Photo-oxidative stress markers reveal absence of physiological deterioration with ageing in *Borderea pyrenaica*, an extraordinarily long-lived herb

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Summary

1. In animals, age-associated disorders are believed to be connected to shifts in the antioxidant/pro-oxidant balance in favour of oxidative stress. However, the contribution of oxidative stress to ageing in long-lived perennials has not been explored to date.
2. Here, we tested age- and sex-related changes in several photo-oxidative stress markers in *Borderea pyrenaica*, a small dioecious geophyte relict of the Tertiary with one of the longest life spans ever recorded for a non-clonal herb (more than 300 years). Given that survival increases with age in *B. pyrenaica*, we hypothesized that oxidative stress does not increase with ageing because the species develops improved anti-oxidant defence.
3. In three field samplings performed during 2008, 2010 and 2011 in the Central Pyrenees (NE Spain), we examined the effects of ageing and sex on photosynthetic pigment levels, PSII integrity (Fv/Fm ratio), lipid peroxidation, and the extent of photo and anti-oxidant protection in chloroplasts. Furthermore, we explored whether age and sex affect plant response to severe natural desiccation.
4. Both male and female plants maintained chlorophyll levels intact, as well as the Fv/Fm ratio and the levels of lipid peroxidation, irrespective of age. This finding suggests the absence of age-associated oxidative stress at the organismal level. Furthermore, photoprotection mechanisms were found to be similarly efficient in the oldest individuals as in juvenile plants, in terms of xanthophyll cycle de-epoxidation and accumulation of low-molecular-weight antioxidants (carotenoids and tocopherols). Indeed, females over 100 years of age were the most resistant to severe desiccation, maintaining higher leaf hydration levels, less chlorophyll degradation and better PSII integrity under stress than females below 100 years, males below or above 100 years, and juveniles.
5. Synthesis. Neither males nor females of the extraordinarily long-lived *B. pyrenaica* show age-dependent signs of oxidative stress. This observation suggests that age-induced oxidative stress is not a universal feature of ageing in perennial plants. Indeed, females older than 100 years showed signs of negative senescence, in that they registered improved physiological performance with increasing age.

Key-words: ageing, anti-oxidants, dioecy, ecophysiology, geophyte, maturity, negative senescence, oxidative stress, perennial herb, photoprotection

Introduction

The ageing of an organism is usually associated with a decline in physiological functions. At advanced stages, this deterioration is reflected by decreased fecundity and increased mortality risk (Ricklefs 2008). Studies on ageing at the organismal level are common in model plants like the annual *Arabidopsis* and rice (Noodén & Leopold 1988; Buchanan-Wollaston et al. 2003; Lim & Nam 2005). However, little attention has been devoted to ageing in perennials, thus limiting our understanding of the process and its extent and severity in the tree of life. In addition, most studies on ageing in perennials have focused on cells or organs (leaves, flowers, fruits or seeds, Franzese & Ghermandi 2011; Arrom & Munné-Bosch 2012; Sun et al. 2012; Wingler et al. 2012). Consequently, our knowledge of senescence at the organismal level in perennials is still very limited (Munné-Bosch 2007, 2012).
2008; Watson & Riha 2010; Thomas 2013). The concept of ageing is widely assumed to be associated with degenerative processes (senescence). However, there is little evidence demonstrating a negative impact of age on the physiology of long-lived perennials (Thomas 2002, 2013; Moncuccini et al. 2005; Munné-Bosch 2007, 2008; Peñuelas & Munné-Bosch 2010; Watson & Riha 2010, 2011). Indeed, some studies suggest that the decrease in age-related performance in perennial plants is negligible (Finch 1998) or even negative (Vaupel et al. 2004).

