Research paper

Mechanisms of piñon pine mortality after severe drought: a retrospective study of mature trees

Monica L. Gaylord[1,3,4], Thomas E. Kolb[1] and Nate G. McDowell[2]

[1]School of Forestry, Northern Arizona University, Flagstaff, AZ 86011, USA; [2]Earth and Environmental Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA; [3]Present address: Forest Health Protection, USDA Forest Service, Flagstaff, AZ 86001, USA; [4]Corresponding author (monicalgaylord@fs.fed.us)

Conifers have incurred high mortality during recent global-change-type drought(s) in the western USA. Mechanisms of drought-related tree mortality need to be resolved to support predictions of the impacts of future increases in aridity on vegetation. Hydraulic failure, carbon starvation and lethal biotic agents are three potentially interrelated mechanisms of tree mortality during drought. Our study compared a suite of measurements related to these mechanisms between 49 mature piñon pine (*Pinus edulis* Engelm.) trees that survived severe drought in 2002 (live trees) and 49 trees that died during the drought (dead trees) over three sites in Arizona and New Mexico. Results were consistent over all sites indicating common mortality mechanisms over a wide region rather than site-specific mechanisms. We found evidence for an interactive role of hydraulic failure, carbon starvation and biotic agents in tree death. For the decade prior to the mortality event, dead trees had twofold greater sapwood cavitation based on frequency of aspirated tracheid pits observed with scanning electron microscopy (SEM), smaller inter-tracheid pit diameter measured by SEM, greater diffusional constraints to photosynthesis based on higher wood $\delta^{13}C$, smaller xylem resin ducts, lower radial growth and more bark beetle (Coleoptera: Curculionidae) attacks than live trees. Results suggest that sapwood cavitation, low carbon assimilation and low resin defense predispose piñon pine trees to bark beetle attacks and mortality during severe drought. Our novel approach is an important step forward to yield new insights into how trees die via retrospective analysis.

Keywords: bark beetles, carbon starvation, hydraulic failure, *Pinus edulis*, resin ducts.

Introduction

Climate models predict more arid conditions for the future in many terrestrial ecosystems due to an increase in frequency and severity of drought driven by higher temperature and by changes in precipitation (Seager et al. 2007, IPCC 2013, Williams et al. 2013). Unusually large rates of tree mortality associated with recent drought have been reported for many global forests and are expected to continue in the future as temperature warms and precipitation is altered (Allen et al. 2010). Research about mechanisms of tree mortality during drought is needed to inform modeling and understanding of future vegetation (e.g., McDowell et al. 2008, 2013a, McDowell and Sevanto 2010, Sala et al. 2010).

The unusually warm global-change-type drought of 2000–03 in the southwestern USA (Breshears et al. 2005) is an example of more arid conditions expected in the future in many ecosystems. A time series of observations by Breshears et al. (2005) in northern New Mexico showed that this drought had 10 consecutive months of dry soil water conditions (15% volumetric water content, soil water potential less than −2.5 MPa) between October 1999 and August 2000, followed by an additional 15 consecutive months of dry soil water conditions between August 2001 and October 2002. Region-wide mortality of piñon pine (*Pinus edulis* Engelm.) during the 2000–03 drought was ~25%, with 90% mortality in some areas (Breshears et al. 2005, Shaw et al. 2005, Williams

et al. 2013). The land area of piñon pine mortality during this drought has been estimated to range between 100,000 and 400,000 ha (Meddens et al. 2012). The many studies of piñon pine mortality in the southwestern USA during this drought are consistent in reporting severe drought, greater mortality of large than small trees and the presence of bark beetles (Coleoptera: Curculionidae) in dying trees (Meddens et al. 2014).

Hypothesized mechanisms of tree mortality during severe drought include hydraulic failure, carbon starvation and lethal biotic attacks (McDowell et al. 2008). Hydraulic failure occurs when strong tension on sapwood water columns sucks air into xylem conduits and the resulting air embolism is not contained and hence spreads through sapwood (Zimmermann 1983, Sperry and Tyree 1990). This containment occurs in most gymnosperms by the aspiration of the valve-like torus-margo (TM) structure to seal pits that connect tracheids, but containment can fail leading to disruption of sapwood water transport (Sperry and Tyree 1990, Hacke and Sperry 2001, Hacke et al. 2004). Catastrophic hydraulic failure during drought may be uncommon for isohydric species such as piñon pine that prevent occurrence of tensions that cause sapwood cavitation by tight stomatal regulation of transpiration (Plaut et al. 2012, Koepke and Kolb 2013, but see McDowell et al. 2013b). This stomatal regulation of isohydric species reduces photosynthesis to near zero during severe drought (Koepke and Kolb 2013, Mitchell et al. 2013), which leads to the second potential mortality mechanism, carbon starvation, in which carbon reserves are depleted by tissue respiration in the absence of assimilation (Adams et al. 2013) and perhaps by hydraulic constraints on phloem transport to carbon sinks (Sala et al. 2010). Chronic carbon starvation and hydraulic failure are thought to be related to the third mechanism, lethal biotic agents, via metabolic costs of synthesizing carbon-rich defenses and the ability to exude them from attack wounds, such as monoterpene-based resins that help protect conifers against bark beetle attacks (Paine et al. 1997, Franceschi et al. 2005). Successful bark beetle attacks often occur on conifers that have small, sparse resin ducts where monoterpenes are synthesized (Kane and Kolb 2010, Gaylord et al. 2013, Ferrenberg et al. 2014). Lethal bark beetle attacks also can occur during drought due to reasons other than tree carbon starvation (McDowell et al. 2011). For example, volatile and auditory emissions from trees during drought may attract beetles (Mattson and Haack 1987, Seybold et al. 2006, Manter and Kelsey 2008, Kelsey et al. 2014), and warm temperatures

that often occur during drought accelerate beetle population growth (Bentz et al. 2010, Creeden et al. 2014).

