UNIVERSITAT DE BARCELONA

Mediterranean forests in a changing environment. Impacts of drought and temperature stress on tree physiology

Dominik Sperlich

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Mediterranean Forests in a Changing Environment - Impacts of Drought and Temperature Stress on Tree Physiology

Tesis Doctoral
Dissertation
by

Dominik Sperlich

May,
2015

Supervised by
Santi Sabaté & Josep Peñuelas

Cover design:

Haifa Debouk

Dominik Sperlich

“Trees are poems the earth writes upon the sky,

We fell them down and turn them into paper,

That we my record our emptiness”

Kahlil Gibran Kahlil

Mediterranean forests in a changing environment - Impacts of drought and temperature stress on tree physiology

Boscos mediterranis en un ambient canviant - Impactes sobre la fisiologia dels arbres causats per la sequera i l'estrés per temperatura

Programa de Doctorat en Ecologia Fonamental i Aplicada

Memòria presentada per Dominik Sperlich
per optar el Grau de Doctor per la Universitat de Barcelona
amb el vist i plau dels directors de tesi:

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To my wife

To my family

Contents

Acknowledgments / Agraïments

This is the story of a dissertation on 284 pages. It is a scientific story. But it is also a very personal story. A story of problems, mistakes, challenges and a lot of learning. A story of ignorance, indecision, procrastination, but also a story of the evolution to expertise, self-assurance and creativeness. Nothing can be read about this on the following 284 pages and nothing is written about all the friends, family, colleagues and my partner although they have all contributed sincerely to make this thesis happen! It is a personal story of a joint venture and here I take the opportunity to thank them all.

First and foremost, I would like to thank my main supervisor Dr. Santi Sabaté and co-supervisor Prof. Josep Peñuelas. Santi, thank you for giving me the chance to pursue this project, for guiding me through the last 4 and a half years, for your great flexibility and trust in me. Josep, thank you for your additional motivation, support and ideas which have greatly enriched my work.

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I am very grateful to the GREENCYCLESII ITN-Marie-Curie project. I found myself in a very diverse group of amazing people that greatly motivated and inspired me and taught me many new things about life and science. I believe the network we have built up will truly last.

Special thanks are devoted to my parents Harald and Barbara and my siblings Julia, Florian and Sebastian who have always been so supportive and interested in what I am doing. Ich bin wirklich dankbar dafür, dass Ihr soviel Interesse zeigt … dafür dass Ihr soviel Verständnis für mich habt … einfach dafür, dass Ihr da seid!!

Dr. Mohamed Debouk w Samira Debouk, shoukran ktir 3ala kil shi. Badde echkerkon 3ala wjoudkon w 3ala da3emkon eleh!

Without you I cannot imagine that I would have taken this path, without you I wouldn’t have started this PhD, without you I wouldn’t have gone through it. You showed so much support, understanding and patience. Haifa, I want to thank you for your love, you have been and you are essential to me and also to this thesis.

Acronyms

Acronym	Unit	Variable name
A_c	µmol CO_2 m^{-2} s^{-1}	Net assimilation rate limited by Rubisco carboxylation
A_j	µmol CO_2 m^{-2} s^{-1}	Net assimilation rate limited by RuPB regeneration
A_{net}	µmol CO_2 m^{-2} s^{-1}	Net assimilation rate
A_o	µmol CO_2 m^{-2} s^{-1}	Net assimilation rate at thermal optimum
A_p	µmol CO_2 m^{-2} s^{-1}	Net assimilation rate limited by triose-phosphate use
c	unitless	Scaling constant
C_a	µmol CO_2 mol air^{-1}	ambient CO_2 concentration
C_{area}	g m^{-2}	Carbon concentration per unit leaf area
C_c	µmol CO_2 mol air^{-1}	Chloroplastic internal CO_2 concentration
C_i	µmol CO_2 mol air^{-1}	Stomatal internal CO_2 concentration
C_{mass}	%	Carbon concentration per unit leaf mass
CUE_i	unitless	foliar intrinsic carbon use efficiency
D	mg cm^{-3}	Leaf density
DBH	cm	Diameter at breast height
DW	mg	Dry weight
F_m	µmol photon m^{-2} s^{-1}	Maximum fluorescence of a dark adapted leaf
F_m'	µmol photon m^{-2} s^{-1}	Maximum fluorescence of a light adapted leaf
F_o	µmol photon m^{-2} s^{-1}	Minimum fluorescence of a dark adapted leaf
F_o'	µmol photon m^{-2} s^{-1}	Minimum fluorescence of a light adapted leaf
F_s	µmol photon m^{-2} s^{-1}	Steady state fluorescence
F_v/F_m	unitless	Maximum quantum efficiency of PSII
FW	mg	Fresh weight
g_m	mol m^{-2} s^{-1} bar^{-1}	Mesophyll internal conductance
g_s	mol H_2O m^{-2} s^{-1}	Stomatal conductance

Table continued

Acronym	Unit	Variable name
H_a	J mol^{-1}	Activation energy
H_d	J mol^{-1}	Deactivation energy
J_{amb}	µmol electron m^{-2} s^{-1}	Electron transport rate at ambient CO_2, saturating light and 25 °C
J_{cf}	µmol electron m^{-2} s^{-1}	Electron transport rate from chlorophyll fluorescence
J_{max}	µmol electron m^{-2} s^{-1}	Maximum electron transport rate
J_{opt}	µmol electron m^{-2} s^{-1}	Electron transport rate at thermal optimum
J_t	kg d^{-1}	Sap flow per tree
K_c	Pa	Michaelis-Menten constant of Rubisco for CO_2
K_o	kPa	Michaelis-Menten constant of Rubisco for O_2
LA	cm^{-2}	Leaf area
LMA	mg cm^{-2}	Leaf mass per area
LT	mm	Leaf thickness
N_{area}	g m^{-2}	Nitrogen concentration per unit leaf area
N_{mass}	%	Nitrogen concentration per unit leaf mass
NPQ	unitless	Nonphotochemical quenching
O	kPa	Partial pressure of O_2 at Rubisco
Ω	K^{-1}	Ohm, difference in temperature from T_{opt} at which J falls to e^{-1} (0.37) of its value at T_{opt}
PPFD	µmol photons m^{-2} s^{-1}	Photonflux density
qP	unitless	Photochemical quenching
R	kJ mol^{-1} K^{-1}	Gas constant
R_d	µmol CO_2 m^{-2} s^{-1}	Day respiration
R_n	µmol CO_2 m^{-2} s^{-1}	Night respiration
S	mg H_2O cm^{-2}	Succulence
SE	unitless	standard error

Table continued

Acronym	Unit	Variable name
SWC	cm^3 cm^{-3}	Soil water content
T_{block}	°C	Block temperature
T_k	Kelvin	Leaf temperature
T_{leaf}	°C	Leaf temperature
T_{opt}	°C	Thermal optimum
TPU	µmol P_i m^{-2} air^{-1}	Triose-phosphate use
$V_{c,max}$	µmol CO_2 m^{-2} s^{-1}	Maximum carboxylation velocity
VPD	kPa	Vapour pressure deficit
WC	%	Leaf water content

Summary

The Mediterranean Basin is a climate-change hotspot of the world. Predicted reductions in annual precipitation, increases in mean temperature, and increases in the variability and occurrence of extreme droughts and heat waves are likely to affect species abundance and distribution. The existence of sympatric plants with different morphological and phenological strategies raises the question how they will respond to novel climate conditions. There is a strong need to improve the mechanistic understanding of key foliar ecophysiological parameters in response to abiotic stressors on a small scale if we are to predict the carbon budget of plant ecosystems in larger scales.

We first reviewed contrasting growth responses to temperature of angio- and gymnosperms in the Iberian Peninsula. Secondly, we studied the seasonal acclimation of different foliar ecophysiological traits in two leaf positions of four Mediterranean tree species in extensive field experiments. We aimed to shed light on the mechanistic understanding of the foliar respiratory and photosynthetic responses to abiotic stress such as drought and temperature.

We found contrasting demographic responses in Mediterranean conifer and angiosperm trees. Widespread forest successional advance of angiosperms and negative growth responses of gymnosperms to temperature are currently occurring in the Iberian Peninsula. Trait-based differences in these two groups contribute to explain their different responses to temperature and their different role during successional processes.

The acclimation behaviour of photosynthetic and morphological traits to seasonal variable growth conditions was strongly pronounced in all tree species. Photosynthetic machineries were resilient to moderate drought, whereas severe drought induced acclimation of morphological traits, photosynthetic downregulation and leaf abscission. The lack of replenishment of soil-water reserves during the early growing season critically enforced the summer drought.

We also observed a notable seasonal acclimation of the thermal optima and of the curvature of temperature responses of photosynthetic assimilation. The photosynthetic system was better acclimated to lower temperatures in winter and to heat stress in the drier and hotter year.

Mild winter temperatures provided a period of growth and recovery that resulted in biochemical recovery, new shoot growth, and moderate transpiration across all evergreen species. High radiation and sudden low temperatures had a combinatory negative effect on the photosynthetic apparatus leading to photoinhibitory stress - especially in sunlit leaves.

Species-specific acclimation partly offset these overall trends in responses to drought and temperature stress. *Quercus ilex* L. and to a lesser extent *Q. pubescens* Wild. showed the highest plasticity in photosynthetic traits whereas *Pinus halepensis* Mill. was most tolerant across the seasons with the most stable temperature response pattern. *Arbutus. unedo* L. was the most vulnerable to drought and photoinhibitory stress in winter. *A. unedo* and *Q. pubescens* had a less sclerophyllic leaf habit and invested the least in acclimation of the morphological structure being most vulnerable to drought-induced leaf abscission. Shaded leaves showed generally a lower photosynthetic potential, but cushioned negative impacts under stress periods.

A long-term rainfall-exclusion experiment in a *Q. ilex* forest increased the foliar carbon-use efficiency and the plasticity of foliar respiratory and photosynthetic traits, but did not affect the biochemical photosynthetic potential. A favourable growth period was thus exploited more efficiently.

Overall, our results indicate that Mediterranean climax-species exhibit a strong acclimatory capacity to warmer and drier conditions, but can be sensitive to extreme drought and extreme temperature stress. The performance of the plants during winter might give important insights in the dynamics of Mediterranean forest communities under novel environmental conditions. Leaf position is an indispensable factor when estimating the canopy carbon balance. Angiosperms and gymnosperms had fundamental different photosynthetic strategies of stress-avoidance versus stress-tolerance, respectivley.

Resumen

El cambio climático aumentará la sequía en la Cuenca Mediterránea y posiblemente afectará a la abundancia y la distribución de especies. Revisamos las respuestas contrastadas del crecimiento a la temperatura de angio- y gimnospermas en la Península Ibérica. Estudiamos la variación de los efectos del estrés térmico y por sequía en rasgos morfológicos, fotosintéticos y de la respiración foliar según la especie y la posición en el dosel. Además, evaluamos el efecto de una sequía crónica sobre la respiración foliar y la fotosíntesis de Quercus ilex L. La maquinaria fotosintética se mostró resiliente frente a la sequía moderada, mientras que la sequía extrema, agravada por las bajas reservas de agua en el suelo, indujo la aclimatación de la morfología foliar, la inhibición de la bioquímica fotosintética y la abscisión foliar. El sistema fotosintético se aclimató mejor a las temperaturas bajas que al estrés por calor. Las temperaturas suaves en invierno derivaron en la recuperación bioquímica, un nuevo crecimiento de los brotes y una transpiración moderada. La elevada radiación y el frío repentino mostraron un efecto combinado negativo, causando estrés fotoinhibitorio. El estrés térmico y por sequía fue más pronunciado en hojas de sol y aminorado en hojas de sombra. Q.ilex y, en menor grado, Q. pubescens Wild. mostraron la plasticidad más elevada de los rasgos fotosintéticos, mientras que Pinus halepensis Mill. fue más tolerante, mostrando la respuesta más estable a la temperatura. Arbutus unedo L. fue la especie más vulnerable a la sequía y al estrés fotoinhibitorio. En respuesta a la sequía crónica, Q. ilex incrementó la eficiencia en el uso del carbono y la plasticidad de los atributos fotosintéticos y de respiración foliar, pero no afectó al potencial fotosintético. En resumen, las especies climácicas mediterráneas se aclimatan frente a condiciones más cálidas y secas, pero pueden ser sensibles ante sequías extremas. El funcionamiento durante el invierno es vital para entender la dinámica de los bosques mediterráneos. La posición de las hojas en la copa es indispensable para estimar el balance de carbono del dosel. Angiospermas y gimnospermas presentan estrategias fotosintéticas contrastadas, de evitación y tolerancia del estrés, respectivamente.

Report by thesis supervisors
Informe dels directors de tesi

In the following a short review is given by the thesis supervisors, Santi Sabaté and Josep Peñuelas on the impact factor of the published articles and the contribution in each article of the PhD student, Dominik Sperlich.

Article 1. Published in *Frontiers in Plant Science*. Impact factor: 3.6

Carnicer J,* Barbeta A*, **Sperlich D***, Coll M, Peñuelas J. 2013. Contrasting trait syndromes in angiosperms and conifers are associated with different responses of tree growth to temperature on a large scale. *Frontiers in Plant Science* 4: 409.

JC, AB and DS shared first authorship. JC, AB and DS developed the initial areas, worked out the hypothesis and synthesized the information in discussion. JC contributed strongly in "Empirical patterns in the Iberian Peninsula: The negative synergistic effects of increased temperatures and forest successional advance". AB contributed strongly in the chapter "A review of the diverse hypotheses that may explain contrasting growth responses to temperature in Mediterranean gymnosperm and angiosperm trees". DS contributed strongly in the chapter "Complex and multiple effects of temperature and drought on the tree physiology". MC contributed with data. JC wrote the final version of the article. JP participated in the work design and helped in correcting and editing manuscript.

Article 2. Published in *Tree Physiology*. Impact factor: 3.4

Sperlich D, Chang CT, Peñuelas J, Gracia C, Sabaté S. 2014. Foliar photochemical processes and carbon metabolism under favourable and adverse winter conditions in a Mediterranean mixed forest, Catalonia (Spain). *Biogeosciences* 11: 5657–5674.

DS carried out the main research tasks: design, field measurements data analyses and writing of the article. CTC contributed in the field campaigns and data analyses. SS participated in the work design. All authors helped in the interpretation of the results, in the discussion and in the correction of the final version of the manuscript.

Article 3. Published in *Biogeosciences*. Impact factor: 3.8

Sperlich D, Chang CT, Peñuelas J, Gracia C, Sabaté S. 2014. Foliar photochemical processes and carbon metabolism under favourable and adverse winter conditions in a Mediterranean mixed forest, Catalonia (Spain). *Biogeosciences* 11: 5657–5674.

This study was conducted in parallel to the work presented in Article 2 and the contributions were the same.

Article 4. Submitted to *New Phytologist*; 18th March 2015. Impact factor: 6.6

Sperlich D, Chang CT, Penuelas J, Gracias C, Sabaté S (2015) Thermal plasticity of photosynthesis in a natural Mediterranean forest. *Submitted to New Phytologist*.

This study was conducted in parallel to the work presented in Article 2 and the contributions were the same.

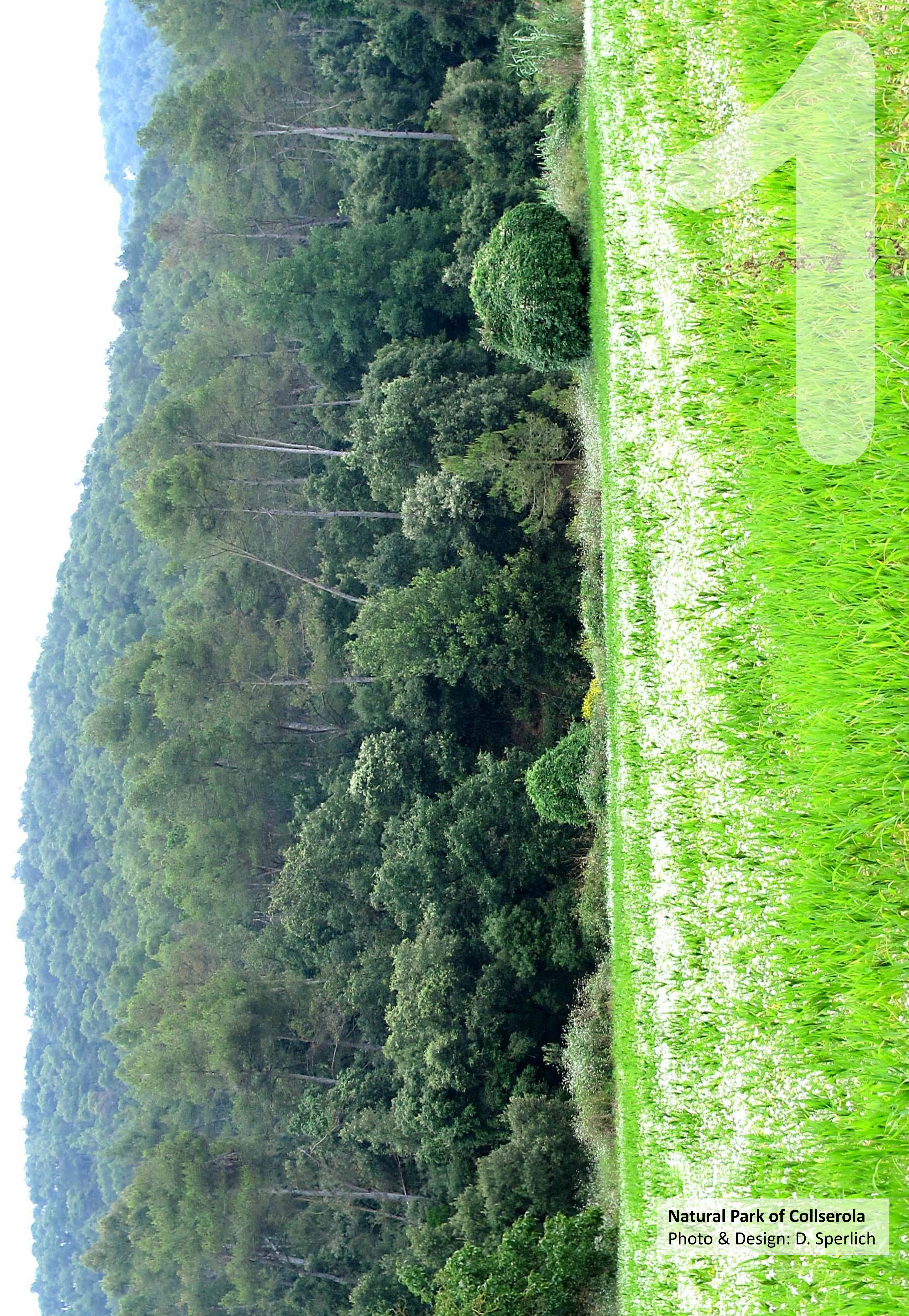
Article 5. Submitted to *New Phytologist*; 24th April 2015. Impact factor: 6.6

Sperlich D, Barbeta A, Ogaya R, Sabaté S, Penuelas J (2015) Balance between carbon uptake and release: impacts of long-term drought on foliar photosynthesis and respiration in *Quercus ilex* L. *Submitted to New Phytologist*.

The PhD student carried out the main research tasks: design, field measurements, data analyses and writing the article. AB and RO contributed in the field campaigns. All authors helped in the interpretation of the results, in the discussion and in the correction of the final version of the manuscript.

Signatures

....	....
Santi Sabaté	Josep Peñuelas



1

Natural Park of Collserola
Photo & Design: D. Sperlich

1 General introduction and objectives

1.1. Global climate change and impacts in the Mediterranean region

Mankind has shaped our environment in an unprecedented manner for the past 250 years. Anthropogenic activities such as unregulated fossil fuel burning, high industrial activity, and widespread deforestation and land use change has led to a **sharp increase in greenhouse gases in the atmosphere as well as to a warming of the climate system** (IPCC, 2013) (Fig. 1.1). A new chronological term was proposed to account for the current geologic epoch – the Anthropocene. Global atmospheric CO_2, for instance, has reached presently concentrations of 400 ppm which is unsurpassed for over 2-million years after oscillating between 180 and 300 ppm and which is 40% higher than pre-industrial levels (IPCC, 2001; Bussotti *et al.*, 2014). Future climate change scenarios predict further increases in the global atmospheric CO_2 concentration and of the Earth mean surface temperature by the end of 2100 (IPCC, 2013). These changes likely strengthen drought events in terms of intensity, frequency and geographic expanse – particularly in arid or semi-arid regions (Somot *et al.*, 2008; Friend, 2010; IPCC, 2013) (Fig. 1.1). In these regions, high evaporative demand and low soil water content during the summer dry period are naturally the main ecological drivers limiting plant growth and productivity (Specht, 1969; Di Castri, 1973). 35-40 % higher air temperatures are predicted in the Mediterranean region relative to global levels within 2050 paralleled by predictions of drastic reductions in precipitation (Giorgi, 2006; Christensen *et al.*, 2007; Sheffield & Wood, 2008). **The Mediterranean Basin was thus defined as one of the "climate- change hotspots" of the world** (Giorgi, 2006). In past decades, ecosystem models on regional or global levels contributed substantially to our understanding of the implications of climate change on a coarse scale where field experiments are limited (Luo, 2007). Much uncertainty, however, remains in the modelled feedback of the global carbon cycle to climatic warming (Booth *et al.*, 2012; Friedlingstein *et al.*, 2014) and in the understanding and modelling of vegetation responses to climate change (Luo, 2007; McDowell *et al.*, 2008; Beaumont *et al.*, 2008). Not only changes in mean climate variables, but also increased climate variability with greater risk of extreme weather events - such as prolonged drought, storms and floods - **question the adaptive capability of forest ecosystems** because the long life-span of trees does not allow for rapid adaptation to environmental changes (Lindner *et al.*, 2010). This is underscored by recent reports showing that most woody species operate generally at narrow hydraulic safety margins against drought-induced mortality (Choat *et al.*, 2012; Choat, 2013). If water stress persist over longer periods, especially when combined with other stress factors such as heat waves or nutrient limitations, it is possible that the amount of fixed CO_2 by photosynthesis does not compensate the amount of CO_2 released by respiration and therefore, a negative annual carbon budget is reached leading eventually to a depletion of carbon reserves (Niinemets, 2010). Increased drought-induced defoliation (Poyatos *et al.*, 2013) associated with the depletion of carbon reserves (Galiano *et al.*, 2012) can finally lead to catastrophic hydraulic failure and tree mortality (Urli *et al.*, 2013; Choat, 2013). Drought induced forest impacts and forest diebacks in the Mediterranean region were reported by numerous studies (Peñuelas *et al.*, 2001; Martínez-Vilalta & Piñol, 2002; Raftoyannis *et al.*, 2008; Allen *et al.*, 2010; Carnicer *et al.*, 2011; Matusick *et al.*, 2013) leading ultimately to vegetation shifts (Jump & Penuelas, 2005; Anderegg *et al.*, 2013) and increasing the risk of forest fires (Piñol *et al.*, 1998; Pausas *et al.*, 2008). **The sensitivity of forest ecosystems to climate change is alarming because they are major players in the global carbon cycle due to their contribution via climate-carbon feedbacks and their regulation of our climate by carbon stores** (Cox *et al.*, 2000; Boisvenue & Running, 2006; Friend, 2010).

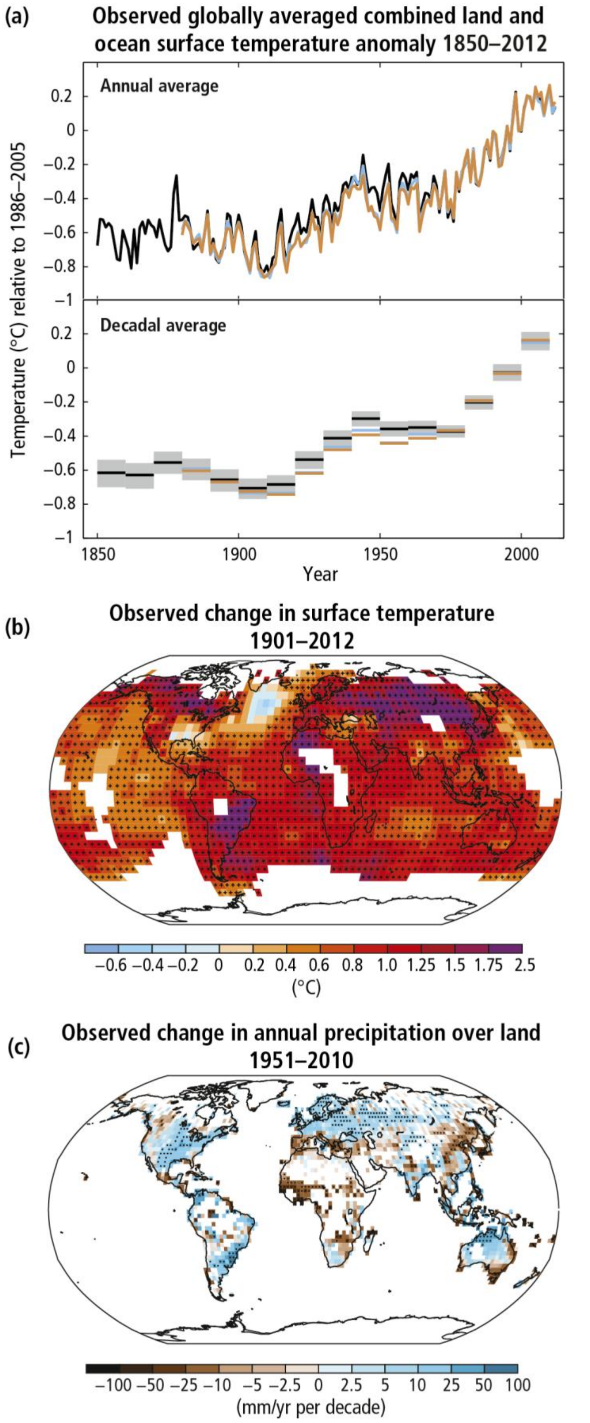


Fig. 1.1. (a) Observed global mean combined land and ocean surface temperature anomalies, from 1850 to 2012 from three data sets. Top panel: annual mean values. Bottom panel: decadal mean values including the estimate of uncertainty for one dataset (black). Anomalies are relative to the mean of 1961-1990. (b) Map of the observed surface temperature change from 1901 to 2012 derived from temperature trends determined by linear regression from one dataset (orange line in panel a). Trends have been calculated where data availability permits a robust estimate (i.e., only for grid boxes with greater than 70% complete records and more than 20% data availability in the first and last 10% of the time period). Other areas are white. Grid boxes where the trend is significant at the 10% level are indicated by a + sign. Modified from IPCC (2013).

1.2. Forests in a changing environment - A walk from the chloroplast to the globe

Forests have recently gained new attention in international debates discussing their potential role in mitigating the climate change (FAO, 2011). A changing environment – what do forests say? Responses of forest ecosystems to changes in precipitation patterns and temperature regimes are inevitably enmeshed in two elementary life processes: photosynthesis and respiration. Photosynthesis converts photochemical solar energy into plant biochemical compounds whereas respiration provides the energy in form of ATP and NADPH and the carbon skeletons for biosynthesis in celluar processes (Fig. 1.2) (Taz & Zeiger, 2010). Carbon dynamics of forest ecosystems form a delicate balance between photosynthetic carbon uptake and respiratory release which are highly sensitive to water deficits and temperature (Flexas *et al.*, 2012; Yamori *et al.*, 2014). They are an integral part of the global carbon cycle and coupled with the climate system because sink-driven processes of atmospheric CO_2 emissions will likely become less efficient under future climate conditions and potentially turn forests into net sources (Friedlingstein *et al.*, 2006; Phillips *et al.*, 2009; Fatichi *et al.*, 2014). Within the climate research community, there is a significant range of uncertainty in predicted temperature, water availability, and atmospheric CO_2 (Friedlingstein *et al.*, 2006; Booth *et al.*, 2012). **Plant photosynthetic and respiratory responses to increasing drought and high temperature were thus identified as key research needs to better understand the climate-carbon feedbacks** (Booth *et al.*, 2012; Bussotti *et al.*, 2014).

Green plants are solar energy collectors converting light energy into chemical energy and supporting the Earth's primary production. Photosynthesis is thus the cornerstone of life as a large fraction of our planet's energy resources result from photosynthetic activity in either recent or ancient times (fossil fuels). The *light reactions* take place in the chloroplast internal thylakoid membranes (Fig. 1.3). Chlorophyll antenna pigments funnel the solar energy down to the reaction centres and initiate a cascade of excited electrons between photosystem (PS) II and I culminating in the synthesis of ATP and NADPH. The maximum potential rate of this electron transport chain is named $J_{\max}$. The end products of the light reactions NADPH and ADP provide the biochemical

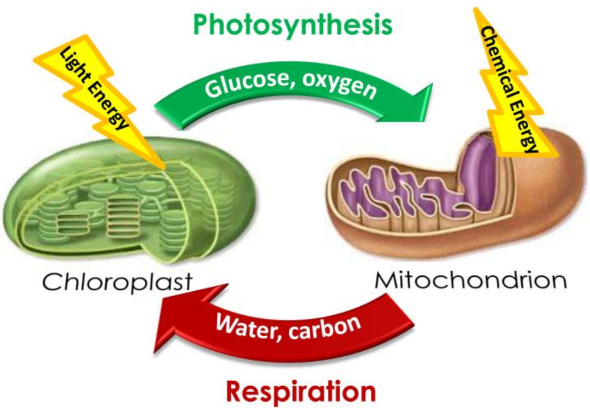


Fig. 1.2 | Elementary life processes: Scheme of chloroplastic photosynthesis and mitochondrial respiration. Pictures of chloroplast and Mitochondrion from http://rmbioblog.blogspot.fi/2012_12_01_archive.html .

energy for the *photosynthetic carbon cycle* in the aqueous stroma of the chloroplasts surrounding the thylakoids where carbon is carboxylated with the crucial enzyme Rubisco (Taz & Zeiger, 2010). Before arriving to the sites of carboxylation, however, carbon crosses rough terrain, as shown in Fig. 1.4: CO_2 has to diffuse from the atmosphere inside the leaf passing the guard cells and stomatal openings (stomatal conductance, g_s). From the stomatal internal air spaces, the journey continues through the mesophyll of the leaf until the CO_2 arrives finally to the chloroplasts where it has to pass gaseous, liquid, and lipid phases (mesophyll conductance, g_m) before it can finally be assimilated and carboxylated by Rubisco (Niinemets *et al.*, 2011). The parameter that describes the maximum velocity of the CO_2 carboxylation by Rubisco is termed $V_{c,max}$. The importance of g_m as a secondary diffusion limitation has only recently been given warrantable attention and recognition (Flexas *et al.*, 2008). g_m can play a significant role under abiotic stress periods highlighting its importance for estimating the whole-carbon gain (Keenan *et al.*, 2010). Terrestrial biosphere models, however, commonly assume infinite values of g_m and the issue of whether (and how) to include g_m in models is actively debated by physiologists and modellers (Rogers *et al.*, 2014). The chloroplasts are thus regarded as the power stations of cells where carbon is assimilated in order to form carbohydrates. **g_s and g_m thus form the diffusion limitation for the net rate of carbon assimilation (A_{net}), and J_{max} and $V_{c,max}$ define the biochemical limitation for A_{net}.** In ecophysiology of terrestrial photosynthesis, these are the key factors to be analysed when dealing with photosynthetic responses, adaptation and acclimation to a changing environment.

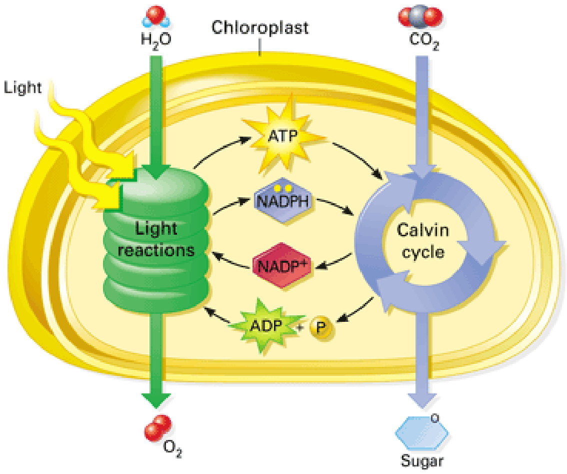


Fig. 1.3 | Power stations of the cells: Scheme of a chloroplast depicting the light reactions in the thylakoid membranes and the carbon reactions of the Calvin cycle. Image from http://www.neshaminy.org/Page/20741 .

Respiration (R) takes place in the mitochondria of the leaves and releases the energy of the photosynthetic carbon compounds in a controlled manner for cellular maintenance and growth. The energy and carbon skeletons provided by R are vital for all cells with several functions for biosynthesis and photosynthesis (Tcherkez & Ribas-Carbó, 2012). R also contributes to significant carbon losses altering the net carbon gain - especially under stress conditions (Van Oijen *et al.*, 2010). Nonetheless, it is difficult to elucidate to which extent the net carbon gain is altered because the mitochondrial-driven CO_2 efflux under dark conditions (night respiration, R_n) is inhibited in the presence of light (day respiration, R_d) as a composite effect of multiple cellular pathways (Heskel *et al.*, 2013). This makes it challenging when trying to measure R_d because the mitochondrial carbon release is masked by the net photosynthetic carbon assimilation

(Haupt-Herting *et al.*, 2001; Pinelli & Loreto, 2003; Yin *et al.*, 2011). **The complete physiological basis behind the inhibition of R_n under light remains not yet fully understood just as there remains a lack of knowledge how seasonality and abiotic stressors affects the balance of R_n with R_d.** This is due to measurement constrains on the one hand, but also lacking research priorities on the other hand (Atkin & Macherel, 2009; Heskel *et al.*, 2014).

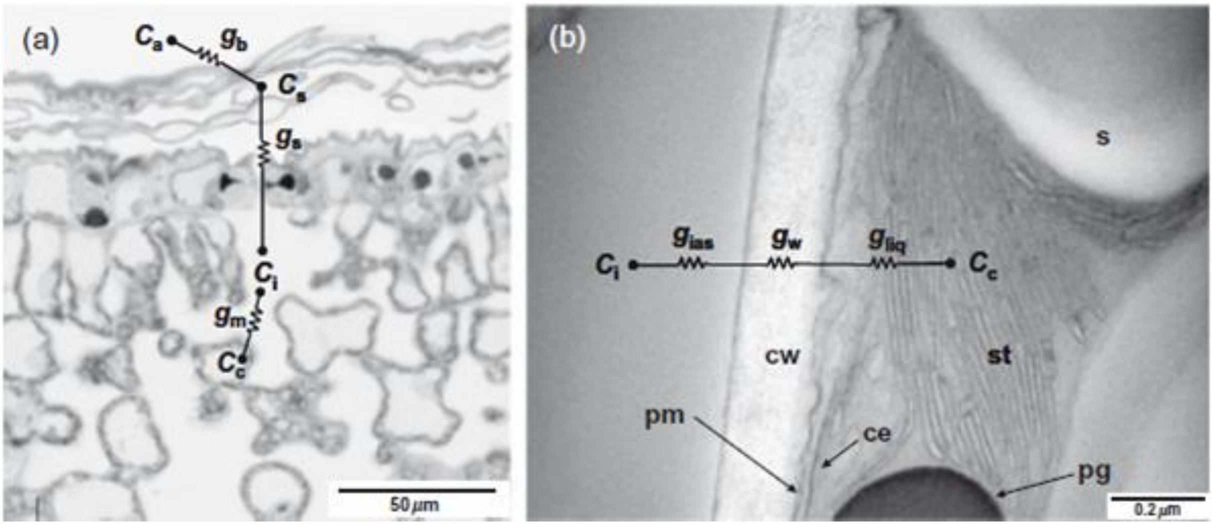


Fig. 1.4 | (a) Micrograph of the abaxial surface of an olive leaf (bottom side up), where the stomata can be seen, as well as the pathway of CO_2 from ambient (C_a) through leaf surface (C_s) and intercellular air spaces (C_i) to the chloroplast (C_c). Boundary layer conductance (g_b), stomatal conductance (g_s) and mesophyll conductance (g_m) are indicated. (b) Electron micrograph of a grapevine leaf where cell wall (c_w), plasma membrane (pm), the chloroplast envelope (ce) and stroma thylakoid (st) can be observed. The pathway of CO_2 from C_i to chloroplastic CO_2 (C_c) is characterized by intercellular air space conductance to CO_2 (g_{ias}), through cell wall (g_w) and through the liquid phase inside the cell (g_{liq}). A grain of starch (s) and a plastoglobule (pg) can be also observed in the picture (photos by A. Diaz-Espejo). Modified from Flexas *et al.* (2008)

The three parameters R, J_{max} and $V_{c,max}$ are critical for scaling up foliar photosynthesis to the canopy level at which global dynamic models operate (Fig. 1.5), but they are not easily measured. So relatively little data of their variability between species or seasons are available (Flexas *et al.*, 2012). R, $V_{c,max}$ and J_{max} are thus often used as constants for various plant functional types and seasons or, in some cases, are derived from other parameters such as leaf nitrogen content (Grassi & Magnani, 2005; Walker *et al.*, 2014). R_d is often taken as half the rate of R_n to account at least for some degree of inhibition (Niinemets, 2014). Besides, little is known on the impact of g_m on photosynthesis in response to abiotic stresses (Keenan *et al.*, 2010). **In this thesis, we try to improve the mechanistic understanding of these parameters on a small scale as they are critical components in modelling the carbon budget of plant ecosystems at larger scales** (Fig. 1.5).

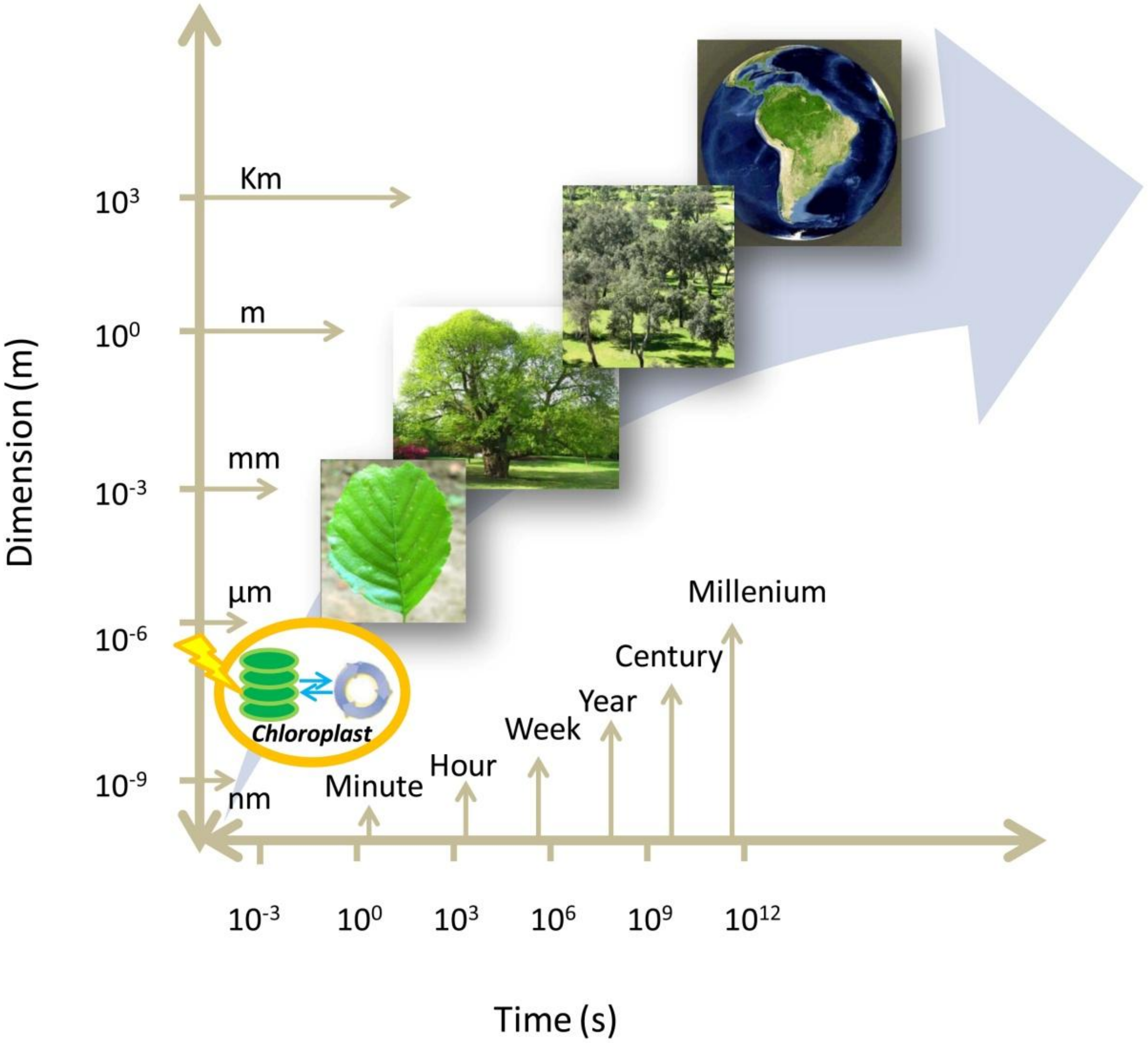


Fig.1.5 | Different time and dimension scales for photosynthetic events (modified from Osmond *et al.*, 2004 and Flexas *et al.*, 2012.

1.3. Drought and temperature stress - “Hot” topics in the Mediterranean

Temperature is a major environmental factor contributing to the natural distribution of species and limiting plant growth and productivity (Mittler, 2006). **The growth environment determines the temperature optimum for photosynthesis and respiration, although changes in the growth temperature can lead to acclimation of this optimum** (Medlyn *et al.*, 2002; Rennenberg *et al.*, 2006; Kattge & Knorr, 2007). Higher plants, particularly evergreen tree species, have a strong capacity for temperature acclimation, including a higher tolerance to heat stress in summer and a capacity for cold hardening in winter (Aschmann, 1973; Orshan, 1983; Blumler, 1991). This ability provides them with a high flexibility throughout the year to benefit from favourable periods, e.g. winter (Gratani, 1996; Ogaya & Peñuelas, 2003). A better understanding of the acclimation of photosynthesis to temperature and the improvement of the predictive capacity of temperature-response models have received renewed interest against the background of climate change and global warming (Medlyn *et al.*, 2002; Kattge & Knorr, 2007; Bunce, 2008; Yamori *et al.*, 2014). Both light and carbon reactions are optimally balanced at the temperature optimum, but limitations occur as the temperature decreases or increases (Taz & Zeiger, 2010). At low temperatures, a decreased enzyme activity and limited phosphate availability can limit net photosynthesis (Sage & Sharkey, 1987; Sage & Kubien, 2007). At higher temperatures, photorespiration is stimulated while photosynthesis is inhibited leading to a decreased quantum yield of CO_2 (Ehleringer & Björkman, 1977; Rennenberg *et al.*, 2006). Additionally, the heat lability of Rubisco activase decreases the CO_2 carboxylation by Rubisco (Law & Crafts-brandner, 1999; Haldimann & Feller, 2004). Nonetheless, these factors are not the primary causes of photosynthetic decline at high temperatures. Rather, the PSII has been characterised as the primary target of heat-induced stress, whereas PSI is comparatively heat resistant (Haldimann & Feller, 2004 and references herein). Few studies have thoroughly assessed the seasonal acclimations of thermal optima and curvatures under natural conditions, especially in the Mediterranean region despite its particular vulnerability to climate changes. The negative effects of temperature on the photosynthetic system and the feedback to global carbon cycle remain a key uncertainty in scenarios of future climate change. In this thesis, we aim to shed light on how seasonal thermal acclimation differs between tree species with different morphological and phenological strategies and under distinct leaf light environments.

Dehydration is constantly affecting plants due to the high evaporative demand of the atmosphere. Plans have evolved several mechanisms to prevent leaf desiccation. As soil water declines, stomata close to minimize water loss and to reduce the risk of hydraulic failure. Stomatal closure, however, impairs the diffusion of the CO_2 needed in the chloroplasts, the sites of carboxylation. The temporary unemployment of Rubisco due to limited substrate availability (CO_2) leads to its de-activation and, during chronic water stress, to its decomposition (Parry, 2002; Chaves & Oliveira, 2004; Lawlor & Tezara, 2009). High incoming radiation that cannot efficiently be dissipated in the Calvin cycle over-excites the photoreaction centres (photoinhibition) and produces reactive oxygen species (ROS) that damage the photosystems and the ATP synthase- needed for the carbon reactions (Epron *et al.*, 1993). Leaves prevent harmful excess of energy with protective actions such as the reorganisation of the thylakoid membrane, closure of reaction centres, and reduced antennal size (Huner *et al.*, 1998; Maxwell & Johnson, 2000; Ensminger *et al.*, 2012; Verhoeven, 2014). These actions reduce PSII efficiency and J_{max}, and enhance alternative energy pathways to prevent damage on the molecular level on the cost of lower carbon assimilation. **The plants thus face a trade-off between reduced carbon fixation and the negative effects of desiccation and over-excitation of the foliar physiology.**

1.4. The ecological context of warming and drought impacts and research needs

The coexistence of plants with different morphological and phenological strategies such as evergreeness and deciduousness, broadleaved and coniferous raises the question how they will respond to contrasting seasonal environmental conditions. In the Iberian Peninsula two big genera from distinct phylogenetic groups dominate the tree species distribution: *Pinus*- species from the gymnosperms, *Quercus*- species from the angiosperm. Which strategy rules out which when facing future climate change scenarios? The demographic performances of trees depend much on their ecophysiological strategies that determine their distribution and abundance in responses to global climate changes. Tree species with evergreen leaves have generally a strong capacity to acclimate and adapt to adverse conditions through photosynthetic downregulation, foliar-trait acclimation, and improved gas exchange (Villar-Salvador *et al.*, 1997; Ogaya & Peñuelas, 2006; Limousin *et al.*, 2009). Shrubby species, in contrast, often show drought-induced leaf abscission and branch dieback, but also a strong resprouting capacity (Ogaya & Peñuelas, 2004). Trees with a deciduous leaf habit have to maximise gas exchange during a shorter growing season. Therefore, they usually show a less conservative hydraulic strategy (Baldocchi *et al.*, 2009). The "low-cost" leaves of the deciduous trees might facilitate drought senescence, so that the reduced transpiratory surface area can effectively avoid damage from hydraulic cavitation and xylem embolism (Ogaya & Peñuelas, 2004, 2006; Barbeta *et al.*, 2012, 2013). Stored non-structural carbohydrates (NSC) strongly determine the recovery of xylem hydraulic conductivity by vessel refilling and the resistance of water transport to drought under prolonged evaporative demand (Ogasa *et al.*, 2013). Depleted NSCs may limit the ability to recover from embolism (Galiano *et al.*, 2012). Pines follow a strategy of water conservation and embolism avoidance, because they have a low capacity to store carbohydrates (Meinzer *et al.*, 2009). Most pines are therefore characterised by an isohydric gas exchange behaviour and strict stomatal control. *Quercus* species, in contrast, show generally more plastic hydraulic features and a good ability in vessel refilling after xylem embolism (Fotelli *et al.*, 2000; Corcuera *et al.*, 2004; Carnicer *et al.*, 2013). Hence, they usually show an anisohydric and water-spending gas exchange behavior. Over extensive areas of the Mediterranean region *Quercus species* form the terminal point of secondary succession whereas pines are usually the pioneers after fire regeneration (Lookingbill & Zavala, 2000). Recent large-scale studies have reported forest successional advances and contrasting responses of growth to temperature in angiosperm and coniferous trees in the Iberian Peninsula that may be attributed to contrasting trait-based ecophysiological strategies (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013).

The scientific background for this thesis was described in this introduction comprising global climate changes and the particular role of forests, the vulnerability of the Mediterranean Basin to climate change, the impacts of abiotic stress on the tree ecophysiology and the demographic performance of Mediterranean forests. In the following, research needs are identified and key research questions are described.

Research question 1

Angiosperm and coniferous ecophysiological strategies may differentially integrate diverse traits such as stomatal sensitivity to vapor-pressure deficit (VPD), hydraulic safety margins and capacity for embolism repair, which in turn are linked to features of the xylem such as NSC content, carbon transfer rates, wood parenchymal fraction and wood capacitance. Are plant growth, development, and survival under environmental stresses dependent on trait-based differences in these two groups? How do these strategies contribute to explain their different responses to temperature and their different role during successional processes in this region?

Research question 2

Characterizing the nature of photosynthetic and morphological responses under different levels of drought and temperature stress is essential to enable the development of accurate models. However, the mechanistic understanding of foliar ecophysiological responses to seasonal changes and abiotic stress is very limited. How do mature Mediterranean trees cope with the highly dynamic seasonality of favourable and unfavourable conditions from summer drought to winter cold? How are key photosynthetic and morphological traits fine-tuned seasonally under natural field conditions and what are the implications for terrestrial biosphere models?

Research question 3

Most ecophysiological studies have been conducted in spring and summer, and winter has been surprisingly overlooked despite its importance for our understanding of the dominance of certain vegetation types and of the responses of vegetation to stress, seasonality and species composition. How do co-occurring species with different functional and structural traits cope with adverse and favourable winter conditions? Are leaves from different crown positions differently affected by winter stress? What role plays winter for evergreen trees in the overall annual carbon balance?

Research question 4

Temperature is a determining factor in the Mediterranean Basin, but surprisingly little information is available for photosynthetic sensitivity and acclimation in Mediterranean tree species. The improvement of the predictive capacity of temperature-response models have received renewed interest against the background of climate change and global warming. How to model best photosynthetic temperature responses? Do thermal optima and curvatures of net assimilation and electron transport acclimate to seasonal changes in a natural Mediterranean forest?

Does seasonal thermal acclimation differ in tree species with different morphological and phenological strategies and in distinct leaf light environments?

Research question 5

In order to elucidate the long-term effects of drought and temperature stress on carbon exchange dynamics in water-limited environments, long-term experiments of partial rainfall exclusion are needed to characterise the respiratory responses to drought relative to photosynthetic carbon gain. How are variations of photosynthetic and respiratory traits of *Q. ilex* affected by seasonal changes in temperature and precipitation from winter over spring to summer? What are the biochemical boundaries and mechanisms of photosynthesis and respiration to seasonal acclimation and drought adaptation? How do foliar intrinsic water and carbon use efficiency (WUE_i and CUE_i) respond to simulated long-term drought?

1.5. Major objectives

This thesis is divided into 8 chapters. Chapter 1 has introduced the general research topics and research questions. The Chapters 2-6 will address the key research questions 1-5 (respectively). Chapter 7 provides an overall discussion over all chapters. Chapter 8 draws the main conclusions from the findings. In the following, we define our major objectives for each chapter to answer the research questions.

Objective 1 (Chapter 2)

The second chapter provides a review about the differences in ecophysiological traits associated with temperature- and drought- induced responses in conifer and angiosperm trees. The main aims in this study are: (i) to list the different hypotheses that may explain contrasting growth responses to temperature in Mediterranean conifer and angiosperm trees and review the differences in ecophysiological traits associated with temperature- and drought- induced responses in these two groups, (ii) to briefly review the multiple effects of temperature on basic tree ecophysiological functions (e.g. photosynthesis, growth, respiration and nutrient uptake and transport), (iii) to analyze the specific case study of forests in the Iberian Peninsula, which present diverging tree growth responses to temperature in angiosperms and conifers, and (iv) to briefly discuss the implications of the findings

Objective 2 (Chapter 3)

The research interests of this chapter is to distinguish the species-specific strategies and to explore the ecophysiological mechanism behind drought responses by examining the fine tuning of foliar photosynthetic potentials/rates and foliar morphological traits. The specific aims were to assess the effect of seasonal environmental changes (above all drought) on (i) photosynthetic and (ii) morphological traits, (iii) to evaluate the extent to which mesophyllic diffusion conductance (g_m) constrains photosynthesis under drought conditions and to investigate how seasonal acclimation varies qualitatively and quantitatively with (iv) species and (v) light environment (leaf canopy position). Ultimately, the aim is to provide a matrix of photosynthetic parameters that could be incorporated into process-based ecosystem models for improving the estimations of carbon flux in the Mediterranean region.

Objective 3 (Chapter 4)

The aims are to (i) investigate the foliar physiology of three evergreen tree species under mild winter conditions, (ii) analyse the effect of sudden changes from favourable to unfavourable conditions on photochemical and non-photochemical processes associated with electron transport, CO_2 fixation and heat dissipation, (iii) determine whether leaves exhibit distinct locational (sunlit or shaded) responses to winter stress, and (iv) identify the species-specific strategies when coping with stress, induced by low temperatures and frost.

Objective 4 (Chapter 5)

In chapter 5, the first goal is to evaluate which formulation models best photosynthetic temperature responses: the peaked function or June's model. The second objective is to answer the question if thermal optima and curvatures of net assimilation and electron transport acclimate to seasonal changes in a natural Mediterranean forest. The third aim is to investigate if seasonal thermal acclimation differs between tree species with different morphological and phenological strategies and under distinct leaf light environments.

Objective 5 (Chapter 6)

Chapter 5 addresses the question how variations of photosynthetic and respiratory traits of *Q. ilex* are affected by seasonal changes in growth temperature and precipitation from winter over spring to summer in combination with a long-term rainfall exclusion experiment. The objective is to study the impact of long-term drought on key limitations of photosynthesis comprising stomatal, mesophyllic and biochemical components as well as mitochondrial respiration at day and night. Our aim is to evaluate the response pattern of the foliar intrinsic water and carbon use efficiency (WUE_i and CUE_i) in order to understand better the boundaries and mechanisms of photosynthesis and respiration to seasonal acclimation and long-term drought adaptation.

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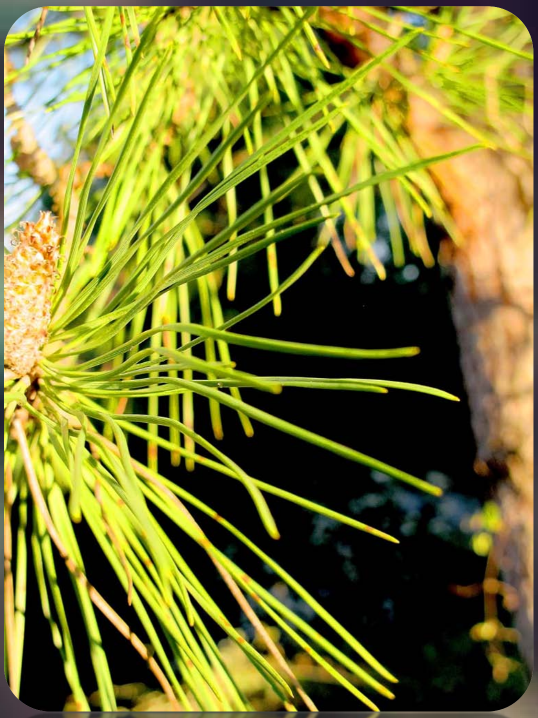

Flower and needles of *Pinus halepensis*
Photo & Design: D. Sperlich

Chapter 2

Contrasting trait syndromes in angiosperms and conifers are associated with different responses of tree growth to temperature on a large scale

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2.1 Abstract

Recent large-scale studies of tree growth in the Iberian Peninsula reported contrasting positive and negative effects of temperature in Mediterranean angiosperms and conifers. Here we review the different hypotheses that may explain these trends and propose that the observed contrasting responses of tree growth to temperature in this region could be associated with a continuum of trait differences between angiosperms and conifers. Angiosperm and conifer trees differ in the effects of phenology in their productivity, in their growth allometry, and in their sensitivity to competition. Moreover, angiosperms and conifers significantly differ in hydraulic safety margins, sensitivity of stomatal conductance to vapor-pressure deficit, xylem recovery capacity or the rate of carbon transfer. These differences could be explained by key features of the xylem such as non-structural carbohydrate content (NSC), wood parenchymal fraction or wood capacitance. We suggest that the reviewed trait differences define two contrasting ecophysiological strategies that may determine qualitatively different growth responses to increased temperature and drought. Improved reciprocal common garden experiments along altitudinal or latitudinal gradients would be key to quantify the relative importance of the different hypotheses reviewed. Finally, we show that warming impacts in this area occur in an ecological context characterized by the advance of forest succession and increased dominance of angiosperm trees over extensive areas. In this context, we examined the empirical relationships between the responses of tree growth to temperature and hydraulic safety margins in angiosperm and coniferous trees. Our findings suggest a future scenario in Mediterranean forests characterized by contrasting demographic responses in conifer and angiosperm trees to both temperature and forest succession, with increased dominance of angiosperm trees, and particularly negative impacts in pines.

2.2 Introduction

The assimilation and allocation of carbon are fundamental processes allowing tree growth, development, survival, reproduction and defense (McDowell, 2011; Sala et al., 2012; Galiano et al., 2012). In addition, non-structural carbohydrates (NSCs) play a variety of functions in tree physiology, providing a temporal buffer to reconcile differences in carbon supply and demand, maintaining hydraulic transport and facilitating osmotic regulation, allowing leaf emergence and bud burst and actively participating in the prevention of frost and drought embolism and repair (Sala et al., 2012). The demographic performance of trees, however, is generally co-limited by other factors that frequently interact in complex ways with the processes of carbon uptake and allocation, such as direct climatic effects on photosynthesis, growth and nutrient uptake (Körner 1998, 2003; Rennenberg et al., 2006), species-specific traits (Wright et al., 2004; Chave et al. 2009; Carnicer et al. 2012) or the impacts of secondary consumers and diseases (Bale et al., 2002).

Recent ecophysiological studies highlight the coupled dynamic links between NSC content in woody tissues and several climate-dependent tree responses such as embolism prevention and repair, growth, bud burst and leaf emergence (Johnson et al., 2012; Sala et al., 2012; Meinzer and McCulloh, 2013). These studies suggest the existence of contrasting trait-based ecophysiological strategies in major plant groups (Johnson et al., 2012; Choat et al., 2012; Meinzer et al., 2013) such as angiosperm and coniferous trees. Arguably, a next necessary step is to analyze how these contrasting ecophysiological strategies may be influencing the distribution and abundance of tree species and their responses to global warming.

Recent large-scale studies have reported contrasting responses of growth to temperature in angiosperm and coniferous trees in Mediterranean forests of the Iberian Peninsula (Gómez-Aparicio et al., 2011; Coll et al., 2013). For example, Gómez-Aparicio et al. (2011) reported a positive effect of rising temperatures on growth of angiosperm trees, but neutral or negative effects on coniferous trees. These contrasting trends between the two phylogenetic groups were later also observed and confirmed by Coll et al. (2013). Critically, whereas a reduction in precipitation was predicted to decrease tree growth in both groups, increases in temperature could produce a performance disadvantage in conifers compared to angiosperm broadleaved trees (Gómez-Aparicio et al., 2011; Coll et al., 2013). Consistent with these empirical findings that associate the negative effects of temperatures and growth in *Pinus* species, palaeoecological studies suggest a persistent link between Pinaceae distributions and low temperatures during the last 100 million years (Millar 1993; Brodribb et al., 2012). Cold periods in the Paleocene and Eocene are associated with an increased abundance of fossils of the genus *Pinus*, and the reverse occurs during warm periods (Millar 1993; Brodribb et al., 2012). Similarly, warm periods during the Miocene and Pliocene are apparently associated with northward contractions of the ranges of Pinaceae species (Millar 1993; Brodribb et al., 2012). Notably, the ecophysiological basis of these contrasting growth and distributional responses to temperature remain poorly discussed and resolved.

Here we review the hypotheses that may contribute to explain the observed contrasting responses of growth to temperature observed in Mediterranean conifers and angiosperms. We review the differences between Mediterranean conifer and angiosperm trees in growth-related traits, including phenology, crown allometry, sensitivity to competition, and drought and winter freezing responses. Furthermore, we hypothesize that angiosperm and coniferous ecophysiological strategies differentially integrate diverse traits such as stomatal sensitivity to vapor-pressure deficit (VPD), hydraulic safety margins and capacity for embolism repair, which in turn are linked to features of the xylem such as NSC content, carbon transfer rates, wood parenchymal fraction and wood capacitance. In sum, our main aims in this study are: i) to list the different hypotheses that may explain contrasting growth responses to temperature in Mediterranean conifer and angiosperm trees and review the differences in eco-physiological traits between Mediterranean conifer and angiosperms trees associated with temperature- and drought-induced responses, ii) to briefly review the multiple effects of temperature on basic tree ecophysiological functions (e.g. photosynthesis, growth, respiration and nutrient uptake and transport), iii) to analyze the specific case study of forests in the Iberian Peninsula, which present diverging tree growth responses to temperature in Angiosperms and Conifers and iv) to briefly discuss the implications of our findings. Below we dedicate a section to each of these objectives (Sections 2.2.1-2.2.4).

2.2.1 A review of the diverse hypotheses that may explain contrasting growth responses to temperature in Mediterranean and angiosperm trees.

Table 2.1 lists the different hypotheses that may explain contrasting growth trends to temperature in Mediterranean conifer and angiosperm trees. The first hypothesis (1.1. *Eco-physiological and hydraulic traits*) states that positive growth responses to increased temperature in angiosperms could be mediated by a less strict stomatal control, allowing them to assimilate carbon for longer during warmer and drier periods. While this could imply that angiosperm could be more vulnerable to xylem cavitation and hydraulic failure, they have a greater capacity for embolism repair. On the other hand, most conifers function with a wider hydraulic safety margin to avoid cavitation but with the cost of lower carbon gain. Beside this specific hypothesis, several other factors could also contribute to explain the differences in growth responses between conifer and angiosperm trees. For example, these two groups differ in the effects of

Table 2.1 | Main hypotheses that may contribute to explain contrasting growth responses to temperature in Iberian Angiosperm and Conifer trees on a large scale.

Hypotheses	**Angiosperms**	**Conifers**	*References*
1.1 Eco-physiological and hydraulic traits	Narrower hydraulic safety margins and higher capacity to reverse embolisms	Wide hydraulic safety margins, early drought-induced stomatal closure and lower carbon gain, low stomatal conductance sensitivity to VPD	Martinez-Ferri et al., 2000, Brodersen et al., 2010, Johnson et al., 2012, Choat et al. 2012 Sala et al., 2012, Epron et al., 2012; Michelot et al., 2012, Meinzer et al., 2013, Brodersen et al., 2013, Ogasa et al. 2013, Coll et al. 2013.
1.2 Phenology	Tree productivity more sensitive to growing season length	Positively affected but less sensitive to growing season length	Churkina et al. 2005, Welp et al., 2007, Piao et al. 2007, Delpierre et al., 2009, Richardson et al., 2010, Gómez-Aparicio et al. 2011, Coll et al. 2013.
1.3 Intra- and inter-specific competition and forest succession	Growth less sensitive to intra and inter-specific stand competition	Growth severely reduced by intra- and inter-specific competence in small, non-dominant trees	Sánchez-Gómez et al., 2008, Gómez-Aparicio et al. 2011, Coll et al. 2013, Vayreda et al 2013, Carnicer et al 2013.
1.4 Size, age and allometry	Different growth allometry and less apical dominance	Peak of crown growth reached at lower sizes	Gómez-Aparicio et al 2011, Poorter et al. 2012
1.5 Drought and temperature	Angiosperm trees are able to maintain substantial transpiration levels during summer drought events	Drought and heat waves often results in early stomatal closure in Mediterranean conifers	Martinez-Ferri et al 2000, Zweifel et al., 2007; de Luis et al 2007, Eilmann et al., 2009, Camarero et al 2010, de Luis et al. 2011, Klein et al., 2011; Poyatos et al., 2013, Coll et al. 2013.
1.6 Winter freezing	Angiosperm trees are more vulnerable to freeze-thaw embolism	Less sensitive to freeze-thaw embolism	Sperry and Sullivan, 1992; Gómez-Aparicio et al. 2011, Brodribb et al., 2012
1.7 Interactions between multiple factors	Yes	Yes	Linares et al., 2010, Gómez-Aparicio et al. 2011, Vayreda et al., 2012, Coll et al., 2013, Ruiz-Benito et al., 2013.
1.8 Local adaptation, individual and provenance variation	Yes	Yes	Rehfeldt, 1978, 1982, Santos et al., 2010; Ramirez-Valiente et al., 2010, 2011, Chmura et al., 2011, Robson et al., 2012, Alberto et al., 2013.
1.9 Phenotypic plasticity	Yes	Yes	Camarero et al., 2010, Nicotra et al., 2010, de Luis et al., 2011.

phenology in their productivity, in the sensitivity of growth to competition, and in growth allometry (Table 2.1, Hypothesis 1.2-1.4). In addition, local adaptation processes and phenotypic plasticity also largely influence tree growth responses to temperature and drought (Table 2.1, Hypothesis 1.5-1.9). Finally, the available empirical evidence suggests that the diverse factors significantly interact in determining growth responses (Table 2.1, Hypothesis 1.7). For example, several studies report strong interactions between tree size, drought and stand density effects in determining large-scale growth patterns in the Mediterranean basin. Below we briefly review the hypotheses listed in Table 2.1 and discuss the experimental tests required to assess their relative importance.

Eco-physiological and hydraulic traits - Different ecophysiological and carbon-allocation strategies in angiosperms and conifers (Hypothesis 1.1)

Table 2.2 summarizes the trait differences between angiosperm and coniferous trees. Key traits that differ between these two groups include stomatal sensitivity to VPD, xylem anatomy, foliar traits, hydraulic safety margins, capacity for embolism repair, NSC content, carbon transfer rates, wood parenchymal fraction and wood capacitance. The available published evidence shows that these diverse traits are functionally related and define two contrasting ecophysiological strategies in conifers and angiosperms. Compared to angiosperms, conifers have a lower stomatal-conductance sensitivity to increased VPD (*sensu* Johnson et al., 2012). In turn, this key difference in stomatal response appears to be tightly related to the different hydraulic safety margins in both groups (Tyree and Sperry, 1988; Nardini et al., 2001; Table 2.2). The wider hydraulic safety margins in conifers thus imply early responses of stomatal closure, which reduce hydraulic conductivity before substantial cavitation occurs. On the other hand, angiosperms can maintain relatively high stomatal conductances even when the xylem pressure caused by high VPD is sufficient to induce extensive cavitation (Johnson et al., 2012; Meinzer et al., 2009; Meinzer et al., 2013).

In support of these trends, Choat et al. (2012) recently reported that species in coniferous forests generally have a higher resistance to drought-induced cavitation and operate with wider hydraulic safety margins than do angiosperms. The minimum xylem pressures in conifers measured in the field were more positive than the xylem pressures causing a 50% loss of hydraulic conductivity, and thus the risk of hydraulic failure by collapse of the water-conducting system was low. In contrast, the hydraulic safety margins reported for angiosperms were narrower, being slightly positive or even negative.

The reported differences in stomatal sensitivity and hydraulic safety margins have in turn been functionally associated with different responses between both groups in the capacity of xylems to recover from embolisms. Recent studies have reported higher capacities in species with narrow safety margins and higher stomatal sensitivities to VPD (see Johnson et al., 2012 for a precise definition of stomatal sensitivity to VPD; Meinzer et al., 2013). The reversal of cavitation has been demonstrated to be feasible on an hourly or daily basis and to occur even under high xylem tension (Hacke and Sperry, 2003; Salleo et al., 2004; Brodersen et al. 2010; Zufferey et al., 2011). Two general but contrasting hydraulic strategies arise: i) high cavitation resistance, low stomatal sensitivity to VPD and low resilience (gymnosperms) and ii) low cavitation resistance but high resilience (angiosperms).

These two basic strategies are notably in turn functionally linked to anatomical differences in cell anatomy, NSC content, wood parenchymal fraction and wood density (Table 2.2). For example, both the percentage of living parenchyma and the concentration of NSCs in the xylem are significantly higher in angiosperms than in conifers (Johnson et al. 2012 and citations therein). During the reversal of embolisms, vessel refilling probably requires an input of energy (Meinzer et al., 2013) and the mobilization of stored carbohydrates. Living wood parenchyma thus acts as a reservoir of both water and carbohydrates. Hence, NSCs stored in cells surrounding vessels are likely to be the source of sugars needed for the maintenance of vascular integrity (Brodersen et al., 2010; Sala et al., 2012). Sugars are possibly transferred from parenchymal cells to embolized vessels for establishing a gradient to drive water away from either the phloem (Nardini et al., 2011) or non-embolized vessels (Brodersen et al., 2010). Furthermore, Améglio et al. (2004) reported the catabolism of starch into sugars and the subsequent efflux from parenchymal cells to the vessels in late winter during the recovery of *Juglans regia* from cavitation induced by the winter freeze-thaw. Likewise, the reported differences between the capacities to reverse embolisms in angiosperms and conifers (Johnson et al., 2012; Brodersen et al., 2013; Meinzer et al., 2013) are likely associated with the differences in sapwood NSC content between these two groups reported by Hoch et al. (2003ab). This empirical evidence suggests that NSC reserves in wood parenchymal cells play a key role in determining the hydraulic strategies of plants, because species with high NSC and parenchymal fractions would have a higher resilience to cavitation and thus could withstand a certain loss of hydraulic conductivity.

Finally, conifers and angiosperms also differ in cell anatomy and wood density (Table 2.2), and several studies suggest functional implications for these traits in climate-induced responses. For example, wood density has been proposed as a good predictor of the resistance of the xylem to drought stress, because species with denser wood tend to have a higher resistance to cavitation (Jacobsen et al., 2007; Pratt et al., 2007). Moreover, Ogasa et al. (2013) found a negative correlation between wood density and xylem recovery in deciduous angiosperm trees (*Salix*, *Betula, Carpinus, Cerasus*), suggesting in turn a negative association between increased cavitation resistance and resilience of xylem function. Wood density in Mediterranean evergreen shrubs was also negatively correlated with the percentage of parenchymal area in the xylem (Jacobsen et al., 2007). This correlation is consistent with the higher capacity of xylems to recover in species with wood of lower density reported by Ogasa et al. (2013), because living xylem

Table 2.2 | Summary of differences in key functional traits between conifers and angiosperms.

Trait	Angiosperms	Conifers	*References*
Wood anatomy	Vessels	Tracheids	Brodribb et al. 2012
	Ring- and diffuse-porous		
	Homogeneous pit membrane	Torus-margo pit membrane	
	Cylindrical phloem sieve elements	Cuboidal phloem sieve elements	Jensen et al. 2012
	Companion cells	Strasburger cells	
Wood parenchymal fraction	High	Low	Nardini et al. 2011, Meinzer and McCulloh 2013
Woody-tissue NSC content	High	Low	Hoch et al 2003ab, Michelot et al. 2012
Wood density	High	Low	Poorter et al. 2012
Xylem embolism recovery capacity	High	Low	Bucci et al. 2003, Salleo et al. 2004, Brodribb et al. 2010
Sensitivity to freeze-thaw embolism	High	Low or absent	Cavender-Bares et al. 2005
Hydraulic safety margins	Narrow or negative	Wide	Choat et al. 2012
Water potential causing 50% loss of hydraulic conductivity	Low	High	Choat et al. 2012
Xylem capacitance	High (ring-porous) Medium (diffuse-porous)	Low	Meinzer and McCulloh 2013
Rate of C transfer	High	Low	Jensen et al. 2012
Sap flow velocity	High	Low	Jensen et al. 2012
Phloem conductivity	High	Low	Jensen et al. 2012
Phloem sieve-element resistance	Low	High	Jensen et al. 2012
Leaf lifespan	Shorter	Longer	Lusk et al. 2003
Shade tolerance	High	Low	Poorter et al. 2012
Interspecific shade-tolerance/drought-tolerance trade-off	Yes	Yes	Niinemets and Valladares 2006
Mesophyllic conductance	High	Low	Niinemets et al. 2011
Photosynthetic capacity	High	Low	Lusk et al. 2003, Flexas et al. 2012
Stomatal density	High	Low	Flexas et al. 2012
Stomatal conductance sensitivity to VPD	High (ring-porous) Medium-low (diffuse-porous)	Low	Johnson et al. 2012, Barbeta et al. 2013, Meinzer et al. 2013, Poyatos et al. 2013
Distal leaf and root embolism and refilling	Rare	Frequent	Johnson et al. 2012.

parenchyma may be involved in the reversal of embolisms (Bucci et al., 2003; Nardini et al., 2011; Zufferey et al., 2011; Brodersen et al., 2010; Brodersen et al., 2013). In addition, low wood density has been associated with high capacitance (McCulloh et al., 2012; Pratt et al., 2007; Sperry et al. 2008). In water-stressed plants, a higher capacitance facilitates the transient release of water stored in living wood cells to the conduit lumen, increasing xylem water potential (Meinzer et al., 2009; Barnard et al., 2011; Zhang et al., 2011).

The higher resistance of conifers to both freeze-thaw and drought-induced cavitation (Sperry and Sullivan, 1992; Wang et al., 1992; Choat et al., 2012) has also been associated with differences in wood anatomy (Table 2.2). The main difference in wood anatomy between angiosperms and gymnosperms is that the latter have tracheids that also provide mechanical strength (Hacke et al., 2001; Poorter et al., 2012). In particular, thick conduit walls providing mechanical strength have been suggested as the factor limiting the size of tracheids in conifers (Pitterman et al., 2006). Small tracheids are less prone to freeze-thaw cavitation in conifers (Tyree and Zimmermann, 1988; Sperry and Sullivan, 1992; Pitterman and Sperry, 2003), as are small vessels in angiosperms (Sperry and Sullivan, 1992), in which other woody cells such as fibers are responsible for mechanical support of the plant. In both groups, however, no direct relationship has been found between conduit size and drought-induced cavitation across species. Pit membrane area, though, must be limited (as it is where air-seeding develops) to achieve a certain level of safety from drought-induced cavitation, which in turn limits the surface area and thus the size of conduit cells (Hacke et al., 2006; Jansen et al., 2009; Brodribb et al., 2012).

We hypothesize that the reported trait differences between conifers and angiosperms Table 2.2) constitute two different strategies that may imply qualitatively different growth responses to increased temperatures and drought in the Mediterranean region. The different stomatal responses to heat waves and summer droughts, inducing drought-avoidance strategies and stomatal closure in conifers, would be key to determining these different growth responses (Martinez-Ferri et al., 2000; Coll et al., 2013; Poyatos et al., 2013). Critically, the higher sensitivity of the stomatal conductance to increases in VPD in conifers may promote near-zero assimilation rates and may strongly limit carbon uptake and photosynthesis over extended drought periods (Martinez-Ferri et al., 2000; Johnson et al., 2012; Poyatos et al., 2013; Meinzer et al., 2013). Summer drought may strongly affect carbon dynamics and NSC mobilization and consumption in both conifers and angiosperms, for example by enhancing the catabolism of starch to soluble sugars for increasing xylem tension and sap osmolarity (Sala et al., 2012), mobilizing NSCs for embolism repair, producing soluble sugars to stabilize cellular proteins and membranes, stopping cell division and tree growth favoring in turn the accumulation of photosynthates in starch (Peñuelas and Estiarte, 1998; Estiarte and Peñuelas, 1999; Körner, 2003) or promoting increased allocation of NSCs in roots and declines in fine-root biomass

(Anderegg, 2012). Even though the coupled effect of these complex processes on the carbon balance of the tree may be quite variable (species and site specific), we suggest that early stomatal closure and the associated larger reductions of assimilation rates in conifers may consistently produce a more negative impact on both carbon balance and growth responses of trees.

On the other hand, increased winter temperatures can reduce the costs associated with the impacts of freeze-thaw embolism and may also differently affect the carbon balance of angiosperms and conifers. Critically, angiosperms have a higher sensitivity to freeze-thaw embolism (Table 2.2) and may experience higher costs. This group could thus benefit more from increased winter temperatures. Higher winter temperatures would thereby entail fewer freeze-thaw cavitations, which are responsible for the almost complete loss of hydraulic conductivity in ring-porous species and for the partial loss in diffuse-porous species by late winter (Sperry and Sullivan, 1992). The restoration of water transport in angiosperms is achieved by the production of earlywood or by vessel refilling, which have carbon demands supplied by NSCs (Barbaroux and Breda, 2002; Epron et al., 2012; Michelot et al., 2012). In contrast, since the xylems of conifers are highly resistant to freeze-thaw cavitation (Sperry and Sullivan, 1992; Brodribb et al., 2012), this group may not have very different NSC costs for the restoration of water transport after mild or cold winters.

Table 2.3 | A brief summary of the seasonal dynamics of NSCs and growth phenology in deciduous broadleaf, evergreen broadleaf and coniferous trees.

	Winter	Spring	Summer	Autumn
Deciduous angiosperm trees	Loss of hydraulic conductivity due to freeze-thaws, being higher in ring-porous than in diffuse-porous species (Sperry and Sullivan, 1992, Wang et al. 1992, Cavender-Bares et al. 2005, Michelot et al. 2012). Before bud burst, some species may refill embolized vessels using NSCs (Améglio et al. 2004).	The onset of radial growth occurs before bud burst in ring-porous species and after bud burst in diffuse-porous species (Michelot et al. 2012). NSCs contribute to growth in both ring- and diffuse-porous species (Epron et al. 2012) but more importantly in ring-porous species (Barbaroux and Breda, 2002, Palacio et al. 2011, Michelot et al. 2012). Starch content decreases in ring-porous trees, and sugars decrease in diffuse-porous trees (Michelot et al. 2012). Milder winter temperatures may favor the formation of wider vessels in ring-porous species in early spring (Matisons and Brumelis 2012). Extended growing season with higher spring temperatures (Peñuelas et al. 2002, Gordo and Sanz 2010).	NSCs in leaves decrease from summer through autumn (Hoch et al. 2003ab). The soluble fraction of NSCs is used to maintain xylem and phloem integrity and cell turgor under drought conditions (Sala et al. 2012). The soluble fraction increases in diffuse-porous species (Michelot et al. 2012). Another study, though, did not observe an increase in soluble fractions or observed reductions (Hoch et al. 2003ab). Higher stomatal conductance and dynamic embolism repair capacity may allow C assimilation even under a certain degree of water deficit (Johnson et al. 2012).	Allocation of carbon to storage (Epron et al. 2012). Extended growing season (Peñuelas et al. 2002, Gordo and Sanz 2009, Vitasse et al. 2009). An increase of drought-induced embolism may also lead to premature leaf abscission (Wang et al. 1992).
Evergreena ngiosperm trees	Reduced losses in hydraulic conductivity caused by freeze-thaws, although evergreen trees are more resistant than deciduous species (Cavender-Bares et al. 2005). C assimilation allocated mainly to storage when temperature is too low for growth (Körner 2003). NSC reserves increase throughout the winter (Rosas et al. 2013). Annual peak in photosynthetic rates for some species (Ogaya and Peñuelas, 2003).	Decline in NSC content by late spring (Rosas et al. 2013), probably invested in growth. As in deciduous trees, vessel diameter is also constrained by winter temperatures (Cavender-Bares et al. 2005). Extended growing season with higher temperatures (Peñuelas et al. 2002, Gordo and Sanz 2009).	NSCs in leaves decrease from summer through autumn (Hoch et al. 2003ab). The soluble fraction of NSCs is used to maintain xylem and phloem integrity and cell turgor under drought conditions (Sala et al. 2012). The soluble fraction peaks in summer in some species (Rosas et al. 2013). Do not close stomata completely even under high evaporative demand and low soil water content (Barbeta et al. 2012, Ogaya and Peñuelas, 2003). Narrower xylem vessels than in deciduous oaks reduce losses of hydraulic conductance (Wang et al. 1992, Sperry and Sullivan 1992 in other species).	Allocation of carbon to storage (Epron et al. 2012, Rosas et al. 2013). Mediterranean evergreens sometimes have a growth peak in autumn (Gutiérrez et al. 2011).

Table continued.

Table 2.3 | Continued.

	Winter	Spring	Summer	Autumn
Conifers	Freeze-thaw resistant species. No accumulated losses in hydraulic conductivity (Wang et al. 1992). Low temperatures may result in an increase of NSCs (Hoch 2008, Gruber et al. 2012, Fajardo et al. 2012, Hoch and Körner, 2012). High minimum temperatures may advance earlywood formation in Mediterranean conifers (Pasho et al. 2012).	Carbohydrate demand of new-leaf cohorts is supplied mainly by older cohorts (Michelot et al. 2012, Eilmann et al. 2010). Growth is apparently not dependent on NSCs (Michelot et al. 2012). High temperatures may lead to an earlier onset of radial growth (Camarero et al. 2010).	NSCs in leaves decrease from summer through autumn (Hoch et al. 2003ab). Peak of starch content before the onset of latewood (Oberhuber et al. 2011). Xylem structure is in general highly resistant to cavitation (Choat et al. 2012, Johnson et al. 2012). Very tight stomatal control may lead to near-zero carbon assimilation (Poyatos et al. 2013).	Mediterranean conifers have a growth peak in autumn (Camarero et al. 2010, Pasho et al. 2012). Allocation of carbon to storage (Epron et al. 2012).

Winter temperature is a major driver for switching carbon allocation either to storage or to growth and respiration (Epron et al., 2012; Körner, 2013) and for the conditioning accumulation of starch (Oleksyn et al., 2000). When temperature is too low for growth, carbon assimilation is still significant, so NSCs derived from winter photosynthesis are mainly allocated to storage during cold periods (Rossi et al., 2008; Fajardo et al., 2012). In addition, the catabolism of starch into soluble carbohydrates during cold periods may possibly maintain intracellular osmotic concentration, which is positively correlated with cold hardiness (Cavender-Bares et al., 2005; Morin et al., 2007). In both conifers and angiosperms, increased winter temperatures are likely to alter cambium activation, growth allocation and the dynamic balance among winter photosynthesis, starch storage and soluble sugar concentrations.

Finally, increased winter, spring and autumn temperatures can significantly influence phenological responses, advancing winter cambium activation, spring bud burst and leaf unfolding or delaying autumn leaf fall (Peñuelas and Filella, 2001). The derived extension of the phenological period could have strong effects on tree height and growth (Vitasse et al., 2009, 2013; Lenz et al., 2013). Both the phenological cycles and the growth-associated carbon dynamics, however, are qualitatively different in conifers, ring-porous deciduous trees, diffuse-porous deciduous trees and evergreen oaks (Epron et al., 2012; Table 3). These differences suggest that these groups may qualitatively differ in the relative effects of increased spring temperatures on carbon dynamics and tree growth. For example, an increase in temperature early in the growing season may also increase vessel diameter in deciduous angiosperms but not in conifers (Matisons and Brumelis, 2012).

Phenology (Hypothesis 1.2)

An average lengthening of the growing season of about 11 days has been detected in Europe from the early 1960s to the end of the 20th century (Menzel and Fabian, 1999, Peñuelas and Filella, 2001, Linderholm, 2006, Menzel et al., 2006). Growing season length has a strong effect on tree productivity, Consequently, the reported temperature-induced changes in phenology could affect tree growth responses (White et al., 1999, Kramer et al., 2000; Picard et al., 2005, Delpierre et al., 2009; Richardson et al., 2009, Vitasse et al., 2009ab, Dragoni et al., 2011, Rossi et al., 2011, Lugo et al., 2012). Empirical evidence in temperate trees suggests that the productivity of evergreen needleleaf forests is less sensitive to phenology than is productivity of deciduous broadleaf forests (Welp et al., 2007, Delpierre et al., 2009, Richardson et al., 2010). For instance, Churkina et al. (2005) reported a different sensitivity of net ecosystem productivity to growing season length in deciduous forests (5.8+ 0.7 g C m-2 d-1), compared with evergreen needle-leaf forests (3.4 + 0.3 g C m-2 d-1). Similarly, Piao et al. (2007) reported different sensitivities of gross ecosystem productivity to growing season length (9.8 + 2.6 g C m^{-2}

d^{-1} in deciduous forests, compared with 4.9 + 2.5 g C m^{-2} d^{-1} in evergreen needle-leaf forests). To our knowledge, it remains untested whether qualitatively different phenology responses in Mediterranean conifers and angiosperm trees may occur and translate into different tree growth responses on a large scale.