Several theories have been proposed to explain the senescence phenomenon in animals. These include the free radical theory [accumulation of reactive oxygen species (ROS), Harman 1956;], the hormonal theory (focused on the loss of the reproductive component of fitness, Bowen & Atwood 2004), the genetic control of senescence (related to telomere shortening caused by a loss of telomerase activity with age, Olovnikov 1973) and evolutionary theories (accumulation of somatic mutations as the organism ages, Medawar 1952), among others. However, none of these theories have provided a universal explanation of senescence, and all of them have been at least partially inconclusive in perennial plants (Ally, Ritland & Otto 2010; Watson & Riha 2010, 2011). Recent studies suggest that, contrary to animals, perennial plants defy senescence mainly because of differences in development, such as the modular growth and the totipotency of plant meristems, which can remain viable after long periods of dormancy (Thomas 2002; Munné-Bosch 2008; Peñuelas & Munné-Bosch 2010).

At the organ level (e.g. leaves, flowers), ageing has been demonstrated to be related to an increase in ROS production (Dhindsa, Plumb-Dhindsa & Thorpe 1981; Sohal & Weindruch 1996; Procházková & Wilhelmová 2007). This oxidative stress is linked to a defence response underlying cellular redox processes. These play an essential role in plant stress tolerance, but at high concentrations, they can also eventually lead to a decrease in membrane stability, an increased rate of lipid peroxidation and reductions in the levels of certain antioxidant enzymes and molecules that confer protection against oxidative damage (Dhindsa, Plumb-Dhindsa & Thorpe 1981; Zimmermann & Zentgraf 2005; Munné-Bosch, Queval & Foyer 2013). However, information in the literature on age-related oxidative stress (considering ageing at the organismal level) is restricted to one species, namely Citrus clusii (Cistaceae), a Mediterranean shrub with a life span of about 15 years (Munné-Bosch & Alegre 2002; Munné-Bosch & Laheza 2007). Results from studies of this perennial plant show that ageing increases the susceptibility of this shrub to oxidative stress, but only in individuals exposed to environmental stress (summer drought). Ageing therefore seems to increase the sensitivity of organisms to oxidative stress, as occurs in humans (Harman 1956).

Another important point to be considered in the ageing process is the role of recombination as a fitness component, which is expected to decrease with age, and the biological reproducti

Ageing in an extraordinarily long-lived herb

endangered by the regional government (Viñales, Sánchez & Alcántara 2007). The aerial part of the plant (stems, leaves and flowers) is performed the previous year and overwinters in a bud, developing quickly in June (Fig. S1 in Supporting Information). Female plants rapidly produce mature fruits during July. The first signs of leaf senescence (chlorosis) appear late in August, and all aerial parts are dead by mid-September. The aerial stem leaves a scar on the bulb, allowing estimation of the age of an individual by counting the number of scars (García & Antor 1995a). Borderae pyrenaica is a strictly sexually reproducing herb, and it has one of the longest life spans ever reported for non-clonal plants (> 300 years old; García & Antor 1995a).

**SAMPLING DESIGN**

The population of *B. pyrenaica* studied is located in the Pineta valley (42°41' N, 0°06' E, 2000 m a.s.l.; Spanish Pyrenees), where several thousand individuals grow in high densities on a north-facing rocky hillside (Fig. S1). The experimental design involved descriptive fieldwork and experimental manipulation in the laboratory. Studies in the field with male and female plants of different ages were performed to evaluate possible age- and sex-related increases in oxidative stress. These experiments were carried out during three campaigns in July of 2008, 2010 and 2011, when plants were flowering and males and females could be easily identified. Leaf samples were taken on clear sunny days. Several indicators of leaf physiological status (water content, oxidative stress and photoprotection mechanisms) were measured in samples collected at midday (at maximum incident photosynthetically active photon flux density (PPFD)). For biochemical analyses, samples were frozen in situ in liquid nitrogen and transported to the laboratory, where they were stored at −80 °C until analyses.

Climatological conditions were monitored throughout the 3-year study to evaluate whether possible age- or gender-related effects were associated with climatic differences between years. The climate conditions during the study were typical of high altitudes (2000 m a.s.l.) in the Central Pyrenees, with a yearly rainfall above 800 mm, mostly falling during the spring and autumn, and low mean monthly temperatures during winter (November–February, Fig. S2 in Supporting Information). The driest year was 2011, particularly during the spring and summer. A similar overall amount of rain was received by plants during 2008 and 2010; however, precipitation patterns differed along the years, being more uniformly distributed in 2008 and more irregularly in 2010 (Fig. S2).