We evaluated the three previously described hypotheses about mechanisms of piñon pine mortality during drought (hydraulic failure, carbon starvation, biotic agents) with unique retrospective measurements on mature trees that died during the 2002 drought and trees that lived at three sites in Arizona and New Mexico. Specifically, we obtained information about xylem cavitation from measurements of tracheid pits, about carbon starvation from measurements of wood carbon isotope discrimination and radial growth, and about biotic agents from measurements of xylem resin duct characteristics and bark beetle attacks. We expected to find no evidence of xylem cavitation in piñon pines that died because isohydric trees such as piñon pine often maintain xylem water potential above a threshold that causes severe cavitation (Linton et al. 1998, Koepke and Kolb 2013; but see McDowell et al. 2013b). We also expected trees that died to show symptoms of carbon starvation, including lower growth and a higher (less negative) ratio of wood C^{13}/C^{12} ($\delta^{13}C$) than trees that lived. Finally, we expected trees that died to have signs of lower bark beetle defenses (fewer and smaller resin ducts) and more bark beetle attacks than trees that lived.

Materials and methods

Study sites

We sampled trees at two sites in Arizona and one site in New Mexico (Table 1): Cherry Canyon (35°13′N, 111°48′E), located 20 km east of Flagstaff, Arizona; Red Mountain (35°51′N, 111°83′E), located 40 km north of Flagstaff, Arizona; and Deer Trap Mesa (35°90′N, 106°27′E), located on the outskirts of Los Alamos, New Mexico. All three locations have a mixture of piñon pine and juniper [*Juniperus monosperma* (Engelm.) Sarg.] and experienced substantial piñon mortality during the 2000–03 drought event (USDA aerial surveys 2003, Breshears et al. 2005, Koepke et al. 2010).

Sample design

We collected field samples from a total of 98 trees (49 live and 49 dead) during the summer of 2009 (Table 1). At each site we selected living and dead mature piñon pines along a belt transect. At the Arizona study sites, the start of each transect was selected based on GPS coordinates originally established by Adams and

Table 1. Description of study sites located in Arizona and New Mexico, USA. Tree diameter was measured ≈15 cm above ground level.

Name, state	Elevation (m)	Soil parent material	Number of dead/live trees	Tree diameter (cm) dead/live mean ± s.e.m.
Cherry Canyon, AZ	2032	Limestone/sandstone	14/14	21.6 ± 1.5/21.3 ± 1.5
Deer Trap Mesa, NM	2195	Hackroy clay/loam	20/20	21.3 ± 1.0/21.3 ± 1.3
Red Mountain, AZ	2092	Basalt/cinders	15/15	18.8 ± 1.5/19.0 ± 1.8

Kolb (2005) in a study of tree growth response to drought. From this start location we established transects in a cardinal direction, roughly perpendicular to the closest main road. We began by selecting trees 50 m beyond the start locations and selected dead trees first. The initial criterion for selection was a high probability that the tree died during the 2000–03 drought event. We based our selection criteria in the field on a visual assessment of decay and the presence/absence of needles and fine branches; this estimated year of death was later verified using dendrochronology (described below). Additional criteria for selection were that dead trees needed to be located within 50 m of our transect line and have a diameter at root collar (DRC) between 10 and 35 cm. The New Mexico study site was located along a narrow mesa, thus we started our transect at the first dead tree we encountered and continued the transect roughly along the center of the mesa using the previously described criteria for dead tree selection. We paired each selected dead tree with the closest live tree (within 100 m) that had a similar DRC (within 1.3 cm), form and canopy position (open grown, dominant or co-dominant). For all trees, we recorded diameter (15 cm above ground level) and sampled sapwood from the main bole. For the dead trees we sampled sapwood by felling the trees and removing two bolts (≈0.3 m in length), one from the bottom of the tree (centered ≈0.5 m above ground level) and a second from approximately mid-bole of the largest stem. For the live trees, we collected one or two large (13 mm) and one or two small (5 mm) diameter sapwood cores from the same lower bole location as described for the dead trees. One of the small diameter cores was immediately placed into a vial with 30% ETOH, and the other cores were stored in straws or plastic bags until arrival at the laboratory, after which they were air dried for 1 week prior to gluing on to wood blocks.

Xylem resin ducts and radial growth

Xylem resin duct characteristics have been associated with pine survival during drought and bark beetle outbreaks (Kane and Kolb 2010, Gaylord et al. 2013, Ferrenberg et al. 2014). We measured resin ducts using methods modified from Kane and Kolb (2010) and used successfully by others on piñon pine (Kläy 2011, Gaylord et al. 2013). We sanded cookies (dead trees) sampled from the lower bole or cores (live trees) using progressively higher grit sand paper (120–400). We scanned each sample to create a high-resolution image (1200 dpi) of a 2 mm path. This path was then analyzed with WinDendro software (Regent Instruments Inc 2009) to assign annual ring boundaries and measure ring widths. We assigned calendar years to growth rings via identification of narrow and wide marker years from a previously constructed master chronology for the sites. Visual cross-dating was confirmed using the statistical software COFECHA (Holmes 1983, Grissino-Mayer 2001).

