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Original Article

How do trees die? A test of the hydraulic failure and carbon starvation hypotheses

Sanna Sevanto[1], Nate G. McDowell[1], L. Turin Dickman[1], Robert Pangle[2] & William T. Pockman[2]

[1]*Earth and Environmental Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA and*
[2]*Department of Biology, University of New Mexico, 219 Yale Blvd., Albuquerque, NM 87131, USA*

ABSTRACT

Despite decades of research on plant drought tolerance, the physiological mechanisms by which trees succumb to drought are still under debate. We report results from an experiment designed to separate and test the current leading hypotheses of tree mortality. We show that piñon pine (*Pinus edulis*) trees can die of both hydraulic failure and carbon starvation, and that during drought, the loss of conductivity and carbohydrate reserves can also co-occur. Hydraulic constraints on plant carbohydrate use determined survival time: turgor loss in the phloem limited access to carbohydrate reserves, but hydraulic control of respiration prolonged survival. Our data also demonstrate that hydraulic failure may be associated with loss of adequate tissue carbohydrate content required for osmoregulation, which then promotes failure to maintain hydraulic integrity.

Key-words: cavitation; forest mortality; hydraulic conductance; non-structural carbohydrates; phloem; xylem.

INTRODUCTION

Climate-related, continental-scale forest mortality events have been observed with increasing frequency during the past 20 years (Allen *et al*. 2010). These events are associated with drought, and have been observed in tropical rainforests (Phillips *et al*. 2009), temperate mountainous and Mediterranean forests (van Mantgem *et al*. 2009; Carnicer *et al*. 2011) and boreal forests (Peng *et al*. 2011).

Current leading hypotheses of plant mortality mechanisms are based on our understanding of plant water relations. Hydraulic failure is expected to occur when water loss from transpiration is sufficiently greater than uptake by roots creating high xylem water tensions, and resulting in progressive cavitation and conductivity loss of the xylem (Sperry *et al*. 1998; McDowell *et al*. 2008). Conversely, carbon starvation is hypothesized to result from avoidance of hydraulic failure through stomatal closure, causing sustained negative carbon balance (McDowell *et al*. 2008). Recent theoretical advances also propose a coupling of carbon starvation and hydraulic failure, suggesting that water stress can cause failure of sugar transport in the phloem, limiting carbohydrate utilization and promoting mortality via either mechanism (McDowell & Sevanto 2010; Sala, Piper & Hoch 2010; McDowell 2011). The mechanism of mortality may impact plant survival rates during drought. Carbon starvation is expected to be a slow process, affecting plants that close their stomata at relatively low xylem water tensions (relatively isohydric plants), and leading to mortality during long drought periods that cause prolonged periods without net photosynthesis. Hydraulic failure, on the other hand, is expected to proceed more rapidly leading to fast mortality in plants that keep their stomata open during drought (relatively anisohydric plants) (McDowell *et al*. 2008). Understanding the mortality mechanism could thus be a key to predicting survival time of different tree species during droughts of different duration and severity. Mechanisms of vegetation mortality during drought and their impact on survival, however, are strongly debated due to a lack of direct tests (Adams *et al*. 2009; McDowell & Sevanto 2010; Sala *et al*. 2010; McDowell 2011; Anderegg *et al*. 2012). Consequently, we do not understand the fundamentals of mortality mechanisms, we lack any benchmarks for assessing mortality processes in natural systems, and we cannot make reliable predictions into the future. This knowledge gap is one of the major shortcomings of dynamic vegetation models (Fisher *et al*. 2010) and induces large uncertainties in climate predictions (Friedlingstein *et al*. 2006; Sitch *et al*. 2008; Fisher *et al*. 2010).

To address these shortcomings, we conducted an experiment specifically designed to promote hydraulic failure and carbon starvation. Our objectives were to (1) identify the physiological changes leading to mortality via hydraulic failure and carbon starvation; (2) determine the key parameters for predicting tree survival time in both mechanisms; and (3) test the importance of phloem transport failure in tree mortality. We hypothesized that (1) trees dying of hydraulic failure would exhibit the greatest loss of hydraulic conductivity and the lowest leaf water potentials, but relatively little loss of tissue carbohydrate content; (2) trees dying of carbon starvation would exhibit the lowest carbohydrate contents, but relatively high leaf water potentials and hydraulic conductivity; and (3) if phloem transport failure was critical to mortality, phloem turgor collapse should determine survival time, and would promote mortality via both hydraulic failure and carbon starvation. It could lead to hydraulic failure through compromised refilling capacity or osmoregulatory failure (McDowell 2011), or to carbon starvation

Correspondence: S. Sevanto. e-mail: sanna@lanl.gov

without considerable utilization of carbohydrate reserves (McDowell & Sevanto 2010; Sala *et al*. 2010).

MATERIALS AND METHODS

We used mature, 2–2.5 m tall piñon pine (*Pinus edulis* L) trees in a greenhouse setting. Piñon pine is a widespread conifer in the Southwestern USA that exhibits stomatal regulation typical of other *Pinus* species worldwide (Martinez-Vilalta, Sala & Piñol 2004) while achieving reproductive maturity at relatively small stature (2–6 m tall depending on location). In the Southwestern USA, this species has undergone large-scale drought-related mortality during the last 10 years (Breshears *et al*. 2009). After replanting in 86 litre pots, the trees were allowed to acclimate in the greenhouse for 3 months. To ensure the best possible soil-root contact, the original soil around the roots was preserved and the empty space in the pots was filled with potting soil. During acclimation the trees were watered daily with no additional fertilization. After acclimation, on 25 March 2010, we divided the trees into three treatments ($n = 4$): (1) severe drought (full light, zero irrigation) to promote hydraulic failure; (2) complete shade (zero light, daily irrigation) to promote carbon starvation; and (3) control (full light, daily irrigation). Shade was generated with cardboard structures built around the trees to prevent light penetration.