Laboratory experiments were also conducted on plants from the same population to examine their response to severe desiccation. For this purpose, we collected a total of 15 juveniles, 22 and 19 males, and 24 and 21 females under and over 100 years old, respectively, during July 2011. The plants were then brought to the laboratory and immediately subjected to severe water stress conditions by keeping them on the bench without a water supply. Plants were desiccated for 2 weeks at constant PPFD values below 10 µmol m−2 s−1 and a relative humidity of 50–60%. Chlorophyll fluorescence was measured every second day up to day 9 after the onset of experimental drought. After that (day 10), a sample of leaves was used to estimate water content while another sample was frozen in liquid nitrogen and later stored at −80 °C until biochemical analyses of oxidative stress and photoprotection mechanisms.

**AGE ESTIMATION**

At the end of the experiment, bulbs were inspected carefully under magnifying glasses to estimate plant age, following García & Antor (1995a; see Fig. S1 for details). Age ranged between 1 and 245 years for individuals studied in the field and between 1 and 280 years for those used in the laboratory experiment. Plants were grouped into three classes for subsequent analysis: juveniles (non-reproductive plants under 50 years), males and females. Juveniles were considered to be plants under 50 years of age and without any reproductive structures (García & Antor 1995a). As all plants were flowering during samplings (all performed during July), these two parameters allowed us to identify juvenile plants as such. It should not be discarded that some juveniles could be confounded with young females that did not bloom the year of sampling; however, this is very unlikely due to size differences. For some analyses, flowering plants were grouped under and over 100 years of age.

**LEAF WATER STATUS**

Leaf water status was measured in the field studies and laboratory experiments to evaluate possible age- and gender-related differences in the physiological status of leaves. Leaf water content was expected to decrease as a result of a physiological deterioration with age and to be dramatically affected in desiccation experiments. Leaf water status was estimated by the following two indexes: the relative water content (RWC) of leaves as RWC (%) = (fresh weight−dry weight)/(turgid weight−dry weight) × 100 and the leaf hydration (H) as H = (fresh weight−dry weight)/dry weight).

**PHOTO-OXIDATIVE STRESS MARKERS**

Measurements of the maximum efficiency of photosystem II photochemistry (Fv/Fm, ratio), levels of photosynthetic pigments, chlorplastic anti-oxidants (carotenoids and tocopherols) and the extent of lipid peroxidation in leaves were used to examine the extent of foliar physiological deterioration with age. The Fv/Fm ratio and the levels of photosynthetic pigments and tocopherols were expected to decrease with photo-oxidative stress in chloroplasts. The extent of lipid peroxidation, as determined by measuring bulk malondialdehyde (MDA) levels, was expected to increase with an overall oxidative stress at the cellular level. The Fv/Fm ratio was calculated following Van Kooten & Snel (1990). For this purpose, we used chlorophyll fluorescence data obtained with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany) in leaves maintained for at least 1 h in darkness. The levels of photosynthetic pigments (chlorophylls and carotenoids) and tocopherols were measured by HPLC, as described by Munné-Bosch & Alegre (2000) and Cela, Chang & Munné-Bosch (2011), respectively. The extent of lipid peroxidation was estimated from the amount of MDA in leaves, following the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

**STATISTICAL ANALYSES**

The relationships between measurements of the response variables obtained in the field (RWC, H, Fv/Fm, ratio, photosynthetic pigments, tocopherols and lipid peroxidation) and age were tested by linear regression using Spearman's rank correlation, separately considering the three reproductive effort groups: juveniles, males and females. In addition, age, group (males, females and juveniles) and year (2008, 2010 and 2011) effects were tested using an analysis of covariance.
Results

