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TESE DE DOUTORAMENTO

# Spatio-temporal study of wood formation of *Quercus robur* L. and *Q. pyrenaica* Willd. in the Northwest of the Iberian Peninsula

Guillermo Guada Prada

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D. **Guillermo Guada Prada**

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## Spatio-temporal study of wood formation of *Quercus robur* L. and *Q. pyrenaica* Willd. in the Northwest of the Iberian Peninsula

D. **Ignacio García González**

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## Abstract

This Ph.D. thesis describes the process of wood formation (xylogenesis) and leaf development during two growing seasons at 14 *Quercus* forests (9 for *Q. robur* and 5 for *Q. pyrenaica*). A retrospective (dendrochronological) study of tree rings and anatomical variables (earlywood conducting vessels) at four out of these 14 sites was also carried out. The sites were located in the Northwest of the Iberian Peninsula, which is key location to study the effects of climatic variations on temperate forest ecosystems from a biogeography point of view, as it is the boundary between the Atlantic and Mediterranean regions. The dynamics of wood formation along the whole growing season are described, as well as the ontogenetic relationship between cambial activity and leaf development in order to know its synchronicity, and the water content of the crown organs (branches, buds, leaves) was also quantified to minimize the observer's subjectivity. The climatic variables affecting wood formation at the 14 sites are also analyzed to understand the adaptive response of the trees to the environmental conditions of the two species. This xylogenetic study provided systematic knowledge on the formation processes of xylem and their associated climatic patterns, which are fundamental to establish a correct interpretation of the cause-effect relationships of the dendrochronological retrospective results in ring-porous species.

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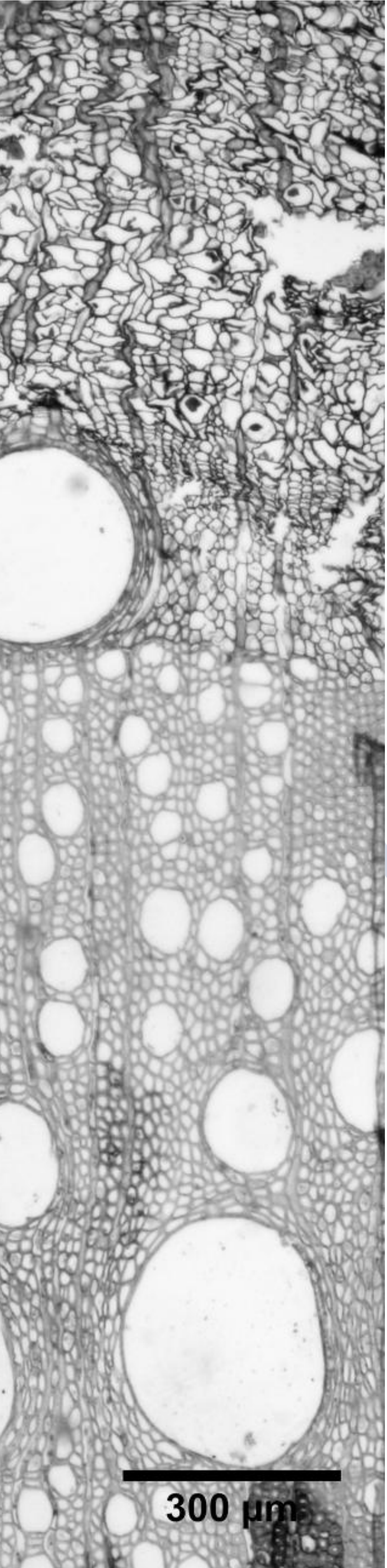
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# Background, objectives and experimental approach

# Introduction

Forests provide adequate habitats for obtaining basic raw materials and multiple benefits due to their ecosystem services, such as avoiding soil erosion, regulating the water cycle, and purifying the air. They are also main reservoirs of biodiversity for both plants and animals. In recent decades, the recognition of the importance of forests has increased because they are essential for the absorption and storage of carbon, and consequently they play a key role in mitigating climate change. The existence of feedbacks between forests and climate has been reflected by changes in physical, chemical, and biological processes that affect planetary energetics, the hydrologic cycle and atmospheric composition (Bonan, 2008;Peñuelas *et al.*, 2009).

The increase in average annual temperature over the last century is modifying the functioning of ecosystems and their composition, which can largely be attributed to exceeding the physiological thresholds of the species involved (Chapin III *et al.*, 2000). For example, the growth of forests in boreal or alpine regions is limited by temperature, and by the water regime in semi-desert zones. Both are prevailing climatic variables that often constrain the growth of the forest (Fritts, 2001), but this does not occur in many European temperate climates (Fonti & Garcia-Gonzalez, 2008), as in the northwest of the Iberian Peninsula; in these cases, conditions are often mesic, and the climatic control of growth is more complex, whereby a single prevailing variable is often not enough to understand climate-growth relationships.

From a biogeographically approach, the Iberian northwest has a key position to study the effects of climate variations on temperate forest ecosystems, since it involves the boundary between the Atlantic and Mediterranean biogeographic regions (Rivas-Martínez, 1987;Loidi, 2017;Souto-Herrero *et al.*, 2018b;a), so that many species reach their distribution limits in this area (Benito Garzón *et al.*, 2008;Rodríguez-Calcerrada *et al.*, 2008). The presence and abundance of these species can be modified over time, particularly as a result of the environmental changes that have been happening for the last decades. This is the case of dominant species in the forest ecosystems of this region, such as *Quercus robur* and *Quercus pyrenaica*. *Q. robur* presents a clear Atlantic behavior, and reaches here the southwestern limit of its world distribution. It is progressively replaced by *Q. pyrenaica* towards those areas with greater Mediterranean influence, since the adaptive response of both species to environmental changes is markedly different. Therefore, a better knowledge of the functional characteristics of dominant species is fundamental to predict the impact of climatic variations.

One of the factors that can be significantly affected by stress conditions in trees is radial growth, i.e., the formation of wood (Bräuning *et al.*, 2017). Apart from the overall increment, the anatomical characteristics of the wood constitute a tool of great interest to understand the adaptive response of trees to variation of environmental conditions. This response can be assessed retrospectively with the help of dendrochronological techniques, but one of their limitations is that the associations between climate and growth are usually analyzed through the statistical correlation between meteorological records and growth variables, without a systematic knowledge of the patterns and processes of xylem formation (Camarero *et al.*, 2010;Pacheco *et al.*, 2015), which are fundamental to establish cause-effect relationships.

The stages of xylem formation cannot be seen with the naked eye, unlike leaves, whose phenological development is easily observable, and is an indicator of the physiological processes linked to the synthesis and mobilization of carbohydrates that are taking place at that moment (Barbaroux & Bréda, 2002;Aloni, 2015). Consequently, a correct interpretation of the dendrochronological results requires a better knowledge of the processes of wood (xylogenesis) and leaf formation (leaf phenology) during the growing season (Fonti *et al.*, 2010).

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# Background

## Xylogenesis: Why and for what?

The etymological meaning of the word ‘xylogenesis’ refers to the development of wood formation (*xylo* = wood*; genesis* = origin, production). The main functions of the wood are support, transport of water and nutrients from the roots to the leaves, distribution of photosynthates, and it also serves as a reservoir of carbohydrates and defensive compounds (Pallardy, 2008). In angiosperm (i.e., *Quercus* species), as opposed to conifers, xylem is usually a more complex and heterogeneous tissue, composed of several types of cells, namely vessels for water conduction, fibers for mechanical support, and parenchyma cells for storage and defense.

Ring-porosity is a very common anatomical feature of dominant tree species in temperate climates. In these plants, the xylem does not have a uniform vessel size throughout the ring, as the larger vessels develop at the beginning of growth, in the so-called earlywood, and there is an abrupt change in vessel size and an increase in fibers later in the season, forming the latewood. During this period, cambial activity usually shows a bimodal pattern with two peaks corresponding to the ring with increments in spring and summer (Pérez-de-Lis *et al.*, 2017).

Earlywood vessels provide more than 90% of the water conductivity of the entire year ring, and are only functional during the current year (Ellmore & Ewers, 1986;Beeckman, 2016). These vessels begin to develop before the leaves unfold, so that accumulated reserves from the previous year (Barbaroux & Bréda, 2002) and climatic conditions at the time of vessel development can explain vessel size variations between years. This is probably the reason why relationships between variations in earlywood vessel features and climate have been satisfactorily shown (García-González *et al.*, 2016).

Wood formation studies are conventionally based on repeated (weekly or bi-weekly) cytological observations of the developing xylem, using microcores (Rossi *et al.*, 2006b) or cambial wounding samples (Gričar *et al.*, 2005;Gričar & Čufar, 2008), extracted from the main trunk at breast height along the growing season. In conifers, the counting of tracheids has allowed establishing the dynamics of secondary growth with a high resolution (Rossi *et al.*, 2012), as well as the kinetics of tracheid development (Cuny *et al.*, 2014), and this information has been successfully used in order to test how different species adjust the timing and length of wood formation to climate. Thus, the application of this methodology in conifers succeeded at identifying strong relations with temperature under cold climate conditions (Rossi *et al.*, 2016), as well as predict the seasonal dynamics of woody biomass production out of a database gathered in the last decades (Cuny *et al.*, 2015), and contributed to understanding forest decline when used as a complement to dendrochronological studies (Camarero *et al.*, 2016;Guada *et al.*, 2016).

However, xylogenetic observations in angiosperms are hampered by the more complex wood structure, and the ongoing ring width is usually measured instead of cell counting (Cufar *et al.*, 2008;Michelot *et al.*, 2012b;Prislan *et al.*, 2013;Pérez-de-Lis *et al.*, 2017), although the area of earlywood vessels has also been occasionally considered for ring-porous species (Alla *et al.*, 2011;Sass-Klaassen *et al.*, 2011;Pérez-de-Lis *et al.*, 2016b). But in spite of some incipient studies (Begum *et al.*, 2010;Kudo *et al.*, 2014), the effect of temperature on the onset, cessation, and duration of wood development is still not well known in native angiosperm forests under natural conditions.

A broader approach, based on the integrative study of the anatomical characteristics of xylem with foliar phenology and tree-ring features, as well as their spatio-temporal variability, should be very useful to assess the adaptive capacity of different oak species to climatic variations.

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## State of the art on wood science in the Northwestern Iberian Peninsula

Dendrochronology is a technique based on the analysis and interpretation of tree rings, as well as in the processes involved in their formation, which can be potentially used as archives or proxies with annual or even intra-annual resolution. Consequently, tree rings allow the retrospective reconstruction of climatic parameters in the past, even spanning for millennia (Fritts, 1976a). Traditionally, dendrochronological methodology involved studying tree-ring width for dating purposes and retrospective climate studies (Fritts & Swetnam, 1989a), but in recent years many studies have focused on obtaining new parameters derived from the anatomy of the rings with the aim of linking climate variability with growth dynamics, especially under climates where the usefulness of ring width is limited to obtain robust results (Fonti *et al.*, 2010;García-González *et al.*, 2016). In the last 15 years, the study of anatomical parameters has been introduced in order to optimize the study in the climatic response and its temporal stability across different areas in Europe (Fonti *et al.*, 2009;Campelo *et al.*, 2010;Gea-Izquierdo *et al.*, 2012;Matisons & Brumelis, 2012;Pritzkow *et al.*, 2016;Rita *et al.*, 2016;Souto-Herrero *et al.*, 2017;2018a). As regards the southwestern distribution boundary of *Q. robur* and *Q. pyrenaica*, the main findings of such analyses show that i) the anatomical features of earlywood are strongly related to the climate, ii) radial increment can be significantly affected by the disturbance regime at each forest stand, masking the prevailing climatic signal (Garcia-Gonzalez & Souto-Herrero, 2017).

In temperate climates, where a single prevailing climatic factor is not enough to explain ring-width variation, the anatomical characteristics of the xylem and their inter-annual and intra-annual variation is of great importance, probably because they have a strong functional meaning (Fonti & Garcia-Gonzalez, 2008). Thus, the number and dimensions of the conductive elements, namely the earlywood vessels in ring-porous trees, determine the capacity of the plant to conduct water, and its vulnerability under water stress (Hacke *et al.*, 2001;Tyree & Zimmermann, 2002).

In addition, the formation of these earlywood vessels begins before leaf unfolding, and therefore before the synthesis of new carbohydrates (Barbaroux & Bréda, 2002). Thus, these processes entirely rely on the reserves accumulated during the previous season. This is probably the reason why the size of the earlywood vessels in porous-ring angiosperms, such as the European deciduous *Quercus* species, are often considered as a powerful climatic proxy (Souto-Herrero *et al.*, 2018a;b), especially in temperate regions where there is not a single limiting factor (García-González & Fonti, 2008). In fact, it is possible to obtain chronologies of earlywood vessels of a great length, up to almost five centuries (Souto-Herrero *et al.*, 2017).

However, for a correct interpretation of the interannual variation provided by these dendrochronological analyses, it is necessary to precisely know the process of wood formation, which is usually studied by monitoring wood growth through xylogenesis (intra-annual variation). Although there is a wide evidence that temperature is one of the main driving forces for plant growth in terrestrial ecosystems, its influence on wood formation has been mainly analyzed on conifers, often at high latitudes or elevations, spanning many sites (Cuny *et al.*, 2015;Rossi *et al.*, 2016).

On the contrary, studies on cambial dynamics of temperate hardwood species such as oaks are limited to very few sites (Lavrič *et al.*, 2017;Pérez-de-Lis *et al.*, 2017;Puchałka *et al.*, 2017). Wood hardiness and difficulties in the sample processing, has required to fine-tune the protocols and make a first approach to the study of xylogenesis of *Q. robur* and *Q. pyrenaica* (González-González, 2012). The procedure has shown a high potential to describe the different phases of the wood formation, and also to associate these processes to foliar phenology observed in the same tree (Sass-Klaassen *et al.*, 2011;González-González, 2012). This allowed Pérez-de-Lis (2016) to establish more robust experimental designs for temperate (*Q. robur*) and sub-Mediterranean oaks (*Q. pyrenaica*), in order to understand their different functional strategies along a water availability gradient, with regard to the durations of the phenological phases of wood and leaves, number of cells of the cambium, ring width, and carbohydrate content. From this study, the authors concluded that *Q. pyrenaica* follows a more conservative growth strategy compared to *Q. robur*, which would be advantageous in unfavorable conditions, but less competitive in optimal conditions.

On the other hand, the absence of quantitative (i.e., less subjective) methods for leaf phenological studies still hampers a clear functional link between primary and secondary growth. Recently quantitative tools have been tested to measure primary growth on shrubs and *Quercus* spp. Thus, Palacio *et al.* (2008) analyzed seasonal changes in dry matter content (DMC; the opposite to water content) at full hydration in different leaf and stem cohorts. Similar high values of DMC were recorded for all species during dormancy, but lower values and significant interspecies differences were observed during the vegetative period, although the concurrence of minimum values with the moment of maximum shoot extension was insufficiently defined. A relationship between minimum DMC and maximum shoot extension can be expected, because plant organs require high amounts of water to maintain the turgor pressure necessary for extension during the growing process (Bradford & Hsiao, 1982). This supports the potential usefulness of DMC as a quantitative tool to identify phenological states.

The methodological synergies of DMC with foliar phenology and the study of xylogenesis provide knowledge on the patterns and processes of xylem formation, which are fundamental for establishing cause-effect relationships obtained from retrospective dendrochronological studies. Only when such as relation has been established, can a growth trait be used as a proxy of past environmental conditions (Bradley, 1986).

## Objectives

This Ph. D. Thesis aims at acquiring information on how primary and secondary growth evolve during the active season in natural conditions, by comparatively studying the seasonal dynamics of wood formation (xylogenesis) and foliar phenology, in order to use this knowledge for the interpretation of past environmental conditions contained in the tree rings. Thus, each phase of foliar phenology, visible to the naked eye, will be associated with the phases of cambial activity that occur simultaneously during the formation of the ring.

It is also intended to establish the causal connection between the variation of daily climatic parameters with the beginning and cessation of the cambial activity of oaks at an intra-annual scale, basing on the hypothesis that temperature is responsible for the advance or delay in the onset, cessation, and consequently the duration, of the vegetation period. This effect is particularly relevant for the study area, where natural forests of *Q. robu*r and *Q. pyrenaica* are at their distribution boundary. Different climatic gradients were selected to embrace the main climatic influences existing in the region.

The improvement of phenological analysis, by exploring the possibility of quantifying primary growth has also been investigated. This was based on the hypothesis that new shoots require a high hydration of the tissues to maintain turgor during the extension phase, which should result in a reduction of their dry matter content.

The research undertaken aims at fulfilling the following specific objectives:

(i) To verify a quantitative method to describe crown phenophases in relation to water content and to secondary growth.

(ii) To describe the main phases of wood formation in an intra-annual cycle, establishing their relationships with the phenological state of the tree, as well as environmental conditions.

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(iii) To identify the main climatic factors that influence the formation of the annual ring during the entire life of the tree, especially that of the earlywood given its functional importance.

(iv) To establish the main differences in the behavior between oak species (*Q. robur* and *Q. pyrenaica*) and geographical areas (Cantabrian, Atlantic, continental inland) in order to infer the potential responses to environmental changes.

## Experimental approach

To achieve the objectives of this Ph. D., three experiments were undertaken on oaks forests in the Northwestern Iberian Peninsula, whereby the one specifically focused on xylogenesis constitutes the main core of the work.

The first experiment (**Experiment 1**) aimed at studying the synchronicity between primary and secondary growth, as well as how this relationship can be modified by climate. It consisted of a two-year monitoring of foliar and cambial phenology at nine forest stands of *Q. robur*, and five of *Q. pyrenaica* located at several elevation gradients under three climatic influences.

A second experiment (**Experiment 2**) involved a dendrochronological study at two sites for both *Q. robur* and *Q. pyrenaica*, which represent some extremes of the gradients, where one of the sites presented more Mediterranean conditions. For *Q. robur*, low-elevation sites were compared between Atlantic and Cantabrian area, which mainly differ in their degree of summer water availability. In the case of *Q. pyrenaica*, the two highest sites, which have a different slope orientation, were compared. For this, increment cores were extracted in order to build up tree-ring chronologies spanning for several decades, which involved tree-ring width and earlywood vessels, and consequently related with climate. The results of the wood formation experiments at each site were used for the interpretation of the results.

In a third experiment (**Experiment 3**), we quantified dry matter content of several organs of one, two and three years old twigs, buds, and leaves at different leaf phenophases in *Q. pyrenaica*, for the growing season on 2013. This study was aimed at testing the usefulness of DMC for quantitative identification of leaf phenological states, avoiding the subjectivity of the observer.

The **first experiment** was conducted along the course of three rivers: Eume and Sor rivers covered by a *Q. robur* forest and Lor river covered by *Q. pyrenaica* woodlands (Fig. 1). These rivers are located in the northwest of the Iberian Peninsula which respectively represent the three main climatic influences, Atlantic, Cantabrian and sub-Mediterranean.

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Both river catchments, covered by *Q. robur,* have an altitudinal gradient from their upper watershed (650 m asl) to their mouths into the Atlantic Ocean or the Cantabrian Sea (150 m asl). The area presents a mild Atlantic climate, with a mean annual temperature ranging 9-13 ºC among sampling sites, and a total precipitation of 900-1,500 mm; maximum rainfall occurs during autumn-winter, and there is varying degree of summer drought depending on elevation or position towards the coast. Nevertheless, these two rivers represent two dominant climatic influences on oak formations in northwestern Iberia. Whereas the Atlantic coast is dominated by a certain Mediterranean trend during summer (higher temperature, more intense drought).

