Simultaneous assessment, through sap flow and stable isotopes, of water use efficiency (WUE) in thinned pines shows improvement in growth, tree-climate sensitivity and WUE, but not in WUEi.

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Abstract

In water-limited regions, adaptive management of forest and water relationships has been put forward, to implement hydrology-oriented silviculture to reduce stand evapotranspiration and, at the tree level, to improve growth and water use efficiency (WUE). The main goal of this study was to evaluate the effect of thinning in the short and medium term on tree growth, climate (drought) sensitivity, WUE performed using growth and sap flow measurements and WUEi performed using $\delta^{13}$C and $\delta^{18}$O isotopes, in a typical semiarid forest. This approach
also evaluated the reliability of isotopes as indicators of the effects of adaptive forest
management. A stagnated Aleppo pine plantation was experimentally thinned at high intensity
(H98) in 1998 and at High (H), Medium (M) and Low (L) intensities in 2008, along with a
control (C). Substantial limitation of tree growth was observed in C. Thinning not only
increased growth, but also changed the tree growth-precipitation relationships, with C trees
depending more on precipitation than thinned trees did. WUEi after thinning was significantly
affected only in the medium term, with C trees being more efficient (94.4 μmolCO₂/molH₂O)
than H98 trees (88.7), especially in dry spells (100.7). WUEi was found to increase when
precipitation decreased, regardless of the treatment. However, WUE increased sharply from C
(1.26 g biomass/L H₂O) to H (3.20 g/L), showing a clear difference with WUEi observed in
the same years. Thinning caused an increase in δ¹⁸O in the short term, but no relationship was
found between δ¹⁸O and tree water use. It can be concluded that forest management improved
WUE in spite of higher tree transpiration, but WUEi remained unchanged, probably due to an
underestimate of photosynthetic capacity. The dual isotope (δ¹³C and δ¹⁸O) conceptual model
was not consistent with our experimental data. Thus, the question of whether stable isotopes
can be used as a tool for addressing the ecophysiological impacts of thinning remains open.

Keywords: Dendroclimatology; Adaptive forest management; Hydrology-oriented
silviculture; Pinus halepensis; Aleppo pine; Tree transpiration.

1. Introduction

Water resource availability in the Mediterranean will be seriously jeopardized in the
foreseeable future (García-Ruiz et al., 2011), which may have a substantial impact on the
semiarid forests growing in the region (Lindner et al., 2010; Torras et al., 2012). To improve
forest resilience, forest managers need adaptive strategies that help make forest management
more efficient and effective under changing water resource availability (Sjölund and Jump,
However, although many studies have addressed this need in recent years, reliable guidelines for adaptive management in line with the eco-regional and social context are still scarce (Fitzgerald et al., 2013).

In water-limited regions, adaptive management usually focuses on forest and water relationships based on hydrology-oriented silviculture (Del Campo et al., 2014; Ungar et al., 2013). Guidelines for this silviculture should be developed through a full approach covering tree to stand scales. Stand scale is related to the hydrologic performance of the physical structure of forests (density, LAI, canopy storage etc.), in which thinning is known to affect water cycle components such as rainfall interception, throughfall, transpiration, soil moisture and deep infiltration (Del Campo et al., 2014; Molina and Del Campo, 2012). On the other hand, tree-scale studies provide fundamental information about how changing forest structure and microclimate might lead to modifications in the ecophysiology of photosynthesis, transpiration and tree water relations (Aussenac, 2000). In this context, tree climate-growth relations, water use (WU) and water use efficiency (WUE) are central topics to be tackled when relating forest management and tree water (Brooks and Mitchell, 2011; Forrester et al., 2012; Kruse et al., 2012; Sohn et al., 2013; Ungar et al., 2013). While WU is the amount of water transpired by trees (Burgess et al., 2001), WUE is the ratio of carbon gain per WU (Brienen et al., 2011). In most of the literature, WUE is discussed either in terms of an instantaneous measurement of the efficiency of carbon gain per water loss, i.e. intrinsic water-use efficiency (WUEi), or as an integral of such efficiency over time, commonly expressed as the ratio of water use to biomass accumulation or to harvestable yield (Dye, 2000; Hubbard et al., 2010).

Plant carbon stable isotope composition ($\delta^{13}$C) provides a time-integrated proxy of WUEi during the growing season (Dawson et al., 2002; Farquhar et al., 1989), because the isotopic carbon discrimination of plants is linearly linked to the $C_i/C_a$ ratio, where $C_i$ is the
partial pressure of CO$_2$ in the leaf intercellular spaces and C$_a$ that of the ambient air (Farquhar et al., 1982; Sheidegger et al., 2000). However, increases in $\delta^{13}$C, interpreted as a reduction in C$_i$, may be the result of either i) reduced stomatal conductance (gs) at a constant photosynthetic capacity (A) or ii) increase in A at a constant gs, i.e. changes in WUE$_i$ are due to changes in transpiration rate or in photosynthesis activity. To overcome this, the dual-isotope model (Sheidegger et al., 2000) was developed to constrain the interpretation of $\delta^{13}$C variations in organic matter by measuring $\delta^{18}$O in the same material. In principle, the latter as a proxy for evaporative demand would be modified by gs but not by A, thus allowing for a better interpretation of WUE$_i$. This model is conceptually sound and many authors have used it to interpret $\delta^{13}$C results measured in tree rings (Brooks and Mitchell, 2011). However, Roden and Siegwolf (2012), on analysing the systematic use of this conceptual model, warned about specific situations in which its applicability might be compromised.