However, other evidence points to complex and species-specific effects of phenology on tree growth. For instance, for both conifer and angiosperm trees, a variety of species-specific responses in bud burst and bud set have been reported along altitudinal and latitudinal gradients, reporting both advances, delays and non-significant clines (Vitasse et al., 2009ab, Alberto et al., 2013, Vitasse et al., 2013). For example, depending on the species considered, Vitasse et al. (2009b) found positive and negative correlations between advanced leaf emergence and annual growth. Moreover, warming can produce complex and counter-intuitive effects on phenology and growth. For example, strong warming in winter could slow the fulfillment of chilling requirements, which may delay spring phenology and growth (Körner and Basler, 2010, Yu et al., 2010) and affect differently early and late successional species (Körner and Basler, 2010).

In the Mediterranean region, mean annual and maximum temperatures have been identified as the major drivers of deciduous tree phenology (Gordo and Sanz, 2010). However, the effects of temperature on the phenology of many conifer and angiosperm tree species in the Mediterranean basin remain yet relatively poorly quantified (Maseyk et al., 2008). It remains also uncertain whether trade-offs between the advance of spring flushing date and the increased risk of frost damage may differ qualitatively between Mediterranean trees (Lechowicz, 1984; Lockhart, 1983). The same applies for trade-offs between delayed autumn leaf fall date, increased autumn photosyntate storage, and increased late-autumn frost damage risk and incomplete leaf nutrient remobilization costs (Lim et al., 2007). Finally, in the Mediterranean basin, drought periods significantly affect both leaf phenology and tree growth in both conifer and angiosperm trees (de Luis et al 2007, Camarero et al 2010, de Luis et al. 2011). For instance, increased leaf retention rate and lifespan have been observed in response to drought in holm oak forests (Bussotti et al., 2003, Misson et al., 2010). Drought also causes foliage to fall earlier and results in incomplete leaf nutrient translocation and increased nutrient concentration in litter (Martinez-Alonso et al., 2007).

Intra-specific competition, inter-specific competition and forest succession (Hypothesis 1.3)

Empirical studies reveal that intra-specific competition acts as a major determinant of growth patterns in Mediterranean forests in both conifer and angiosperm trees (Gómez-Aparicio et al., 2011). Forest densification due to land abandonment and the advance of succession is occurring over extensive areas, increasing competition, reducing tree growth, and increasing mortality (Gómez-Aparicio et al., 2011; Vila-Cabrera et al., 2011; Coll et al., 2013). Coll et al., (2013) reported much higher negative effects of forest stand basal area on conifer growth than in angiosperm trees in both dry and wet extremes of a large-scale rainfall gradient, and these trends were paralleled by higher effects of basal area on small-tree mortality observed in conifers. These results coincide with studies revealing oaks less sensitive to competition than pines in this area (Sánchez-Gómez et al., 2008; Gómez-Aparicio et al., 2011).

Inter-specific competition also plays an important role in determining growth responses in Mediterranean conifer and angiosperm trees. Specifically, large-scale surveys suggest that small-sized conifers are more sensitive to growth suppression by late successional species (Gómez-Aparicio et al., 2011, Zabala et al., 2011; Coll et al., 2013). Angiosperm trees are significantly expanding their distributional ranges, increasing recruitment across extensive areas (Coll et al., 2013, Vayreda et al., 2013). Moreover, during the last decades the expansion of the dominant angiosperm tree *Quercus ilex* has negatively influenced the recruitment success of five *Pinus* species on a large scale in this area (Carnicer et al., 2014).

Size, age and allometry (Hypothesis 1.4)

Mediterranean conifers differ from angiosperm trees in their allometrical relationships between tree size (diameter at breast height) and crown growth variables (Poorter et al., 2012). The peak of crown growth is generally reached at lower sizes in conifers, which also show a much steeper decrease with size than broadleaved species (Poorter et al., 2012). These different allometric relationships are in turn associated with several other traits (maximal height, crown size, shade tolerance, wood density, apical dominance) and also interact with local habitat aridity (Poorter et al., 2012). Similarly, Gómez-Aparicio et al (2011) reported that in Iberian forests competitive effects for conifers scale approximately quadratically with diameter at breast height (DBH^2) and linearly for broadleaved trees. To our knowledge, it remains untested whether these different allometric relationships might be related to the contrasting tree growth responses to temperature reported in Mediterranean conifers and angiosperm trees (Gómez-Aparicio et al., 2011).

Drought and temperature (Hypothesis 1.5)

Large-scale studies demonstrate that drought and increased temperatures significantly limit tree growth in xeric regions of the Mediterranean basin (Andreu et al., 2007; Sarris et al., 2007; Martinez-Alonso et al 2007; Bogino and Bravo 2008; Martínez-Vilalta et al. 2008, Gómez-Aparicio et al., 2011, Vila-Cabrera et al., 2011; Vayreda et al., 2012; Sánchez-Salguero et al., 2012; Candel-Pérez et al., 2012; Coll et al., 2013) and produce qualitatively different ecophysiological responses in Mediterranean conifers and angiosperm trees (Martinez-Ferri et al., 2000; Zweifel et al., 2007; Eilmann et al., 2009). For instance, while drought often results in early stomatal closure in Mediterranean conifers (Martinez-Ferri et al., 2000; Klein et al., 2011; Poyatos et al., 2013), angiosperm trees are able to maintain substantial transpiration levels during summer drought events (Quero et al., 2011).

Drought largely determines cambium growth in Mediterranean forests, producing plastic and seasonally variable patterns, ranging from one single annual peak to markedly bimodal trends (Maseyk et al., 2008; Camarero et al., 2010; de Luis et al., 2011). However, large-scale studies in the Iberian peninsula reveal that competition effects on growth are often stronger than drought effects (Gómez-Aparicio et al., 2011; Coll et al., 2013). Nevertheless, strong interactions between competition and drought effects have been reported, and significantly increase at the edge of climatic gradients (Linares et al., 2010; Vayreda et al., 2012; Coll et al., 2013; Ruiz-Benito et al., 2013). Finally, there is also some evidence of individual predispositions to winter-drought induced tree dieback in *P. sylvestris* (Voltas et al., 2013), local adaptation for water use efficiency in *P. halepensis* (Voltas et al., 2008), and correlations of temperature and genetic variability at candidate loci for drought tolerance in *P. halepensis* and *P. pinaster* (Grivet et al., 2011), suggesting important interactions between individual adaptive traits and drought impacts.

Winter freezing (Hypothesis 1.6)

Angiosperm trees are more vulnerable to freeze-thaw embolism and this may contribute to explain the dominance of conifer trees at high altitudes (Cavender-Bares et al., 2005, Brodribb et al., 2012) and could in turn result in qualitatively different growth responses in conifers and angiosperm trees. For example, Gómez-Aparicio et al. (2011) reported that Atlantic deciduous broadleaved trees in the Iberian peninsula had lower competitive response ability at lower temperatures, in contrast to mountain conifer species. In this study, tree growth of Atlantic deciduous broadleaved trees was negatively affected by low temperatures (Gómez-Aparicio et al., 2011). In line with this, several studies have demonstrated that low winter temperatures directly inhibit cell division and tree growth in cold localities (Körner, 1998; Fajardo et al., 2012; Körner, 2013).

Interactions between multiple factors (Hypothesis 1.7)

Tree growth patterns in the Iberian peninsula have several contributing drivers that interact along geographical gradients (Coll et al., 2013). For instance, Gómez-Aparicio et al. (2011) studied tree growth responses in 15 tree species in Spain and reported that sensitivity to competition increased with decreasing precipitation in all species. Notably, the best predictive models for tree growth in Gómez-Aparcio et al. (2011) included interactions between size, competitive effects and climate variables. Similarly, Coll et al. (2013) modeled growth responses in the Iberian peninsula and reported a significant increase in the strength of interactions between tree size, tree height and climate variables at the drier and wetter edges of rainfall gradients. These interactions could increase with ongoing climate change, and several studies suggest that warming could increase competition for water in Mediterranean forests (Linares et al., 2010).

Local adaptation, individual and provenance variation (Hypothesis 1.8)

Local selection processes may affect the adaptive traits determining the different growth responses to temperature observed in Iberian conifers and angiosperm trees. For example, provenance studies in both conifer and angiosperm trees have revealed genetic differences in growth rates and other growth-related traits (age at reproduction, timing of bud burst and bud set, leaf traits, flowering phenology), suggesting that populations are often adapted to their local conditions of temperature and water availability (Rehfeldt, 1978, 1982, 1988; Borghetti et al., 1993; Climent et al., 2008; Rose et al., 2009; Mátyás et al., 2009; Santos et al., 2010; Ramirez-Valiente et al., 2010, 2011; Chmura et al., 2011; Robson et al., 2012; Alberto et al., 2013). In provenace trial studies, populations from cold environments often cease growth earlier, while populations from warm localities generally grow faster (Alberto et al., 2013). Notably, local selection for increased growth rates may induce lower resistance to drought and frost. For instance, in conifers fast-growing provenances often exhibit lower cold hardiness and/or lower resistance to drought stress (Hannerz et al., 1999; Cregg and Zang, 2001; Chuine et al., 2006). These differences have been attributed to trade-offs between resistance to frost and drought and growth (Chuine et al., 2006, and see Martin St Paul et al., 2012).

Phenotypic plasticity (Hypothesis 1.9)

Mediterranean trees show strong plastic responses in tree growth patterns, which are associated with seasonal climate variability (e.g. Camarero et al., 2010, de Luis et al., 2011). Critically, phenology and growth plasticity responses differ between provenances and species and may determine observed demographic and evolutionary responses to global warming (Nicotra et al., 2010). For example, low elevation provenances often exhibit greater phenological plasticity to temperature than high elevation provenances (Vitasse et al., 2013) and this could in turn influence observed tree growth responses. To our knowledge, it remains untested whether Mediterranean conifers exhibit higher growth plasticity than angiosperm trees, although it has been reported that Iberian conifers show higher growth rates than angiosperm trees in absence of competition (Gómez-Aparicio et al., 2011, Poorter et al., 2012).

Experimental assessment of the relative contribution of the hypotheses (Assessment 1.10)

The available empirical evidence (Sections 1.1-1.9) suggest that several factors interact and seem to determine contrasting growth responses to temperature in Mediterranean conifer and angiosperm trees. Therefore, improved experimental approaches are required to quantitatively assess the relative importance of these factors. While several experimental and observational approaches could be applied, we suggest that reciprocal provenance trial experiments may be especially suited for this purpose. Previous studies assert that multiple common garden experiments located in latitudinal and altitudinal gradients are particularly relevant to study phenology and growth responses to temperature (Reich and Oleksyn, 2008, Vitasse et al., 2010). Furthermore, the inclusion of different provenances in these reciprocal experiments allows the quantification of environmentally induced phenotypically plasticity, genotypic variance and their interaction (e.g. Vitasse et al., 2013). Complementarily, drought effects on growth could be studied by manipulative experiments combined with reciprocal common garden designs (reviewed in Wu et al., 2011, Klein et al., 2011). Similarly, the effects of intra- and inter-specific competition could be studied manipulating tree densities and composition in different experimental groups. Finally, to assess tree size effects and allometric relationships, the study of saplings of different ages would be required. Alternatively, long-term experiments could provide also relevant information to quantify allometric relationships. Finally, in all these experimental designs, the periodic measurement of ecophysiological traits should be implemented to assess their seasonal variation and their putative role in determining growth responses.

2.2.2 Complex and multiple effects of temperature and drought on tree physiology

Climate produces multiple and complex effects on tree physiology. As highlighted in Table 2.1, we expect that multiple physiological processes can simultaneously react to the changes in environmental temperatures and influence growth responses. For example, temperature and drought directly affect several ecophysiological processes such as carbon and nutrient uptake, carbon allocation between tissues, photosynthesis, respiration, processes of embolism prevention and repair, phenological cycles, cambium reactivation, cell division and expansion or carbon transfer rates (Körner, 1998; Rennenberg et al., 2006; Breda et al., 2006; Sanz-Perez et al., 2009; Camarero et al., 2010; Michelot et al., 2012; Epron et al., 2012). Moreover, these direct climatic effects on tree physiology can in turn produce secondary indirect effects, for example the promotion of signaling and regulatory responses, acclimation and phenotypically plastic responses or changes in gene expression (reviewed in Peñuelas et al., 2013).

Table 2.4 | A non-exhaustive and synthetic review of the different effects of temperature (A) and drought (B) on different tree physiological processes.

A. Effects of temperature on tree physiology	*References*
Photosynthesis. Temperatures higher/lower than the optimum decrease photosynthesis and affect multiple biochemical processes. For example, high temperatures can reduce the efficiency of electron transport in the thylakoid membrane of chloroplasts, which in turn down-regulate the content of ribulose-1,5-bisphosphate and deactivate Rubisco. High temperatures also inhibit Rubisco activase, due to their low thermal optimum. The solubility of the two substrates of Rubisco, CO_2 and O_2, is differentially affected by temperature, stimulating photorespiration and inhibiting photosynthesis at high temperatures. · Photosystem II is also sensitive to high temperatures, which stimulate mechanisms to avoid photo-oxidation and membrane denaturation, such as isoprene production and the xanthophyll cycle. · Low temperatures cause a variety of physiological and acclimative responses, including modifications in the structure of the thylakoid membrane in chloroplasts, alleviation of photoinhibition through upregulation of carbon metabolism and increased synthesis of storage carbohydrates, increased production of antioxidants, prevention of intracellular freezing by increased soluble carbohydrates (mobilization of starch to sucrose) and changes in gene expression and signaling pathways. · The growth environment of plants determines the temperature optimum of photosynthesis. In warmer environments, plants acclimate to increase the thermal optimum of the maximum carboxylation velocity ($V_{c,max}$) and the maximum potential rate of electron transport (J_{max}).	(Rennenberg *et al.*, 2006; Morin *et al.*, 2007; Kattge & Knorr, 2007; Chaves *et al.*, 2012; Sharkey & Bernacchi, 2012)
Above the thermal optimum for photosynthesis, the emission of biogenic volatile organic compounds such as isoprene and monoterpenes progressively increases.	(Llusia & Penuelas, 2000; Rennenberg *et al.*, 2006)
Leaf respiration is strongly affected by temperature, increasing at high temperatures (e.g. above 35-40 °C) and peaking at higher temperatures than photosynthesis.	(Rennenberg *et al.*, 2006; Smith & Dukes, 2013)
Temperatures increase net primary production and plant growth. In cold-adapted trees, photosynthesis is less sensitive to low temperatures than is tree growth (cell division and growth, cambium activation). In alpine treelines, new tissue formation is nearly absent at temperatures around 5 °C, but considerable rates of photosynthesis are maintained between 0-10 °C.	(Körner, 1998; Way & Oren, 2010; Wu *et al.*, 2011; Fajardo *et al.*, 2012; Lenz *et al.*, 2013)
Higher temperatures influence foliar phenology, promoting earlier bud burst and delaying leaf fall.	(Peñuelas & Filella, 2001; Penuelas *et al.*, 2002; Vitasse *et al.*, 2009b, 2013)
In the absence of drought, temperature often increases *nutrient-uptake* capacity (NH_4^+, NO_3^-, PO_4^{3-}, K^+). Temperature can also increase both xylem loading of amino compounds and nitrogen allocation in aboveground tissues.	(Rennenberg *et al.*, 2006)
Freezing causes cell dehydration, formation of ice in intracellular spaces and embolism. Buds are more resistant than leaves to frost.	(Morin *et al.*, 2007) (Augspurger, 2009)
Temperature, in absence of drought, positively affects rates of soil respiration and litter decomposition.	(Wu *et al.*, 2011)
Organs, individuals, life stages and species consistently differ in their phenological responses to temperature and sensitivity to damage from frost and drought.	(Niinemets & Valladares, 2006; Morin *et al.*, 2007; Augspurger, 2009).

Table Continued

Table 2.4 | Continued.

B. Effects of drought on tree physiology	*References*
Photosynthesis. Drought limits photosynthesis by stomatal closure, diffusion limitations in the mesophyll and metabolic impairment. It can also limit photosynthesis via secondary effects, such as reduced hydraulic conductance and oxidative stress. Drought activates diverse signaling pathways associated with stomatal closure. For example, it modifies abscisic acid (ABA) signaling in leaves, shoots and roots; increases xylem-sap pH and changes aquaporin concentrations, leaf hydraulic conductance signals and electric signals.	(Chaves *et al.*, 2012; Sharkey & Bernacchi, 2012)
Drought reduces osmotic potential in the soil and predawn leaf water potentials and limits water uptake. To maintain water uptake, plants increase the production of osmolites, down-regulate electron flux and increase the activity of antioxidant enzymes. Drought can also increase the degradation of foliar proteins and the concentration of soluble amino acids and NSCs in the leaves, which may act in turn as osmoprotectants to stabilize proteins and membranes. Drought also promotes an increase in the concentrations of soluble antioxidants.	(Rennenberg *et al.*, 2006)
Severe water stress can produce irreversible or persistent damage in the photosynthetic apparatus of leaves (relative to leaf lifespan).	(Sharkey & Bernacchi, 2012)
Drought reduces tree growth, net primary production, cambium activity, cell division and growth.	(Eilmann *et al.*, 2009; Camarero *et al.*, 2010; Wu *et al.*, 2011; de Luis *et al.*, 2011)
Drought reduces C transfer rates.	(Barthel *et al.*, 2011; Epron *et al.*, 2012)
Drought is associated with acclimative responses such as mid-term reductions in total leaf area and defoliation.	(Ogaya & Peñuelas, 2006; Bréda *et al.*, 2006; Carnicer *et al.*, 2011)
Drought promotes an increase in NSCs in roots and a decrease in fine-root biomass.	(Anderegg, 2012; Anderegg *et al.*, 2013)
Drought alters nutrient-uptake processes, for example promoting increases in ammonification and decreases in denitrification in the soil.	(Geßler *et al.*, 2005)
Isoprenoid emissions can be negatively affected by drought stress and increase during plant recovery after drought.	(Rennenberg *et al.*, 2006; Peñuelas & Staudt, 2010)
Drought can increase the accumulation of ethylene in shoots, in turn reducing shoot growth.	(Chaves *et al.*, 2012)
Water deficit can reduce N uptake from the soil and change N partitioning between roots and shoots, increasing N content in the roots.	(Rennenberg *et al.*, 2006)
Omic studies reveal that drought produces changes in *gene regulation*, for example promoting proline synthesis and down-regulating proline degradation.	(Chaves *et al.*, 2012; Peñuelas *et al.*, 2013)
Negative effects of drought differ between phases of plant development and annual phenophases and are usually stronger during reproductive and leaf-emergence phases in deciduous trees. Drought produces tissue-specific signaling responses in roots, shoots and leaves and tissue-specific interactions between signaling factors. For example, different interactions between ABA and ethylene have been reported in roots and shoots.	(Chaves *et al.*, 2012)

Table 2.4 provides a brief, non-exhaustive description of the diverse effects of temperature and drought on tree physiology. It is important to bear in mind that all these ecophysiological processes often have different sensitivities and thresholds to temperature and water deficit. For example, tree growth and cambium activation are more sensitive to low temperatures than is

photosynthesis (Körner, 1998; Fajardo et al., 2012). In addition, as shown in Table 2.4, responses to climate are often species or tissue specific or depend on developmental stage and seasonal phase and can be influenced by regulatory feedbacks that can often imply multi-tissue coordinated responses. Despite the overwhelming complexity and diversity of the effects of temperature and drought reported in Table 2.4, several studies have demonstrated consistent differences between major plant groups, such as conifers and angiosperms, in climate-induced responses (e.g. Way and Oren, 2010; Gómez-Aparicio et al., 2011; Coll et al., 2013).

2.2.3 Empirical patterns in the Iberian Peninsula: the negative synergistic effects of increased temperatures and forest successional advance.

In the Mediterranean basin, land use changes often negatively interact with increased temperatures and drought events and result, in diverse taxonomic groups, in negative demographic trends detectable on a large scale (Linares et al., 2010, Stefanescu et al., 2011, Carnicer et al., 2013, 2014). In the case of Iberian forests, increased stand competition due to forest successional advance and forest densification has been identified as a major driver of tree demographic responses (Gómez-Aparicio et al. 2011, Carnicer et al., 2014). Notably, stand competition interacts with temperature and drought responses in this region, especially in the drier and wetter edges of rainfall gradients (Linares et al., 2010, Coll et al., 2013). In this section we briefly review the contrasting demographic trends to temperature observed in Conifers and Angiosperm trees in the Iberian peninsula. Forest succession is currently favoring a shift towards an increased dominance of angiosperm trees on a large scale (Coll et al., 2013, Vayreda et al., 2013, Carnicer et al., 2014). On top of this, recent studies (Gómez-Aparicio et al., 2009; Coll et al., 2013) show that tree growth responses to temperature differ between conifers and angiosperms on a large scale in the Mediterranean forests of the Iberian Peninsula. Large-scale empirical patterns of the responses of tree growth to temperature along a gradient of rainfall in Spain are illustrated in Fig. 2.1a, showing contrasting responses in conifers (black dots) and angiosperms (grey dots). Panel (a) depicts the variation of temperature beta estimates on species-specific responses of tree growth in forests located along a gradient of rainfall (Coll et al.,2013). Tree-growth data were obtained from the Spanish National Forest Inventory, which comprises a wide range of forest types, from typically Mediterranean lowland stands to northern temperate forests with strong Atlantic influences to alpine forests located in the Pyrenees (Coll et al., 2013). To analyze the relationship between growth responses to temperature and trait differences between conifers and angiosperms, we used hydraulic safety margins as a key synthetic variable of the hydraulic strategy of each species (Figure 2.1b). Panel (b) depicts two separate linear regressions

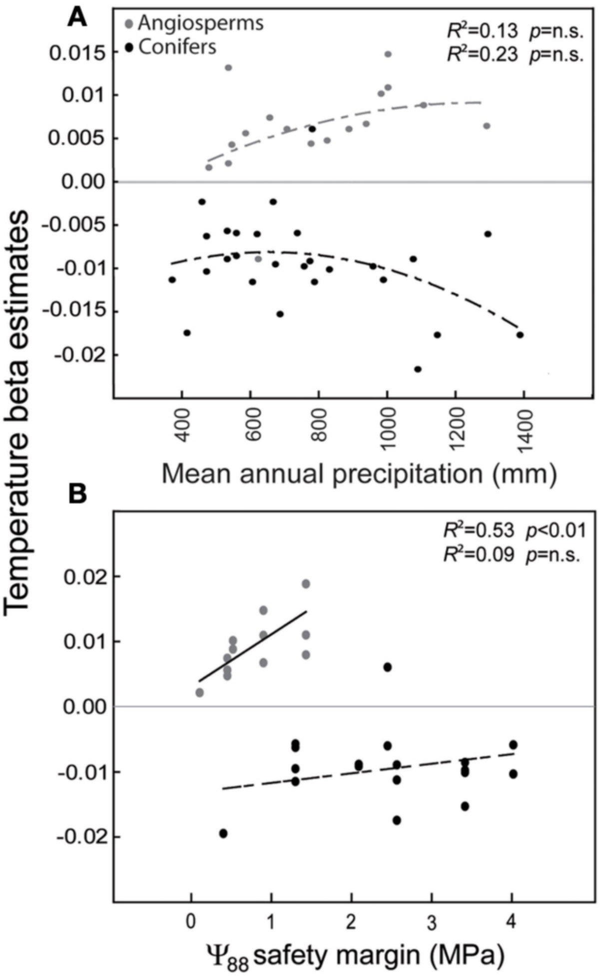

Figure 2.1 | Summary of the variation in the effect of temperature on tree growth along a rainfall gradient (a) and across interspecific differences in hydraulic safety margins (b) in conifers (black dots) and angiosperms (grey dots). The tree species included in the analysis are: *Fagus sylvatica, Quercus ilex, Q. pubescens, Q. pyrenaica, Q. robur, Abies alba, Pinus halepensis, P. nigra, P. pinaster, P. pinea, P. sylvestris* and *P. uncinata. P. radiata* and *Q. suber* were only included in panel (a) due to a lack of data for hydraulic safety margins. Coll et al. (2013) applied generalized linear models (GLM) to study tree growth responses (dependent variable) and assessed the following independent predictors: (i) climate and topography (Emberger water deficit index, mean annual temperature, terrain slope), (ii) forest stand structure (tree density, basal area), (iii) soil (organic layer depth), (iv) individual tree traits (tree height, size (diameter at breast height (DBH)) and (v) management practices (e.g. plantations). Beta estimates in Figure 1a show the reported significant effects of temperature on tree growth in GLM analyses (Coll et al. 2013). n.s. means not significant.

between the temperature beta estimates on growth and the species-specific hydraulic safety margins. Hydraulic safety margins were obtained from Cochard and Tyree (1990), Cochard (1992, 2006), Cochard et al. (1999), Tognetti et al. (1998), Martínez-Vilalta et al. (2002, 2009), Martínez-Vilalta and Piñol (2002), Oliveras et al. (2003), Corcuera et al. (2006) and Choat et al. (2012). A significant linear relationship between growth responses to temperature and species-specific hydraulic safety margins was only observed in angiosperms (Figure 2.1b), and conifers had significantly larger hydraulic safety margins (Figure 2.1b). Across the studied range of hydraulic safety margins, the temperature beta estimates were positive for angiosperms (grey dots) but negative for conifers (black dots), regardless of mean precipitation (Fig. 2.1a). This result is consistent with those of other studies on the effects of climate in the Iberian Peninsula reporting negative significant effects of temperature on tree growth in conifers (Gómez-Aparicio et al., 2011, Candel-Pérez et al., 2012, Büntgen et al., 2013). Figure 2.2 illustrates the specific forest successional context in which the reported contrasting effects of temperature on tree growth previously reported occur. Conifers show a significantly higher percentage of plots characterized by recruitment failure (Figure 2.2a, Carnicer et al., 2014). In contrast, *Quercus* species showed a much larger percentage of recently colonized plots (i.e. plots without adult trees but in which recruits of the focal species were detected, Figure 2.2b, Carnicer et al., 2014). Overall, figures 2.1 and 2.2 suggest that in this area the negative effects of warming and forest successional advance could synergistically impact conifer species during the next decades.

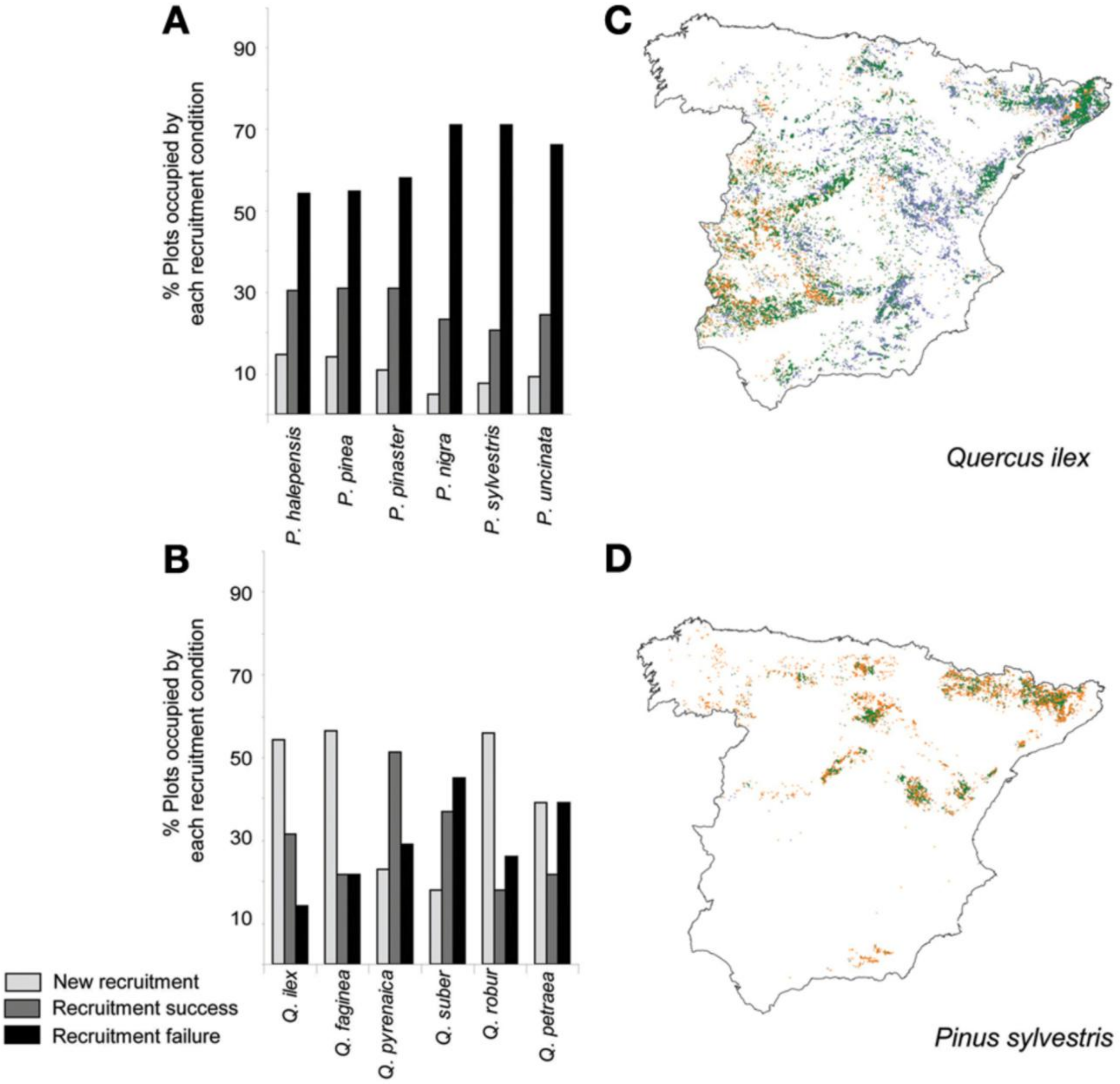

Fig. 2.2 | Contrasting large-scale trends in tree recruitment observed in the Iberian peninsula for small saplings (height <30 cm) in Conifers (*Pinus*) and Angiosperm trees (*Quercus*). a) Variation in the percentage of plots with recruitment success (grey), recruitment failure (black) and new recruitment areas (plots without adult trees of the focal species in which small recruits were detected) in *Pinus* species; b) Variation in the percentage of plots with recruitment success (grey), recruitment failure (black) and new recruitment areas in *Quercus* species. c) Spatial trends in recruitment for the dominant species *Quercus ilex*. Blue areas indicate new recruitment areas (i.e. areas with recruits but absence of adult trees), orange areas illustrate recruitment failure and green areas illustrate recruitment success (i.e. areas characterized by the presence of both adult and small saplings). d) Spatial trends in recruitment for *Pinus sylvestris*. Differences between recruitment trends in *Pinus* and *Quercus* were significant (see Carnicer et al., 2014 for a detailed statistical test. Average proportion of plots with recruitment failure: F = 16.64, P = 0.002; average proportion of plots with new recruitment: F = 35.04, P = 0.0001). Data were obtained from the Spanish National Forest Inventory, consisting in a regular grid of circular plots at a density of 1 plot/km^2.

2.3 Discussion

We have reviewed the different hypotheses that may contribute to explain the recently reported different growth responses to temperature in Mediterranean angiosperm and conifer trees (Table 2.1, Gómez-Aparicio et al., 2011, Coll et al., 2013). Conifer and angiosperm trees differ in the effects of phenology on tree productivity, in their sensitivity to stand competition and in their growth allometry. In addition, they consistently differ in an integrated suite of key traits, including different hydraulic safety margins, stomatal sensitivity, embolism repair capacity and xylem anatomy, suggesting two contrasting ecophysiological strategies to confront drought and extreme temperature events. However, for many Mediterranean conifer and angiosperm trees, detailed empirical studies contrasting the relative effect on tree growth of the factors listed in Table 2.1 are still lacking. For example, it is not clear whether temperature-induced shifts in phenology consistently differ between conifers and angiosperm trees in the Mediterranean region and how these shifts in phenology could differentially alter their productivity. Similarly, the seasonal dynamics of key traits, like cambium growth, tissue NSC content or sap flow, remain yet poorly quantified for many species. So it is clear that improved experimental approaches to contrast and assess the relative effect of the reviewed hypotheses are required (Table 2.1) if we are to explain the contrasting growth trends reported in recent large-scale studies in these two groups (Gómez-Aparicio et al., 2011, Coll et al., 2013, Figure 1).

We have suggested that the relative effects of these factors (Table 2.1) could be contrasted in reciprocal common garden experiments located in altitudinal or latitudinal gradients, that provide an ideal design to estimate temperature effects on phenology and growth, and also allow the estimation of local adaptation and phenotypic plasticity (Vitasse et al., 2009, 2013). In these reciprocal transplant experiments, detailed quantitative analysis of the relationships between growth measures and hydraulic safety margins, stomatal sensitivities to VPD, embolism repair activity and NSC carbon dynamics in wood parenchyma and other tissues would be ideally required to clarify the relative importance of these processes and their dynamic interrelationships (Camarero et al., 2010, de Luis et al., 2011, Pasho et al. 2012, Oberhuber et al., 2011, Michelot et al., 2012).

The available empirical evidence (Gómez-Aparicio et al., 2011, Carnicer et al., 2011, 2013, Coll et al., 2013, Figure 2.2) suggests that increased stand competition associated with successional advance is a primary driver of growth trends in the forests of the Iberian peninsula. So it would be key to simulate this factor in the proposed transplant experiments, manipulating sapling densities and composition. We suggest that mixed pine-oak designs would be especially interesting because recent studies describe the widespread expansion of *Quercus* saplings in the Iberian peninsula and limited recruitment in *Pinus* species (Coll et al., 2013, Carnicer et al., 2014, Vayreda et al., 2013, Figure 2.2). Moreover, *Quercus ilex* seems to act as a keystone species in driving these limited recruitment trends, inhibiting recruitment in five different *Pinus* species (Rouget et al., 2001; Carnicer et al., 2014). In addition, several studies report that pines are more sensitive to competition and their growth can be largely suppressed with the advance of succession, specially on sapling and young stages (e.g. Gómez-Aparicio et al., 2011, Zavala et al., 2011, Coll et al., 2013). Therefore, these processes should be ideally considered in reciprocal transplant experiments, to allow the experimental study of the combined negative synergistic effects of warming and increased successional advance.

Ideally, the experimental approaches tested in these common garden experiments should simulate future forest scenarios in the face of climate change in the Iberian Peninsula. However, future scenarios in this region remain uncertain. For example, the available model predictions vary from important range contractions to substantial range expansions (Benito-Garzón et al., 2011; Keenan et al., 2011; Ruiz-Labourdette et al., 2012; García-Valdés et al., 2013). We have suggested a possible scenario of global change dominated by the widespread expansion of angiosperm broadleaved trees, increased suppression of pine growth and recruitment by *Q. ilex* and specially acute negative demographic trends in mountain pines (*Pinus sylvestris*, *Pinus nigra* and, to a less extent*, P. uncinata*) (Figure 2.2, Carnicer et al., 2014). Other major uncertainties in future forest scenarios are related to non-linear dynamics in fire activity (Loepfe et al., 2012), changes in fire-climate relationships motivated by the generalized advance of forest succession and the expansion of *Quercus* species, that may substantially alter the distribution of forest fuel over extensive areas (Pausas and Paula, 2012; Carnicer et al., 2014), and the future changes in land uses induced by shifts in global energy policies and the increased use of forests as a local energy source (Peñuelas and Carnicer, 2010, Carnicer and Peñuelas, 2012).

In Table 2.3 we have also discussed how tree carbon dynamics may be interacting with climate-induced responses in the seasonal variation of photosynthesis, annual growth cycles, embolism prevention, embolism repair and refilling and stomatal responses. Important gaps in our knowledge remain, and we lack a clear picture of how tissue-specific NSC concentrations vary seasonally, their interspecific variation and how these seasonal variations are connected to the diverse physiological functions examined (i.e. carbon buffer function, winter- and drought-induced embolism repair, embolism prevention, bud burst and leaf unfolding, responses of root and stem growth and respiration) (Hoch et al., 2003ab; Sala et al., 2012; Michelot et al., 2012; Epron et al., 2012). Another aspect that merits more attention in future empirical tests is the putative existence of compensatory dynamics across seasons in the effects of climate on tree physiology. For example, higher temperatures may reduce the costs of winter embolism in broadleaved deciduous trees, lengthen the growing season or increase the production of photosynthates in spring. These changes could in turn allow higher NSC storage in spring, which could increase embolism repair capacity during summer droughts (compensatory seasonal effects).

In summary, a review of the existing empirical evidence suggests that contrasting demographic responses in Mediterranean conifer and angiosperm trees are currently occurring, due to both widespread forest successional advance and to divergent growth responses to temperature. Trait-based differences in these two groups may contribute to explain their different responses to temperature (Table 2.2, Figure 2.1) and their different role during successional processes in this region (Figure 2.2, Table 2.2, reviewed in Zabala et al., 2011, Poorter et al., 2012, Sheffer et al., 2013). Reciprocal common garden experiments may offer a very promising tool to develop integrative tests of the diverse factors reviewed (Table 2.1) and to simulate the synergistic negative effects of forest successional advance and climate warming on conifer species (Carnicer et al., 2014).

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3

Flowers of *Arbutus unedo*
Photo & Design: D. Sperlich

Chapter 3

Seasonal variability of foliar photosynthetic and morphological traits and drought impacts in a Mediterranean mixed forest.

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3.1 Abstract

The Mediterranean region is a hot spot of climate change vulnerable to increased droughts and heat waves. Scaling carbon fluxes from leaf to landscape levels is particularly challenging under drought conditions. We aimed to improve the mechanistic understanding of the seasonal acclimation of photosynthesis and morphology in sunlit and shaded leaves of four Mediterranean trees (*Quercus ilex* L., *Pinus halepensis* Mill., *Arbutus unedo* L., and *Q. pubescens* Willd.) under natural conditions. $V_{c,max}$ and J_{max} were not constant, and mesophyll conductance was not infinite, as assumed in most terrestrial biosphere models, but varied significantly between seasons, tree species, and leaf position. Favourable conditions in winter led to photosynthetic recovery and growth in the evergreens. Under moderate drought, adjustments in the photo/biochemistry and stomatal/mesophyllic diffusion behaviour effectively protected the photosynthetic machineries. Severe drought, however, induced early leaf senescence mostly in *A. unedo, Q. pubescens*, and significantly increased leaf mass per area in *Q. ilex* and *P. halepensis*. Shaded leaves had lower photosynthetic potentials but cushioned negative effects during stress periods. Species-specificity, seasonal variations, and leaf position are key factors to explain vegetation responses to abiotic stress and hold great potential to reduce uncertainties in terrestrial biosphere models especially under drought conditions.

3.2 Introduction

The Mediterranean region is dominated by arid or semi-arid ecosystems where high evaporative demand and low soil-water content during the summer dry period are the main ecological limitations to plant growth (Specht, 1969; Di Castri, 1973). The resilience of plants to drought and heat waves is determined by their frequency and duration, which are projected to become much more severe under current climate change scenarios - particularly in the Mediterranean region (Somot *et al.*, 2008; Friend, 2010; IPCC, 2013). Increased drought-induced defoliation (Poyatos *et al.*, 2013) associated with the depletion of carbon reserves (Galiano *et al.*, 2012) can ultimately lead to catastrophic hydraulic failure and tree mortality (Urli *et al.*, 2013; Choat, 2013). Drought-induced forest impacts and diebacks in the Mediterranean region have been reported in numerous studies (Peñuelas *et al.*, 2001; Martínez-Vilalta & Piñol, 2002; Raftoyannis *et al.*, 2008; Allen *et al.*, 2010; Carnicer *et al.*, 2011; Matusick *et al.*, 2013) and can lead to shifts in vegetation composition (Jump & Penuelas, 2005; Anderegg *et al.*, 2013) and to a higher risk of forest fires (Piñol *et al.*, 1998; Pausas *et al.*, 2008). The challenge in the Mediterranean region in the coming years will be to learn how carbon uptake and growth in species and communities will respond to these changes, and how forest management strategies can be adapted to cushion the negative impacts of climate change on forests (Sabaté, 2002; Bugmann *et al.*, 2010).

In past decades, ecosystem models on regional or global level contributed substantially to our understanding of the implications of climate change on a coarse scale where field experiments are limited (Luo, 2007). Much uncertainty, however, remains in the modelled feedback of the global carbon cycle to climatic warming (Friedlingstein *et al.*, 2014) and in the understanding and modelling of species responses to climate change (Luo, 2007; McDowell *et al.*, 2008; Beaumont *et al.*, 2008). Photosynthesis is generally overestimated in the main Earth system models, with significant regional variations (Anav *et al.*, 2013). Two critical parameters, the maximum rate of carboxylation ($V_{c,max}$) and the maximum rate of electron transport (J_{max}), are a prerequisite for scaling foliar photosynthesis to the canopy level at which global dynamic models operate (Friedlingstein *et al.*, 2006; Friedlingstein & Prentice, 2010). These two parameters describe the biochemical limitations to carbon assimilation, but are not easily measured. So relatively little data of their variability between species or seasons are available. $V_{c,max}$ and J_{max} are thus often used as constants for various plant functional types and seasons or, in some cases, are derived from other parameters such as leaf nitrogen content (Grassi & Magnani, 2005; Walker *et al.*, 2014). Moreover, extreme climatic conditions and inter-annual variability in arid and semi-arid regions are challenging for scaling carbon assimilation patterns from one year to another (Reynolds *et al.*, 1996; Morales *et al.*, 2005; Gulías *et al.*, 2009). Simulations of ecosystem carbon fluxes are consequently limited, first, by underrepresented temporal variability of photosynthetic parameters and soil-water patterns, and second by our limited understanding of the effects of water stress on both carbon uptake and release (Hickler *et al.*, 2009; Niinemets & Keenan, 2014). The modelling performance in Mediterranean-type ecosystems is thus particularly poor and stresses the need for a better mechanistic description of photosynthetic processes under water stress (Morales *et al.*, 2005; Keenan *et al.*, 2011; Zheng *et al.*, 2012; Vargas *et al.*, 2013). Mesophyll conductance, g_m, might play a future key role in improving model performance of photosynthesis under drought conditions (Keenan *et al.*, 2010).

The photosynthetic limitations of Mediterranean vegetation, especially under drought, have been extensively studied (for a review see Flexas *et al.*, 2014), but fewer studies have thoroughly assessed the seasonal behaviour of photosynthesis and morphology under natural conditions in a mixed mature forest. The information gained from seedlings under controlled conditions can only poorly represent the physiological mechanisms of the long-term acclimation to variable environmental conditions in mature trees (Flexas et al., 2006; Mittler, 2006; Niinemets, 2010). Seedlings or saplings are characterised by higher metabolism and enzymatic function, lower leaf dry mass per unit area (LMA), and higher photosynthetic potential relative to mature trees (Johnson & Ball, 1996; Bond, 2000; Niinemets, 2014). Responses to short-term stress are related to the mechanisms of prompt reactions (Flexas et al., 2006). Under natural conditions, however, mature trees acclimate to gradually developing water stress through the photosynthetic pathway (biochemical, stomatal or mesophyllic) (e.g. Martin-StPaul et al. 2013), but also through foliar traits such as nitrogen, LMA etc. (Poorter *et al.*, 2009). Less work has evaluated simultaneously the variations of photosynthetic and morphological traits in response to abiotic stress conditions. The variation of these traits is largely species specific (Orshan, 1983; Chaves *et al.*, 2002; Gratani & Varone, 2004; Krasteva *et al.*, 2013), although within-canopy gradients can play an additional overriding role (Valladares & Niinemets, 2008; Sperlich *et al.*, 2014). Mixed forests provide ideal test conditions where we can observe distinct species-specific strategies coping equally with the yearly variability of environmental conditions.

The aim of this study was to investigate the impact of seasonal environmental changes (above all drought) on foliar photosynthetic and morpholocial traits of the winter-deciduous sub-Mediterranean *Quercus pubescens*, two evergreen sclerophyllous species (*Quercus ilex* and *Arbutus unedo*) and an early-successional drought-adapted conifer, *Pinus halepensis*. *P. halepensis* is characterised as isohydric following a water saving and photoinhibition-tolerant strategy (Martínez-Ferri *et al.*, 2004; Baquedano & Castillo, 2006; Sperlich *et al.*, 2014). *Q. ilex* L. is a late-successional, slow growing, water-spending, photoinhibition-avoiding, anisohydric tree species with a plastic hydraulic and morphological behaviour (Villar-Salvador *et al.*, 1997; Fotelli *et al.*, 2000; Corcuera *et al.*, 2004; Ogaya & Peñuelas, 2006; Limousin *et al.*, 2009). The winter-deciduous anisohydric *Q. pubescens* follows a similar drought- avoiding strategy as *Q. ilex*, but maximizes gas exchange during a shorter growing season (Baldocchi *et al.*, 2009), resulting in high transpiration rates throughout the summer (Poyatos *et al.*, 2008). Over extensive areas of the Mediterranean region *Q. ilex* and *Q. pubescens* form the terminal point of secondary succession (Lookingbill & Zavala, 2000). *A. unedo* - relict of the humid-subtropical Tertiary tree flora (Gratani and Ghia, 2002a and references therein) – is typically occurring as shrub or small tree in the macchia ecosystems and holding a intermediate position concerning stomatal- (Beyschlag *et al.*, 1986; Vitale & Manes, 2005; Barbeta *et al.*, 2012) and photoinhibition-sensitivity (Sperlich *et al.*, 2014). Prolonged climate stress might disadvantage *A. unedo* being more drought sensitive than the companion species (Ogaya & Peñuelas, 2004; Barbeta *et al.*, 2012).

Our particular interests were to distinguish the species-specific strategies and to explore the eco-physiological mechanism behind drought responses by examining the fine tuning of foliar photosynthetic potentials/rates and foliar morphological traits. We hypothesized that i) seasonal environmental changes (above all drought) affect the photosynthetic and ii) morphological traits, iii) mesophyllic diffusion conductance (g_m) strongly constrains photosynthesis under drought conditions, iv) the seasonal acclimation varies qualitatively and quantitatively with species and v) light environment (leaf canopy position). We thus created a matrix of photosynthetic parameters that could be incorporated into process-based ecosystem models to improve estimates of carbon flux in the Mediterranean region.

3.3 Materials and methods

3.3.1 Field site

The experimental site Can Balasc is located in the coastal massif of the Collserola Natural Park (8500 ha), in the province of Barcelona, northeastern Spain (41° 25’ N, 2° 04’ E, 270 m a.s.l.). Seasonal summer droughts, warm temperatures and mild winters characterise the typical Mediterranean climate with a mean August temperature of 22.8 °C and a mean January temperature of 7.9 °C. Mean annual precipitation and temperature are 723 mm and 15.1 °C (1951-2010), respectively (Ninyerola *et al.*, 2007a,b). Sensors for measuring air temperature (HMP45C, Vaisala Oyj, Finland) and solar radiation (SP1110 Skye Instruments Ltd., Powys, UK) were installed at a height of 3 m, in a clearing *ca.* 1 km from the plot.

3.3.2 Stand structure

Our study site is characterised by a dense forest stand (1429 stems ha^{-1}) with a two-layered canopy consisting of a dense layer of *Quercus* species surmounted by shelter trees of the early-successional and fast growing Aleppo Pine (*P. halepensis* Mill.). The mean heights of each layer are 9.9 m and 17.1 m, respectively. The *Quercus* species are the late-successional evergreen Holm Oak (*Q. ilex* L.) and the deciduous Pubescent Oak (*Q. pubescens* Willd.). The Strawberry tree (*A. unedo* L.) grows usually as a shrub being widely abundant in the macchia ecosystems of the Iberian peninsula (Beyschlag *et al.*, 1986; Reichstein *et al.*, 2002). In our study site, however, *A. unedo* occurs scattered in the tree canopy (mean height 8.1 m) enriching the forest diversity with its flowering and fruiting habit. The trees with the biggest dimensions are the pines followed by the two *Quercus* species and at last by *A. unedo* (mean DBH of 33.7, 12.9, 9.6 cm, respectively). The forest succession has reached the final stage: The dense *Quercus* canopy is out-competing the early-successional *P. halepensis* by suppressing the growth of the light demanding pine seedlings and saplings. More details of stand history and field site are described in Sperlich *et al.* (2014).

3.3.3 Sampling method

We conducted eight field campaigns from June 2011 to February 2013. The sampling periods are presented in Table 2 and Figure 1. We avoided difficulties encountered during field measurements such as deviations from the standard temperature (25 °C) or unpredictable plant responses (patchy stomatal conductance) (Mott & Buckley, 1998, 2000) by analysing sampled twigs in the laboratory. We cut twigs with a pruning pull from sunlit and shaded leaf positions, optimally at similar heights. The twigs were immediately re-cut under water in the field, wrapped in plastic bags to minimise transpiration, stored in water buckets, and transported to the laboratory. Five replicates of each leaf position and tree species were collected for the analysis of gas exchange. The twigs were pre-conditioned in the laboratory at room temperature (24-28 °C) in dim light for 1-3 d and were freshly cut every morning. More details and references can be found in Sperlich *et al.* (2014).

3.3.4 Analyses of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, USA). Response curves for foliar net assimilation versus CO_2 concentration were recorded in parallel with the chlorophyll fluorescence measurements. In some cases the sunlit leaves of *Q. ilex* were too small to fill the leaf cuvette (2 cm^2) and so the measured parameters were adjusted after the measurements. For *P. halepensis*, we positioned a layer of needles (ca. 10-15) on the leaf cuvette, avoiding gaps and overlays, and sealed the gaskets with Blu-tack (Bostik SA, La Plaine St Denis, France) to keep the needles in position. The preparation and acclimation of the leaves prior to recording the response curves were conducted as in Sperlich *et al.* (2014).

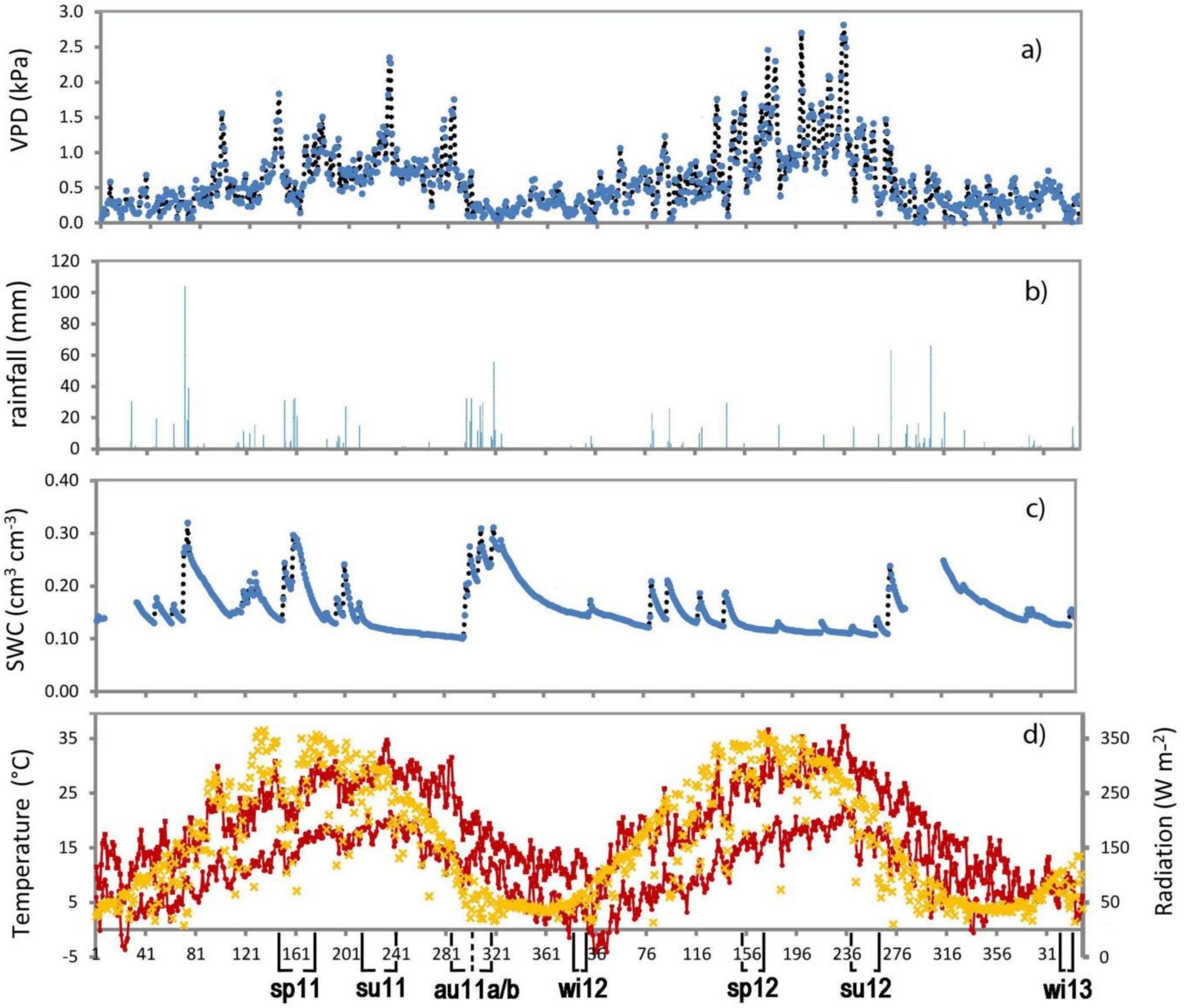

Fig. 3.1 | Environmental variables for the days of the year (DOY) from January 2011 until February 2013; a) atmospheric vapour pressure deficit (VPD), b) rainfall in mm c) soil water content in cm^3 cm^{-3} (gap in data is due to power cut), d) maximum and minimum temperatures in °C on the primary y-axes (in red circles) and radiation in W m^{-2} (in yellow crosses, foreground) on the secondary y-axes. Field campaigns are indicated (acronyms of seasons are detailed in Tab. 3.1).

Table 3.1 | Acronyms for variables utilized in tables and figures.

Campaign	Abbreviation	Date	DOY
Spring 2011	sp11	02.06.11 - 02.07.11	153-183
Summer 2011	su11	17.08.11 - 29.08.11	229-241
Autumn 2011^{a*}	au11a	17.10.11 - 27.10.11	290-300
Autumn 2011^{b*}	au11b	28.10.11 - 11.11.11	301-315
Winter 2012	wi12	09.01.12 - 19.01.12	9-19

[1] The autumn 2011^a campaign was conducted in a period of prolonged summer drought and the autumn 2011^b campaign was conducted after the first rains.

3.3.5 CO$_2$ experiments

The CO$_2$-response curves were recorded at a leaf temperature (T_{Leaf}) of 25 °C and a quantum flux density of 1000 μmol photons m^{-2} s^{-1}. The CO$_2$ concentrations in the leaf chamber (C_a) used to generate the response curves were 400→300→200→150→100→50→400→400→600→800→1200→2000 μmol CO$_2$ mol air^{-1}. The minimum and maximum times for stabilising net assimilation rate (A_{net} in μmol CO$_2$ m^{-2} s^{-1}), stomatal conductance (g_s in mol H$_2$O m^{-2} s^{-1}), and stomatal internal CO$_2$ concentrations (C_i in μmol CO$_2$ mol air^{-1}) for each log were set to 4 and 6 min, respectively.

3.3.6 Calculation of chlorophyll fluorescence parameters

F_m' and F_s were used to estimate the effective quantum yield of photosystem II (Φ_{PSII}, unitless) as:

$$\Phi_{\mathrm{PSII}} = \frac{(\mathrm{F_m}' - \mathrm{F_s})}{\mathrm{F_m}'} \tag{1}$$

where F_s is the steady-state fluorescence of a fully light-adapted sample, and F_m' is the maximal fluorescence yield reached after a pulse of intense light. The effective quantum yield of PSII represents the fraction of photochemically absorbed photons for a light-adapted leaf. The electron-transport rate based on the effective quantum yield of PSII (J_{CF} in μmol electron m^{-2} s^{-1}) was calculated as

$$J_{CF} = \varepsilon * \Phi\mathrm{PSII} * \alpha_{\mathrm{L}} \tag{2}$$

ε is a scaling factor accounting for the partitioning of intercepted light between photosystem I (PSI) and PSII. We assumed that light was equally distributed between both photosystems (ε = 0.5) (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2005). α_L (unitless) is the foliar absorbance; we used the following values: 0.932 for *Q. ilex* and 0.912 for *P. halepensis* for both sunlit and shaded leaves, 0.935 for sunlit leaves of *A. unedo*, 0.917 for shaded leaves of *A. unedo*, 0.939 for sunlit leaves of *Q. pubescens*, and 0.900 for shaded leaves of *Q. pubescens*. For the determination of α_L, foliar reflectance and transmittance were measured at midday in August 2012 using a UniSpec Spectral Analysis System spectroradiometer (PP Systems, Haverhill, USA). The ambient photosynthetic electron transport (J_{amb}) was defined as the value of J_{CF} at a CO_2 concentration of 400 μmol CO_2 mol air^{-1} and a PPFD of 1000 μmol photons m^{-2} s^{-1}. The relationship between J_{amb} and the net assimilation rate (J_{amb}/A_{net}) was used for the analyses of alternative electron sinks other than carbon metabolism. Calculations of F_v/F_m and NPQ can be found in the supplementary material (Note S1).

3.3.7 Estimation of mesophyll conductance

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

$$g_m = \frac{A_{\mathrm{net}}}{C_{\mathrm{i}} - \frac{[\Gamma^{*} * J_{\mathrm{CF}} + 8(A_{\mathrm{net}} + R_{\mathrm{d}})]}{J_{\mathrm{CF}} - 4(A_{\mathrm{net}} + R_{\mathrm{d}})}} \quad (6)$$

where Γ^* is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic CO_2 uptake, and R_d is the mitochondrial respiration of a leaf in light conditions and was estimated from the light-response curves combining gas exchange and measurements with the CF- method proposed by Yin et al. (2009). See supplementary material for details (Note S2). The chloroplastic CO_2 concentration (C_c in μmol CO_2 mol air^{-1}) was determined as:

$$C_c = C_i - \frac{A_{net}}{g_m} \quad (7)$$

3.3.8 Photosynthesis model

The photosynthesis model of Farquhar et al. (1980) considers photosynthesis as minimum of the potential rates of Rubisco activity (A_c) and ribulose-1,5-bisphosphate (RuBP) regeneration (A_j). The model was further complemented with a third limitation (A_p) that considers the limitation by triose-phosphate use (TPU) at high CO_2 concentrations when the CO_2 response shows a plateau or decrease (Sharkey, 1985). However, we rarely detected A_p limitations and TPU was therefore discarded in our analyses. A_{net} was then determined by the minimum of these two potential rates from an A/C_c curve:

$$A_{net} = min\{A_c, A_j\} \quad (8)$$

where

$$A_c = V_{cmax} * \left[\frac{C_c - \Gamma^*}{C_c + K_c\left(1 + \frac{O}{K_O}\right)}\right] - R_d \tag{9}$$

where $V_{c,max}$ (in μmol CO_2 m^{-2} s^{-1}) is the maximum rate of Rubisco carboxylation, K_c is the Michaelis-Menten constant of Rubisco for CO_2, O is the partial pressure of O_2 at Rubisco, and K_o is the Michaelis-Menten constant of Rubisco for O_2, taken from Bernacchi et al. (2002). The equation representing photosynthesis limited by RuBP regeneration is:

$$A_j = J * \left[\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*}\right] - R_d \tag{10}$$

where J (in μmol electron m^{-2} s^{-1}) is the rate of electron transport. We assumed that J becomes J_{max} under light and CO_2 saturation when the maximum possible rate of electron transport is theoretically achieved, although we may have underestimated the true J_{max} (for further details see Buckley & Diaz-Espejo, 2014). $V_{c,max}$ and J_{max} define the biochemical potential to drive photosynthesis and are summarised in the term "photosynthetic potential" (Niinemets *et al.*, 2006). Curves were fit, and diffusion leakage was corrected, as in Sperlich *et al.* (2014).

3.3.9 Foliar morphology, chemical analyses, and assessment of crown condition

Foliar morphological traits were measured on fully expanded leaves (n = 60 per leaf position and species) from the excised twigs in five sampling campaigns in spring and autumn 2011^a (2011^a indicates sampling during a drought), and winter, spring, and summer 2012. Immediately after the gas exchange analyses, we measured fresh weight (FW, mg) and projected leaf surface area (LA, cm^2) (including petioles) with Photoshop from scanned leaves at 300 dpi. We oven-dried the leaves at 70 °C for 48 h and weighed the leaves for dry weight (DW, mg) and measured leaf thickness (LT, mm) with a portable dial thickness gauge (Baxlo Precisión, Barcelona, Spain). We then calculated the percentage of the leaf water content (WC) as [1-(DW/FW)]*100. Leaf mass per area (LMA) (mg cm^{-2}) was calculated as the ratio of DW to LA and leaf tissue density (D, mg cm^{-3}) as the ratio of LMA to LT. Foliar Succulence (S) was calculated as (FW-DW)/LA. We ground the leaves to a fine powder using a MM400 mixer mill (Retsch, Hahn, Germany), encapsulated a sample of 0.7 mg in tin foil and determined carbon and nitrogen contents by EA/IRMS (Elemental Analyzer/Isotope Ratio Mass Spectrometry) and GC/C/IRMS (Gas Chromatography/Combustion/IRMS). The crown condition was assessed using 'International Co-operative

Programme on Assessment and Monitoring of Air Pollution Effects on Forests' (ICP Forests)standards (Eichhorn *et al.*, 2010).

3.3.10 Statistical analyses

We performed the statistical analyses with the R version 3.0.2 (http://www.r-project.org/). The matrix of photosynthetic and morphological traits was subjected to principal component analyses (PCAs) to summarise the principal factors explaining the variation in these parameters. Differences in the parameters between sunlit and shaded leaves were determined with Student's *t*-tests ($P \leq 0.05$). The normality of the data was tested with Shapiro-Wilk tests. If the data were not normally distributed, they were normalised. One-factorial analyses of variance (ANOVAs) with season as the main factor were used to test for differences in the parameters in each species and leaf position. Significant differences were determined at $P \leq 0.05$ with Fisher's least significance difference (LSD) tests. Bonferroni correction was used for familywise error rate. Linear regression analyses were conducted to study the relationships among various leaf traits such as $A_{\mathrm{net}}/g_{\mathrm{s}}$, $A_{\mathrm{net}}/g_{\mathrm{m}}$, $J_{\mathrm{max}}/V_{\mathrm{c,max}}$, $g_{\mathrm{m}}/g_{\mathrm{s}}$, $J_{\mathrm{amb}}/A_{\mathrm{net}}$. With analyses of covariance (ANCOVAs), we tested for differences in regression slopes and intercepts. We applied a non-linear regression analysis using the nls function in R to study the relationship of g_{m}/LMA.

3.4 Results

3.4.1 Environmental and crown conditions

The year 2011 was characterised by 30% more precipitation than the climatic average of 723 mm (1951-2010) (Ninyerola *et al.*, 2007a,b) (Tab. 3, Fig. 1), and no drought-induced leaf shedding was observed. The winter from 1 December 2011 to 31 January 2012 was relatively mild with average maximum and minimum temperatures of 11.8 and 4.2 °C, respectively, coinciding with high photosynthetic potentials and shoot growth. The precipitation in 2012 was 20% lower than the climatic average (Table 3). *A. unedo* and *Q. pubescens* were strongly defoliated during summer 2012; *Q. ilex* and *P. halepensis* to a lesser extent (Table 4). *Q. ilex* showed some discoloration in the more exposed sites. Only one individual of *P. halepensis* showed discoloration. The defoliated *Q. pubescens* trees recovered completely in 2013. In contrast, heavily affected individuals of *A. unedo* showed an irreversible dieback of the main leading branches but also vigorous re-sprouting in 2013.

Table 3.2 | Environmental conditions of two contrasting years (2011 and 2012). Total precipitation, mean temperature, mean soil-water content (SWC), and VPD are listed for each season/year.

	Precipitation (mm)		Temperature (°C)		SWC ($cm^3\ cm^{-3}$)		VPD (kPa)	
Season	2011	2012	2011	2012	2011	2012	2011	2012
Winter	254	25	8.2	7.3	0.17	0.14	0.3	0.4
Spring	197	141	16.6	16.3	0.19	0.15	0.6	0.8
Summer	81	50	22.4	23.4	0.13	0.12	0.9	1.2
Autumn	272	263	13.4	12.6	0.19	0.18	0.4	0.3
Total	804	479	15.3	15.1	0.17	0.14	0.5	0.7

Table 3.3 | Percentages of crown defoliation of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* (*n* = 5, 4, 5, and 5, respectively) assessed during the severe summer 2012 drought, following ICP standards (Eichhorn *et al.*, 2010).

Defoliation (%)	*Q. ilex*	*P. halepensis*	*A. unedo*	*Q. pubescens*
90-95			4	2
85-90			1	1
50-55				2
20-25	2			
10-15	1	1		
0	2	3		

3.4.2 Effect of season, tree species and leaf position on photosynthetic parameters

In Fig. 2a, we present the PCA for the morphological and photosynthetic parameters. No rotation was applied to the space of the PC's. $V_{c,max}$, J_{max}, and g_s were negatively correlated with N_{mass}, C_{mass}, NPQ, and g_m. F_v/F_m, g_s, and water content (WC) were negatively correlated with nitrogen and carbon per unit leaf area (N_{area}, C_{area}), LMA, and density (D). Nitrogen per unit leaf mass (N_{mass}) and g_m correlated well with LT (Fig. 2). A_{net} was correlated negatively with succulence (S) and positively with g_m. PC1 and PC2 explained 37.2 and 20.4% of the variation, respectively. The datapoints within the cluster circles in Fig. 1b-d exhibited similar behaviours in photosynthetic and morphological traits. Leaf positions, seasons, and species could be separated. Sunlit leaves were characterised by higher values on the orthogonal axis. The horizontal axes separated *A. unedo* and *Q. pubescens* from *Q. ilex* and *P. halepensis*. The orthogonal axes separated *Q. ilex* from *P. halepensis* with generally positive values. The seasonality was further investigated for each species and leaf position with ANOVAs for each photosynthetic and morphological parameter.

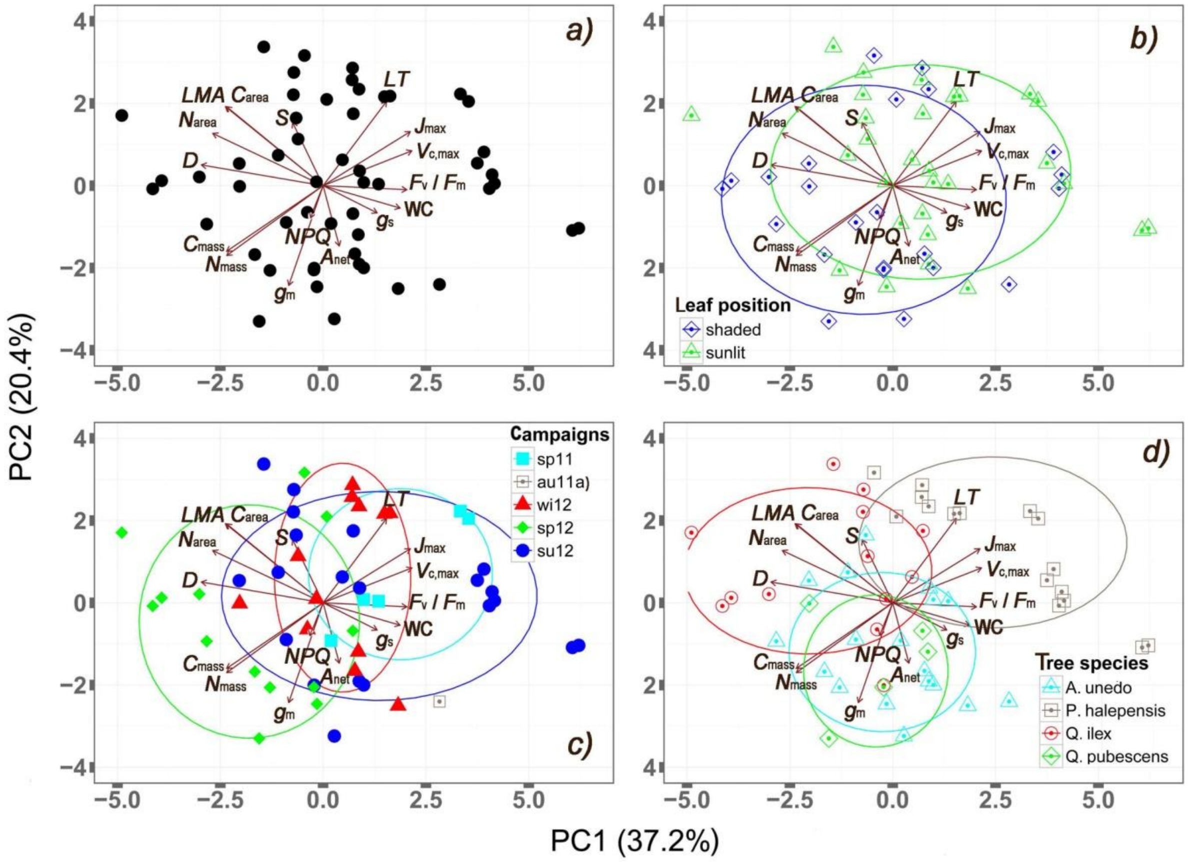

Fig. 3.2 | Principal component analyses (PCA) for a) all trees species, leaf positions, and seasons, b) with differentiation between sunlit and shaded leaves, c) with differentiation between seasonal campaigns, and d) with differentiation between species. We used a subset of all data where both morphological and photosynthetic information was available. Fifteen parameters were used in the PCA: net assimilation rate (A_{net}), stomatal conductance (g_s), mesophyll conductance (g_m), maximum carboxylation rate ($V_{c,max}$), maximum electron transport rate (J_{max}), nonphotochemical quenching (NPQ), maximum quantum efficiency of PSII (F_v/F_m), leaf thickness (LT), leaf mass per area (LMA), leaf density (D), water content (WC), nitrogen content per leaf unit area (N_{area}), nitrogen content per leaf unit mass (N_{mass}), carbon content per leaf unit area (C_{area}), and carbon content per leaf unit mass (C_{mass}). The directions of the arrows indicate the higher levels of the parameters. Principal component (PC) 1 explains 37.2% of the variation, and PC 2 explained 20.4%. The ellipses are normal probability contour lines of 68% for the factors in b) leaf positions, c) seasons, and d) species.

Q. *ilex* had the most plastic response to the environmental conditions. The sunlit leaves of *Q. ilex* exhibited strong declines in several photosynthetic parameters from summer 2011 to autumn 2011^a. $V_{c,max}$, A_{net} and g_s were significantly ($P < 0.05$), and J_{max} and g_m were marginally significantly lower ($P < 0.10$) (Fig. 3 a1-b1). The means of the majority of the photosynthetic parameters recovered after the first rains in autumn 2011^b (2011^b indicates sampling after the drought), reaching pre-drought values, but accompanied by a high standard error. This recovery was thus only significant for J_{max} and g_m. Surprisingly, $V_{c,max}$ and J_{max} peaked in winter and not, as expected, in spring. From that peak we observed significant declines from winter to spring to summer 2012. In contrast to the pattern of $V_{c,max}$ and J_{max}, F_v/F_m, A_{net}, and g_s peaked in spring 2012 (Figs. 3c1, 4a1-b1). These parameters then also declined significantly in summer 2012. Interestingly, g_m peaked in summer 2012 in parallel with a reduction in g_s (Fig. 4c1). The photosynthetic parameters of shaded leaves in *Q. ilex* showed a similar trend, declining after the drought in 2011 and recovering after the autumn rains (Figs. 3, 4). The parameter means of shaded leaves remained relatively stable throughout the season, in contrast to the pattern in sunlit leaves, except for a peak of $V_{c,max}$ and J_{max} in spring 2012. The photosynthetic parameters in *Q. ilex* were significantly lower in shaded leaves. During periods of stress, however, the photosynthetic parameters of sunlit leaves declined and had values similar to those of shaded leaves (Table 6, Figs. 3, 4).

P. halepensis had the highest mean $V_{c,max}$, J_{max}, and F_v/F_m in sunlit leaves than the other species (Figs. 3, 4). The seasonal variation of the photosynthetic potential was not as strongly pronounced as in *Q. ilex*, and mean $V_{c,max}$ and J_{max} remained relatively high and stable in 2011 (Fig. 3a1-b1). The 2012 drought had comparatively stronger effects on $V_{c,max}$ and J_{max} than the 2011 drought. Mean A_{net}, g_s, and g_m, however, were significantly lower in autumn 2011[a] (Fig. 4a1-c1). These values recovered quickly and significantly after the first autumn rains. The relatively high $V_{c,max}$, J_{max}, and F_v/F_m during this period reflected a stronger limitation of g_s and g_m than of the biochemistry imposed on A_{net}. A_{net} recovered in winter 2012 due to the mild conditions (Fig. 4a1). The 2012 summer drought significantly reduced the high values of A_{net} observed in winter 2012, but not as much as after the 2011 drought (Fig. 4a1). Both g_s and g_m remained relatively stable during this period, so the reductions in A_{net} were due to biochemical limitations ($V_{c,max}$ and J_{max}) (Figs. 3, 4). Sunlit and shaded leaves differed the least in *P. halepensis*; only $V_{c,max}$ and J_{max} were significantly different (Table 6). The sunlit and shaded leaves of *P. halepensis* had similar patterns of seasonal variation, but changes between seasonal campaigns were not significant (Fig. 3a1-b1).

A. unedo was similar to *Q. ilex* with seasonally variable photosynthetic parameters, but also with high standard errors (Figs. 3, 4). A_{net} decreased significantly in winter 2012, in contrast to J_{max} and $V_{c,max}$ that peaked in the same campaign (Figs. 3a1-b1 and 4a1). A decline in g_s and g_m in this campaign suggested that they more strongly regulated A_{net} (Fig. 4b1-c1). A_{net}, g_s, and g_m peaked in spring 2012. These increases were significant for A_{net} and g_s and marginally significant for g_m relative to the other field campaigns (Fig. 4a1-4c1). The photosynthetic parameters were generally lower in the shaded leaves of *A. unedo*, but with no clear pattern and high variability (Table 5).

Q. pubescens had much higher photosynthetic potentials than the other species but also had high standard errors (Fig. 3a1-b1). The 2012 summer drought led to a decline of the photosynthetic potentials by approximately one third. These decreases were only significant for the average of spring 2011 and spring 2012 relative to the average of summer 2011 and summer 2012. A_{net} showed a similar trend, with a peak in spring 2012 being reduced significantly by the 2012 summer drought (Fig. 4a1). Stomatal control was more strongly pronounced than mesophyllic control (Fig. 4b1-c1). Shaded leaves had higher A_{net}, g_{m}, and g_{s} means throughout the campaigns, in contrast to lower means of $V_{\mathrm{c,max}}$ and J_{max} (Figs. 3a1-c2, 4a1-b2). Shaded leaves generally showed lower values than sunlit leaves and were less affected by the droughts (Figs. 3a1-c2, 4a1-c2).

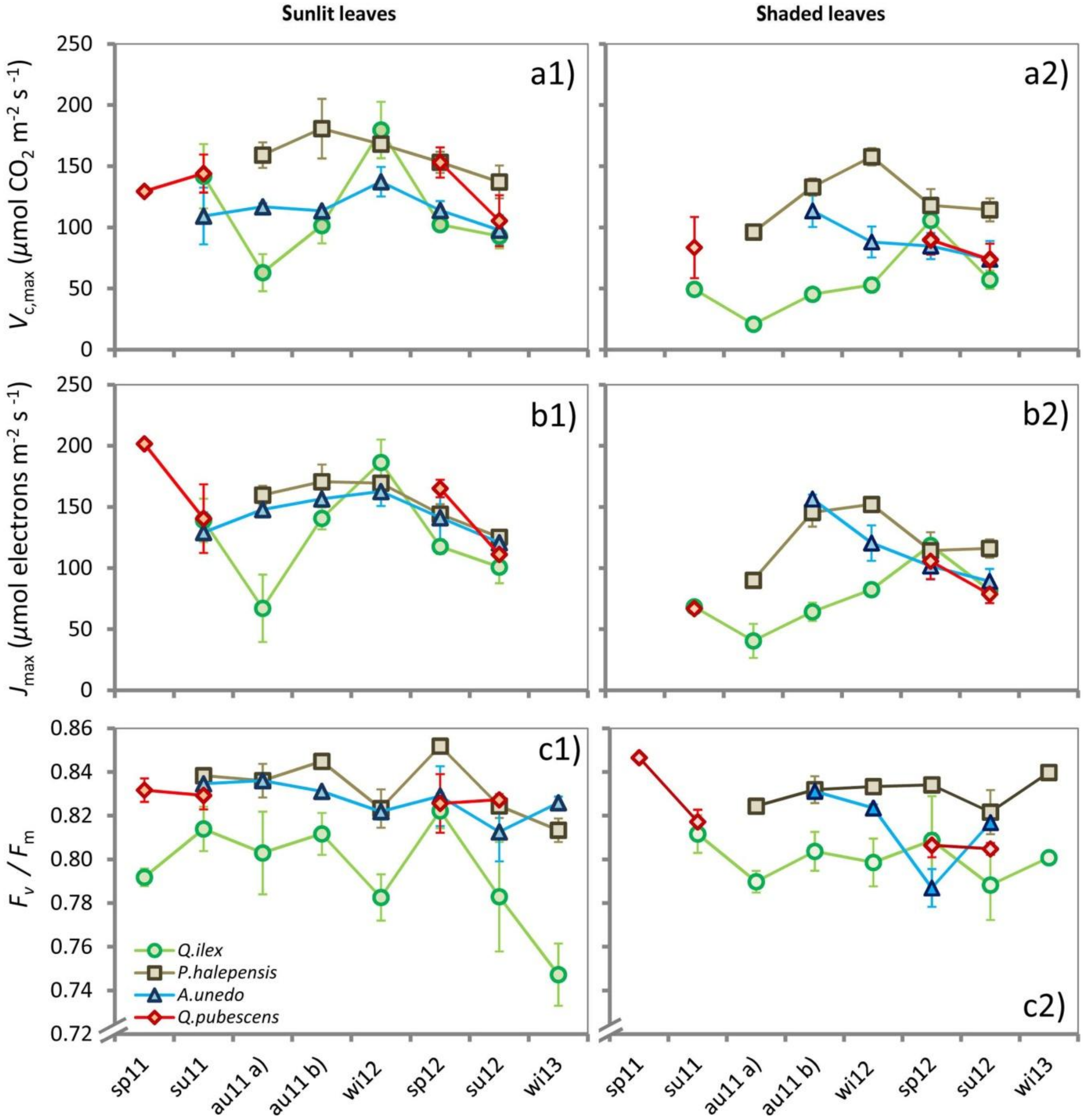

Fig. 3.3 | Line graphs depicting seasonal changes of a) maximum carboxylation rate ($V_{\mathrm{c,max}}$), b) maximum electron-transport rate (J_{max}), and c) maximum quantum efficiency of PSII (F_v/F_m) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Seasonal campaigns were conducted in spring 2011 (sp11), summer 2011 (su11), autumn 2011^a (au11 a), autumn 2011^b (au11b), winter 2012 (wi12), spring 2012 (sp12), summer 2012 (su12), and winter 2013 (wi13). Missing data points were due to limitations of labour and equipment. Vertical bars indicate standard errors of the means (*n* = 3-5).

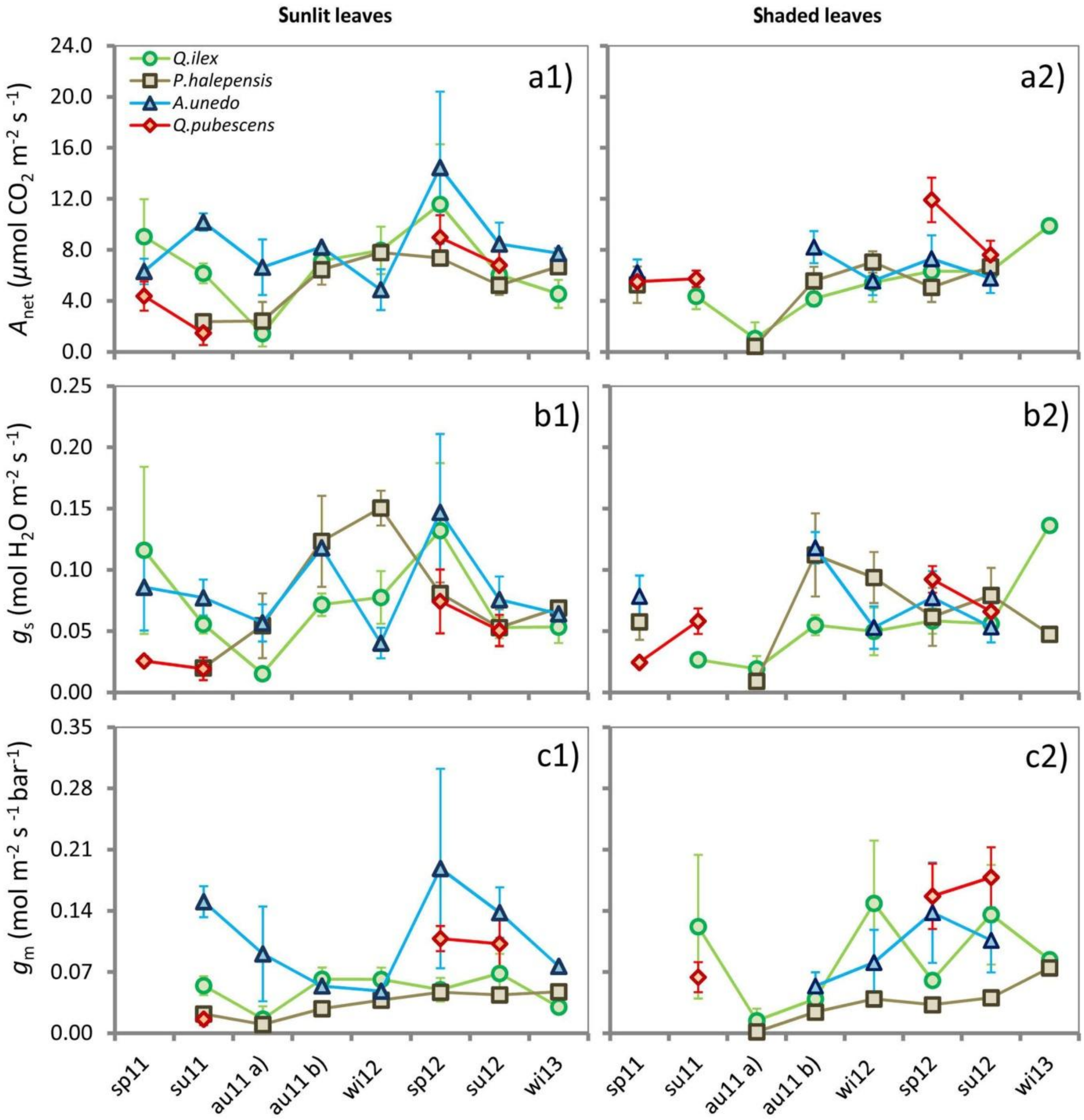

Fig. 3.4 | Line graphs depicting seasonal changes of a) net assimilation (A_{net}), b) stomatal conductance (g_s), and c) mesophyll conductance (g_m) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Seasonal campaigns were conducted in spring 2011 (sp11), summer 2011 (su11), autumn 2011^a (au11 a), autumn 2011^b (au11b), winter 2012 (wi12), spring 2012 (sp12), summer 2012 (su12), and winter 2013 (wi13). Missing data points were due to limitations of labour and equipment. Vertical bars indicate standard errors of the means (*n* = 3-5).

