Balancing the risks of hydraulic failure and carbon starvation

Citation for published version:
Salmon, Y, Torres-Ruiz, JM, Poyatos, R, Martinez-Vilalta, J, Meir, P, Cochard, H & Mencuccini, M 2015, 'Balancing the risks of hydraulic failure and carbon starvation: a twig scale analysis in declining Scots pine' Plant, Cell and Environment. DOI: 10.1111/pce.12572

Digital Object Identifier (DOI):
10.1111/pce.12572

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Plant, Cell and Environment

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Balancing the risks of hydraulic failure and carbon starvation: a twig scale analysis in declining Scots pine

Running title: Twig role in Scots pine drought-induced mortality

Yann Salmon\textsuperscript{1,9*}, José M. Torres-Ruiz\textsuperscript{2,3}, Rafael Poyatos\textsuperscript{4}, Jordi Martinez-Vilalta\textsuperscript{4,5}, Patrick Meir\textsuperscript{1,6}, Hervé Cochard\textsuperscript{7}, Maurizio Mencuccini\textsuperscript{1,8}

*corresponding author (yann.salmon@ed.ac.uk/ysatuniversity@gmail.com)

1: School of Geosciences, University of Edinburgh, Crew Building, The Kings Buildings, West Main Road, EH93JN Edinburgh, UK

2 Université de Bordeaux, BIOGECO, UMR 1202, F-33615 Pessac, France.

3 INRA, UMR 1202 BIOGECO, F-33610 Cestas, France.1202 BIOGECO, 33612, Cestas, France.

4 CREA, Campus de UAB, 08193, Cerdanyola del Vallès, Barcelona, Spain

5 Universitat Autònoma de Barcelona, 08193, Cerdanyola del Vallès, Spain

6 Research School of Biology, Australian National University, Canberra, Australia

7 INRA, UMR547 PIAF, Clermont Université, F-63100 Clermont-Ferrand, France

8 ICREA at CREA, 08193, Cerdanyola del Vallès, Barcelona, Spain

9 Current address: Department of Physics, University of Helsinki, P.O. Box 48, Erik Palménin aukio 1, 00014 University of Helsinki, Finland

Number of words: 7975
Number of Tables: 1
Number of Figures: 6
Abstract

Understanding physiological processes involved in drought-induced mortality is important for predicting the future of forests and for modelling the carbon and water cycles. Recent research has highlighted the variable risks of carbon starvation and hydraulic failure in drought-exposed trees. However, little is known about the specific responses of leaves and supporting twigs, despite their critical role in balancing carbon acquisition and water loss. Comparing healthy (non-defoliated) and unhealthy (defoliated) Scots pine at the same site, we measured physiological variables involved in regulating carbon and water resources. Defoliated trees showed different responses to summer drought compared to non-defoliated trees. Defoliated trees maintained gas-exchange while non-defoliated trees reduced photosynthesis and transpiration during the drought period. At the branch scale, very few differences were observed in non-structural carbohydrate concentrations between health classes. However, defoliated trees tended to have lower water potentials and smaller hydraulic safety margins. While non-defoliated trees showed a typical response to drought for an isohydric species, the physiology appears to be driven in defoliated trees by the need to maintain carbon resources in twigs. These responses put defoliated trees at higher risk of branch hydraulic failure and help to explain the interaction between carbon-starvation and hydraulic failure in dying trees.

Key words: mortality, drought, tree, ecophysiology, NSC, transpiration, photosynthesis, leaf-gas exchange
Introduction

Episodes of tree mortality in response to high temperature and extreme drought have been reported worldwide in the last few decades (e.g., Breshears et al., 2005, Allen et al., 2010, Peng et al., 2011, IPCC, 2014). Since forest species are adapted to their ecological niches, they are at risk when the intensity or length of drought events exceeds their specific limits of resistance. As future climate scenarios under on-going climate change predict that drought frequencies and intensities will increase in several regions worldwide (Collins et al., 2013), the ability to understand, predict and model future tree response and survival to water deficit has become increasingly important.

Based on multiple observational and experimental studies, a theoretical framework has been developed in recent years proposing several mechanisms responsible for drought-induced mortality (cf., recent review by McDowell et al., 2011): 1) C starvation (e.g., Galiano et al., 2011, Adams et al., 2013, Mitchell et al., 2013); 2) hydraulic failure of the plant water transport system (e.g., Anderegg & Anderegg, 2013, Mitchell et al., 2013); and 3) phloem transport failure (e.g., Sala, 2009, Adams et al., 2013, Hartmann et al., 2013, McDowell et al., 2013). In addition, biotic agents (insects, parasites and pathogens) may either make trees more susceptible to mortality by weakening the trees prior to drought stress or they may accelerate an ongoing decline process and eventually kill already weakened trees (e.g., Galiano et al., 2011, Zweifel et al., 2012, Gaylord et al., 2013, Oliva et al., 2014). However, the relative importance and interactions among these processes remain the source of on-going debates (e.g., McDowell et al., 2008, McDowell & Sevanto, 2010, Sala et al., 2010).

Leaves play a crucial role in balancing the risks of C starvation and hydraulic failure, since they are the site of C assimilation and sugar production, as well as the main source of water
loss for trees. However, the role of needle and leaf physiology in drought-induced tree mortality remains poorly understood, especially under field conditions. Several studies have focused on differences in gas-exchange responses or hydraulic properties among different species. In particular, the comparison of drought-sensitive and drought-tolerant species (e.g., Mitchell et al., 2014) or of isohydric and anisohydric species (e.g., Limousin et al., 2013, Woodruff et al., 2015) under increasing water stress have helped gain insights into the role played by photosynthetic organs in the response to drought. A species-specific trade-off between drought-resistance of leaf gas-exchange and leaf physiological efficiency has been reported (Limousin et al., 2013), mirroring the trade-off between xylem vulnerability and conductive efficiency (Tyree et al., 1994). However, this trade-off does not seem to be ubiquitous (Limousin et al., 2013). Most studies report a gas-exchange limitation under drought (Adams et al., 2013, Limousin et al., 2013), but little is known about differences in leaf physiology between healthy and non-healthy trees within a single drought-stressed population.

Scots pine (Pinus sylvestris L.) has become a model species to study physiological and demographic responses to drought (Jackson et al., 1995b, Jackson et al., 1995a, Galiano et al., 2010, Galiano et al., 2011, Zweifel et al., 2012, Poyatos et al., 2013), since it is a geographically widespread species (Critchfield & Little, 1966) that grows under a large range of environments thanks to its ability to adjust its hydraulic system to water availability. The main mechanisms of this adjustment involve tight stomatal control of transpiration and the regulation of the ratio between leaf and sapwood areas (A_L:A_S, Mencuccini & Grace, 1995, Poyatos et al., 2007, Martinez-Vilalta et al., 2009). Nonetheless, mortality of Scots pine has been observed at the xeric border of its distribution in the Mediterranean area (e.g., Martínez-Vilalta & Piñol, 2002, Vilà-Cabrera et al., 2013), central Turkey (Allen et al., 2010) and in
dry alpine valleys (e.g., Bigler et al., 2007, Rigling et al., 2013), and this makes it a suitable model species to study the limits of physiological plasticity and adaptation to drought-stress.

The physiological responses of Scots pine to drought are driven by its isohydric stomatal control (Irvine et al., 1998, Zweifel et al., 2007, Duursma et al., 2008, Poyatos et al., 2008). Under intense drought stress, further limitation of transpiring area in Scots pine is achieved by shedding needles (Kurkela et al., 2009), while chronic and extreme droughts further contribute to long-term defoliation of Scots pine by impairing crown development (Thabeet et al., 2009). The decreased stomatal conductance leads to decreases in photosynthetic C assimilation and thus in non-structural carbohydrate (NSC) availability under drought. Hence, under chronic drought, the isohydric strategy, together with a reduced canopy area, may be associated with an increased reliance on recent photoassimilates in defoliated trees and a possible decline in NSC storage (Eilmann et al., 2010), with the potential consequence of a higher risk of C starvation (McDowell et al., 2008, Breshears et al., 2009). Crown defoliation may thus render trees more vulnerable to subsequent droughts, and indeed, levels of crown defoliation have become a good indicator of the individual vigour of Scots pine under drought stress (Dobbertin et al., 2010, Galiano et al., 2010, Galiano et al., 2011, Barba et al., 2013, Poyatos et al., 2013). In particular, high levels of defoliation (with a crown reduced to less than 50% of its original area) have been associated with reduced NSC availability and higher mortality (Galiano et al., 2011, Poyatos et al., 2013).

However, defoliated Scots pines also show higher sensitivity in soil-to-needle hydraulic conductance to drying soil, despite higher sap flow per needle area during non-stressed conditions (Poyatos et al., 2013). This, therefore suggests that defoliated trees may be more prone to hydraulic failure than non-defoliated trees, despite the isohydric tendency of Scots pine. Xylem embolism caused by high tension during water transport (Tyree & Zimmermann, 2002) can take place in any plant organ depending on its vulnerability to cavitation (Faustino
et al., 2013), and may finally cause tree death due to reduced hydraulic efficiency of the plant (Tyree & Sperry, 1988, McDowell et al., 2008). However, leaves and needles tend to have lower safety margins than stem xylem (Mayr & Cochard, 2003, Bucci et al., 2013, Torres-Ruiz et al., 2014) and can even act as hydraulic circuit breakers either via increased cavitation or conduit collapse (e.g., Zhang et al., 2014). Despite Scots pine being an isohydric species, needles might suffer cavitation during severe drought, which may impair their physiological response to stress.