Studies of WUE$_i$ and of the use of stable isotopes in Aleppo pine have been profuse in the literature. Previous studies used this technique to assess climate-growth relations (Ferrio et al., 2003), intra-specific differences in WUE$_i$ related to seed origin (Voltas et al., 2008) and differences in plantation performance (Querejeta et al., 2008), or to specifically address tree water use in the species (Klein et al., 2013a). Some of these studies provide key information for improving silviculture in the face of increasing water scarcity in Mediterranean regions. However, how forest management affects WUE, how long these changes may last and how stable isotopes can study these effects remain unclear for this species. For instance, Moreno-Gutiérrez et al. (2011, 2012) report no variation in WUE$_i$ in Aleppo pine after reducing forest density; similar results were found by Martín Benito et al. (2010) for black pine. In addition, Gyenge and Fernández (2014) report that thinning increased the amount of water reaching the soil, but that WUE$_i$ was unrelated to growth, nitrogen and light use efficiencies. On the other hand, Querejeta et al. (2008) report a significant effect of the afforestation method on WUE$_i$
of Aleppo pine saplings. In addition, in most studies WUE is addressed by studying δ^{13}C in tree-rings (i.e. WUEi), although some divergences may appear when δ^{13}C results are compared to other techniques such as leaf-scale gas exchange (Klein et al., 2013a).

The present study complements our previously published stand-scale results from a stagnated Aleppo pine plantation (Del Campo et al., 2014) and specifically addresses: i) What are the short- and medium-term effects of thinning intensity on WUE and growth of trees? ii) Do these effects change in drought periods? iii) Is there congruency between WUEi findings with the isotope-based approach and WUE findings obtained from tree growth and tree water use by sap flow measurements? We also addressed the question of whether the dual isotope technique (δ^{13}C and δ^{18}O) could be used to explain further the WUE results.

2. Material and Methods

2.1. Experimental site and design

The experimental site and design have been described elsewhere (Del Campo et al., 2014). Briefly, “La Hunde” site is located in Valencia, Spain (39°5’N; 1°13’W, 943 m a.s.l.). The climate is Mediterranean with an average total annual precipitation of 477 mm and a mean annual temperature of 14.1°C. The soils are shallow (50-60cm) with a sandy-silty texture and basic pH. The area is occupied by *Pinus halepensis* Mill. plantations about 50-60 years old and with high tree density (ca. 1,500 trees/ha) due mainly to low forest management. The experimental set-up encompassed five treatments. One of them was selected within a non-replicated plot heavily thinned in 1998 (H98), leaving approximately 10% of the trees. This 0.2 ha plot was established and sampled to assess the medium-term effects of thinning. Adjacent to this plot, another experimental area was set up. This consisted of a randomized block design with three blocks (0.36 ha each) to assess the short-term effects of thinning. Each block was further divided into four plots (30x30m), three of them with
thinning treatments performed in 2008 at different intensities (High-H, Medium-M and Low-L) and a control plot (C) common to both experimental areas (Table 1).

2.2. Tree growth

Between eight and twelve trees per treatment were cored (north and south) by a 5mm increment borer at 1.30 m height. Each core was mounted on a wooden support and sanded until wood cells were clearly identified under the stereomicroscope. All cores were visually cross-dated and measured to the nearest 0.01mm with a measuring table (LINTAB 6.0, Frank Rinn, Heidelberg, Germany) coupled with the TSAP-Win software package (Rinn, 2011). Cross-dating of the tree-ring width (TRW) series was evaluated by the COFECHA software (Holmes, 1983). Cores with missing rings were ruled out for any further analysis. The average series length was 50.2 (σ=2.51) years; autocorrelation at 1-year average was 0.76 (σ=0.09); and the Gini coefficient, which describes annual changes in the inequality of size and size increment, was 0.42 (σ= 0.08), where 0 indicates perfect equality (the size or growth of all individuals is the same) and 1 indicates perfect inequality. The average series correlation to the master chronology was 0.79 (σ=0.07), excluding the H98 treatment, which was analysed separately, obtaining a correlation of 0.80 (σ=0.09).

The tree ring width (TRW) series were detrended to reduce the systematic noise caused by tree age (Cook and Briffa, 1990), using a cubic smoothing spline function with a wavelength fixed at 67% (Cook et al., 1990) of the length of the series, and a 50% frequency response. In some cores a negative exponential method was used instead. Each measured series was standardized by dividing the observed values by the predicted ones to obtain dimensionless TRW index series (TRWi). TRWi was averaged using a robust bi-weight mean. Additionally, the temporal autocorrelation was removed from each series by an autoregressive model (Cook and Briffa, 1990) to obtain the standard and residual chronology.
To determine the length of the residual chronology for which climatological responses would be tested, a running (20-year) mean of the expressed population signal (EPS) statistic was used to provide an indication of chronology reliability (Wigley et al., 1984).

Then, TRW data were used to estimate annual biomass increment value by an allometric equation developed for *Pinus halepensis* Mill. in the Mediterranean area (Gracia et al., 2004). Factors that might affect allometric relationships in this species vary with stand density, age, site and recent weather conditions (López-Serrano et al., 2005). Because there is no reported study on this species with a similar combination of these factors, we decided to opt for allometric models based on regional forest inventory data (Gracia et al., 2004), which cover a wide ecological range and a good representation of Aleppo pine forest conditions and trends across the landscape. The allometric equations used come from pure or mixed stands (basal area of *Pinus halepensis* >= 70%) with a minimum canopy cover of 40%. The equations allow total aboveground biomass to be calculated (N=2,206 trees, $R^2=0.61$, standard error of estimation 0.74 kg). Belowground biomass was assumed to be 0.30 of the aboveground value, in the range 0.23-0.35 previously reported for this species (Alfaro-Sánchez et al., 2015 and references therein).