For the resin duct analysis, we imported the images into the public domain image-processing program ImageJ (Rasband

1997–2011), and then measured resin duct-area and the number of resin ducts for each year from 1993 to 2002, along the scanned path. Resin duct characteristics, which are described in detail by Kane and Kolb (2010) and Kläy (2011), were then computed and included production (number of ducts), average duct-area (mm^2), resin duct density [number of ducts/area of ring (mm^2)] and percent area (mm^2 of resin ducts/mm^2 of xylem).

We measured annual radial growth for 10 annual rings (1993–2002) and converted raw ring width to annual basal area increment (BAI). Ring widths were converted based on the diameter of the previous year with the diameter taken at the time of sampling as our initial diameter. We then subtracted the growth increments to calculate BAI for each year. We assumed concentric circularity and no change in bark thickness over the 10-year period (Avery and Burkhart 1983). Finally, to further standardize for tree size, we standardized BAI for each tree by dividing the BAI for each year by the tree's basal area at the start of the respective year.

Missing growth rings are a common occurrence in pines in the southwestern USA during drought (Dieterich and Swetnam 1984, Grissino-Mayer et al. 1991, Gaylord et al. 2007). Based on the common occurrence of missing rings, evidence of missing rings in 2002 from our sample of the live trees, and other published reports that document widespread piñon mortality in 2002 (USDA 2003, Shaw et al. 2005), we assumed that trees with the last growth ring cross-dated to 2001 actually died in 2002. Because we were interested in yearly growth and resin duct characteristics, we assigned values of zero for BAI and resin duct characteristics to all missing rings in both live and dead trees. Missing rings occurred most frequently in two notable severe drought years, 1996 and 2002, for both live (missing rings in 38 and 73% of trees for 1996 and 2002, respectively) and dead trees (missing rings in 40% of trees for both 1996 and 2002).

Sapwood $\delta^{13}C$

Because C3 plants discriminate less against C^{13} than C^{12} during partial stomatal closure caused by drought (Farquhar et al. 1989), the $\delta^{13}C$ of wood provides retrospective information about stomatal conductance, an important regulator of carbon assimilation (Dawson and Siegwolf 2007). We measured $\delta^{13}C$ in whole wood of annual rings for both dead and live trees. After cross-dating and measuring the width of each ring as described above, we removed wood in 5-year increments (1993–97 and 1998–2002) from samples. We used 5-year increments to ensure accuracy and adequate sample amount due to the small ring widths. For the dead trees we used a dremel tool (Robert Bosch Tool Corporation, Mount Prospect, IL, USA) with a small rotary cutter to remove fine particles of wood over each 5-year increment. For the live trees, due to difficulty in using the dremel tool on mounted cores and concern about contamination from glue, we sliced the designated years from the mounted cores

using a razor blade. Core samples were then ground to 40 mesh to ensure homogeneity of the samples. A paired t-test found no difference in $\delta^{13}C$ for samples obtained with the dremel tool and samples excised with a razor blade ($P = 0.44$, $n = 10$ pairs). We did not separate early wood from late wood (Livingston and Spittlehouse 1996, Leavitt and Wright 2002) because our hypothesis concerns differences in $\delta^{13}C$ over multiple years, not within years. We used whole wood rather than extracted cellulose because $\delta^{13}C$ of whole wood is highly corrected with $\delta^{13}C$ of cellulose, and wood decay in dead trees has little effect on $\delta^{13}C$ of whole wood (English et al. 2011). We analyzed $\delta^{13}C$ in a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA) coupled with a Thermo Electron Delta Plus Advantage mass spectrometer (Bremen, Germany) operated in continuous flow at the Colorado Plateau Stable Isotope Laboratory, Northern Arizona University, Flagstaff, AZ, USA. Isotope ratios are expressed relative to Vienna Standard Peedee Belemnite. A total of 218 wood samples were analyzed for $\delta^{13}C$ and overall precision was 0.05‰ ($n = 51$).

Scanning electron microscopy analysis

In gymnosperms, water is transported through the xylem by a network of tracheids connected via pits that allow for water movement between the tracheids. Located in each inter-tracheid pit is a unique structure comprised of a central, thickened torus surrounded by a porous margo. When water columns in xylem are exposed to extreme tension during drought, cavitation may occur forming an air embolism in the tracheid (Zimmermann 1983, Sperry and Tyree 1990). Due to the pressure difference on opposite sides of the pit, the torus is aspirated to the side and seals the pit opening (Sperry and Tyree 1990). This response of the torus limits xylem embolism (Hacke and Sperry 2001, Hacke et al. 2004); however, as the pressure increases, the margo may stretch, or even tear, which can lead to displacement of the torus from the pit opening. This allows the embolism to move into the adjacent tracheid (Choat et al. 2008). Continued strong negative pressures on the xylem water column combined with numerous sites of xylem cavitation during severe drought may thus override the regulation capacity of the TM structure, leading to hydraulic failure of the xylem and xylem desiccation (Sperry and Tyree 1988).