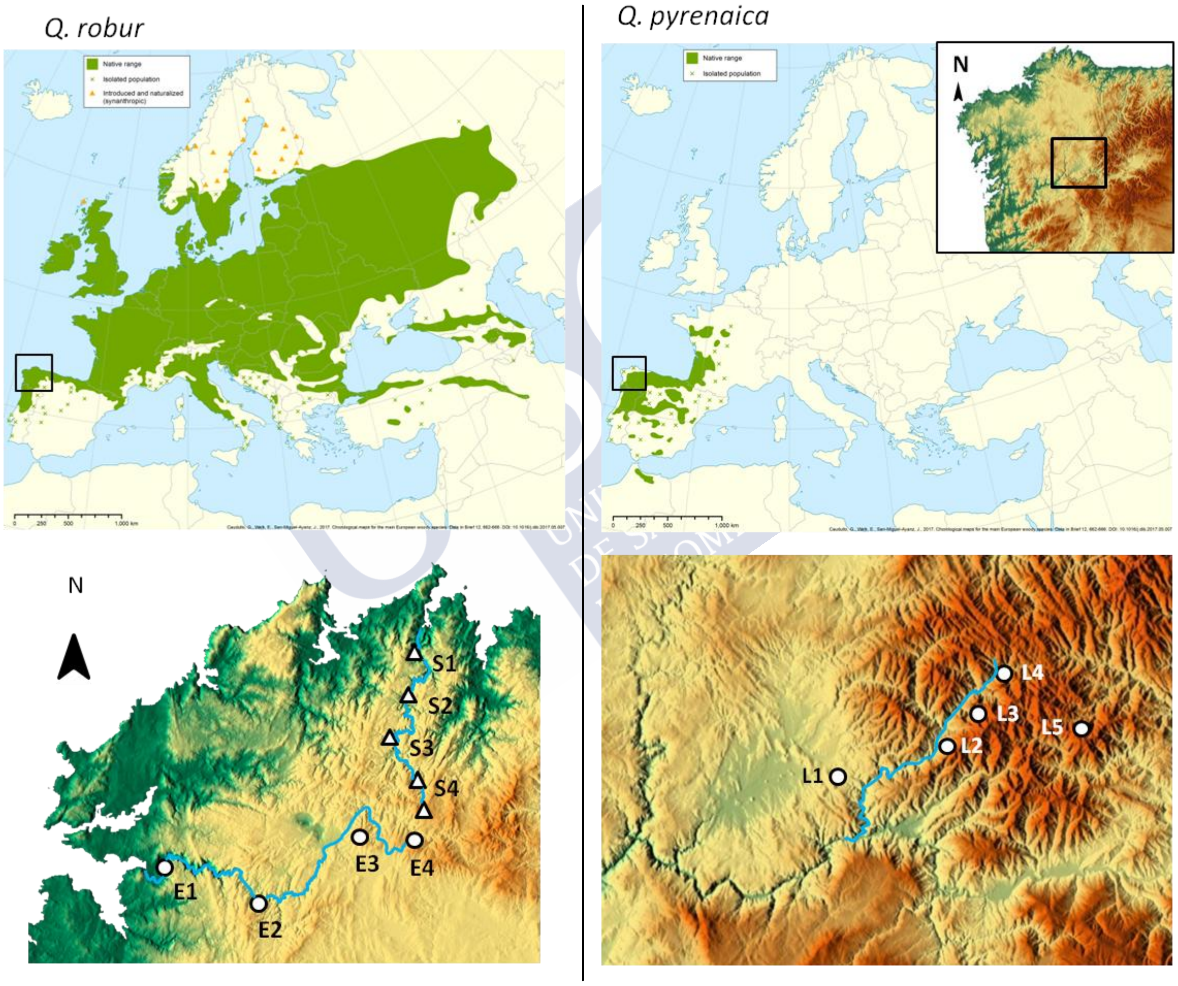

**Fig. 1** Distribution maps of *Quercus robur* and *Q. pyrenaica* (Caudullo *et al.*, 2018). Dots represent study sites along the altitudinal gradient of the three rivers. For *Q. robur,* Eume River (E1-E4) and Sor River (S1-S2), with Atlantic and Cantabrian influences, respectively. For *Q. pyrenaica,* a gradient along the Lor River (L1-L2).

The gradient studied for *Q. pyrenaica* follows an altitudinal gradient from 400 to 1,050 m asl. The sites present an Atlantic climate, but influenced by certain continentality with a Mediterranean distribution of precipitation, characterized by dry summer periods, whereas rainfall is abundant in winter (350-600 mm). The annual temperature ranges 7.5-12.7 ºC among sites. Mediterranean conditions increase as elevation decreases, and most of this area can be considered as the transition from the Atlantic (Eurosiberian) Region.

Along both rivers, we monitored nine locations for *Q. robur* and five for *Q. pyrenaica*, during two consecutive years, from early spring to Autumn in 2012, and 2013. The three climatic gradients were sampled in three consecutive days at intervals of 12-15 days in spring, and 20 days in summer, randomly selecting ten trees per site at each sampling date, with a diameter of 20-40 within an area of ca. 2 ha per forest, summing up a total of 3,182 trees sampled.

In the **Experiment 2,** the two sites covered by *Q. robur* were located under Atlantic and Mediterranean influence, both at the lowest altitudinal gradient, whereas the two sites of *Q. pyrenaica* were located at 1,000-1,500 m a.s.l. in a transition area from Atlantic to Mediterranean climate. In both areas, the two selected sites differed in their orientation, and apparently a notable contrast in its degree of Mediterranean (thermophile) influence.

In the **Experiment 3** we selected the tree species *Q. pyrenaica*, which exhibits a low phenological variation at the individual level in spring. Branches and microcores were collected from 11 trees along one year in order to establish the synchronicity between primary and secondary growth in terms of water content.

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## Thesis Outline

This Thesis contains the three original experiments above described, structured in four chapters following the layout of a scientific article, and a general discussion.

**Chapter I** reports Experiment 3, describing the dynamics of primary and secondary growth in order to test a quantitative method for identifying leaf phenophases using the water content of crown organs, and to relate these phenophases with development of earlywood vessels in the xylem.

**Chapter II** provides data from Experiment 1 of seasonal dynamics of nine *Q. robur* stands, following two altitudinal gradients, which represent the Atlantic and Cantabrian climate influences, with the aim of analyzing the synchronicity between secondary growth and crown development, as well as how temperature affects cambial activity and leaf phenology across this wide range of natural woodlands.

**Chapter III** also reports results from Experiment 1, but focused on five *Q. pyrenaica* stands along a single altitudinal gradient, on a transition from Atlantic to Mediterranean climate and continental influence.

**Chapter IV** describes a dendrochronological study of *Q. robur* and *Q. pyrenaica* (Experiment 2) using quantitative anatomy, in forests located at the extremes of the gradients, which represent the distribution boundary of each species. For *Q. robur*, the two most thermophile sites were compared, therefore sites with a great Mediterranean trend, but slightly differing in their summer xericity (lower plots of the Atlantic and Cantabrian gradients). On the other hand, the two plots with the highest altitude were compared for *Q. pyrenaica*, i.e. those with the strongest Atlantic characteristics, but which also differ in their precipitation regime.

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# Dry-matter content during extension of twigs, buds and leaves reflects hydraulic status related to earlywood vessel development in *Quercus pyrenaica* Willd.

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## Abstract

A quantitative method was tested to describe crown phenophases in relation to water content and to secondary growth in ring-porous species, based on the hypothesis that new shoots require hydrated tissues to maintain the necessary turgor for extension, leading to a reduction in dry matter content (DMC).

We collected a three-year-old branch from 11 *Quercus pyrenaica* Willd. trees at 10-day intervals to estimate DMC of newly developing buds, leaves, and twigs, and processed two opposite stem microcores for xylogenesis. Branch phenophases and shoot length were recorded in the field.

The DMC of all organs decreased during crown development, with a minimum in early June, followed by a gradual increase up to initial values in late September. The shoot extension period concurred with the lowest DMC, but also with the beginning of earlywood maturation in the main stem, suggesting a high tissue hydration only when earlywood vessels become functional to fulfil enough water requirements for shoot and leaf extension.

These results confirm the usefulness of DMC to accurately quantify the phenology of primary growth from bud swelling up to full leaf extension, as a complement to qualitative methods. This variation in DMC appears to be linked to secondary growth as a result of earlywood vessel development.

**Keywords:** crown water status, phenology, ring-porous species, xylem, xylogenesis.

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## Introduction

Earlywood vessels of ring-porous species are considered to transport water only during the year of their formation (Greenidge, 1955;Zasada & Zahner, 1969;Chaney & Kozlowski, 1977;Ellmore & Ewers, 1986;Utsumi *et al.*, 1999;Umebayashi *et al.*, 2008), andtherefore water consumption by crown development should depend on the renewal of the earlywood vessel network every spring. Over the last decades, some works focused on the analysis of the relationships between shoot and cambial phenology in ring-porous species, particularly on the association between earlywood vessel development and shoot phenology (Suzuki *et al.*, 1996;Sass-Klaassen *et al.*, 2011;González-González *et al.*, 2013;Takahashi *et al.*, 2015;Pérez-de-Lis, 2016;Lavrič *et al.*, 2017;Puchałka *et al.*, 2017).

The conventional approach used to quantify secondary growth (i.e., wood formation) relies on xylogenetic studies involving the extraction of a microcore (Rossi *et al.*, 2006a) or cambial wounding (Gričar *et al.*, 2005;Gričar & Čufar, 2008), which are in general performed from the main trunk at breast height. Histological methods allow an accurate monitoring the cell division process, and secondary cell wall deposition in order to sequence these events. In conifers, the number of tracheids is counted to establish the dynamics of secondary growth (Rossi *et al.*, 2012), while in angiosperms the ongoing ring width is usually measured for this purpose, (Cufar *et al.*, 2008;Michelot *et al.*, 2012b;Prislan *et al.*, 2013;Pérez-de-Lis *et al.*, 2017) although earlywood vessel area has been occasionally considered for ring-porous species (Alla *et al.*, 2011;Sass-Klaassen *et al.*, 2011;Pérez-de-Lis *et al.*, 2016b).

Primary growth is more complex to quantify due to the difficult access to the crown of large trees, and to the asynchrony often found among different branches. This problem is usually overcome by estimating phenophases (e.g., swollen buds, bud break,..) following visual observation at the naked eye, binoculars, or photographs (Haggerty & Mazer, 2008;Vilhar *et al.*, 2013). Other authors distinguished between flushing leaves and successive leaves (Takahashi et al., 2015; Takahashi and Takahashi, 2016), even with some attempts to quantify these estimations (González-González *et al.*, 2013). However, visual estimations account for a high degree of subjectivity, and can result in some inaccuracies, especially at defining certain phenophases.

Quantitative approaches including bud size, leaf area index, and shoot elongation measurements have been respectively used to monitor bud, leaf, and shoot development (Cuny *et al.*, 2012;Basler & Korner, 2014;Camarero *et al.*, 2016;Lavrič *et al.*, 2017), but all of them fail to account for the whole primary growth phenophases. Palacio *et al.* (2008) analysed seasonal changes in dry matter content (DMC) at full hydration (the opposite to water content) in different leaf and stem cohorts of several species. Similar high values of DMC were recorded for all species during dormancy, whilst lower values and significant interspecies differences were observed during the vegetative period. Although minimum DMC roughly coincided with the moment of maximum shoot extension, their data were not precise enough to demonstrate the existence of a close relationship between minimum DMC and maximum shoot extension. This relationship can be expected, because plant organs require high amounts of water to maintain the turgor pressure necessary for extension during the growing process (Bradford & Hsiao, 1982). DMC has also been found to be strongly related to dry matter concentration (dry mass per volume of plant organs), but volume estimation is more difficult, particularly in very small organs (Shipley & Vu, 2002).

We hypothesize that the measurement of DMC throughout the growing period will allow a quantitative description of shoot development, where DMC reduction in shoot parts (leaves and stems) will correspond to the peaks of shoot extension. Such hypothesis relies on the ontogenetic processes related to the onset of shoot extension (Davis & Mooney, 1986). Thus, one of the prerequisites for budburst is the increase of hydration of bud and shoots (Bradford & Hsiao, 1982;De Fay *et al.*, 2000). Expanding cells adjust their osmotic potential and cell wall rigidity to maintain adequate turgor pressure throughout the growth process (Boyer, 1988;Van Volkenburgh, 1999). Consequently, the mechanisms used by cells to maintain this pressure may increase the capacity of organs to gain water when they are at full turgidity, hence leading to a DMC reduction in leaves and twigs during shoot extension. Once cell division has ceased, dry matter accumulates in cell walls, causing an increase of DMC associated to a higher volumetric fraction of structure (Roderick *et al.*, 1999). Functional xylem tissues provide water and osmolytes required for bud swelling and budburst (Bonhomme *et al.*, 2010), but the possible mutual interactions between the timing of xylogenesis and shoots DMC remain unexplored.

In order to test this hypothesis by establishing the relationships between primary and secondary growth, we selected the tree species Quercus pyrenaica Willd., which exhibits a short growing period, and a low phenological variation at the individual level in spring. Consequently, the aims of this study were: i) to verify whether the minimum DMC of crown organs in Q. pyrenaica is coupled to the maximum rate of shoot extension, and ii) to quantify variations in DMC of crown organs at different phenological phases, and their relationships with earlywood vessel formation in the stem and crown development.

# Material and Methods

## Study site and tree selection

The study was carried out in the northwestern Iberian Peninsula (43º 1’43.57’’ N, 7º 31’ 5.19’’ W; 500 m a.s.l.), where a dominant woodland of naturally regenerated *Q. pyrenaica* mixed with *Q. robur* L. on a siliceous soil forms a transitional forest from Mediterranean vegetation to Atlantic vegetation. Average annual rainfall is ca. 900 mm, mainly falling between October and February, mean annual temperature is 11.8 ºC, with 18.3 ºC in the hottest month (July), and 5.7 ºC in the coldest (January) (period 2006-2016; http://www.meteogalicia.es).

The selected species *Q. pyrenaica* covers a wide distribution area in the Iberian Peninsula, being fairly common in all inland regions, especially on siliceous soils in the central and western hills between 400 and 1,600 m a.s.l. (Jiménez et al., 1997). The vegetative period mainly extends concomitantly with summer drought, but the strength of the root system allows the maintenance of optimal soil conditions for water retention and protection against erosion (Montserrat, 2008).

Wood micro-cores and twigs were collected at each sampling date from March to September 2013 (day of the year 66-266) from 11 isolated trees to prevent competition effects. We followed a 10-day interval in spring during earlywood vessel formation and crown development; and a 20-day interval in summer; in this way, we accounted for the whole dynamics of primary and secondary growth without inducing crown resprouting. The number of trees sampled was twice the minimum sampling size recommended by Cornelissen *et al.* (2003) for phenological traits, and the heterogeneous size of the selected trees ensured that samples were representative of the study area (Table 1). Trees had a mean diameter of 24.7 ± 4 cm at breast height, with individual values ranging from 19.5 to 32.5 cm, and average height of 8.6 ± 3 m ranging from 6.5 to 16.0 m. The lower canopy limit was at 1.6 to 4.8 m above ground, allowing accessibility for crown sampling.

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**Table 1** Characteristics of *Q. pyrenaica* trees selected for the study. Diameter at breast height (*DBH*), total height (*H*), stem height below the crown (*SBC*) and crown diameter (*CD*). Average and standard error are shown at the bottom.

| Tree (*n*) | DBH (*cm*) | H (*m*) | SBC (*m*) | CD (*m*) |
|---|---|---|---|---|
| **1** | 25.8 | 16 | 4.8 | 5.9 |
| **2** | 19.5 | 6.4 | 3 | 6.75 |
| **3** | 22.4 | 8 | 4 | 7.75 |
| **4** | 23 | 7.2 | 3 | 6.25 |
| **5** | 24.2 | 7.2 | 3.2 | 7.05 |
| **6** | 30 | 12 | 4 | 5.85 |
| **7** | 27.2 | 6.4 | 3.2 | 5.85 |
| **8** | 20.3 | 8 | 2.4 | 5.9 |
| **9** | 24.2 | 5.6 | 1.6 | 5.45 |
| **10** | 23.1 | 6.4 | 1.6 | 6 |
| **11** | 32.5 | 11.2 | 3.2 | 6.8 |
| **Average ± sd** | 24.7 ± 4 | 8.6 ± 3 | 3 ± 1 | 6.3 ± 0.7 |

## Primary and secondary growth measurements

### Dry matter content

We collected one 3-year old branch at each sampling date from each of the 11 selected trees, at sunny orientation below half-crown, always avoiding successive cuttings on the same main branch. No resprouting signals were observed near the cutting wounds. Branches were labelled, bagged, and expeditiously transported to the lab after the end of sampling to be immediately subjected to maximum hydration following Garnier *et al.* (2001). We photographed all branches upon arrival with a digital camera for the visual documentation of the phenophases. Maximum hydration was achieved by immersing the first 3–4 cm of the stem in distilled water, after having cut off underwater the three proximal centimeters of the stem. During hydration, branches were capped by a wet plastic bag, and kept at 5 °C for 24 h.

We measured the full hydration weights from the apical buds, leaves, and twigs of each hydrated branch. When present, apical and top sub-apical buds, leaves, current-year, 1- and 2-year-old cohorts were measured separately. Subsequently, samples were oven-dried at 60 °C for 48 h, and dry weights recorded. All weighing was conducted to the nearest 0.1 mg with a scale (Denver instrument, AA-160, New York, USA). The DMC (mg $g^{-1}$) of leaves and stems was calculated as the ratio between dry ($W_d$) and fresh weight ($W_f$) at full hydration, assuming that the weight difference was due to water:

$$\mathrm{DMC} = W_d/W_f$$

On average, 6.0 samples of current twigs, 5.0 of one-year-old twigs and 2.5 of two-year-old twigs were collected from each tree and sampling date (Table 2), which is about two-fold the sampling size per tree recommended by (Cornelissen *et al.*, 2003).

**Table 2** Average number of samples collected per *Q. pyrenaica* tree over the 12 sampling days. Data provided are the tree identification and number of: current year twigs that were present in the last six dates (*Twigs*), one-year-old twigs (*Twigs - 1*), two-year-old twigs (Twigs - 2), *Buds*, *Small leaves* (those <50% of maximum size; collected during two dates), *Large leaves* (>50% of maximum size; collected in the remaining ten dates), and *cambium* samples. Total sample size is shown at the bottom.

| Tree | Twigs | Twigs - 1 | Twigs - 2 | Buds | Small leaves | Large leaves | Cambium |
|---|---|---|---|---|---|---|---|
| **1** | 5.4±1.1 | 4.8±2.2 | 2.1± 1.4 | 42.7±21.9 | 55±0.0 | 5.5±0.5 | 2 |
| **2** | 7.0±1.5 | 5.5±1.3 | 2.2± 1.2 | 37.5±3.4 | 40±28.3 | 6.0±1.4 | 2 |
| **3** | 5.8±2.3 | 4.9±1.7 | 2.0± 0.9 | 31.3±16.1 | 30±14.1 | 5.5±1.0 | 2 |
| **4** | 5.3±1.5 | 5.0±3.2 | 2.2± 1.4 | 32.6±9.2 | 27.5±10.6 | 5.5±1.0 | 2 |
| **5** | 6.0±1.5 | 4.6±1.7 | 2.8± 1.5 | 40.0±22.5 | 28±2.8 | 5.75±0.5 | 2 |
| **6** | 6.3±1.8 | 5.3±1.3 | 2.6± 1.1 | 37.3±25.7 | 20±0.0 | 5.5±1.0 | 2 |
| **7** | 5.2±1.1 | 4.5±2.4 | 2.5± 1.4 | 32.1±16.7 | 20±0.0 | 4.5±1.2 | 2 |
| **8** | 6.0±0.7 | 5.1±1.9 | 3.4± 1.3 | 28.5±13.4 | 20±0.0 | 6.5±1.2 | 2 |
| **9** | 6.3±2.1 | 4.5±2.6 | 2.2± 1.0 | 39.1±14.8 | 21.5±6.4 | 6.5±1.7 | 2 |
| **10** | 7.0±2.4 | 5.0±2.2 | 2.5± 1.0 | 46.1±14.5 | 56.5±4.9 | 6.5±0.5 | 2 |
| **11** | 5.5±2.0 | 5.2±3.1 | 2.7± 1.6 | 27.5±17.4 | 19.5±4.9 | 6.0±2.0 | 2 |
| **Total samples** | 379 | 658 | 325 | 2474 | 581 | 255 | 264 |

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## Shoot extension

Current-year twigs were measured before drying in order to assess absolute shoot extension rate (SER). This comprised the distance from the insertion point on the proximal scale scar to the tangent line between the apices of the most apical green leaf. Shoot extension rate was calculated for each sampling date as:

$$\mathrm{SER} = (\mathrm{L}_n - \mathrm{L}_{(n-1)})/\mathrm{T}$$

where $\mathrm{L}_n$ (mm) is the mean shoot length of twigs per tree from a sampling date $n$, $\mathrm{L}_{(n-1)}$ (mm) the mean shoot length of each individual in the previous date ($n - 1$), and T the days elapsed between dates ($n - 1$) and $n$.

