GROWTH AND STABLE ISOTOPE SIGNALS ASSOCIATED WITH DROUGHT-RELATED MORTALITY IN SAPLINGS OF TWO COEXISTING PINE SPECIES

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20   Author's contribution: RZ and JC designed experiment; AH performed research (with the exception of isotopic analyses) and analyzed the data; ADH performed carbon isotopic analysis; JIQ contributed to the planning and interpretation of isotope measurements; all authors have contributed to the manuscript.

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25 **Summary**

Drought-induced events of massive tree mortality appear to be increasing worldwide. Species-specific vulnerability to drought-mortality may alter patterns of species diversity and affect future forest composition. We have explored the consequences of the extreme drought of 2005, which caused high sapling mortality (approx. 50%) among

30 10-year-old saplings of two coexisting pine species in the Mediterranean mountains of Sierra Nevada (Spain): boreo-alpine *Pinus sylvestris* and Mediterranean *P. nigra*. Sapling height growth, leaf $\delta^{13}C$ and $\delta^{18}O$, and foliar nitrogen concentration in the four most recent leaf cohorts were measured in dead and surviving saplings. The foliar isotopic composition of dead saplings (which reflects time-integrated leaf gas-exchange

35 until mortality) displayed sharp increases in both $\delta^{13}C$ and $\delta^{18}O$ during the extreme drought of 2005, suggesting an important role of stomatal conductance (g_s) reduction and diffusional limitations to photosynthesis in mortality. While *P. nigra* showed decreased growth in 2005 compared to the previous wetter year, *P. sylvestris* maintained similar growth levels in both years. Decreased growth, coupled with a sharper increase

40 in foliar $\delta^{18}O$ during extreme drought in dead saplings, indicate a more conservative water use strategy for *P. nigra*. The different physiological behavior of the two pine species in response to drought (further supported by data from surviving saplings) may have influenced 2005 mortality rates, which contributed to 2.4-fold greater survival for *P. nigra* over the lifespan of the saplings. This species-specific vulnerability to extreme

45 drought could lead to changes in dominance and distribution of pine species in Mediterranean mountain forests.

Keywords: drought stress, tree mortality, leaf $\delta^{13}C$, leaf $\delta^{18}O$, Mediterranean mountain.

INTRODUCTION

Extreme droughts are recurrent in many climate types worldwide (FIC 2006; Trenberth et al. 2007). Episodes of severe drought can have an impact on woody plant communities in several ways, weakening plant performance and even leading to shrub or tree mortality (Allen and Breshears 1998; Bigler et al. 2006; Miriti et al. 2007). Widespread tree-mortality events are expected to increase in large areas of the planet under the current scenario of climatic change, with elevated temperatures and/or water stress as a common causal factor (Allen et al. 2010). Different vulnerability to drought among species may alter dominance patterns and even accelerate climate-driven distributional shifts, with important implications for forest diversity and composition (Allen and Breshears 1998; Slik 2004).

Studies reporting the effect of extreme droughts on tree mortality have focused largely on adult individuals (Allen et al. 2010 and references within), while studies addressing sapling drought-mortality are scant (but see Suarez and Kitzberger 2008; Floyd et al. 2009; Lingenfelder and Newbery 2009). Sapling sensitivity to extreme drought events may be higher than that reported for adults, given differences in root-system development and hence access to water (Clark 1983), a fact which may mask episodes of mortality that, in the long term, could cancel recruitment pulses of tree species. This may be particularly critical for Mediterranean-type and semi-arid ecosystems, given the growing frequency and severity of extreme droughts that is occurring (and predicted) at regional scales (FIC 2006; Briffa et al. 2009), coupled with the low frequency of windows of opportunity for effective tree recruitment in these ecosystems (Holmgren and Scheffer 2001; García and Zamora 2003).

In this study, we analyze the way in which saplings of two coexisting pine species with contrasting biogeographical origins, a boreo-alpine species (*Pinus*

75   *sylvestris* L.) and a Mediterranean one (*P. nigra* Arnold), respond to an unusual and

extreme drought event. In 2005, the most extreme drought in the last five decades

occurred in Sierra Nevada National Park (SE Spain), affecting 10-year-old pine saplings

of a previous reforestation experiment. This extreme drought can be considered a

"natural experiment" which allowed us to examine the impact of severe water shortage

80   on two coexisting pine species while experimentally controlling for the effects of age

and environmental conditions. Both factors, age-related plant characteristics and spatial

variations in environmental conditions, can severely influence the effect of drought on

plant performance (Donovan and Ehleringer 1991; Cavender-Bares and Bazzaz 2000;

Lloret et al. 2004; Suarez et al. 2004). In order to evaluate the impact of drought on the

85   target species, we measured several ecological and ecophysiological variables linked to

drought stress in saplings of both pine species, including mortality, growth, and leaf

carbon and oxygen isotopic composition ($\delta^{13}C$ and $\delta^{18}O$).

Stable isotope ratios provide time-integrated information on plant

ecophysiological responses to changing abiotic conditions (Dawson et al. 2002) and can

90   also help characterize species-specific plant water use strategies (Moreno-Gutiérrez et

al. 2012). On the one hand, plant $\delta^{13}C$ provides an integrated record of the ratio of

intercellular to atmospheric CO_2 concentrations (c_i/c_a) during the period in which the

carbon (C) was fixed (Farquhar et al. 1982). Factors affecting net photosynthetic rate

(A) and stomatal conductance (g_s) influence c_i and thus, plant carbon isotope ratio.

95   Under drought conditions, low water availability promotes stomatal closure, generally

reducing c_i and increasing $\delta^{13}C$ in plant tissues (Adams and Kolb 2004; Eilmann et al.

2010; Sarris et al. 2013). On the other hand, the evaporative ^{18}O enrichment of leaf

water is imprinted on the assimilated carbohydrates, with plant $\delta^{18}O$ inversely related to

the ratio of atmospheric to intercellular water vapor pressure (e_a/e_i; Dongmann et al.