To ensure no differences in the environment across treatments, trees in each treatment were evenly distributed within the greenhouse space. Air temperature, relative humidity (CS215, Campbell Scientific, Logan, UT, USA), and photosynthetically active photon flux density (PPFD; LI-190 Quantum Sensor; Li-Cor Inc., Lincoln, NE, USA) were measured in the greenhouse in the vicinity of each tree and inside the cardboard structures. We used natural light with temperature control that allowed day-night variation from 15 to 35 °C in the greenhouse. The temperature inside the cardboard structures was ~1 °C lower than in the greenhouse during daytime and ~2 °C higher at night. Relative humidity was on average 7% higher inside the cardboard structures resulting in evaporative demand that was on average 13% lower in the daytime and 5% higher at night-time as compared to the greenhouse. These differences could not explain the variation in survival time of trees, their carbohydrate use or changes in leaf water potentials, sap flow, photosynthesis or respiration rates ($R^2 < 0.1$). Pretreatment tree size (height and stem diameter at ground level), leaf water potential, photosynthesis and sap flow rate were measured to ensure there were no statistically significant differences between the groups (Student's *t*-test). The only observed difference was slightly lower average sap flow rate in control trees than in the two other groups.

To assess physiological mortality mechanisms, we performed continuous sap flow (Heat Ratio Method; Burgess *et al*. 2001) and phloem turgor pressure gradient (Sevanto, Hölttä & Holbrook 2011; Mencuccini *et al*. 2013) measurements, weekly diurnal photosynthesis and nocturnal respiration (LI-6400; Li-Cor Inc.), soil water content (HydroSense; Campbell Scientific Inc.), and pre-dawn leaf water potential (Kaufmann & Thor 1982; pressure chamber Model 1005, PMS Instrument Company, Albany, OR, USA) measurements on each tree. We collected leaf and twig samples for non-structural carbohydrate (NSC) analysis (Hoch, Popp & Körner 2002) weekly, and measured native xylem hydraulic conductivity (Sperry, Donnelly & Tyree 1988) of branches monthly and at the death of each tree. Once shoot respiration ceased, death was confirmed by staining needle and twig samples with 0.1% neutral red (Peterson 1979) to ensure an absence of living cells.

Photosynthesis was measured under constant high light (PPFD 1500 μmol $m^{-2} s^{-1}$), ambient CO_2 concentration (435 ppm), and leaf temperature (25 °C). Before taking the measurements, we allowed the shoots to acclimate to possible changes of light level between the surroundings and the measurement chamber. Measurements were taken only when a steady state in gas exchange had been maintained at least for 2 min. For shaded trees, we also measured leaf gas exchange rate without light at daytime to determine their gas exchange rates under the conditions present inside the cardboard structures. Leaf area was determined with a LI-3100C (Li-Cor Inc.). Parameters were the same for nocturnal respiration measurements, but with no light.

For native xylem hydraulic conductivity measurements, a branch 3–5 mm in diameter was cut from the tree and immediately taken to an adjacent laboratory. There, the branch was cut under water into a 5-cm-long segment, omitting at least 1 cm from the previously cut ends. The section was attached to a tube filled with 100 mM KCl solution, and a gravitational pressure of 0.02 MPa was used to force the solution through the branch. Mass flow rate was measured with a balance. After measurements the samples were flushed with the aid of a vacuum pump to refill any embolized conduits and stained with 1% Safranin-O to determine the initial conductive sapwood area.

NSC analysis

Two twigs were collected weekly from each tree for NSC content analysis. Old needles (older than 1 year), new needles (last year's), xylem, phloem and bark tissues were separated using a razor blade and placed in small coin envelopes. Samples were stored at -70 °C until further processing. After the experiment, we chose NSC samples collected from each tree and the control trees before the treatments, at the time of permanent stomatal closure and at death for analysis. NSCs are defined here as free, low-molecular-weight sugars (glucose, fructose and sucrose) and starch. Upon thawing, the samples were microwaved to stop enzymatic activity, then oven-dried at 65 °C for 24–48 h and ground to fine powder. Approximately 12 mg of plant material was extracted with 1.6 mL distilled water for 60 min in a 100 °C water bath. An aliquot of the extract was incubated with amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA) at 48 °C overnight, to break down total NSC to glucose. The remaining extract was centrifuged and used for the determination of low molecular weight sugars after enzymatic conversion of fructose and sucrose to glucose. The concentration of free glucose was determined photometrically in a 96-well microplate spectrophotometer (Varian Cary 50 UVVis; Varian Medical Systems Inc., Palo Alto, CA, USA) after

enzymatic conversion of glucose to gluconate-6-phosphate. Starch was calculated as total NSC minus low-molecular-weight sugars. All NSC values are expressed as percent dry matter. Leaf and twig combined values are dry mass weighted averages of the NSC content of individual tissues.