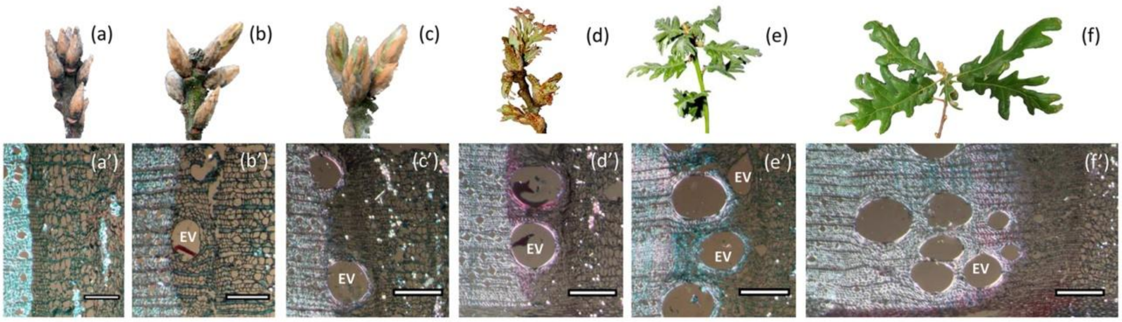

**Fig. 1** Leaf growth images of *Q. pyrenaica* and the corresponding transversal thin sections of earlywood development observed under polarized white light, in a time sequence. The upper row shows primary growth phenophases: (a) bud dormancy, (b) swollen buds, (c) budburst, (d) leaf unfolding, (e) appearance of small leaves and (f) full extension. The lower row shows secondary growth phenophases: (a') dormant cambium, (b') earlywood vessel enlargement, (c') onset of earlywood vessel maturation, (d' and e') earlywood vessel maturation, (f ') cessation of earlywood vessel formation and cell expansion of latewood. Birefringence under the polarizing light indicates secondary cell wall deposition. Scales bars are 150 μm (a', b') and 300 μm (c'- f '). EV, Earlywood vessel.

## Leaf phenological observations

Leaf phenology of the whole crown was recorded for each individual tree during sampling. Observations were made by screening buds of the main shoots from the top to the bottom of the crown using binoculars (10 × magnification), and were expressed as a particular day of year (DOY).

Six phenophases were considered (Fig. 1), namely a) bud dormancy, b) swollen buds, c) budburst, d) leaf unfolding, e) appearance of small leaves, and f) full extension. Bud dormancy corresponds to the overwintering stage, while bud swelling was identified by the apparent separation of buds from the stem, together with the exposition of areas of lighter coloured tissue as a result of the initial extension of cataphylls with the separation of scales. Budburst was characterized by green-coloured expanded buds with no unfolded leaves, and leaf unfolding ended as soon as the leaf blade was clearly visible, but not the petiole. Appearance of small leaves was defined as the moment when at least one leaf was completely out of the bud and current year twigs and petiole could be visually appreciated; and full extension was recorded when leaves attained at least the 50% of their final size. The period of leaf extension spanned from the presence of turgid small leaves (less than the 50% of their final size) until their full extension.

The proportion of each phenophase in the whole canopy of each selected tree was estimated visually. In order to establish the duration of each phenophase within the population, we averaged its proportion considering the whole population of trees (Fig. 2a).

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## Monitoring of xylogenesis

Two microcores of 2-mm diameter were taken using a Trephor device from the northern and southern sides of each tree at 1.3 m above the ground at each sampling day (Rossi et al., 2006). This allowed us to account for the variation in vessel formation between opposite sides, and also to guarantee the presence of enough vessels to correctly identify each cambial phenophase. We took samples on the main stem because vessel formation progresses basipetally, and consequently the presence of mature vessels at this level assures the maturation of the complete vascular path up to the leaves (Aloni, 2013). After extraction, microcores were placed in Eppendorf microtubes with a 50% ethanol solution and stored at 5 °C until processing.

Wood samples were dehydrated by successive immersions in ethanol and xylene, and embedded in paraffin using a tissue processor (Leica TP1020, Wetzlar, Germany). Thereafter, cross-sections of 8–10 µm thickness were obtained from each paraffin block with a manual rotary microtome (Leica RM2125 RTS, Wetzlar, Germany) and placed on microscope slides. After two immersions in xylene for 5-min each to remove the residual paraffin, cross-sections were rehydrated and stained in two consecutive solutions: safranin for 10 minutes and fast green FCF in ethanol (80%) for 30 s, (Cutler *et al.*, 2008). Afterwards, they were newly immersed in xylene to remove water residues, and permanently fixed with Eukitt® resin (O. Kindler GmbH, Reiburg, Germany).

Ring-width measurements and cambial observations of the microcore cross-sections were performed on images taken with a digital camera (Canon EOS 600D, Tokyo, Japan), coupled to a transmitted light microscope (Olympus BX40, Tokyo, Japan), using a white light polarizing filter (40 × magnification). The width of cell expansion and maturation zones was measured on images along three radial lines. We determined each phenological phase of vascular cambial activity, including: onset of earlywood vessel enlargement, onset of earlywood vessel maturation (Fig. 1c'), cessation of earlywood vessel enlargement (Fig. 1f '), and cessation of cell expansion. The onset of earlywood vessel enlargement was considered when the most recent ring contained at least one enlarging vessel, whereas the onset of earlywood vessel maturation was defined when secondary cell wall deposition in vessels was detected as birefringence under the polarizing light; cessation of earlywood formation occurred when earlywood vessel enlargement was no longer detected, but all expanding cells belonged to latewood; and cessation of cell expansion was defined as no more expanding cells were detected, although lignification could be still ongoing. We attributed the corresponding DOY to each cambial phenophase.

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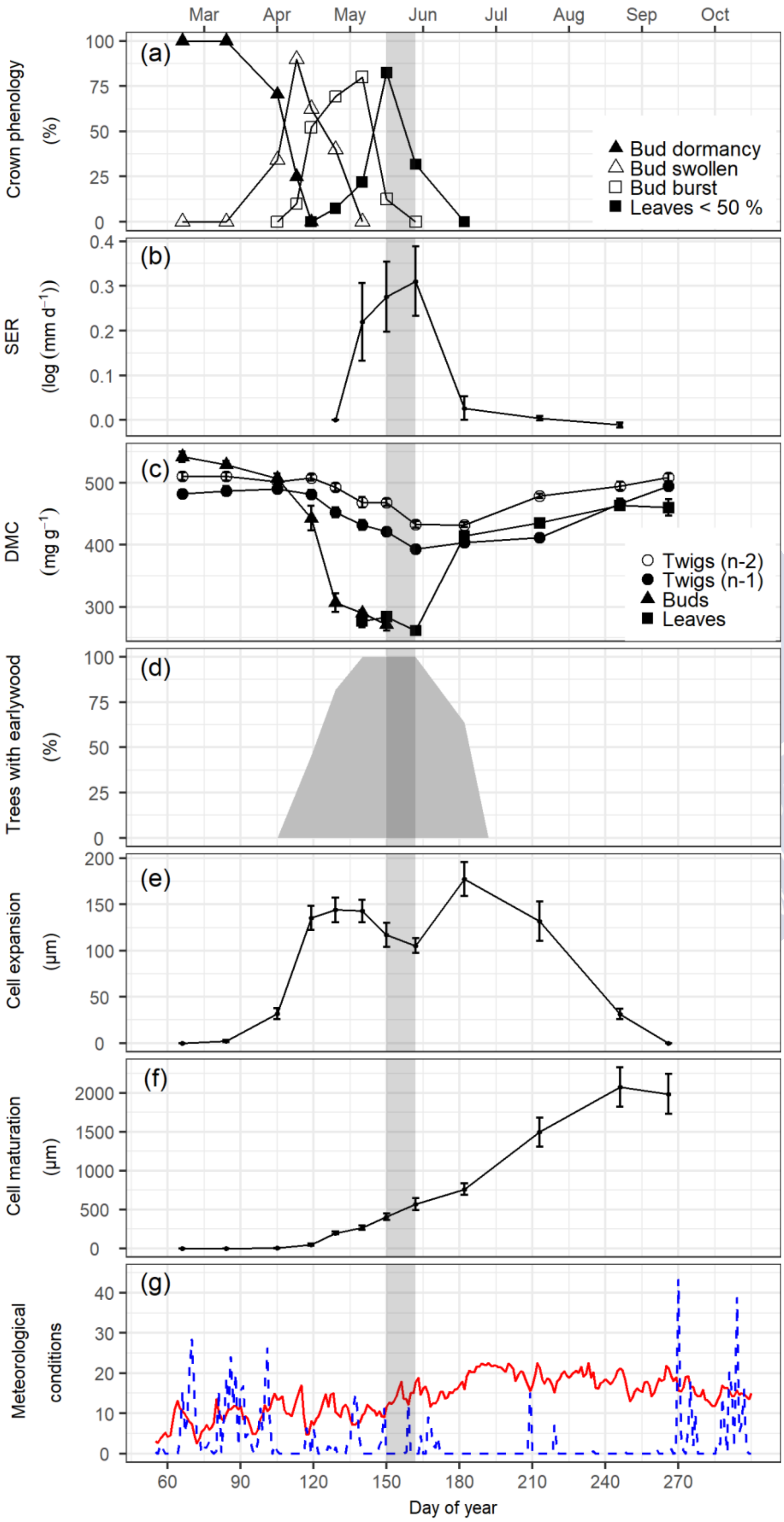

**Fig. 2** Seasonal phenological trends for *Q. pyrenaica*: Phenological diagrams indicated as **(a)** Percentage of crown in the phases of: bud dormancy, swollen buds, budburst, leaves less than 50 % of the leaf size (Leaves < 50 %), **(b)** Shoot extension rate (SER in log(mm d$^{-1}$)), **(c)** Dry matter content (DMC in mg g$^{-1}$) from the buds, leaves, and one- and two-year-old twigs, (n-1) and (n-2) respectively, **(d)** Percentages of trees with mature earlywood, **(e)** Ring-width expansion (μm), **(f)** Wood maturation (μm), **(g)** Meteorological variables: *continuous red line* is the average temperature (°C) and *dashed blue line* the precipitation (mm) (data from the nearest meteorological station, Campus Lugo). Grey bar indicates the period of maximum shoot extension rate (SER). Samples taken from day 66 to 266 of year in 2013.

## Statistical analyses

Raw data of DMC did not follow a normal distribution. Therefore, relationships among the DMC of buds, leaves, and stems of the current and two preceding years were explored using Spearman’s correlation coefficient, which were separately performed for each organ, with data from each sampling day and individual tree.

In order to detect the date of significant variation in DMC on the time course evolution of organs, we applied a linear mixed model analysis with time as fixed factor and tree as random factor. The same analysis was used to characterize leaf DMC during leaf phenological phases, with phenological events as fixed factor and tree as random factor. In both cases, residuals followed a normal distribution according to the Shapiro-Wilks test. Analyses were performed with the R statistical software (R Core Team, 2017) using the ‘nlme’ package (Pinheiro *et al.*, 2016) and "multcomp" package was used to separate least square means.

As previously reported for conifers (Rathgeber *et al.*, 2011) and angiosperms (Pérez-de-Lis *et al.*, 2017), the absence/presence of visually-recorded xylem phenophases were expressed as binary data, and a logistic regression used in order to compute the following four critical dates: beginning of earlywood vessel enlargement (beEW) and beginning of maturation (bmEW), cessation of earlywood vessels enlargement (ceEW), and cessation of ring width expansion (ceRW). The durations of the phases of earlywood with mature vessels (dEW = ceEW - bmEW) and ring in cell extension (deRW = ceRW - beEW) corresponded to the time span between the onset and cessation of these phases. All phases were also compared between northern and southern sides using a Student’s *t*-test. A logistic regression between earlywood phenophases and DMC of buds and leaves was computed to test the ontogeny relations removing 'time' as a factor.

The duration of primary growth phenophases in the whole crown was defined as the time elapsed from the first recording of each event in one or several branches to their appraisal in 100 % of the crown. A logistic regression was also chosen to establish the DOY of each crown phenophase being active in 50% of the tree population, using the first recording and appraisal in 100 % of the crown. The standard deviation of each day expressed as DOY was computed by taking the 2.5% and 97.5% probabilities (Rathgeber *et al.*, 2011).

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# Results

## Crown phenology

The visual appraisal of crown phenology showed overlapping shoot phases during a crown development period of 90 days, from the swollen bud phenophase in early April to the complete leaf extension at the end of June (Fig. 2a). Budburst started between the end of April and early May (117 ± 5.7 DOY for first recording and 128 ± 14 DOY for 100%) and was followed by leaf unfolding in the first fortnight of May (130 ± 14.9 DOY for first recording and 133 ± 10.0 DOY for 100%; Table 3a). Leaf extension took place in the second fortnight of May for first recording of the crown (from 137 ± 19.3 to 154 ± 12.1 DOY) and from the beginning of June to three days before the summer solstice for 100% of the crown (from 150 ± 1.9 to 172 ± 1.6 DOY), coinciding with the maximum shoot extension rate (SER) (Fig. 2b). The time elapsed between the first recording and 100% of the crown was 11 days for budburst, four days for leaf unfolding, and more than 21 days for leaf extension (Table 3a).

**Table 3** Critical dates for primary and secondary growth of *Q. pyrenaica* computed by logistic regression. Phases for primary growth are: computed critical days of bud swelling, budburst, leaf unfolding, less than 50 % and more than 50% of the leaf size for first recording and 100% of crown of each phenophase, and the time elapsed to complete each phase. Phases for secondary growth are: computed critical days for the beginning of earlywood enlargement (beEW) and maturation (bmEW), cessation of earlywood enlargement (ceEW), cessation of ring-width cell expansion (ceRW), duration of earlywood with mature vessels (dEW) and ring-width cell extension (deRW) in the north (N) and south sides (S), as well as the average (S+N).

**(a) Primary growth phenology**

| Crown onset | Bud swelling | Budburst | Leaf unfolding | < 50% leaf size | > 50% leaf size |
|---|---|---|---|---|---|
| **First recording** | 80±2.6 | 117±5.7 | 130± 14.9 | 137±19.3 | 150±1.9 |
| **100% of crown** | 113±1.1 | 128±14.4 | 133±10 | 154±12.1 | 172±1.6 |
| **Time elapsed** | 33±2.8 | 11±15.5 | 4 ±17.9 | 16±22.8 | 21±2.5 |

**(b) Secondary growth phenology**

| Loc | beEW | bmEW | ceEW | ceRW | dEW | deRW |
|---|---|---|---|---|---|---|
| **S** | 97±18.5 | 123±16.2 | 180±3.5 | 247±24 | 57±16.6 | 150±18.8 |
| **N** | 102±21 | 122±14.6 | 183±3.7 | 256±1.4 | 61±15.1 | 154±21 |
| **S+N** | 94±21.8 | 121±15.3 | 182±3.7 | 256±3.9 | 61±15.7 | 162±21.8 |

## Relationship between shoot extension rate and DMC of buds, leaves, and twigs

DMC in leaves, buds, and twigs showed a common decreasing pattern, reaching a minimum in the first half of June (150–162 DOY), followed by a gradual increase until the end of September (Fig. 2c). Maximum SER concurring with the lowest organ DMC suggested the hypothesis of high tissue hydration for

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growth purposes. In addition, time course of DMC variation of individual trees during the sampling period was highly homogeneous (Fig. 3).

SER was negatively correlated with DMC for all organs (Table 4), maximized for current twigs (*r*=–0.590, $P \leq 0.001$), and minimized for 2-year-old twigs ($r = -0.284$ $P \leq 0.023$) due to the lower range of variation in DMC (Table 5). The correlation among DMC of organs ranged from 0.924 to 0.516 ($P < 0.001$ in all cases), which confirms that the change in DMC had a similar pattern for all parts of the crown measured.

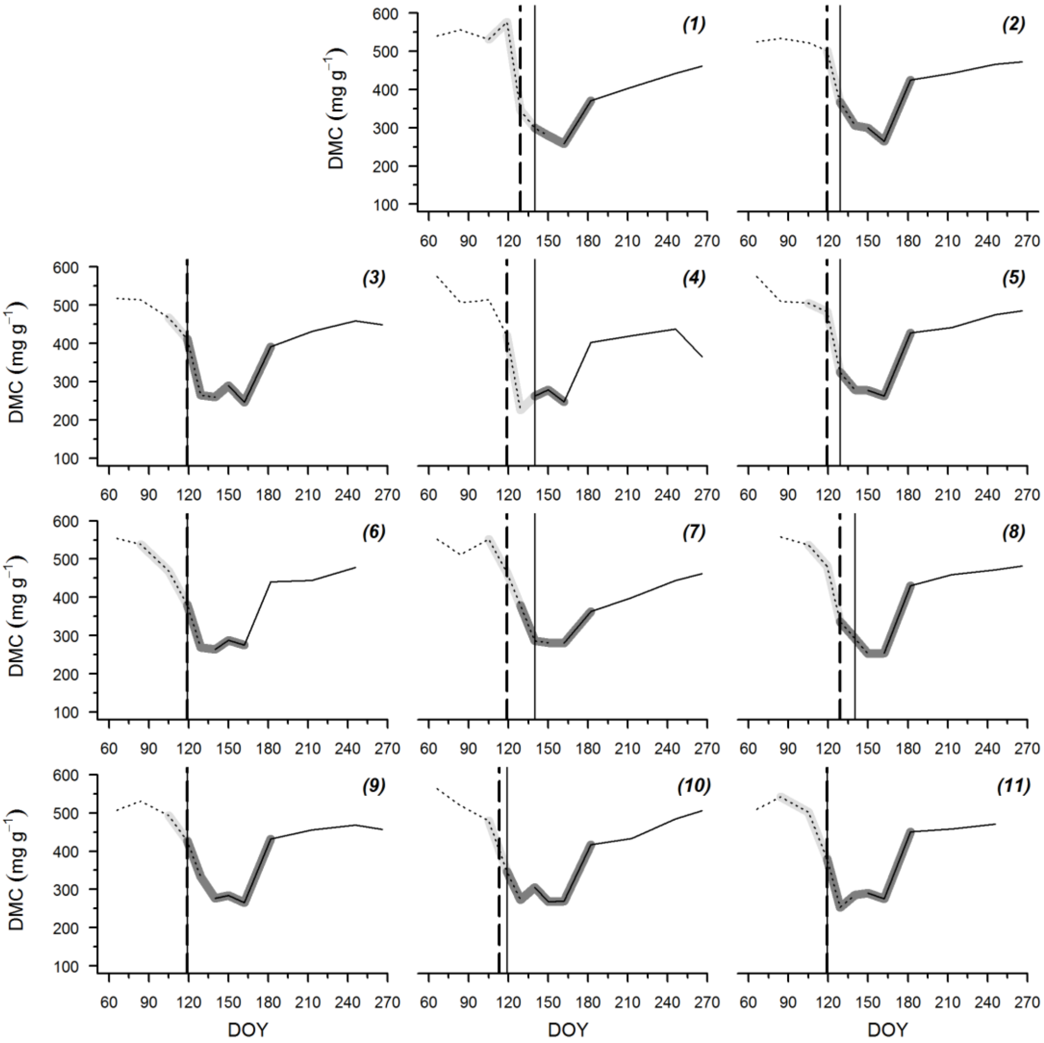

**Fig. 3** Individual seasonal trends of bud swelling and leaf development of 11 *Q. pyrenaica* in 2013 quantified as dry matter content (DMC in mg g$^{-1}$) of buds during swelling (dotted line) and leaves in development (continuous line); the light gray shade shows the period in which all the earlywood vessels were in expansion, while the dark gray shadow shows the overlapping period of some expanding vessels with the deposition of secondary cell wall. Vertical lines indicate the time elapsed from the first recording of budburst (dashed line) in one or several branches to their appraisal in 100 % of the crown (solid line). In brackets the number of each tree. Samples taken from day 66 to 266 of year (DOY) in 2013.