LEAF WATER CONTENT UNDER NATURAL CONDITIONS

Age-related effects on leaf water status were examined separately for juveniles, males and females (the oldest sampled male and female individuals were 245 and 220 years old, respectively), and considering the climatic variability during the 3 years of the study (2008, 2010 and 2011). The RWC was around 80% at all ages, but variability between individuals and gender increased during 2010 and 2011 (Fig. 1). This increase may have been associated with drier conditions during the growing period (May and June) in those years compared with 2008 (Fig. S2). An age-related decrease in RWC was observed only for juvenile and male plants in 2010 (Fig. 1, Table 2). None of the groups showed age-related differences in leaf hydration. A comparative analysis of average RWC values between groups during the study revealed that while RWC values averaged around 80% in males and females, water contents were more variable in juveniles, with RWC values ranging between 70.7% and 89% (Table S1 in Supporting Information). However, analyses of covariance indicated that neither RWC nor H was significantly affected by age or reproductive status (Table 1).

PHOTO-OXIDATIVE STRESS MARKERS UNDER NATURAL CONDITIONS

The effects of age, maturity and sex on photo-oxidative stress at the organismal level were evaluated on the basis of chlorophyll content (Chl \( a+b \)), the Chl \( a/b \) ratio and the maximum efficiency of PSII photochemistry (\( F_{v}/F_{m} \) ratio), together with the levels of MDA, an indicator of the extent of lipid peroxidation in leaves. Chlorophyll levels were not negatively affected by age in males or females in any of the 3 years of study, and only decreased significantly with age in juveniles in 2008 (Fig. 2). Indeed, males showed a significant increase in chlorophyll levels with increasing age during 2011. Nonetheless, this pattern was not observed during 2008 or 2010 (Fig. 2 and Table 2), nor did age influence the Chl \( a/b \) or \( F_{v}/F_{m} \) ratios in juveniles and females in any of the sampling years, but the \( F_{v}/F_{m} \) ratio increased with age in males during 2008 and 2011. This pattern was not confirmed in samples collected during 2010, the year with the smallest variability in this parameter (Fig. 2, Table 2). Similarly, MDA levels did not show any relationship with age for any year sampled, thus indicating constant lipid peroxidation levels within groups and across years (Fig. 2). Analyses of covariance revealed that neither Chl, MDA levels nor the \( F_{v}/F_{m} \) ratio were significantly affected by age or reproductive status (Table 1).

PHOTOPROTECTION MECHANISMS UNDER NATURAL CONDITIONS

Given that the xanthophyll cycle is involved in one of the main photoprotection mechanisms of plants, we examined the...
levels of the xanthophyll cycle pool (VZA, the sum of violaxanthin, antheraxanthin and zeaxanthin), together with the de-epoxidation state of xanthophyll cycle (DPS) in samples collected during 2010 and 2011.

Table 1. P-values of the analysis of covariance (ancova) to test for the effects of year (2008, 2010 and 2011), reproductive status (group: juveniles, males, females), age (as covariate), and group × age interaction, on several indexes of leaf water status, photo-oxidative stress and photoprotection in Bororeoa pyrenaica under field conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age</th>
<th>Group</th>
<th>Year</th>
<th>Group × Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWC</td>
<td>0.291</td>
<td>0.623</td>
<td>0.092</td>
<td>0.014</td>
</tr>
<tr>
<td>H</td>
<td>0.728</td>
<td>0.281</td>
<td>&lt; 0.001</td>
<td>0.339</td>
</tr>
<tr>
<td>Chl a + b</td>
<td>0.902</td>
<td>0.534</td>
<td>&lt; 0.001</td>
<td>0.102</td>
</tr>
<tr>
<td>Chl a/b</td>
<td>0.811</td>
<td>0.805</td>
<td>0.192</td>
<td>0.583</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.494</td>
<td>0.195</td>
<td>&lt; 0.001</td>
<td>0.295</td>
</tr>
<tr>
<td>MDA</td>
<td>0.531</td>
<td>0.518</td>
<td>&lt; 0.001</td>
<td>0.398</td>
</tr>
<tr>
<td>VZA</td>
<td>0.412</td>
<td>0.949</td>
<td>0.001</td>
<td>0.854</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.622</td>
<td>0.922</td>
<td>0.021</td>
<td>0.977</td>
</tr>
<tr>
<td>DPS</td>
<td>0.663</td>
<td>0.938</td>
<td>&lt; 0.001</td>
<td>0.989</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.743</td>
<td>0.783</td>
<td>&lt; 0.001</td>
<td>0.545</td>
</tr>
<tr>
<td>P-Car</td>
<td>0.820</td>
<td>0.837</td>
<td>&lt; 0.001</td>
<td>0.845</td>
</tr>
<tr>
<td>y-Toc</td>
<td>0.980</td>
<td>0.129</td>
<td>0.001</td>
<td>0.050</td>
</tr>
<tr>
<td>a-Toc</td>
<td>0.362</td>
<td>0.084</td>
<td>0.001</td>
<td>0.034</td>
</tr>
</tbody>
</table>