Turgor gradient measurements

To detect possible phloem transport failure, we measured changes in turgor pressure at two heights on each tree and calculated the turgor gradient, the lack of which would be a sign of hindered sugar transport. Phloem turgor gradient was calculated from diurnal diameter variations measured simultaneously on xylem and bark tissues at two heights, 50–80 cm apart, using linear displacement transducers (LVDT; Solartron AX/5.0/S, Solartron Inc., West Sussex, UK) (Sevanto *et al*. 2011). Variation in phloem turgor at each height was calculated as the difference between the whole stem and xylem diameter variations and converted to pressures using Hooke's law (Hölttä, Mencuccini & Nikinmaa 2009) and the assumption of hydraulic equilibrium between the xylem and phloem (Sovonick-Dunford, Lee & Zimmermann 1981). Pre-dawn leaf water potential served as the reference xylem water tension for the upper measurement, and the pressure at the lower measurement accounted for the gravitational pressure difference. The turgor gradient was calculated as the difference in turgor pressure of the upper and lower measurements. Thus, zero turgor gradient here means that turgor pressure at the top of the tree has dropped below the gravitational pressure difference between the sensor locations, and an increase in turgor gradient from initial state is due to phloem at the top of the tree swelling more than at the base. Turgor collapse was defined to occur when the turgor gradient dropped below zero. Note that we measured the turgor pressure of the whole inner bark tissue (phloem conduits and surrounding cells included), and even if the operation of the cells surrounding phloem conduits is linked with sugar transport in the conduits (Turgeon & Wolf 2009), the link to sugar transport velocity is undefined. Thus, collapse of the turgor gradient only indicates loss of functionality of the tissue.

Statistical analysis and calculations

The statistical significance of treatment effects was tested using one-way analysis of variance (ANOVA). The adequacy of the NSC reserves to provide osmoregulation was estimated by calculating the amount of solutes in the bark and phloem tissues required to produce osmotic pressure equal to the pre-dawn leaf water potential using the van't Hoff equation. We assumed hydraulic equilibrium between the xylem and the living tissues surrounding it, and based the minimum carbohydrate requirement on osmotic potential equalling xylem water tension at turgor loss point. For generating the theoretical threshold, we used the initial measured contributions of soluble sugars in these tissues (sucrose 50%, glucose 25% and fructose 25%). To compare with observed bark and phloem NSC content, these values (in mol m^{-3} of water) were converted to sugar content per dry matter using the measured ratio of bark and phloem dry mass to fresh weight. Plants use also other solutes such as potassium (e.g. Talbott & Zeiger 1996) for osmoregulatory purposes. Those were not taken into account here because we did not measure them.

RESULTS

Both treatments caused photosynthesis and sap flow rates to decline rapidly (Fig. 1). The decline was immediate in the shade treatment due to light limitation, but limited photosynthesis persisted under high light for 4 weeks (Fig. 1). Shade trees also continued to transport water for four weeks after treatment initiation despite zero photosynthesis (Fig. 1; non-zero sap flow rate). In the drought treatment, photosynthesis ceased once pre-dawn leaf water potentials reached -1.7 ± 0.2 MPa, 2–7 weeks after treatment initiation. After this point, drought trees maintained leaf water potentials between −2 and −3 MPa, independent of soil water content, which dropped below 10% by the second week of treatments (data not shown). A week before mortality, leaf water potentials declined rapidly to below −6 MPa by death. In contrast, leaf water potentials of the shade trees stayed above −1 MPa until 8 weeks before mortality after which inter-branch variability increased with some branches drying while others retained moisture. Respiration rates declined slowly under both treatments and reached zero at death. All shade trees died in 16–20 weeks, while drought trees died in two distinct sets at 10–15 and 30–44 weeks (hereafter referred to as fast-dying and slow-dying, respectively). Even if survival time varied between the drought trees, all of them showed similar changes in leaf water potential and transpiration rates relative to their lifespan (Fig. 2). Transpiration ceased when 10–20% of survival time had passed, but leaf water potential started declining rapidly only after 80–90% of survival time. Long survival time was thus associated with the ability to maintain high leaf water potentials.

At the time of death, leaf water potentials were lowest in drought trees, while there was no difference between shade and control trees (Fig. 3a,b). Xylem hydraulic conductance of all drought trees was zero during their last week of life regardless of survival time, whereas shade trees maintained hydraulic conductivity above zero even at death (Fig. 3b). The NSC content of leaves and twigs did not decrease significantly in fast-dying drought trees, but decreased substantially in shade trees (Fig. 3c). Consistent with a strategy to reduce carbohydrate consumption, shade trees also shed all but the most vital 1-year-old needles (Freeland 1952) prior to mortality (Supporting Information Fig. S1). Fast-dying drought trees turned completely brown during the last week of life, browning systematically from locations associated with highest xylem water tension (Supporting Information Fig. S1). Intermediate between the fast-dying drought and shade trees, the NSC content of leaves and twigs of slow-dying drought trees dropped significantly (Fig. 3c), and these trees retained a green colour similar to shade trees.

Accessibility and consumption rate of carbohydrate stores were critical to survival regardless of stress from shade or drought. Within each treatment, trees that survived longest had the lowest NSC contents at death (Fig. 4a). Initial