Mean values of DMC (Table 5) had no clear difference for 2-year twigs among sampling dates (DOY), which is in agreement with the lower proportion of growing tissue; whereas buds and 1-year old twigs showed a significant variation ($P < 0.05$) in hydration, which corresponds to bud swelling and earlywood vessel enlargement. This variation in DMC allows a more accurate definition of the onset of crown development than visual methods. Furthermore, significant ($P < 0.05$) increase in DMC of current year twigs and leaves allows identifying the end of shoot elongation.

The variation in DMC of crown organs through time allowed quantifying the visual perception of crown phenology. Shoot phenophases (Table 6) showed low and similar values of DMC (278.9 to 270.9 ± 13.2 mg $g^{-1}$) from leaf unfolding to leaf size > 50%. DMC was maximal during bud dormancy (540.1 ± 13.8 mg $g^{-1}$; $P < 0.05$) and decreased significantly at bud swelling (425.7 ± 13.2 mg $g^{-1}$; $P < 0.05$) and further at budburst (346.9 ± 13.8 mg $g^{-1}$; $P < 0.05$) due to the onset of primordial extension inside buds. In contrast, DMC of leaves increased significantly in early July (DMC 422.3 ± 13.2 mg $g^{-1}$; $P < 0.05$) dating the appearance of a new critical event inappreciable by binoculars or leaf area index measurements, probably the hardening of leaves associated to biomass accumulation and the increased volumetric fraction of structure (Roderick *et al.*, 1999).

**Table 4** Spearman's correlation coefficients between shoot extension rate (SER; log(mm $d^{-1}$) and dry matter content (mg $g^{-1}$) of different crown organs of *Q. pyrenaica*: One year old twigs (Twigs - 1), two years old twigs ( Twigs - 2), current twigs (Twigs), buds and leaves. The corresponding *P* values are also shown at the right side of the table.

| | Twigs - 1 | Twigs - 2 | Twigs | Buds | Leaves | SER |
|---|---|---|---|---|---|---|
| **Twigs - 1** | - | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| **Twigs - 2** | 0.860 | - | < 0.001 | < 0.001 | < 0.001 | 0.023 |
| **Twigs** | 0.785 | 0.778 | - | - | < 0.001 | < 0.001 |
| **Buds** | 0.658 | 0.516 | - | - | - | - |
| **Leaves** | 0.645 | 0.640 | 0.924 | - | - | < 0.001 |
| **SER** | -0.425 | -0.284 | -0.590 | - | -0.502 | - |

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**Table 5** Time course evolution of DMC (mg g$^{-1}$) of buds, one, and two years old twigs, leaves and current year twigs of *Q. pyrenaica* during the crown growing season in 2013. Differences in mean values between DOY ($P$<0.05) are indicated by upper lettering. First values significantly different from previous observations are highlighted in bold.

| DOY | Two years old twigs | One year old twigs | Bud | Leaves | Current year twigs |
|---|---|---|---|---|---|
| 66 | 510.6±8.5 $^{a}$ | 482.4±6.6 $^{a}$ | 543.5±12.6 $^{a}$ | - | - |
| 84 | 510.1±8.5 $^{a}$ | 487.2±6.6 $^{a}$ | 529.4±12.1 $^{a}$ | - | - |
| 105 | 508.8±8.5 $^{a}$ | 490.3±6.6 $^{a}$ | 506.6±12.1 $^{a}$ | - | - |
| 119 | 508.3±8.5 $^{a}$ | 481.7±6.6 $^{a}$ | **442.9±12.1** $^{b}$ | - | - |
| 129 | 478.8±8.5 $^{a, b}$ | **452.4±6.6** $^{b, c}$ | 305.5±12.6 $^{c}$ | - | - |
| 140 | 468.5±8.5 $^{b, c}$ | 431.9±6.6 $^{c, d}$ | 285.9±17.0 $^{c}$ | 276.0±10.2 $^{c}$ | - |
| 150 | 468.3±8.5 $^{b, c}$ | 421.8±6.6 $^{d, e}$ | 259.9±18.8 $^{c}$ | 280.9±7.8 $^{c}$ | 228.0±7.0.$^{a}$ |
| 162 | 432,7±8.5 $^{c}$ | 393.2±6.6 $^{f}$ | - | 259.7±7.2 $^{c}$ | 207.0±6.3.$^{a}$ |
| 182 | 432.4±8.5 $^{c}$ | 404.2±6.6 $^{e, f}$ | - | **413.9±6.9** $^{b}$ | **319.8±6.5.**$^{d}$ |
| 213 | 478.9±9.3 $^{a, b}$ | 411.7±6.6 $^{d, f}$ | - | 435.4±6.9 $^{b}$ | 411.6±6.3.$^{b}$ |
| 246 | 495.4±8.9 $^{a, b}$ | 466.3±6.6 $^{a, b}$ | - | 463.4±6.9 $^{a}$ | 459.6±6.3.$^{c}$ |
| 266 | 509.9±8.9 $^{a}$ | 490.9±6.6 a | - | 464.1±7.2 $^{a}$ | 492.0±6.5.$^{e}$ |

**Table 6** Characterization of phenological phases of crown by dry matter content (mg g$^{-1}$) of *Q. pyrenaica*. Differences in dry matter content between branch phenophases ($P$<0.01) are indicated by upper lettering.

| Branch phenophases | Dry matter content |
|---|---|
| **Bud dormancy** | 540.1±13.8 $^{a}$ |
| **Bud swelling** | 425.7±13.2 $^{b}$ |
| **Budburst** | 346.9±13.8 $^{c}$ |
| **Leaves unfolding** | 278.9±13.2 $^{d}$ |
| **Leave size < 50%** | 277.9±17.6 $^{d}$ |
| **Leaves size > 50%** | 270.9±13.2 $^{d}$ |
| **Leaf hardening** | 422.3±13.8 $^{c}$ |
| **Full mature leaves** | 463.4±13.2 $^{c}$ |

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## Xylogenesis

Crucial events of wood formation did not differ significantly ($P$ > 0.05) between north and south side when logistic curves were compared (Table 3b). Therefore, we averaged data from both sides, and estimated the duration of ring-width increment as 162 ± 21.8 days spanning from early April (94 ± 21.8 DOY) to the end of September (256 ± 3.9 DOY). During this period, cambial activity showed a bimodal pattern (Fig. 2e) with two peaks in ring-width increment in spring (early May; 129 DOY) and summer (early July; 182 DOY). The first one corresponded to earlywood vessel formation, and was lower than the summer peak of latewood, while the decreasing rate in June (150-160 DOY) pointed out the lower rate before the transition from earlywood to latewood formation.

Earlywood maturation spanned 61 ± 15.7 days (Fig. 2d; Table 3b) for 50% of the trees, with the first mature vessels at the beginning of May (121 ± 15.3 DOY), and earlywood vessel enlargement finished at the end of June (182 ± 3.7 DOY). In this period, DMC values dropped below 410 mg $g^{-1}$ in buds and leaves, corresponding to the earlywood development period (Fig. 3, 4).

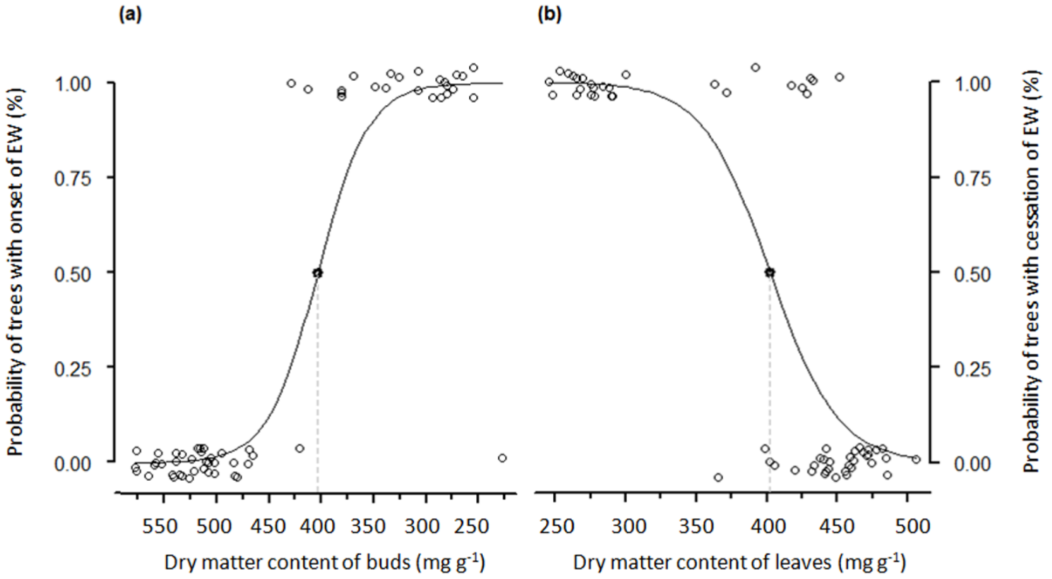

**Fig. 4** Probability of trees reaching each phenophase predicted by logistic regression in relation to bud and leaf dry matter content (DMC in mg $g^{-1}$) in *Q. pyrenaica*. Black point and grey vertical dashed line indicate the DMC when the 50 % of trees reach each earlywood phenophase. **(a)** Dots represent each observation of the onset of earlywood maturation in binary data (0 = no secondary wall deposition in the first earlywood vessel; 1 = at least one vessel has started a secondary wall deposition) in relation to bud DMC (mg $g^{-1}$). **(b)** Dots represent the observation of the cessation of earlywood maturation as binary data (0 = not earlywood enlargement; 1 = at least one early wood vessel in enlargement) in relation to leaf DMC (mg $g^{-1}$).

Significant variation in DMC of buds (Table 5) took place at the end of April (119 DOY; $P < 0.05$) pointing out that bud swelling had already taken place, which coincided with the beginning of earlywood maturation (Table 3b; 121 ± 15.3 DOY; $P < 0.05$). During leaf extension, the significant variation in DMC of leaves and current twigs was detected at the first day of July (182 DOY; $P < 0.05$), at the same time as cessation of earlywood formation.

For all trees, the decline in earlywood enlargement (Fig. 2e), i.e., when earlywood maturation had already started (Fig. 2d,f), coincided with the period of leaf extension (150−170 DOY; Fig. 2a), the lowest DMC of leaves (Fig. 2c), and maximum SER (Fig. 2b).

## Discussion

Our results on *Q. pyrenaica* show significant differences in DMC between dormant and swollen buds, as well as minimum values of DMC from budburst to full extension of leaves. In addition, shoot extension rate (SER) was found to be negatively related to DMC of leaves, and current and preceding year twigs, while the DMC of buds was positively related to that of 1 and 2-year old twigs. These findings are in agreement with seasonal changes in water content of twigs/leaf tissues reported by Davis & Mooney (1986) and Tognetti *et al.* (2000) for several Mediterranean woody species, recording increments in water saturated weight/dry weight ratio during spring growth. This was confirmed by Palacio *et al.* (2008), who also established quantitative relationships between SER and DMC of leaves and twigs.

The sequential changes in DMC allowed us to obtain a more objective dating for the timing of primary growth than the visual inspection by binoculars. The timing of reduction in bud DMC facilitated the monitoring of bud swelling, whereas the increase in leaf DMC indicated the hardening of leaves, which is difficult to identify visually in the practice. This explains why most studies record late phenophases, such as budburst or leaf unfolding, in order to assess the onset of shoot growth. In fact, shoot growth is constituted by two processes, namely the initiation of primordia by the apical meristem, and the extension of these primordia into fully developed organs (Champagnat *et al.*, 1986). The first of them (organogenesis) occurs inside the bud in the vegetative period of the year previous to budburst (Alla *et al.*, 2013). After winter, organogenesis restarts at the onset of budburst, when alteration in cell division at the shoot apical meristem leads to leaf initiation (Sinha, 1999).

Changes in DMC of buds in early spring may be associated with the extension of meristems, which has been attributed to an ontogenic process involving increased hydration (Bradford & Hsiao, 1982;De Fay *et al.*, 2000). Although the early phase of bud swelling is often a part of observation protocols, its precise onset is very difficult to ascertain over multiple buds at regular intervals, especially in the field.

Expanding cells of crown organs adjust their osmotic potential and cell wall elasticity to maintain adequate turgor pressure throughout the growth process (Bradford & Hsiao, 1982;De Fay *et al.*, 2000). The mechanisms used by cells to maintain this pressure may increase the capacity of organs to gain water when set at full turgidity, hence leading to reduced leaf and stem DMC (Palacio *et al.*, 2008). Cessation of this extension, in our case occurs 52 days after budburst, and is concomitant with dry matter accumulated in leaf cells. This contrasts with the

21-day period of leaf unfolding noted for *Q. pubescens* by Lavrič *et al.* (2017) after monitoring leaf area index. A part of this accumulation may come as of non-structural carbohydrates, following the increase of net photosynthetic rates, once respiratory demands associated with growth decrease, and leaves become net sources of carbon (Palacio *et al.*, 2008). For *Q. robur*, Morecroft *et al.* (2003) showed that development of full photosynthetic capacity took place between approximately 50-70 days after bud break. This result is also consistent with the carbohydrate accumulation recently recorded in stems of *Q. pyrenaica* in summer (Pérez-de-Lis *et al.*, 2017).

On average, the phenophases showing the largest increases in water content (the opposite to DMC) were leaf unfolding and leaf extension, with values up to 72%. Bud swelling and budburst were characterized by a lower hydration (57% and 65% respectively), in contrast to only 46% in dormant buds. These low values of hydration in buds are due to the presence of bud scales, which are only dry protective organs. Such increase in the water content of organs need to be supported by either water stored in living cells, (latewood) vessels and vasicentric tracheids from previous years or earlywood vessels from the current year. Therefore, quantification of the water content in twigs along with monitoring of earlywood formation in the stem may be crucial to characterize the progression of crown development in ring-porous hardwood species.

Previous works in deciduous oaks highlighted that some earlywood vessels can be already enlarging at the time of leaf unfolding (Suzuki *et al.*, 1996;Sass-Klaassen *et al.*, 2011;Michelot *et al.*, 2012b;Puchałka *et al.*, 2017). According to González-González *et al.* (2013) and Pérez-de-Lis (2016), earlywood vessel enlargement in the stems of *Q. pyrenaica* takes place before budburst, while secondary wall deposition is initiated at the moment of budburst. This may indicate that new vessels are ready for water transport during leaf development, as evidenced the increasing sap flow recorded upon budburst in *Q. pubescens* (Lavrič *et al.*, 2017). On the other hand, functionality of vessels has been defined by observing the presence of perforations in longitudinal thin sections of *Fraxinus excelsior* L. (Atkinson & Denne, 1988), and *Quercus serrata* Murray and *Robinia pseudoacacia* L. (Kudo *et al.*, 2015). These authors found that perforations in the first earlywood vessels were completed along the entire stem when small leaves were visible to the naked eye. Kitin & Funada (2016) concluded that water requirements of early leaves must be fulfilled by latewood vessels formed in previous years. Moreover, (Basler & Korner, 2014) detected bud swelling and budburst in cuttings of several species (including *Q. petraea*) collected in late winter, which entails that these phenophases may be attained without the presence of newly formed earlywood vessels.

In view of these evidences, our results confirm that new earlywood vessels cannot contribute to budburst, suggesting that water supply should come from already existing living cells and previous year latewood vessels and tracheids. Leaf unfolding is closely coupled with significant changes in DMC of one-year-old twigs, probably associated to the increased hydraulic conductance and new tissues formation, which appears to indicate that new vessels are functional within a few days after the onset of secondary wall deposition.

The difficulties found when defining the cessation of full leaf development may be sorted out by DMC measurements as an index of the cessation of water demand for shoot extension. Low values of DMC in the crown last up to the second fortnight of June, c. 40 days after the maturation of the first new vessels, and coincide with the cessation of earlywood formation. In line with previous studies (Lavrič *et al.*, 2017), our results consequently reveal the required functionality of earlywood vessels to attend the high water demand for shoot and leaf extension to complete the development of the crown. In turn, earlywood vessel development would also influence shoot extension in subsequent years, as the number of leaf primordia in dormant buds appears to be positively related to xylem conductance and vessel size (Cochard *et al.*, 2005).

Consequently, our study highlights the sequence of earlywood development in relation to primary growth events as quantified by the water content of branch organs (Fig. 5). Enlargement of earlywood vessels and bud swelling are concomitant events, suggesting that water demanded by developing buds is supplied by latewood vessels, tracheids or living cells, since new vessels did not reach their final size until budburst. This is followed by leaf unfolding, which coincides with the hydration of 1-year old twigs, probably because the newly formed vessel network is already able to provide enough water, and thus attend the demand for the maximum rate of shoot elongation.