P < 0.0038 (Bonferroni adjusted) shown in bold.

Fig. 2. Chlorophyll (Chl) a + b levels, Chl a/b ratio, maximum efficiency of PSII photochemistry (Fv/Fm ratio) and levels of malondialdehyde (MDA), an indicator of lipid peroxidation, in leaves of juvenile (orange circles), male (brown circles) and female (blue triangles) plants in three independent samplings performed during July 2008, July 2010 and July 2011. The parameters were correlated with plant age by Spearman’s rank correlation. P < 0.05 are considered significant and are given in the insets.

Plant response to simulated severe desiccation

Plant performance after severe desiccation in the laboratory was evaluated by measuring leaf water status, photo-oxidative stress markers and the amount of photoprotective molecules. We tested for differences between juveniles, males and females, grouping individuals above and below 100 years of age. The RWC decreased from around 89% in juveniles and 77% in both males and females under natural conditions (Fig. 1 and Table S1) to around 50% in experimental drought conditions (Fig. 3), and there were no significant differences between the aforementioned groups under water stress (Fig. 3). However, leaf hydration (H) values were higher among the oldest desiccated females (over 100 years of age), than in the remaining groups under similar experimental conditions. Likewise, the Chl a + b levels were higher in centenarian females compared with the other groups. No differences were observed in the Chl alb ratio between plant groups (Fig. 3). The Fv/Fm ratio decreased with length of desiccation, and differences between groups were particularly evident after 7 and 9 days of water stress, centenarian females showing the highest Fv/Fm ratios, followed by females under 100 years of age (Fig. 4). Males were equally as sensitive to severe desiccation as juveniles, irrespective of age (Fig. 4).

Finally, the levels of antioxidant molecules (carotenoids and tocopherols), together with MDA, confirmed that centenarian females were the group with the highest photoprotection capacity, as indicated by higher levels of VZA, and most particularly violaxanthin (Fig. 5). No differences between plant groups were observed in the amount of lutein, β-carotene, tocopherols or MDA (Fig. 5).