3.4.3 Morphological parameters

The foliar traits of *P. halepensis* and *Q. ilex* acclimated most strongly to drought. LMA was significantly higher in *P. halepensis* and *Q. ilex* in both shaded and sunlit leaves in summer 2012 compared to the previous field campaigns (Fig. 5a1-a2). This was similar in *A. unedo* but less pronounced. LMA had no clear pattern in *Q. pubescens*. Elevated LMA was accompanied by higher values of leaf density (D), succulence (S), and carbon content, indicating a more sclerophyllic and succulent structure as response to the drier conditions in 2012 (Figs. S2, S3). N_{mass}

was significantly higher in spring and summer 2012 for *Q. ilex* and *P. halepensis* (shaded and sunlit leaves) and for shaded leaves of *A. unedo*, but not for *Q. pubescens* (Fig. 5b1-b2).

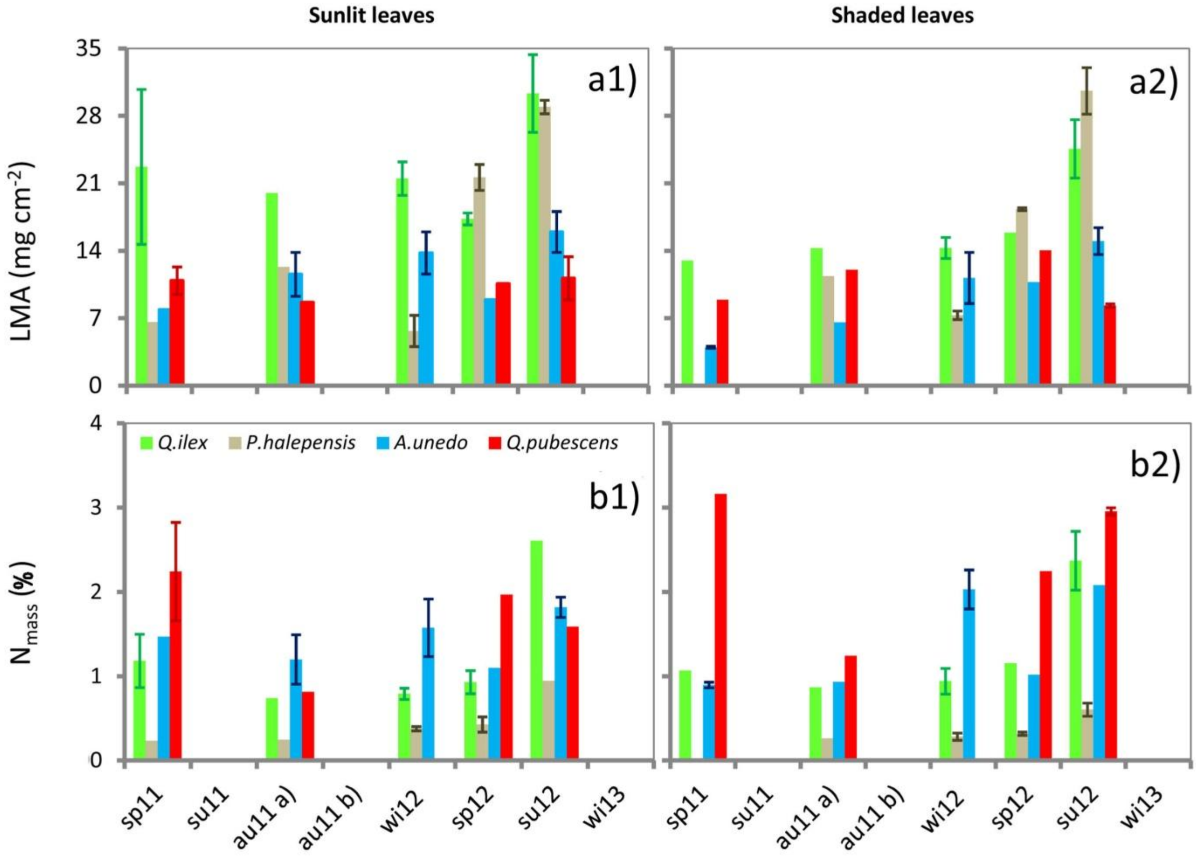

Fig. 3.5 | Bar charts depicting seasonal changes of a) leaf mass per area (LMA) and b) percentage of nitrogen content per unit leaf mass (N_{mass}) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Error bars indicate standard errors of the means (*n* = 3-5).

3.4.4 Relationships of photosynthetic and morphological parameters

In order to analyse the general pattern of several relationships of the photosynthetic parameters and foliar traits, we used ANCOVAs to test for differences in the slopes between seasons across all species.

The slope the A_{net}/g_s relationship was significantly steeper in summer and autumn 2011^a in all species compared to the other field campaigns (Fig. 6a1, Table S1), suggesting an increased intrinsic water-use efficiency during the dry period in 2011. Shaded leaves had a similar conservative water-use strategy in autumn 2011^a (Fig. 6a2). Shallower slopes in autumn 2011^b in both leaf positions represent rapid responses (less than one week) to the post-drought rains easing the strict stomatal control.

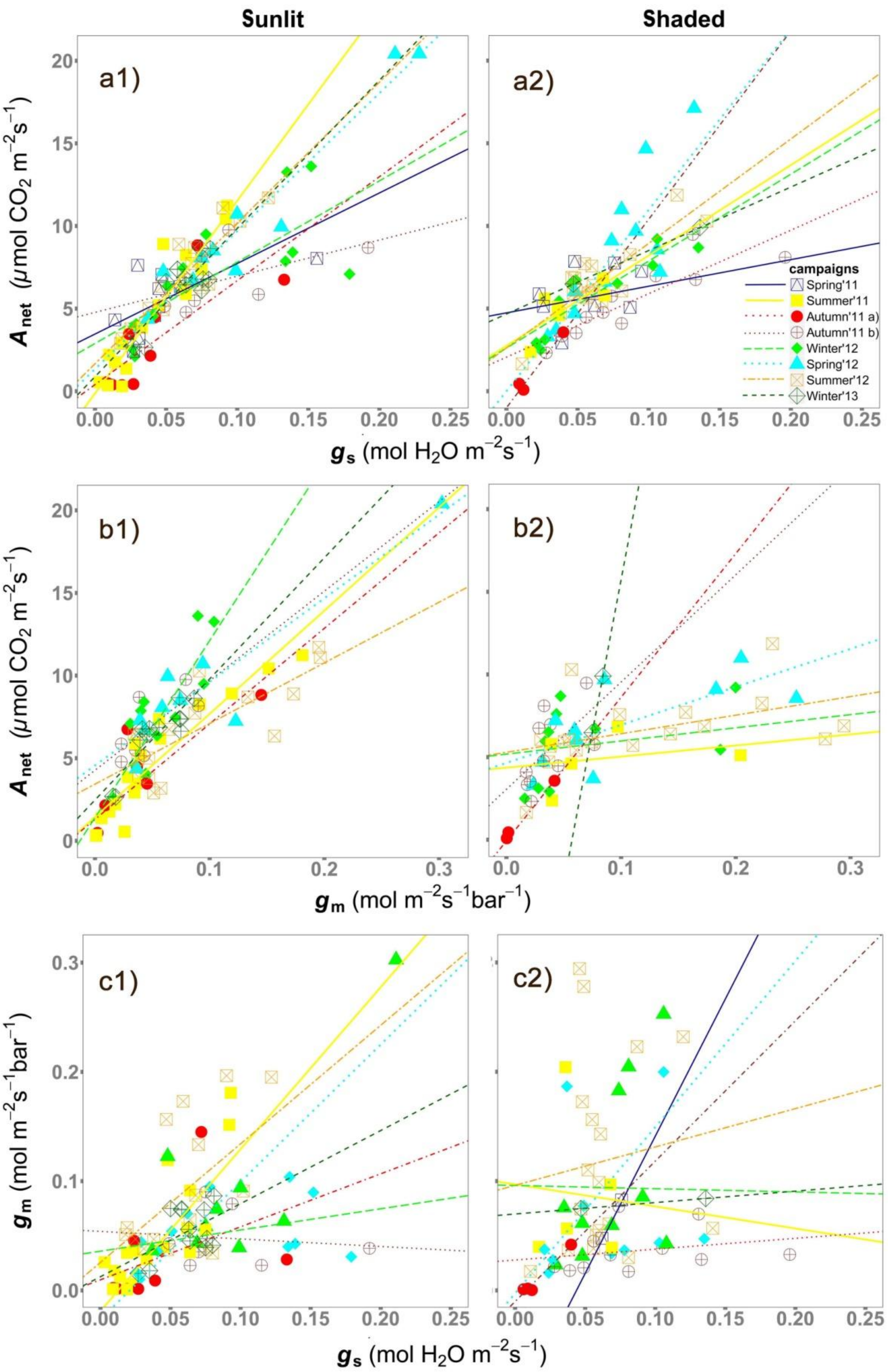

Fig. 3.6 | Seasonal changes of the relationships between a) net assimilation (A_{net}) and stomatal conductance (g_s), b) A_{net} and mesophyll conductance (g_m), and c) g_m and g_s in sunlit (1) and shaded (2) leaves. The regression lines represent the seasonal changes across species. For regression equations see Table S1-3. The relationships are shown as a thin solid line for spring 2011, short dashes for summer 2011, dots-dashes for autumn 2011$^{a)}$, small dots for autumn 2011$^{b)}$, dashes for winter 2012, large dots for spring 2012, large dots-dashes for summer 2012, and a thick solid line for winter 2013. Statistical differences in the slopes between seasonal campaigns were tested by ANCOVAs.

The $A_{\mathrm{net}}/g_{\mathrm{m}}$ relationship in autumn 2011$^{\mathrm{a}}$ also had a significantly steeper slope in both sunlit and shaded leaves recovering after the first rains in autumn 2011$^{\mathrm{b}}$ (Fig. 6b1-2, Table S2). In the drier year 2012, g_{m} imposed less resistance on photosynthetic assimilation compared to the wet year 2011. The slope of the $A_{\mathrm{net}}/g_{\mathrm{m}}$ relationship was significantly higher for winter 2012 than spring and summer 2012, suggesting a stronger control of g_{m} on photosynthesis in winter. The autumn 2011$^{\mathrm{a}}$ and summer 2012 droughts had strong effects on the slope of $A_{\mathrm{net}}/g_{\mathrm{m}}$ in shaded leaves.

With the ANCOVA of the relationship of g_{m} and g_{s}, we investigated the proportional diffusion limitation on photosynthesis. We observed seasonal differences across all species (Fig. 6c, Table S3). Mesophyllic control was stronger in the dry autumn 2011$^{\mathrm{a}}$ and the two winter periods. In contrast, stomatal control was higher than mesophyllic control in the mild 2011 summer drought. This was most strongly pronounced in *P. halepensis* and *Q. ilex* (data not shown).

The slope in the relationship of $V_{\mathrm{c,max}}$ and J_{max} was significantly steeper in autumn 2011$^{\mathrm{a}}$ for both sunlit and shaded (Fig. 7a, Table S4) leaves due to a stronger reduction in $V_{\mathrm{c,max}}$ compared to J_{max}. The overall $J_{\mathrm{max}}/V_{\mathrm{c,max}}$ ratios were 1.09 for sunlit and 1.24 for shaded leaves. The slope of the $J_{\mathrm{amb}}/A_{\mathrm{net}}$ relationship in sunlit and shaded leaves was significantly lower in the more humid periods (autumn 2011$^{\mathrm{b}}$, winter 2012, and winter 2013), indicating lower protective energy dissipation and alternative electron pathways under favourable conditions (Fig. 7b, Table S5).

Increased foliar sclerophylly led to higher LMAs and thus to higher diffusion resistances in the mesophyll, as shown by the relationship between g_{m} and LMA (Fig. 8, Table S6). In spring 2012 and summer 2012, we detected a less negative exponent (hence a gentler curve) (-0.953 and -0.800, respectively) compared to winter 2012 and autumn 2011$^{\mathrm{a)}}$ (-1.486 and -1.533, respectively). This shows that, regardless of the drier conditions and higher LMA in 2012, g_{m} was higher in this period reflecting a regulatory mechanism of g_{m} in the CO_2 diffusion pathway (in line with the results of the $g_{\mathrm{m}}/g_{\mathrm{s}}$ analyses).

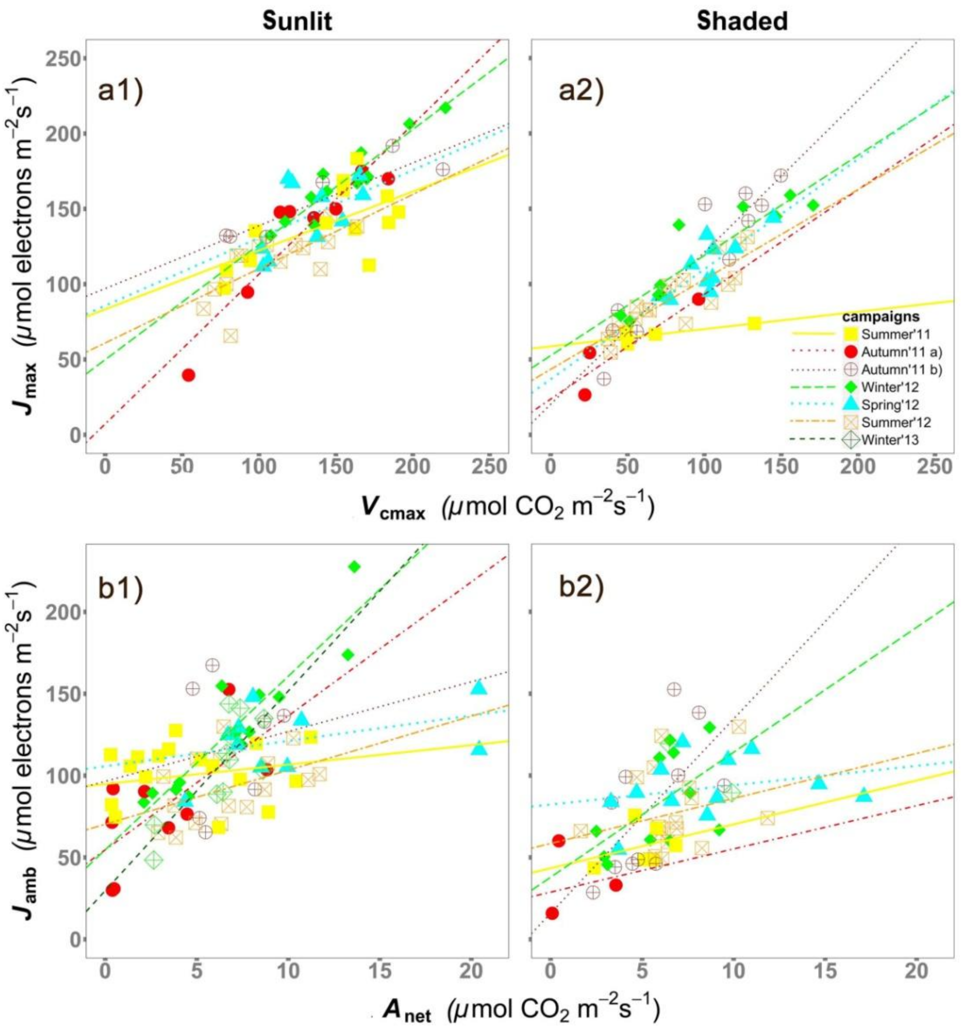

Fig. 3.7 | Seasonal changes of the relationships between a) the maximum electron-transport rate (J_{max}) and the maximum carboxylation rate ($V_{c,max}$) and b) the electron-transport rate from chlorophyllic fluorescence (J_{amb}) and net assimilation (A_{net}) at ambient CO$_2$ concentrations and saturating light in sunlit (a) and shaded (b) leaves. The regression lines represent the seasonal changes across species. For regression equations see Table S4-5. The relationships are shown as a thin solid line for spring 2011, short dashes for summer 2011, dots-dashes for autumn 2011$^{a)}$, small dots for autumn 2011$^{b)}$, dashes for winter 2012, large dots for spring 2012, large dots-dashes for summer 2012, and a thick solid line for winter 2013.

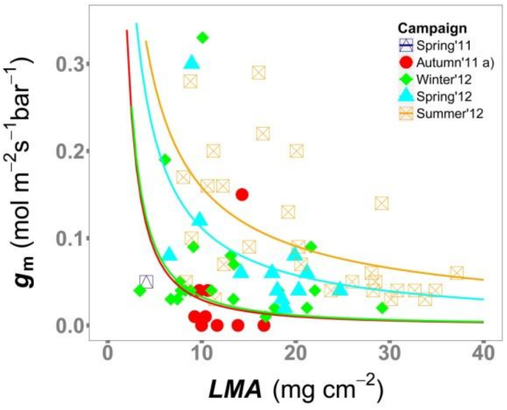

Fig. 3.8 | Seasonal changes of the relationship for all species and leaf positions between a) mesophyll conductance (gm) and leaf mass per area (LMA).We used a subset of morphological and photosynthetic data. Non-linear regression lines of the form $y = x^{-b}$ were fitted to the data. The upper curve is for summer 2012 (b = 0.800), the middle curve is for spring 2012 (b = 0.953) and the lower two overlaying curves are for autumn 2011a) (b = 1.533) and winter 2012 (b = 1.486).

Table 3.5 | Means ± standard errors of a set of photosynthetic parameters and foliar traits for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens*. *P*-values indicate the statistical significance of the differences between sunlit and shaded leaves determined by Student's *t*-tests. Significance is indicated with blue bold text.

Species	*Q. ilex*			*P. halepensis*			*A. unedo*			*Q. pubescens*		
Position	**Sunlit**	**shaded**		**Sunlit**	**Shaded**		**Sunlit**	**Shaded**		**sunlit**	**Shaded**	
Variable	**Mean**	**Mean**	***P***	**Mean**	**Mean**	***P***	**Mean**	**Mean**	***P***	**Mean**	**Mean**	***P***
$V_{c,max}$	121.5±11	53.3±5	**0.000**	158.2±5	128.6±5	**0.001**	111±8	85±7	**0.018**	134±11	81±9	**0.002**
J_{max}	134.6±10	76.5±5	**0.000**	149.7±5	130.6±6	**0.023**	133±8	110±8	**0.045**	135±12	83±7	**0.004**
$J_{max}/V_{c,max}$	1.11±0.05	1.42±0.07	**0.002**	0.98±0.03	1.01±0.02	0.356	1.20±0.03	1.34±0.06	0.064	1.03±0.07	1.09±0.08	0.549
F_v/F_m	0.79±0.01	0.80±0.30	0.302	0.83±0.003	0.827±0.003	0.420	0.81±0.006	0.82±0.004	0.631	0.829±0.003	0.783±0.03	0.131
R_d	1.28±0.11	0.98±0.09	**0.043**	1.72±0.19	1.33±0.16	0.123	1.48±0.11	0.88±0.12	**0.001**	0.99±0.14	0.96±0.11	0.890
A_{net}	6.6±0.7	4.89±0.47	**0.056**	5.5±0.5	5.8±0.5	0.657	7.4±0.7	6.4±0.6	0.267	4.3±0.9	8.4±1.0	**0.004**
g_s	0.070±0.0110	0.049±0.006	0.104	0.083±0.101	0.080±0.012	0.839	0.069±0.008	0.069±0.008	0.967	0.035±0.007	0.068±0.008	**0.005**
g_m	0.048±0.006	0.082±0.018	0.084	0.033±0.003	0.035±0.004	0.771	0.096±0.014	0.095±0.019	0.959	0.060±0.018	0.141±0.023	**0.013**
Φ_{PS2}	0.202±0.02	0.114±0.01	**0.000**	0.252±0.012	0.233±0.015	0.312	0.206±0.013	0.164±0.012	**0.024**	0.200±0.017	0.165±0.011	0.103
NPQ	2.89±0.12	3.14±0.15	0.197	3.17±0.10	3.10±0.14	0.689	3.49±0.15	3.68±0.17	0.432	3.17±0.26	2.54±0.14	**0.050**
LMA	23.8±1.5	19.1±1.5	**0.027**	18.6±2.4	19.7±2.5	0.763	13.1±1.1	11.5±1.3	0.338	10.9±0.6	9.7±0.9	0.239
LT	0.039±0.001	0.030±0.001	**0.000**	0.067±0.001	0.059±0.001	**0.000**	0.028±0.001	0.025±0.001	**0.041**	0.031±0.001	0.029±0.001	**0.038**
C_{area}	105.5±8.3	96.2±8.5	0.440	82.2±16.8	98.2±14.1	0.470	57.6±4.4	59.2±8.7	0.873	49.0±2.8	48.5±5.8	0.942
C_{mass}	35.5±4.1	51.6±	**0.020**	22.3±2.7	20.7±2.4	0.661	62.1±5.6	70.8±8.3	0.395	48.6±4.4	68.6±5.4	**0.010**
N_{area}	3.19±0.29	2.88±0.29	0.451	1.48±0.33	1.89±0.29	0.353	1.39±0.09	1.31±0.19	0.711	1.78±0.16	1.87±0.22	0.743
N_{mass}	1.08±0.14	1.55±0.18	**0.047**	0.41±0.06	0.40±0.05	0.890	1.51±0.14	1.58±0.18	0.763	1.78±0.23	2.71±0.26	**0.015**

3.5 Discussion

3.5.1 Photosynthetic seasonality and effects of drought

We found that $V_{c,max}$ and J_{max} acclimated strongly to the seasonal changes in temperature and water availability in agreement with previous studies (Vitale & Manes, 2005; Corcuera *et al.*, 2005; Misson *et al.*, 2006; Ribeiro *et al.*, 2009; Limousin *et al.*, 2010). High radiation and water stress can have a combinatory negative effect on the photosynthetic apparatus, especially in sunlit leaves. Stomata close to avoid transpiration loss and hydraulic failure, but stomatal closure impairs the diffusion of the CO_2 needed in the chloroplasts, the site of carboxylation. $V_{c,max}$ is a proxy for the maximum potential rate of carboxylation, which is carried out by Rubisco, a costly nitrogen-rich protein. The temporary unemployment of Rubisco due to limited substrate (CO_2) availability leads to its deactivation and, during chronic water stress, to its decomposition (Parry, 2002; Chaves & Oliveira, 2004; Lawlor & Tezara, 2009). High incoming radiation that cannot efficiently be dissipated in the Calvin cycle over-excites the photoreaction centres (photoinhibition) and produces reactive oxygen species (ROS) that damage the photosystems and the ATP synthase- needed for the carbon reactions (Epron *et al.*, 1993). Leaves prevent harmful excess energy with protective actions such as the reorganisation of the thylakoid membrane, closure of reaction centres, and reduced antennal size (Huner *et al.*, 1998; Maxwell & Johnson, 2000; Ensminger *et al.*, 2012; Verhoeven, 2014). These actions reduce PSII efficiency and J_{max}, and enhance alternative energy pathways to prevent damage on the molecular level on the cost of a lower carbon assimilation.

The trees in our study site maintained considerable rates of A_{net} during moderate drought through improved water relations via g_s and g_m control. The relatively stable F_v/F_m values indicate that the protective actions against photoinhibitory stress were effective. The trees showed trunk rehydration after the first autumn rain (Sánchez-Costa *et al.*, 2015) and quickly recovered their photosynthetic potential, suggesting that the Rubisco content remained unaffected by moderate drought. The drought impacts were much more severe in the dry year 2012, illustrating the vulnerability of tree physiology to the depletion of soil-water reserves during the early growing season. The severity of drought strongly determined the relative limitations of g_s and g_m on photosynthesis, especially in *Q. ilex* and *P. halepensis*. Stomatal closure regulated photosynthesis during both the moderate and servere droughts; g_m, in contrast, decreased under moderate, but increased under severe drought. We postulate that altered g_m can ease the leaf internal CO_2 diffusion needed for photosynthesis, especially under chronic water stress when depleted non-structural carbohydrates (NSCs) make plants particularly reliant on photosynthetic products for refinement, repair, and protective actions (Niinemets *et al.*, 2009). Major changes of Φ_{PSII}, F_v/F_m, and photosynthetic potentials across all species reflected these refinements of the photosynthetic apparatus as responses to chronic water stress in summer 2012.

These acclimatisations occurred not only under dry and hot conditions, but also in winter at high radiation and low temperature. Nevertheless, favourable winter conditions in 2012 resulted in biochemical recovery (peak of $V_{c,max}$ and J_{max}), new shoot growth, and moderate transpiration across species (often exceeding summer values) (Sánchez-Costa *et al.*, 2015). Year-round growth patterns with several flushes during the year have also been reported in other studies (Alonso *et al.*, 2003). Under novel climatic conditions, favourable conditions in winter may be crucial in the competition between evergreen and deciduous tree species.

We observed a highly species-specific pattern. *Q. ilex* and *A. unedo* followed a water-spending, anisohydric strategy that maintained A_{net} and g_s in parallel with lower $V_{c,max}$ and J_{max}. In contrast, *P. halepensis* had significantly decreased g_s, consistent with the conservative water-use strategy and strict stomatal control of isohydric species (Borghetti *et al.*, 1998; Martinez-Ferri *et al.*, 2000). *Q. ilex* generally responded most plastically by rapidly adjusting the photosynthetic machinery to the prevailing conditions (García-Plazaola *et al.*, 1997, 1999; Martínez-Ferri *et al.*, 2004). *P. halepensis* was the most tolerant to photoinhibition and had the most robust photosynthetic machinery to combat abiotic stress (Baquedano & Castillo, 2006; Sperlich *et al.*, 2014). The mesophyllic diffusion limitation was lowest in *Q. pubescens* and *A. unedo*, as we claim, due to their deciduous/semi-evergreen foliar habits and lower LMAs (see also Tomás *et al.*, 2014). *Q. pubescens* must maximise gas exchange during a shorter growing season, leading to high photosynthetic potentials, A_{net} (Baldocchi *et al.*, 2009) and transpiration rates throughout the summer (Sánchez-Costa *et al.*, 2015; Poyatos *et al.*, 2008).

3.5.2 Responses specific to leaf position

The seasonality of photosynthetic parameters was qualitatively different between leaf positions (Niinemets *et al.*, 2006; Vaz *et al.*, 2011) and was mostly pronounced in sunlit leaves. Shaded leaves cushioned the negative climatic effects, maintaining their functionality compared to sunlit leaves. Foliar anatomy, morphology, and biochemistry were highly specialised and dependent on the light regime, leading to smaller but also thicker sunlit leaves and broader and thinner shaded leaves (Kull & Niinemets, 1993; Terashima &

Hikosaka, 1995; Niinemets, 2001). Shaded leaves had lower N, photosynthetic potentials, carbon metabolisms and higher $J_{max}/V_{c,max}$ ratio (see also Le Roux *et al.* 2001). Shaded leaves invest in higher J_{max} relative to $V_{c,max}$ in order to increase the light-use efficiency. Responses specific to leaf position, however, differed among tree species due to distinct foliar morphologies and crown architectures. The sun-exposed crown position of *P. halepensis*, surmounting the forest canopy resulted in high photosynthetic potentials and a low $J_{max}/V_{c,max}$ ratio throughout the crown. Pine needles attain nearly saturated photosynthetic rates over a wide range of diurnal and seasonal variation in radiation due to their cylindrical shape and steep angles (Jordan & Smith, 1993; Lusk *et al.*, 2003). Similarly, *Q. pubescens* showed a low differentiation between sunlit and shaded leaves. A low $J_{max}/V_{c,max}$ ratio throughout the crown suggests a higher proportion of sunlit leaves. In contrast, the comparatively higher $J_{max}/V_{c,max}$ ratio of sunlit leaves in *A. unedo* reflects a more shaded growth environment explained by its subordinated position in the forest canopy. The *Q. ilex* canopy was dense with a high proportion of shaded leaves, in line with its shade tolerance. Hence, leaf position specific responses were highest in *Q. ilex*. The comparatively higher photosynthetic values in sunlit leaves decreased partly below the level of shaded leaves under stress conditions (see also Sperlich *et al.*, 2014). Shaded leaves are less exposed to the dramatic changes in radiation and temperature in the outer canopy and can be of particular importance for *Q. ilex* to attain a positive net carbon ratio during stress periods (Valladares *et al.*, 2008). We stress that the solar environment of the leaves is a crucial factor for assessing tree performance, especially in a competitive environment.

3.5.3 Acclimation of foliar morphology

Mediterranean trees acclimate to water deficits with higher investments in structural compounds, thereby increasing leaf density and succulence (Niinemets, 2001; Ogaya & Peñuelas, 2006; Poorter *et al.*, 2009). Foliar traits are known to be good indicators for the ability of Maquis-species to respond to decreases in rainfall under climate change (Gratani & Varone, 2006; Ogaya & Peñuelas, 2007). We confirm that severe water deficit resulted in increased LT and reduced LA and consequently in higher LMA. It was reported that the plasticity of leaf morphology is generally higher than the plasticity of foliar chemistry and assimilation rates over a wide range of woody species (Niinemets, 2001). Under moderate drought, however, foliar morphology was less plastic than foliar chemistry and assimilation rates (Quero *et al.*, 2006); severe water stress affected both to a similar extent. Leaf trait acclimation strongly constrained mesophyll conductance under severe drought, especially in *Q. ilex* and *P. halepensis* (see also Tomás et al. 2013). We postulate that foliar traits served best as proxies for drought acclimation in *Q. ilex* (Grossoni *et* *al.*, 1998; Bussotti *et al.*, 2000) and *P. halepensis* (Alonso *et al.*, 2003), both characterised by high leaf-longevities. These changes may be accompanied by increased leaf vein density that may helped to increase the tolerance to foliar hydraulic dysfunction in Mediterranean plants (Nardini *et al.*, 2014). The foliar traits of *A. unedo* and *Q. pubescens* acclimated the least, so leaves were susceptible to foliar hydraulic dysfunction and drought-deciduousness. We attribute this species-specificity in leaf trait acclimation to functional differences of leaf investment costs and distinct leaf shedding strategies between deciduous / semi-deciduous (*Q. pubescens* and *A. unedo*) to evergreen sclerophyllic species (*Q. ilex* and *P. halepensis*) which we will elaborate further in the following chapter.

3.5.4 Crown defoliation in summer 2012

The lack of rain in early 2012 predisposed the vegetation to leaf senescence observed in summer 2012, with high variability across and within species. Leaf senescence was highest in *A. unedo* and *Q. pubescens* – showing partly completely defoliated crowns. *Q. ilex* and mostly *P. halepensis* overcame this period with marginal leaf shedding. Stored NSCs strongly determine the recovery of xylem hydraulic conductivity by vessel refilling and the resistance of water transport to drought under prolonged evaporative demand (Ogasa *et al.*, 2013). Depleted NSCs may limit the ability to recover from embolisms (Galiano *et al.*, 2012). *A. unedo* is susceptible to hydraulic dysfunction induced by depleted NSC (e.g. Rosas *et al.*, 2013) which might explain the severe branch dieback of *A. unedo* in our study. As shrubby species characteristic of Maquis-biomes (Beyschlag *et al.*, 1986; Harley *et al.*, 1986), *A. unedo* likely faced a trade-off between growing tall and risking hydraulic dysfunction due to high xylem tension under severe soil-water deficits (Choat *et al.*, 2012).Though, *A. unedo* might contend with severe climatic stress through its strong capacity to resprout (see also Ogaya & Peñuelas, 2004).

Pines follow a strategy of water conservation and embolism avoidance, because they have a low capacity to store carbohydrates (Meinzer *et al.*, 2009). *P. halepensis* had a high growth-based water-use efficiency (WUE_{BAI} = Basal area increment/Tree transpiration) during severe drought (Sánchez-Costa *et al.*, 2015), through the combinatory effect of photosynthetic downregulation, foliar-trait acclimation, and improved gas exchange. Thus, this tree species is comparatively the most productive one, especially under drought, confirming its high competitiveness in dry habitats (Zavala & Zea, 2004; Maseyk et al., 2008; de Luis et al., 2011).

Sánchez-Costa *et al*. (2015) observed a higher WUE_{BAI} in *Q. pubescens* compared to *Q. ilex* during the soil-moisture deficit in 2012. The "low-cost" leaves of the deciduous *Q. pubescens* facilitate drought senescence, so that the reduced transpiratory surface area can effectively avoid damage from hydraulic cavitation and xylem embolism (Ogaya & Peñuelas, 2006; Barbeta *et al.*, 2013). Fully refoliated crowns in the following growing season was evidence of its success relative to *A. unedo*. The extraordinarily high photosynthetic potentials in the remaining leaves were probably due to a mechanism to compensate for the reduced total leaf area, as indicated by the higher translocation of leaf nitrogen before leaf shedding.

Q. *ilex* can effectively tolerate the effects of drought by reducing its LMA and by allowing low water potentials (anisohydric behaviour) (Villar-Salvador *et al.*, 1997; Ogaya & Peñuelas, 2006; Limousin *et al.*, 2009). Its hydraulic features are highly plastic, because yearly vessel diameter and recovery are well coupled with annual rainfall (Fotelli *et al.*, 2000; Corcuera *et al.*, 2004). *Q. ilex*, however, was also severely effected in 2012, shedding leaves (Tognetti *et al.*, 1998), reducing radial growth and WUE_{BAI} (Sánchez-Costa *et al.*, 2015). The positive A_{net}, despite the reduced WUE_{BAI}, suggests that photosynthetic products were used for the maintenance and recovery of xylem hydraulic conductivity instead of growth (Castell *et al.*, 1994). In fact, *Quercus* species show generally a good ability in vessel refilling after xylem embolism (Carnicer *et al.*, 2013).

3.5.5 Implications for the global carbon cycle and modelling

There is evidence that the use of seasonally variable photosynthetic potentials reduces uncertainties in modelled ecosystem carbon fluxes relative to the use of constant values (Wilson *et al.*, 2001; Tanaka *et al.*, 2002; Kosugi *et al.*, 2003, 2006; Medvigy *et al.*, 2013). The significant seasonal acclimation of $V_{c,max}$ and J_{max} observed in our study demonstrates that prognostic models should account for seasonal variation, especially in drought-prone areas. Also, the significant role of g_m under abiotic stress periods highlights its importance for estimating the whole-carbon gain. It is now widely accepted that the apparent values of $V_{c,max}$ and J_{max} derived from A/C_i curves are, from a physiological point of view, incorrect. A recent study by Sun *et al.* (2014a) for nearly 130 C_3 species showed that the assumption of infinite g_m in the parameterization of CO_2-response curves underestimates $V_{c,max}$ and J_{max} by up to 75 and 60%, respectively. Terrestrial biosphere models on regional or global scales are most commonly calibrated on A/C_i-based parameters and therefore use apparent values of $V_{c,max}$ and J_{max}. Incorporating values of $V_{c,max}$ and J_{max} parameterised on A/C_c curves would clearly lead to erroneous results, because their use requires the incorporation of g_m and different Rubisco kinetic parameters into the sub-models of photosynthesis. Therefore, the use of consistent equations and parameters when incorporating parameters from experimental studies into vegetation models is inevitable to correctly estimate photosynthesis (Rogers *et al.*, 2014). From a modelling point of view, it might seem questionable why including g_m and A/C_c– based parameters would improve simulation results and not just increase model complexity. Terrestrial biosphere models are currently well calibrated against observational data despite their use of apparent $V_{c,max}$ and J_{max}. Another criticism often raised is that there are still potential errors in various methods to estimate g_m (and subsequently $V_{c,max}$ and J_{max}) including the variable *J*-method (used in this study) (Pons *et al.*, 2009; Tholen *et al.*, 2012; Gu & Sun, 2014)**.** Nonetheless, large uncertainties remain in the simulations of the future CO_2 fluxes of the global carbon cycle (Anav *et al.*, 2013; Friedlingstein *et al.*, 2014). Patterns of temperature and precipitation are highly uncertain in these models due to both a lack of scientific understanding and model representation (Booth *et al.*, 2012).

These uncertainties could partly explain the poor modelling performance for Mediterranean-type ecosystems, because the mechanistic description of the photosynthetic processes under water stress is not very well developed (Morales *et al.*, 2005; Keenan *et al.*, 2011; Zheng *et al.*, 2012; Vargas *et al.*, 2013). As we have shown, the limitations imposed by g_m on photosynthetic assimilation can decrease relatively more than the limitations imposed by g_s or biochemistry ($V_{c,max}$ and J_{max}) under drought or winter stress. This distinction has important consequences for the control of water-use efficiency and holds great potential for improving the estimation of ecosystem carbon fluxes under drought conditions (Niinemets et al. 2009a). As already mentioned above, the issue of whether (and how) to include g_m in models is actively debated by physiologists and modellers (see also Rogers et al. 2014). Keenan *et al.* (2010a) showed that g_m was the missing constraint for accurately capturing the response of terrestrial vegetation productivity to drought. Yet relatively little information is available from modelling exercises that have included g_m in their algorithms, and more research in this field is needed.

Concluding the above, we underline that we need to consider the seasonality of photosynthetic potentials and mesophyll conductance to explain eco-physiological responses to abiotic stress. These two factors should deserves much more attention in terrestrial biosphere modelling because they hold great potential to reduce model uncertainties, especially under Mediterranean climatic conditions.

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3.7 Supporting information

Supplementary figures

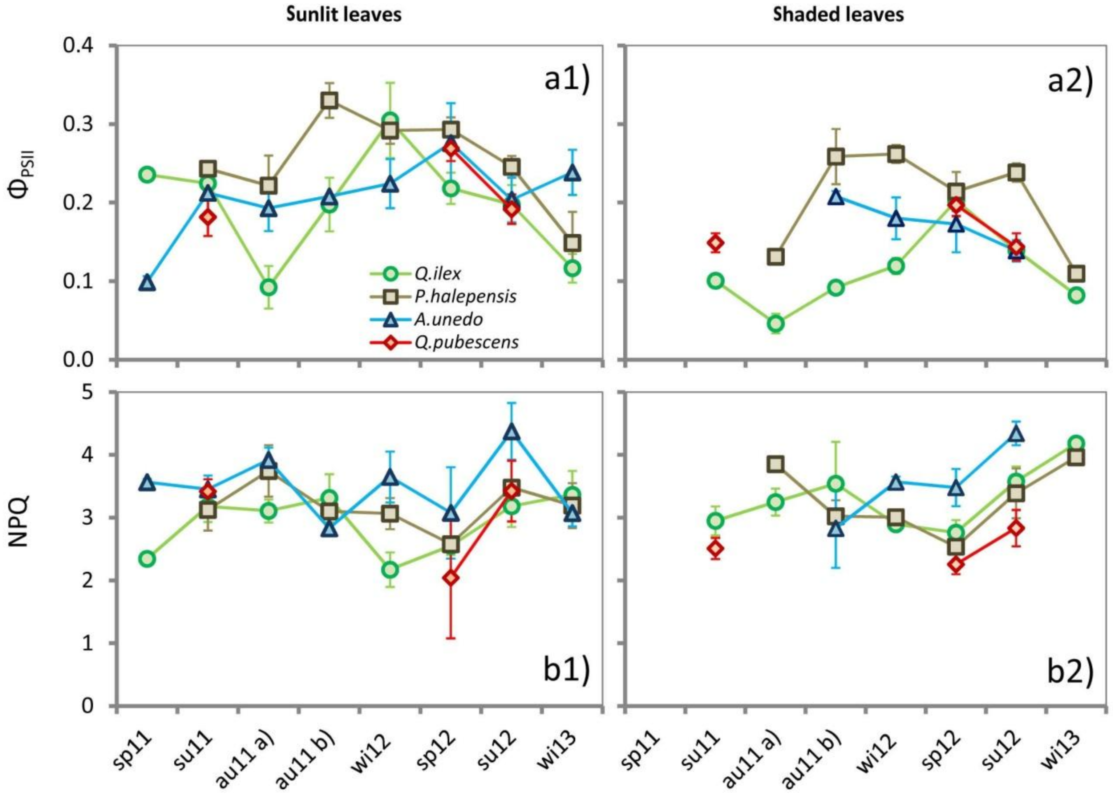

Fig. S3.1 | Line graphs depicting seasonal changes of a) effective quantum efficiency of PSII (Φ_{PSII}), and b) nonphotochemical quenching (NPQ) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Missing data points were due to limitations of labour and equipment. Vertical bars indicate standard errors of the means (*n* = 3-5).

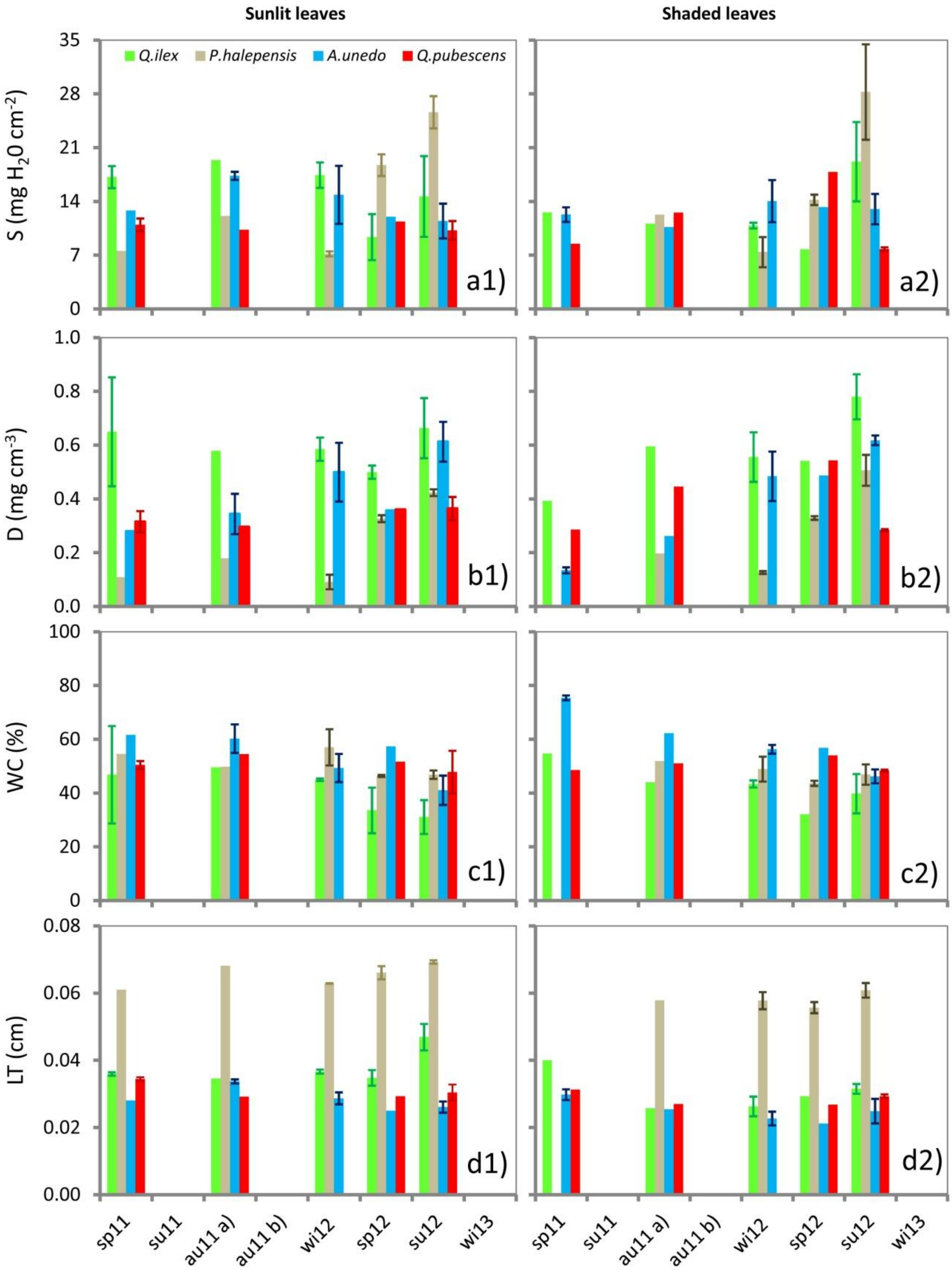

Fig. S3.2 | Bar charts depicting seasonal changes of a) succulence (S), b) leaf density (D), c) water content (WC), and d) leaf thickness (LT) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Error bars indicate standard errors of the means (*n* = 3-5).

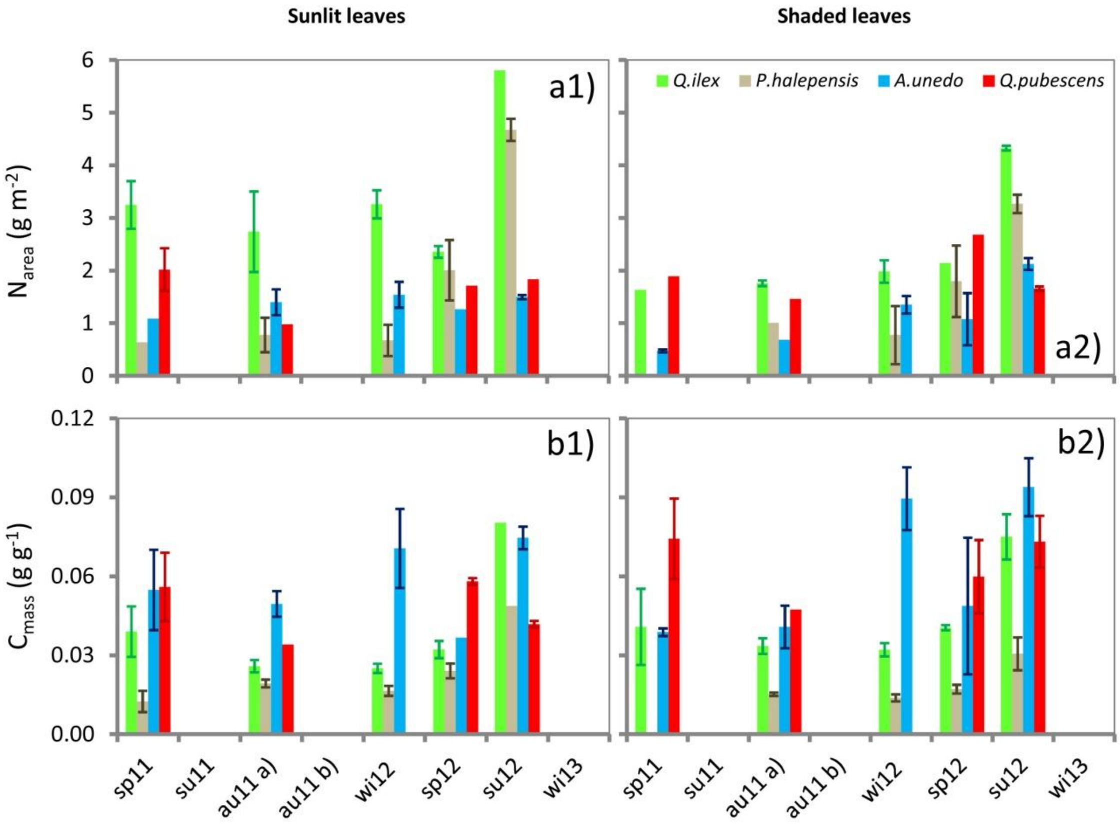

Fig. S3.3 | Bar charts depicting seasonal changes of a) nitrogen per unit leaf area (N_{area}) and b) carbon per unit leaf mass (C_{mass}) for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Error bars indicate standard errors of the means (*n* = 3-5).

Supplementary tables

Table S3.1 | Regression equations and coefficients of determination (R^2) for A_{net}/g_s for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in eight sampling campaigns.

Campaign	Leaf position	*Q. ilex*		*P. halepensis*		*A. unedo*		*Q. pubescens*		All species	
		Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Total	sunlit	$y = 60.7x + 2.4$	0.85	$y = 35.5x + 2.7$	0.61	$y = 80.5x + 1.9$	0.79	$y = 110.5x + 0.5$	0.74	$y = 57.9x + 2.3$	0.69
	shaded	$y = 69.6x + 1.5$	0.72	$y = 37.9x + 2.8$	0.58	$y = 57.7x + 2.4$	0.71	$y = 104.9x + 1.3$	0.78	$y = 57.0x + 2.4$	0.55
Spring 2011	sunlit	$y = 42.7x + 4.1$	0.99			$y = 26.2x + 4.1$	0.72	$y = 31.5x + 3.6$	-0.50	$y = 42.7x + 3.5$	0.89
	shaded			$y = 10.0x + 4.7$	0.98	$y = 62.4x + 1.3$		$y = 42.7x + 4.5$	0.80	$y = 16.1x + 4.7$	-0.11
Summer 2011	sunlit	$y = 93.3x + 1.0$	0.73	$y = 96.3x + 0.5$	0.23	$y = 43.4x + 6.8$	0.79	$y = 92.0x - 0.3$	0.76	$y = 116.6x - 0.1$	0.87
	shaded	$y = 146.9x + 0.5$	0.29					$y = 53.6x + 2.6$	0.52	$y = 54.7x + 2.7$	0.51
Autumn 2011[a]	sunlit	$y = 215.0x - 1.8$	0.91	$y = 56.0x - 0.6$	0.97	$y = 145.3x - 1.6$				$y = 62.9x + 0.4$	0.57
	shaded	$y = 120.7x - 1.3$	0.99							$y = 114.0x - 1.0$	0.96
Autumn 2011[b]	sunlit	$y = 107.0x - 0.6$	0.7	$y = 31.1x + 2.6$	0.96					$y = 21.9x + 4.8$	0.14
	shaded	$y = 70.9x + 0.3$	0.95	$y = 32.4x + 1.9$	0.95	$y = 96.5x - 3.1$				$y = 38.4x + 2.1$	0.73
Winter 2012	sunlit	$y = 85.4x + 1.3$	0.97	$y = -23.3x + 11.3$	0.51	$y = 122.9x - 0.1$	0.89			$y = 49.2x + 2.9$	0.54
	shaded	$y = 73.8x + 1.8$	0.86	$y = 40.3x + 3.3$	0.99	$y = 52.1x + 2.8$	0.49			$y = 52.6x + 2.6$	0.73
Spring 2012	sunlit	$y = 84.3x - 0.5$	0.95	$y = 4.5x + 6.9$	-0.90	$y = 92.9x + 0.8$		$y = 66.9x + 4.0$		$y = 83.5x + 1.3$	0.94
	shaded	$y = 27.6x + 4.7$		$y = 47.6x + 2.1$	0.97	$y = 78.9x + 1.2$	0.73	$y = 150.7x – 2.0$		$y = 110x + 0.03$	0.66
Summer 2012	sunlit	$y = 116.0x - 0.1$	0.86	$y = 50.0x + 2.6$	0.91	$y = 85.9x + 2.0$	0.93	$y = 107.9x + 1.3$	0.67	$y = 85.2x + 1.7$	0.83
	shaded	$y = -49.2x + 9.1$	0.47	$y = 54.0x + 2.4$	0.94	$y = 81.2x + 1.5$	0.69	$y = 78.7x + 2.4$	0.93	$y = 63.2x + 2.6$	0.73
Winter 2013	sunlit	$y = 83.4x + 0.1$	0.96	$y = 5.5x + 6.3$	-0.90	$y = 67.3x + 3.4$	0.93			$y = 89.7x + 0.9$	0.63
	shaded										

Table S3.2 | Regression equations and coefficients of determination (R^2) for A_{net}/g_m for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in eight sampling campaigns.

Campaign	Leaf position	*Q. ilex*		*P. halepensis*		*A. unedo*		*Q. pubescens*		All species	
		Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Total	sunlit	y = 91.2x + 1.4	0.77	y = 113.5x + 1.7	0.50	y = 52.4x + 2.5	0.81	y = 56.8x + 1.3	0.67	y = 54.9x + 2.9	0.65
	shaded	y = 15.6x + 3.6	0.26	y = 2.2x + 107.9	0.52	y = 15.6x + 4.8	0.17	y = 16.1x + 5.3	0.22	y = 16.4x + 4.6	0.24
Spring 2011	sunlit										
	shaded										
Summer 2011	sunlit	y = 67.6x + 2.5	0.81	y = 62.7x + 1.0	0.95	y = 37.8x + 4.5	0.96	y = 121.4x - 0.1	0.44	y = 62.5x + 1.4	0.85
	shaded	y = 16.6x + 1.7						y = 24.7x + 4.2	-0.20	y = 6.7x + 4.4	-0.23
Autumn 2011[a]	sunlit	y = 69.4x + 0.3	0.99	y = 235.3x + 0.1	0.99	y = 40.2x + 3.0				y = 57.8x + 1.3	0.70
	shaded	y = 91.1x - 0.3	0.96							y = 86.9x - 0.1	0.93
Autumn 2011[b]	sunlit	y = 72.2x + 2.7	0.66	y = 217.1x + 0.3	0.86					y = 54.2x + 4.3	0.44
	shaded	y = 47.5x + 2.3	0.61	y = 287.1x - 1.4	0.95					y = 65.0x + 3.1	0.28
Winter 2012	sunlit	y = 133.0x - 0.2	0.91	y = 104.2x + 3.9	0.92	y = 89.8x + 0.6	0.94			y = 107.2x + 1.4	0.72
	shaded	y = 15.2x + 3.2	0.30	y = 206.2x - 1.0	0.99	y = 6.4x + 5.1	-0.40			y = 8.0x + 5.2	0.03
Spring 2012	sunlit	y = 207.0x - 3.2		y = 53.9x + 4.8	0.29	y = 52.2x + 4.6		y = -121.3x + 22.1		y = 50.6x + 4.6	0.83
	shaded	y = -305.3x + 25		y = 204.9x - 1.1	0.99	y = 12.3x + 5.6	-0.70	y = 86.7x -6.8		y = 23.0x + 4.7	0.45
Summer 2012	sunlit	y = 44.3x + 3.0	0.83	y = -143.7x + 11.6	0.98	y = 41.6x + 2.7	0.36	y = 36.2x + 3.1	0.52	y = 36.7x + 3.4	0.59
	shaded	y = 3.7x + 5.8	0.98	y = 189.8x - 1.0	0.66	y = 28.3x + 2.8	0.73	y = 9.0x + 6.0	-0.20	y = 11.2x + 5.3	0.17
Winter 2013	sunlit	y = 138.9x + 0.8	0.98	y = 13.6x + 6.0	-0.60	y = 78.9x + 1.7	-0.10			y = 73.3x + 2.5	0.77
	shaded										

Table S3.3 | Regression equations and coefficients of determination (R^2) for $J_{max}/V_{c,max}$ for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in eight sampling campaigns.

Campaign	Leaf position	*Q. ilex*		*P. halepensis*		*A. unedo*		*Q. pubescens*		All species	
		Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Total	sunlit	$y = 0.92x + 23$	0.85	$y = 0.67x + 45$	0.62	$y = 0.94x + 28$	0.86	$y = 0.62x + 51$	0.38	$y = 0.80x + 35$	0.77
	shaded	$y = 0.89x + 27$	0.73	$y = 0.99x + 3$	0.79	$y = 0.93x + 30$	0.66	$y = 0.42x + 49$	0.23	$y = 0.78x + 33$	0.74
Spring 2011	sunlit										
	shaded										
Summer 2011	sunlit	$y = 0.80x + 42$	0.74	$y = 0.45x + 65$	0.01	$y = 0.72x + 51$	0.99	$y = -3.34x + 686$		$y = 0.39x + 83$	0.35
	shaded							$y = 0.15x + 54$	0.83	$y = 0.12x + 59$	0.47
Autumn 2011[a]	sunlit	$y = 1.44x -38$		$y = 0.64x + 59$	0.65	$y = 0.05x + 142$				$y = 0.99x + 7$	0.82
	shaded	$y = 9.03x - 175$								$y = 0.70x + 24$	0.67
Autumn 2011[b]	sunlit	$y = 0.56x + 83$	0.74	$y = 0.43x + 92$	0.12	$y = 0.27x + 126$				$y = 0.42x + 97$	0.75
	shaded	$y = 0.91x + 22$	-0.03	$y = 1.63x - 70$	0.98	$y = 0.27x + 126$				$y = 1.01x + 20$	0.87
Winter 2012	sunlit	$y = 0.81x + 41$	0.97	$y = 0.55x + 77$	0.86	$y = 0.95x + 32$	0.95			$y = 0.76x + 50$	0.93
	shaded	$y = 0.65x + 46$	0.65	$y = 0.24x + 115$	-0.67	$y = 0.97x + 36$	0.56			$y = 0.67x + 53$	0.81
Spring 2012	sunlit	$y = 3.11x - 200$	0.89	$y = 0.92x + 2$	0.92	$y = 3.40x - 243$		$y = 0.72x + 51$	0.26	$y = 0.45x + 86$	0.22
	shaded	$y = 0.37x + 79$		$y = 1.12x - 17$	0.96	$y = 0.98x + 19$	0.88	$y = 0.98x + 18$	0.45	$y = 0.73x + 37$	0.66
Summer 2012	sunlit	$y = 0.91x + 16$	0.23	$y = 0.16x + 104$	-0.31	$y = 0.55x + 66$	0.79	$y = 0.66x + 42$	0.98	$y = 0.49x + 61$	0.51
	shaded	$y = 0.89x + 31$	0.93	$y = 0.57x + 51$	0.27	$y = 0.57x + 47$	0.59	$y = 0.46x + 45$	0.26	$y = 0.60x + 44$	0.73
Winter 2013	sunlit										
	shaded										

Table S3.4 | Regression equations and coefficients of determination (R^2) for J_{amb}/A_{net} for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in eight sampling campaigns.

Campaign	Leaf position	*Q. ilex*		*P. halepensis*		*A. unedo*		*Q. pubescens*		All species	
		Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Total	sunlit	$y = 5.46x + 59$	0.37	$y = 5.58x + 90$	0.38	$y = 5.40x + 56$	0.35	$y = 2.84x + 539$	0.26	$y = 4.57x + 74$	0.27
	shaded	$y = 6.54x + 23$	0.58	$y = 8.91x + 59$	0.76	$y = 4.83x + 46$	0.26	$y = 3.51x + 81$	0.35	$y = 5.2x + 45$	0.26
Spring 2011	sunlit					$y = 2.10x + 12$				$y = 5.67x - 8$	0.99
	shaded										
Summer 2011	sunlit	$y = -2.95x + 123$	-0.27	$y = 3.87x + 102$	0.70	$y = 18.80x - 92$	0.85	$y = 4.66x + 83$	0.12	$y = 1.19x + 95$	-0.01
	shaded	$y = 1.62x + 39$	0.95					$y = -8.23x + 114$	0.97	$y = 2.68x + 43$	-0.12
Autumn 2011[a]	sunlit	$y = 12.45x + 25$	0.99	$y = 11.30x + 74$	0.89	$y = 6.23x + 49$				$y = 8.18x + 55$	0.39
	shaded	$y = 4.56x + 17$	0.98							$y = 2.64x + 29$	-0.42
Autumn 2011[b]	sunlit	$y = 13.40x - 3.8$	0.81	$y = -6.60x + 193$	0.18	$y = 6.23x + 49$				$y = 3.04x + 97$	-0.17
	shaded	$y = 5.21x + 21$	0.61	$y = 13.17x + 45$	0.74	$y = -2.47x + 117$				$y = 11.87x + 16$	0.34
Winter 2012	sunlit	$y = 10.78x + 56$	0.78	$y = 16.50x + 5$	0.37	$y = 9.08x + 60$	0.98			$y = 10.60x + 55$	0.84
	shaded	$y = 3.08x + 39$	0.91	$y = 5.87x + 79$	0.67	$y = 6.97x + 44$	0.11			$y = 7.63x + 38$	0.24
Spring 2012	sunlit	$y = 1.84x + 80$	0.74	$y = 17.50x + 5$	0.87	$y = 4.01x + 71$		$y = 4.30x + 88$		$y = 1.55x + 106$	0.07
	shaded	$y = -32.90x + 302$		$y = 9.69x + 49$	0.91	$y = 7.70x + 24$	0.57	$y = -1.08x + 110$	-0.38	$y = 1.18x + 82$	-0.02
Summer 2012	sunlit	$y = 5.93x + 41$	0.99	$y = 6.66x + 77$	0.44	$y = 5.24x + 47$	0.58	$y = 3.66x + 65$	-0.15	$y = 3.30x + 70$	0.15
	shaded	$y = 0.75x + 60$	-0.49	$y = 4.90x + 82$	0.49	$y = 0.72x + 61$	-0.30	$y = 3.39x + 42$	-0.05	$y = 2.75x + 58$	0.0002
Winter 2013	sunlit	$y = 10.96x + 29$	0.77	$y = 102.70x - 568$	0.37	$y = -1.09x + 132$	-0.30			$y = 12.17x + 29$	0.60
	shaded										

Table S3.5 | Regression equations and coefficients of determination (R^2) for g_m/g_s for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens* in eight sampling campaigns.

		Q. ilex		*P. halepensis*		*A. unedo*		*Q. pubescens*		All species	
Campaign	Leaf position	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Total	sunlit	y = 0.665x + 0.01	0.53	y = 0.078x + 0.03	0.03	y = 1.44x + 0.001	0.69	y = 1.146x + 0.01	0.34	y = 0.501x + 0.02	0.18
	shaded	y = 0.658x + 0.05	0.01	y = 0.107x + 0.03	0.05	y = 0.58x + 0.06	-0.02	y = 1.090x + 0.07	0	y = 0.199x + 0.07	-0.01
Spring 2011	sunlit										
	shaded										
Summer 2011	sunlit	y = 0.484x + 0.03	0.3	y = 0.096x + 0.02	-0.3	y = 1.065x + 0.07	0.58	y = 0.338x + 0.01	0.08	y = 1.48x - 0.02	0.66
	shaded	y = 8.650x - 0.11						y = 0.319x + 0.05	-0.92	y = -0.202x + 0.10	-0.33
Autumn 2011[a]	sunlit	y = 0.309x + 0.01	0.92	y = 3.613x - 0.12	0.96	y = 0.280x + 0.03				y = 0.485x + 0.01	0.05
	shaded	y = 1.291x - 0.01	0.93							y = 1.284x - 0.01	0.96
Autumn 2011[b]	sunlit	y = 0.442x + 0.05	0.12	y = 0.128x + 0.01	0.7					y = -0.069x + 0.05	-0.18
	shaded	y = 0.936x - 1.13	0.38	y = 0.107x + 0.01	0.84	y = 0.830x + 0.07				y = 0.099x +0.03	-0.04
Winter 2012	sunlit	y = 1.450x - 0.01	0.78	y = 1.240x - 0.002	0.8	y = 0.636x + 0.01	0.68			y = 0.194x + 0.036	0.06
	shaded	y = 1.800x + 0.06	-0.15	y = -0.459x + 0.11	0.99	y = -0.099x + 0.06	-0.43			y = -0.0296x + 0.10	-0.11
Spring 2012	sunlit	y = 3.480x - 0.09		y = 1.783x - 0.07	-0.5	y = 0.561x + 0.041		y = -0.552x + 0.15		y = 1.257x - 0.03	0.5
	shaded	y = -0.091x +0.07		y = 1.861x - 0.01	0.93			y = 3.114x - 0.05		y = 1.520x - 0.002	0.19
Summer 2012	sunlit	y = 0.308x + 0.03	0.45	y = 0.936x + 0.07	0.96	y = 0.401x + 0.02	0.17	y = 1.700x + 0.02	-0.29	y = 1.085x + 0.03	0.25
	shaded	y = -14.1x + 0.93	0.61	y = 2.331x - 0.02	0.39	y = 0.272x + 0.03	0.51	y = 0.728x + 0.131	-0.22	y = 0.351x + 0.01	-0.05
Winter 2013	sunlit	y = 1.62x + 0.01	0.94	y = 0.291x + 0.06	0.12	y = 1.570x - 0.06	0.28			y = 0.669x + 0.01	0.18
	shaded										

Table S3.6 | Non-linear regression equations and coefficients of determination (R^2) for a) g_m/LMA and b) $V_{c,max}$/N_{area} in four seasonal campaigns and for sunlit and shaded leaf positions for *Q. ilex*, *P. halepensis*, *A. unedo*, and *Q. pubescens.*

	g_m/LMA
Campaign	**Equation**
Total	$y = x^{-1.016}$
Autumn 2011[a]	$y = x^{-1.533}$
Winter 2012	$y = x^{-1.486}$
Spring 2012	$y = x^{-0.953}$
Summer 2012	$y = x^{-800}$

Supplementary notes

Note S3.1 | Calculation of maximum quantum yield of PSII and nonphotochemical quenching

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

where F_o is the minimal fluorescence measured under darkness, and F_m is the maximal fluorescence measured after a saturating light pulse. Both parameters were obtained on a dark-adapted leaf with closed PSII reaction centres as described in the previous sections. The F_v/F_m ratio describes the fraction of absorbed photons used in photochemistry under dark conditions and serves as the primary stress indicator of the photosystems. Typical values range between 0.74 and 0.85. Ratios <0.80 are indicative of induced photoprotection (sustained energy dissipation), and ratios <0.74 are indicative of chronic photoinhibition (Björkman & Demmig, 1987; Maxwell & Johnson, 2000; Verhoeven, 2014).

The nonphotochemical quenching (NPQ) is estimated by both dark- and light-adapted fluorescence signals, F_m and F_m', as:

$$NPQ = \frac{(F_m - F_m{}')}{F_m{}'} \tag{3}$$

where F_m and F_m' are the maximal fluorescence of a dark-adapted and light-adapted leaf, respectively.

Note S3.2 | Light experiments and estimation of day respiration

Light-response curves (A/PPFD) were generated at a C_a of 400 μmol CO_2 m^{-2} s^{-1} by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source. To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence: 2500 → 2000 → 1500 → 1000 → 800 → 600 → 500 → 400 → 300 → 200 → 150 → 125→ 100 → 75 → 50 → 40 → 30 → 20 → 10 → 5 → 0 (μmol photons m^{-2} s^{-1}. The minimum and maximum times between each light level for the generation of the A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light during an A/PPFD curve led to a drop in T_{Leaf} as the light decreased. The rapid changes in the light levels prevented the correct adjustment of T_{Leaf} while guaranteeing stable air and water fluxes and avoiding noisy measurements of C_i and g_s. We thus decided to maintain a stable Peltier-block temperature (T_{block}) in the leaf cuvette. T_{block} was adjusted first so that T_{Leaf} was 25 °C at the beginning of the A/PPFD curve and then kept stable throughout the experiment. T_{Leaf} had dropped by approximately 1-3 °C by the completion of the

A/PPFD curve. From this curve, we estimated night and day respiration. The term R_d is sometimes used for dark respiration (Farquhar *et al.*, 1980; Turnbull *et al.*, 2003) but also for day respiration (Yin *et al.*, 2011; Flexas *et al.*, 2012). We will use R_d to represent mitochondrial respiration during the day or under lighted conditions and R_n to represent mitochondrial respiration at night or under dark-adapted conditions. We estimated R_n during the day after darkening the leaf for at least 30 min. R_d was estimated from the light-response curves with the combined GE and CF measurements proposed by Yin et al. (2009), named the CF method. This method amended the Kok method (Kok, 1948) by substituting the A/PPFD relationship with A/PPFD * Φ_{PSII}/4 (Yin *et al.*, 2009).

4

Leaves of *Quercus pubescens*
Photo & Design: D. Sperlich

Chapter 4

Thermal plasticity of photosynthesis in a Mediterranean mixed forest

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4.1 Abstract

Temperature is a major ecological variable that determines the natural distribution of plants. The negative effects of temperature on the photosynthetic system and the feedback to the global carbon cycle remain key uncertainties in scenarios of future climate change, especially in the Mediterranean region. We constructed temperature-response curves for mature trees of four Mediterranean species and recorded the net assimilation rate (A_{net}) in parallel with the electron-transport rate based on chlorophyll fluorescence (J_{cf}) in six seasonal campaigns. We assessed two formulations that modelled the temperature responses: the peaked function (Johnson et al. 1942 cf. Medlyn et al. 2002) and the model by June et al. (2004). The peaked function modelled the observed temperature responses better. The thermal optima of A_{net} and J_{cf} across all species and seasons were 24.7±0.5 and 30.3±0.6 °C, respectively, but varied significantly between seasons. The curvatures of the response curves were only partly affected by seasonal acclimation. The photosynthetic system was generally impeded primarily by high, not low, temperatures and was better acclimated to heat stress in the drier and hotter year. Species-specific acclimation partly offset these general trends. Our results indicate that Mediterranean climax species exhibit a strong capacity to acclimate to warmer and drier conditions.

4.1 Introduction

Temperature is a major environmental factor contributing to the natural distribution of species and limiting plant growth and productivity (Mittler, 2006). The Mediterranean region is characterised by a wide seasonal variation of temperature regimes. Higher plants, particularly evergreen tree species, have a high capacity for temperature acclimation, including a higher tolerance to heat stress in summer and a capacity for cold hardening in winter (Aschmann, 1973; Orshan, 1983; Blumler, 1991). This ability provides them with a high flexibility throughout the year to benefit from favourable periods, e.g. winter (Sperlich *et al.*, 2014). A better understanding of the acclimation of photosynthesis to temperature and the improvement of the predictive capacity of temperature-response models have received renewed interest against the background of climate change and global warming (Medlyn *et al.*, 2002; Kattge & Knorr, 2007; Bunce, 2008; Yamori *et al.*, 2014). These are currently "hot" topics in global change biology because temperature extremes and heat waves are predicted to become much more frequent under future climate change scenarios (IPCC, 2013). Vårhammar *et al.* (2015) recently suggested that montane rainforest climax species may be particularly sensitive to future global warming. Temperature is a determining factor in the Mediterranean Basin, but surprisingly little information is available for photosynthetic sensitivity and acclimation in Mediterranean tree species. An understanding of the species-specific dynamics of leaf temperature is essential for predicting the effects of rising global temperatures on plant growth and species diversity (Bernacchi *et al.*, 2001; Wise *et al.*, 2004; Lin *et al.*, 2012).

Many formulations have been suggested for mathematically describing photosynthetic responses to temperature (Medlyn *et al.*, 2002; Sharkey & Bernacchi, 2012). Opinions differ about how to improve such models by reducing their complexity without suffering a loss of precision. June *et al.* (2004) presented a simple equation, particularly for the temperature response of the electron-transport rate. The simple yet mechanistic nature of the equation has led to its rapid incorporation into terrestrial biosphere models (Friend, 2010).

The mechanism behind the positive correlations of temperature with electron-transport rate and photosynthetic carbon assimilation involves the acceleration of the underlying biochemical processes by higher enzymatic activities (Farquhar *et al.*, 1980; Bernacchi *et al.*, 2001, 2002; Medlyn *et al.*, 2002; Way & Oren, 2010). Excessive radiation and temperature, however, can disrupt the functional integrity of the photosynthetic system. Moderate heat stress (35-45 °C) inhibits photosynthesis through heat lability of Rubisco activase, increased photorespiration, and limitation in electron-transport (Law & Crafts-brandner, 1999; Schrader *et al.*, 2004; Wise *et al.*, 2004; Sage & Kubien, 2007; Yamori *et al.*, 2014). Increasing temperatures above 45 °C primarily damage the chloroplasts and photosystem II (PSII) in the thylakoid membrane (Schrader *et al.*, 2004; Wise *et al.*, 2004; Sharkey & Bernacchi, 2012). Under natural conditions, however, the photosynthetic system is fine-tuned with the growth environment: it maximises CO_2 assimilation at a thermal optimum with decreasing assimilation rates above and below that optimum (Berry & Björkmann, 1980). The harmful effects of excess energy are prevented by reducing PSII efficiency, linear electron-transport rate, and Rubisco activity and by enhancing dissipating energy pathways (Feller *et al.*, 1998; Law & Crafts-brandner, 1999; Haldimann & Feller, 2004). If adverse energy conditions persist over longer periods, plants induce processes of foliar acclimation, such as the reorganisation of the thylakoid membrane, closure of reaction centres, and reduced antennal size (Huner *et al.*, 1998; Maxwell & Johnson, 2000; Verhoeven, 2014). This acclimation occurs most typically over the long-term in response to growth-related changes or seasonal shifts in temperature (Kattge & Knorr, 2007; Gunderson *et al.*, 2009). Thermal acclimation enables the plants to again maximise photosynthetic efficiency under the new growth conditions (Berry and Björkmann 1980, Yamori *et al.* 2014 and references therein).

Ecology is particularly interested in investigating the similarities among plants with different morphological and phenological strategies, such as evergreen and deciduous or broadleaved and coniferous plants, and in studying the specific responses to contrasting seasonal environmental conditions (Blumler, 1991). The responses to temperature may differ between species or between angiosperms and gymnosperms. Méthy, Gillon, and Houssard (1997) showed that the conifer *Pinus halepensis* Mill. had higher PSII photochemical efficiencies and fluorescence quenching within the normal physiological range of temperatures, suggesting higher growth and productivity. In contrast, *Quercus ilex* L. had a higher thermal tolerance after the application of a thermal treatment. Interestingly, in this study a higher proportion of *Q. ilex* leaves recovered, and they also recovered faster than the needles of *P. halepensis*. In a broader context, pine species could be more vulnerable to sudden climate-induced heat waves. Indeed, recent large-scale studies of tree growth in the Iberian Peninsula support this argument, reporting negative growth trends in pines in response to higher temperatures (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013; and also see review by Carnicer *et al.*, 2013). Common garden experiments or field studies in mixed forests are most appropriate for studying the physiological mechanisms of the long-term acclimation to variable environmental conditions to shed light on species-specific strategies. The specific light environment and microclimate of the leaves are key determining factors for the carbon gain of the entire canopy (Valladares & Niinemets, 2008; Vaz *et al.*, 2011).

In this study, we investigated the dynamics of leaf temperature in mature trees of four species (*Q. ilex*, *Q. pubescens* Willd., *P. halepensis*, and *Arbutus unedo* L.) in a mixed forest in Catalonia, northeastern Spain. We characterised the temperature sensitivity and acclimation of sunlit and shaded leaves to the seasonal environmental changes by gas exchange analyses to measure the response of the net assimilation rate (A_{net}) to temperature. We also measured chlorophyll fluorescence to detect the PSII-driven linear electron-transport rate (J_{cf}), because PSII has been characterised as the primary target of heat-induced stress (Haldimann & Feller, 2004 and references herein).

Our first objective was to evaluate the model performance of the peaked function and the function by June *et al.* (2004) to obtain a decisive tool for further analyses. We then characterised the curvatures and the thermal optima of the A_{net} and J_{cf} temperature responses along with the factors leaf position, season, and tree species. Our goals were to determine if the overall performance differed between sunlit and shaded leaves within a species and if the species differed between the different seasonal measurement campaigns. We then also wanted to determine if seasonal mechanisms of acclimation were species-specific and to what extent leaf position would affect these.

4.2 Material and Methods

4.2.1 Field site

The experiment was conducted at the Can Balasc field station in the coastal massif of Collserola Natural Park (8500 ha) in the province of Barcelona, northeastern Spain (41°25′N, 2°04′E; 270 m a.s.l.). The climate is typically Mediterranean, with seasonal summer droughts, warm temperatures, and mild winters. The mean August and January temperatures are 22.8 °C and 7.9 °C, respectively. Mean annual precipitation and temperature are 723 mm and 15.1 °C (1951-2010), respectively (Ninyerola *et al.*, 2007a,b). Sensors for measuring air temperature (HMP45C, Vaisala Oyj, Vantaa, Finland) and solar radiation (SP1110 Skye Instruments Ltd., Powys, UK) were installed at a height of 3 m in a clearing *ca.* 1 km from the study site.

4.2.2 Stand structure

The experimental forest stand is dense (1429 stems ha^{-1}) and consists of a two-layered canopy. *Quercus* species are surmounted by shelter trees of the early-successional and fast growing Aleppo Pine (*P. halepensis*), with mean heights of 9.9 m and 17.1 m, respectively. The *Quercus* species are the late-successional evergreen Holm Oak (*Q. ilex*) and the deciduous Pubescent Oak (*Q. pubescens*). Strawberry trees (*A. unedo*) occur also scattered in the forest stand and surprisingly reach the *Quercus* canopy (mean height of 8.1 m). *A. unedo* is usually widely abundant as a shrub in the macchia ecosystems of the Iberian Peninsula (Beyschlag *et al.*, 1986; Reichstein *et al.*, 2002). The pines have the largest trunks, followed by the two *Quercus* species (same DBH) and then *A. unedo* (mean DBHs of 33.7, 12.9, and 9.6 cm, respectively). The forest succession has reached the final stage: the dense *Quercus* canopy is suppressing the growth of the light-demanding seedlings and saplings of the early-successional *P. halepensis*. Details of the stand history and field site are described by Sperlich *et al.* (2014).

4.2.3 Sampling method

We conducted six field campaigns from June 2011 to September 2012. The sampling periods are presented in Table 1. Modifying leaf temperatures between 10 and 50 °C for constructing the temperature-response curves is not possible under field conditions, so we analyzed sampled twigs in the laboratory. We cut twigs with a pruning pull from sun-exposed and shaded crown positions, optimally at similar heights. The twigs were immediately re-cut under water in the field, wrapped in plastic bags to minimise transpiration, stored in water buckets, and transported to the laboratory. Five replicates of each leaf position and tree species were collected for the analysis of gas exchange. The twigs were pre-conditioned in the laboratory at room temperature (24-28 °C) in dim light for 1-3 d and were freshly re-cut every morning. *Q. pubescens* was only sampled in spring and summer due to its deciduous leaf habit. More details and references can be found in Sperlich *et al.* (2014).

4.2.4 Analyses of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, USA). A_{net} (in μmol CO_2 m^{-2} s^{-1}) was recorded in parallel with the PSII-driven electron-transport rate estimated by chlorophyll fluorescence (J_{cf} in μmol electron m^{-2} s^{-1}). Some sunlit leaves of *Q. ilex* were too small to fill the leaf cuvette (2 cm^2), so the measured parameters were adjusted after the measurements. We positioned a layer of *P. halepensis* needles (*ca*. 10-15) on the leaf cuvette, avoiding gaps and overlays, and sealed the gaskets with Blu-tack (Bostik SA, La Plaine St Denis, France) to keep the needles in position. The leaves were prepared prior to recording the response curves as described by Sperlich *et al.* (2014).

4.2.5 Temperature experiments

The data for the responses of A_{net} and J_{cf} to temperature (A/T and J/T, respectively) were recorded at a controlled CO_2 concentration in the leaf chamber (C_a) of 400 μmol CO_2 mol air^{-1} and a quantum flux density of 1000 μmol photons m^{-2} s^{-1}. The sequence of temperatures in a response curve was 10→ 12.5→ 15→ 17.5→ 20→ 22.5→ 25→ 27.5→ 30→ 32.5→ 35→ 37.5→ 40→ 42.5→ 45→ 47.5→ 50 °C. During the response-curves, the leaf temperature (T_{Leaf}) was not easily adjusted in a correct and efficient manner while guaranteeing stable air and water fluxes and avoiding noisy measurements of stomatal conductance (g_s in mol H_2O m^{-2} s^{-1}) and stomatal internal CO_2 concentration (C_i in μmol CO_2 mol air^{-1}). We thus adjusted the Peltier-block temperature (T_{block}) of the leaf cuvette and used the response of T_{Leaf} in the temperature-response curves. The minimum and maximum acclimation time for each step was set at 8 and 10 min, respectively. The full range of temperatures could rarely be measured, because seasonal weather conditions, in summer or winter for example, impeded high or low T_{Leaf} explaining the high standard error in the extreme range of temperatures as outlined in Fig. 3. The time consuming temperature-response curves combined with unpredictable and inconsistent stomatal behaviour also strongly constrained our target sample size (n = 5), especially for *Q. pubescens*. We had to discard many response curves for this species, so that we did not obtain sufficient data for the seasonal analyses.

4.2.6 Calculation of chlorophyll-fluorescence parameters

The effective quantum yield of PSII (Φ_{PSII}, unitless) was estimated as:

$$\Phi_{PSII} = \frac{(F_m' - F_s)}{F_m'} \tag{1}$$

where F_s is the steady-state fluorescence of a fully light-adapted sample, and F_m' is the maximal fluorescence yield reached after a pulse of intense light. Φ_{PSII} represents the fraction of photochemically absorbed photons for a light-adapted leaf. J_{cf} based on Φ_{PSII} was calculated as:

$$J_{cf} = \varepsilon * \Phi PSII * \alpha_L \tag{2}$$

ε is a scaling factor accounting for the partitioning of intercepted light between photosystem I (PSI) and PSII. We assumed that light was equally distributed between the two photosystems (ε = 0.5) independent of temperature (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2005). This might be a simplification because temperature possibly affects ε. However, limitations in labour and time did not allow for testing the temperature dependence of ε for the studied tree species. α_L is the foliar absorbance. α_L is the foliar absorbance**.** The values of α_L were taken from Sperlich *et al.* (2015).

4.2.7 Modelling the temperature responses

We used two formulations to model the temperature dependence of the photosynthetic parameters, the peaked function (Eq. 3) and June's model (Eq. 4). The peaked function is a modified version of the Arrhenius equation (Johnson *et al.* 1942 cf Medlyn *et al.*, 2002):

$$P(T_k) = P_{opt}\left[\frac{H_d * exp\left(\frac{H_a(T_k - T_{opt})}{T_k * R * T_{opt}}\right)}{H_d - H_a\left(1 - exp\left(\frac{H_d(T_k - T_{opt})}{T_k * R * T_{opt}}\right)\right)}\right] \tag{3}$$

where $P(T_k)$ is the value of parameter P at temperature T_k in Kelvin, R is the gas constant (0.008314, kJ mol^{-1} K^{-1}), P_{opt} is the maximum value of parameter P, T_{opt} is the temperature at which P_{opt} is achieved, H_a (J mol^{-1}) is the exponential increase of the function below the optimum, and H_d (J mol^{-1}) is the rate of decrease above the optimum. This equation and some alternative expressions were first introduced in models of photosynthesis by Tenhunen et al. (1976) and Farquhar *et al.* (1980). Higher values of H_a and H_d translate into sharper ascending and descending arms below and above T_{opt}, respectively, which produce a more peaked curve, whereas lower values of H_a and H_d produce a broader, shallower curve. With the two parameters H_a and H_d, the curve can also adjust to asymmetrical or rapid fall-offs in the temperature responses. The maximum values of A_{net} and J_{cf} at T_{opt} were termed A_{opt} and J_{opt}, respectively. June et al. (2004) suggested a new type of equation for the temperature dependence of the electron-transport rate under high irradiance:

$$J(T_L) = J(T_{opt)}e^{-\left(\frac{T_{Leaf}-T_{opt}}{\Omega}\right)^2} \tag{4}$$

where T_{Leaf} is the leaf temperature (°C), $J(T_{opt})$ is the rate of electron transport at the optimum temperature, T_{opt}, and Ω (K^{-1}) is the difference in temperature from T_{opt} at which J falls to e^{-1} (0.37) of its value at T_{opt}. We also applied this formulation to the temperature response of A_{net}. With only one fitting parameter, Ω, the response curve is less flexible to abrupt fall-offs above or below T_{opt}.

4.2.8 Statistical analyses

We applied the least square fit method using the SOLVER estimator tool in Excel to fit the models to the temperature response curves. The squared errors of the observed points in the temperature-response curve and the modelled points were calculated and summed. Outliers with evidence of an error during the measurements were not included in the curve-fitting procedure. We then estimated the values of H_a and H_d of Eq. 3 with the Excel SOLVER tool and estimated Ω of Eq. 4 in a separate run. SOLVER iteratively changes the parameters to minimise the sum of squares of the deviation from the observation. We then performed further statistical analyses with R version 3.0.2 (http://www.r-project.org/). Two-factorial analyses of variance with season and leaf position as the main factors tested for differences in the parameters in each species. Significant differences were determined at $P \leq 0.05$ with Tukey's honest significant difference tests (HSD).