100 1974; Cernusak et al. 2002). The evaporative enrichment of leaf water is related to stomatal conductance and transpiration rate, because the convection of non-enriched xylem water via the transpiration stream dilutes the back-diffusion of evaporatively enriched leaf water (*Péclet* effect; Wang and Yakir 2000; Barbour 2007). Plant $\delta^{18}O$ can be used to discriminate between biochemical and stomatal limitations to photosynthesis,

105 since it shares the dependence on g_s with plant $\delta^{13}C$, but is unaffected by changes in A (Scheidegger et al. 2000; Grams et al. 2007). Further, simultaneous measurement of bulk leaf $\delta^{13}C$ and $\delta^{18}O$ can also help characterize the species-specific water use strategies of coexisting plant species exposed to the same environmental conditions (Moreno-Gutiérrez et al. 2012). Long leaf lifespans and a single annual flush of needle

110 growth in the target pine species allows comparisons between the $\delta^{13}C$ and $\delta^{18}O$ of successive leaf cohorts formed during contrasting conditions (i.e. extreme drought vs. normal years), in order to evaluate environmental impacts on the physiology of photosynthesis and transpiration.

In the present study, these comparisons were performed on saplings that died

115 during drought as well as on those that survived, in order to elucidate possible drivers of drought-induced mortality. Although in recent years much progress has been made in our understanding of the physiological mechanisms involved in tree death following drought (McDowell et al. 2008; Adams et al. 2009; Breshears et al. 2009), the specific mechanisms leading to tree mortality are far from being resolved (Sala et al. 2010).

120 Hydraulic failure, C starvation, and the effect of biotic agents have been postulated as the main mechanisms of drought-induced mortality (McDowell et al. 2008). Studies analyzing physiological responses of trees to severe drought during the dying process are scant (but see Adams et al. 2009; Breshears et al. 2009), and could help to clarify mechanisms underlying climate-driven vegetation mortality. This is especially true in

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125   studies where critical confounding effects, such variations in age and environmental conditions, are tightly controlled, as in the case of the present work. In particular, the leaf $\delta^{13}C$ and $\delta^{18}O$ values of dead saplings at time of mortality might offer valuable insight into their ecophysiological responses to extreme drought.

The main objective of this study is to assess and compare the responses of two

130   coexisting pine species to an extreme drought event. We hypothesized that the boreo-alpine *P. sylvestris* would be less able to cope with intense drought than the Mediterranean *P. nigra*, thus resulting in more severe water stress (inferred from leaf $\delta^{13}C$ and $\delta^{18}O$), lower growth, and higher mortality rate in the former species. In addition, any difference in the patterns of drought response between dead and surviving

135   saplings is discussed in relation to current knowledge of drought-induced mortality mechanisms. Finally, plausible species-specific differences in drought vulnerability at the sapling stage are examined in the context of future forest composition under an increasing aridity scenario.

140   **MATERIALS AND METHODS**

Study area and species

The study was carried out at the locality of Loma Panaderos (N 37º 04' 50'' W 3º 27' 50'', 1825 m. a.s.l., Sierra Nevada National Park, SE Spain), where the mature forest is dominated by *P. sylvestris* and *P. nigra*. The area was burnt by an accidental wildfire in

145   1983, and was reforested in 1997 with two-year-old saplings of *P. sylvestris* and *P. nigra* that were planted within three fenced areas of ca. 3,000 m² separated by ca. 200 m from each other (see Castro et al. 2002 for details). *Pinus sylvestris* is a boreo-alpine species widely distributed in Eurasia and restricted to high mountains areas in the Mediterranean basin, while *P. nigra* is a widespread Mediterranean species (Barbéro et

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150   al. 1998). Today, *P. sylvestris* and *P. nigra* occupy large areas in the northern
       Mediterranean basin, both growing in mountainous areas at medium to high elevations
       (Barbéro et al. 1998). It is common for populations of the two species to be contiguous,
       usually with *P. sylvestris* at higher elevations or in colder, moister exposures (Blanco et
       al. 1997).

155   Saplings were permanently tagged and monitored for survival since time of
       planting. Mortality was attributed to drought when plants were completely desiccated
       without any visible signs of herbivory, pathogens, or nutrient deficiency (saplings that
       had not survived transplanting were previously excluded). In 1997, first growing season
       after planting, high mortality due to drought was recorded for the two species, according
160   to the described vulnerability to drought during early regeneration stages in
       Mediterranean areas (Mendoza et al. 2009; Matías et al. 2012; Lucas-Borja et al. 2012).
       Mortality in 1997 was significantly higher for *P. sylvestris* (Table 1). Then, drought-
       mortality tended to decrease until 2001, despite the high values recorded in dry 1999
       (Table 1; Fig. 1). Cumulative drought-mortality of saplings until 2001 was significantly
165   higher for *P. sylvestris* (Table 1), whereas no mortality occurred from 2001 to 2004.
       These mortality data are modified from Castro et al. (2004) considering only saplings
       within fenced areas. Finally, during the extreme drought of 2005, high mortality of
       saplings of both species was recorded in late summer (August-September).

              The climate in the experimental area is continental Mediterranean, characterized
170   by a severe summer drought. The annual precipitation is 860 ± 86 mm (mean for 1997-
       2006 period), with a summer mean (months of June, July and August) of 35 ± 9 (mean
       for 1997-2006 period; data from a climatic station placed at La Cortijuela Botanical
       Garden, 800 m away from the study area). The year 2005 was the driest of the last five

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decades (García-Herrera et al. 2007; Online Resource 1), and the summer of 2005 was

175   particularly hot and dry (Online Resource 1 and 2).