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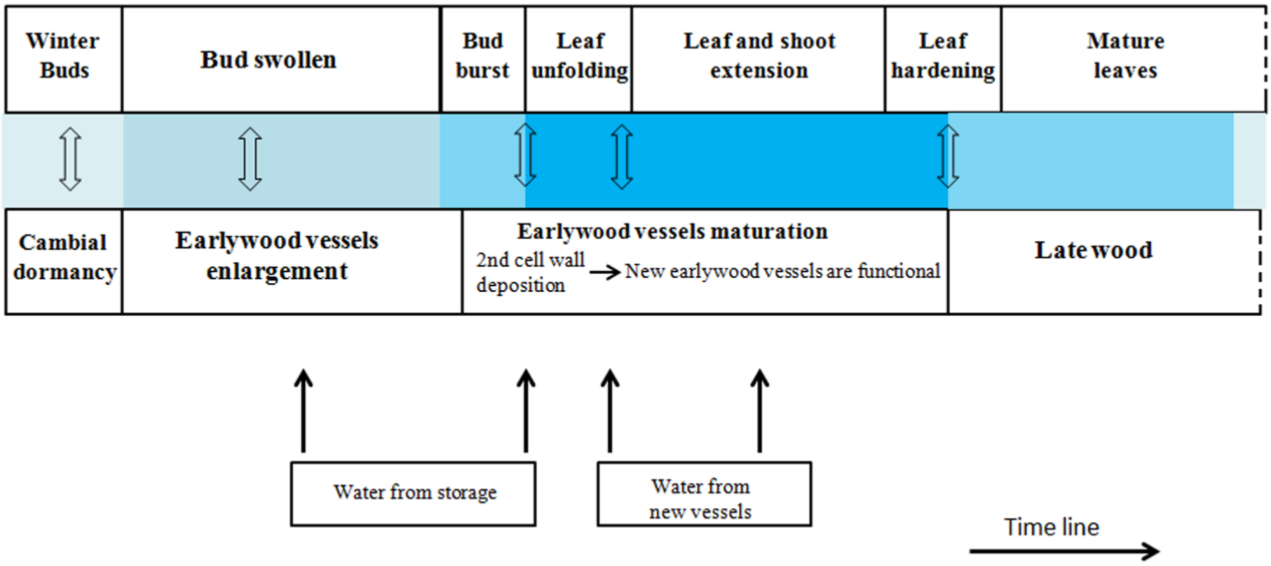

**Fig. 5** Sequence of events during shoot growth of the canopy in relation with the development of earlywood vessel in the stem of *Q. pyrenaica*. Colour density indicates increased water content.

## Conclusions

To our knowledge, we show for the first time that determining crown organ DMC is a powerful tool to quantify primary growth in trees from bud swelling up to full leaf extension, which are the most difficult phenophases to ascertain with visual inspection by binoculars. This method constitutes a simple tool for understanding the dynamics of tissue hydration during crown development, and allows monitoring the water content of buds, twigs, and leaves in relation to secondary growth under field conditions, providing that the branches are easily accessible. Furthermore, the results show that new earlywood vessels need to be functional to fulfil water requirements for shoot extension, but not for budburst. We consequently propose an objective classification of bud and leaf phenophases according to their DMC, which avoids the subjectivity of the observer.

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# Response patterns of xylem and leaf phenology to temperature at the southwestern distribution boundary of *Quercus robur*: A multi-spatial study

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## Abstract

We investigated how temperature patterns affect cambial activity and leaf phenology of oak across a wide range of natural woodlands at its southwestern distribution boundary. Understanding the climatic control of wood formation in dominant species is very relevant to infer tree responses to ongoing environmental changes and their impact on the carbon cycle.

We selected nine sites along two elevation gradients from the coastline in northwestern Iberia, and sampled ten trees per site biweekly during 2012 and 2013. Leaf and cambial phenological phases were related to mean air temperature for 10–60 day running periods along the year to identify the most relevant time windows for cambium and leaf phenophases, and the relationships among them.

The first earlywood vessels expanded before the appearance of small leaves, and subsequently underwent maturation to meet water requirements for full leaf unfolding. The advance or delay of cambial reactivation and budburst varied among sites and years modulated by spring temperature, and were respectively maximized by maximum and minimum values.

Temperature can modify the onset of early phenophases of primary and secondary growth differently, and also the synchronicity between them. However, the maturation of the first earlywood vessels is necessary to undergo full leaf extension.

**Keywords:** budburst, cambium, earlywood, latewood, ring-porous wood, xylogenesis.

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## Introduction

*Quercus robur* L. is a nemoral tree species widely distributed under different climatic conditions throughout Europe (Gilliam, 2016), from the Baltic Sea to its southern distribution boundary in the Iberian Peninsula. Along this boundary, the transition to the Mediterranean region involves its progressive replacement by more drought-tolerant species (Sánchez de Dios *et al.*, 2009), first nemoral oaks, and evergreen oaks under more limiting conditions.

One of these transitional areas is located in northwestern Iberian, where prevailing Atlantic conditions involve high precipitation records mainly during autumn and winter, but summer drought can often occur as a consequence of Mediterranean influence. In coastal areas, winter temperature is not limiting, but warm summer conditions facilitate the occurrence of thermophile species, or even subtropical elements where summer drought is not limiting (Izco *et al.*, 1990). As a result, trees need to couple their patterns of phenology and wood formation to these meteorological conditions.

Activity of primary and secondary meristems follows a distinct annual pattern in crown and cambium, respectively. These processes are constraint to a well-defined time period from spring to autumn, when conditions are favorable for growth and reproduction, whereas trees enter dormancy during winter. Spatial and temporal changes in environmental conditions can modify tree phenology (Menzel *et al.*, 2006;Vitasse *et al.*, 2017), as species adjust the timing and length of their growth and reproduction phases to climate, with variations that depend on specific regional drivers, local adaptations, or individual plasticity (Rossi *et al.*, 2013). Though also interacting with photoperiod (Basler & Korner, 2014), phenological events related to growth resumption are mainly driven by temperature (Begum *et al.*, 2013;Rossi *et al.*, 2016), and consequently changes related to the ongoing global warming have been observed (Menzel *et al.*, 2006).

*Q. robur*, as a deciduous ring-porous hardwood species, needs to yearly renew its xylem vessel network, so that the cambium must build the mechanical and conductive support to fulfill water requirements from roots to leaves. The main pathways of sap flow are the networks of earlywood vessels of the current year, because those from the previous season are no more functional after a year (Chaney & Kozlowski, 1977;Ellmore & Ewers, 1986;Umebayashi *et al.*, 2008). The differentiation of current year’s earlywood vessels has been detected to take place before the onset of bud growth (Sass-Klaassen *et al.*, 2011;Takahashi *et al.*, 2015;Pérez-de-Lis *et al.*, 2016b;Lavrič *et al.*, 2017;Puchałka *et al.*, 2017). However, the importance of their contribution for crown development is still

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under discussion (Kudo *et al.*, 2018). Recent studies on the specific moment when the networks of vessel elements become functional as conduits for water movement suggest that the requirements of the early stages of leaf expansion can still be fulfilled by latewood vessels formed in previous years (Kudo *et al.*, 2015;Kitin & Funada, 2016). Therefore, it is necessary to understand not only the mechanisms of wood formation, but also physiological aspects of the tree growth.

Variations in the meteorological conditions prior or/and at the moment of specific phenological events can affect the synchronization between primary and secondary growth in a different way. Buds, leaves, and flowers that grow from primary meristems are easily visible to the naked eye, and numerous records allow assessing variations in the timing of leaf phenology. For example, Rossi (2015) found that temperature is a predominant factor driving the ecotypic differentiation of budburst in black spruce. Recent studies on different oak provenances in England (Wilkinson *et al.*, 2017) reported an advance in spring budburst across a range of temperatures, and Čufar *et al.* (2012) found that March and April temperatures were related leaf unfolding in beech (*Fagus sylvatica*) in Slovenia. During the growing season, vascular cambium cannot be directly observed to record phenological events, but in the last decades, a great effort has been made to identify stages of wood formation at a cellular level, although this monitoring of xylogenesis is a very time-consuming method.

In conifers, linear and nonlinear patterns of timings and duration of wood phenology showed local annual temperature as the main driver of cambial activity in the northern hemisphere (Rossi *et al.*, 2013;Rossi *et al.*, 2016). Similarly, a phenological advance in xylem phenology linked to temperature was found to modify the production of wood biomass in northeastern France (Cuny *et al.*, 2015). However, the study of xylogenesis in hardwoods has deserved less attention. In the case of ring-porous oaks (*Q. robur* and *Q. pyrenaica* Willd.), Pérez-de-Lis *et al.* (2017) demonstrated that the length of the growing season was modulated by predisposing the number of dormant cambium cells, whereas the size of the first earlywood vessels was affected by the timing of earlywood enlargement (Pérez-de-Lis *et al.*, 2016b). The reactivation of cambial cells by heat treatment was also proven for deciduous species (Begum *et al.*, 2013;Kudo *et al.*, 2014); similarly, differences in the yearly onset can be explained by temperature variations (Prislan *et al.*, 2013).

Although there is much evidence that temperature is one of the main driving forces for plant growth in terrestrial ecosystems, its influence on wood formation has been mainly analyzed on conifers, often at high latitudes or elevations (Lorena *et al.*, 2016;Rossi *et al.*, 2016), whereas studies on cambial dynamics of

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temperate hardwood species such as oaks are limited to very few sites (Lavrič *et al.*, 2017;Pérez-de-Lis *et al.*, 2017;Puchałka *et al.*, 2017). On the other hand, several studies dealt with the variation of phenology across gradients, but were not linked to secondary growth (Vitasse *et al.*, 2017). In order to fill this gap, we intend to evaluate the influence of the temperature regime on the primary and secondary growth of native *Q. robur* forests within a region that covers broad environmental gradients. For this, we compiled a data set of cambial dynamics and leaf phenological phases at nine sites during 2012 and 2013, and related their different phases to temperature. These sites were located towards the most southwestern distribution boundary of oak, and represent Atlantic and Cantabrian influences, from mild coastal areas to high continental inland, which to our knowledge provides the widest range of these data currently available for oak. The aim of our study is to establish the role of temperature on wood formation and crown development by i) comparing the timings of cambial activity and leaf phenological phases, and ii) evaluating how these relationships vary along different microclimatic gradients.

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## Material and methods

### Study site and tree selection

The study was conducted at nine *Q. robur* stands in northwestern Iberia (Table 1). The selected sites covered the course of two rivers Eume (E) and Sor (S), from their upper watershed to their mouths into the Atlantic Ocean or the Cantabrian Sea respectively (Fig. 1a). The area presents a mild Atlantic climate, with a mean annual temperature ranging 9−13 ºC among sites, and a total precipitation of 900−1,500 mm; maximum rainfall occurs during autumn-winter, and there is a varying degree of summer drought depending on elevation or position towards the coast. Oak forests in this area are characterized by moist and warm conditions, which even result in the occurrence of some subtropical plants (Izco *et al.*, 1990), especially at low altitude.

**Table 1** Description of the sites included in the analysis, with their identification codes (ID), the corresponding river catchment (Eume, Atlantic; and Sor, Cantabrian), site name, geographical location, elevation, and diameter at breast height (DBH) of the sampled trees. Normal annual temperature (Temp) and precipitation (Prec) obtained from Rodríguez-Lado *et al.* (2016) (source: digital database at http://rgis.cesga.es).

| ID | River | Latitude (N) | Longitude (E) | Elevation (m a s l) | DBH (cm) | Temp (°C) | Prec (mm) |
|---|---|---|---|---|---|---|---|
| **E1** | Eume | 43.416991 | −8.064395 | 125 | 29.1 ± 4.5 | 13.2 | 1158 |
| **E2** | Eume | 43.372757 | −7.991181 | 350 | 29.0 ± 5.4 | 12.2 | 1268 |
| **E3** | Eume | 43.469576 | −7.788688 | 450 | 29.3 ± 4.4 | 12.0 | 1427 |
| **E4** | Eume | 43.448001 | −7.634295 | 600 | 30.0 ± 4.8 | 9.5 | 1526 |
| **S1** | Sor | 43.676000 | −7.708347 | 125 | 29.5 ± 5.2 | 13.0 | 1194 |
| **S2** | Sor | 43.594782 | −7.721225 | 275 | 28.0 ± 5.4 | 12.9 | 1232 |
| **S3** | Sor | 43.556076 | −7.740287 | 350 | 30.0 ± 5.8 | 12.4 | 1346 |
| **S4** | Sor | 43.529401 | −7.736388 | 500 | 24.8 ± 3.9 | 12.4 | 1361 |
| **S5** | Sor | 43.495749 | −7.739470 | 625 | 25.7 ± 3.6 | 11.0 | 1442 |

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Within each watershed, we followed an altitudinal gradient that covered the whole species distribution, with increasing degrees of precipitation with elevation, and continentality from the coast to the inland mountains. Nevertheless, these two rivers represent two dominant climatic influences on oak formations in northwestern Iberia. Whereas the Atlantic coast is dominated by a certain Mediterranean trend during summer (higher temperature, more intense drought), the Cantabrian coast is exposed to mild northern winds that increase humidity and attemperate maximum temperature (Martínez Cortizas & Pérez Alberti, 1999).

We monitored these forests for two consecutive years, from March to November in 2012 (1,175 trees sampled), and from February to September in 2013 (1,000 trees sampled). For this, 90 trees (9 locations × 10 trees) with a diameter of 20−40 cm were randomly selected at each sampling date within an area of ca. 2 ha per forest. The sampling interval was 12−15 days in spring, and 20 days in summer, because changes during earlywood formation or transition to latewood take place much faster than others later in the season. Trees selected were dominant or codominant in the canopy, and individuals with polycormic stems, partially dead crowns, or evident damage, were avoided. The random selection of trees at each date prevented the study at the individual tree level, but provided a representative sample of the whole forest as a single population, avoiding the bias of a specific tree. In addition, this strategy increased the number of individuals that could be analyzed to relate primary and secondary growth, i.e., to associate leaf and cambium phenophases.

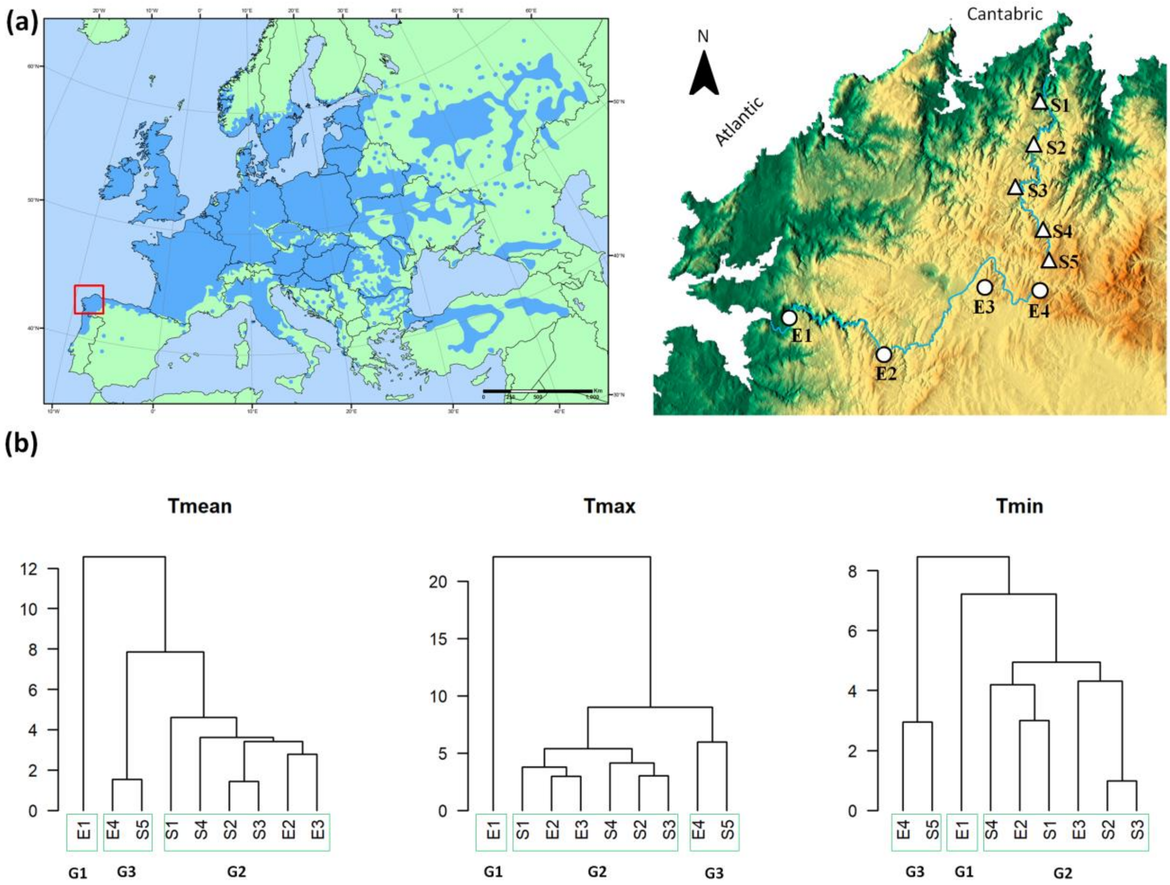

**Figure 1**. Location of the study sites in the northwestern Iberian Peninsula, and corresponding hierarchical classification following temperature regime. **(a)** *Q. robur* distribution map (www.euforgen.org), and the study sites, Eume (E1-E4) and Sor (S1-S5) river. **(b)** Dendrograms of monthly mean, maximum, and minimum temperatures for the nine sites, clustered into three similar climatic conditions G1, G2, G3.

## Site meteorological data

Air temperature was hourly monitored at each site during the sampling period using sensors (iButton DS1922L, San Jose, CA, USA) set at 2 m above the ground surface. We calculated daily mean, maximum, and minimum values, as well as the corresponding thermal amplitudes. Temperature time series were corrected to complete missing data by simple linear regression with daily records from nearby meteorological stations belonging to the weather service Meteogalicia (http://www.meteogalicia.gal/). We compared all study sites according to their monthly mean temperatures for both study years using hierarchical cluster analysis. The Euclidean distance was selected to calculate dissimilarities among sites, whereas the average linkage was used as grouping method (Fig. 1b).

## Xylem sampling and wood phenophases

At each sampling date, a minimum of two microcores (2 mm in diameter, 15 mm in length) was extracted at breast height (1.3 m) out of each selected tree, in perpendicular direction to the slope, by means of a Trephor tool (Rossi *et al.*, 2006a). Samples contained mature and developing xylem of the current year, the cambial zone and adjacent phloem, and at least one previous complete tree ring. After extraction, one microcore per tree was processed by embedding in paraffin, cutting into thin sections with a rotary microtome, and staining following the same protocol as in previous works (Pérez-de-Lis *et al.*, 2016b;Guada *et al.*, 2018).

Observation and width measurements of the wood phenophases of the 2173 microcore cross-sections were performed on images taken with a digital camera (Canon EOS 600D, Tokyo, Japan), coupled to a transmitted light microscope (Olympus BX40, Tokyo, Japan); a white light polarizing filter allowed the detection of secondary cell wall deposition (40 × magnification). We measured the width of cell expansion and maturation zones along three radial lines per image (Fig. S1); and each phase of cambial activity was expressed as particular day of year (DOY), including: beginning of cell enlargement in the earlywood (bE), beginning of earlywood maturation (bM), beginning of latewood maturation (bLW), and cessation of cell expansion (cE). The beginning of cell enlargement was considered when the most recent ring contained at least one enlarging cell; whereas the beginning of earlywood vessel maturation was defined by the deposition of the secondary cell wall in the vessels, detected by birefringence under polarizing light; the cessation cell expansion corresponded to the moment when no expanding cells were further detected, and the beginning of latewood maturation was defined as the moment when earlywood growth had already ceased, and maturation was detected in cells formed beyond the earlywood.

The durations between the onset and cessation of these phases were also calculated for cell expansion (dE= cE − bE), earlywood maturation (dEW = bLW − bM), latewood maturation (dLW = cE − bLW), and cell expansion of first row of earlywood vessels (d1r = bM − bE).

## Leaf phenological observations

Leaf phenology of each sampled tree was recorded in the field. Observations were performed at the upper main branches using binoculars (10 × magnification), and expressed as DOY.