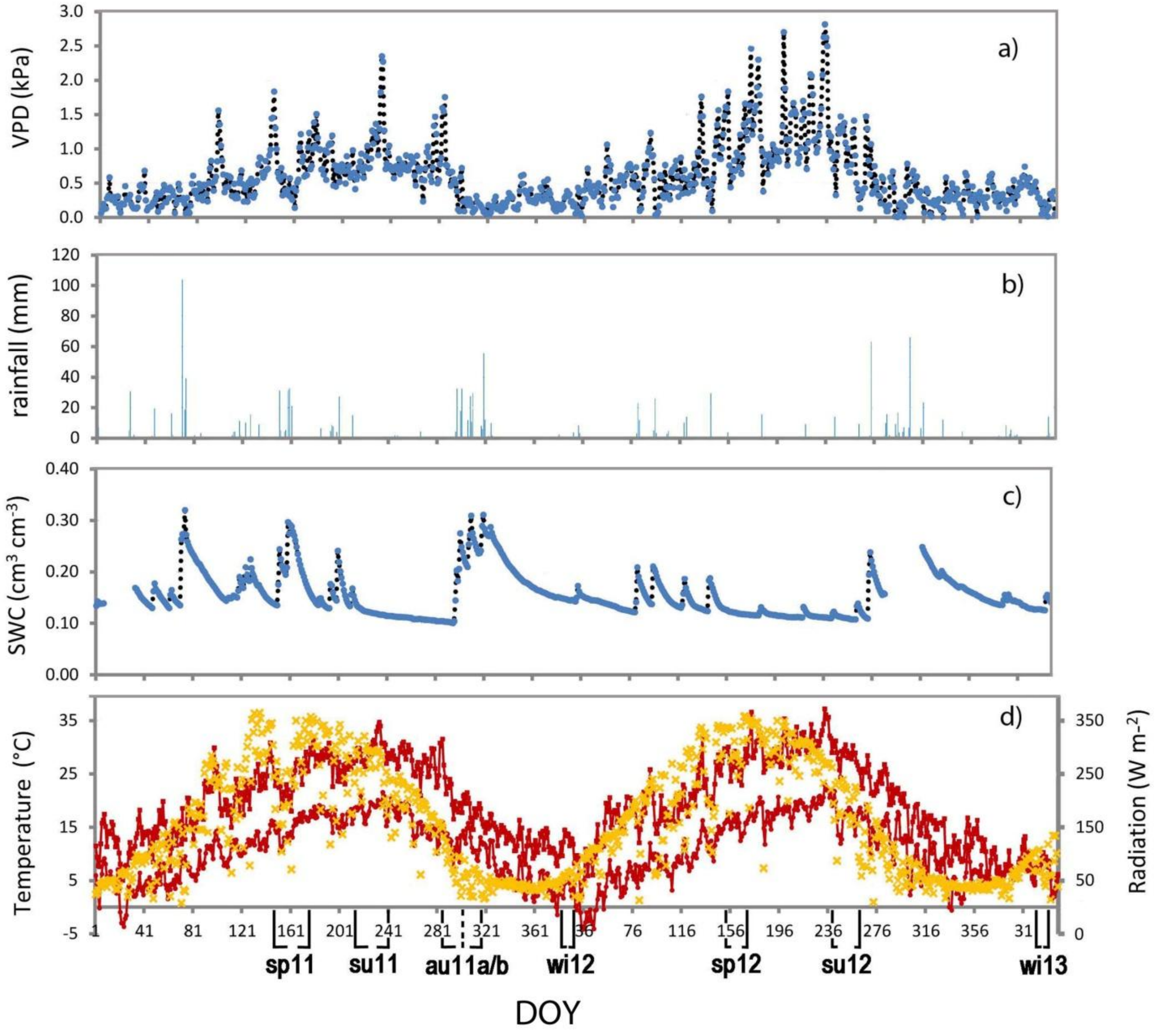

Fig.4.1 | Environmental variables for the day of the year (DOY) from January 2011 to February 2013 a) atmospheric vapour-pressure deficit (VPD), b) rainfall in mm c) soil-water content (gap in the data is due to power cut), and d) maximum and minimum temperatures on the primary y-axes (circles) and radiation (crosses) on the secondary y-axes. The field campaigns are indicated (abbreviations as in Table 1). Modified from Sperlich *et al.* (2015).

4.3 Results

4.3.1 Environmental conditions

The year 2011 was characterised by 30% more precipitation than the climatic average of 723 mm (1951-2010) (Ninyerola *et al.*, 2007a,b) (Fig. 1, Table 2). The seasonal summer drought in 2011 was thus delayed, and the trees experienced relatively humid conditions during our measurement campaign of summer 2011. Our campaign of autumn 2011 was instead at the peak of the drought right before the autumn rains began (Fig. 1). The winter was relatively mild from 1 December 2011 to 31 January 2012, with average maximum and minimum temperatures of 11.8 and 4.2 °C, respectively, coinciding with high photosynthetic potentials and shoot growth. Precipitation in 2012 was 20% lower than the climatic average (Table 2). The spring and summer of 2012 showed 28 % and 38 % lower precipitation (respectively) than the corresponding periods in 2011.

Table 4.1 | Dates and days of the year (DOY) and abbreviation (Abrv.) for the seasonal field campaigns.

Campaign	Abrv.	Date	DOY
Spring 2011	sp11	02.06.11 - 02.07.11	153-183
Summer 2011	su11	17.08.11 - 29.08.11	229-241
Autumn 2011*	au11a	17.10.11 - 27.10.11	290-300
Winter 2012	wi12	09.01.12 - 19.01.12	9-19
Spring 2012	sp12	01.06.12 - 15.06.12	153-167
Summer 2012	su12	24.08.12 - 20.09.12	237-264

*The autumn 2011 campaign was conducted in a period of prolonged summer drought.

Table4.2 | Environmental conditions of two contrasting years (2011 and 2012). Total precipitation, mean temperature, mean soil-water content (SWC), and mean vapour-pressure deficit (VPD) are listed for each season/year.

Season	Precipitation (mm)		Temperature (°C)		SWC ($cm^3\ cm^{-3}$)		VPD (kPa)	
	2011	2012	2011	2012	2011	2012	2011	2012
Winter	254	25	8.2	7.3	0.17	0.14	0.3	0.4
Spring	197	141	16.6	16.3	0.19	0.15	0.6	0.8
Summer	81	50	22.4	23.4	0.13	0.12	0.9	1.2
Autumn	272	263	13.4	12.6	0.19	0.18	0.4	0.3
Total	804	479	15.3	15.1	0.17	0.14	0.5	0.7

4.3.2 Model comparison

The temperature responses of A_{net} and J_{cf} were generally characterised by a shallow increase below, and an abrupt decrease above, the thermal optimum. This asymmetrical response was more accurately modelled by the peaked function, indicated by lower sums of squared errors. Fig. 2a/b illustrates samples of temperature-response curves for A_{net} and J_{cf} for *A. unedo*. The peaked function and June's model were fit to the observed data with the nls method. The squared error was significantly lower for the peaked function than for June's model (Fig. 2a/b). This was also reflected in the mean of the squared error for all data combined that was lower for the peaked function (highly significant at $P < 0.01$) than for June's model for both the A/T (±SE) (6.6±2.4 and 10.4±3.2, respectively) and the J/T (139±28 and 287±73, respectively) response curves.

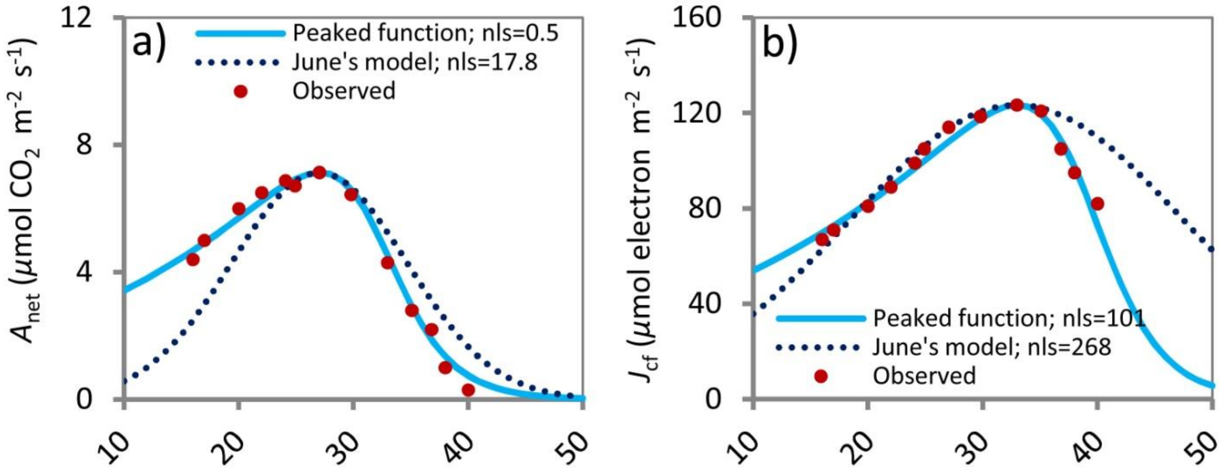

Fig. 4.2 | Observed temperature responses of (a) the net CO_2 assimilation (A_{net}) and (b) the electron-transport rate (J_{cf}) for a sample of *Arbutus unedo*. The peaked function and June's model were fit to the response curves with the non-linear least square (nls) method. The nls presented in the figure represents the summed squared error of each formulation and observation.

4.3.3 Temperature optima of A_{net} and J_{cf} and the role of leaf position

The mean T_{opt} for all data combined was significantly lower for A_{net} (24.7±0.5 °C) than for J_{cf} (30.3±0.6 °C) (Table 3). The T_{opt} of both A_{net} and J_{cf} did not differ significantly between sunlit and shaded leaves. J_{cf} was nevertheless significantly higher in sunlit leaves (Fig. 3). When species were separated, *Q. ilex* and *A. unedo*, but not *P. halepensis* or *Q. pubescens*, had the same J_{cf} pattern (Fig. 3), with significantly higher means of J_{opt} in sunlit leaves (Table 3).

4.3.4 Seasonal trends in the temperature responses across species

In the following we present the results for all species and leaves combined. A_{opt} was highest (8.8±0.7 μmol CO_2 m^{-2} s^{-1}) in spring 2012 and significantly different from A_{opt} in autumn 2011 and winter 2012 (Table 3 and Fig. S2). T_{opt} for A_{net} was highest in spring and summer 2011 (29.3±2.6 and 30.4±2.2 °C, respectively) and significantly different compared to all other field campaigns (Table 3 and Fig. S2). H_a and H_d for A_{net} did not differ significantly between seasons. We thus concluded that the peak of the A/T curve for all species and leaf positions combined shifted both vertically and horizontally due to acclimation of the thermal optimum (Fig. S2).

However, the shape of the curve below and above that thermal optimum remained unchanged. Similarly, the mean J_{opt} was highest in spring 2012 (116±11 µmol electron m^{-2} s^{-1}) (being significantly different from that in autumn 2011 (Table 3 and Fig. S2). The mean T_{opt} of J_{cf} was highest in summer 2011 (34.5±0.8 °C) (Table 3 and Fig. S2) and was significantly different from that in the other field campaigns except spring 2011. The mean T_{opt} of J_{cf} was lowest in autumn 2011 (27.8±0.5 °C) and winter 2012 (28.3±0.9 °C). The mean H_d for J_{cf} was highest in summer 2011 (341±70, unitless), indicated by the steep fall-off of the J/T curve above its thermal optimum (Table 3 and Fig. S2). This mean H_d for J_{cf} was significantly different from those in summer 2012 and winter 2012 that both had the lowest means (185±28 and 181±28, unitless, respectively). In contrast, the ascending arm of the A/T curve did not differ markedly between seasons.

Table 4.3 | Means and standard errors (±SE) of the parameters of the modelled A/T and J/T response curves fitted with the peaked function for four species (*Quercus ilex*, *Pinus halepensis*, *Arbutus unedo*, and *Q. pubescens*) in six seasons (spring 2011, summer 2011,autumn 2011, winter 2012, spring 2012, and summer 2012), and for two leaf positions (sunlit and shaded). T_{opt} is the thermal optimum, A_{opt} (in µmol CO$_2$ m^{-2} s^{-1}) is the net assimilation rate at T_{opt}, J_{opt} (in µmol electron m^{-2} s^{-1}) is the electron-transport rate at T_{opt}, H_a (unitless) is the activation energy representing the ascending arm below T_{opt} and H_d (unitless) is the deactivation energy representing the descending arm above T_{opt}. Limitations in chlorophyll fluorescence equipment led to a gap in the data in spring 2011.

		A/T response curve				J/T response curve			
	Seasons	A_{opt}	T_{opt}	H_a	H_d	J_{opt}	T_{opt}	H_a	H_d
Sunlit	Spring 2011	5.8±0.7	28.0±3.2	104±14	194±23				
	Summer 2011	7.3±0.8	30.3±3.1	54±7	307±41	116±12	34.3±3.6	33±5	347±48
	Autumn 2011	5.9±0.7	23.3±2.5	127±18	314±39	107±12	28.0±3.0	104±18	256±33
	Winter 2012	6.7±0.8	20.2±2.2	60±8	248±29	110±12	28.1±3.1	48±6.2	173±21
	Spring 2012	9.6±1.1	24.2±2.7	46±6	317±40	121±14	29.4±3.3	90±13	164±19
	Summer 2012	7.0±0.8	22.0±2.5	125±16	254±29	105±12	29.9±3.4	75±11	211±26
Shaded	Spring 2011	9.7±1.1	35.4±4.1	100±3.2	105±12				
	Summer 2011	3.4±0.4	32.0±3.7	190±3.1	501±58	61±7	36.9±4.3	43±5	259±48
	Autumn 2011	6.5±0.8	21.7±2.5	73±2.5	249±29	59±7	27.3±3.1	90±17	435±33
	Winter 2012	2.3±0.3	26.5±3.0	27±2.2	375±45	73±9	29.2±3.4	64±8	224±21
	Spring 2012	8.2±0.9	25.5±2.9	73±2.7	378±48	111±12	29.5±3.1	62±12	368±19
	Summer 2012	6.8±0.8	20.0±2.3	100±2.5	192±23	94±11	32.2±3.6	67±11	163±26
All leaves	Spring 2011	6.5±1.4	29.3±2.6	103±26	179±29				
	Summer 2011	7.0±0.8	30.4±2.2	66±15	321±63	112±7	34.5±0.8	34±6	341±70
	Autumn 2011	6.1±0.5	22.8±1.0	111±25	294±37	92±9	27.8±0.5	100±30	312±44
	Winter 2012	6.0±0.7	21.2±1.1	54±13	270±31	104±8	28.3±0.9	51±9	181±27
	Spring 2012	8.8±0.7	24.9±1.2	60±12	349±51	116±11	29.5±2.1	76±19	266±53
	Summer 2012	6.9±0.5	20.9±0.9	111±15	221±22	99±7	31.2±1.4	71±22	185±28
	Sunlit	**7.0±0.3**	**24.9±0.7**	**82±10**	**282±19**	**112±3**	**30.3±0.5**	**66±12**	**247±28**
	Shaded	**7.1±0.6**	**23.9±1.4**	**78±17**	**290±50**	**92±9**	**29.8±0.9**	**73±7**	**302±43**
	All leaves	**7.0±0.3**	**24.7±0.6**	**83±9**	**286±19**	**105±4**	**30.3±0.5**	**67±9**	**265±23**

4.3.5 Species-specific seasonal trends of the temperature-response curves

The response pattern was more differentiated when the species-specific behaviours across the seasons were tested separately (Fig. 4). The seasonality, however, was not very pronounced in shaded leaves, so we will present only the results for sunlit leaves.

The mean A_{opt} of *Q. ilex* was significantly higher in spring 2011 than in summer 2011, autumn 2011 and winter 2012 (Fig. 4a). Similar to the overall trend, its T_{opt} was highest in summer 2011 and significantly higher than in autumn 2011 and summer 2012). In summer 2012, autumn 2011 and winter 2012, *A. unedo* behaved akin to *Q. ilex*: H_a was significantly higher in summer 2012 and autumn 2011 and significantly lower in winter 2012 compared to the other campaigns. In contrast, the mean A_{opt} of *P. halepensis* was significantly higher in winter 2012 than in spring 2011 and marginally significantly higher than in summer 2012 (Fig. 4a). The seasons had otherwise no significant effect on the curvature of A/T in *P. halepensis*. In summary, the humid and warm climates during our measurement campaigns led to higher A_{opt} and T_{opt} in *Q. ilex* and *A. unedo*. The curvature below T_{opt} in these two tree species was more peaked in the dry and warm periods, in contrast to winter when it was shallower. The A/T response of *P. halepensis*, however, remained stable throughout the seasons. For *Q. pubescens* the T_{opt} of A_{net} was higher in summer 2011 than in summer 2012. However, no statistical test could be performed (see Material and Methods).

The mean J_{opt} in *Q. ilex* leaves was significantly higher in spring 2012 than in autumn 2011. J_{opt} did not differ in the other tree species (Fig. 4b1, Table S1, suppl. mat.). *Q. ilex*, *A. unedo*, and *P. halepensis* all had a higher mean T_{opt} of J_{cf} in summer 2011 (Fig. 4b1-b4, Table S1, suppl. mat.). This difference was significant for *Q. ilex* and marginally significant for *P. halepensis* relative to autumn 2011, spring 2012, and winter 2012. The difference for *A. unedo* was significant and marginally significant compared to autumn 2011 and summer 2012, respectively. The mean H_a of J_{cf} in leaves of *Q. ilex* was significantly higher in spring 2012 (similar to H_a of A_{net}) than in summer 2011 and summer 2012 and marginally significantly higher than in autumn 2011 (Fig. 4b1, Table S1, suppl. mat.). For *Q. pubescens* the T_{opt} of J_{cf} was higher in summer 2011 than in summer 2012. Further, a high H_d led to a comparatively stronger decline of J_{cf} in summer 2011 than in summer 2012. However, no statistical test could be performed (see Material and Methods). Statistically significant changes in H_d of J_{cf} were only apparent in the leaves of *P. halepensis*; H_d was significantly higher in summer 2011 than in autumn 2011, summer 2012, and winter 2012 and marginally significantly higher than in spring 2012 (Fig. 4b2, Table S1, suppl. mat.). Overall, all species demonstrated a thermal acclimation for J_{cf}, with a higher T_{opt} in summer 2011. The shape of the J/T curve changed in *Q. ilex*, with a significantly higher H_a in spring 2012 (steeper increase), and in *P. halepensis*, with a significantly higher H_d in summer 2011 (sharper decline).

4.3.5 Species-intercomparison

Across all seasons, *Q. pubescens* generally had the lowest and *A. unedo* the highest mean A_{opt} in both sunlit and shaded leaves (Table S1, suppl. Mat.). *P. halepensis*, however, had the highest mean J_{opt} in both sunlit and shaded leaves (Table S1, suppl. Mat.). In the respective seasons, *A. unedo* had the highest mean A_{opt} for all leaves combined in all campaigns except winter 2012 and spring 2012 (Fig. S1a3 and Table S1, suppl. mat.). In winter 2012, *P. halepensis* had the highest mean A_{opt} (Fig. S1b4 and Table S1, suppl. mat.) and *Q. ilex* in spring 2012 (Fig. S1a5 and Table S1, suppl. mat.). A_{opt} was generally lowest in *Q. pubescens* throughout the seasonal campaigns except in summer 2011 (Fig. S1a1-a5 and Table S1, suppl. mat.). The mean H_a of A_{net} was significantly higher for *A. unedo* in autumn 2011 than for *P. halepensis* and *Q. ilex* (Fig. S1a3 and Table S1, suppl. mat.). The mean H_a of A_{net} in summer 2012 for *Q. ilex* was significantly higher than for *P. halepensis* (significant) and marginally significantly higher than for *A. unedo* (Fig. S1a6, suppl. mat.). The mean H_d of A_{net} in summer 2012 was also marginally significantly higher for *Q. ilex* than for *A. unedo* (Fig. S1a6, suppl. mat.). These results indicated that the A/T response curve for summer 2012 was comparatively more peaked in *Q. ilex* than in the other species. The differences between species were less pronounced in the temperature responses of J_{cf} than of A_{net}. The mean J_{opt} (in μmol electron m^{-2} s^{-1}) of *Q. ilex* (all leaves combined) was particularly low in autumn 2011 (65±9) and significantly different from that of both *P. halepensis* (136±9) and *A. unedo* (102±6) (Fig. S1b3 and Table S1, suppl. mat.). T_{opt}, H_a, and H_d for J_{cf} did not differ significantly across species or leaf positions.

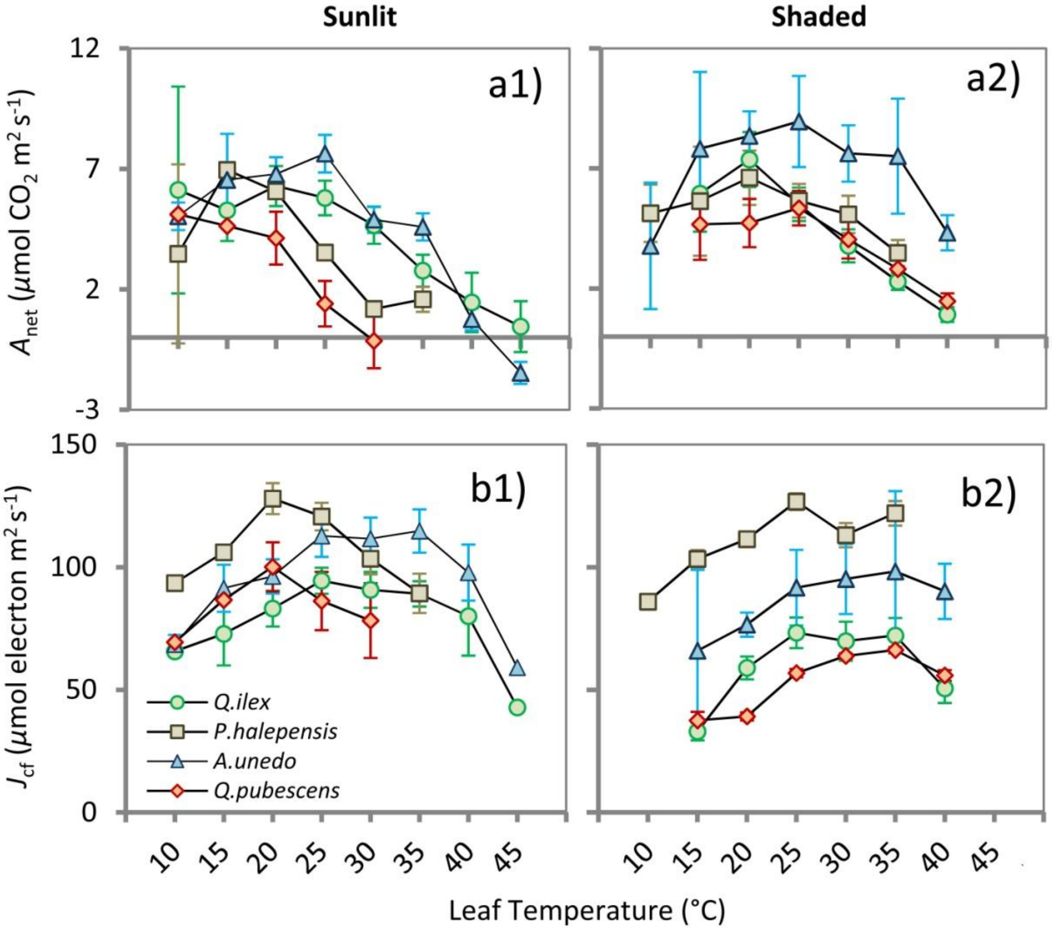

Fig. 4.3 | Line graphs depicting the temperature responses for all seasons combined of (a) the net CO_2 assimilation (A_{net}) and (b) the electron-transport rate (J_{cf}) in (1) sunlit and (2) shaded leaves for four tree species (*Quercus ilex*, *Pinus halepensis*, *Arbutus unedo*, and *Q. pubescens*). The data points are means for temperature increments of 5 °C from 10 to 45 °C. Vertical bars indicate standard errors of the means.

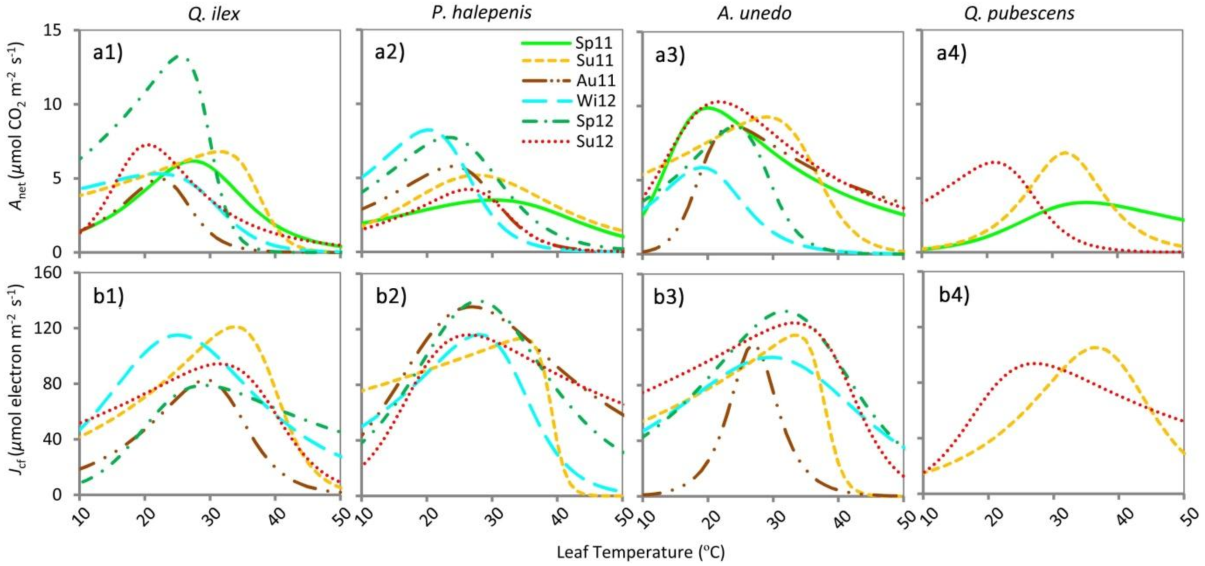

Fig. 4.4 | Temperature-response curves of sunlit leaves for (a) net CO_2 assimilation (A_{net}) and (b) electron transport-rate (J_{cf}) for (1) *Quercus ilex*, (2) *Pinus halepensis*, (3) *Arbutus unedo*, and (4) *Q. pubescens*) for all six seasonal campaigns (abbreviations as in Table 1). *Q. pubescens* was only sampled in spring and summer due to the deciduous leaf habit. Measurement difficulties and limitations in equipment led additionally to a gap in the data for *Q. pubescens* in Sp12 and in Sp11 in J_{cf} for all species. The response curves were computed with the peaked function.

4.4 Discussion

4.4.2 Model comparison

The response curve of photosynthesis to temperature is usually bell-shaped with an ascending and descending arm of the curve (Taz & Zeiger, 2010). The descending arm is often characterised by abrupt fall-offs of the electron-transport or net assimilation rate at high temperatures ranges (e.g. Yamori *et al*. 2014). June's model was suggested as an alternative to reproduce the temperature responses of the electron-transport rate in a simple yet mechanistic manner (June *et al*., 2004) and has been incorporated into terrestrial biosphere models (Friend, 2010). In our study, however, we favoured the peaked function for both the electron-transport rate and the net assimilation rate because the model by June *et al*. (2004) lacked flexibility. June's model uses only one empirical parameter to control either side of the slope in the temperature response, resulting in an extremely high sum of squared errors in cases of abrupt fall-offs of the photosynthetic responses, especially in the higher temperature ranges. The peaked function includes two empirical parameters: H_a is the activation energy and represents the ascending arm of the curve below T_{opt}, and H_d is the deactivation energy above T_{opt}. Higher values of H_a or H_d signify a steeper slope, and lower values signify a more gentle slope. This response function proved to be more flexible in cases of rapid fall-offs of J_{cf} or A_{net} with lower sums of squared errors. Vårhammar *et al*. (2015) showed that ambient temperatures can greatly exceed the photosynthetic optimum temperatures. Mediterranean-type ecosystems are characterised by a strong seasonality of environmental conditions and a high daily variability (Specht, 1969; Aschmann, 1973; Orshan, 1983; Blumler, 1991). The use of the peaked function is thus more appropriate in Mediterranean-type conditions.

4.4.3 Temperature optima

The antenna pigments of the foliar chlorophyll funnel solar energy down to the reaction centres, initiating a cascade of exited electrons between PSII and PSI resulting in the end products ATP and NADPH. The light reactions thus provide the biochemical energy for the photosynthetic carbon cycle where carbon is carboxylated with Rubisco to form glucose. Electron transport in the light reactions and the resulting photosynthetic carbon assimilation are positively correlated with temperature which is explained by a quicker enzyme function accelerating the underlying biochemical processes (Farquhar *et al*., 1980; Bernacchi *et al*., 2001, 2002; Medlyn *et al*., 2002; Way & Oren, 2010). Both light and carbon reactions are optimally balanced at the temperature optimum under the prevailing environmental conditions, but limitations occur as the temperature decreases or increases (Taz & Zeiger, 2010). At low temperatures, a decreased enzyme activity and limited phosphate availability can limit net photosynthesis (Sage & Sharkey, 1987; Sage & Kubien, 2007). At higher temperatures, photorespiration is stimulated leading to a decreased quantum yield of CO_2 (Ehleringer & Björkman, 1977). Additionally, the heat lability of Rubisco activase decreases the CO_2 carboxylation by Rubisco (Law & Crafts-brandner, 1999; Haldimann & Feller, 2004).

Nonetheless, these factors are not the primary causes of photosynthetic decline at high temperatures. Rather, the PSII has been characterised as the primary target of heat-induced stress, whereas PSI is comparatively heat resistant (Haldimann & Feller, 2004 and references herein). With the chlorophyll fluorescence technique that was applied in our study, we measured this PSII-driven electron-transport rate. Hence, electron transport plays an overriding role in limiting whole-leaf photosynthesis following heat stress as demonstrated in several findings (Wise *et al.*, 2004; Vårhammar *et al.*, 2015), which is probably due to proton leakiness across the thylakoid membrane (Sage *et al.*, 2008). The mean temperature optimum in our study was significantly lower for A_{net} (24.6 °C) than for J_{cf} (30.1 °C), typical values reported for most species (Diaz-Espejo *et al.*, 2012). The biochemical reactions of photosynthesis, such as the electron-transport rate, operate more efficiently at higher temperature ranges than the instantaneous CO_2 assimilation rate A_{net} (Taz & Zeiger, 2010). This is because A_{net} depends not only on the biochemical potential of the electron-transport rate and Rubisco activity, but also on photorespiration and the behaviour of stomatal and mesophyllic CO_2 diffusion from the atmosphere to the chloroplasts. These depend strongly on atmospheric vapour-pressure deficit (VPD), foliar hydraulic status, and transpiratory water loss (e.g. Lin *et al.* 2012).

4.4.1 Seasonal acclimation

The growth environment strongly determines the thermal optimum and also the acclimations of this optimum in response to seasonal changes in the growth temperature (Medlyn *et al.*, 2002; Rennenberg *et al.*, 2006; Kattge & Knorr, 2007). The thermal optimum is hereby the maximum value of a parameter whereas T_{opt} is the optimum temperature at which the thermal optimum is achieved. We found a significant seasonal acclimation of the thermal optimum of A_{net}, with higher values in the humid 2011 in both spring and summer. Similarly, the thermal optimum of J_{cf} peaked in the humid summer of 2011. The lowest values were in the dry and cold campaigns of autumn 2011 and winter 2012. The sampled trees were remarkably plastic, with higher thermal optima for both A_{net} and J_{cf} under favourable climatic conditions of high temperatures and humidity. Whereas the acclimation of the thermal optimum describes a vertical shift, the acclimation of T_{opt} describes a horizontal shift of the peak of the temperature response curve. This allows an optimized photosynthetic exploitation for achieving a annual positive carbon balance that is particularly essential for Mediterranean evergreen tree species (García-Plazaola *et al.*, 1999; Martínez-Ferri *et al.*, 2004). These adjustments were paralleled by acclimations of foliar traits such as nitrogen content and leaf mass per area (LMA) as well as of biochemical and diffusion pathways (stomatal and mesophyllic) (Sperlich *et al.*, 2015; see also Martin-StPaul *et al.* 2013 and Poorter *et al.* 2009).

Not only the thermal optimum and T_{opt}, but also the curvature of the temperature response changed seasonally. The shape of the A/T curve changed through H_a in the extreme climatic conditions of our observation period, with a steeper increase in the dry campaigns of summer 2012 and autumn 2011 and a shallower increase in winter 2012. H_d, however, did not change seasonally. The steeper ascent or descent of the temperature curve represents a decreased tolerance to lower or higher temperatures, respectively. We thus concluded that A_{net} was better acclimated to lower temperatures in winter, in contrast to the hottest and driest periods of our measurement campaigns. The curvature of the J/T response had the opposite pattern: H_d but not H_a showed significant seasonal changes. H_d was higher in the campaign of the humid summer of 2011, so that J_{cf} decreased much more drastic above T_{opt} than in the hot and dry periods of autumn 2011 and summer 2012. These results indicate first that the photosynthetic system (mostly PSII) was primarily impeded by high and not low temperatures (Haldimann & Feller, 2004) and second that the photosynthetic system was better acclimated to heat stress in the drier and hotter year. In a recently published companion paper (Sperlich *et al.*, 2015) we showed that the studied plants induced refinements of the photosynthetic apparatus in response to the summer stress period. The findings of the current study underline that these refinements were accompanied by a seasonal acclimation of the thermal optimum and of the curvature of the temperature-response curve. We stress that the photosynthetic responses to temperature should be modelled using plastic functions incorporating an adjustment to account for seasonal acclimation (Gunderson *et al.*, 2009).

4.4.4 Species-intercomparison

The togetherness of plants that grow in the same environment and that exhibit distinct morphological and phenological strategies (evergreen and deciduous, broadleaved and coniferous) raises the question of how they respond to contrasting seasonal environmental conditions. The effects of the yearly variability of environmental conditions on tree physiology are ideally tested in mixed forests for comparing species-specific strategies. We need to better understand the species-specific dynamics of leaf temperature and photosynthetic responses to heat stress when trying to predict the effects of rising global temperatures on plant productivity and growth (Bernacchi *et al.*, 2001; Wise *et al.*, 2004; Lin *et al.*, 2012). Acclimation to the prevalent temperature regime is important, especially in trees with long leaf-lifespans.

High temperatures reduce photosynthetic efficiency by stimulating photorespiration and by damaging the photosynthetic apparatus (Schrader *et al.*, 2004; Wise *et al.*, 2004). Deactivation of Rubisco correlates with a decline in photosynthesis at moderately high temperatures (35-45 °C) (Law & Brandner 1999). Exposure to 60 °C for 30 min completely damages the photosynthetic system of *Q. ilex* (Trabaud & Méthy, 1992). These authors also reported that *Q. ilex* could withstand a temperature of 50 °C with only limited damage to the photosynthetic system. Temperature optima or tolerances of the photosynthetic system can vary among plant functional types (e.g. Méthy *et al.* 1997). A review by Way and Oren (2010), however, reported no differences between deciduous and evergreen tree species. Recent large-scale studies of tree growth in the Iberian Peninsula, on the other hand, have reported negative growth trends in response to rising temperatures in Mediterranean gymnosperms, mostly pines (Gómez-Aparicio *et al.*, 2011; Carnicer *et al.*, 2013; Coll *et al.*, 2013). Our A/T response curves for *Q. ilex* was more peaked in the particularly dry and hot summer of 2012, indicating a quicker downregulation at high temperatures and thus a higher plasticity compared to the other species, confirming the results by Méthy *et al.* (1997).

The response of A_{opt} was very variable between species and seasons. A_{opt} was highest for *A. unedo* in autumn 2011, for *Q. ilex* in spring 2011, and for *P. halepensis* in winter 2012, indicating that the tree species benefitted differently from each season. The species differences were less pronounced for J_{opt}, which was generally highest for *P. halepensis* and lowest for *Q. ilex*. *P. halepensis* performed well under extreme temperatures, especially in winter with the highest J_{opt} of all species (see also Sperlich et al. 2014). The high values of A_{opt} in *A. unedo* during the dry period of autumn 2011 indicated strong anisohydric behaviour (Rosas *et al.*, 2013). *A. unedo* was therefore particularly vulnerable to high xylem pressure under severe soil-water deficits. Indeed, we observed defoliation and branch dieback in response to severe drought in summer 2012 (Sperlich *et al.*, 2015 and see also results by Rosas *et al.*, 2013). *Q. ilex* is also an anisohydric tree species but is more conservative in its water use than *A. unedo*. *Q. ilex* tolerates low water potentials through plastic hydraulic features (Fotelli *et al.*, 2000; Corcuera *et al.*, 2004) and acclimation of foliar traits (Villar-Salvador *et al.*, 1997; Ogaya & Peñuelas, 2006; Limousin *et al.*, 2009), as also observed at our study site; LMA of *Q. ilex* was reduced drastically in the dry 2012 (Sperlich *et al.*, 2015). Moreover, *Q. ilex* behaves highly plastic to abiotic stress showing a pronounced photoinhibition-avoidance (Martinez-Ferri *et al.*, 2000), that is paralleled by a high plasticity of its temperature response behaviour, as shown in this study. *P. halepensis*, as a pine, is a typical isohydric species that follows a strategy of water conservation and embolism avoidance (Meinzer *et al.*, 2009). Photosynthetic downregulation, foliar-trait acclimation, and improved gas exchange enables *P. halepensis* to endure most successfully water and thermal stresses (Sánchez-Costa *et al.*, 2015; Sperlich *et al.*, 2015). *P. halepensis* also strongly benefitted from the mild winter periods (see also Sperlich *et al.*, 2014). On the whole, we found that this tree species is highly resistant to photoinhibition and temperature stress throughout the year, efficient under drought and thus highly competitive in dry habitats, as also found by other studies (Zavala & Zea, 2004; Maseyk *et al.*, 2008; de Luis *et al.*, 2011).

This seems to contrast with the increased dominance of angiosperm trees and negative growth trends in pines over extensive areas of the Iberian Peninsula recently reported in large-scale studies (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013; and also see review by Carnicer *et al.*, 2013). We speculate that age and succession play overriding roles in many old-growth pine stands. Our study site is exemplary for many pine-oak forests in the Iberian Peninsula, where shelter pine trees form the top canopy, followed by a dense layer of *Quercus* species (Zavala *et al.*, 2000). The dense *Quercus* canopy has suppressed the regeneration of the early-successional and light-demanding pine seedlings (Sperlich *et al.*, 2014) that need fire to regenerate (Zavala *et al.*, 2000). Carnicer *et al.* (2014) observed severe limitations of recruitment for most *Pinus* species across extensive areas of the Iberian Peninsula. Pines might face a demographic decline in many pine-oak stands near the end of their life expectancy due to the human fire control, which could account for their vulnerability to abiotic stressors reported in these studies.

All in all, this study has helped to account for the high tolerance to photoinhibition of *P. halepensis* reported by Sperlich *et al.* (2015) showing that the photosynthetic system was highly resistant to temperature stress throughout the seasons. *Q. ilex* and *A. unedo* showed a stronger acclimatory behaviour whereupon *Q. ilex* was characterised with the highest seasonal plasticity, confirming the findings by Sperlich *et al.* (2015). We postulate that the conifer exhibits not only a contrasting morphological and but also distinct photoprotective strategy.

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4.7 Supporting information

Supplementary figures

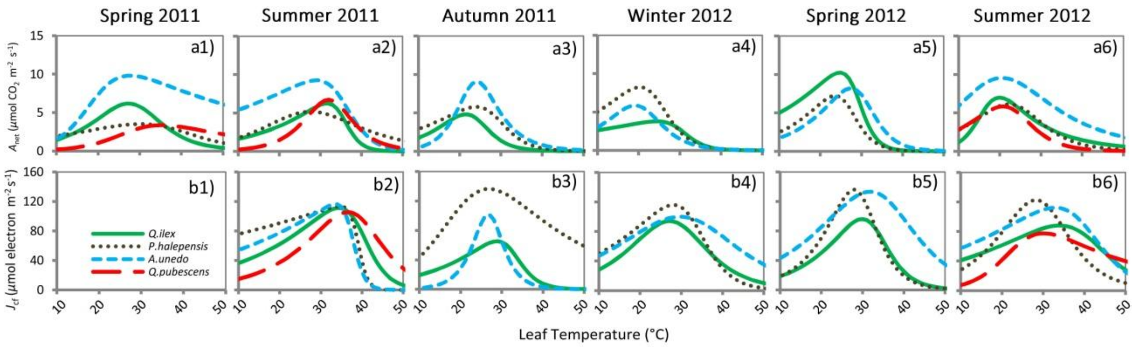

Fig. S4.1 | Temperature-response curves for (a) net CO$_2$ assimilation (A_{net}) and (b) electron-transport rate (J_{cf}) in the six seasonal campaigns (1-6) for *Quercus ilex*, *Pinus halepensis*, *Arbutus unedo*, and *Q. pubescens* and both leaf positions combined. The deciduous *Q. pubescens* was only sampled in spring and summer when leaves were available. Measurement difficulties and limitations in equipment led additionally to a gap in the data for *Q. pubescens* in Sp12. The response curves were computed with the peaked function.

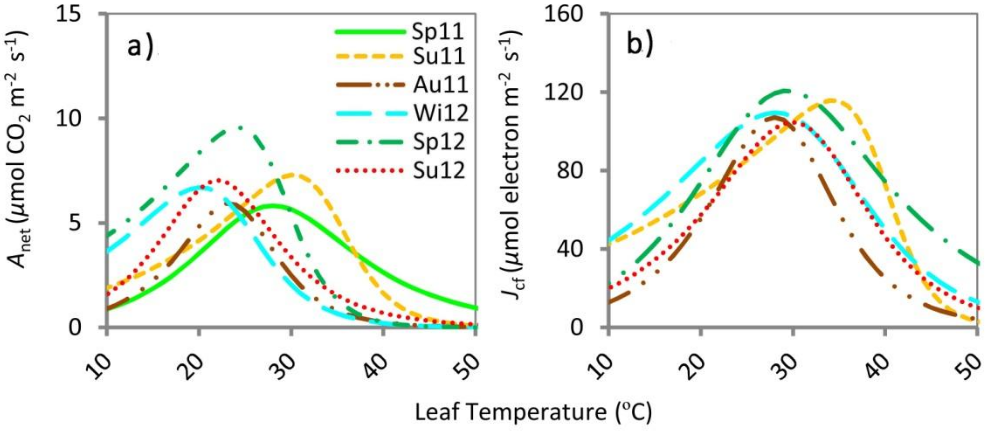

Fig. S4.2 | Average temperature-response curves in sunlit leaves of *Quercus ilex*, *Pinus halepensis*, *Arbutus unedo*, and *Q. pubescens* for (a) net CO$_2$ assimilation (A_{net}) and (b) electron-transport rate (J_{cf}) in the six seasonal campaigns (abbreviations as in Table 1). The response curves were computed with the peaked function.

Supplementary tables

Table S4.1 | Mean values and standard errors (±SE) of the parameters of the modelled A/T and J/T response curves fitted with the peaked function for a) Quercus ilex, b) Pinus halepensis, c) Arbutus unedo, and d) Q. pubescens) in six seasons (spring 2011, summer 2011, autumn 2011, winter 2012, spring 2012, and summer 2012) and for two leaf positions (sunlit and shaded). T_{opt} is the thermal optimum, A_{opt} (in µmol CO_2 m^{-2} s^{-1}) is the net assimilation rate at T_{opt}, J_{opt} (in µmol electron m^{-2} s^{-1}) is the electron-transport rate at T_{opt}, H_a (unitless) is the activation energy representing the ascending arm below T_{opt} and H_d (unitless) is the deactivation energy representing the descending arm above T_{opt}.

a) Q. ilex		**A/T response curve**				**J/T response curve**			
	Seasons	**A_{opt}**	**T_{opt}**	**H_a**	**H_d**	**J_{opt}**	**T_{opt}**	**H_a**	**H_d**
Sunlit	Spring 2011	6.2±1.3	27.3±5.0	78±54	210±40				
	Summer 2011	6.8±1.2	31.6±5.4	21±52	408±88	121±6	33.9±	36±58	303±2
	Autumn 2011	5.0±0.9	22.4±3.9	86±50	342±69	83±11	29.4±	65±54	272±2
	Winter 2012	5.3±1.0	22.2±4.0	16±53	235±42	115±	25.1±	75±25	140±95
	Spring 2012	13.3±2.4	25.6±4.7	37±54	473±90	79±	29.2±	150±33	178±21
	Summer 2012	7.3±1.3	20.5±3.7	191±53	273±49	94±	31.5±	24±46	224±23
Shaded	Summer 2011	3.4±0.6	32.0±5.8	190±56	501±35	61±11	36.9±6.7	43±8	259±47
	Autumn 2011	4.5±0.9	20.0±3.6	71±52	215±14	36±6	27.8±4.9	32±6	425±78
	Winter 2012	2.3±0.4	26.5±4.8	27±54	375±5	73±14	29.2±5.2	64±13	224±44
	Spring 2012	9.0±1.6	24.4±4.2	40±50	346±9	103±18	30.2±5.1	38±8	315±64
	Summer 2012	6.4±1.2	19.0±3.5	278±53	325±51	76±14	39.1±7.1	38±7	69±13
All	Spring 2011	6.2±3.2	27.4±4.2	78±26	210±71				
	Summer 2011	6.3±1.0	31.6±1.1	49±29	423±122	111±15	34.4±0.8	37±8	296±61
	Autumn 2011	4.8±0.6	21.5±1.6	81±13	294±66	65±9	28.8±0.7	53±1	329±61
	Winter 2012	3.8±1.2	24.4±1.7	21±3	305±59	94±18	27.2±1.3	69±15	182±46
	Spring 2012	10.3±1.2	24.8±1.5	40±12	382±62	96±11	29.9±0.9	70±25	276±72
	Summer 2012	7.0±0.9	20.0±1.0	220±33	291±25	88±14	34.0±2.7	29±8	172±87
	Sunlit	**6.8±0.7**	**25.6±1.1**	**66±12**	**341±43**	**99.8±6**	**30.5±0.7**	**63±10**	**248±33**
	Shaded	**6.1±1.0**	**23.8±2.0**	**78±35**	**329±52**	**75.6±11**	**30.7±1.8**	**41±9**	**302±69**
	All leaves	**6.5±0.6**	**24.9±1.0**	**71±12**	**336±34**	**89.4±6**	**30.6±0.7**	**53±8**	**271±31**

Table continued.

Table S4.1 | Continued.

b)P.halepensis		**A/T response curve**				**J/T response curve**			
	Seasons	A_{opt}	T_{opt}	H_a	H_d	J_{opt}	T_{opt}	H_a	H_d
Sunlit	Spring 2011	3.5±0.6	30.2±5.5	29±5	138±25				
	Summer 2011	5.2±0.8	27.6±4.1	70±11	142±21	114±20	34.7±6.2	12±2	745±141
	Autumn 2011	5.8±1.0	24.0±4.2	44±10	258±49	136±24	26.8±4.6	94±21	135±27
	Winter 2012	8.2±1.4	20.3±3.5	46±12	259±48	116±20	28.1±4.9	40±10	238±45
	Spring 2012	7.7±1.4	23.3±4.1	49±10	199±36	140±25	27.9±4.9	78±18	158±29
	Summer 2012	4.2±0.8	26.4±4.8	53±10	282±51	116±21	26.4±4.8	157±29	181±33
Sh.	Spring 2012	6.5±1.2	24.0±53	95±20	530±112	129±23	27.9±5.1	122±30	501±102
	Summer 2012	7.1±1.3	20.2±53	77±14	154±28	126±23	29.2±5.3	34±8	218±43
All leaves	Spring 2011	3.5±	30.2±	29±	138±				
	Summer 2011	5.2±1.8	27.6±9.4	70±29	142±47	114±6	34.7±3	12±2	745±197
	Autumn 2011	5.8±0.4	24.0±1.9	44±19	258±66	136±9	26.8±1	94±42	135±43
	Winter 2012	8.2±0.5	20.3±0.8	46±26	259±50	116±11	28.1±1	40±22	238±59
	Spring 2012	7.2±0.6	23.6±2.1	67±23	331±130	136±8	27.9±2	95±45	295±118
	Summer 2012	6.1±1.0	22.3±2.1	69±16	197±43	123±5	28.2±2	75±43	205±58
	Sunlit	**6.5±0.5**	**23.9±1.0**	**49±9**	**224±24**	**126±5**	**28.9±1.0**	**64±17**	**292±66**
	Shaded	**6.8±0.3**	**22.1±2.6**	**86±25**	**342±169**	**128±9**	**28.5±2.6**	**78±140**	**359±175**
	All leaves	**6.6±0.4**	**23.6±0.9**	**56±9**	**249±38**	**126±4**	**28.9±0.9**	**67±63**	**306±62**

Table continued.

Table S4.1 | Continued.

b)P.halepensis		**A/T response curve**				**J/T response curve**			
	Seasons	A_{opt}	T_{opt}	H_a	H_d	J_{opt}	T_{opt}	H_a	H_d
Sunlit	Spring 2011	3.5±0.6	30.2±5.5	29±5	138±25				
	Summer 2011	5.2±0.8	27.6±4.1	70±11	142±21	114±20	34.7±6.2	12±2	745±141
	Autumn 2011	5.8±1.0	24.0±4.2	44±10	258±49	136±24	26.8±4.6	94±21	135±27
	Winter 2012	8.2±1.4	20.3±3.5	46±12	259±48	116±20	28.1±4.9	40±10	238±45
	Spring 2012	7.7±1.4	23.3±4.1	49±10	199±36	140±25	27.9±4.9	78±18	158±29
	Summer 2012	4.2±0.8	26.4±4.8	53±10	282±51	116±21	26.4±4.8	157±29	181±33
Sh.	Spring 2012	6.5±1.2	24.0±53	95±20	530±112	129±23	27.9±5.1	122±30	501±102
	Summer 2012	7.1±1.3	20.2±53	77±14	154±28	126±23	29.2±5.3	34±8	218±43
All leaves	Spring 2011	3.5±	30.2±	29±	138±				
	Summer 2011	5.2±1.8	27.6±9.4	70±29	142±47	114±6	34.7±3	12±2	745±197
	Autumn 2011	5.8±0.4	24.0±1.9	44±19	258±66	136±9	26.8±1	94±42	135±43
	Winter 2012	8.2±0.5	20.3±0.8	46±26	259±50	116±11	28.1±1	40±22	238±59
	Spring 2012	7.2±0.6	23.6±2.1	67±23	331±130	136±8	27.9±2	95±45	295±118
	Summer 2012	6.1±1.0	22.3±2.1	69±16	197±43	123±5	28.2±2	75±43	205±58
	Sunlit	**6.5±0.5**	**23.9±1.0**	**49±9**	**224±24**	**126±5**	**28.9±1.0**	**64±17**	**292±66**
	Shaded	**6.8±0.3**	**22.1±2.6**	**86±25**	**342±169**	**128±9**	**28.5±2.6**	**78±140**	**359±175**
	All leaves	**6.6±0.4**	**23.6±0.9**	**56±9**	**249±38**	**126±4**	**28.9±0.9**	**67±63**	**306±62**

Table continued.

Table S4.1 | Continued.

c) *A. unedo*		A/T response curve				J/T response curve			
	Seasons	A_{opt}	T_{opt}	H_a	H_d	J_{opt}	T_{opt}	H_a	H_d
Sunlit	Spring 2011	9.9±1.8	20.0±3.7	211±39	250±46				
	Summer 2011	9.3±1.6	29.1±5.1	24±5	270±58	116±20	33.6±5.8	25±5	592±136
	Autumn 2011	8.7±1.6	23.9±4.3	329±59	360±65	110±20	27.0±4.8	224±55	458±82
	Winter 2012	5.9±1.1	19.1±3.4	80±16	245±47	100±18	29.7±5.2	42±8	123±22
	Spring 2012	8.6±1.5	24.2±4.4	52±13	336±64	133±24	31.8±5.7	50±10	160±29
	Summer 2012	10.3±1.9	21.7±4.0	138±25	179±33	125±23	33.3±6.1	18±3	250±46
Shaded	Spring 2011	9.7±1.8	35.4±6.5	100±18	105±19				
	Autumn 2011	9.5±1.7	24.4±4.4	39±10	300±56	94±17	26.5±4.8	176±42	450±83
	Spring 2012	7.4±1.4	34.0±6.2	123±23	234±43				
	Summer 2012	8.8±1.6	19.3±3.5	15±3	92±17	100±18	32.5±5.9	33±6	125±23
All	Spring 2011	9.8±0.1	27.7±7.7	156±56	178±73				
	Summer 2011	9.3±0.4	29.1±2.6	24±11	270±109	116±7	33.6±0.7	25±7	592±279
	Autumn 2011	9.1±0.3	24.2±1.1	184±85	330±38	102±6	26.7±0.6	200±105	454±42
	Winter 2012	5.9±1	19.1±1.9	80±26	245±62	100±15	29.7±2.1	42±7	123±19
	Spring 2012	8.2±0.5	27.5±3.8	75±37	302±71	133±45	31.8±10	50±20	160±55
	Summer 2012	9.5±0.8	20.5±1.2	77±62	136±43	112±13	32.9±0.4	26±8	188±63
	Sunlit	**8.2±0.5**	**23.6±1.5**	**109±30**	**277±37**	**114±6**	**31.1±0.9**	**64±31**	**334±97**
	Shaded	**9.0±0.5**	**27.5±3.1**	**63±24**	**206±52**	**96±5**	**28.5±2.2**	**129±100**	**342±123**
	All leaves	**8.4±0.4**	**24.6±1.4**	**97±23**	**259±31**	**110±5**	**30.6±0.9**	**76±30**	**336±81**

c)*Q.pubescens*		A/T response curve				J/T response curve			
	Seasons	A_{opt}	T_{opt}	H_a	H_d	J_{opt}	T_{opt}	H_a	H_d
Sunlit	Spring 2011	3.4±0.6	35.2±6.4	122±22	160±25				
	Summer 2011	6.7±1.4	32.0±5.8	129±24	295±29	105±20	36.5±6.6	64±14	213±40
	Summer 2012	6.1±1.1	21.2±3.9	50±9	265±48	94±17	27.1±5.0	151±28	178±32
Sh.	Summer 2012	5.7±1.0	20.6±3.8	78±15	214±39	69±12	31.7±5.8	131±29	173±34
All	Spring 2011	3.4±	35.2±	122±	160±				
	Summer 2011	6.7±3.4	32.0±2.6	129±29	295±41	105±32	36.5±4.2	64±39	213±65
	Summer 2012	5.9±0.2	20.8±2.7	69±21	231±25	78±8	30.1±3.3	138±55	175±43
	Sunlit	**5.7±1.6**	**30.1±3.2**	**108±23**	**254±36**	**102±19**	**33.4±4.0**	**93±37**	**201±39**
	Shaded	**5.7±0.3**	**20.6±4.6**	**78±32**	**214±32**	**69±**	**31.7±5.1**	**131±95**	**173±74**
	All leaves	**5.7±1.0**	**27.0±3.1**	**98±18**	**241±26**	**89±13**	**32.7±2.7**	**108±37**	**190±33**

5

Cones of *Pinus halepensis*
Photo & Design: D. Sperlich

Chapter 5

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

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5.1 Abstract

Evergreen trees in the Mediterranean region must cope with a wide range of environmental stresses from summer drought to winter cold. The mildness of Mediterranean winters can periodically lead to favourable environmental conditions above the threshold for a positive carbon balance, benefitting evergreen woody species more than deciduous ones. The comparatively lower solar energy input in winter decreases the foliar light saturation point. This leads to a higher susceptibility to photoinhibitory stress especially when chilly (<12 °C) or freezing temperatures (<0°C) coincide with clear skies and relatively high solar irradiances. Nonetheless, the advantage of evergreen species that are able to photosynthesize all year round where a significant fraction can be attributed to winter months, compensates for the lower carbon uptake during spring and summer in comparison to deciduous species. We investigated the ecophysiological behaviour of three co-occurring mature evergreen tree species (*Quercus ilex* L., *Pinus halepensis* Mill., and *Arbutus unedo* L.). Therefore, we collected twigs from the field during a period of mild winter conditions and after a sudden cold period. After both periods, the state of the photosynthetic machinery was tested in the laboratory by estimating the foliar photosynthetic potential with CO_2 response curves in parallel with chlorophyll fluorescence measurements. The studied evergreen tree species benefited strongly from mild winter conditions by exhibiting extraordinarily high photosynthetic potentials. A sudden period of frost, however, negatively affected the photosynthetic apparatus, leading to significant decreases in key physiological parameters such as the maximum carboxylation velocity ($V_{c,max}$), the maximum photosynthetic electron transport rate (J_{max}), and the optimal fluorometric quantum yield of photosystem II (F_v/F_m). The responses of $V_{c,max}$ and J_{max} were highly species-specific, where *Q. ilex* exhibited the highest and *P.*

halepensis the lowest reductions. In contrast, the optimal fluorometric quantum yield of photosystem II (F_v/F_m) was significantly lower in *A. unedo* after the cold period. The leaf position played an important role in *Q. ilex* showing a stronger winter effect on sunlit leaves in comparison to shaded leaves. Our results generally agreed with the previous classifications of photoinhibition-tolerant (*P. halepensis*) and photoinhibition-avoiding (*Q. ilex*) species on the basis of their susceptibility to dynamic photoinhibition, whereas *A. unedo* was the least tolerant to photoinhibition, which was chronic in this species. *Q. ilex* and *P. halepensis* seem to follow contrasting photoprotective strategies. However, they seemed equally successful under the prevailing conditions exhibiting an adaptive advantage over *A. unedo*. These results show that our understanding of the dynamics of interspecific competition in Mediterranean ecosystems requires consideration of the physiological behaviour during winter which may have important implications for long-term carbon budgets and growth trends.

5.2 Introduction

Mediterranean-type ecosystems are widely associated with broadleaved evergreen sclerophyllous shrubs and trees, the classic vegetation types in climates where hot and dry summers alternate with cool and wet winters (Specht, 1969; Aschmann, 1973; Orshan, 1983; Blumler, 1991). In summer, water is undoubtedly the most important factor limiting growth and survival in the Mediterranean region, whereas spring and autumn provide better growing conditions (Orshan, 1983; Gracia *et al.*, 1999; Sabaté & Gracia, 2011). In winter, the low temperatures and solar radiation limit the amount of energy available for the vegetation, although soil-water contents and water-pressure deficits are favourable. This highly dynamic seasonality of favourable and unfavourable conditions produces a rich diversity of plants in these regions (Cowling *et al.*, 1996). In turn, this features a highly diverse range of traits and taxa that has produced multiple survival strategies which help to explain the abundance and distribution of species (Matesanz & Valladares, 2014). Nonetheless, the predicted reductions in annual precipitation, increases in mean temperature, and increases in the variability and occurrence of extreme droughts and heat waves in arid and semi-arid regions are likely to affect species abundance and distribution (Somot *et al.*, 2008; Friend, 2010; IPCC, 2013). The battle for survival and dominance in plant communities facing these novel changes in their environments evokes great uncertainties and worries in the scientific community concerning the adaptive ability, distribution shifts, or, at worst local extinction of species especially in Mediterranean type ecosystems (Peñuelas *et al.*, 2013; Matesanz & Valladares, 2014).

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

Hereby, mixed forests provide us with an ideal test-bed for investigating the different ecophysiological strategies and their sensitivities to abiotic stresses, because all tree species have to contend equally with the yearly variability of environmental conditions. Nevertheless, most ecophysiological studies have been conducted in spring and summer, and winter has been surprisingly overlooked despite its importance for our understanding of the dominance of certain vegetation-types and of the responses of vegetation to stress, seasonality, and species composition (Orshan, 1983; Tretiach *et al.*, 1997; Oliveira & Peñuelas, 2004). Even though efforts have recently been made to elucidate the behaviour of sclerophyllous ecosystems under variable winter conditions (e.g. García-Plazaola et al., 1999, 1997; Kyparissis et al., 2000; Levizou et al., 2004; Martínez-Ferri et al., 2004; Oliveira and Peñuelas, 2004, 2000), the physiological behaviour of co-occurring species of evergreen trees in the Mediterranean region, including leaf gas exchange (GE) and chlorophyll fluorescence (CF) methods, have been insufficiently studied for understanding the dynamics of photoinhibitory stress and interspecific competition. Therefore, in our study we used an ample set of parameters from GE & CF measurements in order to provide a snapshot in the plant's physiology and in order to characterize in detail the effects on the photosynthetic light and carbon reactions during winter (Flexas *et al.*, 2008; Guidi & Calatayud, 2014). This study was conducted on three species of evergreen trees (*Quercus ilex* L., *Pinus halepensis* Mill., *Arbutus unedo* L.) in northern Catalonia near Barcelona, Spain.

Our aims were to i) investigate the foliar physiology of these three species under mild winter conditions, ii) analyse the effect of sudden changes from favourable to unfavourable conditions on photochemical and non-photochemical processes associated with electron transport, CO_2 fixation, and heat dissipation, iii) determine if leaves exhibit distinct locational (sunlit or shaded) responses to winter stress, and iv) identify the species-specific strategies when coping with stress, induced by low temperatures and frost. These topics are of particular interest due to the recent report of an increased dominance of angiosperm trees and the negative impacts on pines over extensive areas of the Iberian Peninsula (Carnicer *et al.*, 2013). Therefore, we must improve our understanding of the interactions among co-occurring tree species competing for scarce resources and trying to survive and tolerate novel environmental conditions to be able to predict ecosystem responses to global climate change.

5.3 Material and Methods

5.2.1 Field site

Our experiment was conducted at the field station of Can Balasc in Collserola Natural Park, a coastal massif (8500 ha) in the hinterlands of Barcelona, northeastern Spain (41° 25’ N, 2° 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 north-east-facing slope. The climate is characterised by typical Mediterranean seasonal summer droughts and warm temperatures, with a mean August temperature of 22.8 °C. The proximity to the Mediterranean Sea provides mild winters where frosts and snow are rare, as reflected in the mean January temperature of 7.9 °C. Mean annual precipitation and temperature are 723 mm and 15.1 °C (1951-2010), respectively (Ninyerola *et al.*, 2000). The soils have predominantly developed above lithological strata of shales and granite (Sanchez-Humanes & Espelta, 2011). Sensors for measuring air temperature (HMP45C, Vaisala Oyj, Finland) and solar radiation (SP1110 Skye Instruments Ltd., Powys, UK) were installed at a height of 3 m, in a clearing *ca.* 1 km from the plot.

5.2.2 Stand history and composition of tree species

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

5.2.3 Sampling

The sampling of the mild winter period took place between 09.01.-19.01.12 (DOY 9-19). The frosty/chilly period lasted from 19.01.-04.02.12 (DOY 21-35). The sampling period after the frosty/chilly period took place between 14.02.-24.02.12 (DOY 45-55). We obtained sunlit leaves for GE-analyses by sampling five twigs with a pruning pull from the outer part of the upper third of the crown, and shaded leaves by sampling five twigs from the inner part of the crown, optimally at similar heights. In the second field campaign after the frost occurrence, however, we were constrained to sample shaded leaves only from *Q. ilex* due to limitation in labour and equipment. The shaded leaves of *P. halepensis* and *A. unedo* could only be sampled in the first, but not in the second field campaign. The twigs were immediately re-cut under water in buckets in the field and transported to the laboratory retained in plastic bags to minimise transpiration. Five replicates of each species were collected for the analysis of GE. The twigs were preconditioned in the laboratory at a room temperature of 24-28 °C in dim light for 1-3 d and freshly cut the following morning before the measurement of GE (Niinemets *et al.*, 1999, 2005). We intended to avoid the problems we had faced in the field, such as the limited ability of the instruments to reach the standard operating temperature of 25 °C, which was hampered by low ambient temperatures or unpredictable plant responses such as closed stomata or patchy stomatal conductance (Mott & Buckley, 1998, 2000). The pre-conditioned twigs instead had a stable C_i and sufficiently high gs, which are required for conducting a noise-free CO_2-response curve. The method of cutting twigs rehydrated stressed leaves at optimum conditions and allowed us to analyse their long-term acclimation to the environmental conditions from which they were derived. This method has been used in other studies (Epron & Dreyer, 1992; Niinemets *et al.*, 1999, 2005; Laisk *et al.*, 2002; Haldimann & Feller, 2004), and we confirmed that the leaves remained fresh and functional for several days controlled by g_s and fluorescent signals (data not shown). Our ambient values of the GE- and CF-derived parameters accordingly represented the "ambient capacity" of pre-conditioned leaves under near-optimal ambient environmental conditions of CO_2 concentrations and saturating light and at a room temperature of 20-25 °C (Reich *et al.*, 1998).

5.2.4 GE and CF analyses

GE and CF were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA). Response curves for foliar net assimilation versus CO_2 concentration were recorded from five apparently healthy leaves per tree species and leaf position. CF was measured in parallel. *A. unedo* leaves were sufficiently large to cover the leaf cuvette (2 cm^2), whereas sunlit leaves of *Q. ilex* were in some cases too small, and the area of the leaves had to be adjusted after the measurements. For the leaves of *P. halepensis*, we positioned a layer of needles (appr. 10-15) on the leaf cuvette, avoiding gaps and overlays. The putty-like adhesive 'Blu-tack' (Bostik SA, La Plaine St Denis, France) was also used to seal the gaskets and to keep the needles in position.

5.2.5 Preparation and acclimation

Prior to recording the response curves, the temperature of the clamped leaves (T_{Leaf}) was adjusted to 25 °C, and the flow of ambient CO_2 in the leaf chamber (C_a) was set to 400 μmol CO_2 m^{-2} s^{-1} (controlled with a CO_2 mixer). The leaves were dark-adapted for 15-20 min before the measurements, and the data were logged when the GE-derived parameters such as stomatal conductance (g_s), stomatal internal CO_2 concentration (C_i) and mitochondrial respiration in darkness (R_n) had stabilised. For our purposes, dark-adaption did not necessarily mean strict prolonged darkness but referred to a sufficiently low level of ambient background light that did not cause an accumulation of reduced photosystem II (PSII) acceptors, which could be detected as an increase in fluorescence. The leaves were also pre-darkened with special leaf clips or a dark cloth to save time. The chamber light was then turned on at a saturating quantum flux density of 1000 μmol photons m^{-2} s^{-1} (20% blue LED, 80% red LED). The relatively high percentage of blue light stimulated the stomata to open (Farquhar & Sharkey, 1982; Niinemets *et al.*, 2005; Kang *et al.*, 2009). The relative humidity was maintained at 50% (±10%), and the air flow was maintained at 500 μmol s^{-1}. The above conditions were maintained for approximately 20-30 min until the net rate of carbon assimilation (A_{net}), g_s, and C_i of the leaf stabilised.

The GE-derived parameters A_{net}, g_s, and C_i likely require less time to stabilize, especially in healthy and unstressed leaves, but this minimum time range was necessary for the CF-derived parameters to ensure accurate measurement of the efficiency of harvesting light energy by maximal oxidation and therefore open PSII reaction centres under ambient conditions of CO_2

and saturating light, which can be monitored by observing the stability of steady-state fluorescence (F_s). If this stability is not achieved, the effective quantum yield of PSII (Φ_{PSII}) and subsequent calculations of important parameters such as the rate of electron transport based on the CF measurement (J_{CF}) could be underestimated. After all parameters had stabilised, the steady-state GE-derived parameters and several CF-derived parameters in the light-adapted state were recorded simultaneously. F_s followed shortly afterwards by the maximum fluorescence yield in the light-adapted state (F_m') were logged by the emission of a pulse of white light at 10000 mmol m^{-2} s^{-1} to close all PSII reaction centres, followed by a so-called 'dark pulse' for measuring the minimal fluorescence (F_o') of a light-adapted leaf that has been momentarily darkened. The measurement of CO_2 began after the completion of the preparation and acclimation, which required approximately 30 min in unstressed leaves and up to 2 h in stressed leaves.

5.2.6 CO_2 experiments

The CO_2-response curves were recorded at a T_{Leaf} of 25 °C and a quantum flux density of 1000 μmol photons $m^{-2}s^{-1}$. The values of C_a used to generate the response curves were 400 → 300 → 200 → 150 → 100 → 50 → 400 → 400 → 600 → 800 → 1200 → 2000 (in μmol CO_2 m^{-2} s^{-1}). The minimum and maximum times for stabilising A_{net}, g_s, and C_i for each log were set to 4 and 6 min, respectively.

5.2.7 Light experiments

Light response curves (A/PPFD) were generated at a C_a of 400 μmol CO_2 m^{-2} s^{-1} by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source. To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence: 2500 → 2000 → 1500 → 1000 → 800 → 600 → 500 → 400 → 300 → 200 → 150 → 125→ 100 → 75 → 50 → 40 → 30 → 20 → 10 → 5 → 0 (in μmol photons m^{-2} s^{-1}). The minimum and maximum times between each light level for the generation of the A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light during an A/PPFD curve led to a drop in T_{Leaf} as the light decreased. The rapid changes in the light levels prevented the adjustment of T_{Leaf} while guaranteeing stable air and water fluxes and avoiding noisy measurements of C_i and g_s. We thus decided to maintain a stable Peltier-block temperature (T_{block}) in the leaf cuvette. Hence, T_{block} was first adjusted so that T_{leaf} was 25 °C at the beginning of the A/PPFD curve and then kept stable throughout the experiment. T_{Leaf} had dropped by approximately 1-3 °C by the completion of the A/PPFD curve. The calculation of the parameters Fv/Fm, NPQ, qp, and temperature functions, in supplementary material.

5.2.8 Calculation of CF-derived parameters

The maximum efficiency of PSII was calculated by:

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

where F_v is the variable fluorescence of a dark-adapted sample, F_m is the maximal fluorescence measured after a saturating light pulse, and F_o is the minimal fluorescence measured under darkness. These parameters were obtained from dark-adapted leaves with closed PSII reaction centres as described in the previous sections. The F_v/F_m ratio describes the fraction of photochemically absorbed photons under dark conditions. Typical values range between 0.75 and 0.85, depending on age, health, and preconditioning. The F_v/F_m ratio provides information about the maximum or optimum quantum yield and serves as indicator of stress in the photosystems (Buschmann, 2007). Ratios below 0.80 are indicative of induced photoprotection and sustained energy dissipation (Maxwell & Johnson, 2000; Verhoeven, 2014), whereas leaves with ratios below 0.74 are considered to be below the recovery threshold (Björkman & Demmig, 1987). The effective quantum yield of PSII was estimated by:

$$\Phi_{\mathrm{PSII}} = \frac{(\mathrm{F_m}' - \mathrm{F_s})}{\mathrm{F_m}'} \tag{2}$$

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

$$J_{CF} = \varepsilon * \Phi_{\mathrm{PSII}} * \alpha_{\mathrm{L}} \tag{3}$$

where ε is a scaling factor for the partitioning of intercepted light between photosystems I and II. We assumed that light was equally distributed between both photosystems ($\varepsilon = 0.5$) (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2005). α_{L} is the foliar absorbance determined in separate measurements of foliar reflectance and transmittance. The following values of α_{L} were determined: 0.932 for *Q. ilex* and 0.912 for *P. halepensis*, with no differences between sunlit and shaded leaves of these two species, and 0.935 for sunlit leaves of *A. unedo*, and 0.917 for shaded leaves of *A. unedo*. For the determination of these leaf absorptances (α_{L}), foliar reflectance and transmittance were measured at midday in August 2012 using a spectroradiometer UniSpec Spectral Analysis System (PP Systems, Haverhill, MA, USA). The value of J_{CF} at a CO_2 concentration of 400 μmol CO_2 m^{-2} s^{-1} and a PPFD of 1000 μmol photons m^{-2} s^{-1} was termed ambient photosynthetic electron transport (J_{amb}). Its relationship with the net assimilation rate (J_{amb}/A_{net}) was used for the analyses of alternative electron sinks beside carbon metabolism.

5.2.9 Estimation of light respiration and calculation of the effective quantum yield of CO_2 (Φ_{CO2})

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

The effective quantum yield of CO_2 (Φ_{CO2}, unitless) can be calculated using the estimated α_L, R_d, together with A_{net} and PPDF as follows

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

5.2.10 The Farquhar, von Caemmerer, and Berry (1980) photosynthesis model (FvCB)

The FvCB photosynthesis model was employed on the assumption that foliar carbon assimilation was limited either by Rubisco activity (A_c) or by ribulose-1,5-bisphosphate (RuBP) regeneration (A_j) and was driven by light, temperature, and CO_2. The model was further complemented with a third limitation: the photosynthetic rate limited by triose-phosphate use (A_p) (Sharkey, 1985). A_{net} can then be determined by the minimum of these three potential rates from an A/C_c curve:

$$A_{net} = min\{A_c, A_j, A_p,\} \tag{5}$$

where

$$A_c = V_{cmax} * \left[\frac{C_c - \Gamma^*}{C_c + K_c\left(1 + \frac{O}{K_O}\right)}\right] - R_d \tag{6}$$

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

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

where J is the rate of electron transport. The denominator of the above equation represents the stoichiometry of the number of electrons required to regenerate ATP and NADP; we have used four for C_c and eight for Γ^* (Flexas *et al.*, 2012). We assumed that J becomes J_{max} under light and CO_2 saturation when the maximum possible rate of electron transport is theoretically achieved, however acknowledging that the real J_{max} may be somewhat underestimated (Buckley & Diaz-Espejo, 2015).

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

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

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

These three estimated parameters ($V_{\mathrm{c,max}}$, J_{max}, and TPU) define the biochemical capacity to drive the photosynthetic assimilation of CO_2 but are defined here as the photosynthetic potential (Niinemets *et al.*, 2006). The term photosynthetic capacity is here dismissed, despite its frequent use in the literature, to avoid confusion with studies that have used this term for the maximum rate of assimilation under saturating light conditions (e.g. Bertolli and Souza, 2013).

5.2.11 Curve fitting

The procedure for fitting the curves to estimate the photosynthetic parameters $V_{\mathrm{c,max}}$, J_{max}, and TPU applied the least square fit method using the SOLVER estimator tool in Excel. In this procedure, the squared errors of the observed points on the A/C_c curve and the modelled points of Eq.(s) 6, 7, and 8 were calculated and summed. Prior to the fitting procedure, the user must assess the limiting factors, i.e. which points are allocated to which Eq. (6 or 7 or 8). The initial slope of the A/C_c curve is attributed to non-saturating CO_2 conditions when Rubisco activity limits A_{net} (Eq. 6), while the slope of the curve is smoothed at higher CO_2 conditions (usually > 35 Pa), representing the limitation of the regeneration of ribulose-1,5-biphosphate (RuPb) (and hence light is a limiting factor) (Eq. 7). The transition zone (approximately at 25-35 Pa of C_i), however, is a grey zone where one point can be attributed to either one or another limitation.

These points can also introduce noise in the estimations in cases of doubt and are best discarded. Moreover, unusual points with evidence of an error during the measurements were not included in the curve-fitting procedure. At very high CO_2 concentrations, the A/C_c curve plateaus or even decreases slightly. In this case, these points can be attributed to the limitation of triose-phosphate use (Eq. 8). The CO_2 response curves, however, rarely exhibit such a plateau or decrease at high CO_2 concentrations when working on a C_c rather than a C_i basis, so TPU could seldom be estimated in our study. Finally, when attributing all observed points to one or another limitation, we could then estimate the values of $V_{c,max}$ and J_{max} (and possibly TPU) with the SOLVER Excel tool, which iteratively changes the three parameters to minimise the sum of squares of deviation from the observation.

5.2.12 Correction for diffusion leakage

Large gradients between the ambient air and the CO2 concentrations inside the chamber are created during the generation of a carbon-response curve. This leakage is particularly important at the high and low ends of the carbon-response curve when a large CO2-concentration gradient exists between the leaf chamber and the surrounding ambient concentration. Based on the findings by Flexas et al. (2007a), we corrected Anet by subtracting the diffusion leakage for each step of the A/Cc curve obtained from separate response curves with leaves thermally killed in hot water.

5.2.13 Statistical Analyses

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

5.4 Results

5.3.1 Environmental Variables

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

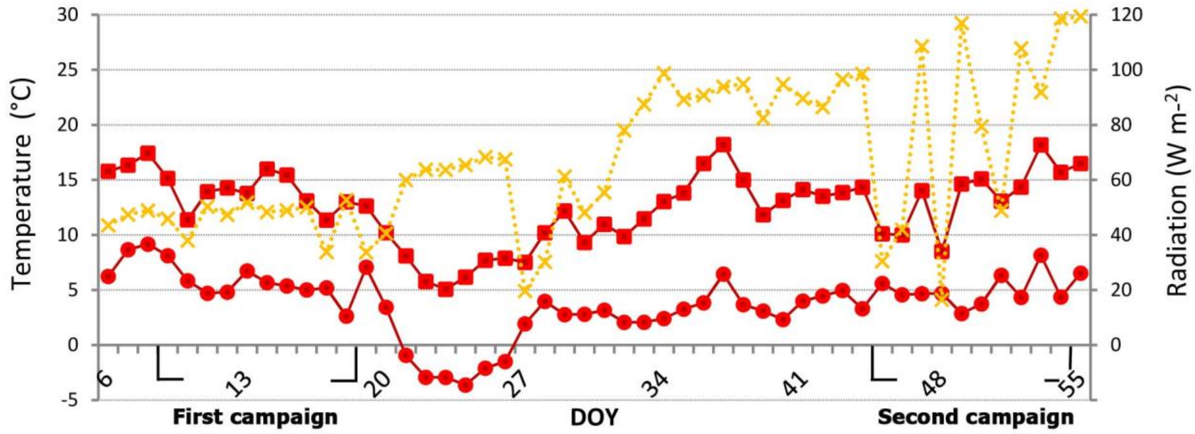

Fig. 5.1 | Maximum and minimum temperatures on the primary y-axes (in red squares and circles, respectively) and radiation (in yellow crosses) on the secondary y-axes are presented for the mild and frost winter period for the day of the year (DOY) in January and February 2012.

5.3.2 Photosynthetic potential

Of the three photosynthetic parameters describing the photosynthetic potential, $V_{c,max}$ and J_{max}, and TPU, only the first two could be satisfactorily estimated from the A/C_c-response curves. The leaves were only occasionally limited by TPU (6 out of 42), despite the excessive CO_2 concentrations in the higher section of the CO_2-response curve. TPU was therefore discarded from further analysis. $V_{c,max}$ and J_{max} were highest in *Q. ilex* but more importantly also decreased most strongly after the period of frost by nearly 50% ($P \leq 0.05$; Fig. 2). The photosynthetic potential of *P. halepensis* was affected the least, reflected by moderate decreases in $V_{c,max}$ and J_{max} (16% and 19%), which were not significant. $V_{c,max}$ and J_{max} were lowest in *A. unedo* during the mild winter period and decreased by approximately 33% after the period of frost. This decrease, however, was not significant due to a large standard error.

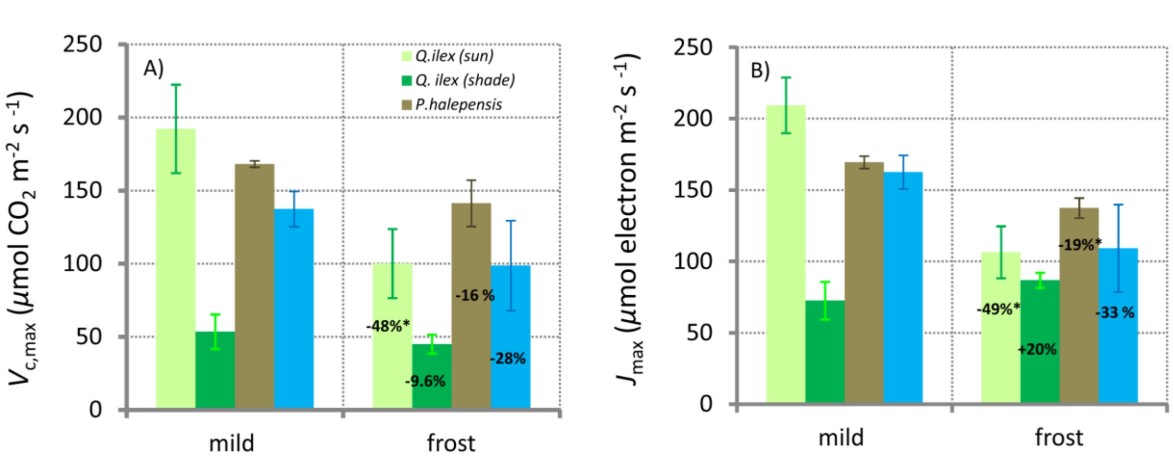

Fig. 5.2 | Bar plot of the effect of a sudden period of frost following a mild winter period in 2012 on A) the maximum velocity of carboxylation ($V_{c,max}$) and B) the maximum rate of electron transport (J_{max}) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

5.3.3 GE-derived parameters under ambient conditions

The period of frost had a strong effect on several GE-derived parameters in *Q. ilex* leaves. The cold temperatures decreased R_n in *Q.ilex* leaves, but the effect was much weaker than for R_d and was not significant (Fig. 3). These parameters responded very weakly to the cold and frost in the leaves of *A. unedo* and *P. halepensis.* A_{net} and Φ_{CO2} were also reduced in *Q. ilex* leaves by approximately 50%. This was significant for the A_{net} (Fig. 4A) and low significant for Φ_{CO2} (Fig. 4B). Further differences were only significant for Φ_{CO2} in *P. halepensis* leaves being reduced by 12 % ($P \leq 0.05$). The CO_2 conductance was more strongly reduced in g_m than in g_s for *Q. ilex* and *A. unedo* leaves which was only significant for the former whereas these parameters seemed unaffected in *P. halepensis* leaves (Fig. 5A and 5B). As a consequence, we observed a tendency of a C_i- increase in parallel with a C_c- decrease in *Q. ilex* and *A. unedo* leaves due to a lower CO_2 uptake in carbon metabolism, but not in *P. halepensis* (Fig. 6A and 6B). The differences observed were not significant ($P \leq 0.05$).

5.3.4 CF-derived parameters under ambient conditions

The GE-derived parameters enabled us to study the immediate responses, but several CF-derived parameters allowed us to determine in more depth the physiological changes in parts of the light-harvesting apparatus, namely PSII. F_v/F_m estimates the maximum quantum yield of PSII and serves as a stress indicator (Fig. 7B). *A. unedo* leaves were most strongly affected by the period of frost, followed by *Q .ilex* leaves, whereas *P. halepensis* leaves were only marginally affected. The changes were not statistically significant in the latter two species ($P \leq$ 0.05). Φ_{PSII} tended to decrease in all species but most strongly in *Q. ilex* leaves (42 %), however insignifcantly (Fig. 7A). NPQ responded very differently in the three species. NPQ did not change much between the two sampling periods in the leaves of *P. halepensis* (6%) but decreased significantly by 25% ($0.05 \leq P \leq 0.1$) in *A. unedo* leaves and tended to increase in *Q. ilex* leaves by 31% ($P \geq 0.05$), however insignificantly (Fig. 8).