To improve our understanding of the physiological responses of Scots pine needles to drought stress, we compared the responses of hydraulic and carbon-related properties of defoliated and non-defoliated trees over a growing season, in a population of northern Spain that has experienced severe annual droughts since the 1990s and where tree mortality has previously been reported (Martínez-Vilalta & Piñol, 2002, Hereş et al., 2012). We measured diurnal courses of gas-exchange by needles, as well as photosynthetic light-response and CO₂-response curves. We also determined the amount of NSC in needles and supporting twigs, the needle predawn and midday water potentials and their osmotic and turgor components. We combined needle transpiration and whole-tree sap flow with water potentials (Ψ) to obtain twig- and tree-level hydraulic conductance. Finally, we measured twig morphological properties and determined the sensitivity of pine needles to embolism, based on Ψ₅₀ and Ψ₈₈, i.e., the water potentials at which 50% and 88% of the cumulative ultrasonic acoustic emissions are recorded. We hypothesized that: 1) defoliated trees show similar gas-exchange performance to non-defoliated trees (per unit leaf area); 2) defoliated trees have lower NSC reserves than non-defoliated trees because of a lower biomass of photosynthetic tissues per twig; and 3) hydraulic properties and safety margins of needles remain unaffected by their level of defoliation.
Material and methods

Site and plant material

The study was conducted in north-eastern Spain at the Poblet nature reserve in the Prades Mountains. The climate is Mediterranean, with a mean annual temperature of 11.3°C (average for 1951–2010) and a mean annual rainfall of 664 mm, peaking in spring and autumn (c.f., Ninyerola et al., 2007b, Ninyerola et al., 2007a). The experimental area is similar to the one of Poyatos et al. (2013), and detailed information about the study site can be found in Hereter & Sanchez (1999). Briefly, the experimental site (41°19′58.05"N, 1°0′52.26"E; 1015 m asl) is located on a 35° Northwest-facing hill slope in the Tillar valley, on fractured schist, which results in fairly rocky and shallow (ca. 40 cm deep) xerochrept soils with a loamy texture and a high gravel content of ca. 44% (Barba et al., 2013).

The vegetation at the site is dominated by Scots pine (54% of the total basal area (BA) and mean diameter at breast height (DBH) of 0.32 m), and the evergreen holm oak (Quercus ilex L. 41% of the total BA and mean DBH of 0.15 m) is the main understorey species (Barba et al., 2013); further details on plot vegetation can be found in Poyatos et al. (2013). The Scots pine population is at least 150 years old and has remained unmanaged for the past 30 years (Hereş et al., 2012). The experimental site is located in an area where Scots pine is affected by drought-induced die-off with a standing mortality higher than 20%, and in which defoliated and non-defoliated individuals co-occur (Martínez-Vilalta & Piñol, 2002, Vilà-Cabrera et al., 2013).

Eight non-defoliated and eight defoliated Scots pines of fairly similar size (Supporting Information Table S1) were selected. Defoliation was expressed as the percentage of green needles, and was visually estimated relative to the healthy canopies of similar sized trees in the same population (Galiano et al., 2010). Individuals with 50% or fewer green needles were
considered to be defoliated (Table S1). It should be noted that the term defoliation can be ambiguous as it also refers to active removal of leaf biomass by predatory agents; in the present study it refers exclusively to the loss of green needle area compared to that of a similar tree growing under optimal conditions. This definition has been extensively used in the context of drought mortality studies (e.g., Bréda et al., 2006, Galiano et al., 2011, Poyatos et al., 2013).

Sampling of twig material for the determination of photosynthetic parameters, total water potentials, turgor and osmotic potentials and carbohydrate concentrations took place during three sampling campaigns during the growing season of 2012 (June, August and November), to represent the environmental conditions typical of warm and fresh springs, the peak of hot summer droughts and the cooler, more mesic autumns, respectively. Sampling of twigs for native levels of gas exchange did not take place in November 2012 because none of the branches within reach received any direct sunlight during the day.

**Meteorological and soil moisture measurements**

Meteorological and soil moisture measurements (Fig.1) were performed using the same system as Poyatos et al. (2013). Briefly, a data acquisition system (CR1000 datalogger and AM16/32 multiplexers, Campbell Scientific Inc., Logan, UT, USA) was used to store 15-min averaged meteorological variables and soil moisture. Sensors for measuring air temperature and air relative humidity (CS215, Campbell Scientific Inc.), precipitation (52203, R.M. Young Company, Traverse City, MI, USA), total solar radiation (SP1110, Skye Instruments Ltd, Llandrindod Wells, Powys, UK) and wind speed (05103-5, R.M. Young Company) were installed at the top of a 16-m-tall tower within 20 m of the plot centre. Average volumetric soil water content (SWC) in the upper 30 cm of soil was monitored using six frequency domain reflectometers (CS616, Campbell Scientific Inc.) randomly distributed within the
plot, and raw measurements were corrected according to Poyatos et al. (2013). Water vapour pressure deficit (VPD) was calculated from air temperature and humidity (Fig. 1). To allow comparison of 2012 with other years, VPD and SWC are also presented for 2010, 2011 and 2013 (Fig. S1).

**Leaf gas-exchange measurements**

To determine *in-situ* native rates of leaf-gas exchange (i.e., transpiration rate $E$, stomatal conductance to water vapour in the light, $g_s$, as well as $CO_2$ assimilation rate, $A_N$), gas-exchange variables were measured every two hours for one day on sun-exposed, mid-canopy twigs with fully expanded needles of three defoliated and three non-defoliated trees. Water use efficiencies (WUE) were calculated as the ratios between $A_N$ and $E$ and between $A_N$ and $g_s$. Measurements were carried out under ambient conditions with a portable photosynthesis system (Li-6400XT, Li-Cor Inc., Lincoln, NE, USA) equipped with a conifer chamber (6400-22L, Li-Cor Inc. The light source was removed to allow sunlight in the chamber through the transparent chamber lid). Note that due to the structure of the conifer chamber, gas-exchange variables were measured at the scale of needles and supporting twigs. Foliage was left in the chamber until steady state gas exchange was reached (*ca.* 5 min), and results were then logged. Five measurements were averaged per tree, time and date.

To determine photosynthetic parameters, on each of the three sampling dates one sun-exposed terminal twig was sampled with a pruning pole from the eight defoliated and eight non-defoliated trees. To limit sampling effects on tree health, samples were shared between measurements of photosynthetic parameters and of water potential (see below). Immediately after sampling, the cut-end of twigs was placed in water and recut to minimise embolism. Photosynthetic responses to $C_i$, the sub-stomatal $CO_2$ concentration (referred to as $CO_2$ response curves or $A-C_i$ curves) and to light availability (light response curves) were
measured with the same photosynthesis measurement system. The light intensity was controlled using a portable light source (6400-22L, Li-Cor Inc.) and the CO₂ supply to the measurement chamber was controlled using the Li-6400 mixer. Due to our sampling method (aimed at minimising the effect of sampling on tree health), a delay between sampling and measuring occurred. However, measurements were performed no later than 48 h after sampling, with most of the samples measured in the first 24 h. To make sure the time between sampling and measurement had little influence on our results, gas exchange response curves were assessed for some randomly selected samples at the beginning and end of our measurement period (differences were typically around 5%; only γ—see below—varied by around 12%).

Each A-Cᵢ curve was performed at a photosynthetically active radiation (PAR) intensity of 1000 µmol photon m⁻² s⁻¹ and at CO₂ concentrations in the cuvette of 300, 200, 150, 125, 100, 75, 50, 40, 400, 600, 900, 1250, 1500, 1750, 2000 µmol mol⁻¹. A minimum of 3 min between steps was given to enable the needles to equilibrate to the new conditions. A-Cᵢ curves were fitted according to Sharkey et al. (2007), using the following parameters: \( V_{c_{\text{max}}} \), maximum carboxylation rate allowed by RuBisCO; \( J \), rate of photosynthetic electron transport (based on NADPH requirement) at the measurement light intensity; \( TPU \), triose phosphate use; \( R_{d} \), day respiration; and \( g_{m} \), mesophyll conductance. In this method, a non-linear curve-fitting method is used to estimate \( g_{m} \) and \( R_{d} \) by minimizing the sum of squared model deviations from the observed data based on both RuBisCO-limited (low internal CO₂ concentration) and RuBP regeneration-limited (intermediate to high internal CO₂ concentration) portions of the A-Cᵢ curve (details and scripts are available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3040.2007.01710.x/suppinfo)

Each light-curve was performed at 400 ppm CO₂ concentration and consisted of 18 PAR levels with measurements taken at 1850, 1500, 1000, 250, 100, 80, 60, 50, 45, 40, 35, 30, 25,
20, 15, 10, 5, 0 µmol\text{photon m}^{-2}\text{s}^{-1}. An additional measurement was taken at 0 µmol\text{photon m}^{-2}\text{s}^{-1} ten minutes after the end of the light-curve to ensure that the dark-respiration values were not influenced by light-enhanced respiration. Light curves were fitted in R (R Core Team, 2014) using the \textit{nls} function to determine the parameters of a Mitscherlich equation ($A_N=A_{\text{max}}*(1-\exp(-\gamma(PAR-LCP)))$, where $A_{\text{max}}$ is the maximum photosynthetic rate (µmol\text{CO}_2\text{m}^{-2}\text{s}^{-1}), PAR the photosynthetically active radiation (µmol\text{m}^{-2}\text{s}^{-1}), LCP the light compensation point (µmol\text{m}^{-2}\text{s}^{-1})$ and $\gamma$ apparent quantum yield or initial slope of the curve). Day and dark respiration ($R_{\text{day}}$ and $R_{\text{dark}}$, respectively) were measured according to the Kok method (as described in Sharp \textit{et al.}, 1984).