We used scanning electron microscopy (SEM) to view the TM structure in order to estimate the occurrence of past xylem cavitation (Sperry and Tyree 1990). We used this technique to compare the amount of xylem cavitation between live and dead trees to better understand the role of hydraulic failure in tree mortality. Because we were concerned that desiccation after tree death might cause the TM to aspirate regardless of the cause of mortality, we conducted a preliminary experiment at the Red Mountain site in Arizona to examine the impact of post-mortality tree desiccation on the position of the TM. On 21 June 2009, we removed two 5-mm diameter increment cores from

the main bole at breast height (1.4 m) of six piñon pine trees. After extraction, the cores were immediately placed in vials containing a solution of 30% ETOH. Midday water potential, measured 1 day prior to core extraction, was -2.02 ± 0.7 MPa, a moderate value for piñon pine that indicates that the sampled trees were not experiencing cavitation from water stress at that time (Linton et al. 1998, Koepke and Kolb 2013). We then baited the trees with pheromone lures to incite piñon ips [*Ips confusus* (LeConte)] attacks (ConTech Enterprises, Victoria, BC, Canada). Five trees were successfully mass attacked. After tree death (>90% needle fade), we waited an additional 2 months and on 24 September 2009, removed an additional two increment cores from the trees following the same procedures described above. We then analyzed these cores to see if there was a higher incidence of TM aspiration in the trees post-mortem than in live trees, using the methods described below for SEM imaging. We determined that a TM had aspirated if the TM structure was visibly pulled through the pit aperture or, if the torus was pressed against the pit opening and the margo fibers were depressed against the primary pit wall (Thomas and Nicholas 1966, Sperry and Tyree 1990) (Figure 1a and b). We found no significant difference in placement of the TM structure (aspirated or unaspirated) ($\chi^2 = 0.93$) between live and dead trees, indicating that the TM did not aspirate 2 months after trees died from a lethal bark beetle attack.

In the main SEM experiment, for the live trees we used the core samples (5 mm) collected and stored in a 30% ETOH solution as previously described. For the dead trees, we extracted cores (5 mm) from the collected bolts (bottom section only) within 3 months post-collection, and placed them into a vial with a 30% ETOH solution. Cores were prepared for SEM analysis by alcohol dehydration (Bozzola and Russell 1999), which consisted of transferring the cores from the original 30% ETOH solution to sequentially increasing ETOH solutions (50, 70, 90 and 100%), allowing the cores to soak for 48 h in each solution. After removal from the final solution, the cores were allowed to air dry. This solvent exchange–dehydration sequence was performed to replace any remaining water in the cells and minimize distortion or damage to the tissue (Exley et al. 1974). For the dead cores we removed the outer 1 mm section of each core with a hand held razor. Cores were sliced along a sagittal plane which allowed us to view the inside of the tracheids and to determine annual tree ring delineation based on tracheid widths (Figure 1c). The core samples were then mounted on aluminum stubs using adhesive tape and the sample was gold sputter coated. We measured the diameters of 10 bordered pits (width at the external wall) in the earlywood of two growth rings (five pits per ring) and determined the placement of the TM membrane as either unaspirated (Figure 1a) or aspirated (Figure 1b). For the dead trees, the two rings were the last 2 years of growth prior to death. For the live trees, the analyzed pits were based on our initial tree pairing. Analyzed pits in the live trees were those
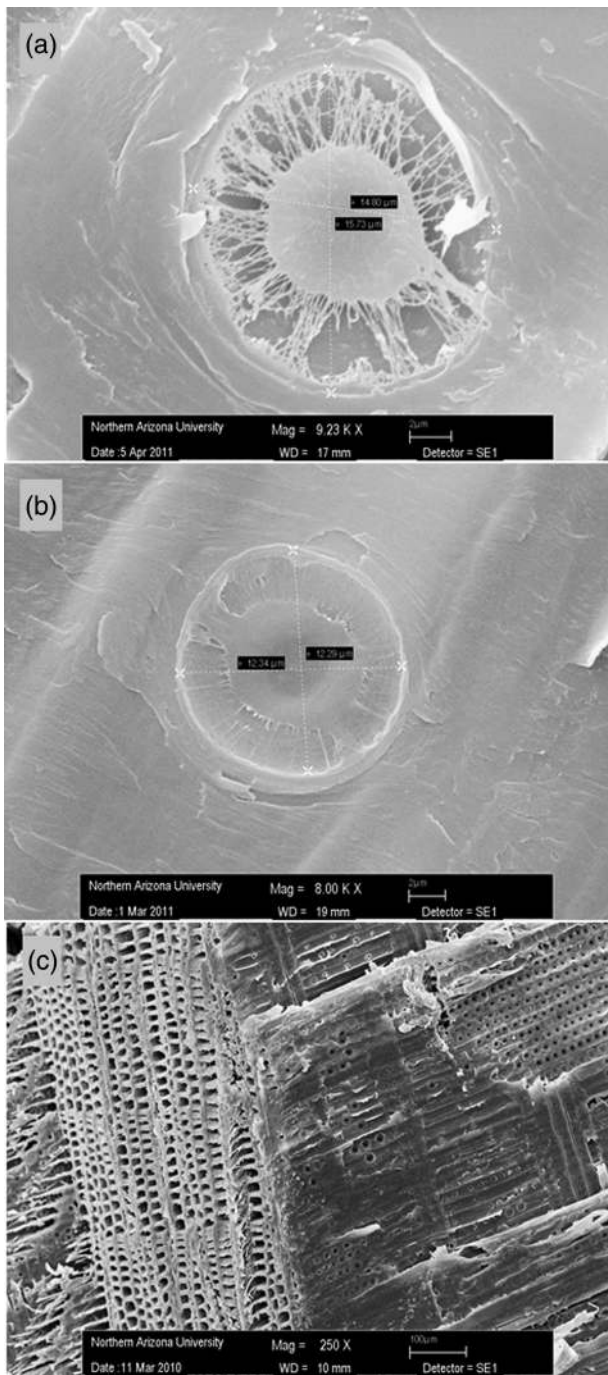
Figure 1. Scanning electron microscopy images used for analysis of TM status. For photos (a) and (b), one side of the pit has been removed to expose the membrane. (a) Unaspirated TM with the central thickened torus and porus outer region (margo) elevated above the pit aperature, (b) aspirated TM with the thickened torus and margo fibers pressed against the wall of the pit chamber and the torus covering the pit aperture, and (c) view of cutting technique used to view growth rings. Torn margo fibers in photo (a) are likely an artifact of SEM preparation techniques.

located in the outer two rings during the year of the paired tree's death. Briefly, after the paired tree death date was determined as previously described, we compared the cores from the live trees

prepared for SEM analysis with the cross-dated cores to determine years of ring growth for our SEM samples. Cores were dissected to allow analysis of the pits in the two growth rings of interest. Because 2002 was often missing, particularly for live trees, the outermost rings were often 2001 and 2000.