Drought index

A drought index (DRI) was calculated for the study site to summarize differences in

moisture conditions among years that cover the entire lifespan of pine saplings (1997-

180   2006). The DRI was calculated using the following formula:

$$DRI = P - PET$$

where P is equal to the sum of the precipitation from August (previous year) to July

(current year), and PET equals the sum of estimated potential evapo-transpiration for

the same period as a function of monthly mean temperatures and geographical latitude

185   (using Thornthwaite (1948) formulation). We chose the same annual period (from

previous August to current July) as Bigler et al. (2006) to calculate annual DRI, given

the similarities in the seasonally dry conditions and the phenology of pine growth for

both study areas. A value of DRI below zero indicates moisture deficit. DRI data are

shown in Figure 1.

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Mortality and growth

In the first week of March 2006, dead and surviving saplings of both species were

counted in the fenced areas to quantify the mortality rate. For each species, 20 live and

20 dead individuals were recorded at random in one of the fenced areas in order to

195   minimize environmental variability. Sapling height, basal diameter, and internode

growth during the last four years (2005, 2004, 2003, and 2002) were measured. Annual

internode growth is easily identifiable by yearly whorls, as both species showed one

flush per year in the study area. Height Growth Index (HGI) values were calculated for

each measured sapling using the following formula:

200    $$HGI = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

where W_2 and W_1 are sapling heights (in mm) at times 2 and 1, respectively. We

calculated annual HGI values for the period 2002-2005, being t_2 and t_1 two consecutive

years.

205   **Leaf $\delta^{13}C$ and $\delta^{18}O$ analyses**

Leaf $\delta^{13}C$ and $\delta^{18}O$ data were analyzed separately for live and dead saplings. While

physiological activity ceased at time of mortality for dead saplings (late summer 2005),

the isotopic composition of foliar tissue in the surviving saplings was subsequently

influenced by environmental conditions during autumn 2005 and winter 2005-2006 as

210   well. Therefore, the data of dead and surviving saplings are not directly comparable.

However, for dead saplings, we expect no major changes in the isotope ratios of needle

tissue between the time of mortality and the time of sampling, given that sampling took

place in winter shortly after the summer drought (ca. 6 months). The decomposition of

organic materials in Mediterranean areas takes place when temperature and humidity

215   requirements are met, resulting in low decomposition rates of pine litter due to dry

summers and cold winters (Kurz et al. 2000; Moro and Domingo 2000). Thus,

decomposition during the dry autumn and cold winter of 2005-2006 must have been

strongly limited by water shortage and low temperature, respectively. It should be noted

that the 2005-2006 autumn and winter were much drier than average for the 1997-2006

220   series (the total precipitation from October 2005 to February 2006 was 87 mm, which is

only 17% of the 506 ± 99 mm mean for the 1997-2006 series). In addition, the needles

of all the cohorts remained attached to twigs and branches in dead saplings, thereby

reducing the potential attack of decomposers. Thus it seems reasonable to assume that the decomposition of needles from pines of sapling death until needle collection for

225   isotope analyses was negligible or very limited. It is also important to note that Grünzweig et al. (2007) found no changes in the bulk leaf $\delta^{13}C$ of *Pinus halepensis* from living leaves to leaf litter in Mediterranean areas. This supports the use of dead needles as trustworthy tissue for stable isotope analyses.

A random subsample of 12 saplings per species and condition (dead/alive) was

230   taken from the above mentioned 20 saplings, in order to perform leaf $\delta^{13}C$ and $\delta^{18}O$ analyses. Needles from the last four cohorts (2005, 2004, 2003, and 2002) were collected at the same time from the main axial stem in the second week of March 2006. The needles were stored in paper envelopes and oven dried at 60ºC for 48 h. Then, needles were ground and analyzed for $\delta^{13}C$ and $\delta^{18}O$. Leaf $\delta^{13}C$ analyses were

235   performed at the Biogeochemistry of Stable Isotopes Laboratory of the Instituto Andaluz de Ciencias de la Tierra (Granada, Spain), using a Carlo Elba 1500NC elemental analyzer interfaced to a mass spectrometer (Thermo Finnigan Delta plus XL). Leaf $\delta^{18}O$ analyses were conducted at the Stable Isotope Facility of the University of California at Davis (USA) using a Heckatech HT Oxygen Analyzer interfaced to a PDZ

240   Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK), following the method described in Kornexl et al. (1999). Leaf samples were converted by pyrolysis in a glassy carbon reactor at 1400°C to CO and H_2O, and oxygen was analyzed as CO.

The abundance of stable isotopes is presented in delta notation (δ), relative to a

245   standard:

$$\delta = \left(\frac{R_{samp}}{R_{st}} - 1 \right) \times 1000\, ‰$$

where R is the molar ratio of the heavy to light isotopes (R= $^{13}C/^{12}C$ or $^{18}O/^{16}O$), R_{samp} refers to the sample, and R_{st} to the international standard Vienna-Pee Dee Belemnite (V-PDB) for $\delta^{13}C$ and Vienna Standard Mean Oceanic Water (V-SMOW) for $\delta^{18}O$. The

250   working standards for $\delta^{13}C$ were sucrose and phthalic acid, and for $\delta^{18}O$ microcrystalline cellulose. The repeated analysis of these internal standards yielded a standard deviation of less than 0.1 ‰ for $\delta^{13}C$ and 0.2 ‰ for $\delta^{18}O$. Analyses of $\delta^{13}C$ and $\delta^{18}O$ were performed on whole-leaf tissue rather than extracted cellulose, because of strong positive correlations observed between $\delta^{13}C$ and $\delta^{18}O$ of whole tissue and

255   cellulose (Sullivan and Welker 2007; Powers et al. 2008).