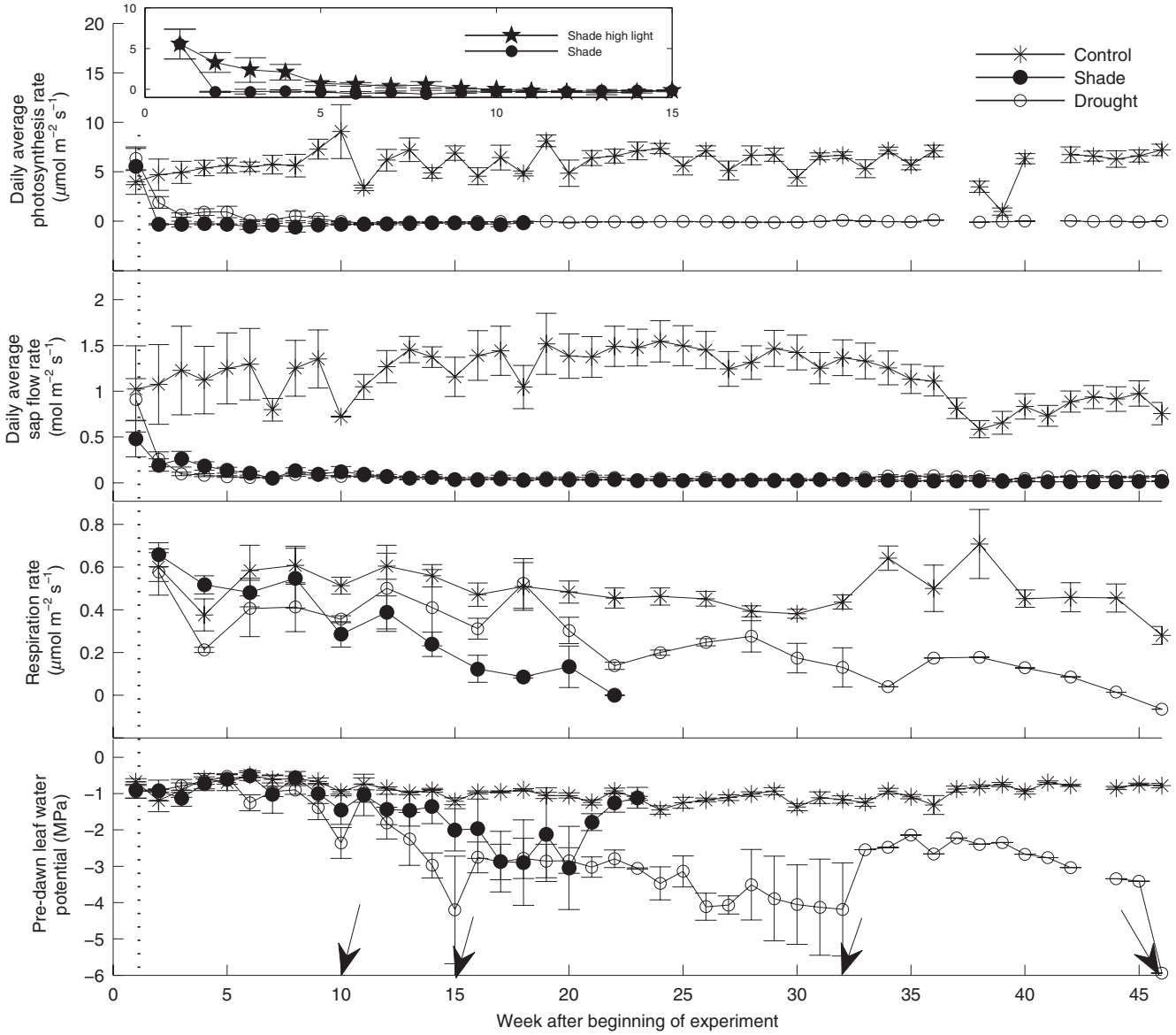

Figure 1. Treatment effects on photosynthesis, sap flow rate, respiration rate and pre-dawn leaf water potential (from top to bottom). The beginning of the experiment is marked by the vertical dotted line. Treatment averages are calculated for the surviving trees at each stage, and therefore the error bars (SE) increase when one tree deviates from the group and dies, and vanish when only one tree in each group remains alive. The arrows in the lowest panel indicate the time when each drought tree died.

glucose, fructose, sucrose and starch content of leaves and twigs correlated poorly with survival ($R^2 < 0.2$), but survival was correlated with initial needle starch content relative to total leaf and twig NSC content in the shade and slow-dying drought trees ($R^2 = 0.94$; Fig. 4b). This indicates that the proportion of needle starch of total NSC reserves was related to the total amount of carbohydrates the trees could utilize during drought. The initial reserves were irrelevant for fast-dying drought trees that were unable to utilize their NSC reserves.

Carbohydrate consumption rate was constrained by water availability. Well-watered shade trees continued depleting NSC reserves and respiring at high rates until NSC content had decreased to ~1 % of dry weight, after which respiration rate decreased rapidly and death ensued (Fig. 5). In contrast, respiration of slow-dying drought trees decreased linearly with decreasing carbohydrate content. Thus, despite more accessible carbohydrate reserves, rapid carbohydrate consumption by shade trees resulted in faster mortality as compared to slow-dying drought trees, but death ensued most rapidly in fast-dying drought trees that had no access to carbohydrate reserves (Fig. 4a).

Phloem turgor seemed to control access to carbohydrate reserves. Phloem turgor collapse preceded mortality for all trees in the drought treatment (Fig. 6a; Supporting Information Fig. S2), but shade trees that most fully utilized their NSC reserves maintained a small turgor gradient until death. Turgor collapse occurred on average 2 weeks prior to

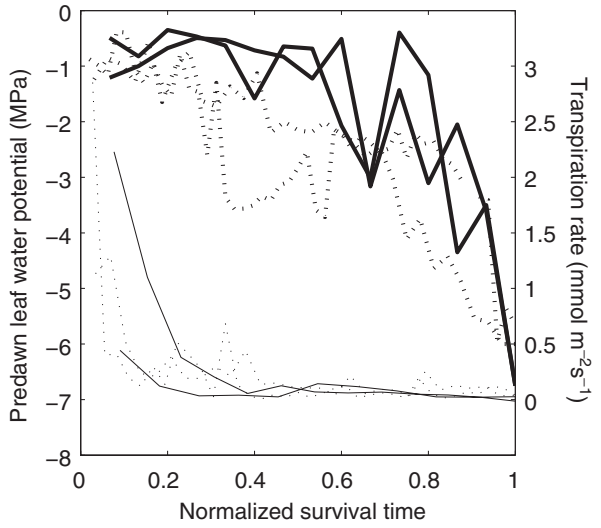

Figure 2. Leaf water potential and shoot transpiration rate of drought trees relative to their survival time. Survival time reaches 1 at mortality. Solid lines represent fast-dying trees, dotted lines slow-dying trees. Thick lines refer to leaf water potential, thin lines to transpiration.