Bark beetle attack

Evidence of bark beetle attacks can be found by visually surveying the exterior bark for pitch tubes, entrance holes, exit holes, boring dust or frass. Furthermore, piñon ips constructs egg laying galleries which leave distinct etchings on the xylem face, visible many years after attack. For all trees, we surveyed two 0.3 m sections on the main bole for evidence of bark beetle attacks (pitch tubes, entrance holes, frass or galleries). We conducted the lower surveys at ≈0.3–0.6 m above ground and the upper survey at approximately mid-bole on the main stem (similar heights as the sapwood collection described above). For the live trees, these surveys were conducted in the field during initial tree selection. Because no evidence of bark beetle attacks was found on any of the live trees, no further bark beetle measurements were conducted on live trees. For the dead trees, we surveyed collected bolts in the laboratory within 2 months post-collection. We quantified bark beetle attack densities on the bolts from the dead trees by removing the outer bark of the tree, counting the number of nuptial chambers and measuring the gallery length. We then calculated the nuptial chamber density (number of nuptial chambers/unit area of wood) and gallery density (total gallery length/total area of wood).

Statistical analysis

For most analyses we used analysis of variance (ANOVA) with tree status (live, dead), site and their interaction as factors. Because the 2000–03 drought and subsequent tree mortality were a region-wide event, we tested whether mortality mechanisms were consistent across sites based on the tree status by site interaction. For three resin duct parameters (number, area and percent area) we analyzed the average of the 5-year period (1998–2002) and the 10-year period (1993–2002) that ended in the drought year of 2002 for each parameter using site, tree status and their interaction as independent variables. We performed the same analysis for standardized BAI (5- and 10-year averages), $\delta^{13}C$ (5- and 10-year averages) and average inter-tracheid pit size (2000 and 2001 or 2001 and 2002). Data were transformed to meet assumptions of normality when needed (Table 2). For duct density, in order to meet assumptions of normality, we compared live and dead trees for each site individually by ANOVA. For TM status, we calculated the percentage of aspirated pits for each tree and then, because the selection of TMs examined in the live trees was based on the initial live-dead tree pairings, used a paired t-test (based on pairs selected in the field) on data pooled over sites to compare live and dead trees. Bark beetle galleries were only present in dead

Table 2. Results of ANOVA (*P* values) and type of data transformation used for resin duct parameters and standardized BAI (BAI/basal area) for live and dead piñon pine trees sampled in Arizona and New Mexico. Duct parameters included: number of ducts, area of ducts, percent area (percent area of ducts/area of xylem growth) and density (number of ducts/area of xylem growth). Variables were averaged for 5- and 10-year periods ending in the year of widespread piñon mortality in the southwestern USA (2002). For all variables other than duct density, we used site, tree status (dead or live) and their interaction as independent variables. Effect of tree status on duct density was analyzed at each site individually in order to meet the normality assumption. *Significant at $\alpha = 0.05$.

Resin duct variable	Site × tree status	Tree status	Site	Data transformation used
Number 1998–2002	0.97	0.93	0.29	None
Number 1993–2002	0.77	0.45	0.94	None
Area 1998–2002	0.09	0.0037*	0.0126*	None
Area 1993–2002	0.3	0.0255*	0.0056*	Square root
% area 1998–2002	0.81	0.03*	<0.0001*	Rank
% area 1993–2002	0.89	0.0124*	<0.0001*	Rank
Density 1998–2002				
Cherry		0.92		Ranked within each site
Deer Trap Mesa		0.66		
Red Mtn		0.93		
Density 1993–2002				
Cherry		0.14		Ranked within each site
Deer Trap Mesa		0.74		
Red Mtn		0.61		
Standardized BAI 1998–2002	0.53	0.0382*	0.53	Log
Standardized BAI 1993–2002	0.44	0.21	0.15	Log

trees, thus, for the dead trees we analyzed the effect of site on bark beetle attack densities using ANOVA.

Results

For 5- and 10-year average duct-area, resin ducts were smaller in dead trees (0.013 ± 0.001 and 0.017 ± 0.001 mm^2, for 5 and 10 years, respectively) than live trees (0.018 ± 0.001 and 0.020 ± 0.001 mm^2, for 5 and 10 years, respectively) (Table 2; Figure 2b). Non-significant tree status by site interactions for duct characteristics shows that differences between live and dead trees were consistent over sites (Table 2). For percent duct-area, dead trees (0.037 ± 0.005 and 0.041 ± 0.005 for 5 and 10 years, respectively) had lower area than live trees (0.045 ± 0.005 and 0.044 ± 0.003 for 5 and 10 years, respectively) (Table 2, Figure 2c). No other resin duct parameters differed significantly between live and dead trees (Table 2, Figure 2). Average standardized BAI for 5 years varied significantly by tree status, with dead trees having lower growth (0.0074 ± 0.0007) than live trees (0.0092 ± 0.0008), but 10-year standardized BAI did not vary by tree status (Table 2, Figure 2e).

