_m with C_i and provides more accurate estimates of photosynthetic
 22 parameters than do A/C_c curves that assume a constant g_m , especially during episodes of water
 23 stress (Flexas et al., 2007). The chloroplastic CO_2 concentration can then be determined using
 24 C_i , A_{net} , and g_m :

$$25 \quad C_c = C_i - \frac{A_{net}}{g_m} \quad (A8)$$

26 where C_c is the chloroplastic CO_2 concentration.

27

1 **Appendix C**

2

3 **Table C1.** The scaling constant (c) and energies of activation (ΔH_a) describing the
4 temperature responses for Rubisco enzyme kinetic parameters K_c , K_o and Γ^* . Taken from
5 Bernacchi et al., (2002).

6

	25°C	c	ΔH_a	unit
K_c	27.24	35.98	80.99	Pa
K_o	16.58	12.38	23.72	kPa
Γ^*	3.74	11.19	24.46	Pa

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