Discussion

Borderea pyrenaica was used to evaluate the effects of individual age and reproductive status on leaf water content, photo-oxidative stress markers and the accumulation of photoprotective molecules. To date, very few studies have considered age as an intrinsic factor triggering physiological degenerative processes at the organismal level in perennial plants (for a complete list to our knowledge, see Munné-Bosch & Alegre 2002; Mencuccini et al. 2005; Munné-Bosch & Lalueza 2007; Oíate & Munné-Bosch 2009; Alí, Ritland & Otto 2010; Hernández, Alegre & Munné-Bosch 2011). The present study is the first specifically designed to test for age-related changes in oxidative stress markers in a very long-lived perennial. The oldest plant used for field studies was 245 years old and while for laboratory experiments, it was 280, although the maximum life span is estimated to be ca. 350 years (M. Oíate, unpubl. data). We evaluated whether demographic, negligible or negative senescence in this long-lived perennial geophyte (Garcia et al. 2010) correlated with improved physiology, and the extent to which photo-oxidative stress markers are indicative of degenerative processes with age in this long-lived herb, as occurs in short-lived annual plants, such as Arabidopsis thaliana (Wingerter et al. 2006; Abreu & Munné-Bosch 2009).
Table 3. Correlation coefficient ($r^2$) and $P$-values (in parentheses) of Spearman's rank correlation analysis to correlate the photoprotection parameters [xanthophyll cycle pool (VZA), zeaxanthin, de-epoxidation state of the xanthophyll cycle (DPS), levels of lutein, $\beta$-carotene, and $\gamma$- and $\alpha$-tocopherols] with plant age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2010</th>
<th></th>
<th></th>
<th>2011</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Juvenile</td>
<td>Male</td>
<td>Female</td>
<td>Juvenile</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>VZA</td>
<td>0.282 (0.175)</td>
<td>-0.159 (0.181)</td>
<td>-0.004 (0.491)</td>
<td>-0.401 (0.062)</td>
<td>-0.212 (0.207)</td>
<td>-0.090 (0.370)</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>-0.110 (0.360)</td>
<td>-0.283 (0.050)</td>
<td>0.406 (0.013)</td>
<td>-0.062 (0.410)</td>
<td>-0.347 (0.086)</td>
<td>-0.028 (0.459)</td>
</tr>
<tr>
<td>DPS</td>
<td>-0.097 (0.377)</td>
<td>-0.176 (0.156)</td>
<td>0.383 (0.018)</td>
<td>-0.064 (0.408)</td>
<td>-0.372 (0.071)</td>
<td>-0.177 (0.256)</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.475 (0.050)</td>
<td>-0.167 (0.168)</td>
<td>0.175 (0.178)</td>
<td>-0.365 (0.082)</td>
<td>0.103 (0.347)</td>
<td>-0.289 (0.139)</td>
</tr>
<tr>
<td>$\beta$-Carotene</td>
<td>0.506 (0.039)</td>
<td>-0.148 (0.198)</td>
<td>0.234 (0.107)</td>
<td>0.016 (0.476)</td>
<td>0.142 (0.293)</td>
<td>-0.293 (0.135)</td>
</tr>
<tr>
<td>$\gamma$-Tocopherol</td>
<td>0.213 (0.243)</td>
<td>0.261 (0.065)</td>
<td>-0.073 (0.356)</td>
<td>0.464 (0.035)</td>
<td>0.002 (0.497)</td>
<td>-0.258 (0.168)</td>
</tr>
<tr>
<td>$\alpha$-Tocopherol</td>
<td>0.177 (0.282)</td>
<td>0.128 (0.232)</td>
<td>-0.032 (0.436)</td>
<td>-0.028 (0.459)</td>
<td>0.114 (0.327)</td>
<td>-0.439 (0.026)</td>
</tr>
</tbody>
</table>

$P < 0.05$ shown in bold.

Fig 3. Relative water content (RWC), hydration (H), chlorophyll (Chl) $a + b$ levels and the Chl $a/b$ ratio of leaves of juvenile and mature plants, considering 2 age groups in the latter group (below and above 100 years), desiccated in the laboratory for 9 days. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with plant age and sex as factors. Different letters indicate differences between age groups using Tukey's post hoc analyses (Tukey's test, $P < 0.05$). Data represent the mean ± SE of $n = 15$ for juveniles, and 22 and 19 males and 24 and 21 females below and above 100 years, respectively. NS, not significant.

Individuals of $B$. pyrenaica did not show any sign of physiological deterioration with age in terms of photo-oxidative stress. Oxidative stress resistance may be behind the absence of age-related degenerative processes in the extremely long-lived herb $B$. pyrenaica. Not only were no signs of physiological deterioration, in terms of photo-oxidative stress markers, observed at advances ages in field studies, but females older than 100 years showed improved performance in response to severe desiccation in laboratory experiments, as indicated by enhanced leaf water status and decreased degradation of photosynthetic pigments. Changes in photosynthetic pigments not only included a higher retention of chlorophylls under stress but also higher levels of xanthophyll cycle pigments, which play an essential role in plant protection against oxidative stress (Dall'Osto et al. 2012). These results could therefore explain the higher PSII integrity in females over 100 years of age in the laboratory experiments.