### 2.3. Water use and meteorological determinations

Sap flow was measured by the heat ratio method (HRM) (Burgess et al., 2001) in four trees per treatment from April 2009 to May 2011, as described in Del Campo et al. (2014). The years 2009 and 2010 from these records were used in this study to calculate water use efficiency (WUE). An artificial neural network model (Fernandes et al., 2015) estimated water use in the months when data were missing or absent. The trees in which sap flow was measured were cored for the growth and isotope analyses. WUE in each tree was calculated for the years 2009 and 2010, as the ratio between annual biomass increment (grams of dry
weight) and tree water use (litres). Daily data on precipitation and temperature were also
available at the site. The long-term series (1960-2008) were obtained from the meteorological
stations nearest to the study site (Ayora-La Hunde from SAIH-CHJ network and Almansa
from SIAR network).

2.4 Carbon and Oxygen isotope analysis

Stable isotopes in the growth rings for the 1995 to 2010 period for the C and H98
treatments were analysed in order to explore the effect of thinning in the medium term. For
the treatments thinned in 2008, the period analysed ran from 2004 to 2010. The samples for
this isotopic analysis were taken in April 2012 from cores that had been previously measured
and dated. The sanded core surfaces were thoroughly cleaned with ethyl alcohol p.a. and
small sections, including both early and late wood, were taken with a surgical blade under the
stereomicroscope. We sampled whole wood with no previous treatment to separate fractions,
in order to minimize analysis time and resources. Recent literature has confirmed that, in
Aleppo pine, whole wood provides more consistent and coherent relationships with climatic
variables (Del Castillo et al., 2015; Klein et al., 2013b). Samples were ground in an agate
mortar and weighed to the nearest 0.001 g in Ag capsules for δ¹⁸O analysis and in Sn capsules
for δ¹³C analysis.

Isotopic signatures of the wood samples were measured on-line by an isotope ratio
mass spectrometer (Finnigan MAT, delta S, delta XL plus, delta XP) via a Conflo II interface
for the combustion/pyrolysis of organic material, at the Paul Scherrer Institut, Ecosystem
Fluxes Research Group, Villigen, Switzerland. Stable isotope data are presented as δ¹³C and
δ¹⁸O, respectively, relative to the international VPDB standard (‰).

2.5 Intrinsic water use efficiency
The isotopic discrimination (\(\Delta\) - Farquhar and Richards, 1984) between carbon from atmospheric CO\(_2\) and plant carbon from C\(_3\) plants, resulting from the preferential use of \(^{12}\)C over \(^{13}\)C during photosynthesis, is defined in Eq. 1.

\[
\Delta = \left( \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{wood}} \right) / \left( 1 + \delta^{13}\text{C}_{\text{wood}} / 1000 \right)
\]  

(1)

where \(\delta^{13}\text{C}_{\text{atm}}\) and \(\delta^{13}\text{C}_{\text{wood}}\) are the isotopic ratios of carbon (\(^{13}\text{C}/^{12}\text{C}\)) in atmospheric CO\(_2\) and plant material, respectively.

\(\Delta\) is linearly related to \(\text{Ci}/\text{Ca}\), which is the ratio of intercellular (\(\text{Ci}\)) to atmospheric (\(\text{Ca}\)) CO\(_2\) mole fractions, by Eq. 2 (Farquhar et al., 1982):

\[
\Delta = a + (b - a)\text{Ci}/\text{Ca}
\]  

(2)

where \(a\) is the fractionation during CO\(_2\) diffusion through the stomata (4.4‰; O’Leary, 1981) and \(b\) is the fractionation associated with reactions by Rubisco and PEP carboxylase (27‰; Farquhar and Richards, 1984). \(\text{Ca}\) and \(\delta^{13}\text{C}_{\text{atm}}\) were obtained for each year, using the relations found by Feng (1999), by means of equations 3 and 4, respectively.

\[
\text{Ca} = 277.78 + 1.35 \times e^{0.01572 \times (\text{year} - 1740)}
\]  

(3)

\[
\delta^{13}\text{C}_{\text{atm}} = -6.429 - 0.0060 \times e^{0.0217 \times (\text{year} - 1740)}
\]  

(4)

We used this additional information, as described in Kruse et al. (2012), to estimate \(\text{Ci}\) and intrinsic water use efficiency (WUEi) for particular years, as affected by stand development and plantation management, following thinning.

\[
\text{Ci} = \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{wood}} - a/b - a
\]  

(5)

\[
\text{WUEi} = A/g = (\text{Ca}/1.5) \times (b - \delta^{13}\text{C}_{\text{atm}} + \delta^{13}\text{C}_{\text{wood}})/(b - a)
\]  

(6)

WUEi is usually expressed in \(\mu\text{molCO}_2/\text{molH}_2\text{O}\).

**2.6. - Data treatment and analysis**
Data of TRW, δ¹⁸O and WUEi were tested for differences between the pre-treatment period (1995-1998 in H98, 2004-2007 in H, M and L treatments) and the post-treatment periods (2008-2010). In all cases the control (C) was also included. Before the analyses, data were tested for normality and variance homogeneity by the Shapiro-Wilk test and Levene tests, respectively. The t-student, ANOVA and Kruskal-Wallis tests were the procedures used for mean comparisons, using the R-package Agricolae (Mendiburu, 2014) and at p-level <0.05.

For the pair H98 and C, these analyses were also conducted in contrasting years by classifying them as dry or wet years. For this purpose, the Standardized Precipitation Index (SPI) (McKee et al., 1993) was calculated by the R-package SPEI index (Beguería and Vicente-Serrano, 2013) to adjust the precipitation series to a given Pearson III distribution probability (Pasho et al., 2011). Positive SPI values indicate greater than median precipitation; and negative values, lower than median precipitation. For the purpose of this study we considered that a drought event started when the SPI value reached -1.0 and ended when the SPI became positive again. A year was considered dry when the sum of the SPI for a 3-month time scale was less than zero; and wet, when it was greater than zero.