Following these measurements, twigs used for gas exchange were sampled from the trees or from the branches used to measure the response curves, and the projected needle area determined (Li-3100C, Li-Cor Inc.). Gas exchange values were recomputed with the exact needle area.

\textbf{Water potentials and hydraulic conductance}

The water potentials $\Psi$ of the terminal tips of branches – typically twigs around 10 cm long and their needles – were measured with a pressure chamber (PMS Instruments, Corvallis, OR, USA) at the same time as the gas exchange measurements in June, August and November. Measurements were taken both at predawn ($\Psi_{\text{pd}}$; just before sunrise at 03:00–05:00 h, solar time) and at midday ($\Psi_{\text{md}}$; 11:00–13:00 h, solar time). On each sampling date, one sun-exposed terminal tip was sampled with a pruning pole from the eight defoliated and eight non-defoliated trees. Prior to measurements, sampled tips were stored in humidified plastic bags to stop water loss from the twigs. The time between sampling and measurement in the field was typically less than 2 h.
To determine levels of osmotic adjustment in our sample trees, a subset of needles was placed in a large insulated box filled with dry-ice for at least 2 hours, to lyse the cells. The needles were then placed in a syringe and pressed to extract the cell content. The solution obtained was analysed in an osmometer (Vapro 5520, Wecor Inc., Logan, UT, USA) to measure the osmotic component of the Ψ, later referred to as osmotic potential with the notation Ψ_{md,o} and Ψ_{pd,o} for midday and predawn, respectively. The turgor component of the Ψ, later referred to as turgor potential with the notation Ψ_{md,t} and Ψ_{pd,t} for midday and predawn, respectively, was calculated as the difference between Ψ and osmotic potential.

Twig level hydraulic conductance (k_{twig}, mmol m^{-2} MPa^{-1} s^{-1}) was calculated as: 
\[ k_{twig} = \frac{E_{md}}{\Psi_{pd} - \Psi_{md}} \]
where \( E_{md} \) is the transpiration rate (E) at midday. Whole-tree hydraulic conductance (k_{tree}, mmol m^{-2} MPa^{-1} s^{-1}) was calculated as: 
\[ k_{tree} = \frac{J_{L,md}}{\Psi_{pd} - \Psi_{md}} \]
where \( J_{L,md} \) is the sap flow per unit needle area at midday (Fig. S2). Sap flow was measured during the three campaigns (June, August and November) on the same 16 trees employed to sample branches for gas exchange parameters and twig water potentials. Two 20 mm long heat dissipation sensors (Granier, 1985) were installed in the trunk at breast height, at opposite azimuths. Sap flow density, corrected for natural temperature gradients within the trunk and for radial variation within the xylem, was converted to sap flow per unit leaf area using tree sapwood depths and leaf areas. See Poyatos et al. (2013) for further details.

**Needle and twig non-structural carbohydrates**

Twigs and needles from terminal tips of branches were sampled for NSC analyses at the same time as the terminal tips for Ψ, i.e. at predawn and midday in June, August and November. Bark was removed from twigs. All samples were microwaved for 180 s within 3 h of collection to stop enzymatic activity, oven-dried for 72 h at 65°C and ground to fine powder. Twigs and needles were separated and analysed independently. Non-structural carbohydrates
(NSC) were defined as free sugars (glucose and fructose), low molecular weight sugars (free sugars and sucrose) plus starch, and were analysed according to Galiano et al. (2011) based on Hoch et al. (2002) with some minor modifications, and expressed as concentration (expressed in g of NSC per g of tissue and referred to as [NSC]).

Morphological properties and vulnerability to cavitation

To determine the morphological properties of the twigs of our studied trees, one primary branch was harvested on four defoliated and four non-defoliated trees in January 2013 and the annual shoot growth measured for the last three years (i.e., 2010, 2011 and 2012). The number of needles per twig and per year and their projected area were measured with a standard desk-top scanner. Twig mass was measured on 10 defoliated and 10 non-defoliated trees in autumn 2014, making sure the sampled twigs were of equal age.

Vulnerability to cavitation of needles of defoliated and non-defoliated trees was determined according to Charra-Vaskou et al. (2012). Briefly, one additional primary branch was harvested on four defoliated and four non-defoliated trees, sealed in dark plastic bags and maintained in contact with water overnight to allow full rehydration. One ultrasonic sensor (150 kHz resonance sensors, R15/C, 80–400 kHz) was attached to a group of 6-10 needles per branch to record ultrasonic emissions (UEs). Sensors were connected to a 20/40/60 dB preamplifier set to 40 dB and to an 8-channel PCI-2 system (PAC 125 18-bit A/D, 3 kHz to 3 MHz PCI2; all components of the UE system from Physical Acoustics Corporation, Wolfsberg, Germany). Water saturated branches were then allowed to dehydrate and the needle Ψ of 2-3 needles per branch was measured at different time intervals with a pressure chamber (Scholander type, PMS Instruments, Corvallis, OR, USA), while acoustic emission measurements were being made. Once the acoustic activity ceased, the cumulative number of UEs was calculated for each Ψ and vulnerability curves of percent cumulative ultrasonic emissions were plotted.
emissions (PCUE) were built accordingly. Curves were fitted using the equation provided by Pammenter & Vander Willingen (1998):

$$PCUE = \frac{100}{1 + \exp(a(\Psi - b))}$$

with $a$ representing a dimensionless parameter controlling the shape of the curve and $b$ the $\Psi$ for a PCUE of 50% (i.e., $\Psi_{50}$). These vulnerability curves enable the determination of the $\Psi$ for a PCUE of 88% ($\Psi_{88}$) and, therefore, enable a comparison of the vulnerability to severe cavitation between defoliated and non-defoliated trees.

**Statistical analyses**

Statistical analyses (water potential and its components, [NSC] and its components, diurnal leaf gas-exchange variables, light and $A-C_i$ curves parameters) were performed in R (R Core Team, 2014) using mixed effect models (package nlme, Pinheiro et al., 2013). These analyses employed dates and health status (defoliation level) as fixed factors and individual tree as a random factor. The interactions between the main factors were included in the initial model, and the model was simplified stepwise based on the corrected Akaike Information Criterion (AICc, for small sample sizes, package MuMIn, Barton, 2014). In each step, we tested whether the simplified model was significantly worse than the original one, using the likelihood ratio test.

Similarly, analyses of NSC and its components were carried out with organs (twig or needles) and time of sampling (predawn and midday) as fixed factors in addition to dates and health status, with their respective interactions also included in the initial model. Analyses of diurnal gas-exchange variables (CO$_2$ assimilation, stomatal conductance and transpiration), also included measurements hours, VPD (water vapour pressure deficit), PAR and air temperature ($T_{air}$) as covariates to reduce noise from varying environmental conditions and help isolate the
effects of the fixed factors (see Table S2). Diurnal gas-exchange variables (CO₂ assimilation, stomatal conductance and transpiration but not WUE) were square-root transformed to reduce heteroscedasticity. Analyses of morphological variables were carried out overall and per year using a t-test to compare defoliated and non-defoliated trees.

Results

Branches morphology

For the three years studied, defoliated trees showed lower annual shoot growth compared to non-defoliated trees. In 2011 and 2012, defoliated trees had shoot growth of 19.1±1.0 mm and 15.1±2.6 mm, respectively, while twigs of non-defoliated trees grew by 35.5±6.5 mm and 32.1±5.9 mm, respectively (Fig. 2A). This difference in growth was marginally significant in 2010. Twig biomass did not differ between defoliated and non-defoliated trees (5.38±1.20 g and 4.95±1.07 g, respectively). In addition, defoliated trees had a lower number of needles per year, with this difference being mostly due to the year 2010 (17±10 vs. 43±8, respectively, Fig. 2B). Similar results were observed for the projected area of the needles, with lower area in defoliated than non-defoliated pines (9.3±5.5 cm² vs. 24.0±2.6 cm², respectively, Fig. 2C).