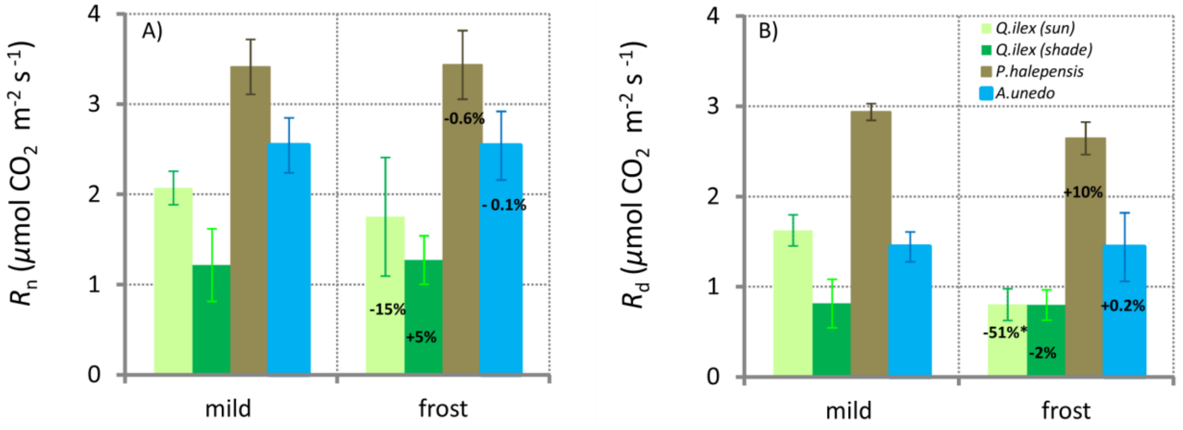

Fig. 5.3 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) nighttime respiration (R_n) and B) daytime respiration (R_d) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

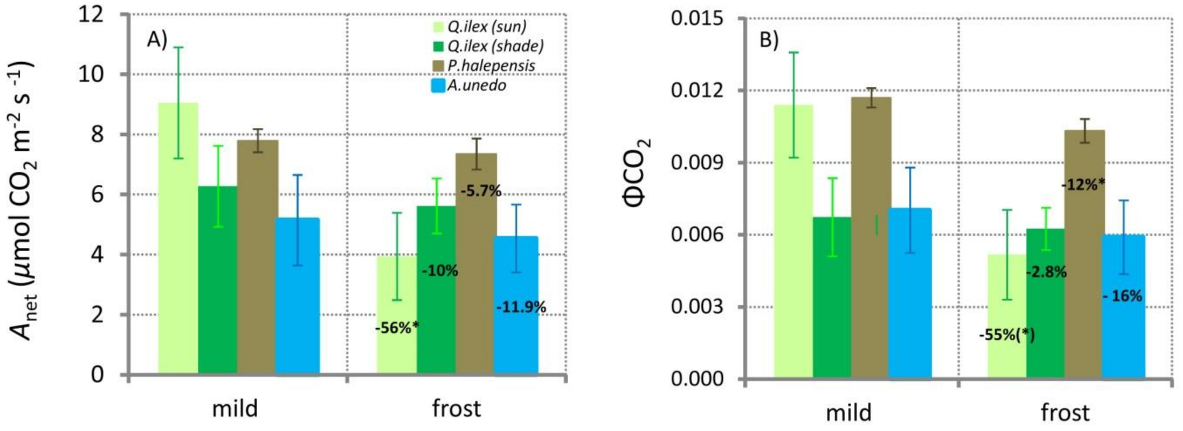

Fig. 5.4 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) net assimilation (A_{net}) and B) the effective quantum yield of net CO_2 assimilation (ΦCO_2) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

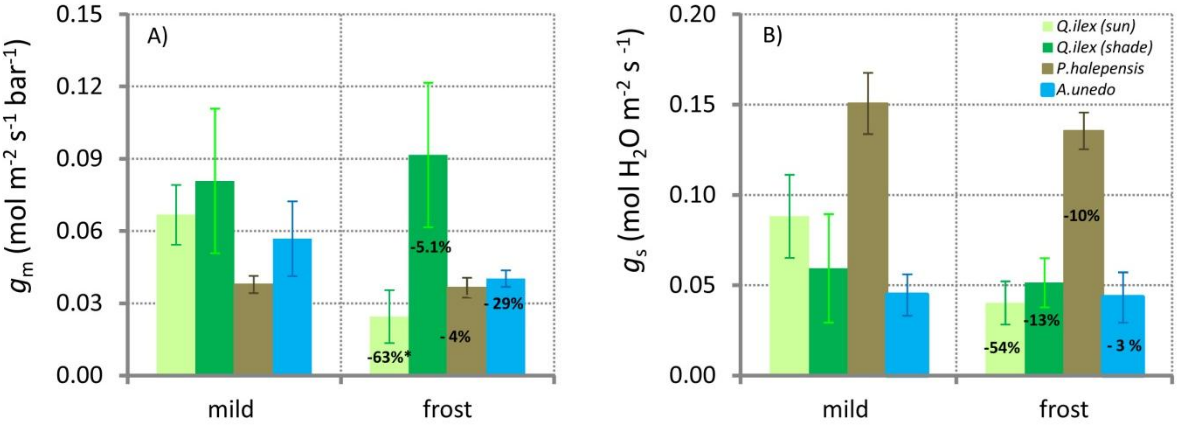

Fig. 5.5 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) mesophyllic conductance (g_m) and B) stomatal conductance (g_s) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

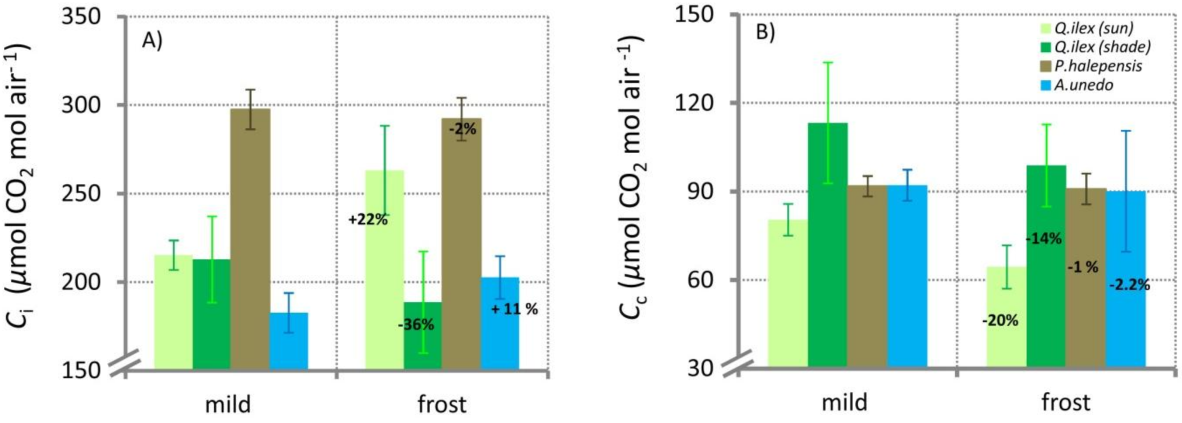

Fig. 5.6 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) the stomatal internal CO$_2$ concentration (C_i) and B) the chloroplastic CO$_2$ concentration (C_c) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

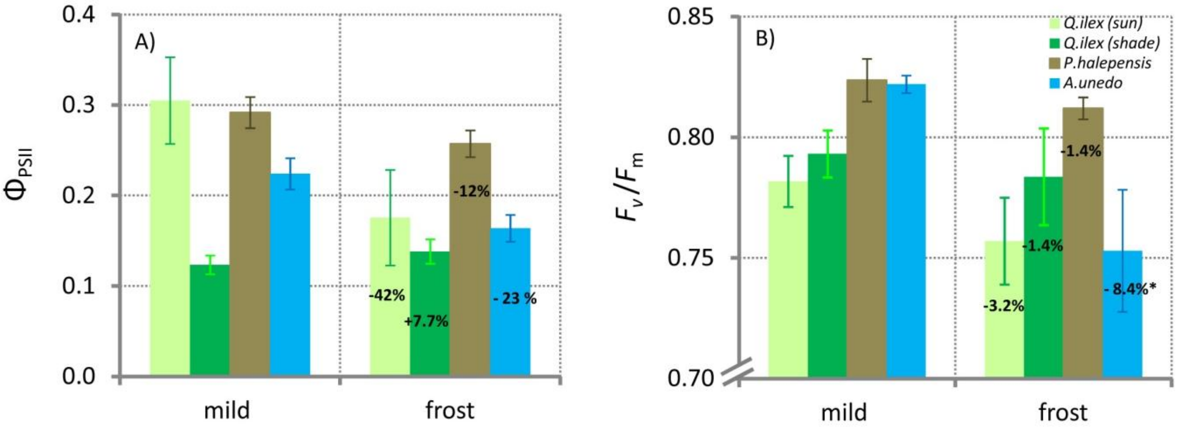

Fig. 5.7 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) the effective quantum yield of photosystem II (Φ_{PSII}) and B) the maximum efficiency of photosystem II (F_v/F_m) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

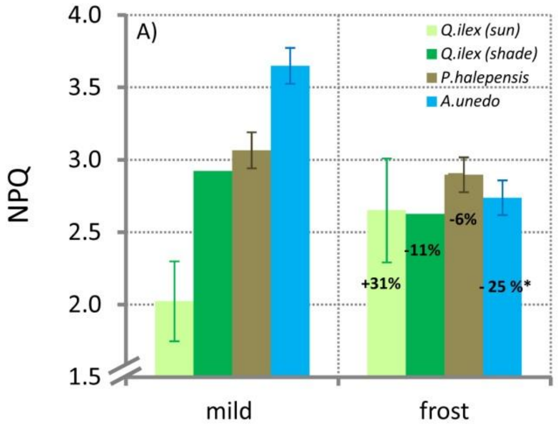

Fig. 5.8 | Bar plot of the effect of a sudden period of frost following a mild winter period on A) non-photochemical quenching (NPQ) and B) photochemical quenching (qp) in sunlit leaves of Q. *ilex* (light green bar), in shaded leaves of Q. *ilex* (dark green bar), *P. halepensis* (beige bar), and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in brackets ($0.05 \leq P \leq 0.1$).

5.3.5 Relationships of foliar photosynthetic variables

The covariance of several relationships of the foliar photosynthetic variables were analysed in an ANCOVA to test for differences in the slopes and intercepts in these relationships. The ANCOVA for the relationship between $V_{\mathrm{c,max}}$ and J_{max} in *Q. ilex* leaves indicated a highly significant ($P \leq 0.01$) reduction in the slope and also intercept showing a similar strong effect on J_{max} than on $V_{\mathrm{c,max}}$ due to the change in weather (Fig. 9A and Table 2). In P. halepensis, the slope was significantly and the intercept marginal significantly reduced (Fig. 9B and Table 2). This shows a comparatively stronger effect on $V_{\mathrm{c,max}}$ than on J_{max} by the cold period. The sunlit leaves of A. unedo and the shaded leaves of Q. ilex did not show any significant changes in the relationship of $V_{\mathrm{c,max}}$ and J_{max} (Fig. 9A, 9C and Table 2). The relationship between the rate of electron transport at ambient conditions derived from CF and the CO_2 assimilation at ambient CO_2 concentrations ($J_{\mathrm{amb}}/A_{\mathrm{net}}$) was similar in all tree species (Fig. 10A,B,C and Table 2). The slopes were higher in response to the stress imposed by the low temperatures but were not significant. When all species were combined the change of the slope was marginally significant, indicating a possible increased alternative electron sink other than carbon metabolism (Table 2).

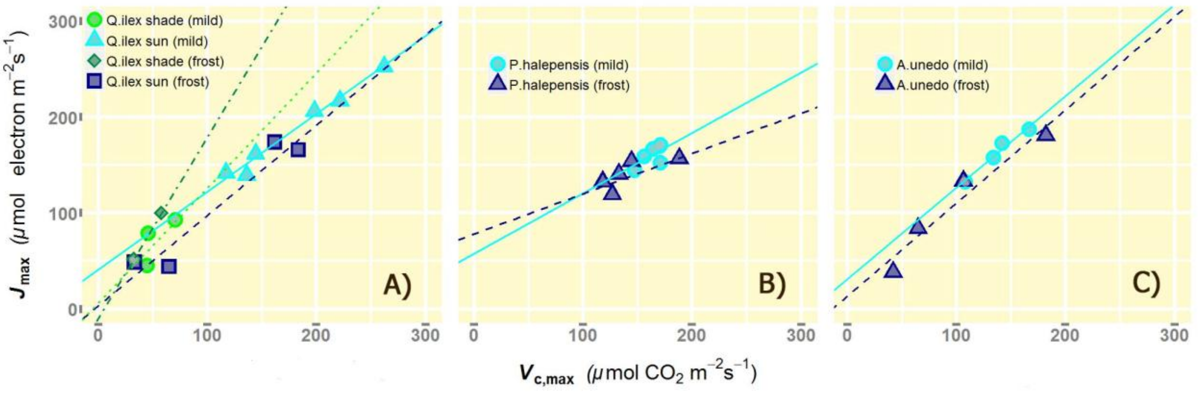

Fig. 5.9. Relationship between the maximum velocity of carboxylation ($V_{\mathrm{c,max}}$) and the maximum rate of electron transport (J_{max}) in *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C), leaves. Leaves measured under mild conditions are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively. Leaves measured after the period of frost are indicated by green diamonds and blue squares in shaded and sunlit locations, respectively.

5.3.6 Role of leaf position

Under mild conditions, the leaves of *Q. ilex* showed the most strongly pronounced differences in the leaf position (data of *P. halepensis* Mill. & *A. unedo* L. not shown). Leaves of *Q. ilex* growing under high irradiances had a more active carbon metabolism (A_{net}, R_d, R_n, and Φ_{CO2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) in all tree species. As described in *Material and Methods* the effect of the leaf position after the sudden cold period was only studied for *Q. ilex*. After the sudden frost period, the photosynthetic potential was much higher in sunlit than in shaded leaves of *Q. ilex*, with both J_{max} and $V_{c,max}$ being highly significant (Fig. 2 and Table 1). These differences disappeared after the cold period, because J_{max} and $V_{c,max}$ in the shaded leaves remained unaffected by the frost. F_v/F_m was generally higher in the shaded leaves, but not significantly ($P \leq 0.05$) (Fig. 8 and Table 1). The photosynthetic parameters under ambient conditions, 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 Table 1). Although not significant, the effects of the cold period on these parameters were stronger in the sunlit leaves. In comparison to these parameters, the leaf position had more pronounced effects on R_n and R_d (Fig. 3 and Table 1). The response of respiration to winter stress, however, differed depending on the location of the leaves. R_n maintained the same balance between sunlit and shaded leaves before and after the cold period, but R_d decreased comparatively more in sunlit leaves due to the period of frost. This pattern was also reflected in Φ_{CO2} (Fig. 4B and Table 1) and in the CF-derived parameters Φ_{PSII} and NPQ, (Fig. 7A, 8 and Table 1) indicating a stronger effect on the photochemical machinery of sunlit leaves than on shaded leaves. Shaded leaves also exhibited a lower J_{amb}/A_{net} ratio, but the ratio increased equally in both leaf positions after the cold period, indicating a similar behaviour of dissipating energy by alternative electron sinks (Fig. 10A and Table 1).

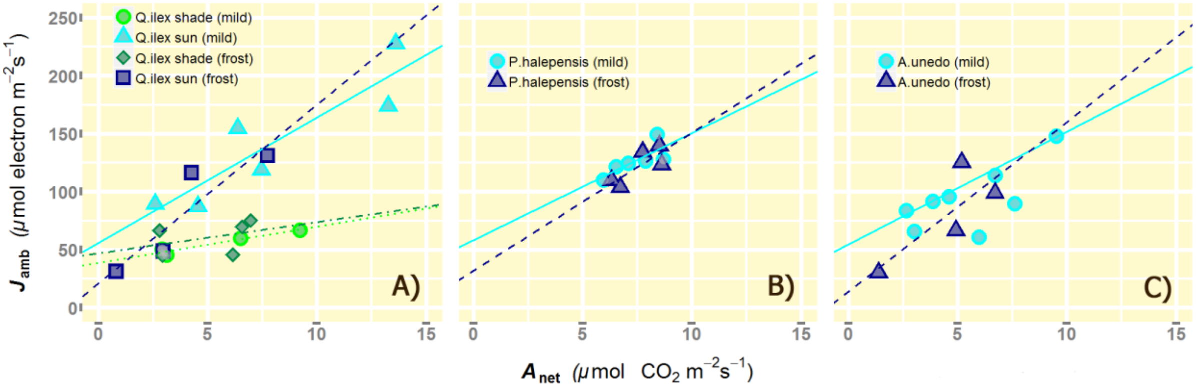

Fig. 5.10 | Relationship between the rate electron transport from chlorophyllic fluorescence (J_{amb}) and net assimilation (A_{net}) at ambient CO_2 concentrations and saturating light (A_{net}) in *Q. ilex* (A), *P. halepensis* (B), *A. unedo* (C), leaves. Leaves measured under mild conditions are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively. Leaves measured after the period of frost are indicated by green diamonds and blue squares in shaded and sunlit locations, respectively

Table 5.1 | *P* values of Student's *t*-tests for the differences between sunlit and shaded leaves of *Q. ilex*.

	Both periods	Mild period	Frost period
$V_{c,max}$	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

Table 5.2 | 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} and $V_{c,max}$

tree species	*Q. ilex*			*Q. ilex*			*P. halepensis*			*A. unedo*			*all species*		
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 = 0.81 x + 41.6	0.97	2E-04	y = 1.2 x + 6.1	0.48	0.193	y = 115.9 x + 148.8	0.04	0.32	y = 0.954 x + 31.5	0.95	0.017	y = 50.2 x + 0.77	0.94	1.4E-07
frost	y = 0.94 x + 3.6	0.89	0.035	y = 1.89 x - 9.19			y = 971 x + 9.9	0.53	0.1	y = 0.97 x + 13.7	0.91	0.029	y = 10.5 x + 0.93	0.90	7.2E-05
p (slope)	5.76E-02			0.83			0.058			0.69			0.072		
p (intercept)	8.91E-09			0.3			0.022			0.28			0.008		

Regression analyses of J_{amb} and A_{net}

tree species	*Q. ilex*			*Q. ilex*			*P. halepensis*			*A. unedo*			*all species*		
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 = 3.1 x + 39	0.91	0.029	y = 9.22 x + 58.3	0.51	0.068	y = 9.7 x + 54.9	0.96	0.005	y = 10.9 x + 51.9	0.84	7.1E-06
frost	y = 15.4 x + 21.1	0.73	0.093	y = 2.7 x + 46.8	-0.13	0.52	y = 11.9 x + 31.9	0.52	0.105	y = 14.6 x + 14.2	0.46	0.200	y = 13.5 x + 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		

5.5 Discussion

5.4.1 Winter in the Mediterranean region

Mediterranean-type ecosystems are exposed to stress from summer droughts but also from low temperatures in winter (Mitrakos, 1980). Less attention, however, has been paid to the degree and extent as well as the wide variation among years and regions of these stress periods, in response to which Mediterranean evergreen species have developed a dynamic photoprotective ability in order to withstand these stressors (Kyparissis *et al.*, 2000; Martínez-Ferri *et al.*, 2004). Despite the occurrence of lower temperatures than in spring conditions, in winter the photosynthetic potential recovered once the leaves became acclimated to the new conditions (Hurry *et al.*, 2000; Dolman *et al.*, 2002). This is important for the plants overall performance because the photosynthetic exploitation of favourable conditions in winter is crucial for achieving a positive carbon balance in Mediterranean evergreen tree species (García-Plazaola *et al.*, 1999b; Martínez-Ferri *et al.*, 2004). We showed how a long lasting comfortable winter period without frost lead to notably high photosynthetic potentials and carbon assimilation in winter being equal to or partly even exceeding spring values (Sperlich et al, unpublished data). As a result, increased winter temperatures influenced phenological responses, advanced winter cambium activation, spring bud burst and leaf unfolding which has been reported in an increasing number of studies (Peñuelas & Filella, 2001). These observations were also reflected in the high sap flow per tree (J_t), ranging for all tree species on average between 5 and 10 kg d^{-1} during the mild winter period (Sánchez et al., unpublished results). Whereas sudden frosts have often been attributed to higher altitudes of the Mediterranean region (Blumler, 1991; Tretiach *et al.*, 1997), we showed that it can also be an important factor for plant growth and distribution in other areas such as the sub-humid Mediterranean climate of our study site (Garcia-Plazaola *et al.*, 2003a). At night when frosts are more likely to occur, we observed the lowest temperatures whereas at daytime the temperatures were often above zero degrees. However, as we showed, not only cool daytime but also cool nighttime temperatures or frosts can affect subsequent daytime photosynthesis and induce photoprotective processes (see also Flexas et al., 1999). In our study, the sudden occurring low temperatures affected strongly the photosynthetic apparatus, although the responses were highly species specific. We will elucidate the physiological mechanism in the following.

5.4.2 PSII – primary target of stress induced by low temperatures

Typically in winter there is an imbalance between light energy absorbed in photochemistry and light energy used in metabolism. This is shown in our data by increased thermal energy dissipation (NPQ) and reduced PSII efficiency (Φ_{PSII}) in order to reduce the harmful effects of excess energy reflecting an inactivation and damage of PSII reaction centres, more precisely, the reaction-centre protein D1 (Demmig-Adams & Adams, 1992; Aro *et al.*, 1993; Mulo *et al.*, 2012). More precise information about the underlying processes that have altered this efficiency is provided by the F_v/F_m ratio. Chronic changes occurring in the F_v/F_m ratio can be related to a cascade of processes which are induced to protect the photosynthetic apparatus including i) re-organisation of the thylakoid membrane, ii) closure of reaction centres, iii) and/or reduced antennal size (Huner *et al.*, 1998; Maxwell & Johnson, 2000; Ensminger *et al.*, 2012; Verhoeven, 2014). The small changes in the F_v/F_m ratio observed in the leaves of *Q. ilex* and *P. halepensis* reflected photoprotective responses without any photodamage. The significantly decline of F_v/F_m in *A. unedo*, however, indicated strong chronic photoinhibition and is an indication of severe photodamage (Martínez-Ferri *et al.*, 2004). We conclude that *A. unedo* suffered most notably from the low temperatures whereas *Q. ilex* and *P. halepensis* were equipped with a good photoprotective capacity able to keep the photosynthetic apparatus intact (Öquist & Huner, 2003). *Q. ilex* showed the most dynamic responses, negating the harmful excitation stress by lowering the photochemical operating efficiency (Φ_{PSII}) and increasing the use of alternative thermal-energy pathways (NPQ). This photoprotective capability represented by a higher NPQ is usually linked to the xanthophyll cycle that responds to environmental factors such as temperature, water deficit, and nutrient availability (Demmig-Adams and Adams, 1996; García-Plazaola et al., 1997). Inter-conversions of the cycle and pool sizes occur following the need to dissipate excess excitation energy in response to summer drought (García-Plazaola *et al.*, 1997; Munné-Bosch & Peñuelas, 2004), but also to winter stress (Kyparissis *et al.*, 2000; Oliveira & Penuelas, 2001; Garcia-Plazaola *et al.*, 2003a; Corcuera *et al.*, 2004). The implicit interpretation of being equipped with a high capacity of photoprotection when NPQ increases was recently questioned by Lambrev et al. (2012). This study reported that quenching and photoprotection were not necessarily linearly related and stated that several possibilities of photoprotective responses other than NPQ of CF existed, such as antennal detachment that could possibly vary with species and growth conditions. The highly dynamic and photoprotective capability of *Q. ilex* leaves, however, was also demonstrated by several other photosynthetic parameters such as $V_{c,max}$, J_{max}, A_{net}, Φ_{CO2}, and R_d, which confirmed this trend and were in accord with the findings by Corcuera *et al.* (2004). Despite reports of several mechanisms of resistance to drought stress in *A. unedo*, including increased levels of zeaxanthin that indicates an enhanced thermal dissipation of excess excitation energy in periods of summer stress (Munné-Bosch & Peñuelas, 2004), we found that *A. unedo* leaves had a lower capacity of photoprotection in response to induced over-excitation of the photosystems by winter stress.

5.4.3 High photosynthetic potentials and strong effects of low temperatures

$V_{c,max}$ and J_{max} were strongly correlated (Wullschleger, 1993), being regulated in a coordinated manner above all in *Q. ilex*. Interestingly, the ANCOVAs indicated that J_{max} decreased more strongly than did $V_{c,max}$. This is because the above described photoprotective adjustments lead to a lower energy-use efficiency in the reaction centres and consequently also to a downregulation of the photosynthetic electron transport J_{max}. The larger decrease of J_{max} relative to $V_{c,max}$ indicated that low temperature stress became manifest first in a hampered pathway of photochemical energy, because PSII complexes are primarily affected by light-induced damage (Maxwell & Johnson, 2000; Taz & Zeiger, 2010; Vass, 2012). Hence, the limitations of the photosynthetic rate by RuBP regeneration are stronger affected by frost and cold induced stress than those by RuBP carboxylation. The relative amounts of photosynthetic proteins can probably explain the differences observed in the $J_{max}/V_{c,max}$ ratio (Hikosaka *et al.*, 1999; Onoda *et al.*, 2005). The physiological responses were highly species-specific. *Q. ilex* leaves responded with significant decreases (approximately 50%) in their photosynthetic potentials (both $V_{c,max}$ and J_{max}). In contrast, $V_{c,max}$ and J_{max} decreased in *P. halepensis* leaves by only 16 and 19%, respectively, and in *A. unedo* leaves by approximately 30% (for both parameters).

5.4.4 Inhibition of carbohydrate metabolism

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

5.4.5 Leaf position specific responses to abiotic stress in winter

It is well know that leaves growing under high irradiances have a more active carbon metabolism (A_{net}, R_{d}, R_{n}, and Φ_{CO2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{\mathrm{c,max}}$) (Taz & Zeiger, 2010). Hereby, *Q. ilex* showed the most strongly pronounced differences between sunlit and shaded leaves. Plants develop leaves with a highly specialised anatomy and morphology for the absorption of the prevailing light in their local environments resulting generally in smaller but also thicker sunlit leaves (Kull & Niinemets, 1993; Terashima & Hikosaka, 1995). Nevertheless, the higher carbon metabolism and photochemical activity of sunlit leaves decreased strongly, partly below the level of shaded leaves, whereas shaded leaves showed little sign of any downregulation but maintained a relatively stable effective quantum yield of CO_2 assimilation in both periods. Furthermore, the photosystems showed no sign of photodamage and generally maintained a higher maximum efficiency than did sunlit leaves. We concluded that foliar-level physiology during winter was better protected in the shaded crown of *Q. ilex* unexposed to the dramatic changes in radiation in the outer canopy, confirming the results by Valladares et al. (2008). We also concluded that *Q. ilex* is a highly dynamic species able to rapidly change its metabolism on the antioxidant and photoprotective level in dependence to its leaf position (García-Plazaola *et al.*, 1997, 1999a; Martínez-Ferri *et al.*, 2004). We show that the foliar plasticity in morphology and anatomy of *Q. ilex* (Valladares *et al.*, 2000; Bussotti *et al.*, 2002) can also be attributed to its biochemical metabolism. We stress that the solar environment of the leaves is a crucial factor when assessing tree performance, especially when comparing tree species in a competitive context.

5.4.6 Ecological context

Q. *ilex* had the most drastic photoprotective response to frost and cool temperatures, whereas *P. halepensis* exhibited a homeostatic behaviour with a very active carbon assimilatory and respiratory metabolism in both periods. *A. unedo* was intermediate, with large decreases in the parameters of carbon metabolism but also a high variability in its response to frost. *A. unedo* also had the lowest photoprotective capability, which might be explained by previous characterisations to be semi-deciduous to drought being at the borderline to evergreen sclerophyllous species (Gratani & Ghia, 2002a,b). Moreover, *A. unedo* occurs naturally most commonly as a shrub and is less frequently found in the forest canopy of mixed forests growing up to 8-10 m tall as in our study site (Beyschlag *et al.*, 1986; Reichstein *et al.*, 2002). Investments in leaves are thus lower and leaf longevity shorter. Leaves of *A. unedo* are more rapidly replaced relative to more sclerophyllic leaves such as those of *Q. ilex*. We postulated that *A. unedo*, considered a relict of the humid-subtropical Tertiary tree flora, was more sensitive to winter stress, which is consistent with its presence mostly in the western Mediterranean basin and its frequent occurrence in coastal zones where humidity and temperature are the main factors determining its geographical distribution (Gratani and Ghia, 2002a and references therein). Our results suggested that *Q. ilex* could greatly benefit from favourable winter conditions exhibiting a high photosynthetic potential and carbon metabolism. Angiosperms are known to make efficiently use of favourable winter periods to recover depleted carbon reserves and embolism induced loss of hydraulic capacity (Carnicer et al., 2013 and references therein). When these relatively favourable conditions changed, *Q. ilex* quickly re-adjusted the photosynthetic machinery to the prevailing conditions, as indicated by the largest decreases in photosynthetic potential and carbon metabolism. Some researchers have proposed the lutein-epoxy cycle in photoprotection of *Quercus* as a mechanism to maintain sustained energy dissipation (Garcia-Plazaola *et al.*, 2003b), which could help to account for the higher tolerance to low temperatures in *Q. ilex* relative to other co-occurring Mediterranean trees or shrubs (Ogaya & Peñuelas, 2003, 2007). *P halepensis* did not suffer a pronounced chronic photoinhibition, confirming the results by Martínez-Ferri *et al.* (2004). Despite a pronounced downregulation of photosynthetic electron transport and an increase in alternative electron sinks, the light-saturated ambient photosynthesis and stomatal conductance remained surprisingly high and constant. *P. halepensis* thus exhibited a successful refinement of photosynthetic electron flow and possibly a successful repair of protein D1 in the PSII reaction centre. The strong downregulation in *Q. ilex* and the homogenous response of *P. halepensis* were possibly due to distinct, previously described strategies. *Q. ilex* has been characterised as a photoinhibition-avoiding species and *P. halepensis* as a photoinhibition-tolerant species (Martinez-Ferri *et al.*, 2000). We have extended this categorisation for *A. unedo*, a less photoinhibition-tolerant tree species, which favoured carbon metabolic processes at the cost of chronic photoinhibition and photodamage. This strategy is similar to those in other semi-deciduous shrubs (Oliveira & Penuelas, 2001; Oliveira & Peñuelas, 2004). The physiological responses of *Q. ilex*, a slowly growing late-successional species, to environmental stressors are highly plastic (Zavala *et al.*, 2000) due to its vegetative activity in a wide range of temperatures and high stomatal control in stressful conditions (Savé *et al.*, 1999; Gratani *et al.*, 2000), high plasticity index and resprouting dynamics (Espelta *et al.*, 1999; Gratani *et al.*, 2000), deep rooting system and large carbohydrate pools (Canadell & Lopez-Soria, 1998; Canadell *et al.*, 1999), and high adaptive variability in foliar phenomorphology (Sabaté *et al.*, 1999). Our findings showed the intra-crown variability in *Q. ilex*, where shaded leaves were widely unaffected by the inhibitory cold stress (Oliveira & Penuelas, 2001). The ability of *Q. ilex* to perform rapid metabolic changes in the antioxidant and photoprotective mechanisms could be of adaptive importance (García-Plazaola *et al.*, 1999a). In contrast, *P. halepensis* is a fast growing conifer that quickly occupies open spaces after disturbances such as fires (Zavala *et al.*, 2000). *P. halepensis*, as do all pines, has a low ability to store carbohydrates and therefore follows a strategy of water conservation and embolism avoidance (Meinzer *et al.*, 2009). High rates of photosynthesis and growth require high concentrations of carboxylation enzymes in the carbon cycle that have high maintenance costs (Valladares & Niinemets, 2008), perhaps accounting for the high respiration rates found in *P. halepensis* leaves. Moreover, differences among the species are also likely to be the result of distinct foliar morphologies and crown architectures. Pine trees are characterised by a relatively low exposure of foliar surface area to direct sunlight due to the cylindrical shape and steep angles of their needles but at the same time are able to exploit a wider range of incident light angles than broadleaved trees. Despite reported flexible adjustments in the orientation of the leaves in several Mediterranean broadleaved sclerophyllic species (Oliveira & Peñuelas, 2000; Werner *et al.*, 2002; Vaz *et al.*, 2011), needle leaves probably still confer some benefits to attain near-saturated photosynthetic rates over a wider range of diurnal and seasonal variation in sun angles (Jordan & Smith, 1993; Lusk *et al.*, 2003), while at the same time showing a high tolerance to photoinhibition. This might account for the good performance of *P. halepensis* under mild winter conditions with moderate abiotic stresses such as in our study. However, under more severe and re-occurring frost events, *P. halepensis* might reach the threshold of its tolerance and severe frost damage can occur. This explains also its absence in mountain regions with more severe winters where *Q .ilex* becomes more competitive. Despite following distinct physiological strategies, both *Q. ilex* and *P. halepensis* seem to cope equally well with the winter conditions they were exposed to whereas the foliar photosynthetic systems of *A. unedo* were more sensitive to sudden frost impacts. Thus, *A. unedo* might have been in a competitive disadvantage for the following growing season.

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

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5.7 Supporting information

Supplementary tables

Table S5.1 | The scaling constant (c) and energies of activation (ΔHa) describing the temperature responses for Rubisco enzyme kinetic parameters K_c, K_o and Γ^*. Taken from Bernacchi et al., (2002).

	25°C	c	ΔHa	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

Supplementary notes

Note S5.1 | Temperature functions

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

$$K_c = e^{\left(c-\left(\frac{\Delta Ha}{R\times(273.15+T_L)}\right)\right)} \tag{S1}$$

and

$$K_o = e^{\left(c-\left(\frac{\Delta Ha}{R\times(273.15+T_L)}\right)\right)} \tag{S2}$$

and

$$\Gamma^* = e^{\left(c-\left(\frac{\Delta Ha}{R\times(273.15+T_L)}\right)\right)} \times \frac{O_2}{20.9} \tag{S3}$$

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

Note S5.2 | CF- parameters

The non-photochemical quenching (NPQ) was estimated by both dark- and light-adapted fluorescent signals F_m and F_m' by:

$$NPQ = \frac{(F_m - F_m')}{F_m'} \tag{S4}$$

where F_m is the maximal fluorescence measured on a dark adapted leaf after a saturating light pulse and F_m' is the maximal fluorescence yield of a light adapted leaf after a pulse of high light. Photochemical quenching (qP) indicates the proportion of open PSII reaction centres and tends to be highest in low light when leaves use light most efficiently (Maxwell & Johnson, 2000). qP was estimated by:

$$qP = \frac{F_m' - F_s}{F_m' - F_o'} \tag{S5}$$

where F_o' is the minimum fluorescence in a light-adapted leaf after a pulse of darkness and F_s is the steady-state fluorescence in a fully light-adapted sample.

Note S5.3 | Estimation of mesophyll conductance

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

$$g_m = \frac{A_{\mathrm{net}}}{C_{\mathrm{i}} - \frac{[\Gamma^* * J_{\mathrm{CF}} + 8(A_{\mathrm{net}} + R_{\mathrm{d}})]}{J_{\mathrm{CF}} - 4(A_{\mathrm{net}} + R_{\mathrm{d}})}} \tag{S6}$$

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

$$C_c = C_i - \frac{A_{net}}{g_m} \tag{S7}$$

where C_c is the chloroplastic CO_2 concentration.

6

Leaves and early acorns of *Q. ilex*
Photo & Design: D. Sperlich

Chapter 6

Balance between carbon uptake and release: impacts of long-term drought on foliar respiration and photosynthesis in *Quercus ilex* L.

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6.1 Abstract

Carbon exchange in terrestrial ecosystems is a key process of the global carbon cycle and consists of a delicate balance between photosynthetic carbon uptake and respiratory release. We have a limited understanding how long-term decreases in precipitation affect the boundaries and mechanisms of photosynthesis and respiration. We examined the seasonality of photosynthetic and respiratory traits and evaluated the adaptive mechanism of the plant carbon balance in response to reduced soil water availability as part of a rainfall-manipulation experiment. This experiment was established in 1999 in a natural forest of *Q. ilex* L. where the soil water was reduced on average by 13% (1999–2013). Day respiration (R_{d}) but not night respiration (R_{n}) was generally higher in the drought treatment leading to an increased $R_{\mathrm{d}}/R_{\mathrm{n}}$. Mesophyll conductance (g_{m}) generally limited photosynthesis more in the drought treatment reflected in a lower $g_{\mathrm{m}}/g_{\mathrm{s}}$. The peak photosynthetic activity of the drought treatment surprisingly occurred in the summer campaign which is usually characterised by a high level of abiotic stress. This was due to atypical favourable conditions in summer underlining the climatic variability in the Mediterranean region. In parallel, the overall trends in summer had a pronounced lower $R_{\mathrm{d}}/R_{\mathrm{n}}$ and higher $g_{\mathrm{m}}/g_{\mathrm{s}}$ in the drought treatment. The plant carbon balance was thus strongly improved through (i) higher photosynthetic rates induced by g_{m} and through (ii) decreased carbon losses mediated by R_{d}. Interestingly, the biochemical photosynthetic potential ($V_{\mathrm{c,max}}$, J_{max}, TPU) was not affected by the drought treatment suggesting a dampening effect on a biochemical level in the long-term. In summary, the trees experiencing a 14 year-long drought treatment adapted through a higher plasticity in photosynthetic and respiratory traits, so that eventually the favourable growth period was exploited more efficiently.

6.2 Introduction

Warmer and drier conditions are expected globally under current climate change scenarios and particularly in the Mediterranean region (Somot *et al.*, 2008; Friend, 2010; IPCC, 2013). Seasonal reoccurring drought is the main natural environmental factor in the Mediterranean region limiting plant growth and yield (Specht, 1969; Di Castri, 1973). Projected water shortages will thus likely intensify the limitations on plant productivity and forest growth and increase the risk of forest fires (Piñol *et al.*, 1998; Pausas *et al.*, 2008). Several studies have already reported drought-induced forest impacts and diebacks in the Mediterranean region (Peñuelas *et al.*, 2001; Martínez-Vilalta & Piñol, 2002; Raftoyannis *et al.*, 2008; Allen *et al.*, 2010; Carnicer *et al.*, 2011; Matusick *et al.*, 2013) as well as shifts in vegetation composition (Jump & Penuelas, 2005; Anderegg *et al.*, 2013). Despite these reports of local and regional impacts, the exact effects and consequences of climatic warming and decreasing precipitation on the global carbon cycle are highly uncertain (Friedlingstein *et al.*, 2014). In fact, the modelling performance in Mediterranean-type ecosystems is particularly poor (Morales *et al.*, 2005; Vargas *et al.*, 2013) owing to underrepresented soil-water patterns and our limited understanding of the effects of water stress on both carbon uptake and release (Hickler *et al.*, 2009; Niinemets & Keenan, 2014). It is known that the drought-induced limitation to plant growth and productivity is mainly caused by reductions in the plant carbon budget, which depends on the balance between photosynthesis and respiration (Flexas *et al.*, 2006). Winter has been somehow overlooked despite its importance for the annual carbon budget, especially for evergreen vegetation (Sperlich *et al.*, 2014, 2015). High variabilities in temperature and precipitation regimes are also characteristic, especially in mountainous areas of the Mediterranean region such as the Prades mountains in northeastern Spain (Barbeta *et al.*, 2013). Climate extremes combined with high inter-annual variability complicate the scaling of carbon dynamics from one year to another (Reynolds *et al.*, 1996; Morales *et al.*, 2005; Gulías *et al.*, 2009).

Although drought responses of the Mediterranean vegetation have been extensively investigated, most studies concern *photosynthetic* responses (for a review see Flexas *et al.*, 2014) whereas *respiratory* responses in leaves have been largely neglected (Niinemets, 2014). Mitochondrial respiration, however, is a central metabolic process that produces energy (ATP, NADPH) and carbon skeletons for cellular maintenance and growth. It also contributes to significant carbon losses - especially under stress conditions - altering the net carbon gain (Van Oijen *et al.*, 2010). The extent to which the net carbon gain is altered when mitochondrial respiration becomes inhibited in light as a composite effect of multiple cellular pathways is nonetheless difficult to elucidate (Heskel *et al.*, 2013). This inhibitory effect of light on respiration has long been known (Pizon, 1902 cf. Tcherkez & Ribas-Carbó, 2012), but the dominance of chloroplastic pyruvate decarboxylation on the respiratory fluxes in light and the strong inhibition of glycolysis and glucose use have only recently been reported (Tcherkez *et al.*, 2005, 2012). However, the complete physiological basis of the inhibition of night respiration (R_n) during the day remains incompletely understood as does the effect of seasonality on the balance of R_n with day respiration (R_d). This is owing to measurement difficulties; R_n can be easily measured by darkening the leaf, but R_d is harder to obtain and is traditionally estimated from carbon- response curves with the Laisk method, from light- response curves with the Kok method, or alternatively with an amended version of the Kok method with chlorophyll fluorescence developped by Yin *et al.* (2009) (see Yin *et al.*, 2011 for a review). Measurement constrains and lacking research priorities can account for the dearth of data on respiratory responses to abiotic stress, particularly drought (Atkin & Macherel, 2009; Heskel *et al.*, 2014). Wright *et al.* (2006) provided evidence that irradiance, temperature and precipitation affect respiration in a wide range of woody species around the world; Mediterranean species, however, were not covered. Catoni *et al.* (2013) recently provided evidence that temperature, and monthly rainfall to a lesser extent, could explain the seasonal variation of R_d in several Mediterranean maquis species. Galmés *et al.* (2007b) noted that the number of studies on plant respiration responses to drought is generally limited- but particularly for Mediterranean species. This is surprising considering the obvious importance of water stress in the Mediterranean region. Seasonal acclimation of respiration is believed to be more important in sclerophyllic perennial leaves (Galmés *et al.*, 2007; Zaragoza-Castells *et al.*, 2007, 2008) than in plants with short-lived leaves (for a review see Atkin & Macherel, 2009). A better characterization of the respiratory responses to drought relative to carbon gain is vital for elucidating the overall effects on carbon exchange dynamics in water-limited environments. Rainfall-manipulation experiments in natural ecosystems are laborious and expensive but highly valuable to more realistically simulate long-term drought. Some studies have recently studied the photosynthetic limitations under long-term drought in natural ecosystems comprising stomatal, mesophyll and biochemical components (Limousin *et al.*, 2010; Martin-StPaul *et al.*, 2012). Since to the best of our knowledge the effects of long-term experimental drought on photosynthesis in parallel with night and day respiration here has not been investigated so far on mature species in natural ecosystems.

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

We investigated the variations of photosynthetic and respiratory traits of *Q. ilex* affected by seasonal changes in growth temperature and precipitation from winter to spring and summer. W also studied the impact of long-term experimental drought on key limitations of photosynthesis comprising stomatal, mesophyllic and biochemical components as well as mitochondrial respiration at day and night. Based on these parameters, we evaluated the response pattern of the foliar intrinsic water- and carbon-use efficiency (WUE_i and CUE_i) with respect to the simulated drought in order to understand better the boundaries and mechanisms of photosynthesis and respiration to seasonal acclimation and adaptation to drought.

6.3 Material and Methods

6.3.1 Experimental site

The experimental site was situated in the Prades Mountains in southern Catalonia (NE Spain; 41°21'N, 1°2'E) at 950 m a.s.l. on a 25% south-facing slope. Temperature, photosynthetically active radiation, air humidity, and precipitation have been continuously monitored with a meteorological station installed at the site. The climate is Mediterranean, with a mean annual rainfall of 609 mm and a mean annual temperature of 12.2 °C (climate data from the meteorological station for 1999–2012). The soil is a Dystric Cambisol over Paleozoic schist with a depth of 35 to 90 cm. The forest is characterised by a dense, multi-stemmed crown dominated by *Q. ilex* and *Phillyrea latifolia* L. with a maximum height of 6–10 m. The understorey is composed of *Arbutus unedo* L., *Erica arborea* L., *Juniperus oxycedrus* L. and *Cistus albidus* L. A long-term rainfall-exclusion experiment has been established and maintained in this forest since 1999 to simulate in-situ projected decreases in precipitation in the Mediterranean region (Peñuelas *et al.*, 2007). Four control and four treatment plots of 15 × 10 m were installed at the same altitude along the mountain slope. In the treatment plots, rain was partially excluded by PVC strips suspended 0.5–0.8 m above the soil (covering 30% of the soil surface). A ditch of 0.8 m in depth around the plots intercepted the runoff water from above the plots and conducted the water around to their bottom. The control plots received no treatment.

6.3.2 Sampling method

We conducted three field campaigns between January 2013 and August 2013 (winter, spring, and summer; dates indicated in Fig. 1). Abiotic stress under field conditions often hampers gas exchange measurements due to deviations from the standard temperature (25 °C) or unpredictable plant responses (e.g. patchy stomatal conductance) (Mott & Buckley, 1998, 2000). We thus cut and analysed rehydrated twigs under standardised conditions (Sperlich *et al.*, 2015). Eight twigs for each drought and control plot (two replicates for each plot) were cut with a pruning pull from the sun-exposed crowns of *Q. ilex* trees. We re-cut the twigs under water in the field, wrapped them in plastic bags to minimise transpiration, and transported them in water buckets to a nearby laboratory. The twigs were pre-conditioned in the laboratory at room temperature (22–26 °C) in dim light for 1–3 d and were freshly re-cut every morning.

6.3.3 Analyses of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, USA). Response curves of net assimilation versus photosynthetic photon-flux density (PPFD) were recorded in parallel with chlorophyll fluorescence measurements on mature, fully expanded leaves. In the summer campaign, we additionally conducted response curves of net assimilation versus CO_2. Some of the *Q. ilex* leaves were too small to fill the leaf cuvette ($2\ cm^2$), so the measured parameters were adjusted after the measurements. The leaves were prepared and acclimated prior to recording the response curves as described in Sperlich *et al.* (2014).

6.3.4 Calculation of chlorophyll fluorescence parameters

The effective quantum yield of photosystem II (Φ_{PSII}, unitless) was estimated as:

$$\Phi_{\mathrm{PSII}} = \frac{(\mathrm{F_m}' - \mathrm{F_s})}{\mathrm{F_m}'} \qquad (1)$$

where F_s is the steady-state fluorescence of a fully light-adapted sample, and F_m' is the maximal fluorescence yield reached after a pulse of intense light. Φ_{PSII} represents the fraction of photochemically absorbed photons for a light-adapted leaf. The electron-transport rate based on the effective quantum yield of PSII (J_{CF} in μmol electron $m^{-2}\ s^{-1}$) was calculated as

$$J_{CF} = \varepsilon * \Phi\mathrm{PSII} * \alpha_{\mathrm{L}} \qquad (2)$$

where ε is a scaling factor accounting for the partitioning of intercepted light between photosystem I (PSI) and PSII. We assumed that light was equally distributed between the two photosystems ($\varepsilon = 0.5$) (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2005). The foliar absorbance (α_L, unitless) was 0.932 for *Q. ilex* (Sperlich *et al.*, 2014). Calculations of F_v/F_m and nonphotochemical quenching (NPQ) can be found in the supplementary material (Note S1).

6.3.5 Light experiments

Light-response curves (A/PPFD) were generated at a leaf chamber internal concentration (C_a) of 400 μmol CO_2 mol air^{-1} by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source. To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence (in μmol photons m^{-2} s^{-1}): 2500→2000→1500→1000→800→600→500→400→300→200→150→125→100→75→50→40→30→20→10→5→0. The minimum and maximum times between each light level for the generation of the A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light during an A/PPFD curve led to a drop in the leaf temperature (T_{Leaf}) as the light decreased. The rapid changes in the light levels prevented the correct adjustment of T_{Leaf} while guaranteeing stable air and water fluxes and avoiding noisy measurements of stomatal conductance (g_s in mol H_2O m^{-2} s^{-1}) and stomatal internal CO_2 concentration (C_i in μmol CO_2 mol air^{-1}). We fixed the Peltier-block temperature (T_{block}) in the leaf cuvette, so that T_{Leaf} was 25 °C at the beginning of the A/PPFD curve. T_{Leaf} had dropped by approximately 1–3 °C by the completion of the A/PPFD curve. We estimated R_n μmol CO_2 m^{-2} s^{-1} after darkening the leaf for at least 30 min. R_d (μmol CO_2 m^{-2} s^{-1}) was estimated from the light-response curves with the method proposed by Yin et al. (2009) combining measurements of gas exchange and chlorophyll fluorescence. This method amended the Kok method (Kok, 1948) by substituting the A/PPFD relationship with A/(PPFD × Φ_{PSII}/4). See Yin et al. (2009) for details on the protocol.

The foliar water-use efficiency (WUE_i) is defined as the amount of carbon gained per unit water used and is estimated with the A_{net}/g_s ratio which is the relationship of net assimilation rate (A_{net}, μmol CO_2 m^{-2} s^{-1}) versus stomatal conductance (Flexas *et al.*, 2013). We calculated the intrinsic carbon use efficiency (CUE_i) as proportion of carbon assimilated per carbon respired (Gifford, 2003), which served as a rough indicator for the foliar carbon balance (Pattison *et al.*, 1998; Galmés *et al.*, 2007)

$$CUE_i = 1 - \frac{A_{net}}{R_d} \qquad (3)$$

where A_{net} is the net assimilation rate at ambient CO_2 (400 μmol CO_2 m^{-2} s^{-1}) and saturating light (1200 μmol photons m^{-2} s^{-1}).

6.3.6 CO_2 experiments

The C_a concentrations used to generate the CO_2-response curves were 400→300→200→150→100→50→400→400→600→ 800→1200→2000 μmol CO_2 mol air^{-1}. T_{Leaf} was set to 25 °C. The saturating PPFD used was 1200 μmol photons m^{-2} s^{-1} based on light-response curves conducted prior to the measurements campaigns. The results of all light-response curves after the measurement campaign, however, indicated a saturating PPFD of 1500 μmol photons m^{-2} s^{-1}. The minimum and maximum times for stabilising A_{net}, g_s, and C_i for each log were set to 4 and 6 min, respectively. Diffusion leakage was corrected as described in Sperlich *et al.* (2014).

6.3.7 Estimation of mesophyll conductance

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

$$g_m = \frac{A_{\mathrm{net}}}{C_{\mathrm{i}} - \frac{[\Gamma^* * J_{\mathrm{CF}} + 8(A_{\mathrm{net}} + R_{\mathrm{d}})]}{J_{\mathrm{CF}} - 4(A_{\mathrm{net}} + R_{\mathrm{d}})}} \tag{4}$$

where Γ^* is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic CO_2 uptake. The chloroplastic CO_2 concentration (C_c in μmol CO_2 mol air^{-1}) was determined as:

$$C_c = C_i - \frac{A_{net}}{g_m} \tag{5}$$

6.3.8 Photosynthesis model

The photosynthesis model of Farquhar *et al.* (1980) considers photosynthesis as the minimum of the potential rates of Rubisco activity (A_c) and ribulose-1,5-bisphosphate (RuBP) regeneration (A_j). A third limitation (A_p) was implemented that considers the limitation by triose-phosphate use at high CO_2 concentrations when the CO_2 response shows a plateau or decrease (Sharkey, 1985). The model was further modified by replacing C_i with C_c for the chloroplast where the actual carboxylation takes place (see for review Flexas *et al.*, 2008). As outlined above, we used the variable-*J* method for the C_c calculation to create A/C_c curves. The modelled assimilation rate A_{mod} was then calculated by the minimum of these three potential rates from the A/C_c curves:

$$A_{mod} = min\{A_c, A_j, A_p\} \tag{6}$$

where

$$A_c = V_{cmax} * \left[\frac{C_c - \Gamma^*}{C_c + K_c\left(1 + \frac{O}{K_o}\right)}\right] - R_d \tag{7}$$

where $V_{c,max}$ (μmol CO_2 m^{-2} s^{-1}) is the maximum rate of Rubisco carboxylation, K_c is the Michaelis-Menten constant of Rubisco for CO_2, O is the partial pressure of O_2 at Rubisco, and K_o is the Michaelis-Menten constant of Rubisco for O_2, taken from Bernacchi et al. (2002). The equation representing photosynthesis limited by RuBP regeneration is:

$$A_j = J * \left[\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*}\right] - R_d \tag{8}$$

where J (in μmol electron m^{-2} s^{-1}) is the rate of electron transport. We assumed that J became J_{max} under light and CO_2 saturation when the maximum possible rate of electron transport was theoretically achieved, although we may have underestimated the true J_{max} (Buckley & Diaz-Espejo, 2015). The limitation of triose phosphate use is estimated as

$$A_p = \frac{3TPU * C_c}{C_c - (1 + 3\alpha_{TPU}) * \Gamma^*} - R_d \tag{9}$$

where TPU is the rate of triose phosphates use at saturating CO_2 concentrations, and α_{TPU} is the proportion of glycerate not returned to the chloroplasts. Eq. 9 is from von Caemmerer (2000) after correcting a typographical error in the expression $3\alpha_{TPU}/2$ to $3\alpha_{TPU}$, as described in Gu *et al.* (2010). This equation fits the A/C_c curve plateau at high CO_2 when a further increase in C_c does not produce any increase of A_{net} anymore or, in some cases, even a decline of A_{net}.

In addition to the A/C_c curves, we replaced C_c with C_i in Eqs. 79 and thus applied the above photosynthesis model to the traditional A/C_i curve. We used an adequate set of kinetic constants from Bernacchi *et al.* (2001). We considered the $V_{c,max}$, J_{max}, and TPU from the A/C_c curve the "true" biochemical potential to drive photosynthesis whereas the parameters from the A/C_i curve where the "apparent" photosynthetic potential.

6.3.9 Statistical analyses

We applied the non-linear least squares (nls) method to fit the models to the measured A/C_c or A/C_i response curves and, with the SOLVER Excel tool, estimated the true and apparent values of $V_{c,max}$, J_{max} and TPU from Eq. 7-9. SOLVER iteratively changes the parameters to minimise the sum of squares of the deviation of observed A_{net} versus modelled A_{mod}. Outliers with evidence of error during the measurements were not included in the curve- fitting procedure. We then performed further statistical analyses with R version 3.0.2 (http://www.r-project.org/). Differences in the parameters between control and drought plots were determined with Student's *t*-tests ($P \leq 0.05$). The normality of the data was tested with Shapiro-Wilk tests, and the data was normalised if not normally distributed. One-factorial analyses of variance with season as the main factor tested for seasonal differences in the parameters. Significant differences were determined at $P \leq 0.05$ with Tukey's Honestly Significant Difference tests. Linear regression analyses were conducted to study the relationships amongst various leaf traits such as $A_{\mathrm{net}}/g_{\mathrm{s}}$, $A_{\mathrm{net}}/g_{\mathrm{m}}$, $J_{\mathrm{max}}/V_{\mathrm{c,max}}$, $g_{\mathrm{m}}/g_{\mathrm{s}}$ and $R_{\mathrm{n}}/R_{\mathrm{d}}$. We tested for differences in regression slopes and intercepts with analyses of co-variance (ANCOVAs).

6.4 Results

6.4.1 Environmental conditions over the sampling period

Frost events were frequent in winter and snowfall was also observed. The maximum temperatures during the day were on an average 4.9 °C (Table 6.1). The spring was humid with a precipitation comparable to that in winter (246 and 269 mm, respectively) and was relatively cold (average of 12 °C) with occasional night frosts (Fig. 6.1). Spring together with winter accounted for nearly 80 % of the annual average precipitation. The summer, in contrast, was dry and warm (total precipitation of 21 mm and average temperature of 20.3 °C), with a vapour-pressure deficit (VPD) nearly twice as high as in spring (0.83 kPa) (Table 6.1). The partial rainfall exclusion reduced the SWC by a total of 13% from the beginning of the experiment in 1999 until the end of our measurement campaign in 2013. For the period of our measurement campaign, the soil water content (SWC) was reduced on average by 14 % by the partial rainfall exclusion (Table 6.1). This difference was highest in spring, with a 24% lower SWC in the drought plots.

6.4.2 Seasonal changes in photosynthetic parameters

We analysed the seasonality of the photosynthetic parameters using the full dataset independent for treatment. Winter had a strong effect on several parameters with lower average values than in spring and summer, except for R_{n} and C_{i} (Table 6.2). A_{net}, g_{s}, g_{m}, $F_{\mathrm{v}}/F_{\mathrm{m}}$ were significantly (at $P < 0.05$) and and R_{d}, C_{c}, CUE_{i} were marginally significantly (at $P < 0.10$) lower in winter than in either spring or summer (Fig. 6.2 and 6.3). In summer, we found surprisingly the highest mean values of A_{net}, g_{s}, g_{m}, and C_{c} that were significantly different to those in spring and winter (Fig. 6.3). $F_{\mathrm{v}}/F_{\mathrm{m}}$ was also highest in summer demonstrating that the photosynthetic systems in spring had not yet fully recovered from the low winter temperatures, but operated at their peak efficiency in summer (Fig. 6.4b). NPQ was lowest in spring (significant to both winter and summer) indicating a low rate of photoinhibitory stress and dissipation of excess energy in the in the relatively cool and wet spring (Fig. 6.4a). Neither Φ_{CO2} nor Φ_{PSII} differed significantly between the seasons (Table 6.2 and Fig. S6.1). The optimum PPFDs for A_{net} and J_{cf} were 1484 and 1552, respectively, and did not change seasonally.

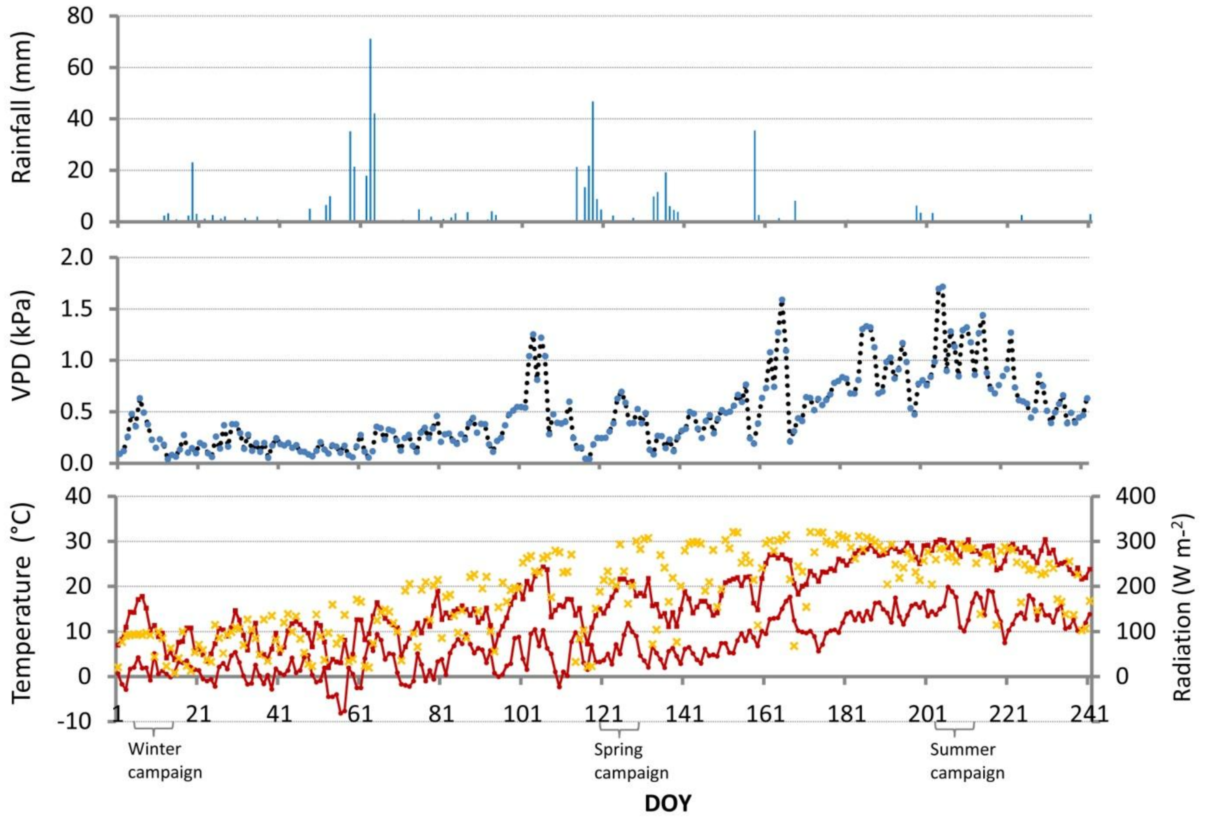

Fig. 6.1 | Environmental variables for the days of the year (DOY) from January to August 2013: a) rainfall, b) atmospheric vapourpressure deficit (VPD), and c) maximum and minimum temperatures in °C on the primary y-axes (red circles) and radiation (yellow crosses) on the secondary y-axes. The field campaigns are indicated.

Table 6.1 | Dates and days of the year (DOY) for each season in 2013 with mean temperature (T), total precipitation (Prec.), mean vapour-pressure deficit (VPD), mean photosynthetic photon flux density (PPFD) and the percentage of the difference in the soil-water content between the control and drought plots (ΔSWC).

Season	date	DOY	T (°C)	Prec. (mm)	VPD (kPa)	PPFD (W m^{-2})	ΔSWC (%)
Winter	01.01.13 - 21.03.13	1-79	4.9	269	0.20	9.1	5.3
Spring	22.03.13 - 21.06.13	79-171	12.0	246	0.45	21.3	23.9
Summer	22.06.13 - 31.08.13	172-242	20.3	21.8	0.83	25.0	7.7
Total	01.01.13 - 31.08.13	1-242	12.1	537	048	18.3	13.5

Several relationships were analysed with ANCOVAs to test if seasonal changes in environmental conditions produced significant differences in slopes (Table 6.3). We analysed the relationship of A_{net}/g_s as an indicator for *WUE*$_i$. The slope of this relationship for the control group was significantly gentler in winter compared to spring and summer - indicating a lower *WUE*$_i$ - but not when all data were combined. The slope of this relationship for the control group was significantly gentler in winter compared to spring and summer, but not when all data were combined. With the relationship of A_{net}/g_m, we analyzed the effect to the mesophyll internal CO_2

diffusion on the net carbon assimilation. This relationship had a significantly steeper slope in winter in comparison to summer in the drought group, but not across all data combined. The relationship of g_m/g_s unveils the relative contribution of stomatal and mesophyll diffusion limitation on the net carbon assimilation. The relationship of g_m/g_s was significantly steeper in the control plot in spring in comparison to summer, but not across all data combined. We analysed the relative importance of day and night mitochondrial respiration with the relationship of R_d/R_n. The slope was significantly steeper in winter compared to spring and summer in both the control and drought plots and also when all data were combined.

Table 6.2 | Means and standard errors (±SE) of a set of photosynthetic parameters and foliar traits for *Q. ilex*. *P*-values indicate the statistical significance of the differences between sunlit and shaded leaves determined by Student's *t*-tests.

		Control			Drought	
Variable	Winter	Spring	Summer	Winter	Spring	Summer
R_n	1.81±0.04	1.49±0.29	1.67±0.18	1.69±0.25	1.69±0.12	1.60±0.14
R_d	1.17±0.19	1.01±0.19	1.20±0.11	1.35±0.29	1.50±0.15	1.15±0.11
R_d/R_n	0.64±0.10	0.69±0.03	0.74±0.05	0.77±0.12	0.88±0.03	0.73±0.06
A_{net}	6.76±1.2	9.43±1.0	10.71±1.0	5.52±2.0	10.17±0.7	13.66±0.9
g_s	0.077±0.032	0.090±0.016	0.116±0.012	0.054±0.021	0.113±0.009	0.161±0.013
g_m	0.054±0.009	0.085±0.014	0.097±0.011	0.047±0.017	0.074±0.017	0.137±0.014
C_i	206±30	198±21	234±8	210±20	227±8	243±6
C_c	74±9	77±3	119±7	61±10	81±5	139±23
NPQ	2.70±0.29	0.82±0.02	2.97±0.26	2.61±0.14	0.80±0.02	2.74±0.31
F_v/F_m	0.80±0.011	0.81±0.007	0.83±0.005	0.78±0.022	0.80±0.007	0.82±0.005
Φ_{CO2}	0.0074±0.0020	0.0092±0.0009	0.0102±0.0014	0.0054±0.0014	0.0097±0.0008	0.0119±0.0018
Φ_{PS2}	0.215±0.045	0.250±0.024	0.206±0.029	0.220±0.009	0.273±0.021	0.218±0.030
$V_{c,max}$			107±9			120±11
J_{max}			132±11			148±12
TPU			9.4±1.2			7.6±1.3

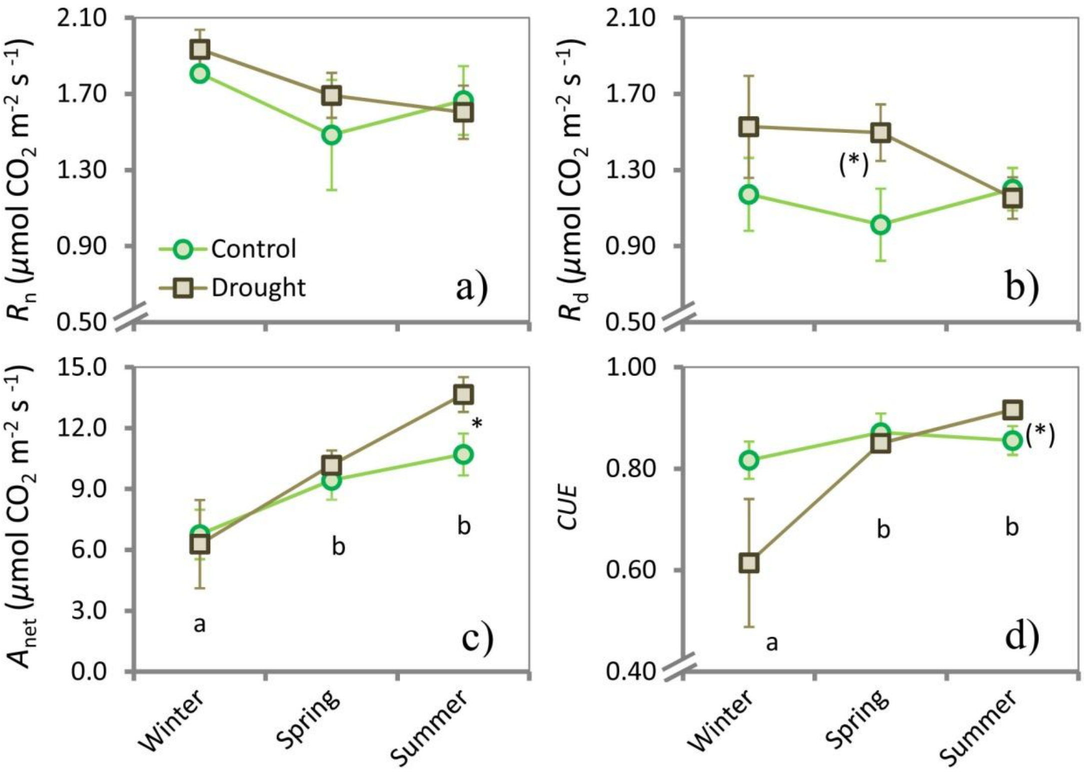

Fig. 6.2 | Line graphs depicting seasonal changes of a) night respiration (R_n), b) day respiration (R_d), c) net assimilation rate (A_{net}), and d) carbonuse efficiency (CUE_i) for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Asterisks and asterisks in brackets indicate significant ($P < 0.05$) and marginally significant ($P < 0.1$) differences between the control and drought plots for each season. Different letters indicate differences between seasons. Vertical bars indicate standard errors of the means (n = 59).

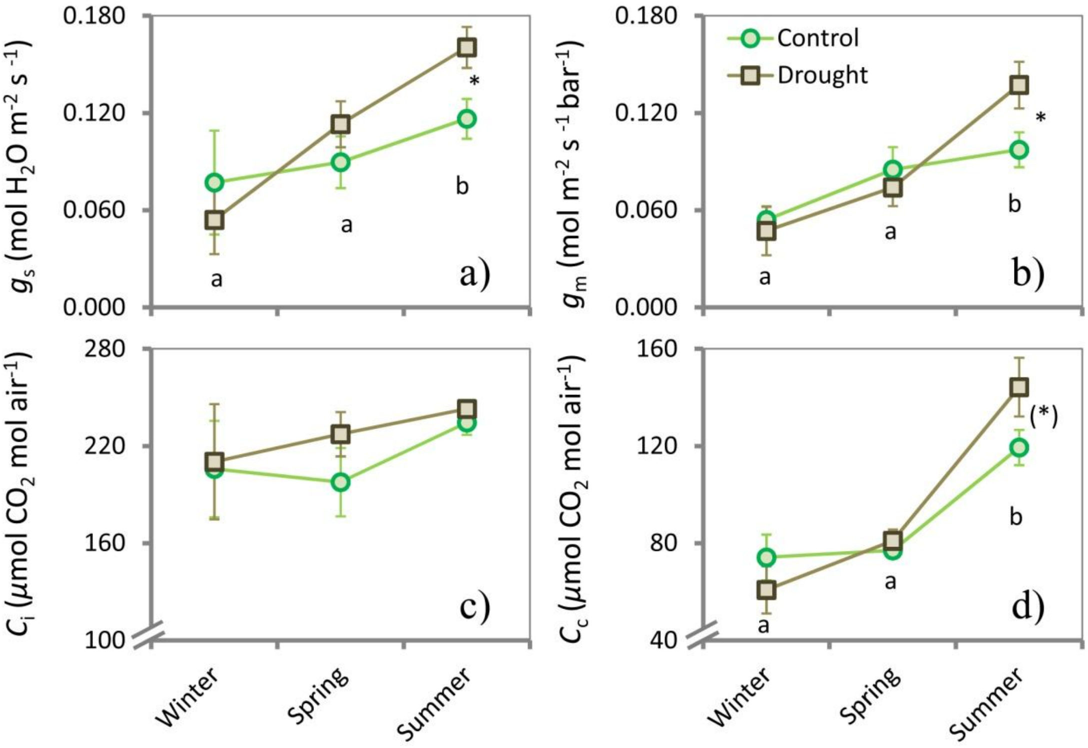

Fig. 6.3 | Line graphs depicting seasonal changes of a) stomatal conductance (g_s), b) mesophyll conductance (g_m), c) stomatal internal CO_2 concentration (C_i), and d) chloroplastic CO_2 concentration (C_c) in sunlit leaves of *Q. ilex*. for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Asterisks and asterisks in brackets indicate significant ($P < 0.05$) and marginally significant ($P < 0.1$) differences between the control and drought plots

for each season. Different letters indicate differences between seasons. Vertical bars indicate standard errors of the means (n = 59).

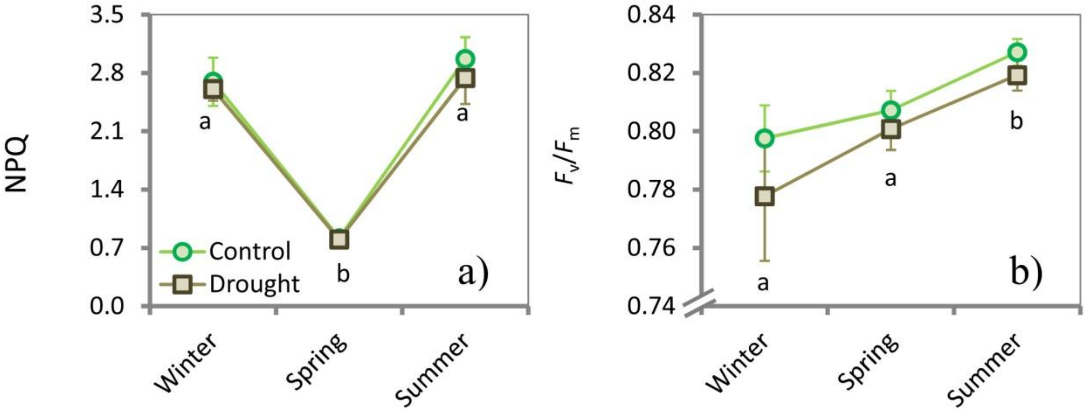

Fig. 6.4 | Line graphs depicting seasonal changes of a) nonphotochemical quenching (NPQ) and b) maximum quantum efficiency of PSII (F_v/F_m) for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Different letters indicate differences between seasons. Vertical bars indicate standard errors of the means (n = 59)

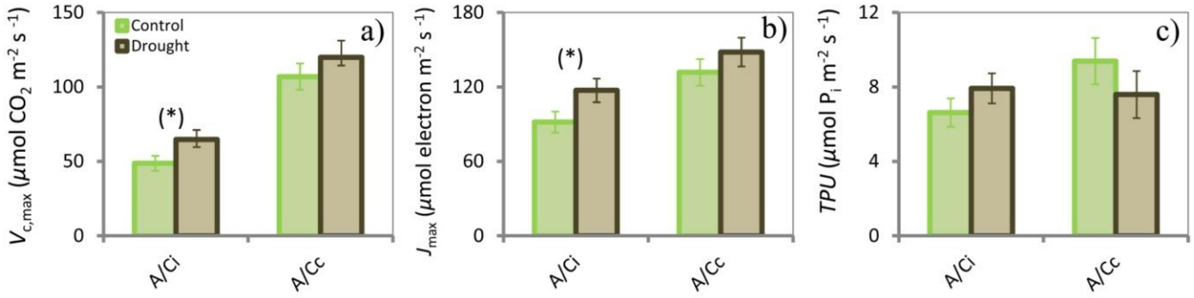

Fig. 6.5 | Bar graphs of a) maximum carboxylation rate ($V_{c,max}$), b) maximum electron-transport rate (J_{max}), and c) triose phosphate use (*TPU*) estimated with CO_2-response curves based on C_i (A/Cc) and C_c (A/Cc) in the control and the drought plots for the summer campaign. Marginal significant differences ($P < 0.1$) between the control and drought plots are marked with asterisks in brackets. Vertical bars indicate standard errors of the means (control n = 7 and drought n = 8).

6.4.3 Effect of experimental drought

R_d/R_n for all seasons combined was significantly higher in the drought treatment (0.79±0.04) compared to the control plots (0.71±0.03). No other general trends were detected. In the respective seasons, however, we found significant effects of the drought treatment with several parameters showing higher average values compared to the control group (Figs. 6.2 and 6.3): A_{net}, g_s and g_m were significantly higher, and CUE_i and C_c were marginal significantly higher in summer, and R_d was marginally significantly lower in spring. We conducted carbon response curves in summer only (see Material and Methods). J_{max}, $V_{c,max}$ and *TPU* were thus only available for the summer campaign. The drought treatment had no significant effect on these photosynthetic potentials when estimated from an A/C_c curve (Fig. 6.5). Additionally, we estimated the apparent photosynthetic potential from A/C_i curves. The drought treatment had a marginal significant effect on the apparent J_{max} and apparent $V_{c,max}$ with lower values in the control plot, but no effect on the apparent *TPU* (Fig. 6.5). A comparison of the photosynthetic potential from A/C_i and A/C_c curves indicated that the foliar internal diffusion limitation imposed by g_m accounted on average for a twofold higher $V_{c,max}$ (54 %) and a threefold higher J_{max} (30 %) and *TPU* (29 %) of the true photosynthetic potential.

The ANCOVAs in the respective seasons identified significant differences in slopes as a result of the experimental drought. The slope of A_{net}/g_s was significantly steeper in the control compared to the treatment group in the winter campaign, indicating a higher WUE_i in the control group (Fig. 6.6). The slope of A_{net}/g_m was significantly steeper in the control group compared to the treatment group in the summer campaign (Fig. 6.6). The overall slope of g_m/g_s was significantly steeper in the control group compared to the treatment group when all seasons were combined (Fig. 6.6). The slope of R_d/R_n was significantly gentler in the control group compared to the treatment group in the spring campaign and when all seasons were combined. Neither season nor treatment significantly affected the slopes of A_{net}/R_d, A_{net}/R_n, J_{amb}/A_{net} and C_c/C_i (Tab. S6.1S6.4, supplementary material).

Table 6.3 | Regression equations and coefficients of determination (R^2) for A_{net}/g_s and A_{net}/g_m (left), and for g_m/g_s and R_d/R_n (right) for *Q. ilex* in three sampling campaigns in the control and drought plots. The *P*-values indicate the significance of the differences between the slopes for the control and drought plots. Equations for non-significant relationships are not displayed.

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

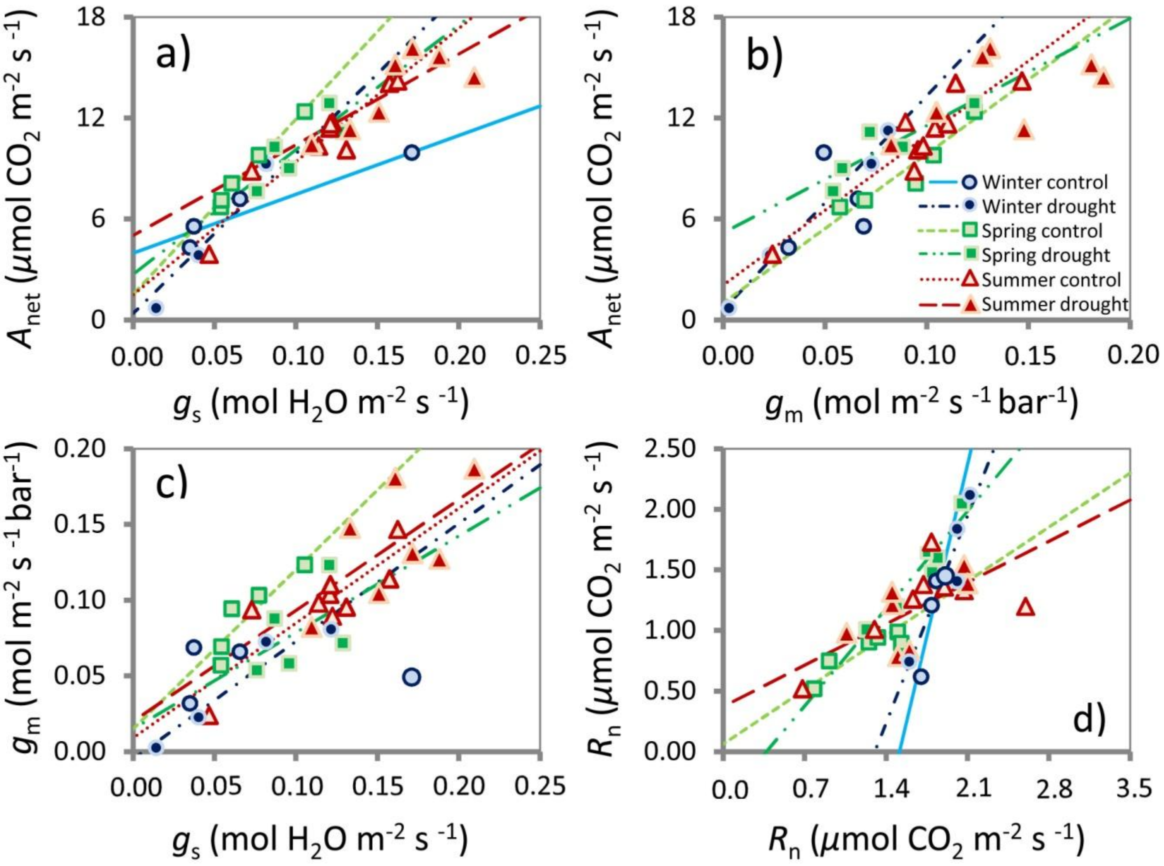

Fig. 6.6 | Scatter plots and regression lines of a) stomatal conductance (g_s) versus net assimilation rate (A_{net}), b) mesophyll conductance (g_m) versus A_{net}, c) g_s versus g_m and d) night respiration (R_n) versus day respiration (R_d) for each season and for control and drought plots. Only the regression lines for significant relationships ($P < 0.05$) are displayed.

6.5 Discussion

The scaling of carbon dynamics from one year to another is particularly challenging in Mediterranean environments due to climate extremes combined with a high inter-annual variability (Reynolds *et al.*, 1996; Morales *et al.*, 2005; Gulías *et al.*, 2009). We investigated the effect of seasonal changes in temperature and precipitation from winter to spring and summer on photosynthetic and respiratory traits of a widely abundant Mediterranean tree species. Our study provides a mechanistic description of seasonal changes of photosynthetic and respiratory processes that can possibly help to improve the modelling performance of future climate change scenarios in Mediterranean-type ecosystems.

6.5.1 Effect of seasonality on photosynthetic and respiratory traits

We found that cold winter temperatures had a stronger negative impact on leaf physiology of *Q. ilex* than summer drought. A_{net} was approximately half the rate in winter compared to the peak found in summer, yet relatively high average winter values were reached (6.5±1.3) that were comparable to those reported in other studies (Gratani, 1996; Ogaya & Peñuelas, 2003). Although both g_m and g_s reduced the CO_2 concentration in the chloroplasts in winter, g_m limited photosynthesis to a greater extent. There is some evidence that g_m acts as a stronger regulator for photosynthesis in winter (Sperlich *et al.*, 2014) although very few studies have examined the behaviour of g_m under natural winter conditions. High water availability and low VPDs and make the reduction of transpiratory water loss through stomatal closure less urgent in the winter period. Low temperatures in winter hamper photosynthetic metabolism and enzymatic activities which may account for the concurrent downregulation of photosynthesis through g_m, as our results indicated. This was paralleled by a drastic decrease in the foliar carbon-use efficiency. In winter, chilly or freezing temperatures often coincide with clear skies and relatively high solar irradiances. The imbalance created between light energy absorbed in photochemistry and light energy used in metabolism increases the susceptibility to photoinhibitory stress (Demmig-Adams & Adams, 1992). This imbalance is particularly problematic for the evergreen vegetation and thermal acclimation to winter conditions is essential to survive these adverse conditions (Blumler, 1991; Öquist & Huner, 2003). As a response, thylakoid membranes are re-organised, reaction centres are closed, and antennal size is reduced in order to protect the photosynthetic apparatus against over-excitation by the incoming radiation (Huner *et al.*, 1998; Ensminger *et al.*, 2012; Verhoeven, 2014). The increased NPQ and decreased F_v/F_m found in our study are good proxies for these photoprotective processes in the thylakoid membranes indicating an increased thermal dissipation of excess energy and a decreased photochemical efficiency (Maxwell & Johnson, 2000). Several studies on *Q. ilex* showed that these processes were accompanied by a higher carotenoid concentration mediated by the xanthophyll cycle (García-Plazaola *et al.*, 1999; Corcuera *et al.*, 2004). These photoprotective mechanisms allow the PSII antenna the primary target for temperature stress to dissipate excessive radiation (Demmig-Adams & Adams, 1996; García-Plazaola *et al.*, 1997). Thus, we found that *Q. ilex* acclimated to the winter conditions with re-occurring night frosts, and exploited the winter period photosynthetically at the cost of lower assimilation rates and a lower carbon-use efficiency (see also Hurry *et al.*, 2000; Dolman *et al.*, 2002; Sperlich *et al.*, 2014). We underline that winter acclimation and exploitation are essential for Mediterranean evergreen tree species in order to achieve a positive annual carbon balance.

Although spring is usually the most active season with respect to photosynthesis and growth, the spring in our study was particularly cool and wet and characterised by a low VPD. Notably lower NPQs in spring in comparison to winter and summer indicate that the photosystems experienced the least amount of photochemical stress in this period. In contrast, NPQ and thus photoinhibitory stress were high in winter and summer. However, the photoprotective mechanism seemed to be effective: The optimal light intensity for net assimilation and the electron transport (approximately 1500 μmol photos m^{-2} s^{-1} for both) and the effective quantum yield of PSII (Φ_{PSII}) (supplementary Fig. S1) did not change between the seasons.

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

6.5.2 Effect of rainfall manipulation on photosynthetic and respiratory traits

Drought experiments with rainfall manipulation under natural conditions can serve as valuable real-time model simulations for scenarios of future climate change. Unfortunately, long-term experiments over several years are costly and laborious and thus particularly scarce. The rainfall manipulation in Prades, maintained since 1999, reduced soil moisture by 13% with respect to ambient conditions and is probably the longest continuous drought experiment worldwide (Wu *et al.*, 2011; Ogaya *et al.*, 2014).