For the dendroclimatic analysis (influence of thinning on the dendroclimatic responses), the residual chronologies (TRWi) from Control and High 98 treatments were examined. The relationship between the residual chronologies and climate data in the time interval 1960-2010 was determined by bootstrapped response functions (Guiot, 1991). To investigate the stability of growth-climate relationships, the moving response by TreeClim R-package was used (Zang and Biondi, 2013, 2015). The coefficients of the response functions were obtained through principal-component regression (Fritts, 1976). Correlation analysis used Pearson’s product moment correlation. The significance of correlation and response function coefficients was tested at 0.05 level using 1,000 bootstrapped estimates, obtained at
random by replacement from the initial data set (Wang et al., 2012; Zang and Biondi, 2015). The temporal window used for calculating growth-climate relations extends from April of the previous year to October of the growth year for the common period (Magruder et al., 2013), which was considered adequate to explain tree-ring width variations. All statistical procedures, except a cross-dating check, were performed using R 3.1.3 GUI 1.62 (R Core Team, 2015).

3. Results

3.1. Influence of thinning in the short and medium term

Tree growth, expressed as TRW, varied a lot in the initial growth of the stand (ca. 15 years), probably related to genetics and environmental conditions. This stage was followed by a steady decrease in both magnitude and variability, probably related to increased competition between trees (Fig. 1). In the following years, TRW values were very small, but still showed responses to climate conditions, especially during dry years (e.g. 1987-1988, 1994-1995 and 2004-2005). Following thinning, the remaining trees showed significant increases in TRW compared with control ones (Fig. 1a, b).

Expressed population signals (EPS) of all TRW residual chronologies were above the critical value of 0.85 (Wigley et al., 1984). In addition, the mean of the correlations between series from each tree over all trees also indicated good agreement (>0.80). In all chronologies there were TRW index reductions in dry years, whereas periods characterized by a substantial increase in radial growth were observed in wet years and after thinning at different scales (Fig. 1c, d). Despite the differences observed after thinning, no significant differences were found in the TRW index before and after thinning (data not shown).

Table 2 summarizes the differences for mean TRW in selected years before and after thinning. There were significant differences after thinning in both the medium-term (between
C and H98) and the short-term analysis, although in this case the lowest-intensity thinning (L) did not differ in growth from the Control.

Values for WUEi ranged from 80.6 to 114.9 μmolCO₂/molH₂O (Table 2), with significant differences after thinning between H98 and C. Values were higher in the Control trees, although this trend was not always consistent over time (Fig. 2a). In contrast, no significant difference was observed in the short-term analysis between any of the thinned treatments and the control treatment (Table 2), with only a marginal difference (p<0.1) between H and C in the single-year comparison (Fig. 2b).

Values for δ¹⁸O ranged from 26.4 to 32.1‰, being very similar in all individual trees before thinning (average = 28.5±0.84‰), and increased after thinning in the short term (2008-2010) (Table 2), with significant differences between H and M treatments relative to C (Fig. 3b-d). In the case of the H98 trees, the δ¹⁸O values (Fig. 3a) were on average 1.8‰ higher than those of the C trees from 1998 to 2004, but this difference dropped in the period 2005-2009 to only 0.3‰ and became non-significant in the 2008-2010 period (Table 2). These results indicate a short-term effect of thinning on δ¹⁸O that then drops in the medium term, although splitting the sample into wet/dry years yielded significant differences (see below).

3.2. Relationships between thinning and climate

Growth, WUEi and δ¹⁸O were also compared between H98 and C trees in specific dry and wet years according to the SPI index (Fig. 1S - supplementary material). In this analysis, significant effects of thinning on the three variables in the drier years and on TRW and δ¹⁸O in the wetter years were found (Table 3), with higher growth and δ¹⁸O in H98 and higher WUEi in C. The other treatments and Control could not be compared because there were not enough wet and dry years before and after thinning. However, there were different slopes on the response of WUEi to annual precipitation after thinning (F=11.03; p=0.021) and
Taking all treatments together, WUEi increased significantly for dry years, regardless of the thinning treatment (Fig. 4).

The static bootstrapped correlation function between tree-ring residual chronologies (TRWi) and climate data (precipitation and temperature) showed different patterns between C and H98 plots, with more uniformity in the sign of correlations in C than in H98: a negative relationship with monthly temperature and a positive one with monthly precipitation (Fig. 2S supplementary material). By seasons, TRWi in C correlated with summer precipitation (June to August; r=0.50) and correlated negatively with summer mean air temperature (r=-0.44), both in the current year. For H98, only autumn temperature correlated with TRWi (September to November; r=0.37) and no significant correlation with precipitation was found.

As growth-climate relationships are not necessarily stable over time, a bootstrapped moving-response function analysis was also performed (Fig. 5). In this case, a similar pattern between monthly precipitation and growth to that described above was observed: higher and positive dependence of growth on precipitation in C trees, which increases over time. That is to say, the H98 plot showed less consistent patterns between precipitation in the current year and growth (only a clear response with May precipitation, Fig. 5b) than the C plot (Fig. 5a), which showed a higher positive response with July to September precipitation of the current year. This pattern was especially clear during the 15 years after the thinning (Fig. 5): temporal windows from 1998 onwards show that the relationship between growth and monthly precipitation in the thinned plot varies between positive and negative, i.e. without a clear pattern (significant correlations were found only in April-May in this period); whereas in the Control the frequency of positive and significant correlations between growth and precipitation, whenever it occurs, is much higher.
3.3 WU, WUE, WUEi and $\delta^{18}$O

Water use, the mean annual transpiration per tree, was greatest in the trees from the H plot, followed by H98>M>L>C, although there was no statistical significance between C, L and M (Fig. 6). On average (years 2009 and 2010), a tree from high-intensity thinning (H) transpired more than three times the amount of water transpired by a control tree. However, water use efficiency (WUE), obtained from both these data and those from the annual increment of biomass in dry weight, showed that trees from both H- and M-intensity thinning were more efficient than the C trees (Fig. 6). The other thinning treatments (L and H98) showed gradually higher WUE than the C one did, but without significant differences (C:1.26, L:1.84, M:2.97, H:3.20 and H98:2.51 g biomass/L water transpired).