Leaf gas-exchange

CO₂ assimilation ($A_N$, Fig. 3A and B), stomatal conductance ($g_s$, Fig. 3C and D) and transpiration per unit leaf area ($E$, Fig. 3E and F) were significantly higher in defoliated than in non-defoliated trees and in June than in August (note that short-term differences in environmental conditions, and especially light and VPD, were accounted for by using them as covariates in the statistical analyses – see above and Table S2). Furthermore, $A_N$, $g_s$ and $E$ were significantly affected by measurement time, showing an increase in the morning to early
afternoon and a decrease in the mid- to late-afternoon. Interactions between health status, measurement dates and time were not significant in either $A_N$, $g_s$ or $E$. WUE (calculated as $A_N / E$) was on average $8.44\pm1.85$ µmol CO$_2$ mmol H$_2$O$^{-1}$ and was only affected by measurement time, whereas WUE (calculated as $A_N / g_s$) was only affected by dates. Interestingly, neither of the two estimates of WUE was affected by the health status of the canopy but the $A_N$ response to $g_s$ was marginally higher ($p=0.08$) in non-defoliated trees than in defoliated trees in both June and August (Fig. S3). This would indicate that non-defoliated trees tend to benefit more from opening their stomata than defoliated trees, although this result might be driven by the smaller range of $g_s$ values experienced by non-defoliated trees compared to defoliated ones.

Estimates of total daily twig-scale assimilation ($A_{day}$ values and description in Table S3) were higher in defoliated than non-defoliated tree, especially in August.Light response curve parameters ($A_{max}$, $LCP$ and $\gamma$) showed no significant differences between defoliated and non-defoliated trees (Table 1). Model selection based on AICc resulted in final models with only health status and dates but no interaction for $A_{max}$, $LCP$ and $\gamma$. $A_{max}$ and $\gamma$ were also unaffected by dates, while $LCP$ was lower in June compared to August and November. As expected, $R_{day}$ was smaller than $R_{dark}$ (by 30 to 60%; Table 1) and $R_{day}$ was significantly affected by measurement date ($p=0.015$), but defoliation class had no effect on either $R_{day}$ or $R_{dark}$.

Results for $A-C_i$ response curves were highly variable (Table S4), and this variability is difficult to reconcile with the comparatively more uniform results from the light response curves (Table 1). For example, in June, $V_{cmax}$ and $J$ in defoliated trees tended to be lower than in non-defoliated trees, but both $V_{cmax}$ and $J$ increased from June to August in defoliated trees and decreased in non-defoliated trees. As a result, $V_{cmax}$ and $J$ (and also TPU and $R_d$) were marginally higher for defoliated trees in August. A post-drought recovery in $V_{cmax}$ and $J$ was more evident in non-defoliated trees in November (Table S4). These seasonal changes in
needle photosynthetic apparatus may be partially explained by the interaction of drought responses and phenological maturation of needle tissues, as previously observed in species from drought-stressed environments (e.g., in Pinus halepensis, Maseyk et al., 2008). However, methodological artefacts in $A-C_i$ response curves cannot be totally excluded. Considering this and that $R_d$ is only the residual of the $A-C_i$ curve fitting, we focus later on $R_{day}$ and $R_{dark}$ calculated with the Kok method.

**Needles and twigs NSC**

Defoliated trees had marginally significantly lower total [NSC] in summer (5.70±0.45%) in needles compared to non-defoliated trees (6.91±0.67%, Fig. 4A and B); while no overall difference was observed between health classes. No significant differences were observed between defoliated and non-defoliated needles and twigs for starch (Fig.4C and D) and glucose+fructose (data not shown). Sucrose in needles was marginally lower in defoliated than non-defoliated trees in summer and thus responsible for the marginally significantly difference in [NSC] observed between tree health class (see above), but otherwise, no differences were observed between defoliated and non-defoliated needles and twigs (Fig. 4E and F). However, defoliation status was kept in the final model selection based on AICc, except for sucrose. Further details on final model structure can be found in Table S5 and estimation of [NSC] per leaf area and total twig NSC are provided in Table S3.

Seasonally, total NSC and starch in both defoliated and non-defoliated pines decreased from June to August (Fig. 4A, B for total NSC and C and D for starch). In August, values for starch were effectively zero for both organs and defoliation classes. For both defoliated and non-defoliated trees, no recovery of total NSC or starch was observed from August to November. The high NSC and starch concentrations in June are probably temporary storage during the favourable months of spring to sustain above and belowground sink activity during
the stress period, but will not be further discussed due to the lack of sink organ data. On the contrary, sucrose increased from spring to autumn in both needles and twigs (Fig. 4E and F). Total NSC was significantly higher in needles than in twigs in spring (Fig. 4A and B), but no differences were observed later on. Glucose plus fructose concentration was higher in twigs (between 4 and 5.5%) than in needles (between 2.9 and 4.6%) and represented the main pool of low molecular weight sugars. In both organs, glucose and fructose concentrations were highest in summer. However, the variability over time of glucose and fructose concentration was much lower (~1.5 fold) than in the other NSC pools, namely starch (>10 fold) and sucrose (> 5 fold).

When reported per needle area, estimates of [NSC] were similar, if not lower, (see note in Table S3 legend) in defoliated than in non-defoliated trees.

Twig water potential components

Ψ_{md} was significantly influenced by health status, sampling dates and their interaction. Ψ_{md} was lower in June in defoliated trees than in non-defoliated trees (Fig. 5A; Table S6). In defoliated trees, Ψ_{md} did not change between June and August, while for non-defoliated trees Ψ_{md} decreased to ca. -2.0 MPa in August, the same value observed for defoliated trees (Fig. 5A, Table S6). Ψ_{md} increased from August to November, reaching values of ca. -1 MPa for both types of trees (Fig. 5A). Ψ_{pd} tracked variations in soil moisture (Fig. 1; Fig. 5B) and decreased from ca. -1.3 MPa in June to ca. -1.8 MPa in August, before increasing to ca. -0.6 MPa in November (Fig. 5B). No overall differences between defoliated and non-defoliated trees were observed for Ψ_{pd}; however, in August, Ψ_{pd} was significantly more negative in defoliated trees than in non-defoliated trees (Fig. 5B; Table S6).

Both predawn and midday osmotic potential (Ψ_{md, o} and Ψ_{pd, o}) increased from June to August and from August to November in both defoliated and non-defoliated trees (Fig. 5C, 5D).
Defoliated trees had lower $\Psi_{md,o}$ and $\Psi_{pd,o}$ than non-defoliated trees (Fig. 5C, 5D). Both predawn and midday turgor potential ($\Psi_{md,t}$ and $\Psi_{pd,t}$) were also significantly different among sampling dates ($p<0.001$). They first decreased from June to August and then increased from August to November in both defoliated and non-defoliated trees (Fig. 5E and F). No overall significant differences were found between defoliated and non-defoliated trees for either $\Psi_{md,t}$ and $\Psi_{pd,t}$.

$k_{twig}$ increased fivefold between June and August in defoliated trees, while it decreased by ca. one third in non-defoliated trees (Table S6). During the same period, $J_{L,md}$ (Fig. S2) was higher during day- and night-time and showed later closure of the stomata during the day in defoliated trees, especially in August and November. As a result, $k_{tree}$ remained stable in defoliated trees while it decreased by ca. three quarters in non-defoliated trees. $k_{tree}$ was significantly affected by date and also tended to be affected by the interactions between defoliation class and date. In June, both $k_{twig}$ and $k_{tree}$ were equal or higher in non-defoliated than in defoliated trees, while in August both $k_{twig}$ and $k_{tree}$ were smaller in non-defoliated than in defoliated trees. $k_{twig}$ was higher than $k_{tree}$ by a factor of 2 in June in both defoliated and non-defoliated trees, while in August $k_{twig}$ was ten times higher than $k_{tree}$ in defoliated trees and five times higher in non-defoliated trees. Note that no statistical tests were performed on $k_{twig}$ since these metrics were calculated using average values by defoliation class because of the different sampling designs for gas-exchange, water potential and sap flow.

**Leaf xylem vulnerability to embolism**

The curves of percent cumulative ultrasonic emission (PCUE) obtained for the two health classes of trees were similar, with a $\Psi_{50}$ at ca. -1.5 MPa in both cases (Fig. 6). However, non-defoliated trees tended to reach a critical level of 88% PCUE at a slightly more negative
needle $\Psi$ than defoliated trees (Fig. 6), although the difference was not statistically significant.

**Discussion**

The physiological responses to drought of needles from defoliated trees clearly differed to those from non-defoliated trees. Contrary to our hypothesis, needles of defoliated trees maintained higher gas-exchange rates during the drought stress period. This presumably allowed needles and terminal twigs of defoliated trees to maintain a similar [NSC] to those of non-defoliated trees. However, the higher gas-exchange rates during drought in defoliated trees came with increased risk of hydraulic failure in needles, because defoliated trees were slightly more sensitive to critical levels of needle embolism and were more water-stressed (more negative water potentials) than non-defoliated trees, especially during June at midday and August at pre-dawn.