Plants face a trade-off under water stress: the closure of the stomates reduces transpiratory water loss but constrains at the same time CO_2 diffusion to the chloroplasts. When chronic water stress begins to deplete stores of non-structural carbohydrates, plants are particularly reliant on photosynthetic products for refinement, repair, and protective actions (Niinemets *et al.*, 2009). Beside g_s, also g_m can act as a second leaf internal valve regulating the CO_2 conductance through carbonic anhydrase and aquaporins (Terashima & Ono, 2002; Lopez *et al.*, 2013; Perez-Martin *et al.*, 2014). We have provided evidence that g_m plays an additional regulatory role in facilitating the CO_2 diffusion to the chloroplasts under long-term drought as shown by the increased g_m/g_s (see also Galmés *et al.*, 2013). Similar results were obtained in *Q. ilex* and *Pinus halepensis* leaves showing an increased g_m in parallel with a decreased g_s under severe drought (Sperlich *et al.*, 2015).

In addition to the importance of the diffusive capacity of stomata and mesophyll for the foliar carbon balance, this balance also depends strongly on the relationship of photosynthesis with respiration. Light inhibits respiration, so R_d is usually lower than R_n (Pizon, 1902 in Tcherkez & Ribas-Carbó, 2012). However, the extent to which R_n or R_d are affected by water scarcity is highly uncertain and seems to depend on the severity of stress (Flexas *et al.*, 2006). Respiration can be reduced under water stress due to the cessation of photosynthesis and growth, but respiration has also been reported to increase under severe water stress (Ghashghaie *et al.*, 2001; Flexas *et al.*, 2006). We found that R_d was approximately 74 % of R_n and that the rainfall exclusion experiment increased the ratio of R_d/R_n (0.79±0.04) compared to the control plot (0.71±0.03). We also showed that foliar respiration in long-lived leaves of *Q. ilex* acclimated to the seasonal changes of growth temperature, irradiance and water availability confirming the results by Zaragoza-Castells *et al.* (2007). However, in contrast to Zaragoza-Castells *et al.*, (2007) this seasonal acclimation was reflected in R_d only, and not in R_n. Although R_d was generally higher in the drought treatment compared to the control group, during summer it decreased in coincidence with the low value of the control group and in coincidence with higher rates of photosynthesis. Thus, the foliar carbon-use efficiency was significantly increased. Against the background of the favorable conditions during our summer campaign, we postulate that the plants acclimated the balance between energy supply versus energy consumption. We identified R_d as the key player in *Q. ilex* for the foliar carbon balance being most responsive to seasons or treatment effects. Griffin and Turnbull (2013) reported that conditions that suppress the light-saturated rate of photosynthetic oxygenation can decrease R_d/R_n. In turn, drier sites rather increase photorespiration through stronger stomatal limitations on gas exchange. Thereafter, the light inhibition of R_n would decrease reflected in an increase of R_d/R_n. The higher rates of R_d in our drought treatment seem to support this hypothesis. However, we did not find any differences in g_s between the drought and control group. Another possibility is that the leaf may exert an acclimation of the respiratory metabolism because the demands for energy (ATP and NADPH) for synthesis of sucrose and carbon skeletons in the cytoplasm are higher under stressful conditions (Flexas *et al.*, 2006; Zaragoza-Castells *et al.*, 2007). R_d provides the basis for building up heat stabilizing components such as heat shock proteins or BVOCs protecting the plant against detrimental effects (Tcherkez and Ribas-Carbó, 2012). Higher photoinhibitory stress can thus increase the respiratory metabolic activity expressed as a higher protein turnover at a given overall protein content (Weerasinghe *et al.*, 2014; Niinemets, 2014) that might explain the higher values of R_d in the drought treatment. A lower stress level would evidently lead to a lower demand for energy and carbon skeletons and hence to a lower protein turnover. This was reflected in effective photoprotective mechanisms and lower rates of R_d in our summer campaign.

We assumed that we would find a lower photosynthetic potential in summer in the drought treatment in response to the combined effect of experimental and seasonal water stress. However, we found that the drought treatment had no significant effect on J_{max}, $V_{c,max}$, or *TPU*. Our results emphasise that the increased photosynthetic activity in the drought treatment in summer was not attributed to a higher potential in the biochemistry of photosynthesis rather than to an increased diffusive capacity of both g_s and g_m. Interestingly, analysis of the apparent J_{max} and apparent $V_{c,max}$ (derived from A/C_i curves) identified marginal significant higher values in the rainfall-exclusion plot. We have provided evidence for the potential confounding effects when using the traditional fitting method based on A/C_i curves to derive the biochemical photosynthetic potential – as also shown in grapevines by Flexas *et al.* (2006). We postulate that the foliar internal diffusion limitation imposed by g_m accounted on average for a twofold higher $V_{c,max}$ (54%) and a threefold higher J_{max} (30%) and *TPU* (29%) of the apparent photosynthetic potential. Similar values were reported for nearly 130 C_3 species in a recent study by Sun *et al.* (2014).

We postulate that, counterintuitively, the trees in the drought plot achieved higher photosynthetic rates and a higher carbon-use efficiency in the drought-prone summer; yet photosynthetic potentials were not affected. The summer presented favourable conditions with high temperatures and radiation but also a high water availability. The individuals under drought treatment benefitted the most from these conditions. Recent findings underscore the high plasticity of *Q. ilex* to seasonal changes in temperature or soil water compared to other Mediterranean species (Sperlich *et al.*, 2015). Moreover, rainfall manipulation was shown to result in a higher stem mortality (Barbeta *et al.*, 2013) and in a reduced leaf area in *Q. ilex* (Ogaya & Peñuelas, 2006) while increasing leaf mass per area. Fewer leaves most certainly disposed of higher biochemical resources and thus compensated for the lower leaf area with higher photosynthetic rates and carbon use efficiency. Also Sperlich *et al.* (2015) found higher photosynthetic potentials in crowns that suffered a reduced total leaf area after a severe drought.

6.5.3 Conclusion

We examined the seasonality of photosynthetic and respiratory traits and evaluated the adaptive mechanism in response to reduced soil water under partial rainfall exclusion. A high climatic variability in the Mediterranean region can lead to counterintuitive effects, with the peak photosynthetic activity in summer which is usually characterised by a high level of abiotic stress. The trees experiencing a 14 year-long drought treatment adapted through a higher plasticity in photosynthetic traits, so that eventually an unexpected favourable growth period in summer was exploited more efficiently - with g_m and R_d as the determining parameters. Drought induced growth declines may be attenuated in the long-term through morphological and physiological acclimation to drought (Leuzinger *et al.*, 2011; Barbeta *et al.*, 2013). Fewer leaves in the drought treatment were compensated by higher net photosynthetic rates. The similarity of photosynthetic potentials in the treatment and control plots suggests a dampening effect also on a biochemical level.

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6.7 Supporting information

Supplementary Figures

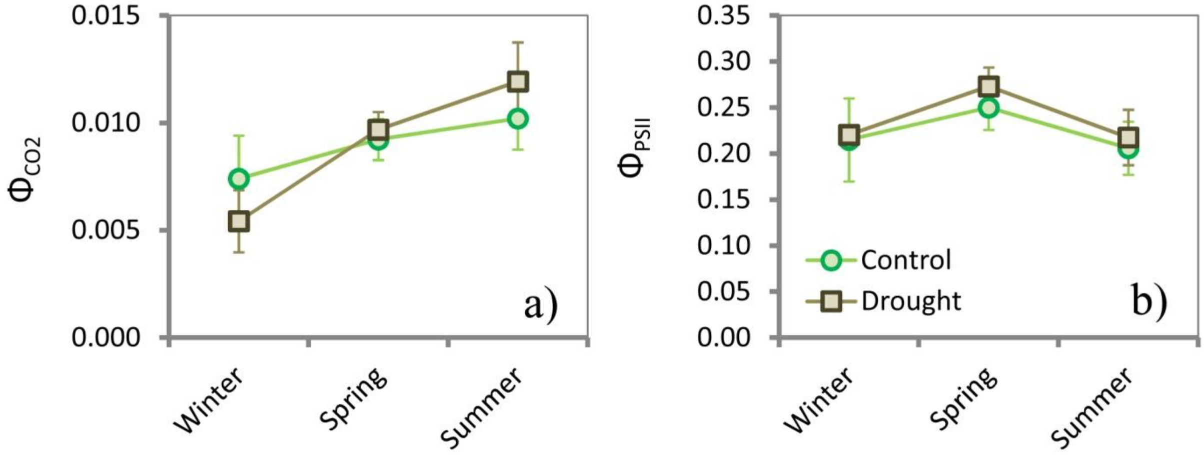

Fig S6.1 | Scatter plots and regression lines of maximum carboxylation rate ($V_{c,max}$) versus maximum rate of electron transport (J_{max}) derived from a) A/C_c and b) A/C_i response curves for control and drought plots in summer 2013. Only the regression lines for significant relationships ($P < 0.05$) are displayed.

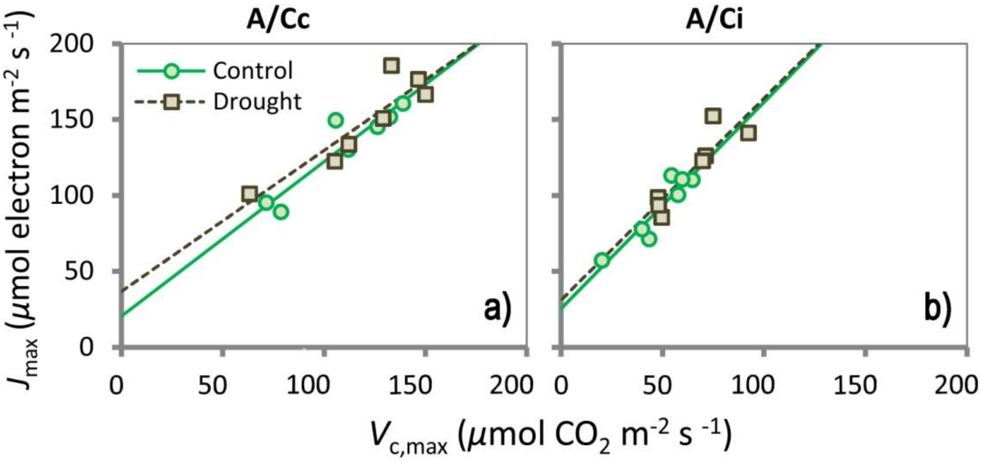

Fig. S6.2 | Line graphs depicting seasonal changes of a) quantum yield of CO_2 (Φ_{CO2}) and b) effective quantum yield of PSII (Φ_{PSII}) for *Q. ilex*. Seasonal campaigns were conducted in winter, spring, and summer 2013. Vertical bars indicate standard errors of the means (n = 59).

Supplementary Tables

Table S6.1 | Regression equations and coefficients of determination (R^2) for a) A_{net}/R_d, b) A_{net}/R_n, c) J_{amb}/A_{net}, and d) C_c/C_i for Q. *ilex* in three sampling campaigns in the control and drought plots. The *P*-values indicate the significance of the differences between the slopes for the control and drought plots. Equations for non-significant relationships are not displayed.

	Campaign	Plot	Equation	R^2	*P*
A) A_{net}/R_d	Total	control	y = -0.98x + 10.41	-0.04	0.513
		drought	y = 1.61x + 8.10	-0.03	
	Winter 2013	control	y = 3.54x + 2.6	-0.04	0.400
		drought	y = 3.26x – 0.25	0.17	
	Spring 2013	control	y = -2.37x + 11.10	-0.02	0.305
		drought	y = 0.89x + 8.4	-0.14	
	Summer 2013	control	y = -2.81x + 14.18	-0.04	0.357
		drought	y = 6.61x + 6.02	0.65	
B) A_{net}/R_n	Total	control	y = -1.46x + 11.69	0.02	0.129
		drought	y = 2.88x + 5.47	0.01	
	Winter 2013	control	y = 22.2x – 33.4	0.45	0.427
		drought	y = 2.91x – 0.62	-0.13	
	Spring 2013	control	y = -1.63x + 11.13	0.02	0.405
		drought	y = 1.43x + 7.39	0.02	
	Summer 2013	control	y = -1.75x + 13.64	-0.04	0.205
		drought	y = 7.96x + 3.55	0.20	
C) J_{amb}/A_{net}	Total	control	y = 4.64x + 87.2	0.29	0.711
		drought	y = 3.88x + 103.6	0.34	
	Winter 2013	control	y = 6.61x + 76.5	0.27	0.738
		drought	y = 8.64x + 85.5	0.68	
	Spring 2013	control	y =8.34 x + 63.8	0.59	0.538
		drought	y = 12.2x + 39.2	0.67	
	Summer 2013	control	y = 4.61x + 78.8	0.16	0.543
		drought	y = 8.12x + 31.2	0.43	
D) C_c/C_i	Total	control	y = 0.18x + 52.9	0.10	0.381
		drought	y = 0.011x + 81.8	-0.10	
	Winter 2013	control	y = 0.171x + 39.0	-0.06	0.435
		drought	y = -0.02x + 57.2	-0.48	
	Spring 2013	control	y =0.053 x + 63.8	-0.14	0.553
		drought	y = 0.12x + 48.3	0.74	
	Summer 2013	control	y = 0.17x + 69.2	0.05	0.499
		drought	y = -1.09x + 344.4	-0.15	

Supplementary Notes

Note S6.1 | Calculation of maximum quantum yield of PSII and nonphotochemical quenching

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

where F_o is the minimal fluorescence measured under darkness, and F_m is the maximal fluorescence measured after a saturating light pulse. Both parameters were obtained on a dark-adapted leaf with closed PSII reaction centres as described in the previous sections. The F_v/F_m ratio describes the fraction of absorbed photons used in photochemistry under dark conditions and serves as the primary stress indicator of the photosystems. Typical values range between 0.74 and 0.85. Ratios <0.80 are indicative of induced photoprotection (sustained energy dissipation), and ratios <0.74 are indicative of chronic photoinhibition (Björkman & Demmig, 1987; Maxwell & Johnson, 2000; Verhoeven, 2014).

The nonphotochemical quenching (NPQ) is estimated by both dark- and light-adapted fluorescence signals, F_m and F_m', as:

$$NPQ = \frac{(F_m - F_m')}{F_m'} \tag{2}$$

where F_m and F_m' are the maximal fluorescence of dark and light-adapted leaves, respectively.

7

Fruits, leaves and flowers of *Arbutus unedo*
Photo & Design: D. Sperlich

7. General discussion

The negative effects of drought and temperature stress on the photosynthetic and respiratory system and the feedback to global carbon cycle remain key uncertainties in scenarios of future climate change, especially in the arid and semi-arid regions of our planet. This dissertation investigates in a review and in field experiments contrasting trait syndromes, seasonal acclimation behaviour of ecophysiological traits, key limitations to photosynthesis in response to drought and temperature stress and impacts of long-term drought on the foliar carbon balance in natural forest ecosystems. In the following, the main research outcomes of each chapter are summarized and discussed (7.1-7.3). Last, we evaluate the implications of our findings for the global carbon cycle and modelling (7.4).

7.1 Contrasting trait syndromes in gymnosperms and angiosperms

Drought and increased temperatures significantly limit tree growth in xeric regions of the Mediterranean basin (Andreu *et al.*, 2007; Sarris *et al.*, 2007; Martínez-Alonso *et al.*, 2007; Bogino & Bravo, 2008; Martínez-Vilalta *et al.*, 2008; Vilà-Cabrera *et al.*, 2011; Gómez-Aparicio *et al.*, 2011; Sánchez-Salguero *et al.*, 2012; Candel-Pérez *et al.*, 2012; Vayreda *et al.*, 2013; Coll *et al.*, 2013) and lead to qualitatively different ecophysiological responses in Mediterranean conifers and angiosperm trees (Martinez-Ferri *et al.*, 2000; Zweifel *et al.*, 2007; Eilmann *et al.*, 2009). Abiotic stress imposed by drought and extreme temperature produce highly complex effects on tree physiology. Multiple physiological processes can simultaneously react to the changes in environmental temperatures and influence growth responses (Table 2.4). For example, temperature and drought directly affect several ecophysiological processes such as carbon and nutrient uptake, carbon allocation between tissues, photosynthesis, respiration, processes of embolism prevention and repair, phenological cycles, cambium reactivation, cell division and expansion or carbon transfer rates (Körner, 1998; Rennenberg *et al.*, 2006; Bréda *et al.*, 2006; Sanz-Pérez *et al.*, 2009; Camarero *et al.*, 2010; Michelot *et al.*, 2012). All these ecophysiological processes have often different sensitivities and thresholds to temperature and water deficit. Tree growth and cambium activation, for instance, are more sensitive to low temperatures than photosynthesis (Fajardo *et al.*, 2012). In addition, responses to climate are often species or tissue specific or depend on developmental stage and seasonal phase. These are regulated by multi-tissue coordinated feedbacks. Despite this complexity, consistent differences between major plant groups, such as conifers and angiosperms, in climate-induced responses were reported (Way & Oren, 2010). **Recent large-scale studies of tree growth in the Iberian Peninsula found negative growth trends in response to temperature in Mediterranean gymnosperm, but positive trends in angiosperm trees** (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013).

Existing empirical evidence reviewed in Chapter 2 underlines that contrasting demographic responses in Mediterranean conifer and angiosperm trees are currently occurring, due to both widespread forest successional advance and due to divergent growth responses to temperature. Consistent with these empirical findings that associate the negative effects of temperatures to growth in *Pinus* species, palaeoecological studies suggest a persistent link between *Pinaceae* distributions and low temperatures during the last 100 million years (Millar, 1993; Brodribb *et al.*, 2012). Notably, the ecophysiological basis of these contrasting growth and distributional responses to temperature remain poorly discussed and resolved. Some scientific evidence points towards contrasting trait-based ecophysiological strategies in these two major plant groups (Johnson *et al.*, 2012; Choat *et al.*, 2012; Meinzer *et al.*, 2013). In Chapter 2, we reviewed and discussed several hypothesis how these contrasting ecophysiological strategies may be influencing the distribution and abundance of Mediterranean conifer and angiosperm trees and their responses to global warming. Table 2.4 summarizes the different effects of temperature and drought on tree physiological processes. Two general but contrasting hydraulic strategies arise: (i) high cavitation resistance, low stomatal sensitivity to VPD and low resilience (gymnosperms) and (ii) low cavitation resistance but high resilience (angiosperms). **Thus, angiosperm and coniferous ecophysiological strategies differentially integrate diverse traits such as stomatal sensitivity to vapor-pressure deficit (VPD), hydraulic safety margins and capacity for embolism repair.** These two basic strategies are in turn functionally linked to anatomical differences of the xylem such as NSC content, carbon transfer rates, wood parenchymal fraction and wood capacitance. Thus, hypothesis one (Table 2.1, Chapter 2) states that positive growth responses to increased temperature in angiosperms could be mediated by a less strict stomatal control, allowing them to assimilate carbon for longer during warmer and drier periods. While this could imply that angiosperm could be more vulnerable to xylem cavitation and hydraulic failure, they have a greater capacity for embolism repair. On the other hand, most conifers function with a wider hydraulic safety margin to avoid cavitation but with the cost of lower carbon gain. Beside this specific hypothesis, several other factors could also contribute to explain the differences in growth responses between conifer and angiosperm trees. **Eventually, we found that multiple factors significantly interact in determining growth responses** (Table 2.1, hypothesis 1.7). For example, several studies report strong interactions between tree size, drought, and stand density effects in determining large-scale growth patterns in the Mediterranean basin (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013). Thus, beside contrasting hydraulic strategies, competition can have a comparatively stronger impact than drought effects (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013). Warming could thus increase competition for water in Mediterranean forests and strengthen these interactions with ongoing climate change, especially at the edge of climate gradients (Linares *et al.*, 2009; Ruiz-Benito *et al.*, 2013; Vayreda *et al.*, 2013; Coll *et al.*, 2013).

Adequate experimental approaches are required to quantitatively assess the relative importance of these factors. Multiple common garden experiments that are located in altitudinal and latitudinal gradients are predestined to study phenology and growth responses to temperature (Reich & Oleksyn, 2008; Vitasse *et al.*, 2009a). We underline that the information gained from seedlings under controlled conditions can only poorly represent the physiological mechanisms of the long-term acclimation to variable environmental conditions in mature trees (Flexas et al., 2006; Mittler, 2006; Niinemets, 2010). Moreover, responses to short-term stress are related to the mechanisms of prompt reactions (Flexas et al., 2006). Under natural conditions, mature trees acclimate to gradually developing water stress through the photosynthetic pathway (biochemical, stomatal or mesophyllic) (e.g. Martin-StPaul et al. 2013), but also through foliar traits such as nitrogen, LMA etc. (Poorter *et al.*, 2009). **Thus, we argue that experiments in natural mixed forests are best suited to study the seasonal acclimation of ecophysiological traits such as photosynthesis or morphology**. Complementarily, drought effects on growth and physiology could be studied by manipulative experiments with rainfall exclusion or combined with reciprocal common garden designs (Wu *et al.*, 2011; Klein *et al.*, 2011).

7.2 Seasonality of photosynthetic and morphological traits in a Mediterranean forest

A major aim of terrestrial ecophysiology is to investigate the mechanism of photosynthetic acclimation and adaptation to understand better the distribution and abundance of species in a changing environment. In order to quantitatively assess the relative importance of the complex and multiple effects of drought and temperature stress on tree physiology, we conducted gas exchange and chlorophyll fluorescence analyses in a natural mixed forest consisting of 4 mature Mediterranean tree species (*Q. ilex*, *Q. pubescens*, *P. halepensis*, *A. unedo*) in two crown positions (sunlit and shaded). The results are presented in chapter 3-5 dealing with the seasonal acclimation-behaviour of photosynthetic and morphological traits in response to abiotic stressors. In the following, a transversal discussion of the three chapters is provided.

7.2.1 Impacts of abiotic stress periods in the Mediterranean

Mediterranean-type climates are characterised with a highly dynamic seasonality where favourable growth periods and adverse conditions strongly alternate throughout the year. The biochemistry of photosynthesis is directly affected by diurnal and also seasonal variations of ambient temperature in both short term and long term (Harley & Baldocchi 1995; June, Evans, and Farquhar 2004). Water is undoubtedly the most important factor limiting growth and survival, particularly in summer, whereas spring and autumn provide better growing conditions (Orshan, 1983; Gracia *et al.*, 1999; Sabaté & Gracia, 2011). In Chapter 3, we showed that **$V_{c,max}$ and J_{max} acclimated strongly to the seasonal changes in temperature and water availability** in agreement with previous studies (Fig. 3.3) (Vitale & Manes, 2005; Corcuera *et al.*, 2005; Misson *et al.*, 2006; Ribeiro *et al.*, 2009; Limousin *et al.*, 2010). When high radiation and temperatures coincide with water stress, they can have a combinatory negative effect on the photosynthetic apparatus, especially in sunlit leaves. This was reflected in the acclimation behaviour of the thermal optimum and also of the curvature of temperature responses of photosynthetic assimilation, as shown in Fig. 4.4 in Chapter 4. The dryer year led to a notably higher thermal optimum of photosynthesis in summer. In the wetter year, photosynthesis was more vulnerable to high temperature stress in summer, but showed a higher tolerance to lower temperatures. The lack of acclimation to very high temperatures is presumably because transpiratory cooling prevents excessive leaf temperatures under more humid conditions. **This shows that the degree of temperature stress depends much on the trees' water status and precipitation regimes.** For example, the rainfalls in late-winter and early spring, determined strongly the severity of drought impacts because trees depend on replenished soil-water reserves to endure summer drought periods. Mediterranean trees can reach deep soil layers (Pinto *et al.*, 2014) or can also benefit from water reservoirs in rock cracks (Barbeta *et al.*, 2015) that allows them to be photosynthetically active also during drier periods (Sánchez-Costa *et al.*, 2015). The depletion of these reserves due to a lack of rainfall in the early year, however, predisposes the vegetation to the summer drought period making them vulnerable to photoinhibition and hydraulic failure, as shown in our study. Beside photosynthetic downregulation, Mediterranean trees can also acclimate to water deficits with higher investments in structural compounds, thereby increasing leaf density and succulence (Niinemets, 2001; Ogaya & Peñuelas, 2006; Poorter *et al.*, 2009). **Our findings underscore that foliar traits such as leaf thickness and leaf area are good indicators for the ability to respond to decreases in rainfall under climate change reflected in the significantly increased LMA under severe drought** (Gratani & Varone, 2006; Ogaya & Peñuelas, 2007). Increased leaf vein density may contribute to increase the tolerance to foliar hydraulic dysfunction in Mediterranean plants (Nardini *et al.*, 2014). The severity of drought also explained the relative limitations on net carbon gain. Stomatal closure regulated photosynthesis during both the moderate and severe droughts; g_m, in contrast, decreased under moderate, but increased under severe drought. **Altered g_m can ease the leaf internal CO_2 diffusion under chronic water stress, especially when depleted non-structural carbohydrates (NSCs) make plants particularly reliant on photosynthetic products for refinement, repair, and protective actions** (Niinemets *et al.*, 2009). Major changes of Φ_{PSII}, F_v/F_m, and photosynthetic potentials across all species reflected these refinements of the photosynthetic apparatus as responses to chronic water stress.

In winter, low temperatures and solar radiation limit the amount of energy available for the vegetation, although soil-water contents and water-pressure deficits are favourable. The above described refinements in bio- and photochemistry not only occurred under dry and hot conditions, but also in winter when high radiation and low temperature led to photoinhibitory stress. Our analyses of temperature response curves in Chapter 4 revealed that the photosynthetic system was primarily impeded by high and not low temperatures, thus showing a strong acclimation to the colder winter condition. In chapter 5, we showed that **mild winter temperatures can provide periods of growth and recovery from stressful summer droughts resulting in biochemical recovery, new shoot growth, and moderate transpiration in evergreen species** (see also Sánchez-Costa *et al.*, 2015). Year-round growth patterns with several flushes during the year have also been reported in other studies (Alonso *et al.*, 2003). The effective acclimation of the photosynthetic apparatus during winter is particularly essential for evergreen tree species to achieve a positive carbon balance and to compensate for the lower photosynthetic rates during the growth period, relative to deciduous species (García-Plazaola *et al.*, 1999; Martínez-Ferri *et al.*, 2004). A sudden frost period, however, had a significant impact on the carbon metabolism and photochemistry. The responses to frost and chilly temperatures differed among species: *Q. ilex* followed a photoinhibition-avoidance strategy whereas *P. halepensis* was rather tolerant to photoinhibition (see also García-Plazaola *et al.*, 1999; Martínez-Ferri *et al.*, 2004). The leaves of *A. unedo*, in contrast, suffered severe photoinhibition. We argue that, a pivotal role devolves on the winter period in Mediterranean-type climates. **The success of evergreen species in future dynamics of competition and novel environmental conditions will not only depend upon the tolerance to withstand abiotic stresses, but also on their effectiveness to benefit rapidly from periods when environmental conditions may be favourable such as in winter.** Hence, the winter period deserves much more attention in current discussions of the (i) adaptive ability, distribution shifts, or potential local extinction of species in Mediterranean-type ecosystems (Peñuelas *et al.*, 2013; Matesanz & Valladares, 2014) and (ii) successional advance of angiosperms and negative growth trends in gymnosperms in the Iberian Peninsula (Gómez-Aparicio *et al.*, 2011; Carnicer *et al.*, 2013; Coll *et al.*, 2013). Under novel climatic conditions, favourable conditions in winter may be crucial in the competition between evergreen and deciduous tree species.

7.2.2 Species-specificity and leaf position

The highly dynamic Mediterranean climate has produced a diverse range of traits, taxa and species characterised with specialised adaptive mechanisms (Blumler, 1991; Öquist & Huner, 2003). The strategies to cope with the consequences of drought stress and to acclimate to variable growth temperatures were highly species-specific. Also, the responses varied notably between sunlit and shaded leaf positions. **We demonstrated that shaded leaves cushioned the effects of abiotic stress and were less affected by temperature extremes.** We postulate that the light regime of leaves in the canopy is a non-neglectable component in whole-canopy photosnythesis (Valladares & Niinemets, 2008; Niinemets, 2014). Responses specific to leaf position, however, differed among tree species due to distinct foliar morphologies and crown architectures.

Q. *ilex* is a late-successional, slow growing, water-spending, photoinhibition-avoiding, anisohydric tree species with a plastic hydraulic and morphological behaviour (Villar-Salvador *et al.*, 1997; Fotelli *et al.*, 2000; Corcuera *et al.*, 2004; Ogaya & Peñuelas, 2006; Limousin *et al.*, 2009). The water-spending, anisohydric strategy was reflected in maintained A_{net} and g_s in parallel with lower $V_{\text{c,max}}$ and J_{max}. The highly plastic hydraulic features were explained by its ability to repair yearly vessel diameter and recover xylem hydraulic conductivity after annual rainfalls (Fotelli *et al.*, 2000; Corcuera *et al.*, 2004). ***Q. ilex* had highly plastic photosynthetic and morphological traits and the most drastic photoprotective responses to extreme temperatures.** The morphological traits were highly adaptive to severe decreases in water availability developing a more succulent leaf structure in the dry year. The leaf position determined strongly the extent and quality of these responses in *Q. ilex*. For example, the comparatively higher photosynthetic values in sunlit leaves decreased partly below the level of shaded leaves under stress conditions. Shaded leaves are less exposed to the dramatic changes in radiation and temperature in the outer canopy and can be of particular importance for *Q. ilex* to attain a positive net carbon ratio during stress periods (Valladares *et al.*, 2008). We stress that the solar environment of the leaves is a crucial factor for assessing tree performance, especially in a competitive environment.

A. *unedo* - relict of the humid-subtropical Tertiary tree flora (Gratani and Ghia, 2002a and references therein) – is typically occurring as shrub or small tree in the macchia ecosystems with an evergreen leaf habit. In our study site it curiously reached the top forest canopy, but grew under domination of the other species. The subordinated position explains the higher proportion of shaded leaves and the low differentiation between sunlit and shaded leaves as also reflected in a high $J_{\text{max}}/V_{\text{c,max}}$ ratio throughout the crown. As we showed in Chapter 5, *A. unedo* is less photoinhibition-tolerant favouring carbon metabolic processes at the cost of chronic photoinhibition and photodamage. The water-spending behaviour is similar than *Q. ilex* maintaining A_{net} and g_s in parallel with lower $V_{\text{c,max}}$ and J_{max} (see also Beyschlag et al. 1986, Vitale and Manes 2005, Barbeta et al. 2012). Nonetheless, *A. unedo* was most vulnerable to drought-induced leaf abscission and branch dieback. *A. unedo* is known to be more susceptible to hydraulic dysfunction induced by depleted NSC (e.g. Rosas *et al.*, 2013). ***A. unedo* likely faced a trade-off between growing tall and risking hydraulic dysfunction due to high xylem tension un-**

der severe soil-water deficits (Choat *et al.*, 2012) **as it usually occurs as shrubby species in Maquis-biomes** (Beyschlag *et al.*, 1986; Harley *et al.*, 1986). Also, we observed a low degree of acclimation in the foliar morphology in the drier year. This underscores its semi-deciduous leaf habit under adverse conditions and tendency for drought-deciduousness.

Q. *pubescens* is a winter-deciduous sub-Mediterranean species that showed a low differentiation between sunlit and shaded leaves, similarly to *A. unedo.* In contrast to *A. unedo*, however, a low $J_{max}/V_{c,max}$ ratio in *Q. pubescens* leaves throughout the crown suggests a higher proportion of sunlit leaves. The leaf morphology showed a low degree of acclimation to drier conditions as also observed in *A. unedo*. The mesophyllic diffusion limitation was lowest in *Q. pubescens* and *A. unedo*, as we claim, due to their deciduous/semi-evergreen foliar habits and lower LMAs (see also Tomás *et al.*, 2014). *Q. pubescens* follows a similar drought- avoiding strategy as *Q. ilex*, but is less anisohydric (Zhou *et al.*, 2014) and maximizes gas exchange during a shorter growing season (Baldocchi *et al.*, 2009), resulting in high transpiration rates throughout the summer (Poyatos *et al.*, 2008; Sánchez-Costa *et al.*, 2015). **We showed that the "low-cost" leaves of the deciduous *Q. pubescens* facilitated drought senescence, so that the reduced transpiratory surface area effectively avoided damage from hydraulic cavitation and xylem embolism** (Ogaya & Peñuelas, 2006; Barbeta *et al.*, 2013). The remaining leaves compensated for the reduced total leaf area with high photosynthetic potentials and re-translocation of leaf nitrogen before leaf shedding. *Q. pubescens* had a higher growth-based water-use efficiency (WUE_{BAI} = Basal area increment/Tree transpiration) than *Q. ilex* during this period (Sánchez-Costa *et al.*, 2015). Fully refoliated crowns in the following growing season after the drought defoliation was evidence of its success relative to *A. unedo*.

P. *halepensis* is an early-successional, fast-growing, heliophilic conifer. The sun-exposed crown of *P. halepensis*, surmounting the forest canopy, resulted in a higher proportion of sunlit leaves. We thus found high photosynthetic potentials and a low $J_{max}/V_{c,max}$ ratio throughout the crown and a low degree of differentiation between sunlit and shaded leaves. *Pine* needles attain nearly saturated photosynthetic rates over a wide range of diurnal and seasonal variation in radiation due to their cylindrical shape and steep angles (Jordan & Smith, 1993; Lusk *et al.*, 2003). ***P. halepensis* was the most tolerant to photoinhibition and had the most robust photosynthetic machinery to combat abiotic stress** (Baquedano & Castillo, 2006; Sperlich *et al.*, 2014). *P. halepensis* exhibited a homeostatic behaviour with a very active carbon assimilatory and respiratory metabolism also under adverse winter conditions. *P. halepensis* showed a conservative water-use strategy and strict stomatal control of isohydric species under drought in order to avoid the loss of hydraulic conductivity through xylem embolism (Borghetti *et al.*, 1998; Martinez-Ferri *et al.*, 2000). *Pines* have a low capacity for storage of carbohydrates and repair of xylem embolism (Meinzer *et al.*, 2009), as explained in detail in Chapter 2. *P. hale-*

pensis had the highest WUE_{BAI} during severe drought (Sánchez-Costa *et al.*, 2015), through the combinatory effect of photosynthetic downregulation, foliar-trait acclimation, and improved gas exchange.

7.2.3 Summary and outlook

In summary, we postulate that the species-specific acclimation of foliar morphologial traits depended on functional differences of leaf investment costs and distinct leaf shedding strategies between deciduous/semi-evergreen (*Q. pubescens* and *A. unedo*) to evergreen sclerophyllic species (*Q. ilex* and *P. halepensis*). *Q. ilex* and *P. halepensis* invested more resources in protecting these leaves against abiotic stressors. The leaves of *A. unedo* and *Q. pubescens* acclimated least to changes in precipitation regimes, and were susceptible to foliar hydraulic dysfunction and drought-deciduousness.

The specific responses of water use efficiency and photosynthetic traits, however, depended rather on the contrasting photosynthetic strategies of the two phylogenetic plant groups than leaf habits. The gymnosperm *P. halepensis* was characterised with the highest stomatal control and most conservative water spending behaviour whereas the angiosperms *Q. ilex*, *Q. pubescens* and *A. unedo* showed a lower stomatal sensitivity. This is because gymnosperms have lower hydraulic safety margins and capacity for embolism repair than angiosperms, as explained in detail in Chapter 2 (Johnson *et al.*, 2012; Choat *et al.*, 2012; Meinzer *et al.*, 2013). *A. unedo* was more drought sensitive than the companion species and might be disadvantaged by prolonged climate stress (Ogaya & Peñuelas, 2004; Barbeta *et al.*, 2012). *Q. ilex*, *Q. pubescens* and *P. halepensis*, however, seemed to be equally successful in coping with drought and temperature stress despite following distinct ecophysiological strategies.

This seems to contrast with recent experimental evidence reporting increased dominance of *Quercus* species and negative growth trends in pines over extensive areas of the Iberian Peninsula, as summarized in Chapter 2 (Gómez-Aparicio *et al.*, 2011; Coll *et al.*, 2013). We speculate that age and succession play overriding roles in many old-growth pine stands. Our study site is exemplary for many pine-oak forests in the Iberian Peninsula, where shelter pine trees form the top canopy, followed by a dense layer of *Quercus* species (Zavala *et al.*, 2000). The dense *Quercus* canopy in our study site has suppressed the regeneration of the early-successional and light-demanding pine seedlings that need disturbances such as fire to regenerate (Zavala *et al.*, 2000). *Q. ilex* and *Q. pubescens* form the terminal point of secondary succession over extensive areas of the Mediterranean region (Lookingbill & Zavala, 2000). Carnicer *et al*. (2014) observed severe limitations of recruitment for most *Pinus* species acros the Iberian Peninsula. Pines might face a demographic decline in many pine-oak stands due to growth declines near the end of their life expectancy and due to recruitment failure because of human fire control. This could account for their vulnerability to abiotic stressors reported in these studies.

7.3 Impacts of long-term drought on photosynthesis and respiration

The carbon exchange of terrestrial ecosystems is one of the key processes of the global carbon cycle and results from a delicate balance between photosynthetic carbon uptake and respiratory release. Experiments under natural conditions with rainfall exclusion in the long-term can be regarded as valuable real-time model simulations for scenarios of future climate change. In Chapter 6, we investigated the concurrent limitations of photosynthesis comprising stomatal, mesophyllic and biochemical components as well as mitochondrial respiration in three seasonal campaigns. We aimed at evaluating the impact of long-term experimental drought on the foliar intrinsic water and carbon use efficiency (WUE_i and CUE_i) in order to understand better the boundaries and mechanisms of photosynthesis and respiration to seasonal acclimation and drought adaptation.

We provide additional evidence to the findings in Chapter 3 that g_m plays an second regulatory role in facilitating the CO_2 diffusion to the chloroplasts under long-term drought as shown by the increased ratio of $g_\mathrm{m}/g_\mathrm{s}$ (see also Galmés *et al.*, 2013). In contrast, the biochemical limitations ($V_\mathrm{c,max}$, J_max, TPU) seemed to have no impact on photosynthesis neither during the seasonal summer drought nor in combination with the long-term rainfall manipulation site. Beside the importance of the diffusive capacity of stomata and mesophyll for the foliar carbon balance, this balance also depends strongly on the relationship of photosynthesis with respiration. *Photosynthetic* responses to drought in Mediterranean vegetation have been extensively investigated, (for a review see Flexas *et al.*, 2014) whereas information about *respiratory* responses are particularly scant (Niinemets, 2014). **We identified R_d as the key player in *Q. ilex* for the foliar carbon balance being highly responsive to seasons or treatment effects.** We found that R_d was smaller than R_n and that the rainfall manipulation experiment increased the ratio of $R_\mathrm{d}/R_\mathrm{n}$ (0.79±0.04) compared to the control plot (0.71±0.03). Respiration can be reduced under water stress due to cessation of photosynthesis and growth, but respiration was also reported to increase under severe water stress (Ghashghaie *et al.*, 2001; Flexas *et al.*, 2006). The higher rates of R_d in the drought treatment are probably the result of an acclimated respiratory metabolism because the demands for energy (ATP and NADPH) for synthesis of sucrose and carbon skeletons in the cytoplasm are higher under stressful conditions (Flexas *et al.*, 2006; Zaragoza-Castells *et al.*, 2007). This is because mitochondrial respiration initiates the construction of heat stabilizing components such as heat shock proteins or BVOCs protecting the plant against detrimental effects (Tcherkez & Ribas-Carbó, 2012; Morfopoulos *et al.*, 2014). We also showed in Chapter 6 that R_d decreased in the drought group during summer and reached the lower value of the control group. This is interesting because R_d was generally higher in the drought treatment compared to the control group. **We thus conclude that the drought treatment increased the foliar carbon use efficiency in summer through high photosynthesis rates concurrent with**

a decrease of R_d. This might be explained with the relatively favourable growth conditions in the summer period characterised with high temperatures and radiation, but also high water availability. The individuals under drought treatment seemed to benefit most strongly from these conditions. Moreover, the treatment effect of the rainfall exclusion in the long-term was shown to result in a higher stem mortality (Barbeta *et al.*, 2013) and in a reduced leaf area in *Q. ilex* (Ogaya & Peñuelas, 2006). Most certainly, fewer leaves disposed of higher biochemical resources and thus compensated for the lower leaf area with higher photosynthetic rates and carbon use efficiency, as also shown in Chapter 3. **We underscore the findings of Chapter 3 attesting *Q. ilex* a high plasticity to respond to changes in temperature or soil water.**

7.4 Implications for the global carbon cycle and for modelling

The significant seasonal acclimation of $V_{c,max}$ and J_{max} observed in our study demonstrates that prognostic models should account for seasonal variation, especially in drought-prone areas. Indeed, it has been shown that the use of seasonally variable photosynthetic potentials can reduce uncertainties in modelled ecosystem carbon fluxes relative to the use of constant values (Wilson *et al.*, 2001; Tanaka *et al.*, 2002; Kosugi *et al.*, 2003, 2006; Medvigy *et al.*, 2013). Also, the significant role of g_m under abiotic stress highlights its importance for estimating the whole-tree carbon gain. There is an ongoing debate on how to include these physiological findings into models. Traditionally, A/C_i curves have been used to derive $V_{c,max}$ and J_{max}. Terrestrial biosphere models are most commonly calibrated on A/C_i-based parameters and therefore use apparent values of $V_{c,max}$ and J_{max}. The ecophysiological community, however, has now recognized that using these apparent values is - physiologically speaking - incorrect. This approach underestimates the true values of $V_{c,max}$ and J_{max} (calculated based on estimates of g_m) up to 75 and 60% (Sun *et al.*, 2014). We confirmed these findings in Chapter 3 and 6. However, incorporating the true values of $V_{c,max}$ and J_{max} parameterised on A/C_c curves into ecosystem carbon flux models would certainly lead to erroneous results, because the sub-models of photosynthesis would require the incorporation of g_m and different Rubisco kinetic parameters. The use of consistent equations and parameters is vital to correctly estimate photosynthesis when parameters from experimental work are integrated into vegetation models (Rogers *et al.*, 2014). From a modeller's point of view, the question arises weather the inclusion of g_m and A/C_c– based parameters would not just increase model complexity without improving simulation accuracy. They would argue that, despite their use of apparent $V_{c,max}$ and J_{max}, terrestrial biosphere models are currently well calibrated against observational data. Also, the potential errors in various methods to estimate g_m (and subsequently $V_{c,max}$ and J_{max}) including the variable *J*- method (used in this study) are often criticized (Pons *et al.*, 2009; Tholen *et al.*, 2012; Gu & Sun, 2014). Nonetheless, there remain large uncertainties in the simulations of the future CO_2 fluxes of the global carbon cycle (Anav *et al.*, 2013; Friedlingstein *et al.*, 2014). In these models, the response pattern of temperature and precipitation are highly uncertain due to both a lack of scientific understanding and model representation (Booth *et al.*, 2012). These uncertainties could be the cause for the poor modelling performance in arid or semi-arid ecosystems. It was shown that the mechanistic description of the photosynthetic processes under water stress is not very well developed (Morales *et al.*, 2005; Keenan *et al.*, 2011; Zheng *et al.*, 2012; Vargas *et al.*, 2013). We have shown that the limitations imposed by g_m on photosynthetic assimilation can decrease relatively more than the limitations imposed by g_s or biochemistry ($V_{c,max}$ and J_{max}) under abiotic stress conditions such as drought or winter. This distinction has important consequences for the control of water-use efficiency. g_m thus holds great potential for improving the estimation of ecosystem carbon fluxes under drought conditions (Niinemets et al. 2009a). For example, Keenan *et al.* (2010a) found that g_m was the missing constraint for accurately capturing the response of terrestrial vegetation productivity to drought. Moreover, Flexas *et al.*, (2013) recently gave a clue on how mesophyll conductance might be coordinated with leaf hydraulic conductance to regulate leaf function and plant performance. As pointed out above, the issue of whether (and how) to include g_m in simulations is actively debated by physiologists and modellers (see also Rogers et al. 2014). Surprisingly relatively little information is available from modelling exercises that have included g_m in their algorithms. Another key player in photosynthesis modelling is foliar respiration. In Chapter 6, we provided evidence that the differentiation between night respiration and day respiration is vital to correctly estimate the foliar carbon balance in different seasons. Currently, most terrestrial biosphere models do neither correctly represent the light inhibition of foliar respiration nor the fractional seasonal changes (Smith & Dukes, 2013; Huntingford *et al.*, 2013). This might have important consequences for the simulated net C exchange and C storage considering that roughly half of plant respiration comes from leaves (Atkin *et al.*, 2007)

In summary, we urge the need to conduct more research on how we can improve carbon flux simulations by including existing empirical evidence such as the seasonality of key foliar respiratory and photosynthetic traits as well as incorporating g_m, the unappreciated but key player in photosynthesis.

7.5 References

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8

Spring in Collserola
Photo & Design: D. Sperlich

8. General conclusion

Chapter 2

Contrasting demographic responses in Mediterranean conifer and angiosperm trees are currently occurring, due to both widespread forest successional advance and to divergent growth responses to temperature. Trait-based differences in these two groups contribute to explain their different responses to temperature and their different role during successional processes. Multiple and interacting factors determine contrasting growth responses to temperature. Reciprocal common garden experiments may offer a very promising tool to develop integrative tests of the diverse factors reviewed and to simulate the synergistic negative effects of forest successional advance and climate warming on conifer species.

Chapter 3

The acclimation behaviour of photosynthetic and morphological traits to seasonal variable growth conditions was strongly pronounced in all tree species. The replenishment of soil-water reserves during the early growing season was critical to endure seasonal drought periods in Mediterranean trees. We postulate that photosynthetic machineries were resilient to moderate drought, whereas severe drought induced foliar trait acclimation, photosynthetic downregulation and leaf abscission. The relative limitation of g_s and g_m on photosynthesis was strongly determined by the severity of the drought. However, the responses to drought and temperature stress were highly species-specific. We underline that we need to consider the seasonality of photosynthetic potentials and mesophyll conductance to explain ecophysiological responses to abiotic stress. These two factors should deserves much more attention in terrestrial biosphere modelling because they hold great potential to reduce model uncertainties, especially under Mediterranean climatic conditions.

Chapter 4

We observed a pronounced seasonal acclimation of the thermal optima and also of the curvature of temperature responses of photosynthesic assimilation. The peaked function modelled the observed temperature responses better than June's model. The mean temperature optima of A_{net} and J_{cf} across all species and seasons were 24.7±0.5 and 30.3±0.6 °C, respectively, but varied significantly between seasons; yet the shapes of the response curves were only partly influenced. Moreover, species-specific acclimation partly offset these overall trends. *Q. ilex* showed the highest plasticity whereas *P. halepensis* was most tolerant with the most stable temperature response pattern. In general, the photosynthetic system was primarily impeded by high, and not low, temperatures and was better acclimated to heat stress in the drier and hotter year. This indicates that Mediterranean climax species exhibit a strong acclimatory capacity to warmer and drier conditions.

Chapter 5

The photosynthetic exploitation of relatively favourable winter conditions might be crucial for evergreen Mediterranean tree species for achieving a positive annual carbon budget. Mild winter temperatures can provide periods of growth and recovery for the evergreen trees from stressful summer droughts that resulted in biochemical recovery, new shoot growth, and moderate transpiration across all evergreen species However, when clear skies and high radiation coincide with low temperatures in winter, they can have a combinatory negative effect on the photosynthetic apparatus leading photoinhibitory stress - especially in sunlit leaves. *A. unedo* was hereby most vulnerable whereas *Q. ilex* and *P. halepensis* seemed to cope equally well with winter stress despite contrasting photoprotective strategies. The winter period might give important insights in the dynamics of Mediterranean forest communities when withstanding increased novel environmental conditions projected in multiple climate change scenarios and benefitting from periods of potential recovery and growth in winter.

Chapter 6

A high climatic variability in the Mediterranean region can lead to counterintuitive effects displaying the peak photosynthetic activity in the summer period which is usually characterised with a high level of abiotic stress. The trees experiencing 14 year-long drought treatment adapted through a higher plasticity in photosynthetic traits, so that eventually an unexpected favourable growth period in summer was exploited more efficiently - with g_{m} and R_{d} as key parameters. On the long-term, drought induced growth declines might be attenuated through morphological and physiological acclimation to drought (Leuzinger *et al.*, 2011; Barbeta *et al.*, 2013). The fact that the photosynthetic potentials in the rainfall manipulation site were not different to the control group seems to underpin the dampening effect also on a biochemical level.

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Published/Accepted

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Chapter 3

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Contrasting trait syndromes in angiosperms and conifers are associated with different responses of tree growth to temperature on a large scale

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Recent large-scale studies of tree growth in the Iberian Peninsula reported contrasting positive and negative effects of temperature in Mediterranean angiosperms and conifers. Here we review the different hypotheses that may explain these trends and propose that the observed contrasting responses of tree growth to temperature in this region could be associated with a continuum of trait differences between angiosperms and conifers. Angiosperm and conifer trees differ in the effects of phenology in their productivity, in their growth allometry, and in their sensitivity to competition. Moreover, angiosperms and conifers significantly differ in hydraulic safety margins, sensitivity of stomatal conductance to vapor-pressure deficit (VPD), xylem recovery capacity or the rate of carbon transfer. These differences could be explained by key features of the xylem such as non-structural carbohydrate content (NSC), wood parenchymal fraction or wood capacitance. We suggest that the reviewed trait differences define two contrasting ecophysiological strategies that may determine qualitatively different growth responses to increased temperature and drought. Improved reciprocal common garden experiments along altitudinal or latitudinal gradients would be key to quantify the relative importance of the different hypotheses reviewed. Finally, we show that warming impacts in this area occur in an ecological context characterized by the advance of forest succession and increased dominance of angiosperm trees over extensive areas. In this context, we examined the empirical relationships between the responses of tree growth to temperature and hydraulic safety margins in angiosperm and coniferous trees. Our findings suggest a future scenario in Mediterranean forests characterized by contrasting demographic responses in conifer and angiosperm trees to both temperature and forest succession, with increased dominance of angiosperm trees, and particularly negative impacts in pines.

Keywords: conifers, angiosperms, functional traits, mediterranean ecosystems, drought, temperature, carbon metabolism, growth

INTRODUCTION

The assimilation and allocation of carbon are fundamental processes allowing tree growth, development, survival, reproduction and defense (McDowell, 2011; Galiano et al., 2012; Sala et al., 2012). In addition, non-structural carbohydrates (NSCs) play a variety of functions in tree physiology, providing a temporal buffer to reconcile differences in carbon supply and demand, maintaining hydraulic transport and facilitating osmotic regulation, allowing leaf emergence and bud burst and actively participating in the prevention of frost and drought embolism and repair (Sala et al., 2012). The demographic performance of trees, however, is generally co-limited by other factors that frequently interact in complex ways with the processes of carbon uptake and allocation, such as direct climatic effects on photosynthesis, growth and nutrient uptake (Körner, 1998, 2003; Rennenberg et al., 2006), species-specific traits (Wright et al., 2004; Chave et al., 2009; Carnicer et al., 2012) or the impacts of secondary consumers and diseases (Bale et al., 2002).

Recent ecophysiological studies highlight the coupled dynamic links between NSC content in woody tissues and several climate-dependent tree responses such as embolism prevention and repair, growth, bud burst and leaf emergence (Johnson et al., 2012; Sala et al., 2012; Meinzer and McCulloh, 2013). These studies suggest the existence of contrasting trait-based ecophysiological strategies in major plant groups (Choat et al., 2012; Johnson et al., 2012; Meinzer et al., 2013) such as angiosperm and coniferous trees. Arguably, a next necessary step is to analyze how these contrasting ecophysiological strategies may be influencing the distribution and abundance of tree species and their responses to global warming.

Recent large-scale studies have reported contrasting responses of growth to temperature in angiosperm and coniferous trees in

Mediterranean forests of the Iberian Peninsula (Gómez-Aparicio et al., 2011; Coll et al., 2013). For example, Gómez-Aparicio et al. (2011) reported a positive effect of rising temperatures on growth of angiosperm trees, but neutral or negative effects on coniferous trees. These contrasting trends between the two phylogenetic groups were later also observed and confirmed by Coll et al. (2013). Critically, whereas a reduction in precipitation was predicted to decrease tree growth in both groups, increases in temperature could produce a performance disadvantage in conifers compared to angiosperm broadleaved trees (Gómez-Aparicio et al., 2011; Coll et al., 2013). Consistent with these empirical findings that associate the negative effects of temperatures and growth in *Pinus* species, palaeoecological studies suggest a persistent link between Pinaceae distributions and low temperatures during the last 100 million years (Millar, 1993; Brodribb et al., 2012). Cold periods in the Paleocene and Eocene are associated with an increased abundance of fossils of the genus *Pinus*, and the reverse occurs during warm periods (Millar, 1993; Brodribb et al., 2012). Similarly, warm periods during the Miocene and Pliocene are apparently associated with northward contractions of the ranges of Pinaceae species (Millar, 1993; Brodribb et al., 2012). Notably, the ecophysiological basis of these contrasting growth and distributional responses to temperature remain poorly discussed and resolved.

Here we review the hypotheses that may contribute to explain the observed contrasting responses of growth to temperature observed in Mediterranean conifers and angiosperms. We review the differences between Mediterranean conifer and angiosperm trees in growth-related traits, including phenology, crown allometry, sensitivity to competition, and drought and winter freezing responses. Furthermore, we hypothesize that angiosperm and coniferous ecophysiological strategies differentially integrate diverse traits such as stomatal sensitivity to vapor-pressure deficit (VPD), hydraulic safety margins and capacity for embolism repair, which in turn are linked to features of the xylem such as NSC content, carbon transfer rates, wood parenchymal fraction and wood capacitance. In sum, our main aims in this study are: (i) to list the different hypotheses that may explain contrasting growth responses to temperature in Mediterranean conifer and angiosperm trees and review the differences in ecophysiological traits associated with temperature- and drought-induced responses in these two groups, (ii) to briefly review the multiple effects of temperature on basic tree ecophysiological functions (e.g., photosynthesis, growth, respiration and nutrient uptake and transport), (iii) to analyze the specific case study of forests in the Iberian Peninsula, which present diverging tree growth responses to temperature in Angiosperms and Conifers, and (iv) to briefly discuss the implications of our findings. Below we dedicate a section to each of these objectives.

A REVIEW OF THE DIVERSE HYPOTHESES THAT MAY EXPLAIN CONTRASTING GROWTH RESPONSES TO TEMPERATURE IN MEDITERRANEAN AND ANGIOSPERM TREES

Table 1 lists the different hypotheses that may explain contrasting growth trends to temperature in Mediterranean conifer and angiosperm trees. The first hypothesis (**Table 1.1**) states that positive growth responses to increased temperature in angiosperms could be mediated by a less strict stomatal control, allowing them to assimilate carbon for longer during warmer and drier periods. While this could imply that angiosperm could be more vulnerable to xylem cavitation and hydraulic failure, they have a greater capacity for embolism repair. On the other hand, most conifers function with a wider hydraulic safety margin to avoid cavitation but with the cost of lower carbon gain. Beside this specific hypothesis, several other factors could also contribute to explain the differences in growth responses between conifer and angiosperm trees. For example, these two groups differ in the effects of phenology in their productivity, in the sensitivity of growth to competition, and in growth allometry (**Table 1.2–1.4**). In addition, local adaptation processes and phenotypic plasticity also largely influence tree growth responses to temperature and drought (**Table 1.5–1.9**). Finally, the available empirical evidence suggests that the diverse factors significantly interact in determining growth responses (**Table 1.7**). For example, several studies report strong interactions between tree size, drought, and stand density effects in determining large-scale growth patterns in the Mediterranean basin. Below we briefly review the hypotheses listed in **Table 1** and discuss the experimental tests required to assess their relative importance.

ECO-PHYSIOLOGICAL AND HYDRAULIC TRAITS. DIFFERENT ECOPHYSIOLOGICAL AND CARBON-ALLOCATION STRATEGIES IN ANGIOSPERMS AND CONIFERS (HYPOTHESIS 1.1)

Table 2 summarizes the trait differences between angiosperm and coniferous trees. Key traits that differ between these two groups include stomatal sensitivity to VPD, xylem anatomy, foliar traits, hydraulic safety margins, capacity for embolism repair, NSC content, carbon transfer rates, wood parenchymal fraction, and wood capacitance. The available published evidence shows that these diverse traits are functionally related and define two contrasting ecophysiological strategies in conifers and angiosperms. Compared to angiosperms, conifers have a lower stomatal-conductance sensitivity to increased VPD (*sensu* Johnson et al., 2012). In turn, this key difference in stomatal response appears to be tightly related to the different hydraulic safety margins in both groups (Tyree and Sperry, 1988; Nardini et al., 2001; **Table 2**). The wider hydraulic safety margins in conifers thus imply early responses of stomatal closure, which reduce hydraulic conductivity before substantial cavitation occurs. On the other hand, angiosperms can maintain relatively high stomatal conductances even when the xylem pressure caused by high VPD is sufficient to induce extensive cavitation (Meinzer et al., 2009, 2013; Johnson et al., 2012).

In support of these trends, Choat et al. (2012) recently reported that species in coniferous forests generally have a higher resistance to drought-induced cavitation and operate with wider hydraulic safety margins than do angiosperms. The minimum xylem pressures in conifers measured in the field were more positive than the xylem pressures causing a 50% loss of hydraulic conductivity, and thus the risk of hydraulic failure by collapse of the water-conducting system was low. In contrast, the hydraulic

Table 1 | Main hypotheses that may contribute to explain contrasting growth responses to temperature in Iberian Angiosperm and Conifer trees on a large scale.

Hypotheses	Angiosperms	Conifers	References
1.1 Eco-physiological and hydraulic traits	Narrower hydraulic safety margins and higher capacity to reverse embolisms	Wide hydraulic safety margins, early drought-induced stomatal closure and lower carbon gain, low stomatal conductance sensitivity to VPD	Martínez-Ferri et al., 2000; Brodersen et al., 2010; Choat et al., 2012; Epron et al., 2012; Johnson et al., 2012; Michelot et al., 2012; Sala et al., 2012; Brodersen and McElrone, 2013; Coll et al., 2013; Meinzer et al., 2013; Ogasa et al., 2013
1.2 Phenology	Tree productivity more sensitive to growing season length	Positively affected but less sensitive to growing season length	Churkina et al., 2005; Piao et al., 2007; Welp et al., 2007; Delpierre et al., 2009; Richardson et al., 2010; Gómez-Aparicio et al., 2011; Coll et al., 2013
1.3 Intra- and inter-specific competition and forest succession	Growth less sensitive to intra and inter-specific stand competition	Growth severely reduced by intra- and inter-specific competence in small, non-dominant trees	Sánchez-Gómez et al., 2008; Gómez-Aparicio et al., 2011; Carnicer et al., 2013a; Coll et al., 2013; Vayreda et al., 2013
1.4 Size, age and allometry	Different growth allometry and less apical dominance	Peak of crown growth reached at lower sizes	Gómez-Aparicio et al., 2011; Poorter et al., 2012
1.5 Drought and temperature	Angiosperm trees are able to maintain substantial transpiration levels during summer drought events	Drought and heat waves often results in early stomatal closure in Mediterranean conifers	Martínez-Ferri et al., 2000; de Luis et al., 2007, 2011; Zweifel et al., 2007; Eilmann et al., 2009; Camarero et al., 2010; Klein et al., 2011; Coll et al., 2013; Poyatos et al., 2013
1.6 Winter freezing	Angiosperm trees are more vulnerable to freeze-thaw embolism	Less sensitive to freeze-thaw embolism	Sperry and Sullivan, 1992; Gómez-Aparicio et al., 2011; Brodribb et al., 2012
1.7 Interactions between multiple factors	Yes	Yes	Linares et al., 2010; Gómez-Aparicio et al., 2011; Vayreda et al., 2012; Coll et al., 2013; Ruiz-Benito et al., 2013
1.8 Local adaptation, individual and provenance variation	Yes	Yes	Rehfeldt, 1978, 1982; Santos et al., 2010; Ramírez-Valiente et al., 2010, 2011; Chmura et al., 2011; Robson et al., 2012; Alberto et al., 2013
1.9 Phenotypic plasticity	Yes	Yes	Camarero et al., 2010; Nicotra et al., 2010; de Luis et al., 2011

safety margins reported for angiosperms were narrower, being slightly positive or even negative.

The reported differences in stomatal sensitivity and hydraulic safety margins have in turn been functionally associated with different responses between both groups in the capacity of xylems to recover from embolisms. Recent studies have reported higher capacities in species with narrow safety margins and higher stomatal sensitivities to VPD (see Johnson et al., 2012 for a precise definition of stomatal sensitivity to VPD; Meinzer et al., 2013). The reversal of cavitation has been demonstrated to be feasible on an hourly or daily basis and to occur even under high xylem tension (Hacke and Sperry, 2003; Salleo et al., 2004; Brodersen et al., 2010; Zufferey et al., 2011). Two general but contrasting hydraulic strategies arise: (i) high cavitation resistance, low stomatal sensitivity to VPD and low resilience (gymnosperms) and (ii) low cavitation resistance but high resilience (angiosperms).

These two basic strategies are in turn functionally linked to anatomical differences in cell anatomy, NSC content, wood parenchymal fraction, and wood density (**Table 2**). For example, both the percentage of living parenchyma and the concentration of NSCs in the xylem are significantly higher in angiosperms than in conifers (Johnson et al., 2012 and citations therein). During the reversal of embolisms, vessel refilling probably requires an input of energy (Meinzer et al., 2013) and the mobilization of stored carbohydrates. Living wood parenchyma thus acts as a reservoir of both water and carbohydrates. Hence, NSCs stored in cells surrounding vessels are likely to be the source of sugars needed for the maintenance of vascular integrity (Brodersen et al., 2010;

Table 2 | Summary of differences in key functional traits between conifers and angiosperms.

Trait	Angiosperms	Conifers	References
Wood anatomy	Vessels Ring-porous and diffuse-porous Homogeneous pit membrane Cylindrical phloem sieve elements Companion cells	Tracheids Torus-margo pit membrane Cuboidal phloem sieve elements Strasburger cells	Brodribb et al., 2012 Jensen et al., 2012
Wood parenchymal fraction	High	Low	Nardini et al., 2011; Meinzer and McCulloh, 2013
Woody-tissue NSC content	High	Low	Hoch et al., 2003; Michelot et al., 2012
Wood density	High	Low	Poorter et al., 2012
Xylem embolism recovery capacity	High	Low	Bucci et al., 2003; Salleo et al., 2004; Brodribb et al., 2010
Sensitivity to freeze-thaw embolism	High	Low or absent	Cavender-Bares et al., 2005
Hydraulic safety margins	Narrow or negative	Wide	Choat et al., 2012
Water potential causing 50% loss of hydraulic conductivity	Low	High	Choat et al., 2012
Xylem capacitance	High (ring-porous) Medium (diffuse-porous)	Low	Meinzer and McCulloh, 2013
Rate of C transfer	High	Low	Jensen et al., 2012
Sap flow velocity	High	Low	Jensen et al., 2012
Phloem conductivity	High	Low	Jensen et al., 2012
Phloem sieve-element resistance	Low	High	Jensen et al., 2012
Leaf lifespan	Shorter	Longer	Lusk et al., 2003
Shade tolerance	High	Low	Poorter et al., 2012
Interspecific shade-tolerance/drought-tolerance trade-off	Yes	Yes	Niinemets and Valladares, 2006
Mesophyllic conductance	High	Low	Niinemets et al., 2011
Photosynthetic capacity	High	Low	Lusk et al., 2003; Flexas et al., 2012
Stomatal density	High	Low	Flexas et al., 2012
Stomatal conductance sensitivity to VPD (m)	High (ring-porous) Medium-low (diffuse-porous)	Low	Johnson et al., 2012; Barbeta et al., 2013; Meinzer et al., 2013; Poyatos et al., 2013
Distal leaf and root embolism and refilling	Rare	Frequent	Johnson et al., 2012

Sala et al., 2012). Sugars are possibly transferred from parenchymal cells to embolized vessels for establishing a gradient to drive water away from either the phloem (Nardini et al., 2011) or non-embolized vessels (Brodersen et al., 2010). Furthermore, Améglio et al. (2004) reported the catabolism of starch into sugars and the subsequent efflux from parenchymal cells to the vessels in late winter during the recovery of *Juglans regia* from cavitation induced by the winter freeze-thaw. Likewise, the reported differences between the capacities to reverse embolisms in angiosperms and conifers (Johnson et al., 2012; Brodersen and McElrone, 2013; Meinzer et al., 2013) are likely associated with the differences in sapwood NSC content between these two groups reported by Hoch et al. (2003). This empirical evidence suggests that NSC reserves in wood parenchymal cells play a key role in determining the hydraulic strategies of plants, because species with high NSC and parenchymal fractions would have a higher resilience to cavitation and thus could withstand a certain loss of hydraulic conductivity.

Finally, conifers and angiosperms also differ in cell anatomy and wood density (**Table 2**), and several studies suggest functional implications for these traits in climate-induced responses. For example, wood density has been proposed as a good predictor of the resistance of the xylem to drought stress, because species with denser wood tend to have a higher resistance to cavitation (Jacobsen et al., 2007; Pratt et al., 2007). Moreover, Ogasa et al. (2013) found a negative correlation between wood density and xylem recovery in deciduous angiosperm trees (*Salix*, *Betula, Carpinus, Cerasus*), suggesting in turn a negative association between increased cavitation resistance and resilience of xylem function. Wood density in Mediterranean evergreen shrubs was also negatively correlated with the percentage of parenchymal area in the xylem (Jacobsen et al., 2007). This correlation is consistent with the higher capacity of xylems to recover in species with wood of lower density reported by Ogasa et al. (2013), because living xylem parenchyma may be involved in the reversal of embolisms (Bucci et al., 2003; Brodersen et al., 2010; Nardini et al., 2011; Zufferey et al., 2011; Brodersen and McElrone, 2013). In addition, low wood density has been associated with high capacitance (Pratt et al., 2007; Sperry et al., 2008; McCulloh et al., 2012). In water-stressed plants, a higher capacitance facilitates the transient release of water stored in living wood cells to the conduit lumen, increasing xylem water potential (Meinzer et al., 2009; Barnard et al., 2011; Zhang et al., 2011).

The higher resistance of conifers to both freeze-thaw and drought-induced cavitation (Sperry and Sullivan, 1992; Wang et al., 1992; Choat et al., 2012) has also been associated with differences in wood anatomy (**Table 2**). The main difference in wood anatomy between angiosperms and gymnosperms is that the latter have tracheids that also provide mechanical strength (Hacke et al., 2001; Poorter et al., 2012). In particular, thick conduit walls providing mechanical strength have been suggested as the factor limiting the size of tracheids in conifers (Pittermann et al., 2006). Small tracheids are less prone to freeze-thaw cavitation in conifers (Tyree and Zimmermann, 1988; Sperry and Sullivan, 1992; Pittermann and Sperry, 2003), as are small vessels in angiosperms (Sperry and Sullivan, 1992), in which other woody cells such as fibers are responsible for mechanical support of the plant. In both groups, however, no direct relationship has been found between conduit size and drought-induced cavitation across species. Pit membrane area, though, must be limited (as it is where air-seeding develops) to achieve a certain level of safety from drought-induced cavitation, which in turn limits the surface area and thus the size of conduit cells (Hacke et al., 2006; Jansen et al., 2009; Brodribb et al., 2012).

We hypothesize that the reported trait differences between conifers and angiosperms (**Table 2**) constitute two different strategies that may imply qualitatively different growth responses to increased temperatures and drought in the Mediterranean region. The different stomatal responses to heat waves and summer droughts, inducing drought-avoidance strategies and stomatal closure in conifers, would be key to determining these different growth responses (Martínez-Ferri et al., 2000; Coll et al., 2013; Poyatos et al., 2013). Critically, the higher sensitivity of the stomatal conductance to increases in VPD in conifers may promote near-zero assimilation rates and may strongly limit carbon uptake and photosynthesis over extended periods (Martínez-Ferri et al., 2000; Johnson et al., 2012; Meinzer et al., 2013; Poyatos et al., 2013). Summer drought may strongly affect carbon dynamics and NSC mobilization and consumption in both conifers and angiosperms, for example by enhancing the catabolism of starch to soluble sugars for increasing xylem tension and sap osmolarity (Sala et al., 2012), mobilizing NSCs for embolism repair, producing soluble sugars to stabilize cellular proteins and membranes, stopping cell division and tree growth favoring in turn the accumulation of photosynthates in starch (Peñuelas and Estiarte, 1998; Estiarte and Peñuelas, 1999; Körner, 2003) or promoting increased allocation of NSCs in roots and declines in fine-root biomass (Anderegg, 2012). Even though the coupled effect of these complex processes on the carbon balance of the tree may be quite variable (species and site specific), we suggest that early stomatal closure and the associated larger reductions of assimilation rates in conifers may consistently produce a more negative impact on both carbon balance and growth responses of trees.

On the other hand, increased winter temperatures can reduce the costs associated with the impacts of freeze-thaw embolism and may also differently affect the carbon balance of angiosperms and conifers. Critically, angiosperms have a higher sensitivity to freeze-thaw embolism (**Table 2**) and may experience higher costs. This group could thus benefit more from increased winter temperatures. Higher winter temperatures would thereby entail fewer freeze-thaw cavitations, which are responsible for the almost complete loss of hydraulic conductivity in ring-porous species and for the partial loss in diffuse-porous species by late winter (Sperry and Sullivan, 1992). The restoration of water transport in angiosperms is achieved by the production of earlywood or by vessel refilling, which have carbon demands supplied by NSCs (Barbaroux and Bréda, 2002; Epron et al., 2012; Michelot et al., 2012). In contrast, since the xylems of conifers are highly resistant to freeze-thaw cavitation (Sperry and Sullivan, 1992; Brodribb et al., 2012), this group may not have very different NSC costs for the restoration of water transport after mild or cold winters.

Winter temperature is a major driver for switching carbon allocation either to storage or to growth and respiration (Epron et al., 2012; Körner, 2013) and for the conditioning accumulation of starch (Oleksyn et al., 2000). When temperature is too low for growth, carbon assimilation is still significant, so NSCs derived from winter photosynthesis are mainly allocated to storage during cold periods (Rossi et al., 2008; Fajardo et al., 2012). In addition, the catabolism of starch into soluble carbohydrates during cold periods may possibly maintain intracellular osmotic concentration, which is positively correlated with cold hardiness (Cavender-Bares et al., 2005; Morin et al., 2007). In both conifers and angiosperms, increased winter temperatures are likely to alter cambium activation, growth allocation and the dynamic balance among winter photosynthesis, starch storage, and soluble sugar concentrations.

Finally, increased winter, spring and autumn temperatures can significantly influence phenological responses, advancing winter cambium activation, spring bud burst and leaf unfolding or delaying autumn leaf fall (Peñuelas and Filella, 2001). The derived extension of the phenological period could have strong effects on tree height and growth (Vitasse et al., 2009a,b, 2013; Lenz et al., 2012). Both the phenological cycles and the growth-associated carbon dynamics, however, are qualitatively different in conifers, ring-porous deciduous trees, diffuse-porous deciduous trees, and evergreen oaks (Epron et al., 2012; **Table 3**). These differences suggest that these groups may qualitatively differ in the relative effects of increased spring temperatures on carbon dynamics and tree growth. For example, an increase in temperature early in the growing season may also increase vessel diameter in deciduous angiosperms but not in conifers (Matisons and Brumelis, 2012).

PHENOLOGY (HYPOTHESIS 1.2)

An average lengthening of the growing season of about 11 days has been detected in Europe from the early 1960s to the end of the twentieth century (Menzel and Fabian, 1999; Peñuelas and Filella,

Table 3 | A brief summary of the seasonal dynamics of NSCs and growth phenology in deciduous broadleaf, evergreen broadleaf and coniferous trees.

	Winter	Spring	Summer	Autumn
Deciduous angiosperm trees	Loss of hydraulic conductivity due to freeze-thaws, being higher in ring-porous than in diffuse-porous species (Sperry and Sullivan, 1992; Wang et al., 1992; Cavender-Bares et al., 2005; Michelot et al., 2012). Before bud burst, some species may refill embolized vessels using NSCs (Améglio et al., 2004).	The onset of radial growth occurs before bud burst in ring-porous species and after bud burst in diffuse-porous species (Michelot et al., 2012). NSCs contribute to growth in both ring- and diffuse-porous species (Epron et al., 2012) but more importantly in ring-porous species (Barbaroux and Bréda, 2002; Palacio et al., 2011; Michelot et al., 2012). Starch content decreases in ring-porous trees, and sugars decrease in diffuse-porous trees (Michelot et al., 2012). Milder winter temperatures may favor the formation of wider vessels in ring-porous species in early spring (Matisons and Brumelis, 2012). Extended growing season with higher spring temperatures (Peñuelas et al., 2002; Gordo and Sanz, 2010).	NSCs in leaves decrease from summer through autumn (Hoch et al., 2003). The soluble fraction of NSCs is used to maintain xylem and phloem integrity and cell turgor under drought conditions (Sala et al., 2012). The soluble fraction increases in diffuse-porous species (Michelot et al., 2012). Another study, though, did not observe an increase in soluble fractions or observed reductions (Hoch et al., 2003). Higher stomatal conductance and dynamic embolism repair capacity may allow C assimilation even under a certain degree of water deficit (Johnson et al., 2012).	Allocation of carbon to storage (Epron et al., 2012). Extended growing season (Peñuelas et al., 2002; Vitasse et al., 2009a,b; Gordo and Sanz, 2010). An increase of drought-induced embolism may also lead to premature leaf abscission (Wang et al., 1992).
Evergreen angiosperm trees	Reduced losses in hydraulic conductivity caused by freeze-thaws, although evergreen trees are more resistant than deciduous species (Cavender-Bares et al., 2005). C assimilation allocated mainly to storage when temperature is too low for growth (Körner, 2003). NSC reserves increase throughout the winter (Rosas et al., 2013). Annual peak in photosynthetic rates for some species (Ogaya and Peñuelas, 2003).	Decline in NSC content by late spring (Rosas et al., 2013), probably invested in growth. As in deciduous trees, vessel diameter is also constrained by winter temperatures (Cavender-Bares et al., 2005). Extended growing season with higher temperatures (Peñuelas et al., 2002; Gordo and Sanz, 2010).	NSCs in leaves decrease from summer through autumn (Hoch et al., 2003). The soluble fraction of NSCs is used to maintain xylem and phloem integrity and cell turgor under drought conditions (Sala et al., 2012). The soluble fraction peaks in summer in some species (Rosas et al., 2013). Do not close stomata completely even under high evaporative demand and low soil water content (Ogaya and Peñuelas, 2003; Barbeta et al., 2012). Narrower xylem vessels than in deciduous oaks reduce losses of hydraulic conductance (Sperry and Sullivan, 1992; Wang et al., 1992 in other species).	Allocation of carbon to storage (Epron et al., 2012; Rosas et al., 2013). Mediterranean evergreens sometimes have a growth peak in autumn (Gutiérrez et al., 2011).

(Continued)

Table 3 | Continued

	Winter	Spring	Summer	Autumn
Conifers	Freeze-thaw resistant species. No accumulated losses in hydraulic conductivity (Wang et al., 1992). Low temperatures may result in an increase of NSCs (Hoch, 2008; Fajardo et al., 2012; Gruber et al., 2012; Hoch and Körner, 2012). High minimum temperatures may advance earlywood formation in Mediterranean conifers (Pasho et al., 2012).	Carbohydrate demand of new-leaf cohorts is supplied mainly by older cohorts (Eilmann et al., 2010; Michelot et al., 2012). Growth is apparently not dependent on NSCs (Michelot et al., 2012). High temperatures may lead to an earlier onset of radial growth (Camarero et al., 2010).	NSCs in leaves decrease from summer through autumn (Hoch et al., 2003). Peak of starch content before the onset of latewood (Oberhuber et al., 2011). Xylem structure is in general highly resistant to cavitation (Choat et al., 2012; Johnson et al., 2012). Very tight stomatal control may lead to near-zero carbon assimilation (Poyatos et al., 2013).	Mediterranean conifers have a growth peak in autumn (Camarero et al., 2010; Pasho et al., 2012). Allocation of carbon to storage (Epron et al., 2012).

2001; Linderholm, 2006; Menzel et al., 2006). Growing season length has a strong effect on tree productivity, Consequently, the reported temperature-induced changes in phenology could affect tree growth responses (White et al., 1999; Kramer et al., 2000; Picard et al., 2005; Delpierre et al., 2009; Richardson et al., 2009; Vitasse et al., 2009a,b; Dragoni et al., 2011; Rossi et al., 2011; Lugo et al., 2012). Empirical evidence in temperate trees suggests that the productivity of evergreen needleleaf forests is less sensitive to phenology than is productivity of deciduous broadleaf forests (Welp et al., 2007; Delpierre et al., 2009; Richardson et al., 2010). For instance, Churkina et al. (2005) reported a different sensitivity of net ecosystem productivity to growing season length in deciduous forests ($5.8 + 0.7\,\mathrm{g\ C\ m^{-2}\ d^{-1}}$), compared with evergreen needleleaf forests ($3.4 + 0.3\,\mathrm{g\ C\ m^{-2}\ d^{-1}}$). Similarly, Piao et al. (2007) reported different sensitivities of gross ecosystem productivity to growing season length ($9.8 + 2.6\,\mathrm{g\ C\ m^{-2}\ d^{-1}}$ in deciduous forests, compared with $4.9 + 2.5\,\mathrm{g\ C\ m^{-2}\ d^{-1}}$ in evergreen needleleaf forests). To our knowledge, it remains untested whether qualitatively different phenology responses in Mediterranean conifers and angiosperm trees may occur and translate into different tree growth responses on a large scale.

However, other evidence points to complex and species-specific effects of phenology on tree growth. For instance, for both conifer and angiosperm trees, a variety of species-specific responses in bud burst and bud set have been reported along altitudinal and latitudinal gradients, reporting both advances, delays and non-significant clines (Vitasse et al., 2009a,b, 2013; Alberto et al., 2013). For example, depending on the species considered, Vitasse et al. (2009b) found positive and negative correlations between advanced leaf emergence and annual growth. Moreover, warming can produce complex and counter-intuitive effects on phenology and growth. For example, strong warming in winter could slow the fulfillment of chilling requirements, which may delay spring phenology and growth (Körner and Basler, 2010; Yu et al., 2010) and affect differently early and late successional species (Körner and Basler, 2010).

In the Mediterranean region, mean annual and maximum temperatures have been identified as the major drivers of deciduous tree phenology (Gordo and Sanz, 2010). However, the effects of temperature on the phenology of many conifer and angiosperm tree species in the Mediterranean basin remain yet relatively poorly quantified (Maseyk et al., 2008). It remains also uncertain whether trade-offs between the advance of spring flushing date and the increased risk of frost damage may differ qualitatively between Mediterranean trees (Lockhart, 1983; Lechowicz, 1984). The same applies for trade-offs between delayed autumn leaf fall date, increased autumn photosyntate storage, and increased late-autumn frost damage risk and incomplete leaf nutrient remobilization costs (Lim et al., 2007). Finally, in the Mediterranean basin, drought periods significantly affect both leaf phenology and tree growth in both conifer and angiosperm trees (de Luis et al., 2007, 2011; Camarero et al., 2010). For instance, increased leaf retention rate and lifespan have been observed in response to drought in holm oak forests (Bussotti et al., 2003; Misson et al., 2010). Drought also causes foliage to fall earlier and results in incomplete leaf nutrient translocation and increased nutrient concentration in litter (Martínez-Alonso et al., 2007).

INTRA-SPECIFIC COMPETITION, INTER-SPECIFIC COMPETITION AND FOREST SUCCESSION (HYPOTHESIS 1.3)

Empirical studies reveal that intra-specific competition acts as a major determinant of growth patterns in Mediterranean forests in both conifer and angiosperm trees (Gómez-Aparicio et al., 2011). Forest densification due to land abandonment and the advance of succession is occurring over extensive areas, increasing competition, reducing tree growth, and increasing mortality (Gómez-Aparicio et al., 2011; Vilà-Cabrera et al., 2011; Coll et al., 2013). Coll et al. (2013) reported much higher negative effects of forest stand basal area on conifer growth than in angiosperm trees in both dry and wet extremes of a large-scale rainfall gradient, and these trends were paralleled by higher effects of basal area on small-tree mortality observed in conifers. These results coincide with studies revealing oaks less sensitive to competition than pines in this area (Sánchez-Gómez et al., 2008; Gómez-Aparicio et al., 2011).

Inter-specific competition also plays an important role in determining growth responses in Mediterranean conifer and angiosperm trees. Specifically, large-scale surveys suggest that small-sized conifers are more sensitive to growth suppression by late successional species (Gómez-Aparicio et al., 2011; Zavala et al., 2011; Coll et al., 2013). Angiosperm trees are significantly expanding their distributional ranges, increasing recruitment across extensive areas (Coll et al., 2013; Vayreda et al., 2013). Morover, during the last decades the expansion of the dominant angiosperm tree *Quercus ilex* has negatively influenced the recruitment success of five *Pinus* species on a large scale in this area (Carnicer et al., 2013a).

SIZE, AGE, AND ALLOMETRY (HYPOTHESIS 1.4)

Mediterranean conifers differ from angiosperm trees in their allometrical relationships between tree size (diameter at breast height) and crown growth variables (Poorter et al., 2012). The peak of crown growth is generally reached at lower sizes in conifers, which also show a much steeper decrease with size than broadleaved species (Poorter et al., 2012). These different allometric relationships are in turn associated with several other traits (maximal height, crown size, shade tolerance, wood density, apical dominance) and also interact with local habitat aridity (Poorter et al., 2012). Similarly, Gómez-Aparicio et al. (2011) reported that in Iberian forests competitive effects for conifers scale approximately quadratically with diameter at breast height (dbh^2) and linearly for broadleaved trees. To our knowledge, it remains untested whether these different allometric relationships might be related to the contrasting tree growth responses to temperature reported in Mediterranean conifers and angiosperm trees (Gómez-Aparicio et al., 2011).

DROUGHT AND TEMPERATURE (HYPOTHESIS 1.5)

Large-scale studies demonstrate that drought and increased temperatures significantly limit tree growth in xeric regions of the Mediterranean basin (Andreu et al., 2007; Martínez-Alonso et al., 2007; Sarris et al., 2007; Bogino and Bravo, 2008; Martínez-Vilalta et al., 2008; Gómez-Aparicio et al., 2011; Vilà-Cabrera et al., 2011; Candel-Pérez et al., 2012; Sánchez-Salguero et al., 2012; Vayreda et al., 2012; Coll et al., 2013) and produce qualitatively different ecophysiological responses in Mediterranean conifers and angiosperm trees (Martínez-Ferri et al., 2000; Zweifel et al., 2007; Eilmann et al., 2009). For instance, while drought often results in early stomatal closure in Mediterranean conifers (Martínez-Ferri et al., 2000; Klein et al., 2011; Poyatos et al., 2013), angiosperm trees are able to maintain substantial transpiration levels during summer drought events (Quero et al., 2011).

Drought largely determines cambium growth in Mediterranean forests, producing plastic and seasonally variable patterns, ranging from one single annual peak to markedly bimodal trends (Maseyk et al., 2008; Camarero et al., 2010; de Luis et al., 2011). However, large-scale studies in the Iberian peninsula reveal that competition effects on growth are often stronger than drought effects (Gómez-Aparicio et al., 2011; Coll et al., 2013). Nevertheless, strong interactions between competition and drought effects have been reported, and significantly increase at the edge of climatic gradients (Linares et al., 2010; Vayreda et al., 2012; Coll et al., 2013; Ruiz-Benito et al., 2013). Finally, there is also some evidence of individual predispositions to winter-drought induced tree dieback in *P. sylvestris* (Voltas et al., 2013), local adaptation for water use efficiency in *P. halepensis* (Voltas et al., 2008), and correlations of temperature and genetic variability at candidate loci for drought tolerance in *P. halepensis* and *P. pinaster* (Grivet et al., 2011), suggesting important interactions between individual adaptive traits and drought impacts.