These data on WUE did not show any correlation with WUEi, which showed no differences between treatments (except in drier years in the medium-term analysis). In fact, there was a clear trend for the C trees to have higher values of WUEi in the years 2009 and 2010 (Fig. 2). The units of WUEi, $\mu$mol C fixed per mol H$_2$O, are used as a relative indicator rather than an absolute value. However, it was thought useful to compare WUE and WUEi for the same trees and the same years and thus show the effect of treatments.

4. Discussion

Hydrology-oriented silviculture might assist in adapting semiarid forests to climate changes. However, to achieve this goal requires better understanding and quantification of tree-water relations and in particular of tree water use efficiency. Here, the effects of thinning in the short and medium term were studied by focusing on water use efficiency and climate-growth relationships in a semiarid, planted pine forest.
4.1. Influence of thinning in the short and medium term

Thinning induced differences in tree growth rates, as expected (Del Campo et al., 2014). The results on the isotopes showed a medium-term effect of thinning on WUEi (higher for C trees) and a short-term effect on $\delta^{18}O$ (lower for C trees). Similar findings were reported by Brooks and Mitchell (2011), with a significant increase in $\delta^{18}O$ up to 6 years after thinning (thinning removed 50% of basal area), i.e. in the short term, and with little or no response on WUEi in both the long and the short term. Independence of WUEi on stand density reduction in the short term was reported for Aleppo pine by Moreno-Gutiérrez et al. (2011). These results suggest that medium to high thinning does not improve WUEi in spite of the important physiological changes induced in the remaining trees, as seen for growth. This may be because, under our experimental conditions, the years after thinning were very similar in terms of overall precipitation, despite the differences in net rainfall between plots (Del Campo et al., 2014) and the different seasonal distribution of precipitation (Fig. 3S and 4S - Supplementary materials). This is supported by the results found for dry years (Table 3) and the significant relation between WUEi and precipitation (Fig. 4). Strong correlation between WUEi, $\delta^{18}O$ and growth with inter-annual changes in water availability is known in Pinus halepensis (Moreno-Gutiérrez et al., 2012).

Differences in $\delta^{18}O$ were also enhanced in dry years in our study. However, our $\delta^{18}O$ results do not corroborate those reported by Moreno-Gutiérrez et al. (2011), in which the remaining Aleppo pine trees in the heavily thinned treatment had lower leaf $\delta^{18}O$ than the moderately thinned ones. These authors attribute this to higher stomatal conductance due to decreased inter-tree competition for water, which is supported by Farquhar et al. (2007). In contrast, other authors (Martín-Benito et al., 2010) reported that changes in canopy microclimate after thinning (decrease in relative humidity and increase in leaf temperature) are the likely drivers for $\delta^{18}O$ cell increases, which is also supported by Farquhar et al.
(2007). Our results corroborate this latter theory, suggesting that increases in δ¹⁸O might be
due to the hotter and drier environment induced in the stand after thinning, but that this
increase might not be related to changes in stomatal conductance.

4.2. Climate growth relationships after thinning

Results have demonstrated that thinning makes trees less sensitive to water
shortages, indicating that trees in the C plot need to rely more heavily on current year
precipitation than those in H98. These results are common for several species (Martín-Benito
et al., 2010; Simonin et al., 2007; Sohn et al., 2013; Zhang et al., 2013), although no data have
been reported specifically for Aleppo pine, to the author’s knowledge. Other authors have
analysed climate-growth relations for this species (De Luis et al., 2011; Moreno-Gutiérrez et
al., 2011, 2012; Pasho et al., 2012), but no management effect was assessed. As such, our
main finding is the observed temporal trend of growth-climate relationship, varying over the
trees’ lifetime (Herrero et al., 2013; Magruder et al., 2013), especially after thinning. The
dynamics of cambial activity in Aleppo pine is characterized by two major growth phases,
one in spring (March-May) and another in autumn (September-November), interrupted in the
summer period (De Luis et al., 2011), which in turn also affects tree-ring growth (Moreno-
Gutiérrez et al., 2012). In our study, tree growth in the thinned plot (H98) showed a positive
association with the previous summer’s temperature and with May precipitation in the same
year, thus taking advantage of the rainfall increase recorded in May during the last 15 years
(Fig. 4S - Supplementary materials). On the other hand, the C plot was constrained by water
deficit (Del Campo et al., 2014): any precipitation in the growing season fostered growth,
with its relationship becoming significantly more positive as time passed and competition
among trees increased. However, these relationships were lower in H98 as a consequence of
less competition due to thinning, emphasizing the importance of water supply when the
vessels are formed (González and Eckstein, 2003).
As these results confirm that thinning increases the resilience of Aleppo pine trees to climate variations, it should be seen as an effective use of adaptive silviculture to face climate changes. However, it seems that when thinning is delayed, the results might be less effective due to more prolonged stagnation, as observed in our contrasting experimental plots (H vs H98, Fig. 2).