We considered four different phenophases, namely budburst (BB), leaf unfolding (LU), appearance of small leaves (SL; leaves < 50% of their apparent

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final size), and full extension (FL; leaves > 50% of their apparent final size). Bud dormancy corresponded to the overwintering stage, while bud swelling was identified by the apparent separation of buds from the stem, together with the exposition of areas of lighter colored tissue as a result of the initial extension of cataphylls with the separation of scales. Budburst was characterized by green-colored expanded buds with no unfolded leaves, and leaf unfolding ended as soon as the leaf blade was clearly visible, but not the petiole. Appearance of small leaves was defined as the moment when at least one leaf was completely out of the bud, and current year twigs and petiole could be visually appreciated; and full extension was recorded when leaves attained at least the 50% of their apparent final size.

## Statistical analyses

In order to assess the critical dates of xylem and leaf phenology, the information on the absence or presence of each phenological event per site and year was expressed as binary data (0 no active phenophase; 1 active phenophase). Afterwards, we performed logistic regressions by GLM for each phenophase using the DOY as independent variable, and considering a probability of 0.5 as the most likely DOY for the activity of a given event (Rathgeber *et al.*, 2011). The effects of year, site, and their interaction were studied by comparing the best logistic regression fit when these factors were included isolated or in combination as classification variables. Independent variables considered in the models tested were as follows: DOY + year; DOY+ site; DOY + year + site; DOY + year × site. The model providing the lowest corrected Akaike's Information Criterion (AICc) for each event was selected as the best fit, and a multiple comparison between years and sites was performed using the Tukey test.

Relationships among the dates of wood phenological events estimated from the logistic regression and their durations were related using Pearson’s correlations. The DOYs of cessation of cell expansion among sites diverged significantly from normality, and were transformed using the $Y^2$ formula.

We used two approaches to establish relationships between primary and secondary growth at tree scale, and specifically to look for a possible link between vessel formation and budbreak. First, we selected the trees achieving the expansion of first row of earlywood vessels, i.e., those showing enlarging vessels adjacent to the previous annual ring, but still no maturation, and classified them according to their leaf phenology in order to obtain the proportion of trees that started crown development before earlywood maturation. The second approach consisted of selecting the trees with already mature

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earlywood vessels for each phenological crown phase, and consequently we established the proportion of trees that had started earlywood maturation for each stage of leaf development.

In order to detect the time window that influenced primary and secondary growth, we obtained moving averages of climatic data, and compared them to the DOYs of each phenological event along the growing season. Thus, we preliminary used 30-day averages of maximum and minimum temperatures, as well as thermal amplitude, and correlated them to each phenological event every 15 days. A more detailed analysis of the most relevant variables involved running temperature means of 10 to 60 days, shifted in only one day. For these analyses, we assumed that the increase of temperature advanced along with tree growth, and performed a linear model between the temperature before the event and the timing of each event per site, including the year as class variable. For each linear model, the *p*-value of a Type I test, and the corresponding percentage of variation explained, were provided for the temperature effect and for the difference between years. We also estimated temperature thresholds from those in the DOY predicted by the GLM logistic curve per site and year. All statistical analyses were performed using the R statistical software (R Core Team, 2017).

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## Results

### Temperature across sites

The two-year temperature records show a temperate regime, with an average variation of annual mean of 3.8 ºC across the whole area, from 10.1 ºC at S5 (continental influence) to 13.8 ºC at E1 (Atlantic influence). All sites (Table 1) exhibited a mild winter with an overall range of daily mean temperatures from −0.1 ºC to 15.4 ºC, respectively corresponding to the continental sites and to the sites closest to sea level; values below 0 ºC were only one day in February at the two most continental and highest locations. The minimum absolute temperature of all sites ranged from −3.5 to −0.25 ºC, with a period below 0 ºC of only four days at the most Atlantic site (E1), and up to 30 days under continental influence (E4). The maximum absolute temperature ranged between 25 ºC in July at S3, and 41 ºC in June at E1.

The sites were grouped into three main clusters based on their monthly mean temperatures of the two study years (Fig. 1b). The first cluster included only one site (E1), namely the lowest location (100 m a.s.l.) at the Atlantic side, where the monthly mean temperature ranged between 7.8 and 21 ºC. The second cluster held the lowest Cantabrian site S1(100 m a.s.l.), and all sites at mid elevation (200−500 m a.s.l.) regardless of being at the Atlantic or Cantabrian side; the average temperature range in this group was from 5.6 to 19 ºC. The remaining locations belonged to the third cluster, and included the two highest sites (650 m a.s.l.) of both watersheds, with monthly mean temperatures ranging from 4.6 to 17.6 ºC, being the coldest and most continental sites.

As regards differences between the altitudinal gradients along both rivers, mean monthly temperatures differed more among the locations on the Atlantic side (western exposure) than those towards the Cantabrian sea (northern exposure). There were also differences between both study years, mainly an increase in March maximum temperature in 2012, yielding an increment of the thermal amplitude due to the absence of precipitations (Fig. S2).

## Relationships between wood phenological timings and the duration of wood formation

Many of the dates and durations of cambium phenological events were correlated to each other (Table 2). Dates of detection of the first enlarging earlywood vessels were positively correlated with those of the beginning of maturation in both earlywood ($r$= 0.821; $P < 0.001$), and latewood ($r = 0.560$; $P < 0.05$). Thus, a late onset of vessel enlargement was associated to a delayed onset of vessel maturation. In contrast, no correlation was observed between beginning of cell enlargement and its cessation of expansion in latewood.

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**Table 2** Pearson's correlation matrix between the date of cambium phenology events (DOY) and the duration of wood maturation phases (days). The events are: beginning of cell enlargement (bE); earlywood maturation (bM); latewood maturation (bLW); and cessation of cell expansion (cE with a normal-transformation $Y^2$). The durations are referred to cell expansion (dE), earlywood maturation (dEW), latewood maturation (dLW), and cell expansion of first row of earlywood vessels (d1r).

| | **bE** | **cE** | **bM** | **bLW** | **dE** | **dEW** | **dLW** | **d1r** |
|---|---|---|---|---|---|---|---|---|
| **bE** | | | **< 0.001** | **< 0.05** | **< 0.05** | | | |
| **cE** | 0.170 | | | | **< 0.001** | | **< 0.001** | |
| **bM** | 0.821 | 0.358 | | | | | | |
| **bLW** | 0.560 | −0.022 | 0.562 | | | **< 0.01** | **< 0.01** | |
| **dE** | −0.478 | 0.791 | −0.197 | −0.357 | | | **< 0.001** | |
| **dEW** | −0.086 | −0.349 | −0.244 | 0.665 | −0.240 | | **< 0.01** | |
| **dLW** | −0.270 | 0.726 | −0.138 | −0.703 | 0.808 | −0.700 | | |
| **d1r** | −0.192 | 0.361 | 0.402 | 0.069 | 0.428 | −0.282 | 0.197 | |

The duration of cell enlargement was negatively correlated to its onset ($r = -0.478$; $P < 0.05$), and positively to the date of cessation of cell expansion ($r = 0.791$; $P < 0.001$), with a higher correlation for the latter. The duration of earlywood maturation was positively correlated to the beginning of latewood maturation ($r = 0.665$; $P < 0.01$), while there was no correlation to the onset of cell maturation or earlywood cell enlargement although the present definition of latewood maturation has to be borne in mind. The duration of latewood had a strong positive correlation to both duration ($r = 0.808$; $P < 0.001$) and cessation ($r = 0.726$; $P < 0.001$) of cell expansion, while it was negatively correlated to the duration of earlywood formation ($r = -0.700$; $P < 0.01$), and to the beginning of latewood maturation ($r = -0.703$; $P < 0.01$). Consequently, the durations of both cell enlargement and latewood maturation were more closely associated to the cessation than to the onset of cell enlargement.

## Phenological timings of xylem and leaves

The best fit of the logistic regression for all events, except for the cessation of xylem growth (cE), was achieved by the models including the interaction between year and site (Table 3). This indicates that the effect of the year on the delay or advancement of xylem and crown growth varied across sites. For the cessation of xylem growth, the best fit was provided by the model with year and site as independent main effects.

**Table 3** Generalized linear models to evaluate the effect of site and year on the occurrence of the main phenological events. AIC increments (DAICc) for each model are shown as compared to those of the model with the lowest score (the best-fitted model). Selected models are highlighted in bold. Beginning of cell enlargement (bE); beginning of maturation (bM); beginning of latewood maturation (bLW); cessation of cell expansion (cE); budburst (BB); leaf unfolding (LU); <50% of its apparent final leaf size (SL); >50% of its apparent final leaf size (FL); d.f. refers to degrees of freedom.

| | | **Xylogenesis** | | | | **Leaf phenology** | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fixed effects** | **d.f.** | **bE** | **bM** | **bLW** | **cE** | **BB** | **LU** | **SL** | **FL** |
| **DOY + Site x Year** | 19 | **0** | **0** | **0** | 3.50 | **0** | **0** | **0** | **0** |
| **DOY + Site + Year** | 11 | 30.94 | 11.32 | 6.52 | **0** | 84.29 | 4.01 | 21.34 | 13.92 |
| **DOY + Site** | 10 | 66.06 | 19.69 | 52.80 | 5.98 | 106.54 | 5.05 | 19.45 | 23.63 |
| **DOY + Year** | 3 | 130.39 | 87.88 | 43.53 | 20.97 | 208.96 | 170.68 | 199.68 | 231.26 |

The beginning of cell enlargement started on average 10 days earlier in 2012 than in 2013, but while the differences were significant at six out of the nine sites (E1, E3, E4, S2, S4, and S5), ranging from 23 days at E4 ($Z = -3.17$, $P < 0.01$) to 11 days at S2 ($Z = -2.34$, $P < 0.05$); this was not the case for the other three sites (E2, S1, and S3), which ranged from one day at S3 ($Z = 0.34$, $P = 0.73$) to five days at S1 ($Z = 1.03$, $P = 0.30$).

According to the post-hoc Tukey's test, the beginning of cell enlargement started on average six days earlier in 2012 than in 2013, although differences were only significant at three sites (E1, E3, and S5), and ranged from 15 days at S5 ($Z = -2.59$, $P < 0.01$) to 20 days at E3 ($\mathrm{Z} = -2.92$, $P < 0.01$). At the other six

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sites (E2, E4, S1, S2, S3, and S4) the differences between years ranged from one day at S4 ($Z = 0.17$, $P = 0.86$) to nine days at E4 ($Z = -1.53$, $P = 0.13$).

With regard to leaf phenology, budburst occurred on average 20 days later than the beginning of cell expansion in 2012 ($P < 0.001$), but synchronously in 2013 ($P = 0.57$). Differences in the onset between years were significant at five sites (E4, S2, S1, S3, and S4) ranging from 12 days at S4 ($Z = -3.4$, $P < 0.001$) to 29 days at S2 ($Z = 6.46$, $P < 0.001$ while they were only from one day at E3 ($Z = -0.16$, $P = 0.87$) to seven days at E2 ($Z = 1.51$, $P = 0.13$) at the other four sites (E1, E2, E3, S5).

The onset of leaf unfolding was synchronous with that of earlywood maturation (bM) in 2012 ($Z = 0.03$, $P \approx 1$). Differences between years were only non-significant at S1 (13 days, $Z = 2.41$, $P = 0.01$) and S2 (11 days, $Z = 2.21$, $P = 0.02$), while the appearance of small leaves was synchronous with earlywood maturation in 2013 ($Z = 0.579$, $P = 0.98$) and did differ between years ($Z = -0.028$, $P = 0.97$).

## Relationship between primary and secondary growth

Although the chronology of phenological events derived from the logistic regression provided some information on the relationship between primary and secondary growth, the random selection of trees per date allowed a further insight into this relationship, with a high number of samples at tree level.

A total of 314 individuals from the whole data set showed the cambial phenophase ‘cell expansion’, but had not started earlywood vessel maturation yet; we consequently calculated the proportion of these individuals with different crown phenophases (Fig. 2a). Out of the 174 trees sampled in 2012, only one showed expanded leaves (more than 50% of their apparent final size), while there were eight trees out of 140 in 2013. The number of trees at earlier phenological phases of crown development was higher, reaching 42 at budbreak in 2012 and 61 in 2013, while even a higher number (107 in 2012; 30 in 2013) still presented close buds.

Our second approach aimed at defining which proportion of trees with mature earlywood vessels showed each of the different crown phenophases, out of a total of 750 observations (Fig. 2b). The results showed an increasing proportion of trees with mature cells during the successive phenophases of crown development. The evolution was similar in both years, and trees with visible leaves were those that had a greater proportion of mature earlywood cells.

These analyses point to a synchronization between primary and secondary growth, as practically no expanding leaves existed at the moment earlywood cells began to enlarge. A percentage of 91% out of 314 trees enlarging the first

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cells did not show any phenological phases that were more advanced than leaf unfolding, whereas ca. 75% of 103 trees had begun the maturation of the first row of vessels concomitantly to the appearance of small leaves.

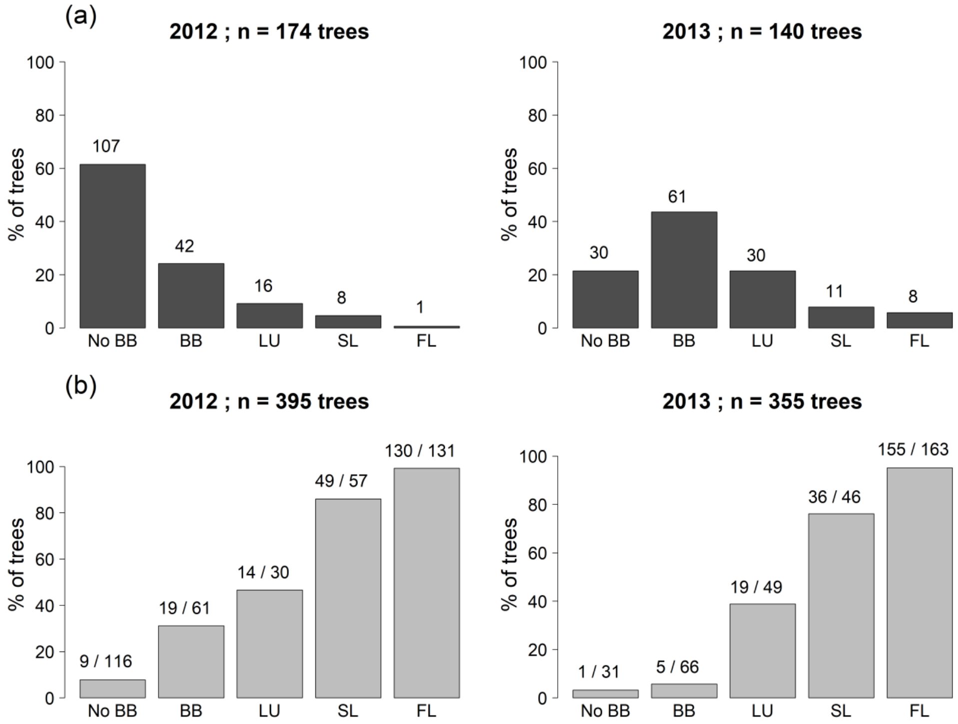

**Figure 2** Relationship of primary and secondary growth at tree scale. **(a)** Percentage of different crown phenophases at the moment of beginning of cell enlargement; number of trees per each crown phenophase are indicated above the column. **(b)** Percentage of trees with vessel maturation for each crown phenophase in spring; the number of trees with maturation out of the total amount trees for each specific crown phenophase is indicated above the column. No budburst (No BB), budburst (BB), leaf unfolding (LU), small leaves (SL), full leaf expansion (FL).

## Temperature and tree growth relationships

The sole mean annual temperature did not explain the differences in the onset and cessation of primary and secondary growth among these nearby sites, but the adjusted $R^2$ of their linear regression with progressive monthly temperatures highlighted the relevance of certain periods prior to the phenological events (Fig. 3 and 4). The adjusted $R^2$ ranged between 0.00 and 0.61; the highest values corresponded to the period between the second fortnight of February and the second fortnight of April for maximum, minimum, and mean temperatures on the beginning of cell enlargement; and from mid-March to mid-April for the effect of minimum temperature on the beginning of budburst (Fig. 4). On the contrary, the cessation of cell enlargement did not show any significant correlation. The thermal amplitude was only significant for the beginning of vessel enlargement, from February to the second fortnight of April, which is the period with the greatest variations between years, as can be seen for March temperatures (Fig. S2).

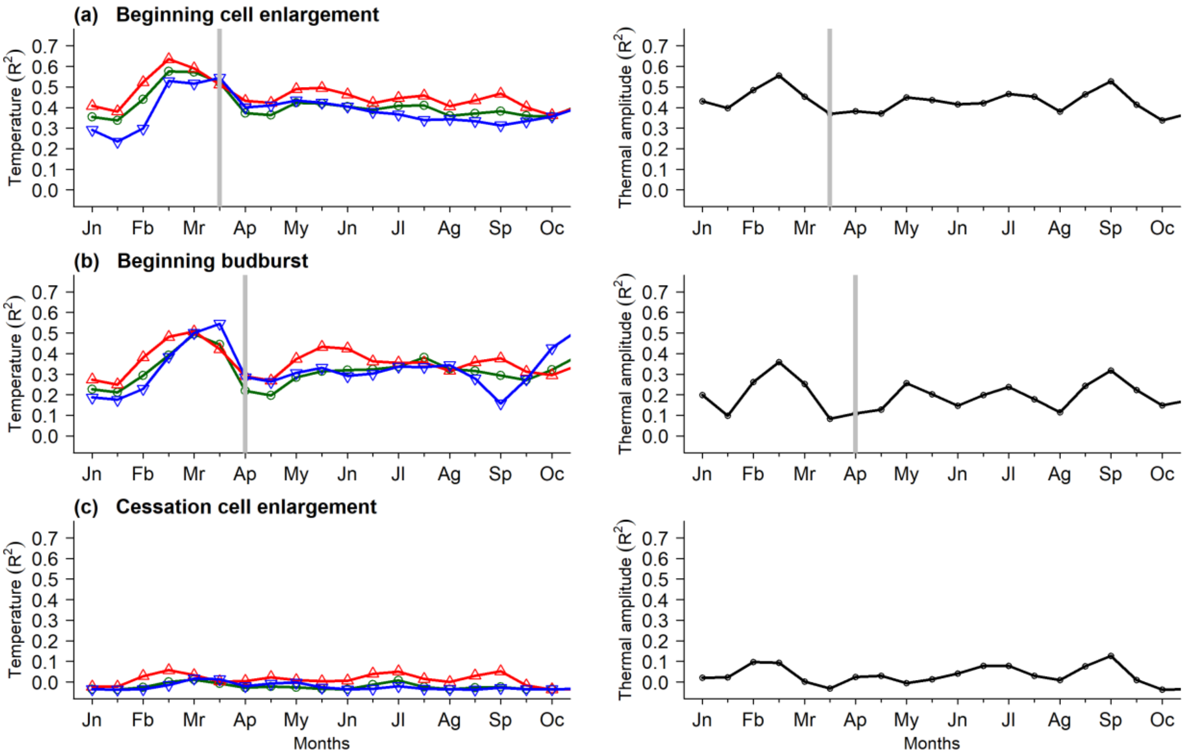

**Figure 3** Adjusted R-square of the linear regression of 30-day average temperate at each site on the dates of beginning and cessation of cell enlargement and budburst. Regressions were calculated for progressive periods separated in 15 days. *Line (without points)*: thermal amplitude; *blue line (triangle down)*: minimum temperature; *green line (circle)*: mean temperature; and *red line (triangle up)*: maximum temperature; the *grey vertical line* indicates the period when the phenological event occurs. Each point corresponds to the date that is represented in the graph as the last day of the period analyzed.