Dead trees had more positive wood δ^{13}C than live trees for both 5- and 10-year periods (Figure 3a). The difference between dead and live trees was significant for the 10-year average ($P = 0.0478$, DF = 1, F ratio = 4.03; live -22.69 ± 0.10‰; dead -22.39 ± 0.11‰) but not for the 5-year average ($P = 0.07$, DF = 1, F ratio = 3.34). Pit diameter was significantly smaller in dead (12.8 ± 0.2 µm) than live trees (13.2 ± 0.2 µm; $P = 0.0477$, DF = 1, F ratio = 4.038; Figure 3b). The interaction between site and tree status was not significant for pit diameter

($P = 0.26$, DF = 2, F ratio = 1.37) or in either period for wood δ^{13}C (5 year: $P = 0.78$, DF = 2, F ratio = 0.25; 10 year: $P = 0.98$, DF = 2, F ratio = 0.02), indicating that differences between live and dead trees were consistent over sites.

There was a significant effect of tree status on the percent of aspirated pits per tree ($P < 0.0001$, DF = 47, t ratio = 9.61). The percentage of aspirated pits was almost twofold greater for dead trees ($93.5 \pm 2.5\%$) than live trees ($47.2 \pm 3.9\%$; Figure 3c).

Site was included in our analyses to evaluate whether differences between live and dead trees were consistent over sites by testing the tree status by site interaction. This interaction was never significant, but several parameters differed significantly over sites (Table 2, Table S1 available as Supplementary Data at *Tree Physiology* Online).

As reported earlier, there was no evidence of beetle attacks (successful or unsuccessful entrance holes) on any of the live trees we surveyed. In contrast, 96% of the dead trees had evidence of bark beetle attacks. There were significant differences among sites for bark beetle attack densities (see Table S2, Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Discussion

Our results advance understanding of the mechanisms that cause widespread tree mortality during severe drought, which is predicted to increase in the future as climate warms (Seager et al. 2007, Williams et al. 2013). The retrospective approach we applied to mature piñon pine trees exposed to recent severe
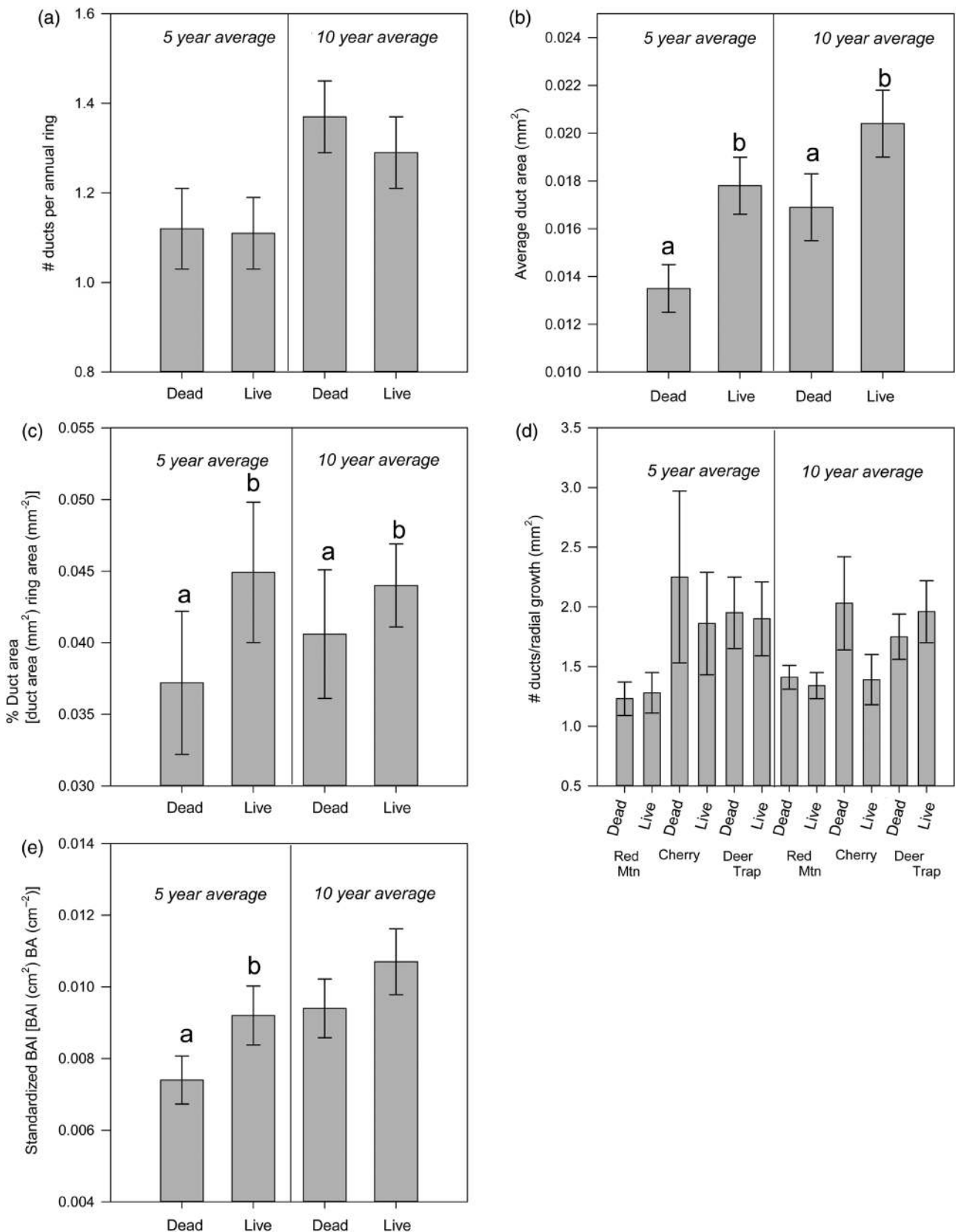
Figure 2. Bar graphs (mean ± 1 s.e.m.) for resin duct parameters and standardized BAI for live and dead piñon trees (*Pinus edulis*) in Arizona and New Mexico. Parameters shown are (a) average number of ducts, (b) average area of ducts, (c) average % duct area, (d) average duct density and (e) average standardized BAI, for 5 years (1998–2002) and 10 years (1993–2002) prior to the year of high tree mortality (2002) in the southwestern USA. Bars labeled with different letters are significantly different within each date range as determined by ANOVA at $\alpha = 0.05$.
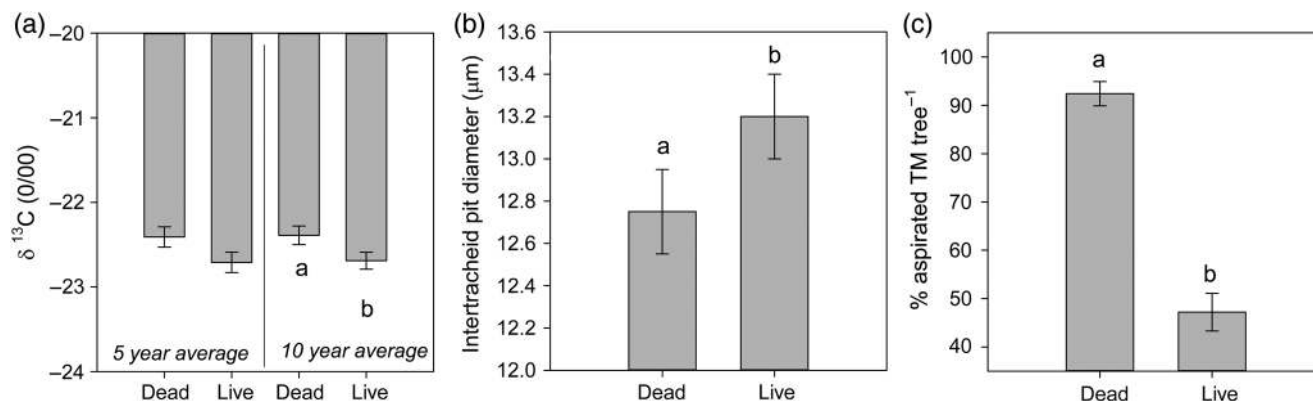
Figure 3. Bar graphs (mean ± 1 s.e.m.) for (a) wood $\delta^{13}C$, (b) inter-tracheid pit diameter and (c) percentage of TM structures aspirated in live and dead piñon trees sampled in New Mexico and Arizona. Bars labeled with different letters are significantly different as determined by ANOVA (a and b) or paired t-test (c) at $\alpha = 0.05$ within each date range.