4.3. Interpreting trends in WUE, WUEi and $\delta^{18}O$

Our WUE results for 2009 and 2010, estimated from water use through sap flow and biomass increment measurements, indicated that all thinning treatments improved the way trees used the soil water reservoir, but to different extents. WUE grew proportionally with decreasing forest cover, as indicated by a strong increase from C to M trees and similar values for M and H trees. Moreover, a high-intensity thinning treatment (H98) also showed (though non-significant) improvement in WUE 10 years after the intervention (ca. two-fold compared with C trees), but progressively decreased when compared to H. As the biomass term in the estimation of WUE is derived from diameter-based allometric equations, we suspect that some of our treatments (especially those with low densities) are out of the range covered by the equations. This is why we used up to six allometric equations from different authors (Jenkins et al. 2003; Grünzweig et al., 2007; Alfaro-Sánchez et al., 2015 and references therein), including models derived from low-density stands. Estimates of total tree biomass indicated that variations between treatments in our equations were similar and proportional within the different models (data not shown). In a nearby region with similar site, same species and different thinning treatments, Alfaro-Sánchez et al. (2015) reported that root growth was comparatively lower (root/shoot ratio) under high density and no management. If this occurred in our study, it would enhance the differences between treatments.

In terms of hydrology-oriented silviculture, the most interesting result was found when comparing tree water use, which clearly increased from C to H trees, to tree WUE,
which had an opposite trend. This indicates that trees in the M treatment, with a moderate soil water uptake between the C, L trees and the H trees, offset their water use by high growth rates, probably due to changes in A (photosynthetic rate), as may be expected from this forest structure reduction. Hale (2003) found that light environment below canopy was exponentially modified by basal area reduction through thinning in a Sitka spruce plantation. Therefore, and considering the hydrological data for these trees at the stand scale (Del Campo et al., 2014), we hypothesize that the opening created by the M treatment represents the initial stage in which substantial modifications in microclimatic conditions are produced, affecting leaf photosynthesis by improving light intensity and leaf temperature (Aussenac, 2000). Moreover, through the H, H98 and C comparisons, we observed that WUE was still higher 10 years after thinning (H98 versus C), but less than the short-term increase (H98 versus H). This result indicates that the efficiency of silvicultural interventions on tree status may change over time (Aussenac, 2000), as could be expected, because of increased forest cover due to crown growth.

In contrast, the results from stable isotopes showed a non-significant effect of thinning on WUEi in the short term. Since WUEi estimated from $\delta^{13}C$ may change due to alterations either in A (photosynthetic rate) or gs (stomatal conductance) or both, $\delta^{18}O$ was complementarily used as an indicator of gs (Scheidegger et al., 2000). These authors described that $\delta^{18}O$ in plant tissues may reflect three different sources of variation, i) $\delta^{18}O$ in the source of water, ii) leaf water enrichment and iii) biochemical fractionation during the synthesis of organic matter. In this respect, several thinning studies have considered the $\delta^{18}O$ in the source of water and the biochemical fractionation during the synthesis of organic matter to be the same in control and thinned trees, when trees are collocated and are from the same species (Brooks and Mitchell, 2011; Martín-Benito et al., 2010). Therefore, gs changes are directly related to the leaf water enrichment led by thinning. This would suggest that our
δ^{18}O-enriched treatments (H, M and H98) showed higher gs and consequently higher water use, which is consistent with our experimental data on sapflow. However, other authors have pointed out that it is difficult to correlate δ^{18}O and gs (Barnard et al., 2012), since the environmental factors affecting leaf water enrichment should be constant among the sampled trees (Roden and Siegwolf, 2012). Our thinning treatments increased considerably the amount of water reaching the soil surface by reducing rainfall interception, but also increased evaporation from the soil (Del Campo et al., 2014; Molina and Del Campo, 2012). Therefore, we hypothesize that the assumption of constant δ^{18}O in the soil water between the sampled trees may be violated by thinning causing evaporation to increase during rainfall and by higher direct evaporation from the soil surface. However, Moreno-Gutiérrez et al. (2011) found no differences in δ^{18}O in xylem water (proxy for soil water) between moderate and heavy thinning treatments under similar experimental conditions.

To address the discrepancies we found between WUE and WUEi, we posited no differences in our source of water (as reported by Moreno-Gutiérrez et al., 2011) and thus assumed higher gs in our δ^{18}O-enriched trees (which was consistent with the sapflow results). Then, the lack of changes in WUEi as derived from δ^{13}C might be due to an underestimation of A (photosynthetic rate), which must have increased proportionally more than gs in order to agree with the WUE observed from biomass growth. Therefore, the question concerning the use of stable isotopes as a tool for determining the physiological impact of thinning remains open. Our experimental data do not support predictions drawn from the dual-isotope conceptual model (Scheidegger et al., 2000) (Table 4), assuming stomatal conductance as a proxy for WU and photosynthetic capacity as a proxy for biomass growth. Following Roden and Siegwolf (2012), this disagreement might be grounded in (i) the differences in ambient humidity and leaf temperature for different thinning treatments; the δ^{18}O enrichment of soil water could be a source of uncertainty, as previously discussed; (ii) that the ratio of vapour
pressure deficit (D) to gs was not previously studied for *P. halepensis*, making it difficult to ascertain how potential humidity variation would translate into changes in gs; (iii) both isotopes being measured in tree rings, which could show dampened signals when compared to leaf δ^{18}O; (iv) the ambient humidity/D ratios between treatments and years not remaining constant - this may well be the greatest limitation on using this model in plots with different treatments; and (v) the drought signal (common in Mediterranean summers) in isotope composition in leaves might not be well represented in tree ring material because of shut-off periods in secondary growth.

Other studies in this species also found discrepancies between WUEi from leaf-scale gas exchange measurements and from the integrated tree ring-based values (Klein et al., 2013a). To the best of our knowledge, our study is the first study in semiarid forests to compare WUE measurements at stand level, with thinning treatments, and WUEi in tree rings as a time-integrated proxy of WUE during the growing season. Consequently, more studies will be needed to clarify to what extent stable isotopes are suitable tools in forest management.

**Conclusions**

While some of the effects of thinning have been pointed out in other studies, this paper introduces a novel contribution relating water use efficiency (WUE), as measured by sap flow sensors, and intrinsic water use efficiency (WUEi) derived from stable isotope analyses in the same trees in a Mediterranean Aleppo pine plantation subjected to different thinning intensities.