permanent stomatal closure suggesting a possible feedback to stomatal control as suggested by Nikinmaa *et al*. (2013). Turgor collapse could have been related to an inability to osmoregulate living cells, as indicated by strong correlations between the turgor gradient and both NSC storage in the bark tissue, as well as sucrose transported in the phloem (Fig. 6b). Also, the observed bark and phloem soluble sugar content was adequate for osmoregulatory needs until permanent stomatal closure, which occurred shortly after phloem turgor collapse (Fig. 7). For drought trees, the timing of turgor collapse also correlated with carbohydrate utilization and survival suggesting that sugar transport was involved in the mortality process: a one-week delay in phloem turgor collapse lead to four weeks of additional survival time ($R^2 = 0.96$; data not shown). In contrast, shade trees were not limited by sugar transport capacity, and maintained a small turgor gradient until death because readily available water reduced the NSC demand for turgor maintenance. Interestingly, during the mortality process, leaf water potentials of drought trees did not change with soil water content, transpiration rate (see Fig. 2) or stomatal conductance ($R^2 < 0.1$) but correlated best with changes in phloem thickness (Fig. 8) suggesting a link between phloem turgor maintenance and the progress of hydraulic failure.

DISCUSSION

Drought is one of the main natural disturbances expected to lead to vegetation changes in the future (Allen *et al*. 2010; Choat *et al*. 2012). Different strategies for coping with drought (e.g. isohydric versus anisohydric) may make trees susceptible to dying through different physiological mechanisms, and the mechanisms might determine how fast trees die (McDowell *et al*. 2008). Recent evidence contrasting three species across the iso-anisohydry continuum strongly supported this hypothesis (Mitchell *et al*. 2013).

Our results show that all of the hypothesized mortality mechanisms can occur in trees of the same, relatively isohydric species, but both the progress of symptoms and survival time during drought may vary with individual trees even in similar environmental conditions. Our results are consistent with that the fast-dying drought trees succumbed to outright hydraulic failure (relatively rapid decline in leaf water potential, zero hydraulic conductivity and relatively untouched carbohydrate reserves at death), the shade trees died of carbon starvation (lowest carbohydrate reserves, no change in leaf water potential and only small change in hydraulic conductivity at death), and the slow-dying drought trees experienced both processes (slow decline in leaf water potential, zero

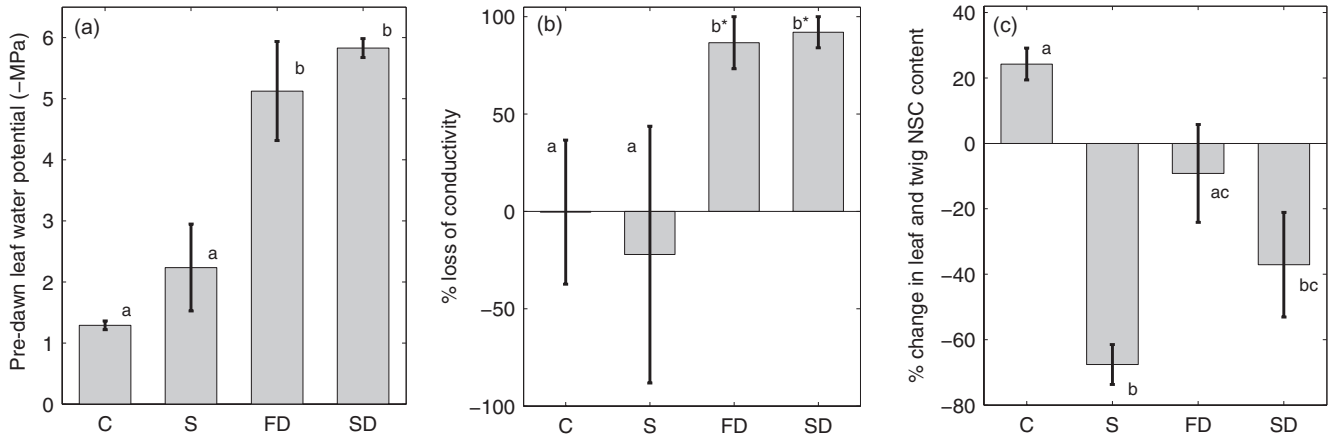

Figure 3. Leaf water potential at death (a); change in xylem hydraulic conductivity between pretreatment and death (b); and the change in leaf and twig total non-structural carbohydrate (NSC) content (soluble sugars and starch) between pretreatment and death (c). C, control; S, shade; FD, fast-dying drought; SD, slow-dying drought. Lower case letters indicate groups that are statistically significantly different from each other [analysis of variance (ANOVA) $P < 0.01$]. In panel (b), values statistically significantly different from zero at death are marked with an asterisk (Student's *t*-test $P < 0.001$).