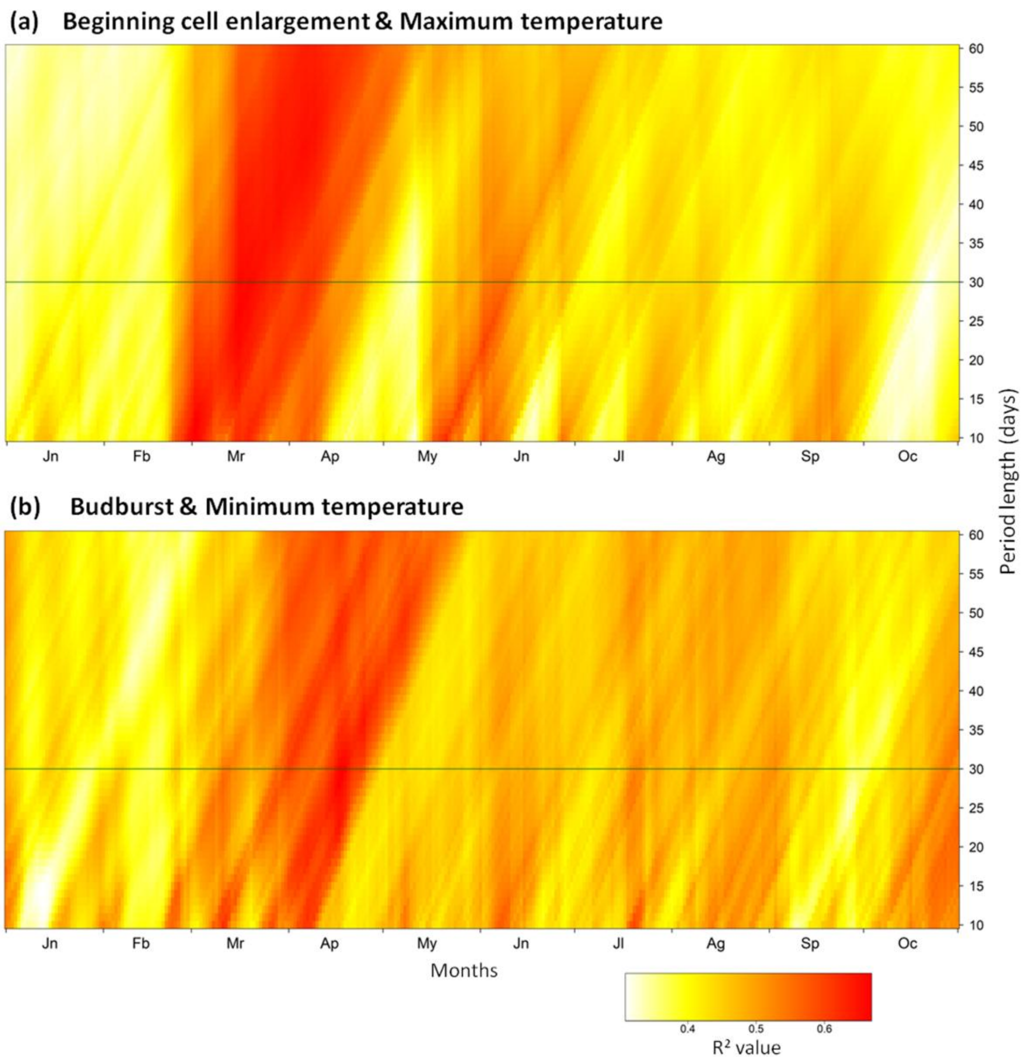

**Figure 4** Adjusted R-square of linear regression relating the dates of **(a)** maximum temperature on beginning of cell enlargement, and **(b)** minimum temperature on budburst. Running means were calculated for periods of 10 to 60 days, shifted in one day. Horizontal line indicates the average for a 30-day period. Each dot shown in the graph corresponds to last date of the period analyzed.

In order to verify if these variations appeared to drive the start of the active period, the onset of each phenological event was related to March temperature at each site, including the effect of year as a fixed factor (Table 4). For all linear regressions, the interaction between year and temperature was not significant, suggesting that the response to temperature was similar in both years.

Budburst was not affected ($P > 0.05$) by maximum temperature from mid-March to mid-April, which only explained 15% of the total variation, while mean temperature explained 47% and 53% of total and the within-year variation (Table 4). Early-spring minimum temperature explained 56% of the variation in the onset of budburst among sites, which averaged 107 ± 12.8 and 98 ± 9.3 DOY in 2012 and 2013, associated to mean minimum temperatures of 5.3 and 5.8 ºC respectively. Dates of budburst predicted by these temperatures were 107 ± 2.5

and 104 ± 2.5 DOY in 2012 and 2013 respectively, decreasing by 7.22 days ºC$^{-1}$ (Table 4). The beginning of the successive phenophases (leaf unfolding, leaves < 50% of their final size, and > 50% of their final size) was also explained by mid-March to mid-April temperature, because the delay in budburst affected the successive leaf phenophases (Fig. 3).

**Table 4** Significant ($P < 0.05$) statistical values of the linear regression for the different events, expressed as day of the year (DOY) or duration in days as dependent variable; and the mean, amplitude (Ampl.) minimum (Min.), and maximum (Max.) site temperature for the period of each event as independent variable; year is considered as fixed factor. The values shown are: intercept (a); slope (b); residual standard error of the regression (RSE); and adjusted $R^2$ of the model, including temperature and year effects (Adj. $R^2$). The *P*-value of Type I test is provided for the temperature effect, as well as the difference between years, expressed in days, whenever this effect was significant. Variables with the highest $R^2$ are shown in bold.

| Events | Period | Temp. | a | b | RSE | Year effect | Type I *P* | Adj. $R^2$ |
|---|---|---|---|---|---|---|---|---|
| Beginning of cell enlargement (bE) | March | Mean | 137.99 | −5.09 | 6.68 | ns | < 0.001 | 0.5219 |
| | | Max. | **130.69** | **−2.82** | **5.99** | **ns** | **< 0.001** | **0.6161** |
| | | Ampl. | 122.31 | −3.90 | 6.24 | ns | < 0.001 | 0.5821 |
| Budburst (BB) | Mid-March to mid-April | Mean | 165.90 | −6.40 | 8.94 | −10 | < 0.01 | 0.472 |
| | | Min. | **145.79** | **−7.22** | **8.06** | **ns** | **< 0.001** | **0.567** |
| Beginning of cell maturation (bM) | March | Mean | **172.51** | **−5.54** | **6.90** | **ns** | **< 0.001** | **0.555** |
| | | Min. | 147.78 | −5.37 | 8.21 | 9 | < 0.05 | 0.3719 |
| | | Max. | 190.16 | −4.48 | 6.36 | −12 | < 0.001 | 0.6225 |
| Duration of 1st vessels enlargement (d1r) | April | Mean | 53.98 | −2.72 | 4.79 | ns | < 0.01 | 0.367 |
| | | Min. | 46.62 | −2.83 | 5.13 | ns | < 0.05 | 0.271 |
| | | Max. | **57.77** | **−2.20** | **4.52** | **ns** | **< 0.01** | **0.434** |
| Duration of latewood (dLW) | October | Max. | −27.103 | 5.78 | 14.26 | −23 | 0.05 | 0.45 |

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March maximum temperature explained 62% of the variation in the onset of cell enlargement, which averaged 86 ± 7.8 and 98 ± 7.7 DOY in 2012 and 2013 respectively. The predicted values were 86 ± 1.9 and 97 ± 1.9 DOY, advancing by 2.82 days ºC$^{-1}$ (Table 4), for mean maximum temperatures in March of 15.8 and 11.7 ºC in 2012 and 2013.

The beginning of latewood cell maturation was also explained by March temperature (Table 4), because the delay in cell enlargement affected the onset of cell maturation and latewood, as shown by the strong correlation between both events (Table 2 and Fig. 5). However, the interval between these two phases, which is the duration of enlargement of the first row of vessels (d1r), was neither correlated ($P > 0.05$) to the onset cell expansion nor to maturation (Table

2). Nevertheless, the linear model showed that 43% of the variation recorded in this interval (d1r) was explained by the average maximum temperature during its duration (April) with an advance of 2.22 days ºC$^{-1}$ (Table 4). The average maximum temperature in April across all sites was 11.3 ºC in 2012, and 13.3 ºC in 2013; and predicted durations of enlargement of the first vessels row were 32 ± 1.5 and 28 ± 1.5 days, with recorded averages of 33.1 ± 5.9 and 27.6 ± 2 days, respectively.

The duration of maturation of both earlywood ($P > 0.5$) and latewood could not be explained by the variation in temperature, although latewood duration tended ($P = 0.05$) to respond to October maximum temperature (Table 4), probably pointing out the existence of a late pulse of growth in autumn if weather conditions permit.

As a result, it seems that the thermal influences of late winter (March) are transferred to the successive phenological events of primary and secondary growth, because one phenological phase leads to comparable shifts of the successive phases (Fig. 5).

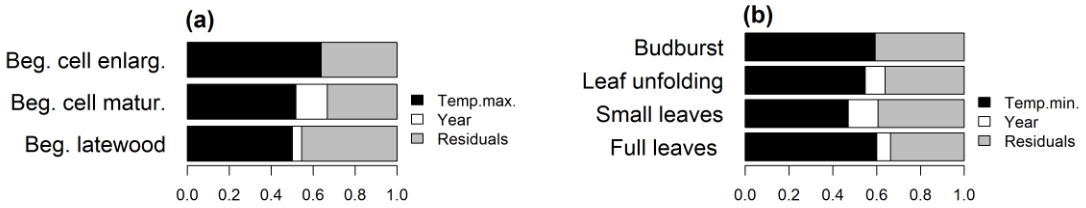

**Figure 5** Variance partition of phenological events with temperature and year as independent variables. **(a)** Contribution of the March average maximum temperature to total variability of the phases of the xylem (beginning of cell enlargement, beginning cell vessel maturation, and beginning latewood maturation). **(b)** Contribution of the average minimum temperature from mid-March to mid-April to total variability of the phases of the crown (budburst, leaf unfolding, small leaves, and full leaf extension).

## Discussion

### Linear patterns between events and durations of xylogenesis

The correlations between xylem timings and durations let us verify the existence of common relationships, and also support the evidence of internal and external (mainly thermal) drivers. Wood growth dynamics seemed to be homogeneous among sites, and the sequence of dates of successive phenological timings and durations are closely interconnected.

The beginnings of the different recorded events of xylogenesis (bE, bM, bLW) were positively correlated. This was somehow expected, since they are consecutive timings where the first phenological phase (bE) leads to comparable shifts of the successive phases, as previously reported for *Q. pyrenaica* and *Q.*

*robur* under temperate climates by Pérez-de-Lis *et al.* (2016b), and for conifers in cold environments (Rossi *et al.*, 2013). In contrast, the onset and cessation of cell enlargement were not directly related. To this respect, it should be noted that onset and end of xylogenesis involve different parts of the xylem, i.e., the first and the last cell produced (Rossi *et al.*, 2013). Nevertheless, both phenological events showed significant correlation with the duration of cell enlargement. Although cessation of xylem growth explained more variation in the relation between events and durations (62%) than the beginning of cell enlargement (23%), the latter becomes important because the variation of spring phenology is more closely linked to favorable conditions for photosynthesis than to variations in autumnal growth season (Chuine, 2010).

The duration of vessel expansion in the first row as associated to spring temperature (mainly maximum), but was neither correlated to the onset of vessel expansion nor to the beginning of cell maturation. These results do not support the existence of a causal relationship between overall duration of vessel expansion and the dates of their onset and cessation. However, Pérez-de-Lis *et al.* (2016b) found that longer periods of vessel expansion were related to higher hydraulic diameters in the first row of vessels for *Q. robur*, while Souto-Herrero *et al.* (2017) analyzed long vessel chronologies of *Q. robur*, showing that environmental conditions in early spring were also linked to vessel diameter in this first row. Consequently, we suggest that it is spring temperature that controls the duration of vessel expansion, which in turn determines the final vessel size.

## Primary and secondary growth relationships

There is an increasing interest in the relationships between primary and secondary growth in ring-porous trees (e.g.,Sass-Klaassen *et al.* (2011)), in order to understand the influence of climate on the development of earlywood vessels, because it has been shown to be a powerful dendroclimatic proxy (Souto-Herrero *et al.*, 2017). This linkage between crown phenology and intra-annual dynamics of xylem formation has been investigated for different ring-porous hardwood species, as summarized by Kitin & Funada (2016). According to the literature, the enlargement of the first vessel elements starts before budburst, but their maturation onset differs among studies (Kudo *et al.*, 2015;Pérez-de-Lis *et al.*, 2016b;Puchałka *et al.*, 2017); in fact, Puchałka *et al.* (2017) attributed such differences to genetic variability. But the number of individuals was very limited in previous studies; in constrat, our study based on a sample size of 750 trees randomly selected during spring in two years. We observed that only 38−46% of the individuals had started earlywood vessel maturation at the moment of leaf unfolding, and even most of the trees with small leaves (76−85%) had already

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undergone the maturation of the first vessel row. In addition, out of a sample of 314 trees with earlywood vessel enlargement and still no secondary wall, more than the half had not achieved budburst yet (75% in 2012, 56% in 2013), and a small proportion (14% in 2012, 22% in 2013) was at the stage of budburst; later leaf phenophases were hardly detected at this stage of vessel enlargement.

In view of these findings, we hypothesize that the newly-formed vessels are not capable of supplying enough water for bud swelling and leaf unfolding in most trees yet. In this sense, Pérez-de-Lis *et al.* (2016b) also reported that secondary wall deposition was not initiated at the moment of budburst in some *Q. robur* trees, Kudo *et al.* (2015) found that the first earlywood vessels were completed along the entire stem only when small leaves were visible to the naked eye in *Q. serrata*; and Guada *et al.* (2018) described the maturation of the first vessel row to be concomitant with the leaf phenophases having the greatest demand for water, such as leaf unfolding and extension. Kitin & Funada (2016) concluded that water requirements for the initial leaf development needed to be fulfilled by latewood vessels formed in previous years. Therefore, these latewood vessels are probably enough to provide water before the appearance of small leaves, as requirements at this stage are minimal in contrast to the amount of water needed for full leaf expansion, which should require new functional earlywood vessels for more water conduction (Lavrič *et al.*, 2017). Thus, Guada *et al.* (2018) quantified the water content of different leaf phenophases in *Q. pyrenaica*, and found that water transport of newly-formed earlywood vessels was required to maintain the turgidity of extending leaves and stems, but was not fundamental for the earliest phenological stages (bud swelling and leaf unfolding).

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## Phenology and temperature

The relationship between cambial activity and leaf development previously described help us understand the role of new earlywood vessels on the successful completion of crown development in ring-porous hardwoods. However, this internal relationship can be modified by external factors such as climatic conditions, which can somehow lead to advances or delays in the different phenological events.

Spring temperature is the major driving force for cambium and crown reactivation, but most investigations on its relation to xylogenesis were based on conifers at high latitudes or elevations (Rossi *et al.*, 2008;Rossi *et al.*, 2016). Our results based on a temperate ring-porous hardwood suggest that resumption of primary growth requires a minimum threshold temperature, which is probably related to the need of a certain thermal accumulation (Wilkinson *et al.*, 2017).

On the other hand, expansion of cambial derivatives is more dependent on concomitant warm temperatures. The fact that budburst is more linked to minimum temperatures while cell enlargement relies on maximum values causes that the process of vessel maturation can take place slightly before or after leaf unfolding depending on the conditions of each specific year. In fact, we estimate that minimal temperature from mid-March to mid-April increases budburst by 7.2 DOYs $^{\circ}C^{-1}$, while March maximum temperature anticipates cell enlargement by 2.8 DOYs $^{\circ}C^{-1}$, so that the variations between both processes are not coupled. However, the new functional earlywood vessels are required for full shoot and leaf expansion (Guada *et al.*, 2018).

The influence of temperature on cambium reactivation has also been confirmed for other deciduous hardwoods. Thus, Kudo *et al.* (2014) showed that localized heating for six weeks induced earlier cambial reactivation in seedlings of *Q. serrata* Thumb., while a similar result was found for a 4-week heating in poplar (*Populus sieboldii* x *P. grandidentata*) (Begum *et al.*, 2007). In both studies, elevated temperature (20 ± 5 ºC) caused earlier xylem differentiation, suggesting that the variation of temperature modifies the onset of cell enlargement. Our study evidenced that the maximum temperature and thermal amplitude (i.e., the difference between maximum and minimum daily temperatures) anticipated cell enlargement in 2012 compared to 2013, as shown by the average of 90 trees. In contrast, the minimum temperature was not able to explain the differences in cell enlargement between years. According to Begum *et al.* (2010), cambial reactivation can be predicted considering the daily maximum temperature; likewise, we are the opinion that an increasing temperature (and thermal amplitude) is an important factor for cambial differentiation of the first vessels elements in the ring-porous hardwood *Q. robur*.

Although crown development has been reported to require a minimum threshold to break dormancy (Caffarra & Donnelly, 2011;Prislan *et al.*, 2013;Wilkinson *et al.*, 2017), budburst is considered to be also induced by photoperiod (Basler & Korner, 2014). Yet, photoperiod differences are small in our study due to the latitudinal proximity among all sites; as a result, minimum spring temperature appears to be the main factor affecting budburst of *Q. robur* in this study area. This is consistent with the results reported by Wilkinson *et al.* (2017) for the south of England, who found a negative correlation between the date of budburst and the mean daily air temperature for *Q. robur* and *Q. petraea (Matt)Liebl.*, whereby spring warming had a considerably larger effect on budburst than winter chilling.

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While wood formation responds to temperature increments probably by increasing its growth rate, it is the variation in the minimum and maximum temperatures that advances or delays the onset of primary and secondary growth differently across years and sites (Fig. **6a**). The broad sampling strategy involving nine sites and two study years allowed us to estimate the average threshold temperature for each event (Fig. 6b). The different timing for the beginning of cell expansion can be explained by the considerable variation in maximum temperature between years; but this is not the case for leaf unfolding, which hardly varied between 2012 and 2013, probably due to the similar values of minimum temperature.

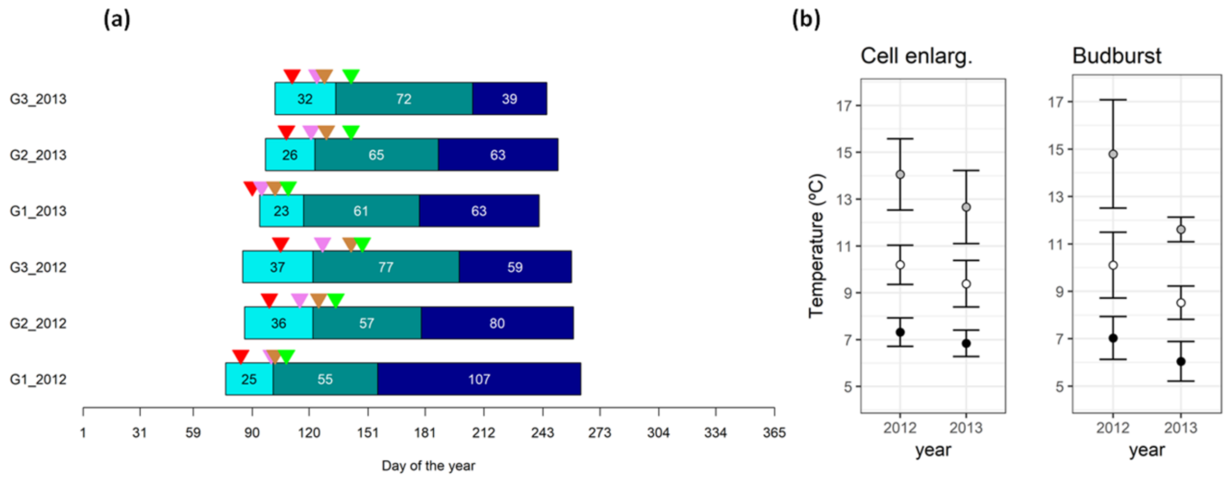

**Figure 6** (a) Sequence of the timings for the events of wood formation and crown phenology for groups and years. G1 (Atlantic influence; N=10), G2 (intermediate; N=60), G3 (continental influence; N=20) in 2012 and 2013. Wood formation events are indicated by boxes in the bar diagrams (pale: cell enlargement; intermediate: earlywood maturation; dark: latewood maturation; triangles refer to leaf phenology, with lighter colors progressively referring to later stages (budburst, leaf unfolding, leaves with <50% of its apparent final size, and leaves with >50% of its apparent final size. (b) Thresholds of temperatures (minimum (black dots), mean (white dots) and maximum (grey dots)), corresponding with the 0.5-probability of appreciation of cell enlargement and budburst for *Q. robur* in 2012 and 2013. Error bars indicate the standard deviation among the nine sites.