**Carbon assimilation and water loss**

Despite having a smaller needle area per twig, defoliated trees showed higher $A_N$ and $A_{day}$ during the growing season than non-defoliated trees. The latter showed a typical response to drought in Scots pines: decreased C assimilation during periods of low water availability (e.g., Berninger *et al.*, 1996, Irvine *et al.*, 1998). The unusual gas-exchange response to water deficit in an isohydric species observed in the defoliated trees suggests that while non-defoliated trees can afford a period of low C intake, defoliated trees need to maintain C-assimilation during the period of peak stress. The higher $A_N$ in defoliated trees came at a cost in terms of water loss, because of higher $E$ throughout the growing season. In addition, defoliated trees showed no improvement of WUE and smaller $A_N$ gain per unit $g_s$ increase than non-defoliated trees. This result is further supported by the higher sap flow per needle area, later closure of the stomata during the day and higher night-time sap flow in defoliated
trees, especially in August and November. Poyatos et al. (2013) observed similar sap flow patterns under favourable hydrological conditions in 2011. At the peak of the drought in 2011, both types of tree achieved very low sap flow rates, while in the present study higher sap flow was maintained in defoliated trees throughout the growing season. This is possibly due to the differences in drought stress development between years: 2011 had a mild but extremely long drought, while 2012 was a very hot and dry summer during which VPD and thus evaporative demand reached extreme values for the site (Fig. S1).

The differences in $E$ between defoliated and non-defoliated trees were associated with differences in the $g_s$ response to drought. Non-defoliated trees closed stomata to limit water loss under drought, in agreement with existing knowledge of Scots pine needle physiology, thus exhibiting tight stomatal control in order to avoid critically low needle $\Psi$ values (Irvine et al., 1998, Zweifel et al., 2007, Duursma et al., 2008, Poyatos et al., 2008). The $g_s$ decrease in non-defoliated trees with decreasing water availability is also consistent with previous comparison of irrigated and non-irrigated isohydric species, which showed gas-exchange limitations under drought stress for both treatments albeit with a stronger limitation in non-irrigated trees (e.g., Limousin et al., 2013, Torres-Ruiz et al., 2013). Counter to expectation, the defoliated trees here showed a surprising stomatal response by maintaining $A_N$, $E$ and $g_s$ during the dry period; while their twig water potentials fell to values consistent with substantial losses of hydraulic conductance (see discussion about hydraulic risk).

**Carbon reserves and use**

Despite their smaller number of needles and their smaller area, defoliated trees were able to maintain an almost identical [NSC] in needles and supporting twigs compared to non-defoliated trees. The measured values of [starch] and [low molecular weight sugars], i.e. 2 to 15%, are comparable to those reported for Scots pine needles under drought in a dry alpine
valley of Austria (Gruber et al., 2012). However, a more defoliated population in the same forest at Prades (with on average, 26% green leaves instead of 42% in the present study) had lower [NSC] in branches and leaves than in the non-defoliated trees (Aguadé et al., 2015).

Thus, at medium levels of defoliation (i.e. this study), higher values of $A_N$ allow partially defoliated trees to maintain their terminal branch [NSC]. However, with increasing defoliation, the remaining needles do not appear to be sufficient to keep the branch [NSC] at a level similar to that of non-defoliated trees. This explanation is further supported by the marginal difference observed in August in needle [NSC], which is consistent with the onset of a defoliation and drought effect on needle [NSC].

The [NSC] in needles and supporting twigs depends not only on C input (photosynthesis, see above) but also on C output (respiration, growth, and export). Estimates of [NSC] concentrations per needle area were similar, if not lower, in defoliated than in non-defoliated trees, despite higher $A_{day}$, suggesting that an equal or larger fraction of the daily assimilated C is transferred to the supporting twigs. Total twig [NSC] decreased from June to August in both defoliated and non-defoliated trees, but more in the former than in the latter (Table S3), supporting the hypothesis that higher gas-exchange in defoliated trees is a response to lower NSC availability. Growth and growth respiration can be considered negligible at the three measurements dates, because stopping growth is the first response of plants to water shortage (Sala et al., 2010) during drought-stressed periods (June and August) when C uptake is limited due to stomatal closure, and because of phenological considerations (and lack of direct sunlight due to the north-facing slope) in November. Thus, maintenance respiration is the main form of catabolism during our study and it does not differ between health classes.

The lower $R_{day}$ in August compared to June and November is probably related to the down-regulation of processes with high C-respiratory cost, e.g., protein synthesis, turnover and growth (Gibon et al., 2009, Hummel et al., 2010) during the stress period.
A set of 21 trees including the present 16 trees showed significantly lower trunk [NSC] in defoliated than non-defoliated trees (0.7% and 1.05%, respectively; Poyatos et al., 2013). Thus, despite the lack of differences in [NSC] at the needle and twig level in the studied population, differences in [NSC] in the stem were observed. This result is consistent with Norway spruce responses to drought, in which needle [NSC] did not differ between dying and surviving trees, although dying trees had significantly lower amount of NSC in their roots (Hartmann et al., 2013). More severely defoliated trees from the same population also showed significantly lower [NSC] in trunk and roots (Aguadé et al., 2015), in further agreement with an additional Scots pine population in Northern Spain (Galiano et al., 2011).

Although the defoliated trees studied here were able to maintain enough NSC reserves in the needles and twigs, they could potentially suffer from C-starvation in non-photosynthetic organs as the result of two non-mutually-exclusive mechanisms: 1) the total amount of photosynthetic area does not permit enough NSC to be produced to maintain a C balance similar to that of the non-defoliated trees (Galiano et al., 2011); and 2) phloem transport from needles to C-sink organs might be impaired, due to the competition for water between phloem transport and transpiration (Nikinmaa et al., 2013). Recent modelling of the same Scots pine population (Mencuccini et al., in press and in review) suggests that direct phloem failure by high viscosity is unlikely to play a role in long-term mortality risks, as it would require extremely negative twig $\Psi$ (Sevanto, 2014, Sevanto et al., 2014). However, this does not rule out a temporary loss of phloem transport capacity in defoliated trees, as a result of loss of cell turgor during periods of very low twig $\Psi$ and high $E$. Indeed, at midday, needle turgor was only 0.3-0.4 MPa, while $\Psi_{md}$ averaged around -2.0 MPa. By comparison, values of -2.5 MPa in both defoliated and non-defoliated trees were reported for $\Psi_{md}$ during the longer drought of 2011 (Poyatos et al., 2013). Nonetheless, the relatively high $A_N$ and $E$ in defoliated trees, coupled with low absolute values of [NSC] in leaves and twigs, suggest that
potential turgor impairment of phloem transport must be a temporary, not a permanent, phenomenon.

**Hydraulic risk**

As needles from defoliated trees maintained $A_N$ at the cost of losing more water (Fig. 3 and S1), more negative $\Psi_{md}$ values were reached in June. These low $\Psi_{md}$ were maintained throughout the summer in defoliated trees, while non-defoliated trees reached similar values only at the peak of drought in August. Summer $\Psi$ values were similar to those reported from the same site in 2010 and 2011. However, even lower values were measured in mid-October 2011 after an extraordinarily long drought (Poyatos et al., 2013). Hence, in the short intense drought of summer 2012 (Fig. S1), Scots pine maintained higher $\Psi$ levels than under less intense, but longer, drought conditions. Between June and August in 2012, defoliated trees increased $k_{\text{twig}}$ and kept $k_{\text{tree}}$ constant, while both $k_{\text{twig}}$ and, to a lesser extent, $k_{\text{tree}}$ decreased in non-defoliated trees. These results are superficially different from those in Poyatos et al. (2013), who observed a decrease in $k_{\text{tree}}$ in both tree types between June and August in 2010 and 2011. This apparent discrepancy may be partly explained by the low soil moisture observed in June 2012 compared to other years (SWC in June 2012 is close to SWC usually measured later on in summer, Fig. S1). The high evaporative demand and the maintenance of gas-exchange in summer 2012 in defoliated trees might have required adjustment of their hydraulic transport capacity. Liu et al. (2014) showed an association between experimental defoliation and increase in hydraulic conductance through changes in aquaporin expression. Although the causes of defoliation differ between the two studies (and thus the physiological mechanisms to respond to it are possibly different), changes in aquaporin expression may have played a role in the increase in $k_{\text{twig}}$ in defoliated trees.
Additionally, defoliated trees tended to risk reaching extreme levels of embolism (88% PCUE, $\Psi_{88}$) at higher (less negative) $\Psi$ than non-defoliated trees (Fig. 6). Values of $\Psi_{nd}$ measured in the field (Fig. 5) cannot be directly compared to needle xylem vulnerability curves obtained from PCUE (Fig. 6), because of uncertainties on the source of UAE and of intra-needle $\Psi$ gradients. However, $\Psi_{nd}$ measured in defoliated trees in June and August were close to the $\Psi_{88}$ values in needles, suggesting a substantial risk of losses of leaf hydraulic conductance at midday, while $\Psi_{nd}$ in non-defoliated trees decreased at the peak of the drought to values similar to those of defoliated trees, but without reaching $\Psi_{88}$. Therefore, defoliated trees possibly operate within smaller safety margins than non-defoliated trees, in agreement with similar observations from several angiosperm species, in which desiccated shoots had lower safety margins ($\Psi - \Psi_{50}$) than non-desiccated ones (Nardini et al., 2013).