Leaf N analysis

Leaf nitrogen concentration (N) is considered an indicator of maximum carboxylation capacity (Field and Mooney 1986) and is positively related to photosynthetic capacity

260   (Reich et al. 1995), as the greatest part of foliar N is linked to photosynthetic machinery (Evans and Seemann 1989). Therefore, we analyzed leaf N to test for differences in carboxylation capacity among species (separately for live and dead saplings). Leaf N was measured in the same samples used to analyze $\delta^{13}C$ and $\delta^{18}O$ (see above), using an elemental analyzer Leco CNH TrueSpec (Michigan, USA).

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Data analysis

Differences in mortality rate between the two species were analyzed using a nominal logistic regression. Height, basal diameter and HGI were compared between living and dead individuals, as saplings' growth ended prior to the mortality event (authors'

270   personal observation). Differences in height and basal diameter were examined using a one-way ANOVA. HGI data were analyzed using a multivariate ANOVA with the

dependent variable as a repeated measure. *Leaf cohort* was the repeated factor, whereas *species*, *condition* (live vs. dead), *species x condition*, and all interactions with *leaf cohort* were sources of variation. As mentioned above leaf $\delta^{13}C$, $\delta^{18}O$ and N data were

275   analyzed separately for live and dead saplings. Leaf $\delta^{13}C$, $\delta^{18}O$, and N data were subjected to a multivariate ANOVA with *leaf cohort* as the repeated factor, and *species* and its interaction with *leaf cohort* as sources of variation.

Post hoc comparisons between species for different leaf cohorts in HGI, leaf $\delta^{13}C$ and $\delta^{18}C$, and foliar N were performed using t-tests and applying a Bonferroni

280   correction to a significance level of 0.05 (4 variables * 4 leaf cohorts * 2 conditions = 32 comparisons; $\alpha = 0.0016$). In addition, Pearson's correlation coefficient was used to analyze paired relationships between HGI, leaf $\delta^{13}C$, and leaf $\delta^{18}O$, considering each pine sapling and each leaf cohort separately for the two species and conditions. Slopes for significant correlations were compared between species using a standardized major-

285   axis test. All the analyses were performed using JMP 7.0 (SAS Institute Inc.), except comparisons of correlations' slopes, which were performed using SMATR software (Falster et al. 2006). All results throughout this paper are given as mean ± standard error.

290   **RESULTS**

Mortality and growth

After the 2005 extreme drought, cumulative sapling mortality associated with drought since planting (1997-2005) was significantly higher for *P. sylvestris* than for *P. nigra* (Table 1), thus resulting in 2.4-fold greater survival for *P. nigra* than for *P. sylvestris*.

295   Mortality during the extreme drought year of 2005 was also higher for *P. sylvestris* than for *P. nigra*, although this difference was not statistically significant (Table 1).

Within pine species, there were no significant differences in height, basal diameter or HGI between living and dead saplings (P > 0.05 for both species). Mean height of the saplings at the end of the 2005 growing season was 79.75 ± 4.05 cm for *P.*

300   *sylvestris* and 74.36 ± 2.68 cm for *P. nigra*, whereas the mean basal trunk diameter was 2.69 ± 0.14 cm and 2.86 ± 0.14 cm, respectively. Differences between species in HGI varied among years (significant *year x species* interaction, F = 12.23, *P* < 0.0001; see Fig. 2). HGI during the extreme drought year of 2005 was significantly greater for *P. sylvestris* than for *P. nigra* (P < 0.0016, Fig. 2). The HGI of *P. nigra* during the extreme

305   drought year of 2005 decreased relative to that observed during the previous year. In contrast, *P. sylvestris* maintained similar HGI values in both years (Fig. 2).

Leaf δ^{13}C, δ^{18}O and N

Foliar δ^{13}C and δ^{18}O varied between annual leaf cohorts for dead saplings, with sharp

310   increases in both parameters in the 2005 cohort relative to the 2004 cohort (Table 2, Fig. 2). Leaf δ^{13}C in dead saplings was not significantly different between *P. nigra* and *P. sylvestris* (Table 2). However, dead *P. nigra* saplings showed significantly higher δ^{18}O than dead *P. sylvestris* across leaf cohorts (Table 2, Fig. 2), and particularly for the 2005 cohort (*Post hoc* comparisons, Fig. 2). There were no significant differences in foliar N

315   between dead saplings of the two species (Table 2). Foliar N in dead saplings ranged from 0.27 % in the 2002 leaf cohort to 1.70 % in the 2005 leaf cohort.

Surviving saplings of *P. nigra* showed significantly higher δ^{13}C for the leaf cohort produced during the extreme drought of 2005, whereas there were no significant differences in leaf δ^{18}O between species (Table 2, Fig. 2). Foliar N in surviving saplings

320   showed no significant differences between species (Table 2), ranging from 0.62 % in the 2002 leaf cohort to 2.06 % in the 2005 leaf cohort.

Relationships between leaf $\delta^{13}C$, $\delta^{18}O$ and HGI

Across leaf cohorts, $\delta^{13}C$ was strongly positively correlated with $\delta^{18}O$ in dead saplings

325   of both pine species (Fig. 3). In addition, leaf $\delta^{13}C$ was tightly negatively associated

with HGI in dead saplings of both pine species (Fig. 4). Leaf $\delta^{18}O$ was also negatively

correlated with HGI for dead *P. nigra* (r = -0.49, *P* = 0.0005) and dead *P. sylvestris* (r =

-0.33, *P* = 0.029) saplings. Correlation coefficients were in all cases higher for *P. nigra*

than for *P. sylvestris*, but no significant differences in slopes were found between the

330   two species (*P* > 0.05 for the three comparisons).

For live saplings of both pine species, the positive correlation between leaf $\delta^{13}C$

and $\delta^{18}O$ was only marginally significant (Fig. 3). Similar to dead saplings, the negative

correlation between leaf $\delta^{13}C$ and HGI was significant for live saplings of the two

species (Fig. 4), with no significant differences in slopes (SMATR test statistic = 0.008,

335   *P* = 0.929). No significant correlations were found between leaf $\delta^{18}O$ and HGI for live

saplings of *P. sylvestris* (r = 0.05, *P* = 0.7532) and *P. nigra* (r = -0.20, *P* = 0.1883).