drought at multiple sites in Arizona and New Mexico is an essential complement to recent experimental manipulations of water availability to sapling size piñon pines in greenhouses (Adams et al. 2009, 2013, Sevanto et al. 2014) and to larger trees in rainfall exclusion studies (Gaylord et al. 2013). Our comparison between co-occurring trees that lived and trees that died provides insights on mechanisms and tree characteristics that determine mortality during severe drought under actual field conditions (e.g., Galiano et al. 2011, Herrero et al. 2013, Macalady and Bugmann 2014). Our study is unique in retrospectively evaluating three major hypotheses of drought-induced tree mortality (hydraulic failure, carbon starvation and biotic agents) in mature field-grown trees exposed to drought. The results from our study are consistent with recent manipulative studies on piñon pine that implicate multiple interacting mechanisms of tree mortality during extreme drought (McDowell et al. 2013b, Sevanto et al. 2014). Non-significant tree status by site interactions in our study, in all analyses, strongly suggests consistent region-wide mechanisms of mortality of mature pinon pines in northern Arizona and northern New Mexico rather than site-specific mechanisms.

Based on studies of the response of piñon pine xylem water potential to drought published, or in progress, at the start of our study (Linton et al. 1998, Plaut et al. 2012), we did not expect hydraulic failure to be an important mechanism of mortality. Contrary to this expectation, we found almost twofold greater percentage of aspirated pits in dead trees (94%) than live trees (47%). This result suggests that trees that died in our study experienced more sapwood cavitation during the 2002 drought event than trees that lived. One explanation for this result is that the dead trees died during drought due to other mechanisms, such as bark beetle attacks, and cavitation occurred during or after death as the dead trees dried. We found no evidence for this explanation in our pilot study (detailed in the Materials and methods section), which showed that mortality of piñon pine from a rapid mass attack of bark beetles followed by 2 months

of drying of the dead tree on site does not cause TM aspiration. Similarly, bark beetles are known to vector fungi, which occlude water conduction in the xylem (Långström et al. 1993, Hubbard et al. 2013). We observed no fungal hyphae in tracheids or pits in the many SEM images we examined. Thus, occlusion of water conduction in sapwood by fungi vectored by bark beetles does not explain our results. Our finding of twofold greater xylem cavitation in trees that died during drought compared with trees that lived suggests a role of hydraulic failure in drought-induced piñon pine mortality. Consistent with this, later studies such as McDowell et al. (2013b) found strong evidence for substantial hydraulic failure in mature piñon pine exposed to experimental drought manipulations. Our results, combined with those of McDowell et al. (2013b), suggest that a rethinking of the hydraulic failure paradigm for isohydric species (McDowell et al. 2008) is strongly merited.