WINTER FREEZING (HYPOTHESIS 1.6)

Angiosperm trees are more vulnerable to freeze-thaw embolism and this may contribute to explain the dominance of conifer trees at high altitudes (Cavender-Bares et al., 2005; Brodribb et al., 2012) and could in turn result in qualitatively different growth responses in conifers and angiosperm trees. For example, Gómez-Aparicio et al. (2011) reported that Atlantic deciduous broadleaved trees in the Iberian peninsula had lower competitive response ability at lower temperatures, in contrast to mountain conifer species. In this study, tree growth of Atlantic deciduous broadleaved trees was negatively affected by low temperatures (Gómez-Aparicio et al., 2011). In line with this, several studies have demonstrated that low winter temperatures directly inhibit cell division and tree growth in cold localities (Körner, 1998, 2013; Fajardo et al., 2012).

INTERACTIONS BETWEEN MULTIPLE FACTORS (HYPOTHESIS 1.7)

Tree growth patterns in the Iberian peninsula have several contributing drivers that interact along geographical gradients (Coll et al., 2013). For instance, Gómez-Aparicio et al. (2011) studied tree growth responses in 15 tree species in Spain and reported that sensitivity to competition increased with decreasing precipitation in all species. Notably, the best predictive models for tree growth in Gómez-Aparicio et al. (2011) included interactions between size, competitive effects and climate variables. Similarly, Coll et al. (2013) modeled growth responses in the Iberian peninsula and reported a significant increase in the strength of interactions between tree size, tree height and climate variables at the drier and wetter edges of rainfall gradients. These interactions could increase with ongoing climate change, and several studies suggest that warming could increase competition for water in Mediterranean forests (Linares et al., 2010).

LOCAL ADAPTATION, INDIVIDUAL- AND PROVENANCE VARIATION (HYPOTHESIS 1.8)

Local selection processes may affect the adaptive traits determining the different growth responses to temperature observed in Iberian conifers and angiosperm trees. For example, provenance studies in both conifer and angiosperm trees have revealed genetic differences in growth rates and other growth-related traits (age at reproduction, timing of bud burst and bud set, leaf traits, flowering phenology), suggesting that populations are often adapted to their local conditions of temperature and water availability (Rehfeldt, 1978, 1982, 1988; Borghetti et al., 1993; Climent et al., 2008; Mátyás et al., 2009; Rose et al., 2009; Ramírez-Valiente et al., 2010, 2011; Santos et al., 2010; Chmura et al., 2011; Robson et al., 2012; Alberto et al., 2013). In provenance trial studies, populations from cold environments often cease growth earlier, while populations from warm localities generally grow faster (Alberto et al., 2013). Notably, local selection for increased growth rates may induce lower resistance to drought and frost. For instance, in conifers fast-growing provenances often exhibit lower cold hardiness and/or lower resistance to drought stress (Hannerz et al., 1999; Cregg and Zhang, 2001; Chuine et al., 2006). These differences have been attributed to trade-offs between resistance to frost and drought and growth (Chuine et al., 2006 and see Martin St Paul et al., 2012).

PHENOTYPIC PLASTICITY (HYPOTHESIS 1.9)

Mediterranean trees show strong plastic responses in tree growth patterns, which are associated with seasonal climate variability (e.g., Camarero et al., 2010; de Luis et al., 2011). Critically, phenology and growth plasticity responses differ between provenances and species and may determine observed demographic and evolutionary responses to global warming (Nicotra et al., 2010). For example, low elevation provenances often exhibit greater phenological plasticity to temperature than high elevation provenances (Vitasse et al., 2013) and this could in turn influence observed tree growth responses. To our knowledge, it remains untested whether Mediterranean conifers exhibit higher growth plasticity than angiosperm trees, although it has been reported that Iberian conifers show higher growth rates than angiosperm trees in absence of competition (Gómez-Aparicio et al., 2011; Poorter et al., 2012).

EXPERIMENTAL ASSESSMENT OF THE RELATIVE CONTRIBUTION OF THE HYPOTHESES

The available empirical evidence suggest that several factors interact and seem to determine contrasting growth responses to temperature in Mediterranean conifer and angiosperm trees. Therefore, improved experimental approaches are required to quantitatively assess the relative importance of these factors. While several experimental and observational approaches could be applied, we suggest that reciprocal provenance trial experiments may be especially suited for this purpose. Previous studies assert that multiple common garden experiments located in latitudinal and altitudinal gradients are particularly relevant to study phenology and growth responses to temperature (Reich and Oleksyn, 2008; Vitasse et al., 2010). Furthermore, the inclusion of different provenances in these reciprocal experiments allows the quantification of environmentally induced phenotypic plasticity, genotypic variance and their interaction (e.g., Vitasse et al., 2013). Complementarily, drought effects on growth could be studied by manipulative experiments combined with reciprocal common garden designs (reviewed in Klein et al., 2011; Wu et al., 2011). Similarly, the effects of intra- and inter-specific competition could be studied manipulating tree densities and composition in different experimental groups. Finally, to assess tree size effects and allometric relationships, the study of saplings of different ages would be required. Alternatively, long-term experiments could provide also relevant information to quantify allometric relationships. Finally, in all these experimental designs, the periodic measurement of ecophysiological traits should be implemented to assess their seasonal variation and their putative role in determining growth responses.

COMPLEX AND MULTIPLE EFFECTS OF TEMPERATURE AND DROUGHT ON TREE PHYSIOLOGY

Climate produces multiple and complex effects on tree physiology. As highlighted in **Table 1**, we expect that multiple physiological processes can simultaneously react to the changes in environmental temperatures and influence growth responses. For example, temperature and drought directly affect several ecophysiological processes such as carbon and nutrient uptake, carbon allocation between tissues, photosynthesis, respiration, processes of embolism prevention and repair, phenological cycles, cambium reactivation, cell division and expansion or carbon transfer rates (Körner, 1998; Bréda et al., 2006; Rennenberg et al., 2006; Sanz-Pérez et al., 2009; Camarero et al., 2010; Epron et al., 2012; Michelot et al., 2012). Moreover, these direct climatic effects on tree physiology can in turn produce secondary indirect effects, for example the promotion of signaling and regulatory responses, acclimation and phenotypically plastic responses or changes in gene expression (reviewed in Peñuelas et al., 2013b). **Table 4** provides a brief, non-exhaustive description of the diverse effects of temperature and drought on tree physiology. It is important to bear in mind that all these ecophysiological processes often have different sensitivities and thresholds to temperature and water deficit. For example, tree growth and cambium activation are more sensitive to low temperatures than is photosynthesis (Körner, 1998; Fajardo et al., 2012). In addition, as shown in **Table 4**, responses to climate are often species or tissue specific or depend on developmental stage and seasonal phase and can be influenced by regulatory feedbacks that can often imply multi-tissue coordinated responses. Despite the overwhelming complexity and diversity of the effects of temperature and drought reported in **Table 4**, several studies have demonstrated consistent differences between major plant groups, such as conifers and angiosperms, in climate-induced responses (e.g., Way and Oren, 2010; Gómez-Aparicio et al., 2011; Coll et al., 2013).

Table 4 | A non-exhaustive and synthetic review of the different effects of temperature (A) and drought (B) on different tree physiological processes.

	References
(A) EFFECTS OF TEMPERATURE ON TREE PHYSIOLOGY	
Photosynthesis. Temperatures higher/lower than the optimum decrease photosynthesis and affect multiple biochemical processes. For example, high temperatures can reduce the efficiency of electron transport in the thylakoid membrane of chloroplasts, which in turn down-regulate the content of ribulose-1,5-bisphosphate and deactivate Rubisco. High temperatures also inhibit Rubisco activase, due to their low thermal optimum. The solubility of the two substrates of Rubisco, CO_2, and O_2, is differentially affected by temperature, stimulating photorespiration and inhibiting photosynthesis at high temperatures. Photosystem II is also sensitive to high temperatures, which stimulate mechanisms to avoid photo-oxidation and membrane denaturation, such as isoprene production and the xanthophyll cycle. Low temperatures cause a variety of physiological and acclimative responses, including modifications in the structure of the thylakoid membrane in chloroplasts, alleviation of photoinhibition through upregulation of carbon metabolism and increased synthesis of storage carbohydrates, increased production of antioxidants, prevention of intracellular freezing by increased soluble carbohydrates (mobilization of starch to sucrose) and changes in gene expression and signaling pathways. The growth environment of plants determines the temperature optimum of photosynthesis. In warmer environments, plants acclimate to increase the thermal optimum of the maximum carboxylation velocity (Vcmax) and the maximum potential rate of electron transport (Jmax).	Rennenberg et al., 2006 Morin et al., 2007 Kattge and Knorr, 2007 Chaves et al., 2012 Flexas et al., 2012; Sharkey and Bernacchi, 2012
Above the thermal optimum for photosynthesis, the emission of biogenic volatile organic compounds such as isoprene and monoterpenes progressively increases.	Llusià and Peñuelas, 2000; Rennenberg et al., 2006
Leaf respiration is strongly affected by temperature, increasing at high temperatures (e.g., above 35–40°C) and peaking at higher temperatures than photosynthesis.	Rennenberg et al., 2006; Smith and Dukes, 2013
High temperatures often increase net primary production and plant growth. In cold-adapted trees, photosynthesis is less sensitive to low temperatures than is tree growth (cell division and growth, cambium activation). In alpine treelines, new tissue formation is nearly absent at temperatures around 5°C, but considerable rates of photosynthesis are maintained between 0 and 10°C.	Körner, 1998; Way and Oren, 2010; Wu et al., 2011; Fajardo et al., 2012; Lenz et al., 2012
Higher temperatures influence foliar phenology, promoting earlier bud burst and delaying leaf fall.	Peñuelas and Filella, 2001; Peñuelas et al., 2002; Vitasse et al., 2009a,b, 2013
In the absence of drought, temperature often increases *nutrient-uptake* capacity (NH_4^+, NO_3^-, PO_{43}^-, K^+). Temperature can also increase both xylem loading of amino compounds and nitrogen allocation in aboveground tissues.	Rennenberg et al., 2006
Freezing causes cell dehydration, formation of ice in intracellular spaces and embolism. Buds are more resistant than leaves to frost.	Morin et al., 2007; Augspurger, 2009
Temperature, in absence of drought, positively affects rates of soil respiration and litter decomposition.	Wu et al., 2011
Organs, individuals, life stages and species consistently differ in their phenological responses to temperature and sensitivity to damage from frost and drought.	Niinemets and Valladares, 2006; Morin et al., 2007; Augspurger, 2009
(B) EFFECTS OF DROUGHT ON TREE PHYSIOLOGY	
Photosynthesis. Drought limits photosynthesis by stomatal closure, diffusion limitations in the mesophyll and metabolic impairment. It can also limit photosynthesis via secondary effects, such as reduced hydraulic conductance and oxidative stress. Drought activates diverse signaling pathways associated with stomatal closure. For example, it modifies abscisic acid (ABA) signaling in leaves, shoots and roots; increases xylem-sap pH and changes aquaporin concentrations, leaf hydraulic conductance signals and electric signals.	Chaves et al., 2012 Sharkey and Bernacchi, 2012
Drought reduces osmotic potential in the soil and predawn leaf water potentials and limits water uptake. To maintain water uptake, plants increase the production of osmolites, down-regulate electron flux and increase the activity of antioxidant enzymes. Drought can also increase the degradation of foliar proteins and the concentration of soluble amino acids and NSCs in the leaves, which may act in turn as osmoprotectants to stabilize proteins and membranes. Drought also promotes an increase in the concentrations of soluble antioxidants.	Rennenberg et al., 2006.

(Continued)

Table 4 | Continued

	References
(B) EFFECTS OF DROUGHT ON TREE PHYSIOLOGY	
Severe water stress can produce irreversible or persistent damage in the photosynthetic apparatus of leaves (relative to leaf lifespan).	Sharkey and Bernacchi, 2012
Drought reduces tree growth, net primary production, cambium activity, cell division and growth.	Eilmann et al., 2009; Camarero et al., 2010; Wu et al., 2011; de Luis et al., 2011
Drought reduces C transfer rates.	Barthel et al., 2011; Epron et al., 2012
Drought is associated with acclimative responses such as mid-term reductions in total leaf area and defoliation.	Bréda et al., 2006; Ogaya and Peñuelas, 2006; Carnicer et al., 2011
Drought promotes an increase in NSCs in roots and a decrease in fine-root biomass.	Anderegg, 2012; Anderegg et al., 2012
Drought alters nutrient-uptake processes, for example promoting increases in ammonification and decreases in denitrification in the soil.	Gessler et al., 2005
Isoprenoid emissions can be negatively affected by drought stress and increase during plant recovery after drought.	Rennenberg et al., 2006; Peñuelas and Staudt, 2010
Drought can increase the accumulation of ethylene in shoots, in turn reducing shoot growth.	Chaves et al., 2012
Water deficit can reduce N uptake from the soil and change N partitioning between roots and shoots, increasing N content in the roots.	Rennenberg et al., 2006
Omic studies reveal that drought produces changes in *gene regulation*, for example promoting proline synthesis and down-regulating proline degradation.	Chaves et al., 2012; Peñuelas et al., 2013a
Negative effects of drought differ between phases of plant development and annual phenophases and are usually stronger during reproductive and leaf-emergence phases in deciduous trees. Drought produces tissue-specific signaling responses in roots, shoots and leaves and tissue-specific interactions between signaling factors. For example, different interactions between ABA and ethylene have been reported in roots and shoots.	Chaves et al., 2012

EMPIRICAL PATTERNS IN THE IBERIAN PENINSULA: THE NEGATIVE SYNERGISTIC EFFECTS OF INCREASED TEMPERATURES AND FOREST SUCCESSIONAL ADVANCE

In the Mediterranean basin, land use changes often negatively interact with increased temperatures and drought events and result, in diverse taxonomic groups, in negative demographic trends detectable on a large scale (Linares et al., 2010; Stefanescu et al., 2011; Carnicer et al., 2013b). In the case of Iberian forests, increased stand competition due to forest successional advance and forest densification has been identified as a major driver of tree demographic responses (Gómez-Aparicio et al., 2011; Carnicer et al., 2013a). Notably, stand competition interacts with temperature and drought responses in this region, especially in the drier and wetter edges of rainfall gradients (Linares et al., 2010; Coll et al., 2013). In this section we briefly review the contrasting demographic trends to temperature observed in Conifers and Angiosperm trees in the Iberian peninsula. Forest succession is currently favoring a shift toward an increased dominance of angiosperm trees on a large scale (Carnicer et al., 2013a; Coll et al., 2013; Vayreda et al., 2013). On top of this, recent studies (Gómez-Aparicio et al., 2011; Coll et al., 2013) show that tree growth responses to temperature differ between conifers and angiosperms on a large scale in the Mediterranean forests of the Iberian Peninsula. Large-scale empirical patterns of the responses of tree growth to temperature along a gradient of rainfall in Spain are illustrated in **Figure 1A**, showing contrasting responses in conifers (black dots) and angiosperms (gray dots). Panel **(A)** depicts the variation of temperature beta estimates on species-specific responses of tree growth in forests located along a gradient of rainfall (Coll et al., 2013). Tree-growth data were obtained from the Spanish National Forest Inventory, which comprises a wide range of forest types, from typically Mediterranean lowland stands to northern temperate forests with strong Atlantic influences to alpine forests located in the Pyrenees (Coll et al., 2013). To analyze the relationship between growth responses to temperature and trait differences between conifers and angiosperms, we used hydraulic safety margins as a key synthetic variable of the hydraulic strategy of each species (**Figure 1B**). Panel **(B)** depicts two separate linear regressions between the temperature beta estimates on growth and the species-specific hydraulic safety margins. Hydraulic safety margins were obtained from Cochard and Tyree (1990), Cochard (1992, 2006), Tognetti et al. (1998), Cochard et al. (1999), Martínez-Vilalta and Piñol (2002), Martínez-Vilalta et al. (2002, 2009), Oliveras et al. (2003), Corcuera et al. (2006), and Choat et al. (2012). A significant linear relationship between growth responses to temperature and species-specific hydraulic safety margins was only observed in angiosperms (**Figure 1B**), and conifers had significantly larger hydraulic safety margins (**Figure 1B**). Across the studied range of hydraulic safety margins, the temperature beta estimates were positive for angiosperms (gray dots) but negative for conifers (black dots), regardless of mean precipitation (**Figure 1A**). This result is consistent with those of other studies on the effects of climate in the Iberian Peninsula reporting negative significant effects of temperature on tree growth in conifers (Gómez-Aparicio et al., 2011; Candel-Pérez et al., 2012; Büntgen et al., 2013). **Figure 2** illustrates the specific forest successional context in which the reported contrasting effects of temperature on tree growth previously reported occur. Conifers show a significantly higher percentage of plots characterized by recruitment failure (**Figure 2A**; Carnicer et al., 2013a). In contrast, *Quercus* species showed a much larger percentage of recently colonized plots and/or resprouting areas (i.e., plots without adult trees but in which recruits and/or resprouts of the focal species were detected, **Figure 2B**, Carnicer et al., 2013a). Overall **Figures 1**, **2** suggest that in this area the negative effects of warming and forest successional advance could synergistically impact conifer species during the next decades.

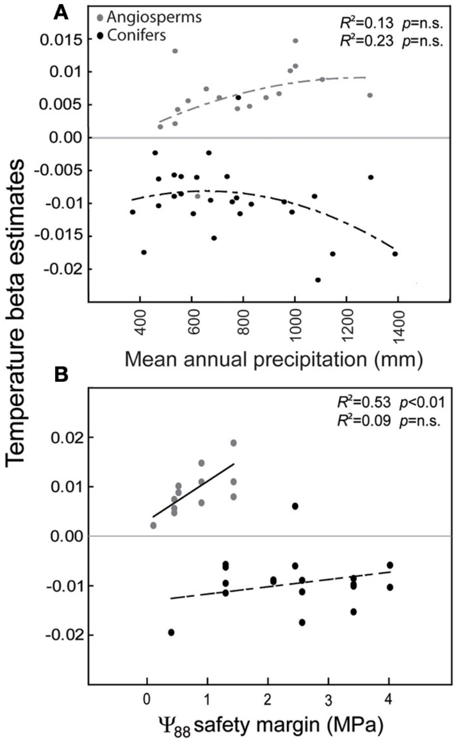

FIGURE 1 | Summary of the variation in the effect of temperature on tree growth along a rainfall gradient (A) and across interspecific differences in hydraulic safety margins (B) in conifers (black dots) and angiosperms (gray dots). The tree species included in the analysis are: *Fagus sylvatica, Quercus ilex, Q. pubescens, Q. pyrenaica, Q. robur, Abies alba, Pinus halepensis, P. nigra, P. pinaster, P. pinea, P. sylvestris,* and *P. uncinata. P. radiata* and *Q. suber* were only included in panel **(A)** due to a lack of data for hydraulic safety margins. Coll et al. (2013) applied generalized linear models (GLM) to study tree growth responses (dependent variable) and assessed the following independent predictors: (i) climate and topography (Emberger water deficit index, mean annual temperature, terrain slope), (ii) forest stand structure (tree density, basal area), (iii) soil (organic layer depth), (iv) individual tree traits [tree height, diameter at breast height (DBH)], and (v) management practices (e.g., plantations). Beta estimates in panels **(A)** and **(B)** show the reported significant effects of temperature on tree growth in GLM analyses (Coll et al., 2013). n.s. means not significant.

DISCUSSION

We have reviewed the different hypotheses that may contribute to explain the recently reported different growth responses to

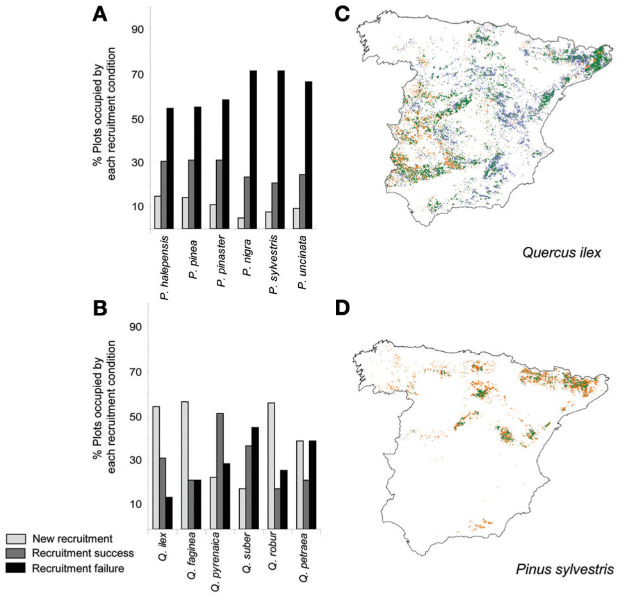

FIGURE 2 | Contrasting large-scale trends in tree recruitment observed in the Iberian peninsula for small saplings (height <30 cm) in Conifers (*Pinus*) and Angiosperm trees (*Quercus*). (A) Variation in the percentage of plots with recruitment success (gray), recruitment failure (black) and new recruitment areas (plots without adult trees of the focal species in which small recruits or resprouts were detected) in *Pinus* species; **(B)** Variation in the percentage of plots with recruitment success (gray), recruitment failure (black) and new recruitment areas in *Quercus* species. **(C)** Spatial trends in recruitment for the dominant species *Quercus ilex*. Blue areas indicate new recruitment areas (i.e., areas with recruits but absence of adult trees), orange areas illustrate recruitment failure and green areas illustrate recruitment success (i.e., areas characterized by the presence of both adult and small saplings). **(D)** Spatial trends in recruitment for *Pinus sylvestris*. Differences between recruitment trends in *Pinus* and *Quercus* were significant (see Carnicer et al., 2013a for a detailed statistical test. Average proportion of plots with recruitment failure: $F = 16.64$, $P = 0.002$; average proportion of plots with new recruitment: $F = 35.04$, $P = 0.0001$). Data were obtained from the Spanish National Forest Inventory, consisting in a regular grid of circular plots at a density of 1 plot/km^2.

temperature in Mediterranean angiosperm and conifer trees (**Table 1**; Gómez-Aparicio et al., 2011; Coll et al., 2013). Conifer and angiosperm trees differ in the effects of phenology on tree productivity, in their sensitivity to stand competition and in their growth allometry. In addition, they consistently differ in an integrated suite of key traits, including different hydraulic safety margins, stomatal sensitivity, embolism repair capacity and xylem anatomy, suggesting two contrasting ecophysiological strategies to confront drought and extreme temperature events. However, for many Mediterranean conifer and angiosperm trees, detailed empirical studies contrasting the relative effect on tree growth of the factors listed in **Table 1** are still lacking. For example, it is not clear whether temperature-induced shifts in phenology consistently differ between conifers and angiosperm trees in the Mediterranean region and how these shifts in phenology could differentially alter their productivity. Similarly, the seasonal dynamics of key traits, like cambium growth, tissue NSC content or sap flow, remain yet poorly quantified for many species. So it is clear that improved experimental approaches to contrast and assess the relative effect of the reviewed hypotheses are required (**Table 1**) if we are to explain the contrasting growth trends reported in recent large-scale studies in these two groups (Gómez-Aparicio et al., 2011; Coll et al., 2013; **Figure 1**). We have suggested that the relative effects of these factors (**Table 1**) could be contrasted in reciprocal common garden experiments located in altitudinal or latitudinal gradients that provide an ideal design to estimate temperature effects on phenology and growth, and also allow the estimation of local adaptation and phenotypic plasticity (Vitasse et al., 2009a,b, 2013). In these reciprocal transplant experiments, detailed quantitative analysis of the relationships between growth measures and hydraulic safety margins, stomatal sensitivities to VPD, embolism repair activity and NSC carbon dynamics in wood parenchyma and other tissues would be ideally required to clarify the relative importance of these processes and their dynamic inter-relationships (Camarero et al., 2010; de Luis et al., 2011; Oberhuber et al., 2011; Michelot et al., 2012; Pasho et al., 2012).

The available empirical evidence (Gómez-Aparicio et al., 2011; Coll et al., 2013; **Figure 2**) suggests that increased stand competition associated with successional advance is a primary driver of growth trends in the forests of the Iberian peninsula. So it would be key to simulate this factor in the proposed transplant experiments, manipulating sapling densities and composition. We suggest that mixed pine-oak designs would be especially interesting because recent studies describe the widespread expansion of *Quercus* saplings and resprouts in the Iberian peninsula and limited recruitment in *Pinus* species (Carnicer et al., 2013a; Coll et al., 2013; Vayreda et al., 2013; **Figure 2**). Moreover, *Quercus ilex* seems to act as a keystone species in driving these limited recruitment trends, inhibiting recruitment in five different *Pinus* species (Rouget et al., 2001; Carnicer et al., 2013a). In addition, several studies report that pines are more sensitive to competition and their growth can be largely suppressed with the advance of succession, specially on sapling and young stages (e.g., Gómez-Aparicio et al., 2011; Zavala et al., 2011; Coll et al., 2013). Therefore, these processes should be ideally considered in reciprocal transplant experiments, to allow the experimental study of the combined negative synergistic effects of warming and increased successional advance.

Ideally, the experimental approaches tested in these common garden experiments should simulate future forest scenarios in the face of climate change in the Iberian Peninsula. However, future scenarios in this region remain uncertain. For example, the available model predictions vary from important range contractions to substantial range expansions (Benito Garzón et al., 2011; Keenan et al., 2011; Ruiz-Labourdette et al., 2012; García-Valdés et al., 2013). We have suggested a possible scenario of global change dominated by the widespread expansion of angiosperm broadleaved trees, increased suppression of pine growth and recruitment by *Q. ilex* and specially acute negative demographic trends in mountain pines (*Pinus sylvestris*, *Pinus nigra* and, to a less extent, *P. uncinata*) (**Figure 2**; Carnicer et al., 2013a). Other major uncertainties in future forest scenarios are related to nonlinear dynamics in fire activity (Loepfe et al., 2012), changes in fire-climate relationships motivated by the generalized advance of forest succession and the expansion of *Quercus* species that may substantially alter the distribution of forest fuel over extensive areas (Pausas and Paula, 2012; Carnicer et al., 2013a), and the future changes in land uses induced by shifts in global energy policies and the increased use of forests as a local energy source (Peñuelas and Carnicer, 2010; Carnicer and Peñuelas, 2012).

In **Table 3** we have also discussed how tree carbon dynamics may be interacting with climate-induced responses in the seasonal variation of photosynthesis, annual growth cycles, embolism prevention, embolism repair and refilling and stomatal responses. Important gaps in our knowledge remain, and we lack a clear picture of how tissue-specific NSC concentrations vary seasonally, their interspecific variation and how these seasonal variations are connected to the diverse physiological functions examined (i.e., carbon buffer function, winter- and drought-induced embolism repair, embolism prevention, bud burst and leaf unfolding, responses of root and stem growth and respiration) (Hoch et al., 2003; Epron et al., 2012; Michelot et al., 2012; Sala et al., 2012). Another aspect that merits more attention in future empirical tests is the putative existence of compensatory dynamics across seasons in the effects of climate on tree physiology. For example, higher temperatures may reduce the costs of winter embolism in broadleaved deciduous trees, lengthen the growing season or increase the production of photosynthates in spring. These changes could in turn allow higher NSC storage in spring, which could increase embolism repair capacity during summer droughts (compensatory seasonal effects).

In summary, a review of the existing empirical evidence suggests that contrasting demographic responses in Mediterranean conifer and angiosperm trees are currently occurring, due to both widespread forest successional advance and to divergent growth responses to temperature. Trait-based differences in these two groups may contribute to explain their different responses to temperature (**Table 2**, **Figure 1**) and their different role during successional processes in this region (**Figure 2**, **Table 2**, reviewed in Zavala et al., 2011; Poorter et al., 2012; Sheffer, 2012). Reciprocal common garden experiments may offer a very promising tool to develop integrative tests of the diverse factors reviewed (**Table 1**) and to simulate the synergistic negative effects of forest successional advance and climate warming on conifer species (Carnicer et al., 2013a).

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Research paper

Seasonal variability of foliar photosynthetic and morphological traits and drought impacts in a Mediterranean mixed forest

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The Mediterranean region is a hot spot of climate change vulnerable to increased droughts and heat waves. Scaling carbon fluxes from leaf to landscape levels is particularly challenging under drought conditions. We aimed to improve the mechanistic understanding of the seasonal acclimation of photosynthesis and morphology in sunlit and shaded leaves of four Mediterranean trees (*Quercus ilex* L., *Pinus halepensis* Mill., *Arbutus unedo* L. and *Quercus pubescens* Willd.) under natural conditions. $V_{c,max}$ and J_{max} were not constant, and mesophyll conductance was not infinite, as assumed in most terrestrial biosphere models, but varied significantly between seasons, tree species and leaf position. Favourable conditions in winter led to photosynthetic recovery and growth in the evergreens. Under moderate drought, adjustments in the photo/biochemistry and stomatal/mesophyllic diffusion behaviour effectively protected the photosynthetic machineries. Severe drought, however, induced early leaf senescence mostly in *A. unedo* and *Q. pubescens*, and significantly increased leaf mass per area in *Q. ilex* and *P. halepensis*. Shaded leaves had lower photosynthetic potentials but cushioned negative effects during stress periods. Species-specificity, seasonal variations and leaf position are key factors to explain vegetation responses to abiotic stress and hold great potential to reduce uncertainties in terrestrial biosphere models especially under drought conditions.

Keywords: abiotic stress, *Arbutus unedo*, J_{max}, leaf position, leaf traits, *Pinus halepensis*, *Quercus ilex*, *Quercus pubescens*, seasonality, $V_{c,max}$.

Introduction

The Mediterranean region is dominated by arid or semi-arid ecosystems where high evaporative demand and low soil-water content (SWC) during the summer dry period are the main ecological limitations to plant growth (Specht 1969, Di Castri 1973). The resilience of plants to drought and heat waves is determined by their frequency and duration, which are projected to become much more severe under current climate change scenarios—particularly in the Mediterranean region (Somot et al. 2008, Friend 2010, IPCC 2013). Increased drought-induced defoliation (Poyatos et al. 2013) associated with the depletion of carbon reserves (Galiano et al. 2012) can ultimately lead to catastrophic hydraulic failure and tree mortality (Choat 2013, Urli et al. 2013). Drought-induced forest impacts and diebacks in the Mediterranean region have been reported in numerous studies (Peñuelas et al. 2001, Martínez-Vilalta and Piñol 2002, Raftoyannis et al. 2008, Allen et al. 2010, Carnicer et al. 2011, Matusick et al. 2013) and can lead to shifts in vegetation composition (Jump and Penuelas 2005, Anderegg et al. 2013) and to a higher risk of forest fires (Piñol et al. 1998, Pausas et al. 2008). The challenge in the Mediterranean region in the coming years will be to learn how carbon uptake and growth in species and communities will respond to these changes, and how forest management strategies can be adapted to cushion the negative impacts of climate change on forests (Sabaté et al. 2002, Bugmann et al. 2010).

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In past decades, ecosystem models on regional or global levels have contributed substantially to our understanding of the implications of climate change on a coarse scale where field experiments are limited (Luo 2007). Much uncertainty, however, remains in the modelled feedback of the global carbon cycle to climatic warming (Friedlingstein et al. 2014) and in the understanding and modelling of species' responses to climate change (Luo 2007, Beaumont et al. 2008, McDowell et al. 2008). Photosynthesis is generally overestimated in the main Earth system models, with significant regional variations (Anav et al. 2013). Two critical parameters, the maximum rate of carboxylation ($V_{c,max}$) and the maximum rate of electron transport (J_{max}), are a prerequisite for scaling foliar photosynthesis to the canopy level at which global dynamic models operate (Friedlingstein et al. 2006, Friedlingstein and Prentice 2010). These two parameters describe the biochemical limitations to carbon assimilation, but are not easily measured, so relatively few data regarding their variability between species or seasons are available. $V_{c,max}$ and J_{max} are thus often used as constants for various plant functional types and seasons or, in some cases, are derived from other parameters such as leaf nitrogen content (Grassi and Magnani 2005, Walker et al. 2014). Moreover, extreme climatic conditions and inter-annual variability in arid and semi-arid regions are challenging for scaling carbon assimilation patterns from one year to another (Reynolds et al. 1996, Morales et al. 2005, Gulías et al. 2009). Simulations of ecosystem carbon fluxes are consequently limited, first, by underrepresented temporal variability of photosynthetic parameters and soil-water patterns, and second by our limited understanding of the effects of water stress on both carbon uptake and release (Hickler et al. 2009, Niinemets and Keenan 2014). The modelling performance in Mediterranean-type ecosystems is thus particularly poor and stresses the need for a better mechanistic description of photosynthetic processes under water stress (Morales et al. 2005, Keenan et al. 2011, Zheng et al. 2012, Vargas et al. 2013). Mesophyll conductance, g_m, might play a future key role in improving model performance of photosynthesis under drought conditions (Keenan et al. 2010).

The photosynthetic limitations of Mediterranean vegetation, especially under drought, have been extensively studied (for a review see Flexas et al. 2014), but fewer studies have thoroughly assessed the seasonal behaviour of photosynthesis and morphology under natural conditions in a mixed mature forest. The information gained from seedlings under controlled conditions can only poorly represent the physiological mechanisms of the long-term acclimation to variable environmental conditions in mature trees (Flexas et al. 2006, Mittler 2006, Niinemets 2010). Seedlings or saplings are characterized by higher metabolism and enzymatic function, lower leaf dry mass per unit area (LMA) and higher photosynthetic potential relative to mature trees (Johnson and Ball 1996, Bond 2000, Niinemets 2015). Responses to short-term stress are related to the mechanisms of prompt reactions (Flexas et al. 2006). Under natural conditions, however, mature trees acclimate to gradually developing water stress through the photosynthetic pathway (biochemical, stomatal or mesophyllic) (e.g., Martin-StPaul et al. 2013), but also through foliar traits such as nitrogen, LMA etc. (Poorter et al. 2009). Less work has evaluated simultaneously the variations of photosynthetic and morphological traits in response to abiotic stress conditions. The variation of these traits is largely species specific (Orshan 1983, Chaves et al. 2002, Gratani and Varone 2004, Krasteva et al. 2013), although within-canopy gradients can play an additional overriding role (Valladares and Niinemets 2008, Sperlich et al. 2014). Mixed forests provide ideal test conditions where we can observe distinct species-specific strategies coping equally with the yearly variability of environmental conditions.

The aim of this study was to investigate the impact of seasonal environmental changes (above all drought) on foliar photosynthetic and morphological traits of the winter-deciduous sub-Mediterranean *Quercus pubescens* Willd., two evergreen sclerophyllous species (*Quercus ilex* L. and *Arbutus unedo* L.) and an early-successional drought-adapted conifer, *Pinus halepensis* Mill. *Pinus halepensis* is characterized as isohydric following a water saving and photoinhibition-tolerant strategy (Martínez-Ferri et al. 2004, Baquedano and Castillo 2006, Sperlich et al. 2014). *Quercus ilex* is a late-successional, slow growing, water-spending, photoinhibition-avoiding, anisohydric tree species with a plastic hydraulic and morphological behaviour (Villar-Salvador et al. 1997, Fotelli et al. 2000, Corcuera et al. 2005*a*, Ogaya and Peñuelas 2006, Limousin et al. 2009). The winter-deciduous anisohydric *Q. pubescens* follows a similar drought-avoiding strategy to *Q. ilex*, but maximizes gas exchange during a shorter growing season (Baldocchi et al. 2010), resulting in high transpiration rates throughout the summer (Poyatos et al. 2008). Over extensive areas of the Mediterranean region *Q. ilex* and *Q. pubescens* form the terminal point of secondary succession (Lookingbill and Zavala 2000). *Arbutus unedo*— a relict of the humid-subtropical Tertiary tree flora (Gratani and Ghia 2002 and references therein)—typically occurrs as shrub or small tree in the macchia ecosystems and holds an intermediate position concerning stomatal (Beyschlag et al. 1986, Vitale and Manes 2005, Barbeta et al. 2012) and photoinhibition sensitivity (Sperlich et al. 2014). Prolonged climate stress might disadvantage *A. unedo*, being more drought sensitive than the companion species (Ogaya and Peñuelas 2004, Barbeta et al. 2012).

Our particular interests were to distinguish the species-specific strategies and to explore the eco-physiological mechanism behind drought responses by examining the fine tuning of foliar photosynthetic potentials/rates and foliar morphological traits. We hypothesized that seasonal environmental changes (above all drought) affect the (i) photosynthetic and (ii) morphological traits, (iii) mesophyllic diffusion conductance (g_m) strongly constrains photosynthesis under drought conditions, and the seasonal acclimation varies qualitatively and quantitatively with (iv) species and (v) light environment (leaf canopy position). We thus created a matrix of photosynthetic parameters that could be incorporated into process-based ecosystem models to improve estimates of carbon flux in the Mediterranean region.

Materials and methods

Field site

The experimental site Can Balasc is located in the coastal massif of the Collserola Natural Park (8500 ha), in the province of Barcelona, northeastern Spain (41°25′N, 2°04′E, 270 m above sea level). Seasonal summer droughts, warm temperatures and mild winters characterize the typical Mediterranean climate with a mean August temperature of 22.8 °C and a mean January temperature of 7.9 °C. Mean annual precipitation and temperature are 723 mm and 15.1 °C (1951–2010), respectively (Ninyerola et al. 2007*a*, 2007*b*). Sensors for measuring air temperature (HMP45C, Vaisala Oyj, Vantaa, Finland) and solar radiation (SP1110 Skye Instruments Ltd, Powys, UK) were installed at a height of 3 m, in a clearing ~1 km from the plot.

Stand structure

Our study site is characterized by a dense forest stand (1429 stems ha^{-1}) with a two-layered canopy consisting of a dense layer of *Quercus* species surmounted by shelter trees of the early-successional and fast growing Aleppo Pine (*P. halepensis* Mill.). The mean heights of each layer are 9.9 and 17.1 m, respectively. The *Quercus* species are the late-successional evergreen Holm Oak (*Q. ilex* L.) and the deciduous Pubescent Oak (*Q. pubescens* Willd.). The Strawberry tree (*A. unedo* L.) usually grows as a shrub, being widely abundant in the macchia ecosystems of the Iberian Peninsula (Beyschlag et al. 1986, Reichstein et al. 2002). In our study site, however, *A. unedo* occurs scattered in the tree canopy enriching the forest diversity with its flowering and fruiting habit. The trees with the biggest dimensions are the pines followed by the two *Quercus* species and last by *A. unedo* (mean DBH of 33.7, 12.9, 9.6 cm, respectively). The forest succession has reached the final stage: the dense *Quercus* canopy is out-competing the early-successional *P. halepensis* by suppressing the growth of the light-demanding pine seedlings and saplings. More details of stand history and field site are described in Sperlich et al. (2014).

Sampling method

We conducted eight field campaigns from June 2011 to February 2013. The sampling periods are presented in Figure 1 and Table 2. We avoided difficulties encountered during field measurements such as deviations from the standard temperature (25 °C) or unpredictable plant responses (patchy stomatal conductance) (Mott and Buckley 1998, 2000) by analysing sampled twigs in the laboratory. We cut twigs with a pruning pull from sunlit and shaded leaf positions, optimally at similar heights. The twigs were immediately re-cut under water in the field, wrapped in plastic bags to minimize transpiration, stored in water buckets and transported to the laboratory. Five replicates of each leaf position and tree species were collected for the analysis of gas exchange. The twigs were pre-conditioned in the laboratory at room temperature (24–28 °C) in dim light for 1–3 days and were freshly cut every morning. More details and references can be found in Sperlich et al. (2014).

Analyses of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA). Response curves for foliar net assimilation versus CO_2 concentration were recorded in parallel with the chlorophyll fluorescence measurements. In some cases the sunlit leaves of *Q. ilex* were too small to fill the leaf cuvette (2 cm^2) and so the measured parameters were adjusted after the

Table 1. Acronyms for variables utilized in tables and figures.

Acronym	Unit	Variable name
PPFD	μmol photons m^{-2} s^{-1}	Photosynthetic photon flux density
$V_{c,max}$	μmol CO_2 m^{-2} s^{-1}	Maximum carboxylation velocity
J_{max}	μmol electron m^{-2} s^{-1}	Maximum electron-transport rate
F_v/F_m	Unitless	Maximum quantum efficiency of PSII
R_d	μmol CO_2 m^{-2} s^{-1}	Day respiration
A_{net}	μmol CO_2 m^{-2} s^{-1}	Net assimilation rate
C_i	μmol CO_2 mol air^{-1}	Stomatal internal CO_2 concentration
C_c	μmol CO_2 mol air^{-1}	Chloroplastic internal CO_2 concentration
g_s	mol H_2O m^{-2} s^{-1}	Stomatal conductance
g_m	mol m^{-2} s^{-1} bar^{-1}	Mesophyll internal conductance
Φ_{PS2}	Unitless	Effective quantum yield of PSII
NPQ	Unitless	Nonphotochemical quenching
LT	mm	Leaf thickness
FW	mg	Fresh weight
DW	mg	Dry weight
LMA	mg cm^{-2}	Leaf mass per area
LT	mm	Leaf thickness
S	mg H_2O cm^{-2}	Succulence
D	mg cm^{-3}	Leaf density
WC	%	Leaf water content
C_{area}	g m^{-2}	Carbon concentration per unit leaf area
C_{mass}	%	Carbon concentration per unit leaf mass
N_{area}	g m^{-2}	Nitrogen concentration per unit leaf area
N_{mass}	%	Nitrogen concentration per unit leaf mass

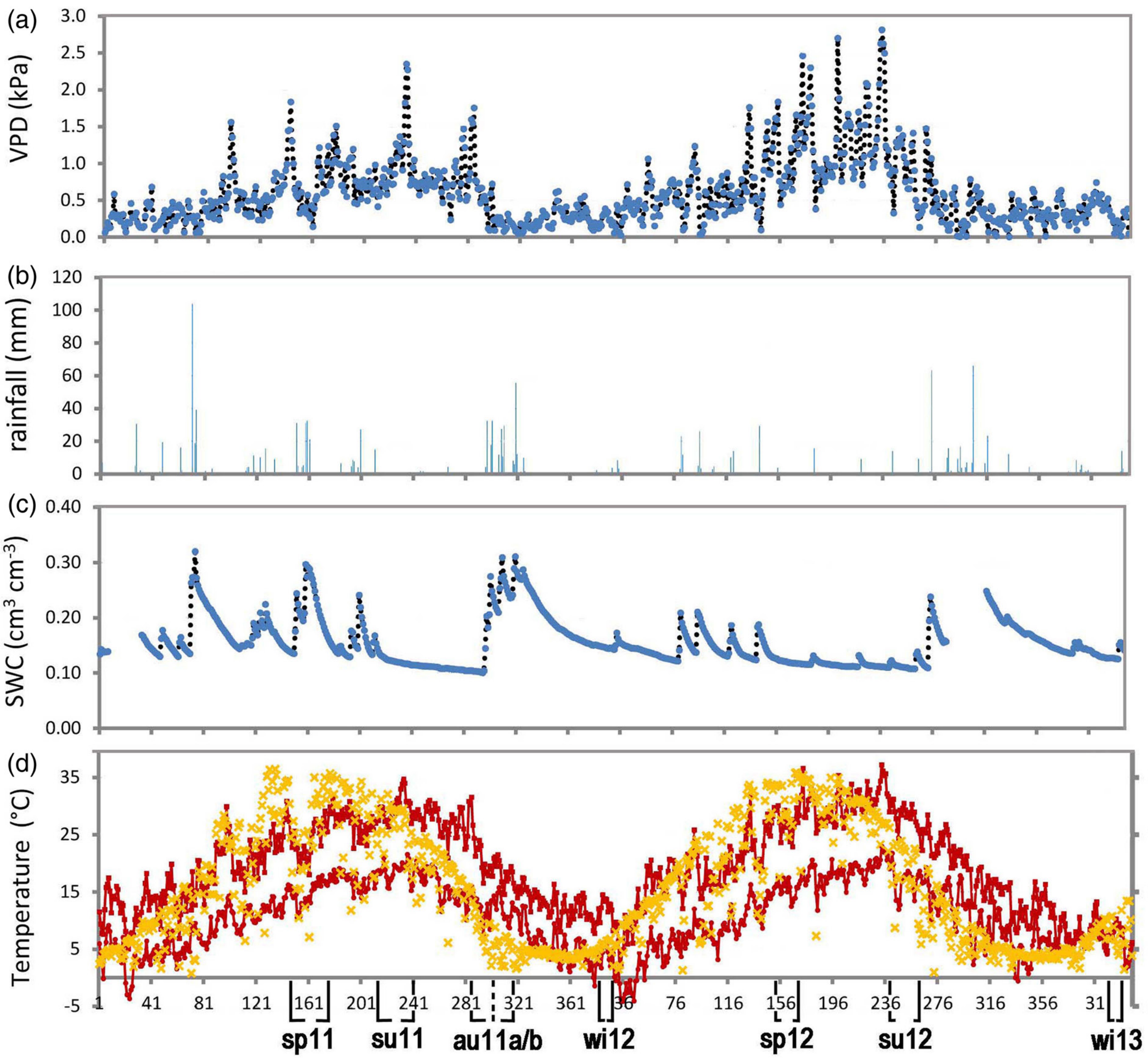

Figure 1. Environmental variables are presented for the day of the year (DOY) from January 2011 until February 2013; (a) atmospheric vapour pressure deficit (VPD), (b) rainfall in mm (c) soil-water content (SWC) in cm^3 cm^{-3} (gap in data is due to power cut), (d) maximum and minimum temperatures in °C on the primary *y*-axes (circles) and radiation in W m^{-2} (crosses, foreground) on the secondary *y*-axes. Field campaigns are indicated (acronyms of seasons are detailed in Table 1).

Table 2. Dates and days of the year (DOY) of seasonal field campaigns.

Campaign	Abbreviation	Date	DOY
Spring 2011	sp11	02.06.11–02.07.11	153–183
Summer 2011	su11	17.08.11–29.08.11	229–241
Autumn 2011^{a1}	au11^{a}	17.10.11–27.10.11	290–300
Autumn 2011^{b1}	au11^{b}	28.10.11–11.11.11	301–315
Winter 2012	wi12	09.01.12–19.01.12	9–19
Spring 2012	sp12	01.06.12–15.06.12	153–167
Summer 2012	su12	24.08.12–20.09.12	237–264
Winter 2013	wi13	11.02.13–21.02.13	42–52

[1] The autumn 2011^{a} campaign was conducted in a period of prolonged summer drought and the autumn 2011^{b} campaign was conducted after the first rains.

measurements. For *P. halepensis*, we positioned a layer of needles (~10–15) on the leaf cuvette, avoiding gaps and overlays and sealed the gaskets with Blu-tack (Bostik SA, La Plaine St Denis, France) to keep the needles in position. The preparation and acclimation of the leaves prior to recording the response curves were conducted as in Sperlich et al. (2014).

CO_2 experiments

The CO_2-response curves were recorded at a leaf temperature (T_{Leaf}) of 25 °C and a quantum flux density of 1000 μmol photons m^{-2} s^{-1}. The CO_2 concentrations in the leaf chamber (C_a) used to generate the response curves were 400 → 300 → 200 → 150 → 100 → 50 → 400 → 400 → 600 → 800 → 1200 → 2000 μmol CO_2 m^{-2} s^{-1}. The minimum and maximum times for stabilizing net assimilation rate (A_{net} in μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s in mol H_2O m^{-2} s^{-1}) and stomatal internal CO_2 concentrations

(C_i in µmol CO$_2$ mol air^{-1}) for each log were set to 4 and 6 min, respectively.

Calculation of chlorophyll fluorescence parameters

F'_m and F_s were used to estimate the effective quantum yield of photosystem II (Φ_{PSII}, unitless) as:

$$\Phi_{PSII} = \frac{(F'_m - F_s)}{F'_m}, \quad (1)$$

where F_s is the steady-state fluorescence of a fully light-adapted sample, and F'_m is the maximal fluorescence yield reached after a pulse of intense light. The effective quantum yield of PSII represents the fraction of photochemically absorbed photons for a light-adapted leaf. The electron-transport rate based on the effective quantum yield of PSII (J_{CF} in µmol electron m^{-2} s^{-1}) was calculated as

$$J_{CF} = \varepsilon \times \Phi PSII \times \alpha_L. \quad (2)$$

ε is a scaling factor accounting for the partitioning of intercepted light between photosystem I (PSI) and PSII. We assumed that light was equally distributed between both photosystems ($\varepsilon = 0.5$) (Bernacchi et al. 2002, Niinemets et al. 2005). α_L (unitless) is the foliar absorbance; we used the following values: 0.932 for *Q. ilex* and 0.912 for *P. halepensis* for both sunlit and shaded leaves, 0.935 for sunlit leaves of *A. unedo*, 0.917 for shaded leaves of *A. unedo*, 0.939 for sunlit leaves of *Q. pubescens* and 0.900 for shaded leaves of *Q. pubescens*. For the determination of α_L, foliar reflectance and transmittance were measured at midday in August 2012 using a UniSpec Spectral Analysis System spectroradiometer (PP Systems, Amesbury, MA, USA). The ambient photosynthetic electron transport (J_{amb}) was defined as the value of J_{CF} at a CO$_2$ concentration of 400 µmol CO$_2$ mol air^{-1}, a PPFD of 1000 µmol photons m^{-2} s^{-1} and at a T_{Leaf} of 25 °C. The relationship between J_{amb} and the net assimilation rate (J_{amb}/A_{net}) was used for the analyses of alternative electron sinks other than carbon metabolism. Calculations of F_v/F_m and nonphotochemical quenching (NPQ) can be found in the Note S1 available as Supplementary Data at *Tree Physiology* Online.

Estimation of mesophyll conductance

We estimated g_m (in mol m^{-2} s^{-1} bar^{-1}) using the variable-J method by Harley et al. (1992):

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

where Γ^* is the CO$_2$ concentration at which the photorespiratory efflux of CO$_2$ equals the rate of photosynthetic CO$_2$ uptake, and R_d is the mitochondrial respiration of a leaf in light conditions and was estimated from the light-response curves combining gas exchange and measurements with the CF-method proposed by Yin et al. (2009) (see Note S2 available as Supplementary Data at *Tree Physiology* Online). The chloroplastic CO$_2$ concentration (C_c in µmol CO$_2$ mol air^{-1}) was determined as:

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

Photosynthesis model

The photosynthesis model of Farquhar et al. (1980) considers photosynthesis as the minimum of the potential rates of Rubisco activity (A_c) and ribulose-1,5-bisphosphate (RuBP) regeneration (A_j). The model was further complemented with a third limitation (A_p) that considers the limitation by triose-phosphate use (TPU) at high CO$_2$ concentrations when the CO$_2$ response shows a plateau or decrease (Sharkey 1985). However, we rarely detected A_p limitations and TPU was therefore discarded in our analyses. A_{net} was then determined by the minimum of these two potential rates from an A/C_c curve:

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

where

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

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

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

where J (in µmol electron m^{-2} s^{-1}) is the rate of electron transport. We assumed that J becomes J_{max} under light and CO$_2$ saturation when the maximum possible rate of electron transport is theoretically achieved, although we may have underestimated the true J_{max} (for further details see Buckley and Diaz-Espejo 2015). $V_{c,max}$ and J_{max} define the biochemical potential to drive photosynthesis and are summarized in the term 'photosynthetic potential' (Niinemets et al. 2006). Curves were fit, and diffusion leakage was corrected, as in Sperlich et al. (2014).

Foliar morphology, chemical analyses and assessment of crown condition

Foliar morphological traits were measured on fully expanded leaves ($n = 60$ per leaf position and species) from the excised twigs in five sampling campaigns in spring and autumn 2011^a (2011^a indicates sampling during a drought), and winter, spring and summer 2012. Immediately after the gas exchange analyses, we measured fresh weight (FW, mg) and projected leaf surface area (LA, cm^2) (including petioles) with Photoshop from scanned leaves at 300 dpi. We oven-dried the leaves at 70 °C for 48 h and weighed the leaves for dry weight (DW, mg) and measured leaf thickness (LT, mm) with a portable dial thickness gauge (Baxlo Precisión, Barcelona, Spain). We then calculated the percentage of the leaf WC as $[1 - (DW/FW)] \times 100$. Leaf mass per area (LMA) ($mg\ cm^{-2}$) was calculated as the ratio of DW to LA and leaf tissue density (D, $mg\ cm^{-3}$) as the ratio of LMA to LT. Foliar succulence (S) was calculated as (FW–DW)/LA. We ground the leaves to a fine powder using a MM400 mixer mill (Retsch, Hahn, Germany), encapsulated a sample of 0.7 mg in tin foil and determined carbon and nitrogen contents by EA/IRMS (Elemental Analyzer/Isotope Ratio Mass Spectrometry) and GC/C/IRMS (Gas Chromatography/Combustion/IRMS). The crown condition was assessed using 'International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests' (ICP Forests) standards (Eichhorn et al. 2010).

Statistical analyses

We performed the statistical analyses with R version 3.0.2 (http://www.r-project.org/). The matrix of photosynthetic and morphological traits was subjected to principal component analyses (PCAs) to summarize the principal factors explaining the variation in these parameters. Differences in the parameters between sunlit and shaded leaves were determined with Student's t-tests ($P \leq 0.05$). The normality of the data was tested with Shapiro–Wilk tests. If the data were not normally distributed, they were normalized. One-factorial analyses of variance (ANOVAs) with season as the main factor were used to test for differences in the parameters in each species and leaf position. Significant differences were determined at $P \leq 0.05$ with Fisher's least significance difference (LSD) tests. Bonferroni correction was used for familywise error rate. Linear regression analyses were conducted to study the relationships among various leaf traits such as A_{net}/g_s, A_{net}/g_m, $J_{max}/V_{c,max}$, g_m/g_s and J_{amb}/A_{net}. With analyses of co-variance (ANCOVAs), we tested for differences in regression slopes and intercepts. We applied a non-linear regression analysis using the nls function in R to study the relationship of g_m/LMA.

Results

Environmental and crown conditions

The year 2011 was characterized by 30% more precipitation than the climatic average of 723 mm (1951–2010) (Ninyerola et al. 2007*a*, 2007*b*) (Table 3, Figure 1), and no drought-induced leaf shedding was observed. The winter from 1 December 2011 to 31 January 2012 was relatively mild with average maximum and minimum temperatures of 11.8 and 4.2 °C, respectively, coinciding with high photosynthetic potentials and shoot growth. The precipitation in 2012 was 20% lower than the climatic average (Table 3). *Arbutus unedo* and *Q. pubescens* were strongly defoliated during summer 2012, *Q. ilex* and *P. halepensis* to a lesser extent (Table 4). *Quercus ilex* showed some discolouration in the more exposed sites. Only one individual of *P. halepensis* showed discolouration. The defoliated *Q. pubescens* trees recovered completely in 2013. In contrast, heavily affected individuals of *A. unedo* showed an irreversible dieback of the main leading branches but also vigorous re-sprouting in 2013.

Table 3. Environmental conditions of two contrasting years (2011 and 2012). Total precipitation, mean temperature, mean soil-water content (SWC) and mean VPD are listed for each season/year.

	Precipitation (mm)		Temperature (°C)		SWC ($cm^3\ cm^{-3}$)		VPD (kPa)	
Season	2011	2012	2011	2012	2011	2012	2011	2012
Winter	254	25	8.2	7.3	0.17	0.14	0.3	0.4
Spring	197	141	16.6	16.3	0.19	0.15	0.6	0.8
Summer	81	50	22.4	23.4	0.13	0.12	0.9	1.2
Autumn	272	263	13.4	12.6	0.19	0.18	0.4	0.3
Total	804	479	15.3	15.1	0.17	0.14	0.5	0.7

Table 4. Percentages of crown defoliation of *Q. ilex*, *P. halepensis*, *A. unedo* and *Q. pubescens* ($n = 5$, 4, 5 and 5, respectively) assessed during the severe summer 2012 drought, following ICP standards (Eichhorn et al. 2010).

Defoliation (%)	*Q. ilex*	*P. halepensis*	*A. unedo*	*Q. pubescens*
90–95			4	2
85–90			1	1
50–55				2
20–25	2			
10–15	1	1		
0	2	3		

Effect of season, tree species and leaf position on photosynthetic parameters

In Figure 2a, we present the PCA for the morphological and photosynthetic parameters. No rotation was applied to the space of the PCs. $V_{c,max}$, J_{max} and g_s were negatively correlated with N_{mass}, C_{mass}, NPQ and g_m. F_v/F_m, g_s and WC were negatively correlated with nitrogen and carbon per unit leaf area (N_{area}, C_{area}), LMA and density (D). Nitrogen per unit leaf mass (N_{mass}) and g_m correlated well with LT (Figure 2). A_{net} was correlated negatively with succulence (S) and positively with g_m. PC1 and PC2 explained 37.2 and 20.4% of the variation, respectively. The datapoints within the cluster circles in Figure 2b–d exhibited similar behaviours in photosynthetic and morphological traits. Leaf positions, seasons and species could be separated. Sunlit leaves were characterized by higher values on the orthogonal axis. The horizontal axes separated *A. unedo* and *Q. pubescens* from *Q. ilex* and *P. halepensis*. The orthogonal axes separated *Q. ilex* from *P. halepensis* with generally positive values. The seasonality was further investigated for each species

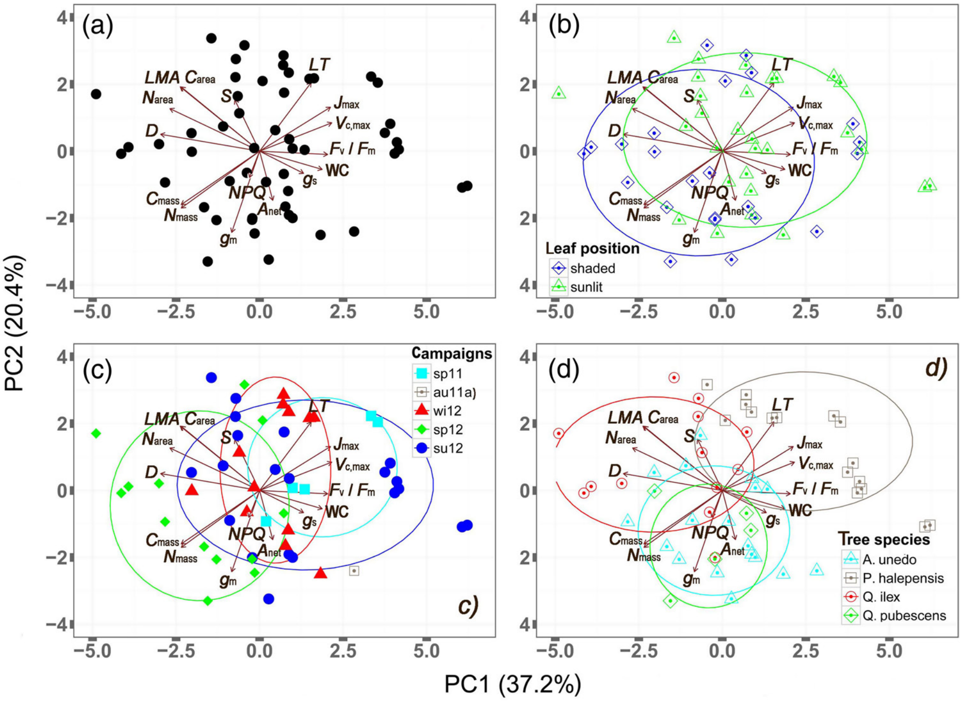

Figure 2. PCA for (a) all trees species, leaf positions and seasons, (b) with differentiation between sunlit and shaded leaves, (c) with differentiation between seasonal campaigns and (d) with differentiation between species. We used a subset of all data where both morphological and photosynthetic information was available. Fifteen parameters were used in the PCA: net assimilation rate (A_{net}), stomatal conductance (g_s), mesophyll conductance (g_m), maximum carboxylation rate ($V_{c,max}$), maximum electron transport rate (J_{max}), NPQ, maximum quantum efficiency of PSII (F_v/F_m), LT, LMA, leaf density (D), WC, nitrogen content per leaf unit area (N_{area}), nitrogen content per leaf unit mass (N_{mass}), carbon content per leaf unit area (C_{area}) and carbon content per leaf unit mass (C_{mass}). The directions of the arrows indicate the higher levels of the parameters. Principal component (PC) 1 explains 37.2% of the variation, and PC2 explained 20.4%. The ellipses are normal probability contour lines of 68% for the factors in (b) leaf positions, (c) seasons and (d) species.

and leaf position with ANOVAs for each photosynthetic and morphological parameter.

Quercus ilex *Quercus ilex* had the most plastic response to the environmental conditions. The sunlit leaves of *Q. ilex* exhibited strong declines in several photosynthetic parameters from summer 2011 to autumn 2011^{a}. $V_{c,max}$, A_{net} and g_s were significantly ($P < 0.05$), and J_{max} and g_m were marginally significantly lower ($P < 0.10$) (Figure 3a1–b1). The means of the majority of the photosynthetic parameters recovered after the first rains in autumn 2011^{b} (2011^{b} indicates sampling after the drought), reaching pre-drought values, but accompanied by a high standard error. This recovery was thus only significant for J_{max} and g_m. Surprisingly, $V_{c,max}$ and J_{max} peaked in winter and not, as expected, in spring. From that peak we observed significant declines from winter to spring to summer 2012. In contrast to the pattern of $V_{c,max}$ and J_{max}, F_v/F_m, A_{net} and g_s peaked in spring 2012 (Figures 3c1 and 4a1–b1). These parameters then also declined significantly in summer 2012. Interestingly, g_m peaked in summer 2012 in parallel with a reduction in g_s (Figure 4c1). The photosynthetic parameters of shaded leaves in *Q. ilex* showed a similar trend, declining after the drought in 2011 and recovering after the autumn rains (Figures 3 and 4). The parameter means of shaded leaves remained relatively stable throughout the season, in contrast to the pattern in sunlit leaves, except for a peak of $V_{c,max}$ and J_{max} in spring 2012. The photosynthetic parameters in *Q. ilex* were significantly lower in shaded leaves. During periods of stress, however, the photosynthetic parameters of sunlit leaves declined and had values similar to those of shaded leaves (Table 5, Figures 3 and 4).

Pinus halepensis Mean $V_{c,max}$, J_{max} and F_v/F_m were generally higher in sunlit leaves of *P. halepensis* than the other species (Figures 3 and 4). The seasonal variation of the photosynthetic potential was not as strongly pronounced as in *Q. ilex*, and mean $V_{c,max}$ and J_{max} remained relatively high and stable in 2011 (Figure 3a1–b1). The 2012 drought had comparatively stronger effects on $V_{c,max}$ and J_{max} than the 2011 drought. Mean A_{net}, g_s and g_m, however, were significantly lower in autumn 2011^{a} (Figure 4a1–c1). These values recovered quickly and significantly after the first autumn rains. The relatively high $V_{c,max}$, J_{max} and F_v/F_m during this period reflected a stronger limitation of g_s and g_m than of the biochemistry imposed on A_{net}. A_{net} recovered in winter 2012 due to the mild conditions (Figure 4a1). The 2012 summer drought significantly reduced the high values of A_{net} observed in winter 2012, but not as much as after the 2011 drought (Figure 4a1).

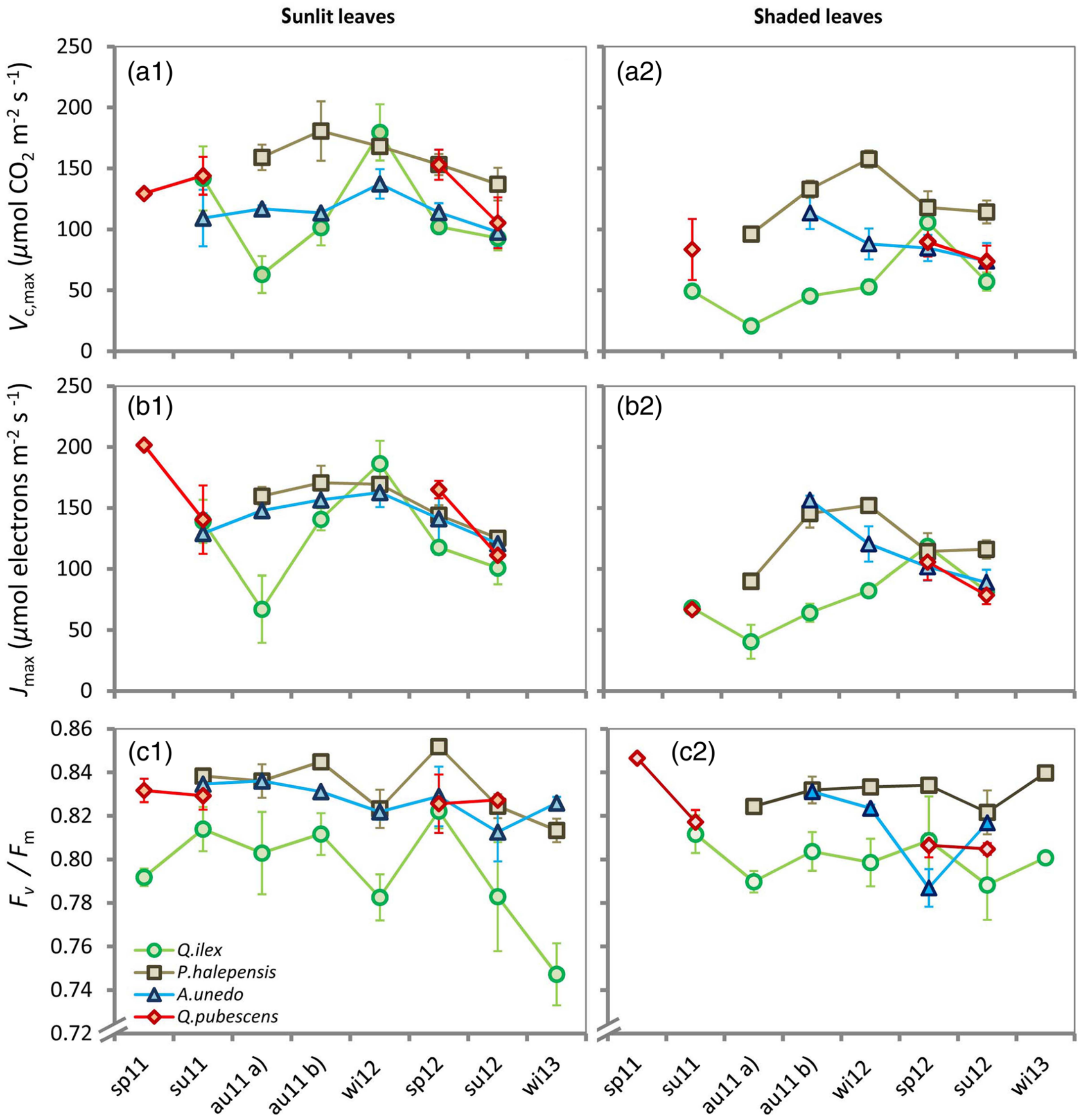

Figure 3. Line graphs depicting seasonal changes of (a) maximum carboxylation rate ($V_{c,max}$), (b) maximum electron-transport rate (J_{max}) and (c) maximum quantum efficiency of PSII (F_v/F_m) for *Q. ilex*, *P. halepensis*, *A. unedo* and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Seasonal campaigns were conducted in spring 2011 (sp11), summer 2011 (su11), autumn 2011^a (au11^a), autumn 2011^b (au11^b), winter 2012 (wi12), spring 2012 (sp12), summer 2012 (su12) and winter 2013 (wi13). Missing data points were due to limitations of labour and equipment. Vertical bars indicate standard errors of the means ($n = 3$–5).

Both g_s and g_m remained relatively stable during this period, so the reductions in A_{net} were due to biochemical limitations ($V_{c,max}$ and J_{max}) (Figures 3 and 4). Sunlit and shaded leaves differed the least in *P. halepensis*, only $V_{c,max}$ and J_{max} were significantly different (Table 5). The sunlit and shaded leaves of *P. halepensis* had similar patterns of seasonal variation, but changes between seasonal campaigns were not significant (Figure 3a1–b1).

Arbutus unedo Similar to *Q. ilex*, the photosynthetic parameters in *A. unedo* varied strongly seasonally but had high standard errors (Figures 3 and 4). A_{net} decreased significantly in winter 2012, in contrast to J_{max} and $V_{c,max}$ that peaked in the same campaign (Figures 3a1–b1 and 4a1). A decline in g_s and g_m in this campaign suggested that they more strongly regulated A_{net} (Figure 4b1–c1). A_{net}, g_s and g_m peaked in spring 2012. These increases were significant for A_{net} and g_s and marginally significant for g_m relative to the other field campaigns (Figure 4a1–4c1). The photosynthetic parameters were generally lower in the shaded leaves of *A. unedo*, but with no clear pattern and high variability (Table 5).

Quercus pubescens The photosynthetic potentials were much higher in *Q. pubescens* than in the other species but also had high standard errors (Figure 3a1–b1). The 2012 summer drought led to a decline of the photosynthetic potentials by approximately one-third. These decreases were only significant for the average of spring 2011 and spring 2012 relative to the average of summer 2011 and summer 2012. A_{net} showed a similar trend, with a

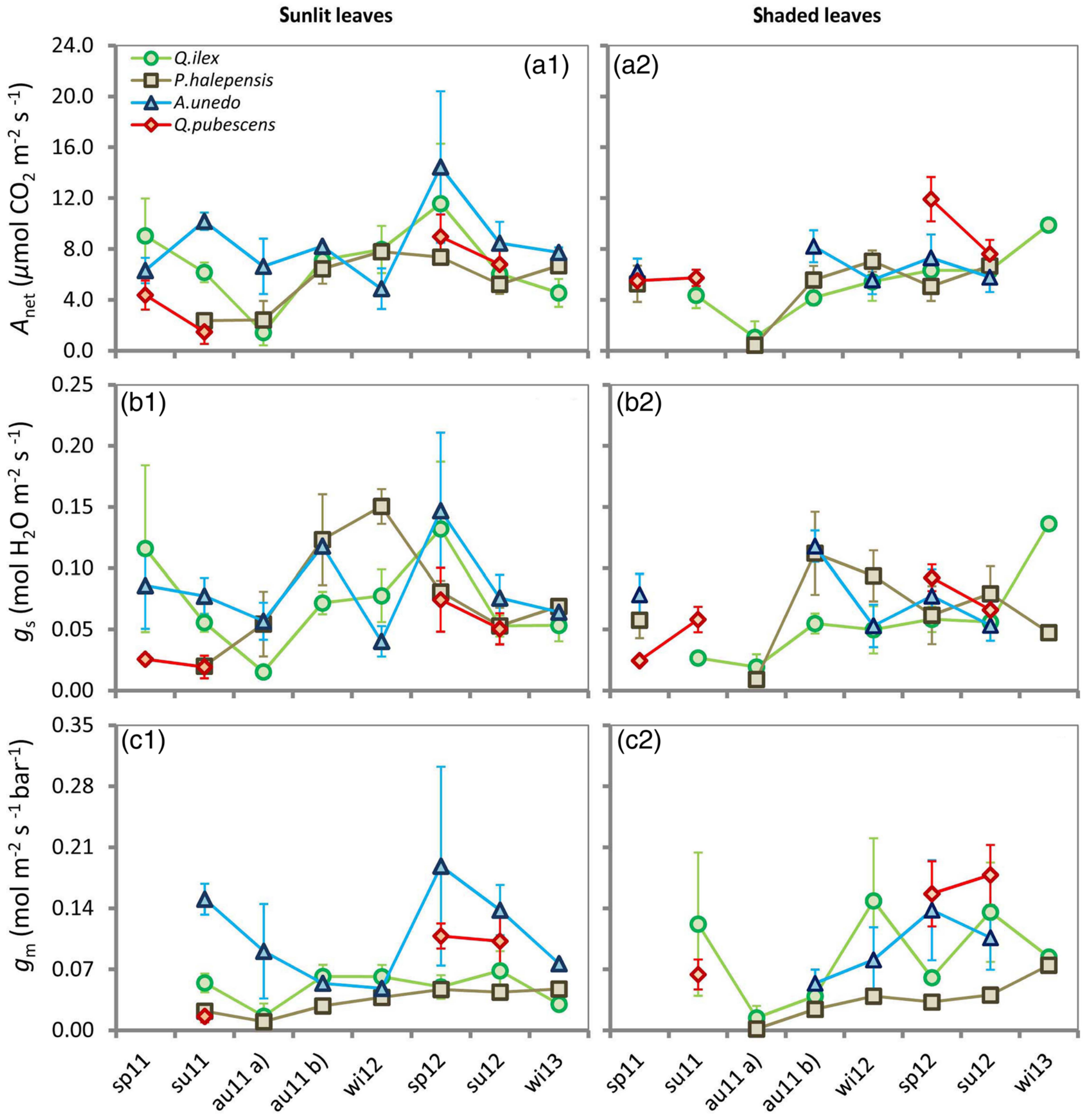

Figure 4. Line graphs depicting seasonal changes of (a) net assimilation (A_{net}), (b) stomatal conductance (g_s) and (c) mesophyll conductance (g_m) for *Q. ilex*, *P. halepensis*, *A. unedo* and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Seasonal campaigns were conducted in spring 2011 (sp11), summer 2011 (su11), autumn 2011^a (au11^a), autumn 2011^b (au11^b), winter 2012 (wi12), spring 2012 (sp12), summer 2012 (su12) and winter 2013 (wi13). Missing data points were due to limitations of labour and equipment. Vertical bars indicate standard errors of the means ($n = 3$–5).

peak in spring 2012 being reduced significantly by the 2012 summer drought (Figure 4a1). Stomatal control was more strongly pronounced than mesophyllic control (Figure 4b1–c1). Shaded leaves had higher A_{net}, g_m and g_s means throughout the campaigns, in contrast to lower means of $V_{c,max}$ and J_{max} (Figures 3 and 4a1–b2). Shaded leaves generally showed lower values than sunlit leaves and were less affected by the droughts (Figures 3 and 4).

Morphological parameters

The foliar traits of *P. halepensis* and *Q. ilex* acclimated most strongly to drought. Leaf mass per area was significantly higher in *P. halepensis* and *Q. ilex* in both shaded and sunlit leaves in summer 2012 compared with the previous field campaigns (Figure 5a1–a2). This was similar in *A. unedo* but less pronounced. LMA had no clear pattern in *Q. pubescens*. Elevated LMA was accompanied by higher values of leaf density (*D*), succulence (S) and carbon content, indicating a more sclerophyllic and succulent structure as a response to the drier conditions in 2012 (Figures S2 and S3 available as Supplementary Data at *Tree Physiology* Online). N_{mass} was significantly higher in spring and summer 2012 for *Q. ilex* and *P. halepensis* (shaded and sunlit leaves) and for shaded leaves of *A. unedo*, but not for *Q. pubescens* (Figure 5b1–b2).

Relationships of photosynthetic and morphological parameters

In order to analyse the general pattern of several relationships of the photosynthetic parameters and foliar traits, we used

Table 5. Means ± standard errors of a set of photosynthetic parameters and foliar traits for sunlit and shaded leaves of *Q. ilex*, *P. halepensis*, *A. unedo* and *Q. pubescens*. *P*-values indicate the statistical significance of the differences between sunlit and shaded leaves determined by Student's *t*-tests. Significance is indicated with bold text.

Species	*Q. ilex*		*P*	*P. halepensis*		*P*	*A. unedo*		*P*	*Q. pubescens*		*P*
Leaf position	Sunlit	Shaded		Sunlit	Shaded		Sunlit	Shaded		Sunlit	Shaded	
Variable	Mean	Mean		Mean	Mean		Mean	Mean		Mean	Mean	
$V_{c,max}$	121.5 ± 11	53.3 ± 5	**0.000**	158.2 ± 5	128.6 ± 5	**0.001**	111 ± 8	85 ± 7	**0.018**	134 ± 11	81 ± 9	**0.002**
J_{max}	134.6 ± 10	76.5 ± 5	**0.000**	149.7 ± 5	130.6 ± 6	**0.023**	133 ± 8	110 ± 8	**0.045**	135 ± 12	83 ± 7	**0.004**
$J_{max}/V_{c,max}$	1.11 ± 0.05	1.42 ± 0.07	**0.002**	0.98 ± 0.03	1.01 ± 0.02	0.356	1.20 ± 0.03	1.34 ± 0.06	0.064	1.03 ± 0.07	1.09 ± 0.08	0.549
F_v/F_m	0.79 ± 0.01	0.80 ± 0.30	0.302	0.83 ± 0.003	0.827 ± 0.003	0.420	0.81 ± 0.006	0.82 ± 0.004	0.631	0.829 ± 0.003	0.783 ± 0.03	0.131
R_d	1.28 ± 0.11	0.98 ± 0.09	**0.043**	1.72 ± 0.19	1.33 ± 0.16	0.123	1.48 ± 0.11	0.88 ± 0.12	**0.001**	0.99 ± 0.14	0.96 ± 0.11	0.890
A_{net}	6.6 ± 0.7	4.89 ± 0.47	**0.056**	5.5 ± 0.5	5.8 ± 0.5	0.657	7.4 ± 0.7	6.4 ± 0.6	0.267	4.3 ± 0.9	8.4 ± 1.0	**0.004**
g_s	0.070 ± 0.0110	0.049 ± 0.006	0.104	0.083 ± 0.101	0.080 ± 0.012	0.839	0.069 ± 0.008	0.069 ± 0.008	0.967	0.035 ± 0.007	0.068 ± 0.008	**0.005**
g_m	0.048 ± 0.006	0.082 ± 0.018	0.084	0.033 ± 0.003	0.035 ± 0.004	0.771	0.096 ± 0.014	0.095 ± 0.019	0.959	0.060 ± 0.018	0.141 ± 0.023	**0.013**
Φ_{PS2}	0.202 ± 0.02	0.114 ± 0.01	**0.000**	0.252 ± 0.012	0.233 ± 0.015	0.312	0.206 ± 0.013	0.164 ± 0.012	**0.024**	0.200 ± 0.017	0.165 ± 0.011	0.103
NPQ	2.89 ± 0.12	3.14 ± 0.15	0.197	3.17 ± 0.10	3.10 ± 0.14	0.689	3.49 ± 0.15	3.68 ± 0.17	0.432	3.17 ± 0.26	2.54 ± 0.14	**0.050**
LMA	23.8 ± 1.5	19.1 ± 1.5	**0.027**	18.6 ± 2.4	19.7 ± 2.5	0.763	13.1 ± 1.1	11.5 ± 1.3	0.338	10.9 ± 0.6	9.7 ± 0.9	0.239
LT	0.039 ± 0.001	0.030 ± 0.001	**0.000**	0.067 ± 0.001	0.059 ± 0.001	**0.000**	0.028 ± 0.001	0.025 ± 0.001	**0.041**	0.031 ± 0.001	0.029 ± 0.001	**0.038**
C_{area}	105.5 ± 8.3	96.2 ± 8.5	0.440	82.2 ± 16.8	98.2 ± 14.1	0.470	57.6 ± 4.4	59.2 ± 8.7	0.873	49.0 ± 2.8	48.5 ± 5.8	0.942
C_{mass}	35.5 ± 4.1	51.6 ±	**0.020**	22.3 ± 2.7	20.7 ± 2.4	0.661	62.1 ± 5.6	70.8 ± 8.3	0.395	48.6 ± 4.4	68.6 ± 5.4	**0.010**
N_{area}	3.19 ± 0.29	2.88 ± 0.29	0.451	1.48 ± 0.33	1.89 ± 0.29	0.353	1.39 ± 0.09	1.31 ± 0.19	0.711	1.78 ± 0.16	1.87 ± 0.22	0.743
N_{mass}	1.08 ± 0.14	1.55 ± 0.18	**0.047**	0.41 ± 0.06	0.40 ± 0.05	0.890	1.51 ± 0.14	1.58 ± 0.18	0.763	1.78 ± 0.23	2.71 ± 0.26	**0.015**

ANCOVAs to test for differences in the slopes between seasons across all species.

The slope the A_{net}/g_s relationship was significantly steeper in summer and autumn 2011^a in all species compared with the other field campaigns (Figure 6a1, Table S1 available as Supplementary Data at *Tree Physiology* Online), suggesting an increased intrinsic water-use efficiency during the dry period in 2011. Shaded leaves had a similar conservative water-use strategy in autumn 2011^a (Figure 6a2). Shallower slopes in autumn 2011^b in both leaf positions represent rapid responses (<1 week) to the post-drought rains easing the strict stomatal control.

The A_{net}/g_m relationship in autumn 2011^a also had a significantly steeper slope in both sunlit and shaded leaves recovering after the first rains in autumn 2011^b (Figure 6b1–b2, Table S2 available as Supplementary Data at *Tree Physiology* Online). In the drier year 2012, g_m imposed less resistance on photosynthetic assimilation compared with the wet year 2011. The slope of the A_{net}/g_m relationship was significantly higher for winter 2012 than spring and summer 2012, suggesting a stronger control of g_m on photosynthesis in winter. The autumn 2011^a and summer 2012 droughts had strong effects on the slope of A_{net}/g_m in shaded leaves.

With the ANCOVA of the relationship of g_m and g_s, we investigated the proportional diffusion limitation on photosynthesis. We observed seasonal differences across all species (Figure 6c1–c2, Table S3 available as Supplementary Data at *Tree Physiology* Online). Mesophyllic control was stronger in the dry autumn 2011^a and the two winter periods. In contrast, stomatal control was higher than mesophyllic control in the mild 2011 summer drought. This was most strongly pronounced in *P. halepensis* and *Q. ilex* (data not shown).

The slope in the relationship of $V_{c,max}$ and J_{max} was significantly steeper in autumn 2011^a for both sunlit and shaded (Figure 7a1–a2, Table S4 available as Supplementary Data at *Tree Physiology* Online) leaves due to a stronger reduction in $V_{c,max}$ compared with J_{max}. The overall $J_{max}/V_{c,max}$ ratios were 1.09 for sunlit and 1.24 for shaded leaves. The slope of the J_{amb}/A_{net} relationship in sunlit and shaded leaves was significantly lower in the more humid periods (autumn 2011^b, winter 2012 and winter 2013), indicating lower protective energy dissipation and alternative electron pathways under favourable conditions (Figure 7b1–b2, Table S5 available as Supplementary Data at *Tree Physiology* Online).