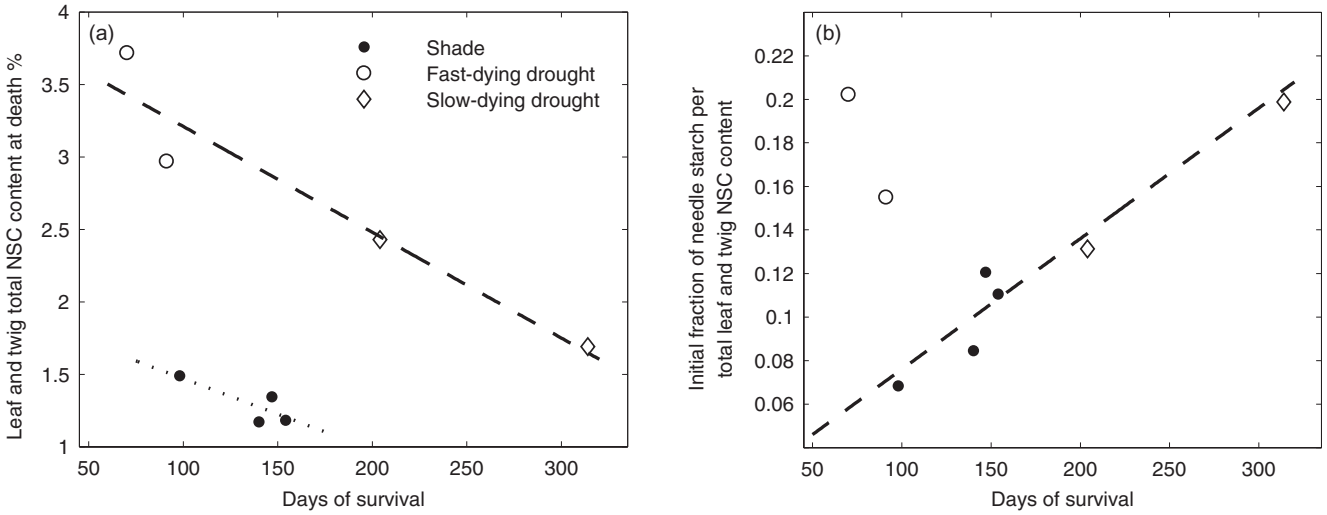

Figure 4. The relationship between tree survival time and leaf and twig total non-structural carbohydrate (NSC) content at death (a); and initial needle starch content relative to initial leaf and twig NSC content (b). The slopes for drought and shaded trees in (a) ($y = -0.0073x + 3.94$; $R^2 = 0.92$; and $y = -0.0049x + 1.96$; $R^2 = 0.67$, respectively) are not statistically significantly different. The offset is caused by the ability of the well-watered shaded trees to utilize a larger portion of their NSC reserves than drought trees. In (b), the initial NSC content of fast-dying drought trees does not show the relationship to survival time seen in shade and slow-dying drought trees ($y = 0.006x + 0.016$; $R^2 = 0.94$). The fraction for contol trees was 0.08 ± 0.02.These results support the carbon-water interdependency hypothesis; the more carbohydrates a tree can consume (a), and the larger the initial NSC reserve pool (starch) it has (b), the longer the tree lives. But water availability is critical for controlling carbon consumption (a).

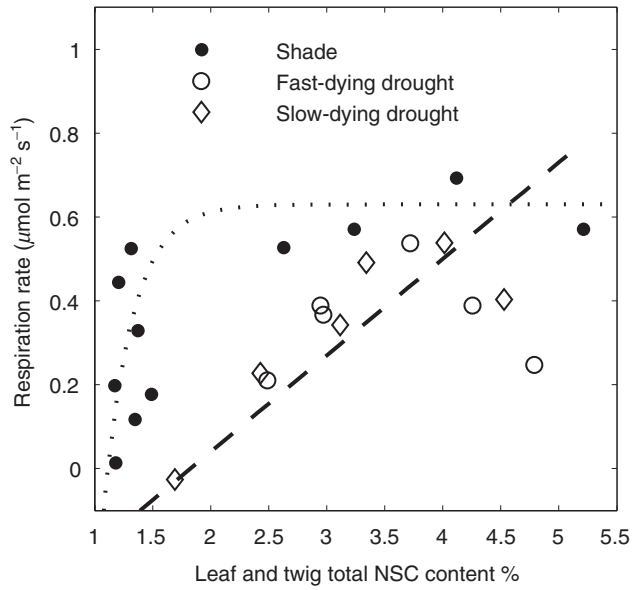

Figure 5. The relationship between respiration rate and non-structural carbohydrate (NSC) content of drought and shade trees. Respiration rate of shade trees remained high until NSC content had decreased to ~1% ($y = -54/\exp(4x) + 0.63$; $R^2 = 0.57$). In the slow-dying drought trees, respiration rate decreased linearly with decreasing NSC content ($y = 0.23x - 0.42$; $R^2 = 0.72$). The NSC content of fast-dying drought trees did not change during the experiment, and thus, the respiration rate for these trees decreased independent of NSC content.

hydraulic conductivity but also relatively depleted carbohydrate reserves at death), suggesting a coupling of hydraulic failure and carbon starvation (McDowell 2011; Fig. 3). While hydraulic failure may have been induced by xylem tension exceeding cavitation thresholds due to progressive water loss, for example, through bark and cuticular tissues, it seems that carbohydrates played a strong role in determining when the final, fast decline in water potential occurred, and how long the trees survived (Figs 2 & 4). Typical of pine trees (Martinez-Vilalta *et al.* 2004), our trees behaved relatively isohydrically (Tardieu & Simonneau 1998), and declines in leaf water potentials and hydraulic conductivity occurred only just prior to mortality, weeks after permanent stomatal closure and zero transpiration rates (Figs 1 & 2), and independent of changes in soil water content, suggesting hydraulic isolation (Plaut *et al.* 2012). Our data thus imply that hydraulic failure could be associated with loss of adequate tissue carbohydrate content required for osmoregulation (Figs 7 & 8). Carbohydrates share the task of osmoregulation with other solutes such as potassium, sodium, calcium and amino acids (Khanna-Chopra *et al.* 1994; Talbott & Zeiger 1996). We did not measure any of these substances, and therefore, conclusive proof of osmoregulatory failure and its connection with hydraulic failure requires further studies.