Hydraulic conductance and sap flow results also suggest a decoupling between whole tree sap flow and needle transpiration in defoliated trees during intense drought, probably mediated by water storage. The observations of a large increase in $k_{twig}$ in defoliated trees in August, of differences in $\Psi_{pd}$ between defoliated and non-defoliated trees in August, and of a sustained higher nocturnal sap flow in defoliated trees in August and November, all collectively suggest that defoliated trees may have been unable to restore capacitance reserves during the night to allow their needles $\Psi_{pd}$ to equilibrate with the soil, and are consistent with the use of stored water to sustain transpiration during the day.

These adjustments of hydraulic conductance (and probably capacitance) allowed defoliated trees to successfully maintain their cell turgor at a level similar to that of non-defoliated trees. This was however only achieved by maintaining more negative osmotic pressures through the day and through the year (i.e. accumulating higher solute concentrations). Based on the assumption that soluble sugars would be the only solute, we estimated that the lower osmotic potential in defoliated trees could cost approximately a 0.05 to 0.10 mol.L$^{-1}$ increase in
soluble sugars concentration in defoliated trees compared to non-defoliated trees (Table S7). Plants use other solutes (e.g. potassium, calcium, sodium, amino acids) in addition to NSC to maintain osmotic pressure (e.g., Talbot and Zeiger 1996). Although not measured in the present study, it is unlikely that defoliated trees have better access to those solutes than non-defoliated trees since their acquisition is energy costly. Consequently, despite having similar [NSC], defoliated trees were likely to have less sugar available for physiological activity such as respiration, export to the sink organs, repair of damage, growth, defence, and needle xylem reinforcement. In other words, in order to maintain needle cell integrity (i.e., turgor), defoliated trees needed to employ a higher fraction of their [NSC] for osmoregulatory purposes (Table S7) and were consequently at increased risk of C-starvation.

Conclusion

Our results offer new insights regarding the ongoing interactions between physiological processes leading to death in a Mediterranean Scots pine population. To understand how the present results generated insight for our interpretation of tree survival under drought, one should keep in mind that mortality happening at the branch scale negatively impacts the survival of the whole tree, while potential recovery of a branch (e.g., as measured by $\Psi_{pd}$ and $\Psi_{md}$ in autumn) does not necessarily translate into the recovery of the whole trees (i.e. other branches might have died). Defoliated and non-defoliated trees differ in their carbon-acquisition-for-water-loss trade-off characteristics. Defoliated trees increase carbon uptake under drought stress at the cost of higher water loss, while non-defoliated trees do the opposite. Indeed, non-defoliated trees showed a typical response for an isohydric species (i.e., closing stomata to maintain $\Psi$ above critical levels), which, under the chronic drought conditions of the study site, may eventually lead to severe carbon deficits, but by doing so they increase their chance of survival to shorter and/or more intense drought by limiting the risk of hydraulic failure. In contrast, defoliated trees showed a less usual physiological
response. Probably as a result of the smaller amount of photosynthetic tissue (leaf area) available, and the need to maintain C resources, they maintained higher gas-exchange activity in the remaining needles. At moderate levels of defoliation (i.e. 35-50% of green leaves), defoliated trees maintained needle [NSC] similar to that of non-defoliated trees, at least in twigs. However, it is unlikely that defoliated trees can maintain a favourable whole-tree C balance, especially since they have to maintain more negative osmotic pressures to maintain cell turgor, a process which itself draws upon energy reserves. Overall, their smaller hydraulic safety margins and higher water losses also put them at higher risk of critical levels of embolism in terminal twigs and, therefore, expose them to higher chances of branch mortality by hydraulic failure. In turn, this results in further loss of photosynthetic tissues triggering a feedback loop between C-starvation and hydraulic failure that increases the likelihood of subsequent death.

Acknowledgments

Thanks to all the staff at PNIN de Poblet for allowing us to carry out research at the ‘Barranc del Tillar’ nature reserve and for their logistic support in the field. We would like to thank Lucia Galiano, David Aguadé, Teresa Rosas and Josep Barba for their help in the field. We also thank Teresa Rosas for help with NSC analysis. Thanks to Dr. K. Charra-Vaskou and Dr. G. Charrier for helping with the acoustics measurements. Thanks to Dr. X. Li for proof-reading the manuscript. This research was funded by Natural Environment Research Council (NERC) grant NE/I011749/1 to M.M. and P.M. and Spanish MICINN grants CGL2010-16373 and CSD2008-0004; P.M. was also supported by ARC grant FT110100457. YS was funded by NERC (RA0929 to MM) and RP by a Juan de la Cierva postdoctoral fellowship. Jose M. Torres-Ruiz and part of this work were supported by a STSM Grant from COST Action FP1106 (STReESS). Finally, we would like to thanks two anonymous reviewer
and the editor of *Plant, Cell and Environment* for their useful suggestions that helped improve the manuscript.

**References**

Adams H.D., Germino M.J., Breshears D.D., Barron-Gafford G.A., Guardiola-Claramonte M., Zou C.B. & Huxman T.E. (2013) Nonstructural leaf carbohydrate dynamics of *Pinus edulis* during drought-induced tree mortality reveal role for carbon metabolism in mortality mechanism. *New Phytologist*, **197**, 1142–1151.

Aguadé D., Poyatos R., Gómez M., Oliva J. & Martínez-Vilalta J. (2015) The role of defoliation and root rot pathogen infection in driving the mode of drought-related physiological decline in Scots pine (*Pinus sylvestris* L.). *Tree Physiology*, **35**, 229-242.

Allen C.D., Macalady A.K., Chenchouni H., Bachelet D., McDowell N., Vennetier M., . . . Cobb N. (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest Ecology and Management*, **259**, 660-684.

Anderegg W.R.L. & Anderegg L.D.L. (2013) Hydraulic and carbohydrate changes in experimental drought-induced mortality of saplings in two conifer species. *Tree Physiology*, **33**, 252-260.

Barba J., Curiel Yuste J., Martínez-Vilalta J. & Lloret F. (2013) Drought-induced tree species replacement is reflected in the spatial variability of soil respiration in a mixed Mediterranean forest. *Forest Ecology and Management*, **306**, 79-87.

Barton K. (2014) MuMIn: Multi-model inference.

Berninger F., Mäkelä A. & Hari P. (1996) Optimal control of gas exchange during drought: empirical evidence. *Annals of Botany*, **77**, 469-476.

Bigler C., Gavin D.G., Gunning C. & Veblen T.T. (2007) Drought induces lagged tree mortality in a subalpine forest in the Rocky Mountains. *Oikos*, **116**, 1983-1994.

Bréda N., Huc R., Granier A. & Dreyer E. (2006) Temperate forest trees and stands under severe drought: a review of ecophysiological responses, adaptation processes and long-term consequences. *Ann. For. Sci.*, **63**, 625-644.

Breshears D.D., Cobb N.S., Rich P.M., Price K.P., Allen C.D., Balice R.G., . . . Meyer C.W. (2005) Regional vegetation die-off in response to global-change-type drought. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 15144-15148.

Breshears D.D., Myers O.B., Meyer C.W., Barnes F.J., Zou C.B., Allen C.D., . . . Pockman W.T. (2009) Tree die-off in response to global change-type drought: mortality insights from a decade of plant water potential measurements. *Frontiers in Ecology and the Environment*, **7**, 185-189.

Bucci S.J., Scholz F.G., Peschiutta M.L., Arias N.S., Meinzer F.C. & Goldstein G. (2013) The stem xylem of Patagonian shrubs operates far from the point of catastrophic dysfunction and is additionally protected from drought-induced embolism by leaves and roots. *Plant, Cell & Environment*, **36**, 2163-2174.
Charra-Vaskou K., Badel E., Burlett R., Cochard H., Delzon S. & Mayr S. (2012) Hydraulic efficiency and safety of vascular and non-vascular components in Pinus pinaster leaves. Tree Physiology, 32, 1161-1170.

Collins M., Knutti R., Arblaster J., Dufresne J.-L., Fichefet T., Friedlingstein P., . . . Wehner M. (2013) Long-term Climate Change: Projections, Commitments and Irreversibility. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (eds T.F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, & P.M. Midgley), pp. 1029-1136. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Crittfield W.B. & Little E.L. (1966) Geographic distribution of pines of the world (vol. Miscellaneous Publication 991). USDA Forest Service

Dobbertin M., Eilmann B., Bleuler P., Giuggiola A., Graf Pannatier E., Landolt W., . . . Rigling A. (2010) Effect of irrigation on needle morphology, shoot and stem growth in a drought-exposed Pinus sylvestris forest. Tree Physiology, 30, 346-360.

Duursma R.A., Kolari P., Perämäki M., Nikinmaa E., Hari P., Delzon S., . . . Mäkelä A. (2008) Predicting the decline in daily maximum transpiration rate of two pine stands during drought based on constant minimum leaf water potential and plant hydraulic conductance. Tree Physiology, 28, 265-276.