Thinning induced differences in tree growth rates and changed the tree growth-climate relationships, showing higher dependence in the non-thinned plot on monthly precipitation in the current year whatever the season, whereas tree growth in the thinned plot
was more related to the expected ecophysiological pattern, i.e. precipitation in May. These results are more consistent as time has elapsed since the first thinning in 1998, creating a significant improvement in forest resilience to climate changes. The earlier high-intensity thinning treatment (H98) had a higher initial growth rate than the similar 2008 thinning treatment (H), showing that the delay in the decision to thin hampers initial growth rates.

Measurements of stable isotopes in tree rings have been said to provide powerful information about the physiological and environmental factors that control WU and WUEi. However, our study indicated slight or no changes in WUEi after thinning, and only dry years seemed to enhance WUEi, especially in the high-competing stands (control). According to $\delta^{18}$O data and the Sheidegger et al. (2000) model, this enhancement is due to an improved photosynthetic rate. These results do not corroborate the experimental ones when the sapflow technique was employed, in which thinned plots had higher water use and also higher WUE than control plots due to a significant increase in tree growth after thinning, which seemed to be underestimated when using WUEi estimated from $\delta^{13}$C. This conclusion points to the need for more detailed studies along these lines, incorporating a broader sample of years and species, before general assumptions can be made about changes in WUE in Mediterranean forests, as derived from adaptive treatments.

**Acknowledgements**

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allowing the use of the La Hunde experimental forest and for their assistance in carrying out the fieldwork. We express our gratitude to Professor R. Montes for constructive criticism and suggestions on an earlier version of the paper. The first author thanks the Mundus 17 Programme, coordinated by the University of Porto (Portugal).

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Table 1. Forest structure variables in each plot studied. DBH is average Diameter at Breast Height. Adapted from Molina and Del Campo (2012) and Del Campo et al. (2014).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cover (%)</th>
<th>Density (trees ha(^{-1}))</th>
<th>DBH (cm)</th>
<th>Mean height (m)</th>
<th>Basal Area (m(^2) ha(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>84</td>
<td>1489</td>
<td>17.8</td>
<td>11.5</td>
<td>40.1</td>
</tr>
<tr>
<td>Low intensity (L)</td>
<td>68</td>
<td>744</td>
<td>21.2</td>
<td>12.2</td>
<td>27.2</td>
</tr>
<tr>
<td>Medium intensity (M)</td>
<td>50</td>
<td>478</td>
<td>21.7</td>
<td>11.3</td>
<td>18.2</td>
</tr>
<tr>
<td>High intensity (H)</td>
<td>22</td>
<td>178</td>
<td>20.4</td>
<td>12.2</td>
<td>9.4</td>
</tr>
<tr>
<td>High intensity-1998 (H98)</td>
<td>41</td>
<td>155</td>
<td>25.2</td>
<td>12.6</td>
<td>13.6</td>
</tr>
</tbody>
</table>
Table 2. Mean values of Tree Ring Width (TRW, cm), intrinsic Water Use Efficiency (WUEi, μmolCO2/molH2O) and oxygen isotopic signature (δ18O, ‰). Mean values followed by the same letter did not differ significantly either at short-term or at mid-term (p<0.05).

<table>
<thead>
<tr>
<th>Time span</th>
<th>Thinning</th>
<th>Treatment (thinning intensity)</th>
<th>TRW*</th>
<th>WUEi</th>
<th>δ18O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-term (1995-2010)</td>
<td>Before ('95-'97)</td>
<td>Control</td>
<td>0.09 (a)</td>
<td>98.5 (a)</td>
<td>28.7 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High98</td>
<td>0.06 (a)</td>
<td>97.4 (a)</td>
<td>28.9 (a)</td>
</tr>
<tr>
<td></td>
<td>After ('08-'10)</td>
<td>Control</td>
<td>0.06 (b)</td>
<td>94.4 (a)</td>
<td>28.2 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High98</td>
<td>0.17 (a)</td>
<td>88.7 (b)</td>
<td>29.0 (a)</td>
</tr>
<tr>
<td>Short-term (2004-2010)</td>
<td>Before ('04-'07)</td>
<td>Control</td>
<td>0.05 (a)</td>
<td>100.4 (a)</td>
<td>28.2 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>0.07 (a)</td>
<td>101.8 (a)</td>
<td>28.3 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>0.06 (a)</td>
<td>96.4 (a)</td>
<td>28.1 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0.05 (a)</td>
<td>100.5 (a)</td>
<td>28.2 (a)</td>
</tr>
<tr>
<td></td>
<td>After ('08-'10)</td>
<td>Control</td>
<td>0.07 (c)</td>
<td>94.4(a)</td>
<td>28.2 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>0.42 (a)</td>
<td>97.6 (a)</td>
<td>29.3(a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>0.19 (b)</td>
<td>94.1 (a)</td>
<td>29.4 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0.12 (bc)</td>
<td>92.1 (a)</td>
<td>28.5 (ab)</td>
</tr>
</tbody>
</table>

* Mean and differences in TRW refer only to those trees sampled for isotopes analyses.
Table 3. Mean values of Tree Ring Width (TRW, cm), intrinsic Water Use Efficiency (WUEi, μmolCO2/molH2O) and oxygen isotopic signature (δ18O, ‰). Mean values between treatments followed by the same letter did not differ significantly. * and ** indicate significant differences at p<0.05 and p<0.01 respectively.