Extrinsic factors play a critical role in physiological ageing. A study of the shrub $C$. elusii, with a life span of about 15 years, demonstrated that photo-oxidative stress in leaves increased with age only during summer droughts, a typical Mediterranean combination of water deficit and high solar...
radiation (Munné-Bosch & Latueza 2007). In the present study, no signs of photo-oxidative stress were observed within the unusually long life span of *B. pyrenaica*, despite the fact that field samplings were performed on clear sunny days and therefore at high solar light intensities of around 2000 μmol m⁻² s⁻¹, and temperature may exceed 35 °C on scree surfaces. Furthermore, the pattern observed was consistent across years despite substantial differences in natural conditions (2010 and 2011 were drier than 2008), which may increase ROS production in chloroplasts. Indeed, juvenile plants and males showed a decrease in RWC with age during 2010. This reduction could be associated with decreased water availability during May, June and July, a common pattern described in other species (Bray 1997; Larcher 2003). These adverse conditions did not lead to increased photo-oxidative stress in the oldest individuals, thus supporting the notion that this species is not subjected to senescence. For instance, the oldest males did not show increased photooxidative stress compared with their younger counterparts, and the oldest females did not show any sign of water stress despite their higher reproductive effort and the possible sink effect exerted by large developing fruits. Indeed, females showed increased xanthan and DPS values with age during 2010, which suggests increased photo-protection as ageing progresses. These observations therefore indicate that females were not only more efficient than males at maintaining water homeostasis under changing climatic conditions, but also displayed greater higher photoprotection capacity. It is also noteworthy that the *Fv/Fm* ratio rose with increasing age in males during 2008 and 2011 (*P* = 0.049 and 0.001, respectively). This increase was due to decreased PSII integrity (smaller *Fv/Fm* ratios) in younger individuals compared with the oldest ones, thus suggesting that the oldest males responded similarly (2010) or even better (2008 and particularly 2011) than their younger counterparts in periods of photo-oxidative stress.

Plant performance after severe experimental desiccation in the laboratory supported the pattern found in the field. Although the biological significance of results obtained in this way is limited by the fact that de-rooted bulbs do not necessarily reflect what would happen in nature, these results give us an idea of the potential of this particular species to respond to extreme desiccation. The oldest females (aged above 100 years) showed the greatest resistance to desiccation as indicated by the time course evolution of *Fv/Fm* ratios, followed by females younger than 100 years, while males and juveniles showed a similar response. Females also displayed a greater capacity to withstand extreme desiccation, as indicated by higher RWC values, *Fv/Fm* ratios and an increased retention of xanthophylls under severe stress. Given that similar results were obtained in de-rooted plants and in aerial parts without bulbs (data not shown), we propose that the resistance to simulated severe stress, like that performed in laboratory conditions, lies partly in the somatic (leaf) tissues, rather than in an increased accumulation of water and/or nutrient in bulbs in females. The bulb and the root system also play a major role in controlling water homeostasis in this species. Males and females with larger bulbs and roots showed less variation in RWC values between years compared with juveniles, which may be more susceptible to variable climatic conditions.

Apart from the capacity of plants to respond to variable climatic conditions, several factors may additionally contribute to the extraordinary life span of *B. pyrenaica* and other small perennial geophytes. Several studies propose that size is a key determinant in age-related decline in growth vigour of mature trees, such as the Scots pine (*Pinus sylvestris*, Mencuccini et al. 2005). Therefore, it is likely that limited size protects small geophytes from some size-related physiological deterioration that occurs with ageing in several other larger perennials, such as shrubs and particularly trees. In *Urtica dioica*, size was not found to be responsible for the maturity-related declines in growth vigour (Oñate & Munné-Bosch 2009), and reproduction was proposed as the main cause for such a decline and the shift to perenniality of non-reproductive shoots. In *B. pyrenaica*, the alternative use of 5 meristems (only one is activated each year) may allow a 5-fold reduction in the potential physiological deterioration with age, implying that a 200-year-old plant has a 'physiological' age of 40 years in terms of meristem ageing (Oñate, García & Munné-Bosch 2012). This could explain the absence of photo-oxidative stress in this species and the several-fold reduction in 'physiological' ageing, which has been considered of capital importance in decreasing mortality rates in both plants and animals, including human populations in the change from hunter-gatherers to today's populations with the lowest ever mortality (Burger, Bandisch & Vaupel 2012). The alternate use of meristems may decrease the potential harmful effects of deleterious mutations and seasonal shoot development (with leaves, flowers and fruits appearing during spring and summer only,
and being absent during most of the year, except the bulb), thus preventing potential physiological deterioration above-ground during most of the year. Furthermore, B. pyrenaica is one of the slowest growing plants ever reported, because bulbs of centenarian individuals weigh less than 5 g (García & Antor 1995a). Therefore, the amount of biomass accumulated over centuries is very small, and consequently, the need to maintain permanent viable structures is limited to the bulb only. Furthermore, this bulb is protected inside rock crevices and thus is protected from predation. All these factors can explain the extraordinary longevity of this small geophyte.