Eilmann B., Buchmann N., Siegwolf R., Saurer M., Cherubini P. & Rigling A. (2010) Fast response of Scots pine to improved water availability reflected in tree-ring width and δ13C. Plant, Cell & Environment, 33, 1351-1360.

Faustino L.I., Bulfe N.M.L., Pinazo M.A., Monteoliva S.E. & Graciano C. (2013) Dry weight partitioning and hydraulic traits in young Pinus taeda trees fertilized with nitrogen and phosphorus in a subtropical area. Tree Physiology, 33, 241-251.

Galiano L., Martinez-Vilalta J. & Lloret F. (2010) Drought-induced multifactor decline of Scots pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems, 13, 978-991.

Galiano L., Martinez-Vilalta J. & Lloret F. (2011) Carbon reserves and canopy defoliation determine the recovery of Scots pine 4 yr after a drought episode. New Phytologist, 190, 750-759.

Gaylord M.L., Kolb T.E., Pockman W.T., Plaut J.A., Yepez E.A., Macalady A.K., . . . McDowell N.G. (2013) Drought predisposes piñon-juniper woodlands to insect attacks and mortality. New Phytologist, 198, 567–578.

Gibon Y., Pyl E.-T., Sulpice R., Lunn J.E., Höhne M., Günther M. & Stitt M. (2009) Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when Arabidopsis is grown in very short photoperiods. Plant, Cell & Environment, 32, 859-874.

Granier A. (1985) Une nouvelle méthode pour la mesure du flux de sève brute dans le tronc des arbres. Ann. For. Sci., 42, 193-200.

Gruber A., Pirkebner D., Florian C. & Oberhuber W. (2012) No evidence for depletion of carbohydrate pools in Scots pine (Pinus sylvestris L.) under drought stress. Plant Biology, 14, 142-148.

Hartmann H., Ziegler W., Trumbore S. & Knapp A. (2013) Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy. Functional Ecology, 27, 413-427.

Hereş A.-M., Martínez-Vilalta J. & Claramunt López B. (2012) Growth patterns in relation to drought-induced mortality at two Scots pine (Pinus sylvestris L.) sites in NE Iberian Peninsula. Trees, 26, 621-630.
Hereter A. & Sánchez J. (1999) Experimental areas of Prades and Montseny. In: Ecology of Mediterranean Evergreen Oak Forests. Springer Berlin & Heidelberg, Germany.

Hoch G., Popp M. & Körner C. (2002) Altitudinal increase of mobile carbon pools in Pinus cembra suggests sink limitation of growth at the Swiss treeline. Oikos, 98, 361-374.

Hummel I., Pantin F., Sulpice R., Piques M., Rolland G., Dauzat M., ... Muller B. (2010) Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. Plant Physiology, 154, 357-372.

IPCC (2014) Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Irvine J., Perks M.P., Magnani F. & Grace J. (1998) The response of Pinus sylvestris to drought: stomatal control of transpiration and hydraulic conductance. Tree Physiology, 18, 393-402.

Jackson G.E., Irvine J. & Grace J. (1995a) Xylem cavitation in Scots pine and Sitka spruce saplings during water stress. Tree Physiology, 15, 783-790.

Jackson G.E., Irvine J. & Grace J. (1995b) Xylem cavitation in two mature Scots pine forests growing in a wet and a dry area of Britain. Plant, Cell & Environment, 18, 1411-1418.

Kurkela T., Drenkhan R., Vuorinen M. & Hanso M. (2009) Growth response of young Scots pines to needle loss assessed from productive foliage. Forestry Studies, 50, 5-22.

Limousin J.-M., Bickford C.P., Dickman L.T., Pangle R.E., Hudson P.J., Boutz A.L., ... McDowell N.G. (2013) Regulation and acclimation of leaf gas exchange in a pinon–juniper woodland exposed to three different precipitation regimes. Plant, Cell & Environment, 36, 1812-1825.

Liu J., Equiza M.A., Navarro-Rodenas A., Lee S.H. & Zwiazek J.J. (2014) Hydraulic adjustments in aspen (Populus tremuloides) seedlings following defoliation involve root and leaf aquaporins. Planta, 240, 553-564.

Martínez-Vilalta J., Cochard H., Mencuccini M., Sterck F., Herrero A., Korhonen J.F.J., ... Zweifel R. (2009) Hydraulic adjustment of Scots pine across Europe. New Phytologist, 184, 353-364.

Martínez-Vilalta J. & Piñol J. (2002) Drought-induced mortality and hydraulic architecture in pine populations of the NE Iberian Peninsula. Forest Ecology and Management, 161, 247-256.

Maseyk K.S., Lin T., Rotenberg E., Grünzweig J.M., Schwartz A. & Yakir D. (2008) Physiology-phenology interactions in a productive semi-arid pine forest. New Phytologist, 178, 603-616.

Mayr S. & Cochard H. (2003) A new method for vulnerability analysis of small xylem areas reveals that compression wood of Norway spruce has lower hydraulic safety than opposite wood. Plant, Cell & Environment, 26, 1365-1371.

McDowell N., Pockman W.T., Allen C.D., Breshears D.D., Cobb N., Kolb T., ... Yepez E.A. (2008) Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? New Phytologist, 178, 719-739.

McDowell N.G., Beerling D.J., Breshears D.D., Fisher R.A., Raffa K.F. & Stitt M. (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality. Trends in Ecology and Evolution, 26, 523-532.

McDowell N.G., Fisher R.A., Xu C., Domec J.C., Hölttä T., Mackay D.S., ... Pockman W.T. (2013) Evaluating theories of drought-induced vegetation mortality using a multimodel–experiment framework. New Phytologist, 200, 304-321.
McDowell N.G. & Sevanto S. (2010) The mechanisms of carbon starvation: how, when, or does it even occur at all? New Phytologist, 186, 264-266.

Mencuccini M. & Grace J. (1995) Climate influences the leaf area/sapwood area ratio in Scots pine. Tree Physiology, 15, 1-10.

Mencuccini M., Minunno F., Salmon Y., Martínez-Vilalta J. & Hölttä T. (in press) Coordination of physiological traits involved in drought-induced mortality. New Phytologist.

Mitchell P.J., O'Grady A.P., Tissue D.T., White D.A., Ottenschlaeger M.L. & Pinkard E.A. (2013) Drought response strategies define the relative contributions of hydraulic dysfunction and carbohydrate depletion during tree mortality. New Phytologist, 197, 862-872.

Mitchell P.J., O’Grady A.P., Tissue D.T., Worledge D. & Pinkard E.A. (2014) Co-ordination of growth, gas exchange and hydraulics define the carbon safety margin in tree species with contrasting drought strategies. Tree Physiology, 34, 443-458.

Nardini A., Battistuzzo M. & Savi T. (2013) Shoot desiccation and hydraulic failure in temperate woody angiosperms during an extreme summer drought. New Phytologist, 200, 322-329.

Nikinmaa E., Hölttä T., Hari P., Kolari P., Mäkelä A., Sevanto S. & Vesala T. (2013) Assimilate transport in phloem sets conditions for leaf gas exchange. Plant, Cell & Environment, 36, 655-669.

Ninyerola M., Pons X. & Roure J.M. (2007a) Monthly precipitation mapping of the Iberian Peninsula using spatial interpolation tools implemented in a Geographic Information System. Theoretical and Applied Climatology, 89, 195-209.

Ninyerola M., Pons X. & Roure J.M. (2007b) Objective air temperature mapping for the Iberian Peninsula using spatial interpolation and GIS. International Journal of Climatology, 27, 1231-1242.

Oliva J., Stenlid J. & Martínez-Vilalta J. (2014) The effect of fungal pathogens on the water and carbon economy of trees: implications for drought-induced mortality. New Phytologist, 203, 1028-1035.

Pammenter N.W. & Van der Willigen C. (1998) A mathematical and statistical analysis of the curves illustrating vulnerability of xylem to cavitation. Tree Physiology, 18, 589-593.

Peng C., Ma Z., Lei X., Zhu Q., Chen H., Wang W., . . . Zhou X. (2011) A drought-induced pervasive increase in tree mortality across Canada's boreal forests. Nature Clim. Change, 1, 467-471.

Pinheiro J., Bates D., DebRoy S., Sarkar D. & R Development Core Team (2013) nlme: linear and nonlinear mixed effects models.

Poyatos R., Aguadé D., Galiano L., Mencuccini M. & Martínez-Vilalta J. (2013) Drought-induced defoliation and long periods of near-zero gas exchange play a key role in accentuating metabolic decline of Scots pine. New Phytologist, 200, 388-401.

Poyatos R., Llorens P., Piñol J. & Rubio C. (2008) Response of Scots pine (Pinus sylvestris L.) and pubescent oak (Quercus pubescens Willd.) to soil and atmospheric water deficits under Mediterranean mountain climate. Ann. For. Sci., 65, 306.