DISCUSSION

The high mortality rates observed in 2005 are unusual for 10-year-old pine saplings well

340   past their vulnerable young-sapling stage, which demonstrates the severity of the 2005

drought. The leaf $\delta^{13}C$ and $\delta^{18}O$ values of dead saplings at time of mortality may offer

valuable insights into their ecophysiological responses to extreme drought. In this

respect, it is important to note that $\delta^{13}C$ in *Pinus* does not differ between living leaves

and leaf litter in dry Mediterranean ecosystems (Grünzweig et al. 2007), which allows

345   the use of both live and dead needles for stable isotope measurements. The increase in

$\delta^{13}C$ observed in the 2005 leaf cohort of both pine species indicates a reduced c_i/c_a ratio,

and appeared to be driven by reduced CO_2 exchange between leaf and atmosphere due to partial stomatal closure in response to low water availability. This increase in $\delta^{13}C$ must have been the result of a decrease in c_i, because c_a was similar for both pine

350 species. The decrease in c_i likely resulted from a stronger reduction in g_s than in A, as drought conditions promote stomatal closure, especially in isohydric species like *P. sylvestris* and *P. nigra* (Irvine et al. 1998; Cinnirella et al. 2002).

The concurrent increase in $\delta^{18}O$ observed in the 2005 leaf cohort of dead saplings points to reduced stomatal conductance and transpiration rates under severe

355 drought conditions in both species. Models describing leaf water enrichment in ^{18}O include the *Péclet* effect (Wang and Yakir 2000; Barbour 2007), which refers to the ratio of back-diffusion of enriched water from the sites of evaporation to convective influx of non enriched xylem water through transpiration flow. In this context, the increase in $\delta^{18}O$ in the 2005 leaf cohort likely reflects a decreased dilution effect by the

360 non-enriched transpiration stream on the enriched leaf water $\delta^{18}O$, due to lower stomatal conductance and transpiration in both pine species (which is subsequently imprinted in new assimilates). Moreno-Gutiérrez et al. (2011) showed that decreased stomatal conductance indeed led to significant bulk leaf $\delta^{18}O$ enrichment in drought-stressed *Pinus halepensis* trees growing in a semiarid Mediterranean ecosystem. However, it

365 should be noted that more intense evaporative enrichment of source (soil) water and/or lower mean relative humidity (RH) during the drought of 2005 than during previous years may have also contributed to much higher $\delta^{18}O$ values in the 2005 leaf cohort compared to previous cohorts (Barbour 2007).

Stomatal conductance is not the only variable controlling CO_2 diffusion into the

370 leaf that can be affected by drought. Previous studies have shown that leaf mesophyll conductance (which determines the resistance to diffusion of CO_2 from the substomatal

cavity to the sites of carboxylation in the chloroplast) is also affected by water availability (Flexas et al. 2012 and references within). Thus, reductions in both g_s and mesophyll conductance (g_m) in response to drought may have contributed to decreased

375   c_i/c_a and increased $\delta^{13}C$ in the 2005 leaf cohort. Sharp increases in $\delta^{13}C$ and $\delta^{18}O$ in the 2005 leaf cohort of dead saplings and positive correlations between $\delta^{13}C$ and $\delta^{18}O$ indicate strong diffusional limitations on photosynthesis (exerted by both g_s and g_m, *sensu* Grassi and Magnani 2005) in both pine species in response to drought. This interpretation is further supported by the negative correlations between $\delta^{13}C$ and HGI

380   across individuals and leaf cohorts (see Fig. 4), as height growth is very sensitive and responsive to climatic factors in tree saplings (Chinn and Wang 2002; Gamache and Payette 2004; Reich and Oleksyn 2008).

The unusually high $\delta^{13}C$ values of the 2005 leaf cohort in *P. sylvestris* dead saplings (with maximum values close to -22 ‰) were much higher than those

385   previously reported for *P. sylvestris* seedlings under experimental drought conditions (Cregg and Zhang 2001). Foliar $\delta^{13}C$ values in dead saplings of both pine species in 2005 were actually similar to or even higher than those reported for other pine species from much drier ecosystems than the study area (annual rainfall of 300 mm or less; Van de Water et al. 2002; Oliveras et al. 2003; Querejeta et al. 2008; Moreno-Gutiérrez et al.

390   2011), including reports of extreme drought conditions which triggered widespread drought-induced mortality events (Adams and Kolb 2004; Koepke et al. 2010).

Although the saplings of the two species that died during the 2005 summer responded to the extreme drought conditions with a decrease of g_s/g_m relative to previous years, *P. sylvestris* and *P. nigra* displayed species-specific ecophysiological

395   responses that could help explain the observed differences in cumulative mortality rates between species. Firstly, *P. nigra* showed decreased HGI in 2005 compared to the

previous year, while *P. sylvestris* maintained similar growth levels in the two years. Secondly, dead *P. nigra* saplings showed higher $\delta^{18}O$ in the 2005 leaf cohort than did *P. sylvestris* (see Fig. 2), indicating a greater reduction in g_s and transpiration during the

400   extreme drought in the former species. It is important to note that dead saplings of the two pine species were exposed to the same environmental conditions (including factors known to affect plant $\delta^{18}O$ such as temperature, rainfall amount and isotopic composition, RH, etc) and thus must have used source water with same or very similar $\delta^{18}O$ values (assuming no major disparities in rooting depth, which are unlikely given

405   that saplings of both species had the same size). Therefore, we think it is both reasonable and safe to interpret the observed differences in foliar $\delta^{18}O$ between dead saplings of the two pine species as indicative of differences in stomatal conductance and transpiration.