<table>
<thead>
<tr>
<th>Time</th>
<th>Year type</th>
<th>Treatment</th>
<th>TRW</th>
<th>WUEi</th>
<th>δ^{18}O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before thinning</td>
<td>Dry</td>
<td>Control</td>
<td>0.06 (a)</td>
<td>101.4 (a)</td>
<td>28.5 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H98</td>
<td>0.06 (a)</td>
<td>99.3 (a)</td>
<td>29.3 (a)</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>Control</td>
<td>0.12 (a)</td>
<td>94.8 (a)</td>
<td>28.7 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H98</td>
<td>0.07 (a)</td>
<td>93.8 (a)</td>
<td>28.9 (a)</td>
</tr>
<tr>
<td>After thinning</td>
<td>Dry</td>
<td>Control</td>
<td>0.04 (b)**</td>
<td>100.7 (a)**</td>
<td>28.4 (b)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H98</td>
<td>0.33 (a)**</td>
<td>96.7 (b)**</td>
<td>29.8 (a)**</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>Control</td>
<td>0.06 (b)**</td>
<td>97.3 (a)</td>
<td>28.2 (b)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H98</td>
<td>0.37 (a)**</td>
<td>94.2 (a)</td>
<td>29.5 (a)*</td>
</tr>
</tbody>
</table>
Table 4. Results of the dual-isotope model (Scheidegger et al. 2000) after applied to our experimental data (2009-2010): relative variation in $\delta^{13}$C, $\delta^{18}$O, tree water use (WU) and biomass growth between the different thinning treatments and the control. Up or downward arrows represent increasing or decreasing values, $\approx$ indicates insignificant changes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>This paper’s data (2009-2010)</th>
<th>Relative variation to the Control (treatment value / control value)</th>
<th>Sheidegger et al. (2000) Theoretical variation expected for stomatal conductance, $g$ (proxy for WU) and photosynthetic capacity, $A_{\text{max}}$ (proxy for biomass growth)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta^{13}$C</td>
<td>$\delta^{18}$O</td>
<td>WU</td>
</tr>
<tr>
<td>Low</td>
<td>1.004</td>
<td>1.022</td>
<td>1.109</td>
</tr>
<tr>
<td>Medium</td>
<td>1.003</td>
<td>1.060</td>
<td>1.054</td>
</tr>
<tr>
<td>High</td>
<td>0.994</td>
<td>1.044</td>
<td>3.592</td>
</tr>
<tr>
<td>High 98</td>
<td>1.020</td>
<td>1.037</td>
<td>2.347</td>
</tr>
</tbody>
</table>
Figure captions

Fig. 1. Temporal dynamics of mean Tree Ring Width (TRW, cm; a: 1960-2010, b: 1995-2010) and the residual chronologies (TRW index, c: 1960-2010, d: 1995-2010) in each treatment. Arrows indicate the thinning dates.

Fig. 2. Intrinsic Water Use efficiency (WUEi, μmolCO2/molH2O) by single years. a: Control and High-intensity thinning in 1998, b: Control and High-intensity thinning in 2008, c: Control and Medium-intensity thinning and d: Control and Low-intensity thinning. Data shown are average values ± SD. A star near the axis indicates significant difference at p<0.05; a double star indicates significant difference at p<0.01; and a dot indicates marginal difference (p<0.1) in that year.

Fig. 3. Tree-ring Oxygen isotopic signature (δ18O, ‰). a: Control and High-intensity thinning in 1998, b: Control and High-intensity thinning in 2008, c: Control and Medium-intensity thinning and d: Control and Low-intensity thinning. Data shown are average values ± SD. A star near the axis indicates significant difference at p<0.05; a double star indicates significant difference at p<0.01; and a dot indicates marginal difference (p<0.1) in that year.

Fig. 4. Relationships between mean intrinsic water use efficiency (WUEi, μmolCO2/molH2O) and total precipitation (Ppt, mm) for all treatments and years together. The black line and equation refer to all treatments together.

Fig. 5. Moving response functions of precipitation (ppt) from April of previous (prev) year to October of current (curr) year for a residual tree-ring chronology (TRWi). The window size is fourteen years; the windows have been offset by one year. a: Control plot; b: Thinned plot (H98). In each cell, the intensity of the colour represents the magnitude of the correlation.
(positive or negative) between growth and monthly precipitation for that temporal window. Asterisks in a cell indicate significant correlations at p<0.05. A clear change in trend can be observed in the thinned plot after treatment took place (1998). For a better interpretation of colours in this Figure, see the web version of this article.

Fig. 6. Mean annual WUE (g of dry weight increment / L of water transpired per tree) and water use (L of water transpired per tree) for the years 2009 and 2010 in the different treatments. Treatments with different letters indicate significant differences at p<0.05. Different case letters mean different analyses.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.

\[ WUEi = 109.21 - 0.0244 \times \text{ppt} \]

\[ R^2 = 0.48 \quad \text{p-value} < 0.001 \]
Fig. 6.
Fig. 1S. Evolution of the drought index (SPI) at different time scales (1, 3, 12 months respectively) in the study area for the period 1961 to 2010. Grey-shaded area refers to years 2009 and 2010 corresponding to the period of measurement of water use (tree transpiration) in this study.
Fig. 2S. Static bootstrapped correlation function (Zang and Biondi, 2013) for temperature and precipitation from April of previous year to October of current year. a: Control plot, b: Thinned plot (H98). The darker bars indicate a coefficient significant (p<0.05); the lines represent the 95%-confidence interval. Lowercase letters represent the month of the previous year (April to December) and uppercase letters, month of the current year (January to October)
Fig. 3S. Seasonal values of total precipitation during the period 1960-2011, following the atmospheric circulation patterns as described in Pasho et al. (2011). Spring (April-May), Summer (June-August), Autumn (September-November) and winter (December-March).

Fig. 4S. Climatic diagram at La Hunde site. Grey and dark grey bars indicate total monthly precipitation (mm) in the period 1960 to 2010 and 1995 to 2010 respectively. Continuous and dashed line represent monthly mean temperature (°C) in the period 1960 to 2010 and 1995 to 2010 respectively.