Poyatos R., Martínez-Vilalta J., Čermák J., Ceulemans R., Granier A., Irvine J., . . . Mencuccini M. (2007) Plasticity in hydraulic architecture of Scots pine across Eurasia. Oecologia, 153, 245-259.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

31
Rigling A., Bigler C., Eilmann B., Feldmeyer-Christe E., Gimmi U., Ginzler C., Dobbertin M. (2013) Driving factors of a vegetation shift from Scots pine to pubescent oak in dry Alpine forests. *Global Change Biology*, 19, 229-240.

Sala A. (2009) Lack of direct evidence for the carbon-starvation hypothesis to explain drought-induced mortality in trees. *Proceedings of the National Academy of Sciences*, 106, E68-E68.

Sala A., Piper F. & Hoch G. (2010) Physiological mechanisms of drought-induced tree mortality are far from being resolved. *New Phytologist*, 186, 274-281.

Sevanto S. (2014) Phloem transport and drought. *Journal of Experimental Botany*, 65, 1751-1759.

Sevanto S., McDowell N.G., Dickman L.T., Pangle R. & Pockman W.T. (2014) How do trees die? A test of the hydraulic failure and carbon starvation hypotheses. *Plant, Cell & Environment*, 37, 153-161.

Sharkey T.D., Bernacchi C.J., Farquhar G.D. & Singsaas E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant, Cell & Environment*, 30, 1035-1040.

Sharp R.E., Matthews M.A. & Boyer J.S. (1984) Kok effect and the quantum yield of photosynthesis. *Plant Physiology*, 75, 95-101.

Thabeet A., Vennetier M., Gadbin-Henry C., Denelle N., Roux M., Caraglio Y. & Vila B. (2009) Response of *Pinus sylvestris* L. to recent climatic events in the French Mediterranean region. *Trees*, 23, 843-853.

Torres-Ruiz J.M., Diaz-Espejo A., Morales-Sillero A., Martín-Palomo M.J., Mayr S., Beikircher B. & Fernández J.E. (2013) Shoot hydraulic characteristics, plant water status and stomatal response in olive trees under different soil water conditions. *Plant and Soil*, 373, 77-87.

Tyree M.T. & Zimmermann M.H. (2002) *Xylem structure and the ascent of sap*. Springer, Berlin, Germany.

This article is protected by copyright. All rights reserved.
Zweifel R., Steppe K. & Sterck F.J. (2007) Stomatal regulation by microclimate and tree water relations: interpreting ecophysiological field data with a hydraulic plant model. *Journal of Experimental Botany, 58*, 2113-2131.
Table 1: Variables (average ± S.E., n=7 or 8) from the light response curves and calculation of day and dark respirations according to the Kok method ($R_{\text{day}}$ and $R_{\text{dark}}$, respectively) of needles from defoliated (D) and non-defoliated (ND) Scots pine in June, August and November. $A_{\text{max}}$, maximum photosynthetic rate; $LCP$, light compensation point and $\gamma$ apparent quantum yield. Different letters indicate significant difference (p≤0.05) between defoliated and non-defoliated trees within a month, while different letter with a minus sign indicates marginally significant differences (p≤0.1).

| Light response curve | June          | August        | November     |
|----------------------|---------------|---------------|--------------|
|                      | D            | ND            | D            | ND            | D            | ND            |
| $A_{\text{max}}$ (µmol CO$_2$ m$^{-2}$ s$^{-1}$) | 9.6 ± 2.4$^a$ | 10.9 ± 0.3$^a$ | 11.7 ± 1.8$^a$ | 12.1 ± 4.8$^a$ | 10.1 ± 0.7$^a$ | 10.5 ± 2.3$^a$ |
| $LCP$ (µmol photon m$^{-2}$ s$^{-1}$)  | 122.5 ± 19.0$^a$ | 76.5 ± 69.2$^a$ | 30.3 ± 23.3$^a$ | 21.3 ± 20.4$^a$ | 62.9 ± 9.7$^a$ | 93.7 ± 19.2$^a$ |
| $\gamma$ (nmol CO$_2$ µmol photon$^{-1}$) | 3 ± 0.1$^a$ | 2.3 ± 1.0$^a$ | 4.3 ± 0.6$^a$ | 5.4 ± 1.8$^a$ | 5.1 ± 0.7$^a$ | 3.3 ± 0.5$^a$ |
| Kok method           |               |               |              |               |              |               |
| $R_{\text{day}}$ (µmol m$^{-2}$ s$^{-1}$)  | 2.52 ± 0.49 | 2.93 ± 1.98 | 1.13 ± 0.17 | 1.80 ± 0.77 | 2.02 ± 0.36 | 2.49 ± 0.39 |
| $R_{\text{dark}}$ (µmol m$^{-2}$ s$^{-1}$) | 4.02 ± 0.51 | 4.47 ± 2.23 | 2.89 ± 0.59 | 3.62 ± 0.72 | 3.36 ± 0.45 | 3.51 ± 0.95 |
Figure 1: Meteorological conditions at the experimental site in 2012 as a function of day of the year (DOY): precipitation, solar radiation, air temperature, water vapour pressure deficit (VPD) and soil water content. The three grey areas represent the three sampling periods (J, A and N for June, August and November, respectively).

Figure 2: Annual shoot growth/length (A), number of needles (B) and needles projected area (C) of defoliated (white) and non-defoliated (black) Scots pine for the year 2010, 2011 and 2012. Each point represents the average value (n= 4) for a tree class. Error bars indicate ±1SE, * indicate significant differences between defoliated and non-defoliated trees (p≤0.05), while open triangle (∇) indicates marginal significances (p<0.1).

Figure 3: Diurnal changes in CO₂ assimilation (A and B), stomatal conductance (C and D) and transpiration (E and F) in June (A, C and E) and August (B, D and F) in defoliated (white) and non-defoliated (black) Scots pines. The variability of PAR and VPD values at times of measurements in June (G) and August (H) are also presented to allow interpret the variability in gas-exchange. Each point represents the average value for a tree class at one date (n=3). Error bars indicate ±1SE. Symbols after the panel letter indicate significant (*, p≤0.05) or marginally significant (∇, p≤0.1) difference between defoliated and non-defoliated trees in the panel. Differences are typically more significant if only the midday data are considered (i.e. first and last measurements excluded).

Figure 4: Seasonal changes in total non-structural carbohydrates (A and B) and its components: starch (C and D) and sucrose (E and F), in defoliated (white) and non-defoliated (black) Scots pine twigs (A, C, and E) and needles (B, D and F). Sample taken at predawn and midday are pooled together, and consequently each point represents the average value for a tree type at one date (n=14 to 16). Error bars indicate ±1SE. Open triangles (∇) indicate marginal significant differences (p≤0.1) between defoliated and non-defoliated trees.

Figure 5: Seasonal changes in water potential (A and B) and its main components: osmotic potential (C and D) and turgor potential (E and F), in defoliated (white) and non-defoliated (black) Scots pine at midday (A, C, and E) and predawn (B, D and F). Each point represents the average value for a tree type at one date (n=7 or 8). Error bars indicate ±1SE. * symbols after the panel letter indicate level of significance (p≤0.05) between defoliated and non-defoliated trees in the panel. * in the plots indicate significant differences (p≤0.05) between defoliated and non-defoliated trees at the considered date.

Figure 6: Xylem vulnerability curves of needles from defoliated (grey) and non-defoliated (black) Scots pines represented as the percentage of cumulative number of ultrasonic emissions (UEcum) as a function of needle water potential (ψ). Values of the needle water potential inducing 50% (ψ₅₀) and 88% (ψ₈₈) of cumulative ultrasonic acoustic emissions from needles.
Solar radiation
Soil Water Content
Air temperature
Precipitation

![Graphs showing the relationship between DOY and various environmental variables.](image)
A

Annual shoot growth (mm)

B

Number of needles per twig

C

Projected needle area per twig (cm²)

Years

2010 2011 2012

Defoliated

Non-defoliated

Accepted Article
June

- Panel A: Net CO2 assimilation rate (µmol CO2 m⁻² s⁻¹)
- Panel C: Stomatal conductance (mol H2O m⁻² s⁻¹)
- Panel E: Transpiration rate (mmol H2O m⁻² s⁻¹)

August

- Panel B: Stomatal conductance (mol H2O m⁻² s⁻¹)
- Panel D: Stomatal conductance (mol H2O m⁻² s⁻¹)
- Panel F: Stomatal conductance (mol H2O m⁻² s⁻¹)

Defoliated vs. Non-defoliated
A) $\Psi_{md}$

B) $\Psi_{pd}$

C) $\Psi_{md,o}$

D) $\Psi_{pd,o}$

E) $\Psi_{md,t}$

F) $\Psi_{pd,t}$

Water potential (MPa)

Osmotic potential (MPa)

Turgor potential (MPa)

Month

Defoliated

Non-defoliated
\( \Psi \) (MPa) vs. UE \text{cum} (%)

Defoliated

(\( \Psi_{50} = -1.56 \) MPa; \( \Psi_{88} = -2.12 \) MPa)

Non-defoliated

(\( \Psi_{50} = -1.54 \) MPa; \( \Psi_{88} = -2.44 \) MPa)