Declining carbohydrate reserves and failing phloem transport could also cause hydraulic failure through impaired refilling (McDowell & Sevanto 2010; Sala *et al.* 2010; McDowell 2011). Carbohydrates may play an important role in refilling of embolized conduits (Secchi & Zwieniecki 2011), and if they cannot be transported to sites where they are needed, refilling could fail. However, in addition to solutes, refilling always

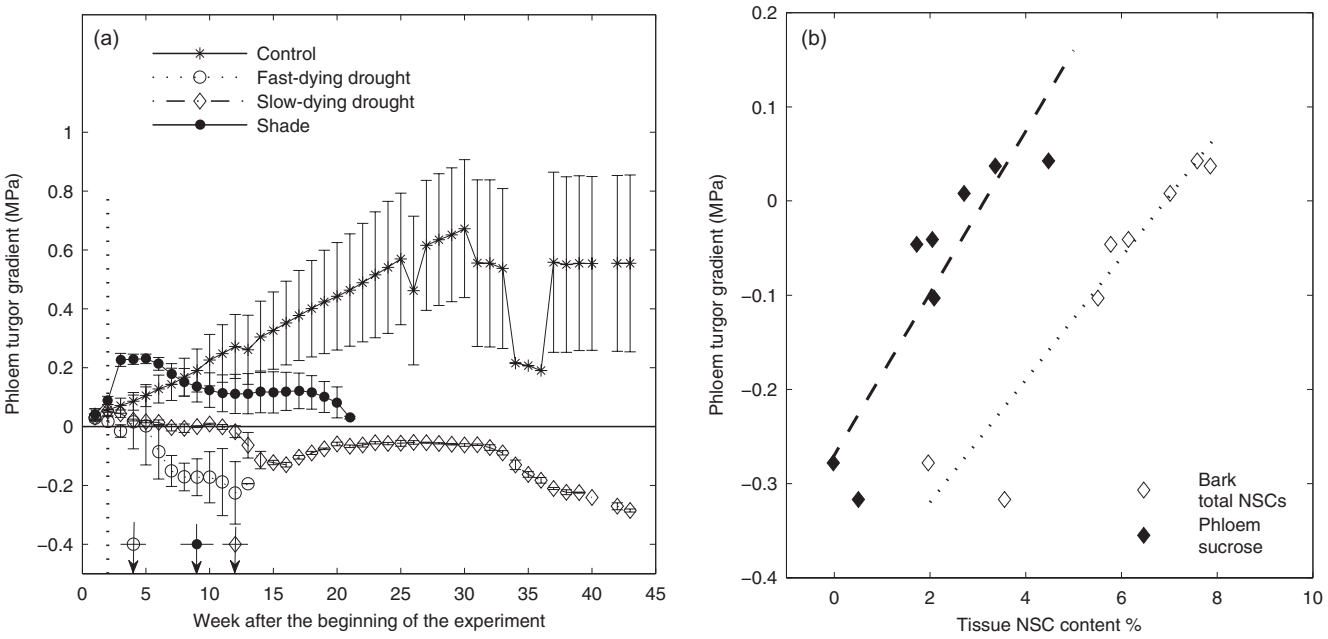

Figure 6. Changes in phloem turgor gradient during the experiment (average ± SE) (a) and the relationship of turgor gradient in drought trees with total non-structrual carbohydrate (NSC) content of bark ($y = 0.065x - 0.45$; $R^2 = 0.90$) and with sucrose content of phloem ($y = 0.086x - 0.27$; $R^2 = 0.82$) (b). Positive turgor gradient indicates higher turgor pressure at the top of the tree than at the base, and is necessary for maintainance of operational phloem. Turgor collapse occurs when the pressure at the top of the tree drops below the gravitational pressure difference between sensor locations (i.e. turgor gradient crosses zero line). Negative gradient is due to phloem turgor collapse at the top of the tree (Supporting Information Fig. S2), and does not indicate upward sugar flow. In (a), the vertical dotted line marks the beginning of the treatments and the arrows indicate the average (±SE) timing of permanent stomatal closure for each treatment. The decrease in the turgor gradient of the control trees in weeks 32–34 is due to a failure in the irrigation system resulting in some water stress. The steady increase is due to increasing NSC content and swelling of the phloem at the top of the tree.

requires water (Holbrook & Zwieniecki 1999; Secchi & Zwieniecki 2011). Hydraulic isolation limits xylem water reserves, and refilling one conduit could just lead to cavitation of an adjacent one. This could exhaust carbohydrate reserves, but would not lead to additional loss of conductivity unless water leaks outside the initial apoplast-symplast volume. Thus, mortality of living cells associated with carbon limitations could temporarily lead to additional water available for refilling elsewhere in the plant. However, if cell mortality promotes leakage outside the plant (through, e.g. leaves, bark or roots), it would lead to rapid hydraulic failure, which is consistent with what we saw in the last week of life of the drought trees.

In both treatments, survival time was strongly influenced by hydraulic limitations on carbohydrate transport and utilization. Critically, both access to carbohydrate reserves (Figs 3 & 4) and their utilization rate (Fig. 5) were controlled by water availability (compare drought and shade treatments) providing evidence that carbohydrate use is controlled by water potential (Boyer 1968) and the functionality of the transport system (Atkin & Macherel 2009) rather than by photosynthate production. In agreement with the phloem transport failure hypothesis, collapse of phloem turgor seems to promote mortality during drought by limiting access to carbohydrate reserves (McDowell & Sevanto 2010; Sala *et al*. 2010; McDowell 2011), and consistent with a hydraulic constraint on NSC consumption (McDowell & Sevanto 2010; Sala *et al*. 2010; McDowell 2011), respiration of slow-dying drought trees decreased with decreasing carbohydrate content (Fig. 5). For trees that could access their carbohydrate reserves (shade and slow-dying drought trees), the initial leaf starch content relative to total NSC content seemed also to be important for survival (Fig. 4b), which is consistent with the critical nature of starch supply during prolonged periods of low photosynthesis (Stitt & Zeeman 2012). However, turgor collapse occurred months before actual death. Therefore, analogous to stomatal closure, even if promoting mortality, phloem failure may not be the final step leading to death. It is unknown how large a volume loss affects phloem function. At the time of turgor collapse, our trees had lost ~20% of phloem volume (Supporting Information Fig. S2), which is 25% of the total loss shown at death. If phloem failure requires a higher volume loss, the estimated timing of phloem collapse relative to permanent stomatal closure is incorrect. Turgor collapse, however, was so rapid in every case that the correlation with survival time remains the same.