Increased foliar sclerophylly led to higher LMAs and thus to higher diffusion resistances in the mesophyll, as shown by the relationship between g_m and LMA (Figure 8, Table S6 available as Supplementary Data at *Tree Physiology* Online). In spring 2012 and summer 2012, we detected a less negative exponent (hence a gentler curve) (−0.953 and −0.800, respectively) compared with winter 2012 and autumn 2011^a (−1.486 and −1.533, respectively). This shows that, regardless of the drier conditions and higher LMA in 2012, g_m was higher in this period

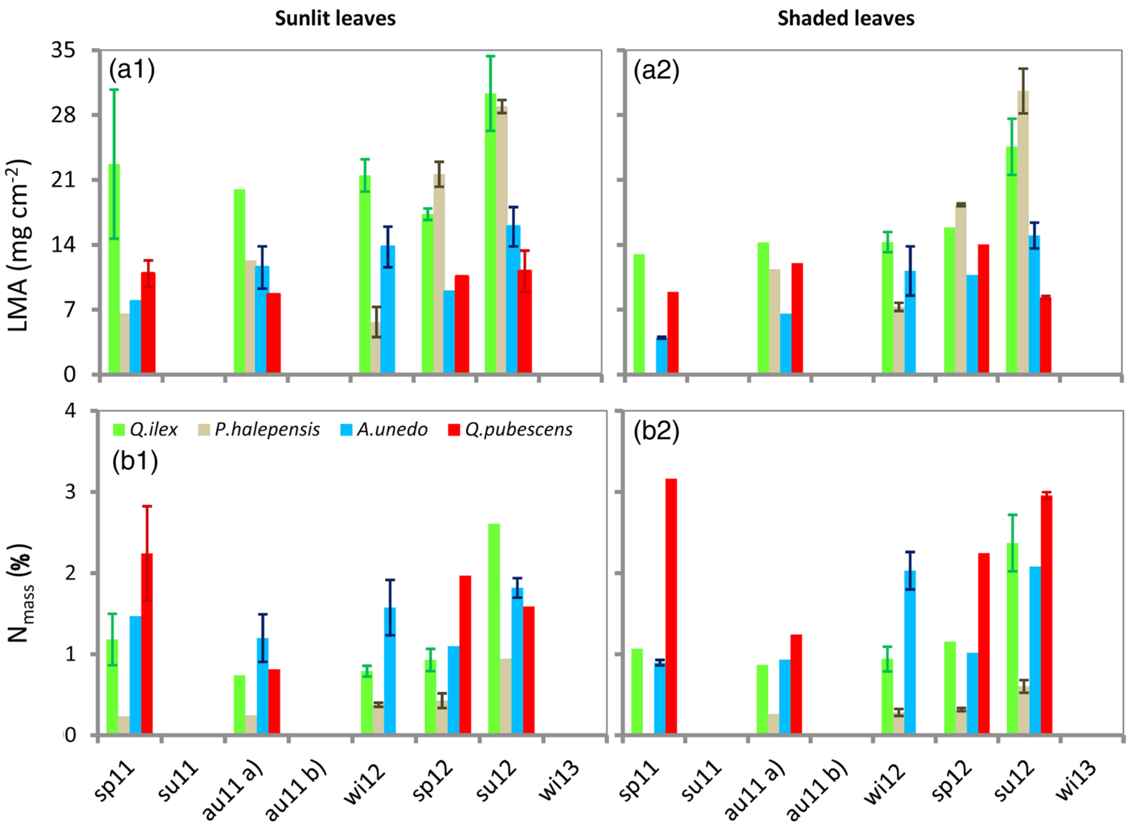

Figure 5. Bar charts depicting seasonal changes of (a) leaf mass per area (LMA) and (b) percentage of nitrogen content per unit leaf mass (N_{mass}) for *Q. ilex*, *P. halepensis*, *A. unedo* and *Q. pubescens* in sunlit (1) and shaded (2) leaves. Error bars indicate standard errors of the means ($n = 3$–5).

reflecting a regulatory mechanism of g_m in the CO_2 diffusion pathway (in line with the results of the g_m/g_s analyses).

Discussion

Photosynthetic seasonality and effects of drought

We found that $V_{c,max}$ and J_{max} acclimated strongly to the seasonal changes in temperature and water availability in agreement with previous studies (Corcuera et al. 2005*b*, Vitale and Manes 2005, Misson et al. 2006, Ribeiro et al. 2009, Limousin et al. 2010). High radiation and water stress can have a combined negative effect on the photosynthetic apparatus, especially in sunlit leaves. Stomata close to avoid transpiration loss and hydraulic failure, but stomatal closure impairs the diffusion of the CO_2 needed in the chloroplasts, the site of carboxylation. $V_{c,max}$ is a proxy for the maximum potential rate of carboxylation, which is carried out by Rubisco, a costly nitrogen-rich protein. The temporary unemployment of Rubisco due to limited substrate (CO_2) availability leads to its de-activation and, during chronic water stress, to its decomposition (Parry 2002, Chaves and Oliveira 2004, Lawlor and Tezara 2009). High incoming radiation that cannot efficiently be dissipated in the Calvin cycle over-excites the photoreaction centres (photoinhibition) and produces reactive oxygen species that damage the photosystems and the ATP synthase needed for the carbon reactions (Epron et al. 1993). Leaves prevent harmful excess energy with protective actions such as the reorganisation of the thylakoid membrane, closure of reaction centres and reduced antennal size (Huner et al. 1998, Maxwell and Johnson 2000, Ensminger et al. 2012, Verhoeven 2014). These actions reduce PSII efficiency and J_{max}, and enhance alternative energy pathways to prevent damage on the molecular level on the cost of a lower carbon assimilation.

The trees in our study site maintained considerable rates of A_{net} during moderate drought through improved water relations via g_s and g_m control. The relatively stable F_v/F_m values indicate that the protective actions against photoinhibitory stress were effective. The trees showed trunk rehydration after the first autumn rain (Sánchez-Costa et al. 2015) and quickly recovered their photosynthetic potential, suggesting that the Rubisco content remained unaffected by moderate drought. The drought impacts were much more severe in the dry year 2012, illustrating the vulnerability of tree physiology to the depletion of soil-water reserves during the early growing season. The severity of drought strongly determined the relative limitations of g_s and g_m on photosynthesis, especially in *Q. ilex* and *P. halepensis*. Stomatal closure regulated photosynthesis during both the moderate and severe droughts; g_m, in contrast, decreased under moderate, but increased under severe drought. We postulate that altered g_m can ease the leaf

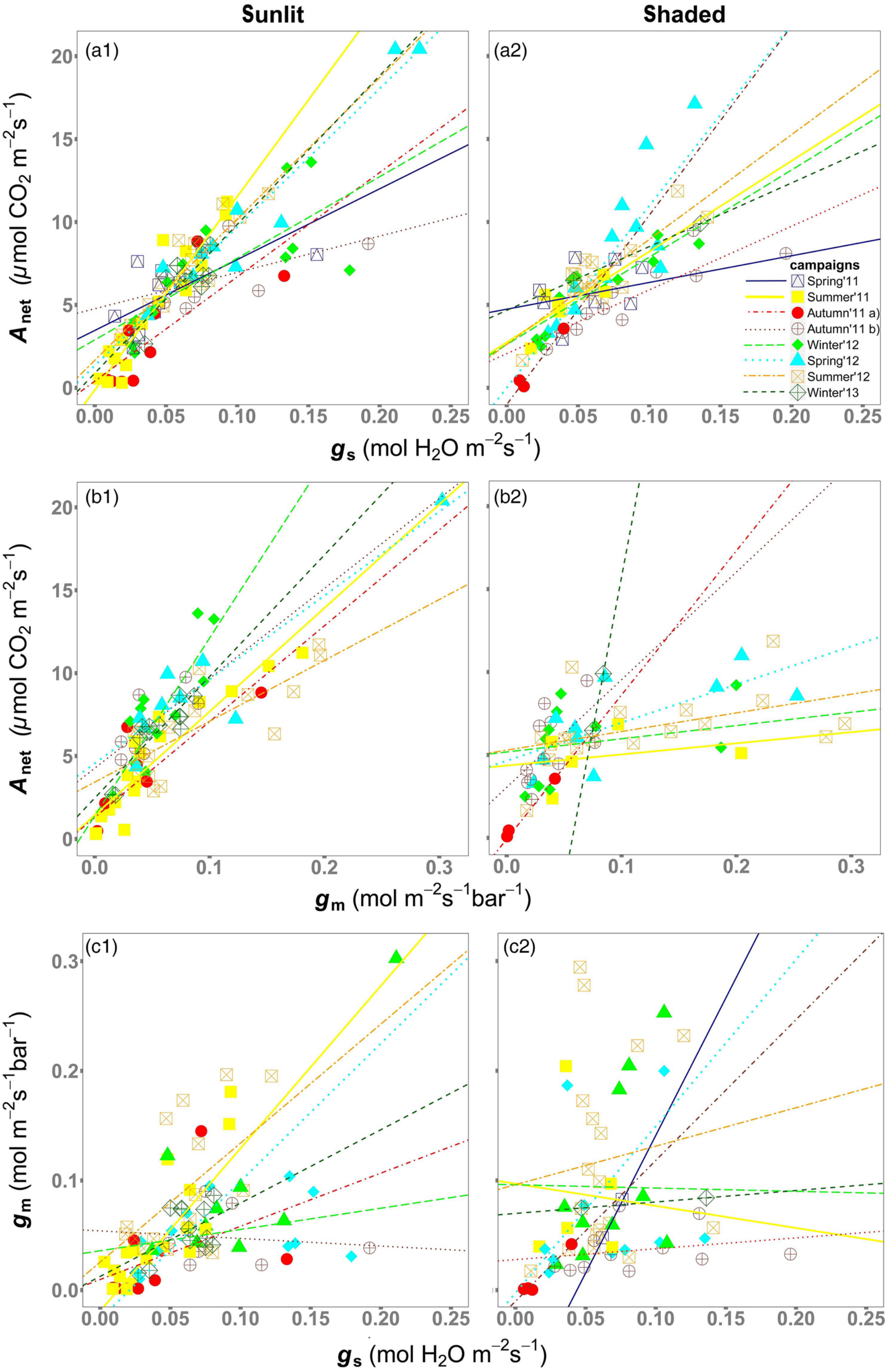

Figure 6. Seasonal changes of the relationships between (a) net assimilation (A_{net}) and stomatal conductance (g_s), (b) A_{net} and mesophyll conductance (g_m) and (c) g_m and g_s in sunlit (1) and shaded (2) leaves. The regression lines represent the seasonal changes across species. For regression equations see Tables S1–S3 available as Supplementary Data at *Tree Physiology* Online. The relationships are shown as a solid thin line for spring 2011, solid thick line for summer 2011, dots-dash for autumn 2011^a, small dots for autumn 2011^b, dashes for winter 2012, thick dots for spring 2012, short dashes-large dashes for summer 2012, and short dashes for winter 2013. Statistical differences in the slopes between seasonal campaigns were tested by ANCOVAs.

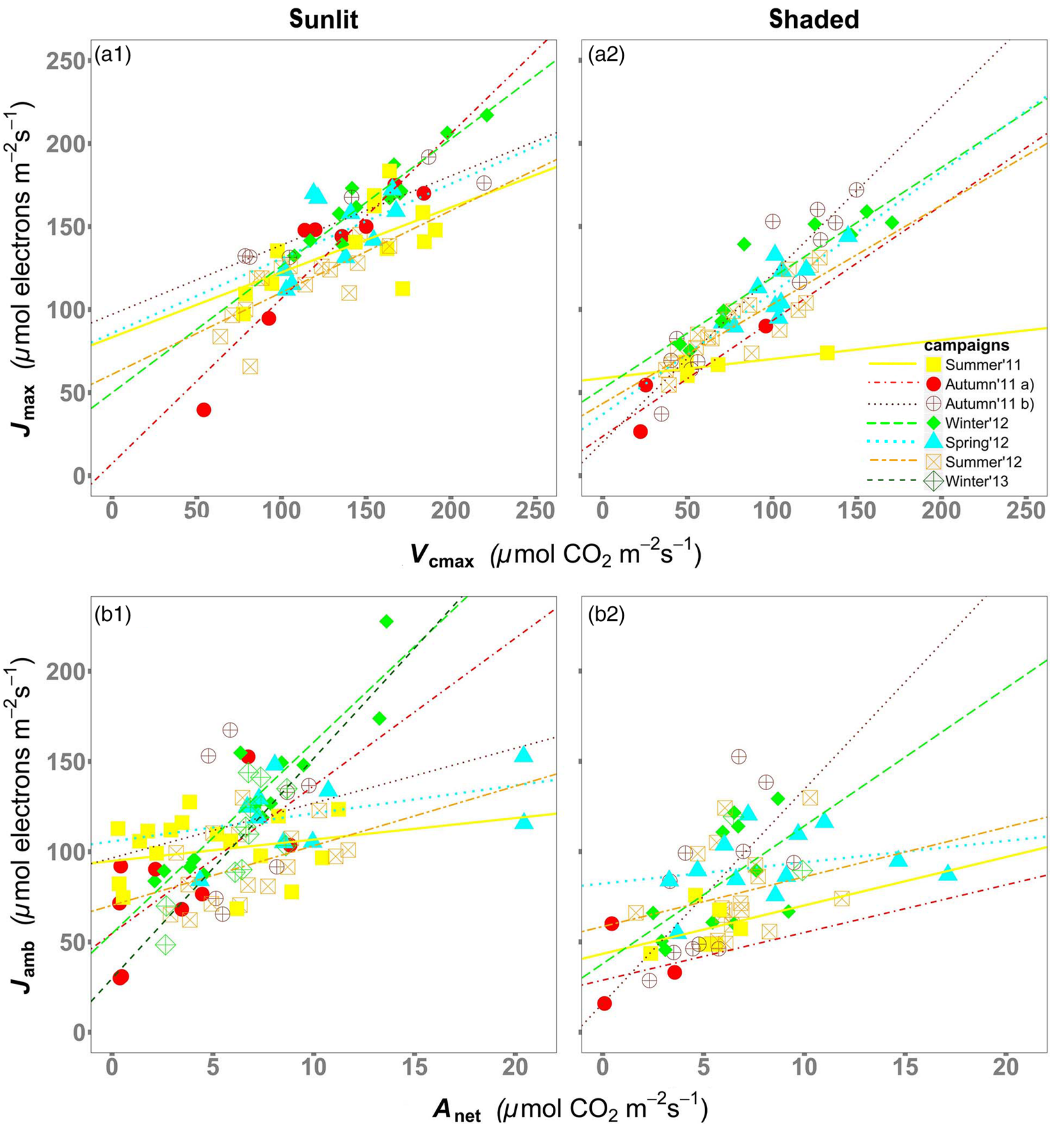

Figure 7. Seasonal changes of the relationships between (a) the maximum electron-transport rate (J_{max}) and the maximum carboxylation rate ($V_{c,max}$) and (b) the electron-transport rate from chlorophyllic fluorescence (J_{amb}) and net assimilation (A_{net}) at ambient CO$_2$ concentrations and saturating light in sunlit (a) and shaded (b) leaves. The regression lines represent the seasonal changes across species. For regression equations see Tables S4 and S5 available as Supplementary Data at *Tree Physiology* Online. The relationships are shown as a solid thick line for summer 2011, dots-dash for autumn 2011[a], small dots for autumn 2011[b], dashes for winter 2012, thick dots for spring 2012, short dashes-large dashes for summer 2012, and short dashes for winter 2013.

internal CO$_2$ diffusion needed for photosynthesis, especially under chronic water stress when depleted non-structural carbohydrates (NSCs) make plants particularly reliant on photosynthetic products for refinement, repair and protective actions (Niinemets et al. 2009). Major changes of Φ_{PSII}, F_v/F_m and photosynthetic potentials across all species reflected these refinements of the photosynthetic apparatus as responses to chronic water stress in summer 2012.

These acclimatizations occurred not only under dry and hot conditions, but also in winter at high radiation and low temperature. Nevertheless, favourable winter conditions in 2012 resulted in biochemical recovery (peak of $V_{c,max}$ and J_{max}), new shoot growth and moderate transpiration across species (often exceeding summer values) (Sánchez-Costa et al. 2015). Year-round growth patterns with several flushes during the year have also been reported in other studies (Alonso et al. 2003). Under novel climatic conditions, favourable conditions in winter may be crucial in the competition between evergreen and deciduous tree species.

We observed a highly species-specific pattern. *Quercus ilex* and *A. unedo* followed a water-spending, anisohydric strategy that maintained A_{net} and g_s in parallel with lower $V_{c,max}$ and J_{max}. In contrast, *P. halepensis* had significantly decreased g_s, consistent with the conservative water-use strategy and strict stomatal control of isohydric species (Borghetti et al. 1998, Martinez-Ferri

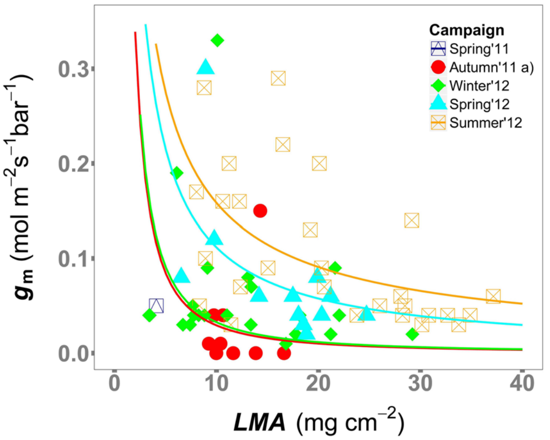

Figure 8. Seasonal changes of the relationship for all species and leaf positions between (a) mesophyll conductance (g_m) and leaf mass per area (LMA). We used a subset of morphological and photosynthetic data. Non-linear regression lines of the form $y = x^{-b}$ were fitted to the data. The upper curve is for summer 2012 ($b = 0.800$), the middle curve is for spring 2012 ($b = 0.953$) and the lower two overlaying curves are for autumn 2011^a ($b = 1.533$) and winter 2012 ($b = 1.486$).

et al. 2000). *Quercus ilex* generally responded most plastically by rapidly adjusting the photosynthetic machinery to the prevailing conditions (García-Plazaola et al. 1997, 1999, Martínez-Ferri et al. 2004). *Pinus halepensis* was the most tolerant to photoinhibition and had the most robust photosynthetic machinery to combat abiotic stress (Baquedano and Castillo 2006, Sperlich et al. 2014). The mesophyllic diffusion limitation was lowest in *Q. pubescens* and *A. unedo*, as we claim, due to their deciduous/semi-evergreen foliar habits and lower LMAs (see also Tomás et al. 2014). *Quercus pubescens* must maximize gas exchange during a shorter growing season, leading to high photosynthetic potentials, A_{net} (Baldocchi et al. 2010) and transpiration rates throughout the summer (Poyatos et al. 2008, Sánchez-Costa et al. 2015).

Responses specific to leaf position

The seasonality of photosynthetic parameters was qualitatively different between leaf positions (Niinemets et al. 2006, Vaz et al. 2011) and was mostly pronounced in sunlit leaves. Shaded leaves cushioned the negative climatic effects, maintaining their functionality compared with sunlit leaves. Foliar anatomy, morphology and biochemistry were highly specialized and dependent on the light regime, leading to smaller but also thicker sunlit leaves and broader and thinner shaded leaves (Kull and Niinemets 1993, Terashima and Hikosaka 1995, Niinemets 2001). Shaded leaves had lower N, photosynthetic potentials, carbon metabolisms and higher $J_{max}/V_{c,max}$ ratio (see also Le Roux et al. 2001). Shaded leaves invest in higher J_{max} relative to $V_{c,max}$ in order to increase the light-use efficiency. Responses specific to leaf position, however, differed among tree species due to distinct foliar morphologies and crown architectures. The sun-exposed crown position of *P. halepensis* surmounting the forest canopy resulted in high photosynthetic potentials and a low $J_{max}/V_{c,max}$ ratio throughout the crown. Pine needles attain nearly saturated photosynthetic rates over a wide range of diurnal and seasonal variation in radiation due to their cylindrical shape and steep angles (Jordan and Smith 1993, Lusk et al. 2003). Similarly, *Q. pubescens* showed a low differentiation between sunlit and shaded leaves. A low $J_{max}/V_{c,max}$ ratio throughout the crown suggests a higher proportion of sunlit leaves. In contrast, the comparatively higher $J_{max}/V_{c,max}$ ratio of sunlit leaves in *A. unedo* reflects a more shaded growth environment explained by its subordinated position in the forest canopy. The *Q. ilex* canopy was dense with a high proportion of shaded leaves, in line with its shade tolerance. Hence, leaf position specific responses were highest in *Q. ilex*. The comparatively higher photosynthetic values in sunlit leaves decreased partly below the level of shaded leaves under stress conditions (see also Sperlich et al. 2014). Shaded leaves are less exposed to the dramatic changes in radiation and temperature in the outer canopy and can be of particular importance for *Q. ilex* to attain a positive net carbon ratio during stress periods (Valladares et al. 2008). We stress that the solar environment of the leaves is a crucial factor for assessing tree performance, especially in a competitive environment.

Acclimation of foliar morphology

Mediterranean trees acclimate to water deficits with higher investments in structural compounds, thereby increasing leaf density and succulence (Niinemets 2001, Ogaya and Peñuelas 2006, Poorter et al. 2009). Foliar traits are known to be good indicators for the ability of Maquis-species to respond to decreases in rainfall under climate change (Gratani and Varone 2006, Ogaya and Peñuelas 2007). We confirm that severe water deficit resulted in increased LT and reduced LA and consequently in higher LMA. It was reported that the plasticity of leaf morphology is generally higher than the plasticity of foliar chemistry and assimilation rates over a wide range of woody species (Niinemets 2001). Under moderate drought, however, foliar morphology was less plastic than foliar chemistry and assimilation rates (Quero et al. 2006); severe water stress affected both to a similar extent. Leaf trait acclimation strongly constrained mesophyll conductance under severe drought, especially in *Q. ilex* and *P. halepensis* (see also Tomás et al. 2013). We postulate that foliar morphological traits served best as proxies for drought acclimation in *Q. ilex* (Grossoni et al. 1998, Bussotti et al. 2000) and *P. halepensis* (Alonso et al. 2003), both characterized by high leaf longevities. These changes may be accompanied by increased leaf vein density that may help to increase the tolerance to foliar hydraulic dysfunction in Mediterranean plants (Nardini et al. 2014). The foliar morphological traits of *A. unedo* and *Q. pubescens* acclimated the least, so leaves were susceptible to foliar hydraulic dysfunction and drought deciduousness. We attribute this species-specificity in acclimation of foliar morphology to functional differences of leaf investment costs and distinct leaf shedding strategies between deciduous/semi-deciduous (*Q. pubescens* and *A. unedo*) and evergreen sclerophyllic species (*Q. ilex* and *P. halepensis*), which we will elaborate further in the following section.

Crown defoliation in summer 2012

The lack of rain in early 2012 predisposed the vegetation to leaf senescence observed in summer 2012, with high variability across and within species. Leaf senescence was highest in *A. unedo* and *Q. pubescens*—showing partly completely defoliated crowns. *Quercus ilex* and mostly *P. halepensis* overcame this period with marginal leaf shedding. Stored NSCs strongly determine the recovery of xylem hydraulic conductivity by vessel refilling and the resistance of water transport to drought under prolonged evaporative demand (Ogasa et al. 2013). Depleted NSCs may limit the ability to recover from embolisms (Galiano et al. 2012). *Arbutus unedo* is susceptible to hydraulic dysfunction induced by depleted NSC (e.g., Rosas et al. 2013), which might explain the severe branch dieback of *A. unedo* in our study. As a shrubby species characteristic of Maquis biomes (Beyschlag et al. 1986, Harley et al. 1986), *A. unedo* likely faced a trade-off between growing tall and risking hydraulic dysfunction due to high xylem tension under severe soil-water deficits (Choat et al. 2012). However, *A. unedo* might contend with severe climatic stress through its strong capacity to resprout (see also Ogaya and Peñuelas 2004).

Pines follow a strategy of water conservation and embolism avoidance, because they have a low capacity to store carbohydrates (Meinzer et al. 2009). *Pinus halepensis* had a high growth-based water-use efficiency (WUE_{BAI} = Basal area increment/Tree transpiration) during severe drought (Sánchez-Costa et al. 2015), through the combined effect of photosynthetic downregulation, foliar-trait acclimation and improved gas exchange. Thus, this tree species is comparatively the most productive one, especially under drought, confirming its high competitiveness in dry habitats (Zavala and Zea 2004, Maseyk et al. 2008, de Luis et al. 2011).

Sánchez-Costa et al. (2015) observed a higher WUE_{BAI} in *Q. pubescens* compared with *Q. ilex* during the soil-moisture deficit in 2012. The 'low-cost' leaves of the deciduous *Q. pubescens* facilitate drought senescence, so that the reduced transpiratory surface area can effectively avoid damage from hydraulic cavitation and xylem embolism (Ogaya and Peñuelas 2006, Barbeta et al. 2013). Fully refoliated crowns in the following growing season was evidence of its success relative to *A. unedo*. The extraordinarily high photosynthetic potentials in the remaining leaves were probably due to a mechanism to compensate for the reduced total leaf area, as indicated by the higher translocation of leaf nitrogen before leaf shedding.

Quercus ilex can effectively tolerate the effects of drought by reducing its LMA and by allowing low water potentials (anisohydric behaviour) (Villar-Salvador et al. 1997, Ogaya and Peñuelas 2006, Limousin et al. 2009). Its hydraulic features are highly plastic, because yearly vessel diameter and recovery are well coupled with annual rainfall (Fotelli et al. 2000, Corcuera et al. 2005*a*). *Quercus ilex*, however, was also severely effected in 2012, shedding leaves (Tognetti et al. 1998), reducing radial growth and WUE_{BAI} (Sánchez-Costa et al. 2015). The positive A_{net}, despite the reduced WUE_{BAI}, suggests that photosynthetic products were used for the maintenance and recovery of xylem hydraulic conductivity instead of growth (Castell et al. 1994). In fact, *Quercus* species show generally a good ability in vessel refilling after xylem embolism (Carnicer et al. 2013).

Implications for the global carbon cycle and modelling

There is evidence that the use of seasonally variable photosynthetic potentials reduces uncertainties in modelled ecosystem carbon fluxes relative to the use of constant values (Wilson et al. 2001, Tanaka et al. 2002, Kosugi et al. 2003, 2006, Medvigy et al. 2013). The significant seasonal acclimation of $V_{c,max}$ and J_{max} observed in our study demonstrates that prognostic models should account for seasonal variation, especially in drought-prone areas. Also, the significant role of g_m under abiotic stress periods highlights its importance for estimating the whole-carbon gain. It is now widely accepted that the apparent values of $V_{c,max}$ and J_{max} derived from A/C_i curves are, from a physiological point of view, incorrect. A recent study by Sun et al. (2014) for nearly 130 C_3 species showed that the assumption of infinite g_m in the parameterization of CO_2-response curves underestimates $V_{c,max}$ and J_{max} by up to 75 and 60%, respectively. Terrestrial biosphere models on regional or global scales are most commonly calibrated on A/C_i-based parameters and therefore use apparent values of $V_{c,max}$ and J_{max}. Incorporating values of $V_{c,max}$ and J_{max} parameterized on A/C_c curves would clearly lead to erroneous results, because their use requires the incorporation of g_m and different Rubisco kinetic parameters into the submodels of photosynthesis. Therefore, the use of consistent equations and parameters when incorporating parameters from experimental studies into vegetation models is inevitable to correctly estimate photosynthesis (Rogers et al. 2014). From a modelling point of view, it might seem questionable why including g_m and A/C_c-based parameters would improve simulation results and not just increase model complexity. Terrestrial biosphere models are currently well calibrated against observational data despite their use of apparent $V_{c,max}$ and J_{max}. Another criticism often raised is that there are still potential errors in various methods to estimate g_m (and subsequently $V_{c,max}$ and J_{max}) including the variable *J*-method (used in this study) (Pons et al. 2009, Tholen et al. 2012, Gu and Sun 2014). On the other hand, although this may not represent a perfectly accurate approach, we claim that representing g_m is preferable to neglecting g_m completely (Parkhurst 1994) as large uncertainties remain in the simulations of the future CO_2 fluxes of the global carbon cycle (Anav et al. 2013, Friedlingstein et al. 2014).

Patterns of temperature and precipitation are highly uncertain in these models due to both a lack of scientific understanding and model representation (Booth et al. 2012).

These uncertainties could partly explain the poor modelling performance for Mediterranean-type ecosystems, because the mechanistic description of the photosynthetic processes under water stress is not very well developed (Morales et al. 2005, Keenan et al. 2011, Zheng et al. 2012, Vargas et al. 2013). As we have shown, the limitations imposed by g_m on photosynthetic assimilation can decrease relatively more than the limitations imposed by g_s or biochemistry ($V_{c,max}$ and J_{max}) under drought or winter stress. This distinction has important consequences for the control of water-use efficiency and holds great potential for improving the estimation of ecosystem carbon fluxes under drought conditions (Niinemets et al. 2009). As already mentioned above, the issue of whether (and how) to include g_m in models is actively debated by physiologists and modellers (see also Rogers et al. 2014). Keenan et al. (2010) showed that g_m was the missing constraint for accurately capturing the response of terrestrial vegetation productivity to drought. Yet relatively little information is available from modelling exercises that have included g_m in their algorithms, and more research in this field is needed.

Concluding the above, we underline that we need to consider the seasonality of photosynthetic potentials and mesophyll conductance to explain eco-physiological responses to abiotic stress. These two factors should deserve much more attention in terrestrial biosphere modelling because they hold great potential to reduce model uncertainties, especially under Mediterranean climatic conditions.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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Biogeosciences

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

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Abstract. Evergreen trees in the Mediterranean region must cope with a wide range of environmental stresses from summer drought to winter cold. The mildness of Mediterranean winters can periodically lead to favourable environmental conditions above the threshold for a positive carbon balance, benefitting evergreen woody species more than deciduous ones. The comparatively lower solar energy input in winter decreases the foliar light saturation point. This leads to a higher susceptibility to photoinhibitory stress especially when chilly ($<12\,°C$) or freezing temperatures ($<0\,°C$) coincide with clear skies and relatively high solar irradiances. Nonetheless, the advantage of evergreen species that are able to photosynthesize all year round where a significant fraction can be attributed to winter months, compensates for the lower carbon uptake during spring and summer in comparison to deciduous species. We investigated the ecophysiological behaviour of three co-occurring mature evergreen tree species (*Quercus ilex* L., *Pinus halepensis* Mill., and *Arbutus unedo* L.). Therefore, we collected twigs from the field during a period of mild winter conditions and after a sudden cold period. After both periods, the state of the photosynthetic machinery was tested in the laboratory by estimating the foliar photosynthetic potential with CO_2 response curves in parallel with chlorophyll fluorescence measurements. The studied evergreen tree species benefited strongly from mild winter conditions by exhibiting extraordinarily high photosynthetic potentials. A sudden period of frost, however, negatively affected the photosynthetic apparatus, leading to significant decreases in key physiological parameters such as the maximum carboxylation velocity ($V_{c,max}$), the maximum photosynthetic electron transport rate (J_{max}), and the optimal fluorometric quantum yield of photosystem II (F_v/F_m). The responses of $V_{c,max}$ and J_{max} were highly species specific, with *Q. ilex* exhibiting the highest and *P. halepensis* the lowest reductions. In contrast, the optimal fluorometric quantum yield of photosystem II (F_v/F_m) was significantly lower in *A. unedo* after the cold period. The leaf position played an important role in *Q. ilex* showing a stronger winter effect on sunlit leaves in comparison to shaded leaves. Our results generally agreed with the previous classifications of photoinhibition-tolerant (*P. halepensis*) and photoinhibition-avoiding (*Q. ilex*) species on the basis of their susceptibility to dynamic photoinhibition, whereas *A. unedo* was the least tolerant to photoinhibition, which was chronic in this species. *Q. ilex* and *P. halepensis* seem to follow contrasting photoprotective strategies. However, they seemed equally successful under the prevailing conditions exhibiting an adaptive advantage over *A. unedo*. These results show that our understanding of the dynamics of interspecific competition in Mediterranean ecosystems requires consideration of the physiological behaviour during winter which may have important implications for long-term carbon budgets and growth trends.

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1 Introduction

Mediterranean-type ecosystems are widely associated with broadleaved evergreen sclerophyllous shrubs and trees, the classic vegetation types in climates where hot and dry summers alternate with cool and wet winters (Aschmann, 1973; Blumler, 1991; Orshan, 1983; Specht, 1969). In summer, water is undoubtedly the most important factor limiting growth and survival in the Mediterranean region, whereas spring and autumn provide better growing conditions (Gracia et al., 1999; Orshan, 1983; Sabaté and Gracia, 2011). In winter, the low temperatures and solar radiation limit the amount of energy available for the vegetation, although soil water contents and water pressure deficits are favourable. This highly dynamic seasonality of favourable and unfavourable conditions produces a rich diversity of plants in these regions (Cowling et al., 1996). In turn, this features a highly diverse range of traits and taxa that has produced multiple survival strategies which help to explain the abundance and distribution of species (Matesanz and Valladares, 2014). Nonetheless, the predicted reductions in annual precipitation, increases in mean temperature, and increases in the variability and occurrence of extreme droughts and heat waves in arid and semi-arid regions are likely to affect species abundance and distribution (Friend, 2010; IPCC, 2013; Somot et al., 2008). The battle for survival and dominance in plant communities facing these novel changes in their environments evokes great uncertainties and worries in the scientific community concerning the adaptive ability, distribution shifts, or, at worst, local extinction of species especially in Mediterranean type ecosystems (Matesanz and Valladares, 2014; Peñuelas et al., 2013).

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

Hereby, mixed forests provide us with an ideal test-bed for investigating the different ecophysiological strategies and their sensitivities to abiotic stresses, because all tree species have to contend equally with the yearly variability of environmental conditions. Nevertheless, most ecophysiological studies have been conducted in spring and summer, and winter has been surprisingly overlooked despite its importance for our understanding of the dominance of certain vegetation types and of the responses of vegetation to stress, seasonality and species composition (Oliveira and Peñuelas, 2004; Orshan, 1983; Tretiach et al., 1997). Even though efforts have recently been made to elucidate the behaviour of sclerophyllous ecosystems under variable winter conditions (e.g. García-Plazaola et al., 1999, 1997; Kyparissis et al., 2000; Levizou et al., 2004; Martínez-Ferri et al., 2004; Oliveira and Peñuelas, 2004, 2000), the physiological behaviour of co-occurring species of evergreen trees in the Mediterranean region, including leaf gas exchange (GE) and chlorophyll fluorescence (CF) methods, have been insufficiently studied for understanding the dynamics of photoinhibitory stress and interspecific competition. Therefore, in our study we used an ample set of parameters from GE & CF measurements in order to provide a snapshot in the plant's physiology and in order to characterize in detail the effects on the photosynthetic light and carbon reactions during winter (Flexas et al., 2008; Guidi and Calatayud, 2014). This study was conducted on three species of evergreen trees (*Quercus ilex* L., *Pinus halepensis* Mill., *Arbutus unedo* L.) in northern Catalonia near Barcelona, Spain.

Our aims were to (i) investigate the foliar physiology of these three species under mild winter conditions, (ii) analyse the effect of sudden changes from favourable to unfavourable conditions on photochemical and non-photochemical processes associated with electron transport, CO_2 fixation and heat dissipation, (iii) determine whether leaves exhibit distinct locational (sunlit or shaded) responses to winter stress, and (iv) identify the species-specific strategies when coping with stress, induced by low temperatures and frost. These topics are of particular interest due to the recent report of an increased dominance of angiosperm trees and the negative impacts on pines over extensive areas of the Iberian Peninsula (Carnicer et al., 2013). Therefore, we must improve our understanding of the interactions among co-occurring tree species competing for scarce resources and trying to survive and tolerate novel environmental conditions to be able to predict ecosystem responses to global climate change.

2 Material and methods

2.1 Field site

Our experiment was conducted at the field station of Can Balasc in Collserola Natural Park, a coastal massif (8500 ha) in the hinterlands of Barcelona, northeastern Spain (41°25′ N, 2°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 northeast-facing slope. The climate is characterized by typical Mediterranean seasonal summer droughts and warm temperatures, with a mean August temperature of 22.8 °C. The proximity to the Mediterranean Sea provides mild winters where frosts and snow are rare, as reflected in the mean January temperature of 7.9 °C. Mean annual precipitation and temperature are 723 mm and 15.1 °C (1951–2010), respectively (Ninyerola et al., 2000). The soils have predominantly developed above lithological strata of shales and granite (Sanchez-Humanes and Espelta, 2011). Sensors for measuring air temperature (HMP45C, Vaisala Oyj, Finland) and solar radiation (SP1110 Skye Instruments Ltd, Powys, UK) were installed at a height of 3 m, in a clearing ca. 1 km from the plot.

2.2 Stand history and composition of tree species

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

2.3 Sampling

The sampling of the mild winter period took place in the period 9–19 January 2012 (DOY 9–19). The frosty/chilly period lasted from 19 January–4 February 2012 (DOY 21–35). The sampling period after the frosty/chilly period took place between 14 and 24 February 2012 (DOY 45–55). We obtained sunlit leaves for GE analyses by sampling five twigs with a pruning pull from the outer part of the upper third of the crown, and shaded leaves by sampling five twigs from the inner part of the crown, optimally at similar heights. In the second field campaign after the frost occurrence, however, we were constrained to sample shaded leaves only from *Q. ilex* due to limitation in labour and equipment. The shaded leaves of *P. halepensis* and *A. unedo* could only be sampled in the first, but not in the second field campaign. The twigs were immediately re-cut under water in buckets in the field and transported to the laboratory retained in plastic bags to minimize transpiration. Five replicates of each species were collected for the analysis of GE. The twigs were pre-conditioned in the laboratory at a room temperature of 24–28 °C in dim light for 1–3 d and freshly cut the following morning before the measurement of GE (Niinemets et al., 1999, 2005). We intended to avoid the problems we had faced in the field, such as the limited ability of the instruments to reach the standard operating temperature of 25 °C, which was hampered by low ambient temperatures or unpredictable plant responses such as closed stomata or patchy stomatal conductance (Mott and Buckley, 1998, 2000). The pre-conditioned twigs instead had a stable C_i and sufficiently high g_s, which are required for conducting a noise-free CO_2-response curve. The method of cutting twigs rehydrated stressed leaves at optimum conditions and allowed us to analyse their long-term acclimation to the environmental conditions from which they were derived. This method has been used in other studies (Epron and Dreyer, 1992; Haldimann and Feller, 2004; Laisk et al., 2002; Niinemets et al., 1999, 2005), and we confirmed that the leaves remained fresh and functional for several days controlled by g_s and fluorescent signals (data not shown). Our ambient values of the GE- and CF-derived parameters accordingly represented the “ambient capacity” of pre-conditioned leaves under near-optimal ambient environmental conditions of CO_2 concentrations and saturating light and at a room temperature of 20–25 °C (Reich et al., 1998).

2.4 GE and CF analyses

GE and CF were measured with a Li-Cor LI-6400XT Portable Photosynthesis System equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA). Response curves for foliar net assimilation versus CO_2 concentration were recorded from five apparently healthy leaves per tree species and leaf position. CF was measured in parallel. *A. unedo* leaves were sufficiently large to cover the leaf cuvette (2 cm^2), whereas sunlit leaves of

Q. ilex were in some cases too small, and the area of the leaves had to be adjusted after the measurements. For the leaves of *P. halepensis*, we positioned a layer of needles (approx. 10–15) on the leaf cuvette, avoiding gaps and overlays. The putty-like adhesive "Blu-tack" (Bostik SA, La Plaine St Denis, France) was also used to seal the gaskets and to keep the needles in position.

2.4.1 Preparation and acclimation

Prior to recording the response curves, the temperature of the clamped leaves (T_{Leaf}) was adjusted to 25 °C, and the flow of ambient CO_2 in the leaf chamber (C_{a}) was set to 400 µmol CO_2 m^{-2} s^{-1} (controlled with a CO_2 mixer). The leaves were dark-adapted for 15–20 min before the measurements, and the data were logged when the GE-derived parameters such as stomatal conductance (g_{s}), stomatal internal CO_2 concentration (C_{i}) and mitochondrial respiration in darkness (R_{n}) had stabilized. For our purposes, dark-adaption did not necessarily mean strict prolonged darkness but referred to a sufficiently low level of ambient background light that did not cause an accumulation of reduced photosystem II (PSII) acceptors, which could be detected as an increase in fluorescence. The leaves were also pre-darkened with special leaf clips or a dark cloth to save time. The chamber light was then turned on at a saturating quantum flux density of 1000 µmol photons m^{-2} s^{-1} (20 % blue LED, 80 % red LED). The relatively high percentage of blue light stimulated the stomata to open (Farquhar and Sharkey, 1982; Kang et al., 2009; Niinemets et al., 2005). The relative humidity was maintained at 50 % (±10 %), and the air flow was maintained at 500 µmol s^{-1}. The above conditions were maintained for approximately 20–30 min until the net rate of carbon assimilation (A_{net}), g_{s}, and C_{i} of the leaf stabilized.

The GE-derived parameters A_{net}, g_{s}, and C_{i} likely require less time to stabilize, especially in healthy and unstressed leaves, but this minimum time range was necessary for the CF-derived parameters to ensure accurate measurement of the efficiency of harvesting light energy by maximal oxidation and therefore open PSII reaction centres under ambient conditions of CO_2 and saturating light, which can be monitored by observing the stability of steady-state fluorescence (F_{s}). If this stability is not achieved, the effective quantum yield of PSII (Φ_{PSII}) and subsequent calculations of important parameters such as the rate of electron transport based on the CF measurement (J_{CF}) could be underestimated. After all parameters had stabilized, the steady-state GE-derived parameters and several CF-derived parameters in the light-adapted state were recorded simultaneously. F_{s}, followed shortly afterwards by the maximum fluorescence yield in the light-adapted state (F'_{m}), was logged by the emission of a pulse of white light at 10 000 mmol m^{-2} s^{-1} to close all PSII reaction centres, followed by a so-called "dark pulse" for measuring the minimal fluorescence (F'_{o}) of a light-adapted leaf that has been momentarily darkened. The measurement of CO_2 began after the completion of the preparation and acclimation, which required approximately 30 min in unstressed leaves and up to 2 h in stressed leaves.

2.4.2 CO_2 experiments

The CO_2-response curves were recorded at a T_{Leaf} of 25 °C and a quantum flux density of 1000 µmol photons $m^{-2}s^{-1}$. The values of C_{a} used to generate the response curves were 400 → 300 → 200 → 150 → 100 → 50 → 400 → 400 → 600 → 800 → 1200 → 2000 (in µmol CO_2 m^{-2} s^{-1}). The minimum and maximum times for stabilizing A_{net}, g_{s}, and C_{i} for each log were set to 4 and 6 min, respectively.

2.4.3 Light experiments

Light-response curves (A/PPFD) were generated at a C_{a} of 400 µmol CO_2 m^{-2} s^{-1} by automatically applying changes in the photosynthetically active radiation with the LI-6400XT light source. To obtain precise responses at the low range of the light gradient for estimating the daily mitochondrial respiration by the Kok effect (Kok, 1948), we used the following PPFD sequence: 2500 → 2000 → 1500 → 1000 → 800 → 600 → 500 → 400 → 300 → 200 → 150 → 125 → 100 → 75 → 50 → 40 → 30 → 20 → 10 → 5 → 0 (in µmol photons m^{-2} s^{-1}). The minimum and maximum times between each light level for the generation of the A/PPFD curves were set to 1 and 2 min, respectively. The gradient from high to low light during an A/PPFD curve led to a drop in T_{Leaf} as the light decreased. The rapid changes in the light levels prevented the adjustment of T_{Leaf} while guaranteeing stable air and water fluxes and avoiding noisy measurements of C_{i} and g_{s}. We thus decided to maintain a stable Peltier-block temperature (T_{block}) in the leaf cuvette. Hence, T_{block} was first adjusted so that T_{Leaf} was 25 °C at the beginning of the A/PPFD curve and then kept stable throughout the experiment. T_{Leaf} had dropped by approximately 1–3 °C by the completion of the A/PPFD curve.

The calculation of the parameters NPQ, qP and temperature functions can be found in the supplementary material.

2.5 Calculation of CF-derived parameters

The maximum efficiency of PSII was calculated by:

$$\frac{F_{\mathrm{v}}}{F_{\mathrm{m}}} = \frac{(F_{\mathrm{m}} - F_{\mathrm{o}})}{F_{\mathrm{m}}}, \tag{1}$$

where F_{v} is the variable fluorescence of a dark-adapted sample, F_{m} is the maximal fluorescence measured after a saturating light pulse, and F_{o} is the minimal fluorescence measured under darkness. These parameters were obtained from dark-adapted leaves with closed PSII reaction centres as described in the previous sections. The $F_{\mathrm{v}}/F_{\mathrm{m}}$ ratio describes the fraction of photochemically absorbed photons under dark conditions. Typical values range between 0.75 and 0.85, depending on age, health and preconditioning. The $F_\mathrm{v}/F_\mathrm{m}$ ratio provides information about the maximum or optimum quantum yield and serves as an indicator of stress in the photosystems (Buschmann, 2007). Ratios below 0.80 are indicative of induced photoprotection and sustained energy dissipation (Maxwell and Johnson, 2000; Verhoeven, 2014), whereas leaves with ratios below 0.74 are considered to be below the recovery threshold (Björkman and Demmig, 1987). The effective quantum yield of PSII was estimated by

$$\Phi_\mathrm{PSII} = \frac{\left(F'_\mathrm{m} - F_\mathrm{s}\right)}{F'_\mathrm{m}}, \tag{2}$$

where F_s is the steady-state fluorescence in a fully light-adapted sample, and F'_m is the maximal fluorescence yield after a pulse of high light. The Φ_PSII is the counterpart of the optimum quantum yield and represents the fraction of photochemically absorbed photons in a light-adapted leaf (Maxwell and Johnson, 2000):

$$J_\mathrm{CF} = \varepsilon \times \Phi_\mathrm{PSII} \times \alpha_\mathrm{L}, \tag{3}$$

where ε is a scaling factor for the partitioning of intercepted light between photosystems I and II. We assumed that light was equally distributed between both photosystems ($\varepsilon = 0.5$) (Bernacchi et al., 2002; Niinemets et al., 2005). α_L is the foliar absorbance determined in separate measurements of foliar reflectance and transmittance. The following values of α_L were determined: 0.932 for *Q. ilex* and 0.912 for *P. halepensis*, with no differences between sunlit and shaded leaves of these two species, and 0.935 for sunlit leaves of *A. unedo*, and 0.917 for shaded leaves of *A. unedo*. For the determination of these leaf absorptances (α_L), foliar reflectance and transmittance were measured at midday in August 2012 using a spectroradiometer UniSpec Spectral Analysis System (PP Systems, Haverhill, MA, USA). The value of J_CF at a CO_2 concentration of 400 μmol CO_2 m^{-2} s^{-1} and a PPFD of 1000 μmol photons m^{-2} s^{-1} was termed ambient photosynthetic electron transport (J_amb). Its relationship with the net assimilation rate ($J_\mathrm{amb}/A_\mathrm{net}$) was used for the analyses of alternative electron sinks beside carbon metabolism.

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

In the literature, the term R_d was sometimes used for dark respiration (Farquhar et al., 1980; Turnbull et al., 2003), but also for day respiration (Flexas et al., 2012; Yin et al., 2011). We will use R_d to represent mitochondrial respiration during the day or under lighted conditions and R_n to represent mitochondrial respiration at night or under dark-adapted conditions. We estimated R_n during the day after darkening the leaf for at least 30 min. R_d was estimated from the light-response curves with the combined GE and CF measurements proposed by Yin et al. (2009), named the CF method. This method amended the Kok method (Kok, 1948) by substituting the A/PPFD relationship with A/(PPFD × Φ_PSII/4)). See Yin et al. (2009) for details.

The effective quantum yield of CO_2 (Φ_{CO_2}, unitless) can be calculated using the estimated α_L, R_d, together with A_net and PPDF as follows:

$$\Phi_{CO_2} = \frac{(A_\mathrm{net} - R_\mathrm{d})}{\mathrm{PPFD} \times \alpha_\mathrm{L}}. \tag{4}$$

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

The FvCB photosynthesis model was employed on the assumption that foliar carbon assimilation was limited either by Rubisco activity (A_c) or by ribulose-1,5-bisphosphate (RuBP) regeneration (A_j) and was driven by light, temperature, and CO_2. The model was further complemented with a third limitation: the photosynthetic rate limited by triose-phosphate use (A_p) (Sharkey, 1985). A_net can then be determined by the minimum of these three potential rates from an A/C_c curve:

$$A_\mathrm{net} = \min\left\{A_\mathrm{c}, A_\mathrm{j}, A_\mathrm{p},\right\}, \tag{5}$$

where

$$A_\mathrm{c} = V_\mathrm{c,max} \times \left[\frac{C_\mathrm{c} - \Gamma^*}{C_\mathrm{c} + K_\mathrm{c}\left(1 + \frac{O}{K_\mathrm{o}}\right)}\right] - R_\mathrm{d}, \tag{6}$$

where $V_\mathrm{c,max}$ represents the maximum rate of Rubisco carboxylation, K_c is the Michaelis–Menten constant of Rubisco for CO_2, O is the partial pressure of O_2 at Rubisco, and K_o is the Michaelis–Menten constant of Rubisco for O_2 (Table B1, see Appendix B) and C_c determined with the variable-J method (Eqs. A7 and A8). The equation representing photosynthesis limited by RuBP regeneration is

$$A_j = J * \left[\frac{C_\mathrm{c} - \Gamma^*}{4C_c + 8\Gamma^*}\right] - R_\mathrm{d}, \tag{7}$$

where J is the rate of electron transport. The denominator of the above equation represents the stoichiometry of the number of electrons required to regenerate ATP and NADP; we have used four for C_c and eight for Γ^* (Flexas et al., 2012). We assumed that J becomes J_max under light and CO_2 saturation when the maximum possible rate of electron transport is theoretically achieved (see also Buckley and Diaz-Espejo, 2014).

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

$$A_\mathrm{p} = \frac{3\mathrm{TPU} \times C_\mathrm{c}}{\Gamma^*\left[C_\mathrm{c} - \left(\frac{1+3\alpha_\mathrm{TPU}}{2}\right)\right]} - R_\mathrm{d}, \tag{8}$$

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

These three estimated parameters ($V_\mathrm{c,max}$, J_max and TPU) define the biochemical capacity to drive the photosynthetic assimilation of CO_2 but are defined here as the photosynthetic potential (Niinemets et al., 2006). The term photosynthetic capacity is here dismissed, despite its frequent use in the literature, to avoid confusion with studies that have used this term for the maximum rate of assimilation under saturating light conditions (e.g. Bertolli and Souza, 2013).

2.8 Curve fitting

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

2.9 Correction for diffusion leakage

Large gradients between the ambient air and the CO_2 concentrations inside the chamber are created during the generation of a carbon-response curve. This leakage is particularly important at the high and low ends of the carbon-response curve when a large CO_2-concentration gradient exists between the leaf chamber and the surrounding ambient concentration. Based on the findings by Flexas et al. (2007a), we corrected A_net by subtracting the diffusion leakage for each step of the A/C_c curve obtained from separate response curves with leaves thermally killed in hot water.

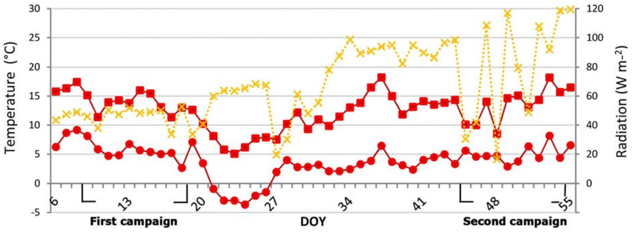

Figure 1. Maximum and minimum temperatures on the primary y-axes (in red squares and circles, respectively) and radiation (in yellow crosses) on the secondary y-axes are presented for the mild and frost winter period for the day of the year (DOY) in January and February 2012.

2.10 Statistical analyses

All statistical analyses were performed using the R software package, version 3.0.2 (http://www.r-project.org/). Differences in the parameters between the mild and cold winters were determined with Student's t-tests ($P \leq 0.05$). Shapiro–Wilk tests of normality tested for normality of the data. Data were normalized at $P \leq 0.1$. One-factorial analyses of variance (ANOVAs) with tree species as the main factor tested for differences between tree species of the parameters in the sampling periods. Significant differences were determined at $P \leq 0.05$ with Tukey's HSD tests. Regression analyses were conducted to study the relationship between J_max and $V_\mathrm{c,max}$ and between J_amb and A_net. Analyses of covariance (ANCOVAs) tested for differences in slopes and intercepts.

3 Results

3.1 Environmental variables

Collserola Natural Park experienced extremely mild winter conditions in November and December 2011 and January 2012, when average minimum temperatures (10.4 °C in November, 5 °C in December, and 3.4 °C in January) remained above 0 °C and no frosts occurred. Average maximum temperatures were 16.3 °C in November, 12.2 °C in December, and 11.4 °C in January. All species had considerable shoot growth of up to 15 cm during this mild period. Sudden low temperatures, however, led to frost on 6 consecutive days and a minimum average temperature of −2.3 °C (day of the year, DOY 21–26) followed by 8 days of cool temperatures averaging +2.6 °C (DOY 27–35) (Fig. 1). The average radiation during first field campaign (DOY 9–19) was 46 and during the period of frost was 58 W m^{-2}.

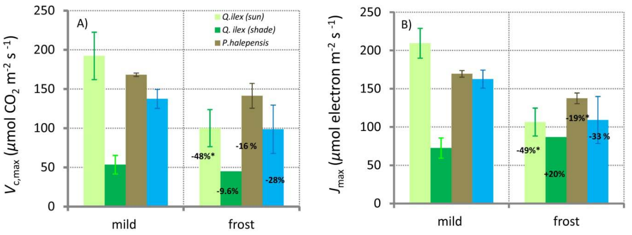

Figure 2. Bar plot of the effect of a sudden period of frost following a mild winter period in 2012 on **(a)** the maximum velocity of carboxylation ($V_{\mathrm{c,max}}$) and **(b)** the maximum rate of electron transport ($J_{\max}$) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

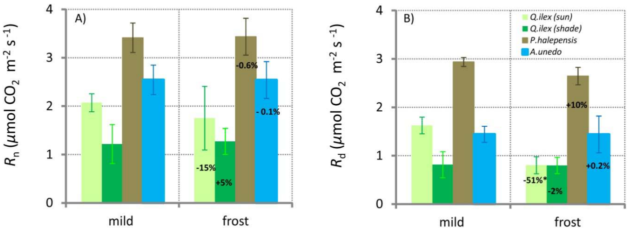

Figure 3. Bar plot of the effect of a sudden period of frost following a mild winter period on **(a)** nighttime respiration (R_{n}) and **(b)** daytime respiration (R_{d}) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

3.2 Photosynthetic potentials

Of the three photosynthetic parameters describing the photosynthetic potential, $V_{\mathrm{c,max}}$, $J_{\max}$ and TPU, only the first two could be satisfactorily estimated from the A/C_{c}-response curves. The leaves were only occasionally limited by TPU (6 out of 42), despite the excessive CO_2 concentrations in the higher section of the CO_2-response curve. TPU was therefore discarded from further analysis. $V_{\mathrm{c,max}}$ and $J_{\max}$ were highest in *Q. ilex* but more importantly also decreased most strongly after the period of frost by nearly 50 % ($P \leq 0.05$; Fig. 2). The photosynthetic potential of *P. halepensis* was affected the least, reflected by moderate decreases in $V_{\mathrm{c,max}}$ and $J_{\max}$ (16 and 19 %), which were not significant. $V_{\mathrm{c,max}}$ and $J_{\max}$ were lowest in *A. unedo* during the mild winter period and decreased by approximately 33 % after the period of frost. This decrease, however, was not significant, due to a large standard error.

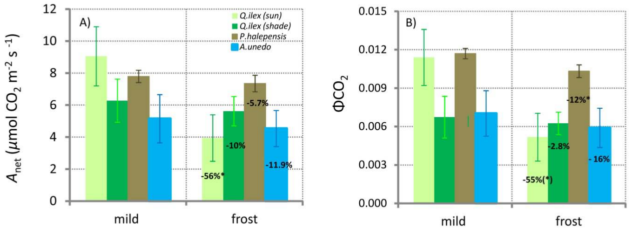

Figure 4. Bar plot of the effect of a sudden period of frost following a mild winter period on **(a)** net assimilation (A_{net}) and **(b)** the effective quantum yield of net CO_2 assimilation (ΦCO_2) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$) and marginal significance with an asterisk in parentheses ($0.05 \leq P \leq 0.1$).

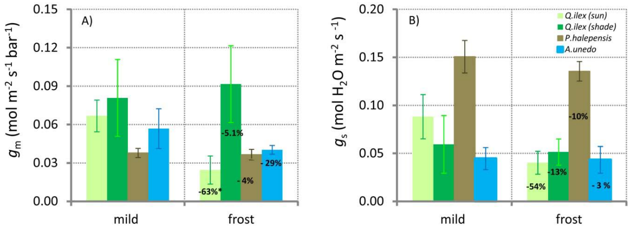

Figure 5. Bar plot of the effect of a sudden period of frost following a mild winter period on **(a)** mesophyllic conductance (g_{m}) and **(b)** stomatal conductance (g_{s}) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

3.3 GE-derived parameters under ambient conditions

The period of frost had a strong effect on several GE-derived parameters in *Q. ilex* leaves. The cold temperatures decreased R_{n} in *Q. ilex* leaves, but the effect was much weaker than for R_{d} and was not significant (Fig. 3). These parameters responded very weakly to the cold and frost in the leaves of *A. unedo* and *P. halepensis*. A_{net} and $\Phi_{\mathrm{CO_2}}$ were also reduced in *Q. ilex* leaves by approximately 50 %. This was significant for the A_{net} (Fig. 4a) and of low significance for $\Phi_{\mathrm{CO_2}}$ (Fig. 4b). Further differences were only significant for $\Phi_{\mathrm{CO_2}}$ in *P. halepensis* leaves being reduced by 12 % ($P \leq 0.05$). The CO_2 conductance was more strongly reduced in g_{m} than in g_{s} for *Q. ilex* and *A. unedo* leaves which was only significant for the former whereas these parameters seemed unaffected in *P. halepensis* leaves (Fig. 5a and b). As a consequence, we observed a tendency of a C_{i} increase in parallel with a C_{c} decrease in *Q. ilex* and *A. unedo* leaves due to a lower CO_2 uptake in carbon metabolism, but not in *P. halepensis* (Fig. 6a and b). The differences observed were not significant ($P \leq 0.05$).

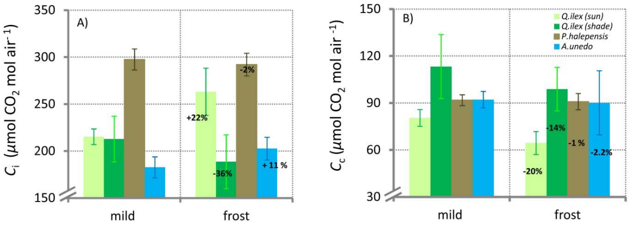

Figure 6. Bar plot of the effect of a sudden period of frost following a mild winter period on **(a)** the stomatal internal CO_2 concentration (C_i) and **(b)** the chloroplastic CO_2 concentration (C_c) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

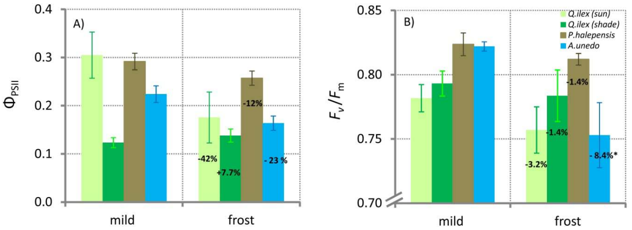

Figure 7. Bar plot of the effect of a sudden period of frost following a mild winter period on **(a)** the effective quantum yield of photosystem II (Φ_{PSII}) and **(b)** the maximum efficiency of photosystem II (F_v/F_m) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

3.4 CF-derived parameters under ambient conditions

The GE-derived parameters enabled us to study the immediate responses, but several CF-derived parameters allowed us to determine in more depth the physiological changes in parts of the light-harvesting apparatus, namely PSII. F_v/F_m estimates the maximum quantum yield of PSII and serves as a stress indicator (Fig. 7b). *A. unedo* leaves were most strongly affected by the period of frost, followed by *Q. ilex* leaves, whereas *P. halepensis* leaves were only marginally affected. The changes were not statistically significant in the latter two species ($P \leq 0.05$). Φ_{PSII} tended to decrease in all species but most strongly in *Q. ilex* leaves (42 %), however insignificantly (Fig. 7a). NPQ responded very differently in the three species. NPQ did not change much between the two sampling periods in the leaves of *P. halepensis* (6 %) but decreased significantly by 25 % ($0.05 \leq P \leq 0.1$) in *A. unedo* leaves and tended to increase in *Q. ilex* leaves by 31 % ($P \geq 0.05$), however insignificantly (Fig. 8).

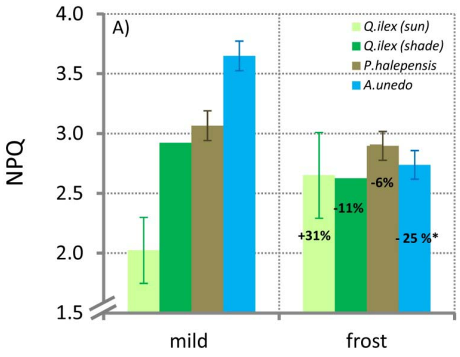

Figure 8. Bar plot of the effect of a sudden period of frost following a mild winter period on non-photochemical quenching (NPQ) in sunlit leaves of *Q. ilex* (light green bar), in shaded leaves of *Q. ilex* (dark green bar), *P. halepensis* (beige bar) and *A. unedo* (blue bar). The error bars represent the standard error, and the percentages indicate the change between periods where significance is indicated with an asterisk ($P \leq 0.05$).

3.5 Relationships of foliar photosynthetic variables

The covariance of several relationships of the foliar photosynthetic variables were analysed in an ANCOVA to test for differences in the slopes and intercepts in these relationships. The ANCOVA for the relationship between $V_{c,max}$ and J_{max} in *Q. ilex* leaves indicated a highly significant ($P \leq 0.01$) reduction in the slope and also intercept showing a similar strong effect on J_{max} than on $V_{c,max}$ due to the change in weather (Fig. 9a and Table 2). In *P. halepensis*, the slope was significantly reduced and the intercept was marginally significantly reduced (Fig. 9b and Table 2). This shows a comparatively stronger effect on $V_{c,max}$ than on J_{max} by the cold period. The sunlit leaves of *A. unedo* and the shaded leaves of *Q. ilex* did not show any significant changes in the relationship of $V_{c,max}$ and J_{max} (Fig. 9a, c and Table 2). The relationship between the rate of electron transport at ambient conditions derived from CF and the CO_2 assimilation at ambient CO_2 concentrations (J_{amb}/A_{net}) was similar in all tree species (Fig. 10a, b, c and Table 2). The slopes were higher in response to the stress imposed by the low temperatures but were not significant. When all species were combined, the change of the slope was marginally significant, indicating a possible increased alternative electron sink other than carbon metabolism (Table 2).

3.6 Role of leaf position

Under mild conditions, the leaves of *Q. ilex* showed the most strongly pronounced differences in the leaf position (data of *P. halepensis* Mill. and *A. unedo* L., not shown). Leaves of *Q. ilex* growing under high irradiances had a more active carbon metabolism (A_{net}, R_d, R_n, and Φ_{CO_2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) in all tree species. As described in Sect. 2, the

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

	Both periods	Mild period	Frost period
$V_{c,max}$	0.001	0.002	0.172
J_{max}	0.006	0.002	0.553
$J_{max}/V_{c,max}$	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_d	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_{CO_2}	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

effect of the leaf position after the sudden cold period was only studied for *Q. ilex*. After the sudden frost period, the photosynthetic potential was much higher in sunlit than in shaded leaves of *Q. ilex*, with both J_{max} and $V_{c,max}$ being highly significant (Fig. 2 and Table 1). These differences disappeared after the cold period, because J_{max} and $V_{c,max}$ in the shaded leaves remained unaffected by the frost. F_v/F_m was generally higher in the shaded leaves, but not significantly ($P \leq 0.05$) (Fig. 8 and Table 1). The photosynthetic parameters under ambient conditions, such as A_{net}, g_s, C_i, C_c and g_m, were not affected much by the leaf position (Figs. 4–6 and Table 1). Although not significant, the effects of the cold period on these parameters were stronger in the sunlit leaves. In comparison to these parameters, the leaf position had more pronounced effects on R_n and R_d (Fig. 3 and Table 1). The response of respiration to winter stress, however, differed depending on the location of the leaves. R_n maintained the same balance between sunlit and shaded leaves before and after the cold period, but R_d decreased comparatively more in sunlit leaves due to the period of frost. This pattern was also reflected in Φ_{CO_2} (Fig. 4b and Table 1) and in the CF-derived parameters Φ_{PSII} and NPQ, (Figs. 7a, 8 and Table 1) indicating a stronger effect on the photochemical machinery of sunlit leaves than on shaded leaves. Shaded leaves also exhibited a lower J_{amb}/A_{net} ratio, but the ratio increased equally in both leaf positions after the cold period, indicating a similar behaviour of dissipating energy by alternative electron sinks (Fig. 10a and Table 1).

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

Tree species	*Q. ilex* sunlit			*Q. ilex* shaded			*P. halepensis* sunlit			*A. unedo* sunlit			All species sunlit		
Leaf position	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
Regression analyses of J_{max} and $V_{c,max}$															
Mild	$y = 0.81x + 41.6$	0.97	2×10^{-4}	$y = 1.2x + 6.1$	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	1.4×10^{-7}
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)	5.76×10^{-2}			0.83			0.022			0.69			0.072		
p (intercept)	8.91×10^{-9}			0.3			0.058			0.28			0.008		
Regression analyses of J_{amb} and A_{net}															
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	7.1×10^{-6}
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	1.7×10^{-4}
p (slope)	0.337			0.72			0.59			0.322			0.098		
p (intercept)	0.51			0.45			0.31			0.29			0.071		

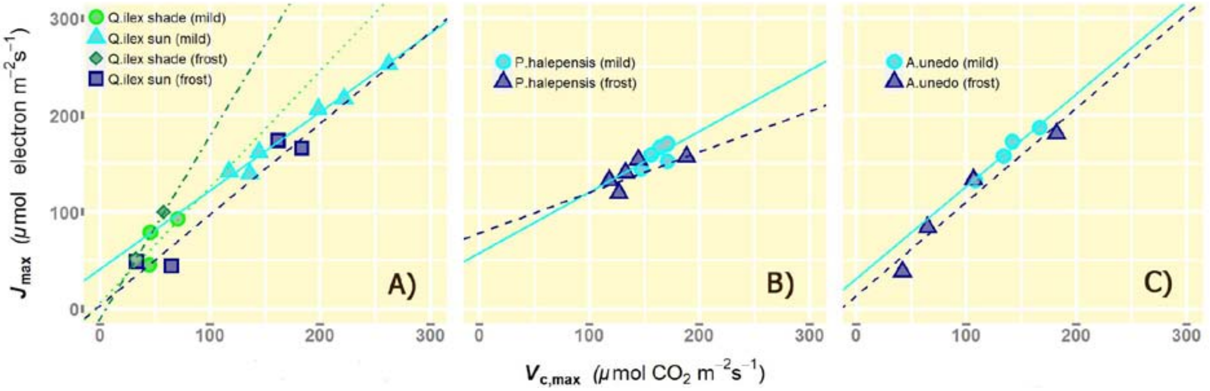

Figure 9. Relationship between the maximum velocity of carboxylation ($V_{c,max}$) and the maximum rate of electron transport (J_{max}) in **(a)** *Q. ilex*, **(b)** *P. halepensis* and **(c)** *A. unedo* leaves. Leaves measured under mild conditions are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively. Leaves measured after the period of frost are indicated by green diamonds and blue squares in shaded and sunlit locations, respectively.

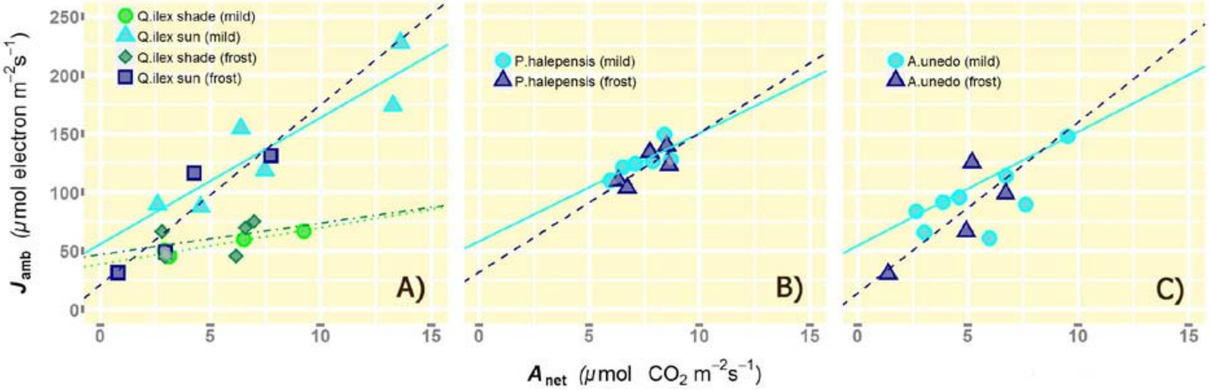

Figure 10. Relationship between the rate electron transport from chlorophyllic fluorescence (J_{amb}) and net assimilation (A_{net}) at ambient CO$_2$ concentrations and saturating light (A_{net}) in **(a)** *Q. ilex*, **(b)** *P. halepensis* and **(c)** *A. unedo* leaves. Leaves measured under mild conditions are indicated by green circles and cyan triangles in shaded and sunlit locations, respectively. Leaves measured after the period of frost are indicated by green diamonds and blue squares in shaded and sunlit locations, respectively.

4 Discussion

4.1 Winter in the Mediterranean region

Mediterranean-type ecosystems are exposed to stress from summer droughts but also from low temperatures in winter (Mitrakos, 1980). Less attention, however, has been paid to the degree and extent as well as the wide variation among years and regions of these stress periods, in response to which Mediterranean evergreen species have developed a dynamic photoprotective ability in order to withstand these stressors (Kyparissis et al., 2000; Martínez-Ferri et al., 2004). Despite the occurrence of lower temperatures than in spring conditions, in winter the photosynthetic potential recovered once the leaves became acclimated to the new conditions (Dolman et al., 2002; Hurry et al., 2000). This is important for the plants' overall performance because the photosynthetic exploitation of favourable conditions in winter is crucial for achieving a positive carbon balance in Mediterranean evergreen tree species (García-Plazaola et al., 1999b; Martínez-Ferri et al., 2004). We showed how a long lasting comfortable winter period without frost lead to notably high photosynthetic potentials and carbon assimilation in winter being equal to or partly even exceeding spring values (Sperlich et al, unpublished data). As a result, increased winter temperatures influenced phenological responses, advanced winter cambium activation, spring bud burst and leaf unfolding which has been reported in an increasing number of studies (Peñuelas and Filella, 2001). These observations were also reflected in the high sap flow per tree (J_t), ranging for all tree species on average between 5 and 10 kg d^{-1} during the mild winter period (Sánchez et al., unpublished results). Whereas sudden frosts have often been attributed to higher altitudes of the Mediterranean region (Blumler, 1991; Tretiach et al., 1997), we showed that it can also be an important factor for plant growth and distribution in other areas such as the sub-humid Mediterranean climate of our study site (Garcia-Plazaola et al., 2003a). At night when frosts are more likely to occur, we observed the lowest temperatures whereas at daytime the temperatures were often above zero degrees. However, as we showed, not only cool daytime but also cool nighttime temperatures or frosts can affect subsequent daytime photosynthesis and induce photoprotective processes (see also Flexas et al., 1999). In our study, the sudden occurring low temperatures affected strongly the photosynthetic apparatus, although the responses were highly species specific. We will elucidate the physiological mechanism in the following.

4.2 PSII – primary target of stress induced by low temperatures

Typically in winter there is an imbalance between light energy absorbed in photochemistry and light energy used in metabolism. This is shown in our data by increased thermal energy dissipation (NPQ) and reduced PSII efficiency (Φ_{PSII}) in order to reduce the harmful effects of excess energy reflecting an inactivation and damage of PSII reaction centres – more precisely, the reaction-centre protein D1 (Aro et al., 1993; Demmig-Adams and Adams, 1992; Mulo et al., 2012). More precise information about the underlying processes that have altered this efficiency is provided by the $F_\mathrm{v}/F_\mathrm{m}$ ratio. Chronic changes occurring in the $F_\mathrm{v}/F_\mathrm{m}$ ratio can be related to a cascade of processes which are induced to protect the photosynthetic apparatus including (i) re-organization of the thylakoid membrane, (ii) closure of reaction centres, (iii) and/or reduced antennal size (Ensminger et al., 2012; Huner et al., 1998; Maxwell and Johnson, 2000; Verhoeven, 2014). The small changes in the $F_\mathrm{v}/F_\mathrm{m}$ ratio observed in the leaves of *Q. ilex* and *P. halepensis* reflected photoprotective responses without any photodamage. The significant decline of $F_\mathrm{v}/F_\mathrm{m}$ in *A. unedo*, however, indicated strong chronic photoinhibition and is an indication of severe photodamage (Martínez-Ferri et al., 2004). We conclude that *A. unedo* suffered most notably from the low temperatures whereas *Q. ilex* and *P. halepensis* were equipped with a good photoprotective capacity able to keep the photosynthetic apparatus intact (Öquist and Huner, 2003). *Q. ilex* showed the most dynamic responses, negating the harmful excitation stress by lowering the photochemical operating efficiency (Φ_{PSII}) and increasing the use of alternative thermal-energy pathways (NPQ). This photoprotective capability represented by a higher NPQ is usually linked to the xanthophyll cycle that responds to environmental factors such as temperature, water deficit and nutrient availability (Demmig-Adams and Adams, 1996; García-Plazaola et al., 1997). Inter-conversions of the cycle and pool sizes occur following the need to dissipate excess excitation energy in response to summer drought (García-Plazaola et al., 1997; Munné-Bosch and Peñuelas, 2004), but also to winter stress (Corcuera et al., 2004; Garcia-Plazaola et al., 2003a; Kyparissis et al., 2000; Oliveira and Penuelas, 2001). The implicit interpretation of being equipped with a high capacity of photoprotection when NPQ increases was recently questioned by Lambrev et al. (2012). This study reported that quenching and photoprotection were not necessarily linearly related and stated that several possibilities of photoprotective responses other than NPQ of CF existed, such as antennal detachment that could possibly vary with species and growth conditions. The highly dynamic and photoprotective capability of *Q. ilex* leaves, however, was also demonstrated by several other photosynthetic parameters such as $V_{\mathrm{c,max}}$, $J_{\max}$, A_{net}, $\Phi_{\mathrm{CO_2}}$ and R_d, which confirmed this trend and were in accord with the findings by Corcuera et al. (2004). Despite reports of several mechanisms of resistance to drought stress in *A. unedo*, including increased levels of zeaxanthin that indicates an enhanced thermal dissipation of excess excitation energy in periods of summer stress (Munné-Bosch and Peñuelas, 2004), we found that *A. unedo* leaves had a lower capacity of photoprotection in response to induced over-excitation of the photosystems by winter stress.

4.3 High photosynthetic potentials and strong effects of low temperatures

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

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

4.4 Inhibition of carbohydrate metabolism

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

4.5 Leaf position specific responses to abiotic stress in winter

It is well known that leaves growing under high irradiances have a more active carbon metabolism (A_{net}, R_d, R_n and Φ_{CO_2}), photochemical efficiency (Φ_{PSII}), and photosynthetic potential (high J_{max} and $V_{c,max}$) (Taz and Zeiger, 2010). In this regard, *Q. ilex* showed the most strongly pronounced differences between sunlit and shaded leaves. Plants develop leaves with a highly specialized anatomy and morphology for the absorption of the prevailing light in their local environments resulting generally in smaller but also thicker sunlit leaves (Kull and Niinemets, 1993; Terashima and Hikosaka, 1995). Nevertheless, the higher carbon metabolism and photochemical activity of sunlit leaves decreased strongly, partly below the level of shaded leaves, whereas shaded leaves showed little sign of any downregulation but maintained a relatively stable effective quantum yield of CO_2 assimilation in both periods. Furthermore, the photosystems showed no sign of photodamage and generally maintained a higher maximum efficiency than did sunlit leaves. We concluded that foliar-level physiology during winter was better protected in the shaded crown of *Q. ilex* unexposed to the dramatic changes in radiation in the outer canopy, confirming the results by Valladares et al. (2008). We also concluded that *Q. ilex* is a highly dynamic species able to rapidly change its metabolism on the antioxidant and photoprotective level in dependence to its leaf position (García-Plazaola et al., 1997, 1999a; Martínez-Ferri et al., 2004). We show that the foliar plasticity in morphology and anatomy of *Q. ilex* (Bussotti et al., 2002; Valladares et al., 2000) can also be attributed to its biochemical metabolism. We stress that the solar environment of the leaves is a crucial factor when assessing tree performance, especially when comparing tree species in a competitive context.

4.6 Ecological context

Q. ilex had the most drastic photoprotective response to frost and cool temperatures, whereas *P. halepensis* exhibited a homeostatic behaviour with a very active carbon assimilatory and respiratory metabolism in both periods. *A. unedo* was intermediate, with large decreases in the parameters of carbon metabolism but also a high variability in its response to frost. *A. unedo* also had the lowest photoprotective capability, which might be explained by previous characterisations to be semi-deciduous to drought being at the borderline to evergreen sclerophyllous species (Gratani and Ghia, 2002a, 2002b). Moreover, *A. unedo* occurs naturally most commonly as a shrub and is less frequently found in the forest canopy of mixed forests growing up to 8–10 m tall as in our study site (Beyschlag et al., 1986; Reichstein et al., 2002). Investments in leaves are thus lower and leaf longevity shorter. Leaves of *A. unedo* are more rapidly replaced relative to more sclerophyllic leaves such as those of *Q. ilex*. We postulated that *A. unedo*, considered a relict of the humid-subtropical Tertiary tree flora, was more sensitive to winter stress, which is consistent with its presence mostly in the western Mediterranean basin and its frequent occurrence in coastal zones where humidity and temperature are the main factors determining its geographical distribution (Gratani and Ghia, 2002a and references therein). Our results suggested that *Q. ilex* could greatly benefit from favourable winter conditions exhibiting a high photosynthetic potential and carbon metabolism. Angiosperms are known to make efficient use of favourable winter periods to recover depleted carbon reserves and embolism-induced loss of hydraulic capacity (Carnicer et al., 2013 and references therein). When these relatively favourable conditions changed, *Q. ilex* quickly re-adjusted the photosynthetic machinery to the prevailing conditions, as indicated by the largest decreases in photosynthetic potential and carbon metabolism. Some researchers have proposed the lutein-epoxy cycle in photoprotection of *Quercus* as a mechanism to maintain sustained energy dissipation

(Garcia-Plazaola et al., 2003b), which could help to account for the higher tolerance to low temperatures in *Q. ilex* relative to other co-occurring Mediterranean trees or shrubs (Ogaya and Peñuelas, 2003, 2007). *P. halepensis* did not suffer a pronounced chronic photoinhibition, confirming the results by Martínez-Ferri et al. (2004). Despite a pronounced downregulation of photosynthetic electron transport and an increase in alternative electron sinks, the light-saturated ambient photosynthesis and stomatal conductance remained surprisingly high and constant. *P. halepensis* thus exhibited a successful refinement of photosynthetic electron flow and possibly a successful repair of protein D1 in the PSII reaction centre. The strong downregulation in *Q. ilex* and the homogeneous response of *P. halepensis* were possibly due to distinct, previously described strategies. *Q. ilex* has been characterized as a photoinhibition-avoiding species and *P. halepensis* as a photoinhibition-tolerant species (Martinez-Ferri et al., 2000). We have extended this categorization for *A. unedo*, a less photoinhibition-tolerant tree species, which favoured carbon metabolic processes at the cost of chronic photoinhibition and photodamage. This strategy is similar to those in other semi-deciduous shrubs (Oliveira and Penuelas, 2001; Oliveira and Peñuelas, 2004). The physiological responses of *Q. ilex*, a slowly growing late-successional species, to environmental stressors are highly plastic (Zavala et al., 2000) due to its vegetative activity in a wide range of temperatures and high stomatal control in stressful conditions (Gratani et al., 2000; Savé et al., 1999), high plasticity index and resprouting dynamics (Espelta et al., 1999; Gratani et al., 2000), deep rooting system and large carbohydrate pools (Canadell and Lopez-Soria, 1998; Canadell et al., 1999), and high adaptive variability in foliar phenomorphology (Sabaté et al., 1999). Our findings showed the intra-crown variability in *Q. ilex*, where shaded leaves were widely unaffected by the inhibitory cold stress (Oliveira and Penuelas, 2001). The ability of *Q. ilex* to perform rapid metabolic changes in the antioxidant and photoprotective mechanisms could be of adaptive importance (García-Plazaola et al., 1999a). In contrast, *P. halepensis* is a fast growing conifer that quickly occupies open spaces after disturbances such as fires (Zavala et al., 2000). *P. halepensis*, as do all pines, has a low ability to store carbohydrates and therefore follows a strategy of water conservation and embolism avoidance (Meinzer et al., 2009). High rates of photosynthesis and growth require high concentrations of carboxylation enzymes in the carbon cycle that have high maintenance costs (Valladares and Niinemets, 2008), perhaps accounting for the high respiration rates found in *P. halepensis* leaves. Moreover, differences among the species are also likely to be the result of distinct foliar morphologies and crown architectures. Pine trees are characterized by a relatively low exposure of foliar surface area to direct sunlight due to the cylindrical shape and steep angles of their needles but at the same time are able to exploit a wider range of incident light angles than broadleaved trees. Despite reported flexible adjustments in the orientation of the leaves in several Mediterranean broadleaved sclerophyllic species (Oliveira and Peñuelas, 2000; Vaz et al., 2011; Werner et al., 2002), needle leaves probably still confer some benefits to attain near-saturated photosynthetic rates over a wider range of diurnal and seasonal variation in sun angles (Jordan and Smith, 1993; Lusk et al., 2003), while at the same time showing a high tolerance to photoinhibition. This might account for the good performance of *P. halepensis* under mild winter conditions with moderate abiotic stresses such as in our study. However, under more severe and reoccurring frost events, *P. halepensis* might reach the threshold of its tolerance and severe frost damage can occur. This explains also its absence in mountain regions with more severe winters where *Q. ilex* becomes more competitive. Despite following distinct physiological strategies, both *Q. ilex* and *P. halepensis* seem to cope equally well with the winter conditions they were exposed to whereas the foliar photosynthetic systems of *A. unedo* were more sensitive to sudden frost impacts. Thus, *A. unedo* might have been in a competitive disadvantage for the following growing season.

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

Appendix A

A1 Temperature functions

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

$$K_c = e^{\left(c-\left(\frac{\Delta \mathrm{Ha}}{R\times(273.15+T_L)}\right)\right)} \tag{A1}$$

$$K_o = e^{\left(c-\left(\frac{\Delta \mathrm{Ha}}{R\times(273.15+T_L)}\right)\right)} \tag{A2}$$

$$\Gamma^* = e^{\left(c-\left(\frac{\Delta Ha}{R\times(273.15+T_L)}\right)\right)} \times \frac{O_2}{20.9}, \tag{A3}$$

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

A2 CF parameters

The non-photochemical quenching (NPQ) was estimated by both dark- and light-adapted fluorescent signals F_m and F'_m by

$$\mathrm{NPQ} = \frac{\left(F_m - F'_m\right)}{F'_m}, \tag{A4}$$

where F_m is the maximal fluorescence measured on a dark adapted leaf after a saturating light pulse and F'_m is the maximal fluorescence yield of a light adapted leaf after a pulse of high light. Photochemical quenching (qP) indicates the proportion of open PSII reaction centres and tends to be highest in low light when leaves use light most efficiently (Maxwell and Johnson, 2000). qP was estimated by

$$\mathrm{qP} = \frac{F'_m - F_s}{F'_m - F'_o}, \tag{A5}$$

where F'_o is the minimum fluorescence in a light-adapted leaf after a pulse of darkness and F_s is the steady-state fluorescence in a fully light-adapted sample.

A3 Estimation of mesophyll conductance

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

$$g_m = \frac{A_{\mathrm{net}}}{C_i - \frac{[\Gamma^* \times J_{\mathrm{CF}} + 8(A_{\mathrm{net}} + R_d)]}{J_{\mathrm{CF}} - 4(A_{\mathrm{net}} + R_d)}}, \tag{A6}$$

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

$$C_c = C_i - \frac{A_{\mathrm{net}}}{g_m}, \tag{A7}$$

where C_c is the chloroplastic CO_2 concentration.

Appendix B

Table B1. The scaling constant (c) and energies of activation (ΔHa) describing the temperature responses for Rubisco enzyme kinetic parameters K_c, K_o and Γ^*. Taken from Bernacchi et al. (2002).

	25 °C	c	ΔHa	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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