Another novel finding from the SEM analysis was that inter-tracheid pit diameter was significantly smaller for trees that died during drought than trees that lived. This finding is surprising given that many earlier studies, mainly of angiosperms, suggest that vulnerability to cavitation increases with diameter of xylem conduits or pits (Crombie et al. 1985, Sperry and Tyree 1988, Hacke et al. 2004, Pallardy 2008). Previous work in conifers has suggested that protection against air-seeding-induced cavitation in conifers is more due to specific tracheid and pit structure and high elasticity, porosity and mechanical strength of the TM membrane than pit size alone (Hacke and Sperry 2001, Pittermann et al. 2006, Choat et al. 2008). Nonetheless, the tendency for trees with smaller pits to cavitate more during drought in our study can be partly explained by using the Ohm's Law analogy for plant water flow: flow = water potential gradient/resistance (Pallardy 2008). Small pits increase resistance and hence reduce water flow through sapwood with other factors equal. Low sapwood water flow in trees with small pits has two consequences pertinent to the tendency of such trees to die during drought. First, sapflow could increase by a larger water

potential gradient, but xylem vulnerability curves for piñon pine show that this larger gradient would increase xylem tension and hence increase cavitation and hydraulic failure (Linton et al. 1998, Koepke and Kolb 2013). Second, if water flow through sapwood does not increase, reduced sapwood hydraulic conductance would accelerate stomatal closure (Hubbard et al. 2001), which in turn reduces photosynthesis and would promote carbon starvation.

The results partly support our prediction that trees that died during drought would show more symptoms of carbon starvation than trees that lived. The small, but significant difference between wood $\delta^{13}C$ for dead (−22.39) and live (−22.69) trees suggests lower stomatal conductance and greater diffusional limitation to photosynthesis for trees that died during drought, consistent with other comparisons of wood $\delta^{13}C$ between pine trees that died and trees that lived during severe drought (McDowell et al. 2010, Herrero et al. 2013). Additional calculations provide further support for greater carbon limitation in dead trees than in live. For instance, solving for C_i/C_a (concentration of carbon dioxide inside the leaf/outside the leaf; assuming $\delta^{13}C_a = -8‰$; Ehleringer 1989, Farquhar et al. 1989) results in 0.47 for live trees and 0.46 for dead trees. In addition, estimating C_i using a C_a value of 380 ppm results in C_i values of 178.6 ppm for live trees and 174.8 ppm for dead trees. These values of C_i occur in the steepest portion of the A–C_i curve for many plants, where assimilation rates are low and small differences in C_i can result in substantial differences in carbon limitation (Sharkey 1985, Wullschleger 1993, Lawlor and Cornic 2002). Lower standardized BAI for dead trees than live trees for the 5 years prior to death is consistent with both our initial hypothesis about carbon starvation and results from another study of piñon pine (Macalady and Bugmann 2014). Our finding of a weaker relationship between piñon pine mortality and growth for the 10 years prior to death than 5 years prior to death is consistent with a recent report of temporal shifts in tree growth–mortality relationships for piñon pine at some sites (Macalady and Bugmann 2014), and highlights the importance of evaluating mortality mechanisms with measurements of multiple characteristics.

We found strong support for the hypothesis that lethal biotic agents, which are intertwined with hydraulic failure and carbon starvation, contribute to drought-induced mortality. The connection between carbon limitation and biotic agents in our study system arises because metabolically expensive carbon-based monoterpenes are a likely primary defense of piñon pine against ips bark beetle attacks. Severe water stress reduces size and abundance of resin ducts in piñon pine sapwood and this reduction is positively associated with successful bark beetle attacks (Gaylord et al. 2013). In our study, trees that died had smaller resin ducts and a lower percent of xylem area in ducts than trees that lived, consistent with other recent comparisons between trees that die and trees that survive drought and bark beetle attacks (Kane and Kolb 2010, Ferrenberg et al. 2014). Almost all trees that died (96%) were successfully attacked by bark beetles whereas trees that lived had no evidence of attacks. These results show that certain trees within woodland populations are predisposed to successful attack by bark beetles because of low investment in resin ducts that produce physical and chemical defenses. Resin duct traits are known to be under genetic control in conifers (Rosner and Hannrup 2004) in addition to environmental control by drought and perhaps other factors (Gaylord et al. 2013). We speculate that genotype by environmental interaction in defense against drought-associated lethal biotic agents is an important contributor to tree-to-tree variation in mortality within sites in piñon pine and likely other genetically diverse conifers.

Conclusion

Our unique retrospective study produced five major results that inform understanding of mortality mechanisms during severe drought in a widespread tree of semi-arid woodlands. First, evidence for a role of hydraulic failure in death was unexpectedly strong for the highly isohydric piñon pine. Second, small intertracheid pits were strongly associated with death, perhaps because they reduced sapwood hydraulic conductance. Third, death was associated with chronic diffusional constraints to photosynthesis as shown by higher wood $\delta^{13}C$. Fourth, death was associated with slow recent radial growth. Fifth, death was associated with low defense against bark beetle attacks as shown by smaller xylem resin ducts. Our results support a role of multiple intertwined mechanisms of drought-induced mortality. Our study provides an innovative approach to examine mechanisms of tree mortality from drought long after the death process has occurred.

Supplementary data

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

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Conflict of interest

None declared.

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