Determining when a tree dies is a challenging task (Anderegg, Berry & Field 2012). We used zero shoot respiration and needle colour change as indicators of mortality confirmed by staining of leaf and twig samples. Zero shoot respiration was chosen because of practicality, and because of the mechanistic foundation metabolism plays in life. For piñon pine, this is also a safe final point because this species does not resprout. From a wider perspective, zero shoot

respiration indicates that the trees did not contribute much to gas exchange with the atmosphere even if it is possible that some roots and bark tissue close to the ground level were still alive at this stage in the well-watered shade trees. In natural conditions, it is also possible that shoot respiration ceases to the detection limit of our devices, but the trees still might be able to recover if precipitation occurs. In our case, the xylem water potential of shade trees declined rapidly after death (after zero shoot respiration) indicating post-mortem hydraulic failure, despite that soil moisture content was still at field capacity. This emphasizes the importance of correct definition of mortality, and timing of measurements when determining the final steps of the mortality process. While the definition of mortality may affect our interpretation of the mortality mechanism, the clarity of the definition allows repeatability.

Our data does not answer directly why fast-dying drought trees lost phloem turgor and died so much faster than slow-dying drought trees. These two groups of trees did not differ initially from each other in any variable measured here. Our data point towards cellular-level controls of sugar and water dynamics that could have affected stress responses of osmoregulation and respiration rate (Atkin & Macherel 2009), but unfortunately, we were unable to identify any difference in source populations or location of origin that could hint to possible genetic differences.

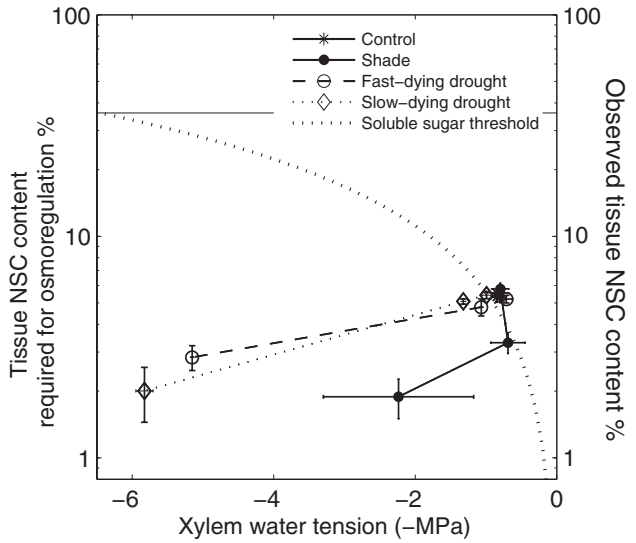

Figure 7. The concentration of soluble sugars (sucrose, glucose and fructose) required for osmoregulation at different xylem water tensions assuming hydraulic equilibrium between the xylem and the phloem (dotted line); and the observed soluble sugar content (±SE) at the beginning of the treatment, at zero stomatal conductance, and at death for each treatment group. Time can be read here from right to left, as xylem water tension increased monotonously with time. The soluble sugar content of all trees was at or above the osmoregulation threshold at pretreatment, but below the threshold at permanent stomatal closure and at death. The horizontal line marks the sucrose solubility threshold for our samples.

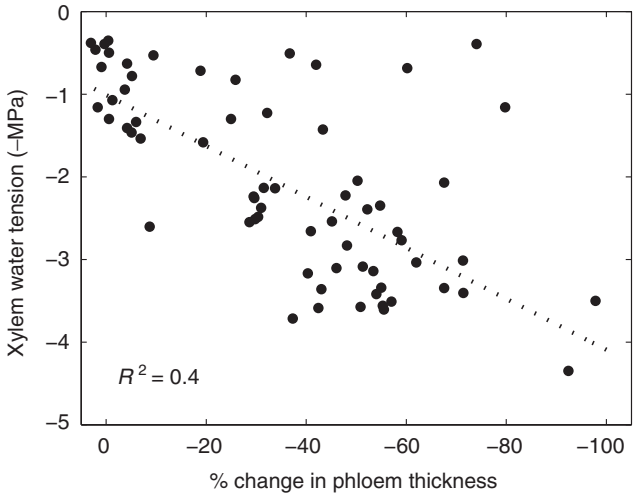

Figure 8. Decline in xylem water tension in drought treatment was best explained by shrinkage in the phloem at the top of the tree, which was related to declining carbohydrate content (Fig. 6).

Our results provide a means of identifying the dominant mortality mechanism. It is clear that trees can die of hydraulic failure and carbon starvation, and during drought, the dominance of these mechanisms determines survival time. In natural drought conditions, pure carbon starvation, as seen in the shade trees, might be rare because of the impacts of drought on utilization of carbohydrate reserves. Mortality can thus be expected at relatively high tissue carbohydrate contents as seen in slow-dying drought trees. Phloem function seems to play a key role in drought mortality, but because we did not address cellular-level controls of metabolism, the controls of the exact cascade of processes leading to mortality remain a critical question for further study. Our results emphasize the importance of hydraulic constraints on plant carbohydrate balance for predicting the timing of tree mortality.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Images showing progression of needle response, from initiation of response to death.
Figure S2. Change in phloem and xylem thickness at the base and top of the trees after treatment initiation.