Another point to be considered in the present study is the age range studied and the maximum life span of a given species. The maximum age of the plants in this study was
280 years of 350 years noted as the maximum life span (i.e. 80% of the maximum age). Therefore, it cannot be excluded that senescence occurs in plants only after 80% of the maximum life span is reached, that is, beyond the limits of our study. In any case, if senescence does not occur, what causes these plants to die? Indeed, only very few studies have shown increased mortality rates associated with physiological deterioration at the organisinal level in perennial plants (Picó & Retana 2008; Herrera & Jovani 2010). It cannot be excluded that the ageing effect in these studies was masked, at least partly, by changing climatic conditions and/or plant size throughout the years of study. In this regard, it has been shown that regional warming and pathogen attack rather than ageing are the most likely contributors to tree mortality rates (Silvertown, Franco & Pérez-Ishiwara 2001; Allen et al. 2009). Therefore, it appears that up to the age addressed here, mortality in *B. pyrenaica* is due to factors extrinsic to the plant, that is, biotic or abiotic stressors that can lead to the death of these individuals in the Central Pyrenees, such as pathogens, regional warming during the summer or more stochastic events of freezing during the winter.

Conclusions

*Bordea pyrenaica* does not show age-related signs of oxidative stress. In contrast to plants with shorter life spans, this species has a great capacity to deal with the potential degenerative effects of ageing. This finding therefore demonstrates that age-induced oxidative stress is not a universal feature of ageing in plants. Given the much higher reproductive effort and longevity, it is not surprising that these plants are more prone to age-related signs of senescence and that age-related oxidant stress is not a universal feature of long-lived plants. In contrast, plants with shorter life spans, this species has a great capacity to deal with the potential degenerative effects of ageing. This finding therefore demonstrates that age-induced oxidative stress is not a universal feature of ageing in plants. Given the much higher reproductive effort and longevity, it is not surprising that these plants are more prone to age-related signs of senescence and that age-induced oxidant stress is not a universal feature of long-lived plants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Borderline pyrenes plants in the lab bench (A), with a
detail of the scars used to age individuals (B), the size of a bulb (C),
and plants growing in the Central Pyrenees (D).

Figure S2. Climatological conditions during the 3 years of samplings
(2008, 2010 and 2011) at Pineta valley, including monthly precipita­
tion and monthly mean air temperature.

Figure S3. Levels of the xanthophyll cycle pool (VZA) and zeaxan­
thin (Z), and de-epoxidation state of the xanthophyll cycle (DPS) in
leaves of juvenile plants (orange circles), males (brown circles) and
females (blue triangles) in three independent samplings performed

Figure S4. Levels of lutein, β-carotene, γ-tocopherol and α-tocoph­
erol per unit of chlorophyll in leaves of juvenile plants (orange cir­
cles), males (brown circles) and females (blue triangles) in three
independent samplings performed during July 2008, July 2010 and
July 2011.

Table S1. Relative water content (RWC) of leaves of juvenile plants,
male and female plants during the 3 years of samplings (2008, 2010 and
2011) at Pineta valley. Data represent the mean ± SE and the number of
individuals analyzed (n).
Neither males nor females of the extraordinarily long-lived Borderea pyrenaica show age-dependent signs of oxidative stress. This observation suggests that age-induced oxidative stress is not a universal feature of ageing in perennial plants. Indeed, females older than 100 years showed signs of negative senescence, in that they registered improved physiological performance with increasing age.