In contrast to the pattern observed for foliar $\delta^{18}O$, dead saplings of the two

410   species showed similar $\delta^{13}C$ in the 2005 leaf cohort (see Fig. 2). The absence of differences in leaf N between the two species suggests little or no differences in maximum carboxylation capacity (Field and Mooney 1986). In a greenhouse study examining the influence of light intensity on photosynthetic rates, *P. sylvestris* and *P. nigra* displayed very similar photosynthetic behaviors, with minor differences in A

415   under well-watered conditions (Fernández and Tapias-Martín 2004). Thus, it seems reasonable to assume that under the extreme drought conditions of 2005, *P. nigra* had lower g_s and g_m than *P. sylvestris,* which also led to lower photosynthesis in the former. This would explain the similar foliar $\delta^{13}C$ in the two species but higher $\delta^{18}O$ in *Pinus nigra* (Scheidegger et al. 2000; Grams et al. 2007; Moreno-Gutiérrez et al. 2011). This

420   interpretation of isotope data is consistent with the growth reduction observed in 2005 in *P. nigra* (but not in *P. sylvestris*) and may indicate different strategies of the two

coexisting species in their response to severe drought conditions (Moreno-Gutiérrez et al. 2012): *P. nigra* exhibits a more conservative water use strategy characterized by a tighter control of g_s in response to severe water shortage, whereas *P. sylvestris* shows a

425   more prodigal water use strategy with less tight control over g_s during drought.

Species-specific physiological responses to drought stress could influence mortality rates and affect the establishment success of the two pine species. Although mortality rates observed in 2005 were not significantly different between species, mortality was higher in *P. sylvestris*, in accordance with previous patterns of drought-

430   mortality observed during the young-sapling stages, which contributed to significant between-species differences in cumulative drought-mortality since time of planting (see Table 1). The greater stomatal responsiveness to drought of *P. nigra* compared to *P. sylvestris* could be an important trait influencing species-specific vulnerability to drought-mortality. Tighter stomatal control of transpiration is considered an

435   advantageous characteristic in dry ecosystems, leading to a more conservative water use (e.g. Yin et al. 2005). However, persistently low g_s under protracted drought conditions would decrease C assimilation, thus eventually pushing saplings close to C starvation. A reduction in g_m can aggravate this situation, further reducing C assimilation through limiting photosynthesis (Flexas et al. 2012). In contrast, the lower stomatal

440   responsiveness to extreme drought conditions exhibited by *P. sylvestris* could increase its vulnerability to cavitation and the risk of mortality from hydraulic failure (McDowell et al. 2008). Overall the data indicate that the boreo-alpine species *P. sylvestris* is less well adapted and less resistant to drought than the Mediterranean *P. nigra*.

The saplings that survived the extreme drought of 2005 showed results similar to

445   those of dead saplings in many aspects. An increase in $\delta^{13}C$ in the 2005 leaf cohort in response to extreme drought was also observed in surviving saplings, particularly for *P.*

nigra. However, no concomitant increases in $\delta^{18}O$ were recorded in the 2005 leaf cohort relative to previous cohorts, possibly due to the highly dynamic nature of the oxygen isotope composition of leaves (particularly the non-structural, soluble carbohydrates) in

450   living saplings. Surviving saplings likely underwent additional drought stress after the massive mortality event of the summer 2005, due to dry autumn 2005 and winter 2005-2006 conditions, which were much drier than average for the 1997-2006 series (the total precipitation from October 2005 to February 2006 was 87 mm, which is only 17% of the 506 ± 99 mm mean for the 1997-2006 series). Live saplings of *P. nigra* showed

455   lower height growth in 2005 and higher $\delta^{13}C$ in the 2005 leaf cohort than live saplings of *P. sylvestris*, which further supports a more conservative water use strategy in the former species.

        Apparent increases in drought-induced forest mortality events across different biomes (Allen et al. 2010) have triggered interest in the mechanisms of tree mortality

460   during drought. In this respect is important to note that the absence of any pest or pathogen problems in the present study avoided the confounding effects of biotic agents in drought-induced mortality, such as insect outbreaks that could cause mortality in trees weakened by drought (McDowell et al. 2008). For species showing isohydric behavior, such *P. sylvestris* and *P. nigra*, plant-carbon balance seems to play an

465   important role in mortality (McDowell et al. 2008; Sala et al. 2010). Higher C demand boosted by high summer temperatures (due to higher respiration costs; Hartley et al. 2006) coupled with low C assimilation because of diminished g_s/g_m, may lead to sapling death because of cellular-level C starvation (associated with impaired floem transport; Sala et al. 2000) and/or whole-plant C starvation (as suggested by several studies;

470   Eilmann et al. 2010; Galiano et al. 2011; Klein et al. 2011). Nevertheless, catastrophic hydraulic failure cannot be ruled out as a cause of sapling mortality in our study. In fact,

C metabolism and hydraulic functioning are highly interdependent processes (McDowell et al. 2011) that are strongly influenced by g_s.

In the context of increasing widespread drought-induced tree mortality events, our results support the idea that massive mortality may also occur at the sapling stage, even if adults survive the extreme drought; in fact, adult tree mortality was not observed in the study area during the 2005 drought (authors' personal observation) despite the high sapling mortality. Sapling vulnerability to extreme drought events may be higher than that of adults due to a shallower root system and lower C reserves. This may be particularly true for open areas near forest edges, where the forest has the potential for expansion, but where an adult tree canopy that could buffer climatic extremes is absent.

The different responses of both pine species to the 2005 extreme drought and the associated differences in cumulative drought-mortality could have important consequences for forest dynamics and the distribution of these currently coexisting pine species. Massive sapling mortality events could delay and/or altogether prevent regeneration in seasonally dry forests that may already be threatened by difficulties in seedling establishment (Mendoza et al. 2009). In fact, *P. sylvestris* has its southernmost distribution at the study site, and its recruitment in these Mediterranean mountains is already seriously constrained by summer drought (Castro et al. 2005; Mendoza et al. 2009; Matías et al. 2012). In contrast, *P. nigra* may exhibit comparatively greater survival and establishment success than *P. sylvestris* under a global-warming context. In addition, *P. nigra* has higher seed dispersal and greater capacity for colonization of open habitats than *P. sylvestris* (Debain et al. 2007). Altogether, this could lead to a shift in regeneration capacity towards *P. nigra* in areas where the two species currently coexist, potentially altering patterns of species dominance and distribution in Mediterranean forest ecosystems.

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Table 1 Annual and cumulative mortality rate due to drought for *P. sylvestris* and *P. nigra* from 1997 to 2005 (only years with mortality are showed). Annual mortality rate was measured shortly after summer (October), except in 2005, when it was measured in the following March. Mortality was attributed to drought because the plants were completely desiccated without any visible signs of herbivory, pathogens, or nutrient deficiency. Bold numbers indicate significant differences in drought-mortality between species. Changes in n values from year to year also account for frost mortality, which only occur during the first two years (less than 15%, data not shown). By this way, frost mortality does not affect the calculation of mortality rates due to drought. 1997-2000 data was modified from Castro et al. (2004), considering only saplings inside fences

	Annual mortality rate				Cumulative mortality rate			
	P. sylvestris		*P. nigra*		*P. sylvestris*		*P. nigra*	
Event	%	n	%	n	%	n	%	n
1997	**45.5**	378	**34.6**	402	**45.5**	378	**34.6**	402
1998	14.2	176	9.4	245	**56.6**	378	**42.2**	402
1999	**31.4**	137	**11.5**	200	**71.9**	378	**51.1**	402
2000	**11.7**	94	**4.5**	177	**75.2**	378	**53.3**	402
2005	62.8	83	48.3	169	**89.8**	378	**76.0**	402

Table 2 Summary of the multivariate ANOVA results for foliar carbon and oxygen isotopic composition (δ^{13}C and δ^{18}O, ‰), and foliar nitrogen content (N, %) for dead and live saplings

Source	Dead			Live		
	df	F	*P*	df	F	*P*
Foliar δ^{13}C						
Between subjects						
Species	1	0.07	0.7996	1	5.57	**0.0275**
Within subjects						
Time (Leaf cohort)	3	410.06	**<0.0001**	3	87.38	**<0.0001**
Time x Species	3	2.61	0.0801	3	3.77	**0.0271**
Foliar δ^{18}O						
Between subjects						
Species	1	17.45	**0.0006**	1	0.06	0.8173
Within subjects						
Time (Leaf cohort)	3	69.48	**<0.0001**	3	8.52	**0.0015**
Time x Species	3	2.97	0.0629	3	1.70	0.2099
Foliar N						
Between subjects						
Species	1	1.68	0.2090	1	0.13	0.7247
Within subjects						
Time (Leaf cohort)	3	36.06	**<0.0001**	3	25.94	**<0.0001**
Time x Species	3	0.71	0.5585	3	1.70	0.2001

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Figures

Fig. 1 Drought index for 1998-2006 series, the time period covering the lifespan of pine saplings

Fig. 2 Height Growth Index (HGI), leaf δ^{13}C, and leaf δ^{18}O for dead and live saplings of *Pinus sylvestris* and *P. nigra* in the last four years (2002-2005). Annual HGI was calculated as the difference in height (in mm) between two consecutive years in a logarithmic scale. (*) indicate significant differences between groups for the *Post hoc* analysis ($P < 0.0016$). Bars indicate the standard errors of calculated means. n = 20 for HGI, and n=12 for leaf δ^{13}C and δ^{18}O

Fig. 3 Pearson's correlations between leaf δ^{13}C and δ^{18}O for dead and live saplings of *Pinus sylvestris* and *P. nigra* considering each pine sapling. n = 48

Fig. 4 Pearson's correlations between leaf δ^{13}C and Height Growth Index (HGI) for dead and live saplings of *Pinus sylvestris* and *P. nigra* considering each pine sapling. Annual HGI was calculated as the difference in height (in mm) between two consecutive years in a logarithmic scale. n = 48

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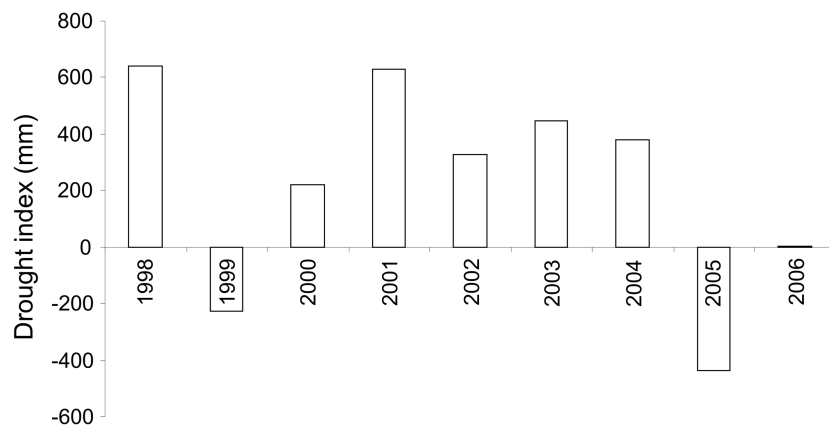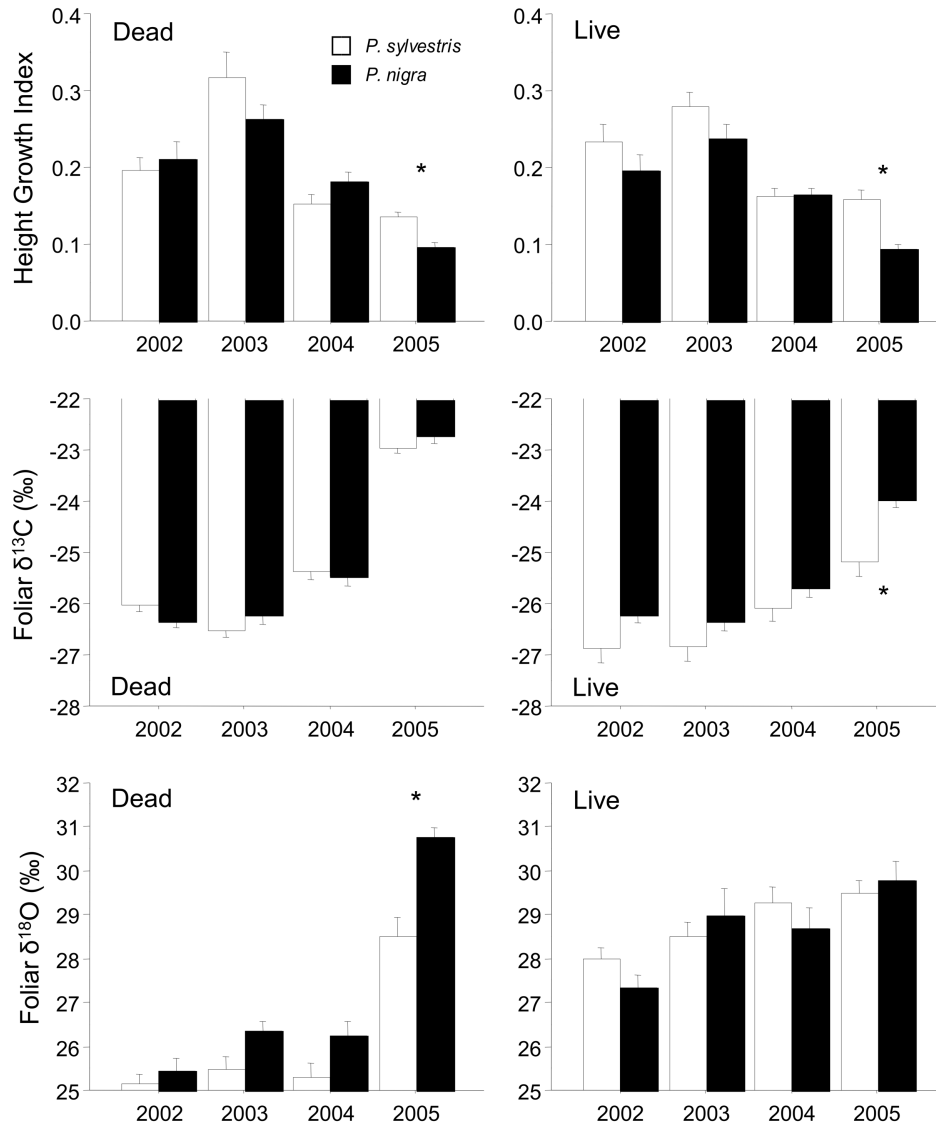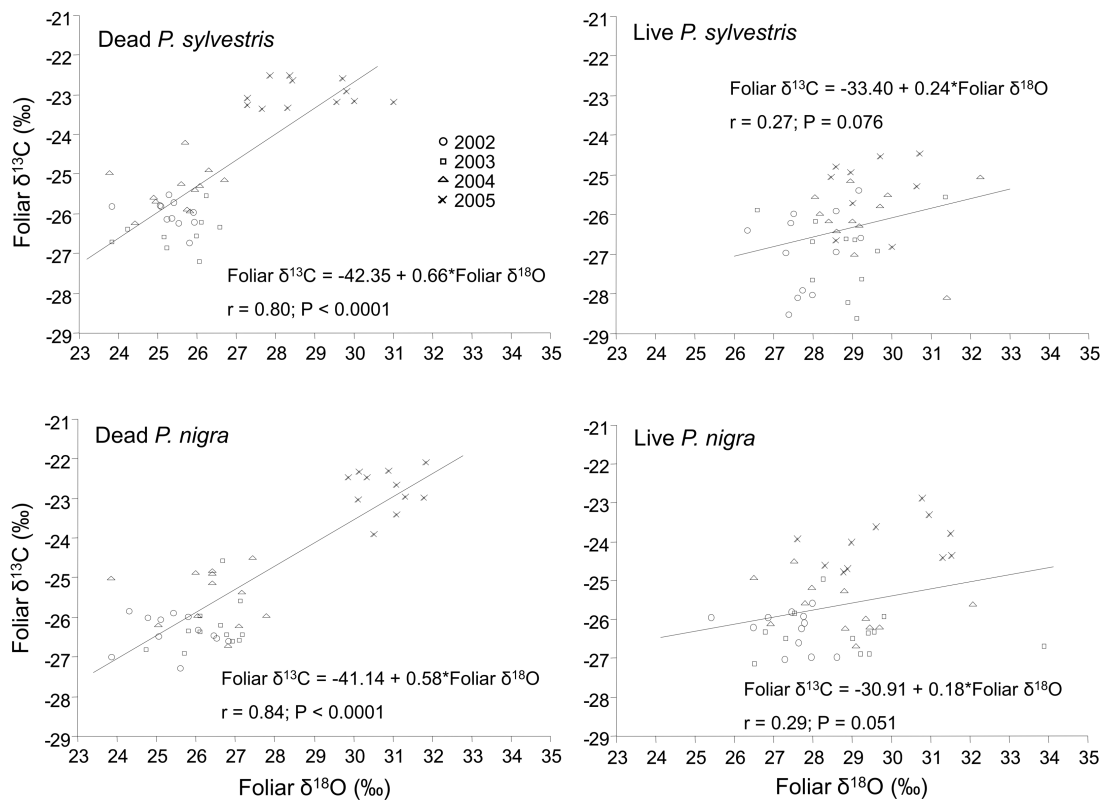
Figure 1

Figure 2

Figure 3

Figure 4