## Conclusions

Primary and secondary growth of *Q. robur* at its southwestern distribution boundary was closely linked to the moment of budburst and vessel enlargement. Practically all trees began earlywood vessel enlargement before budburst, and only when at least the first new vessels had completed maturation, did current leaves undergo full expansion. Therefore, previous year’s vessels are sufficient to fulfill water requirements for budburst and initial leaf growth, but current year’s vessels are needed to provide enough water for further leaf and shoot development.

Differences in spring (or late winter) temperature regime between years and among sites were able to explain the advancement and delay of leaf and earlywood development. However, shoot and cambium activity were not controlled by the same environmental conditions, because minimum temperature determined budburst, whereas maximum temperature was responsible for the onset of vessel formation. Consequently, understanding the environmental control of growth resumption in *Q. robur* is not straightforward, because not only the timings, but also the relationship between primary and secondary growth, can be modified by external factors.

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# Supporting Information

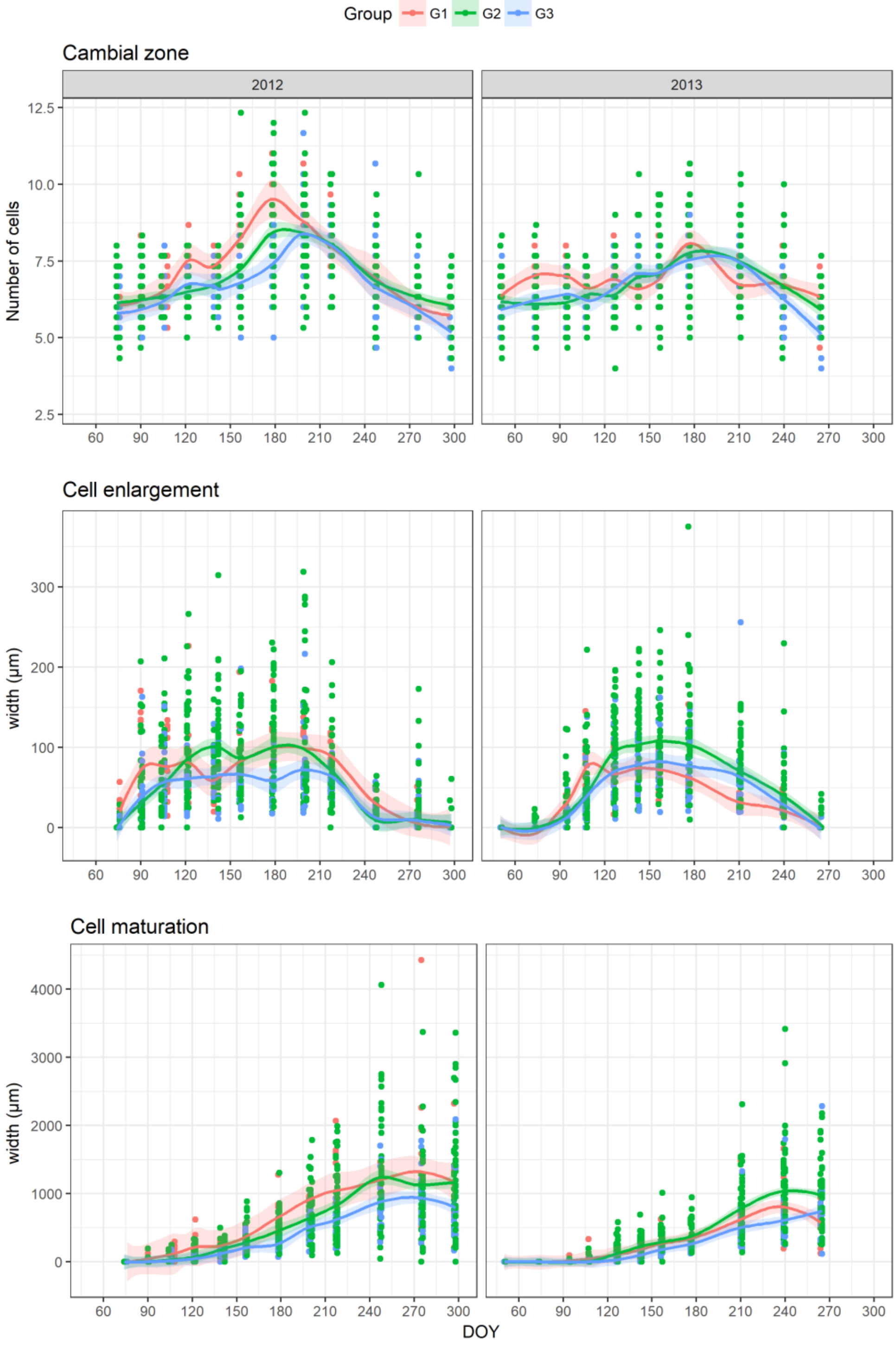

**Fig S1** Seasonal dynamics of radial growth for *Q. robur* grouped by the corresponding hierarchical classification following temperature regime. Groups: G1 (Atlantic influence; 10 sites), G2 (continental influence; 20 sites), G3 (intermediate; 60 sites) in 2012 and 2013.

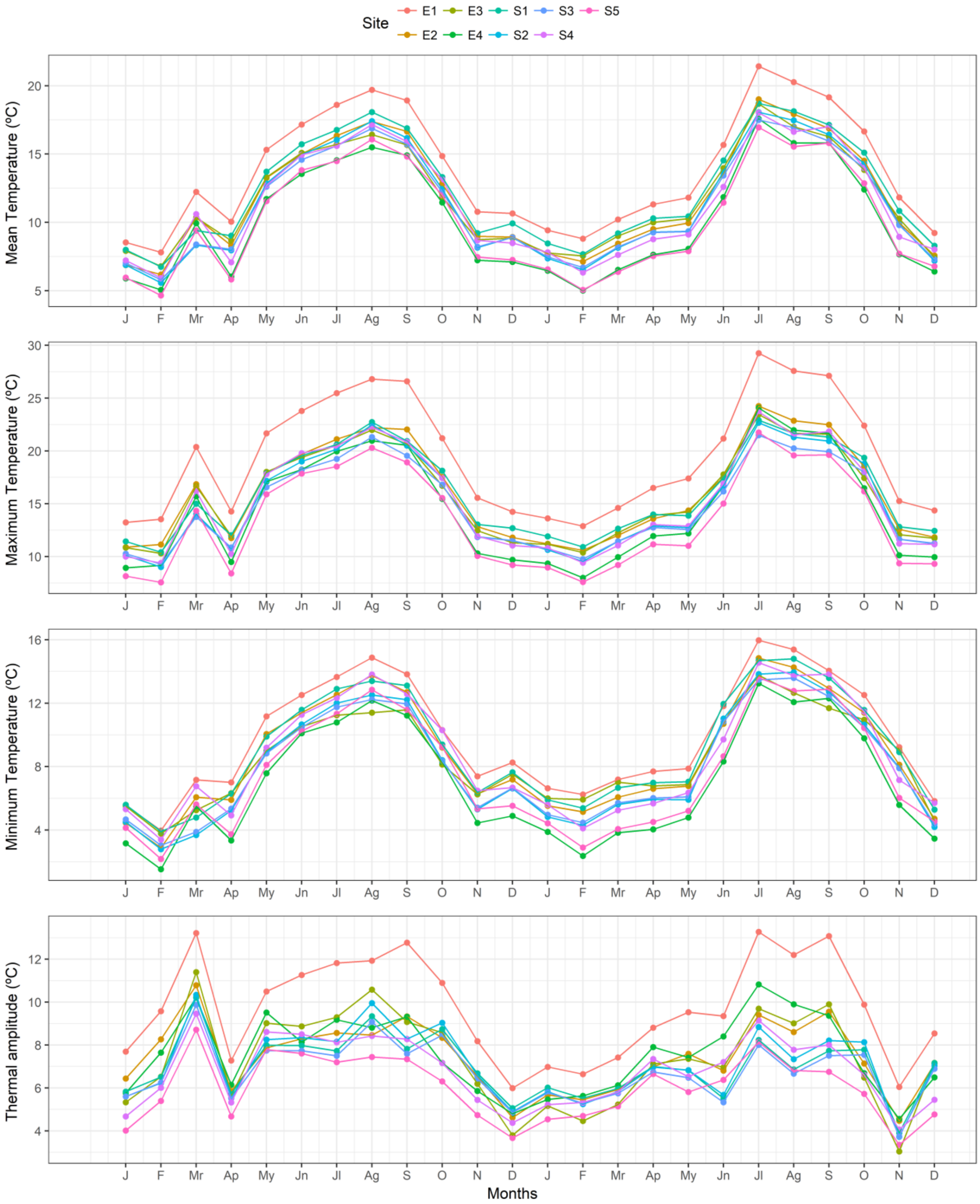

**Fig S2** Monthly variation of mean, maximum, and minimum temperature, as well as thermal amplitude, at the nine study sites during 2012 and 2013.

# Temperature regime controls the cessation of wood formation rather than the beginning in a sub-Mediterranean ring-porous oak

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## Abstract

We studied the relation between xylem and leaf phenology, and how the environmental factor can modify the growth rhythm of *Quercus pyrenaica* Willd. in the boundary of its northwestern distribution.

For this, wood formation and leaf phenology were monitored in 2012 and 2013 along five sites along an altitudinal gradient. Ten trees were randomly sampled at each site every 10-20 days to record leaf and cambial phenological phases. These events were related to each other, as well as to air temperature for 10-60 day running periods along the year, in order to identify the association between processes, and the most relevant time windows for the onset and cessation of tree growth.

The first earlywood begin the vessels expanded before budburst, and leaf unfolding occurred when at least the first row of earlywood vessel had undergone maturation. However, the temperature regime does not explain the beginning of cell maturation, whereas the cessation of cell enlargement was delay by maximum temperature, and to a greater extent by thermal amplitude.

Primary and secondary growth of *Q. pyrenaica* were clearly synchronized at all sites, confirming that the maturation of the first earlywood vessels is essential for crown development. This strong synchronicity points out that the beginning of tree growth is probably controlled by photoperiod rather than temperature; on the contrary, the cessation of wood formation appears to be more thermally dependent.

**Keywords:** budburst, cambium, earlywood, latewood, xylogenesis, *Quercus*.

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## Introduction

Many oak species (*Quercus* spp.) are the dominant trees in European natural deciduous forests. Nemoral oaks (*Q. robur* L., *Q. petraea* (Matt.) Liebl.) spread from northern Europe until the boundary of the Mediterranean region, where they are progressively replaced by more drought-tolerant species. In the Iberian Peninsula, western France, and north Africa, this transitional role is undertaken by sub-Mediterranean marcescent oaks, among which *Q. pyrenaica* Willd. is probably the most important species (Sánchez de Dios *et al.*, 2009). It mainly occurs in mountain areas of western Iberia, on acidic nutrient-poor soils, and under a subhumid to humid (occasionally hyperhumid) climate regime.

The relatively low value of its wood, charcoal, and acorns compared to other oak species such as *Q. ilex* and *Q. suber*, as well as its outstanding resprouting vigour have historically restricted *Q. pyrenaica* to silvopastoral uses in mountainous areas (Kalcsits *et al.*, 2009;Salomon *et al.*, 2017). Consequently, *Q. pyrenaica* disappeared from fertile and flat lands, where it was replaced by more productive oak species (Ruiz de la Torre, 2006). But this species is becoming more interesting as a forest species due to a great resprouting capacity after periods of drought and fire, and plays a crucial role in long-term vegetation dynamics (Clarke *et al.*, 2010). Nowadays, it is considered a species of a considerable resilience to be an important carbon sin0.k for the future (Cañellas *et al.*, 2017). For these reason, recent studies have been recently performed, as e.g., investigations on genetics (Valbuena-Carabana & Gil, 2017), and drought tolerance (Fernández-de-Uña *et al.*, 2017).

European white oaks, such as *Q. pyrenaica*, present ring-porous anatomy, characterized by larger vessels at the beginning of the growth, in the part or the ring called earlywood. These large vessels provide more than 90% of the water flow of the entire annual ring, and are only functional during the current year (Ellmore & Ewers, 1986). At least the first row of vessels is formed prior to full crown development, and therefore conditions that affect the balance of reserves accumulated in the previous year (Michelot *et al.*, 2012a), or operating directly at the time of vessel formation may determine their development. For this reason, the formation of earlywood vessels in such species has been observed to be associated to environmental conditions (García-González & Eckstein, 2003;Souto-Herrero *et al.*, 2018b), either to the temperature or the precipitation prevailing before or at the time of vessel formation in spring (Fonti & Garcia-Gonzalez, 2004;Fonti *et al.*, 2007;Fonti & Garcia-Gonzalez, 2008). Time series of earlywood vessel features evidenced strong climate relationships of *Q. pyrenaica* with temperature and soil water saturation (González-González *et al.*, 2014;Garcia-Gonzalez & Souto-Herrero, 2017). In addition, geomorphic events causing stem burial and root exposure (Gärtner, 2007;Ouden *et al.*, 2007) and flooding (e.g., (St. George & Rannie, 2003;Tardif & Conciatori, 2006;Tardif *et al.*, 2010) can induce striking changes in vessel size. But the understanding of the environmental control of earlywood vessels, as well as the reasons for the specific time windows observed requires complementary studies to be carried out. Leaf phenology has been sometimes used as an indicator of the stages of vessel formation, but it is not consistent across all ring-porous species (Sass-Klaassen *et al.*, 2011), and therefore, it should probably be established for each species separately. Recently, the association between xylogenesis and leaf phenology has been quantitatively assessed for *Q. pyrenaica* (Pérez-de-Lis *et al.*, 2016b;Pérez-de-Lis *et al.*, 2017;Guada *et al.*, 2018), but not related to its variations along with climate under natural conditions.

Temperature and photoperiod are the main factors that drive the timings of seasonal wood and leaf formation events. These variations are most visible in foliar phenology, and it has been described that the phenological variation fits the warming pattern (Menzel *et al.*, 2006;Cleland *et al.*, 2007;Fu *et al.*, 2015), and this response also varies among *Quercus* species at the southern limit of their distribution (Wilkinson *et al.*, 2017).

The study of the primary and secondary growth of trees, as well as their association, is crucial to know the species-specific differences, and to understand the diverse responses to environmental changes. Unfortunately, detailed studies linking leaf and cambial phenology for ring-porous trees, involving comparisons between contrasting sites or among climatic gradients are very scarce. A xylogenesis study by Guada *et al.* (2019), involving a large number of *Q. robur* natural woodland throughout two seasons showed that the annual mean temperature cannot be considered an indicator of phenological shifting in temperate climate conditions and the time window of a year, the advancement or delay of phenology need to be considered.

Tree species adjust their phenology to climate by modulating the length of the growing season according to specific regional environmental drivers, local adaptations and individual plasticity (Cuny *et al.*, 2015;Rossi *et al.*, 2016). *Q. pyrenaica*, a typical sub-Mediterranean species, i.e., a species occurring at the boundary from the Atlantic to the Mediterranean region, seems to undergo a different strategies than *Q. robur*, because it avoids late frosts, and stands prolonged periods of drought (Pérez-de-Lis *et al.*, 2017). Apart from other adaptations, this is respectively achieved by delaying budburst in spring, and bearing more xerophile leaves, but probably some processes to optimize reserve balance for reactivation after a long dormant season are probably involved.

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However, how the advancement or delay of phenological events are affected by temperature along the growing season is unknown for this ring-porous species. In order to understand these temperature effects we compiled, a data set on cambial dynamics and leaf phenological phases at five *Q. pyrenaica* sites along an altitudinal gradient in the northwest of the Iberian Peninsula during 2012 and 2013, and we proceeded to i) accurately relate cambial and leaf phenological phases to each other, and ii) determine how the onset and cessation of wood formation and crown development vary along the temperature gradient.

## Material and Methods

### Study site and tree selection

We selected five stands (L1, L2, L3, L4, L5) dominated by *Q. pyrenaica* Willd. in the northwestern Iberian Peninsula, following an altitudinal gradient from 400 to 1,000 m a.s.l. (Table 1), along the watershed of the River Lor (Fig 1). The prevailing climatic conditions at the study sites can be considered as Atlantic, with a certain continentality due to the inland and mountain location; and annual temperature ranges 7.5–12.7 ºC among sites. Precipitation is high, but irregularly distributed throughout the year, especially for dry summer periods, whereas it is abundant in winter (350–600 mm). These somehow Mediterranean trend imposed by the precipitation pattern increases as elevation decreases, and for this reason most of the area can be considered as the transition from the Atlantic Region. In fact, the sub-Mediterranean *Q. pyrenaica* is a typical transitional species (Sánchez de Dios *et al.*, 2009). It grows on siliceous soils, usually under continental climate, and its distribution mainly spans mountain areas in the Mediterranean region, or along the transition from Mediterranean to Atlantic vegetation (Eurosiberian Region).

We monitored these forests for two consecutive years, from April to November in 2012, and from March to September in 2013. Simultaneously, 50 trees (5 sites × 10 trees) with a diameter of 20-40 cm were randomly selected at each sampling date within an area of ca. 2 ha per forest. The sampling interval was 12–15 days in spring, and 20 days in summer, because changes during earlywood formation or transition to latewood take place much faster than others later in the season. Trees selected were dominant or codominant in the canopy, and individuals with polycormic stems, partially dead crowns, or evident damage, were avoided. The random selection of trees at each date prevented the study at the individual tree level, but provided a representative sample of the whole forest as a single population, avoiding the bias of a specific tree. In addition, this strategy increased the number of individuals that could be analyzed

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to relate primary and secondary growth, i.e., to associate leaf and cambium phenophases.

**Table 1** Description of the sites included in the analysis, with their identification codes (ID), geographical location, elevation, and diameter at breast height (DBH) of the sampled trees. Normal annual temperature (Temp) and precipitation (Prec) values obtained from Rodríguez-Lado et al. (2016) (source: digital database at http://rgis.cesga.es).

| ID | Latitude (N) | Longitude (E) | Altitude (m.a.s.l.) | DBH (cm) | Temp (°C) | Prec (mm) |
|---|---|---|---|---|---|---|
| **L1** | 42.567856 | -7.398149 | 400 | 23.3 ± 3.3 | 12.7 | 859 |
| **L2** | 42.556641 | -7.216217 | 600 | 25.6 ± 4.5 | 11.5 | 1045 |
| **L3** | 42.650625 | -7.121141 | 875 | 25.5 ± 4.9 | 10.4 | 1261 |
| **L4** | 42.684528 | -7.094356 | 950 | 25.8 ± 4.4 | 8.5 | 1428 |
| **L5** | 42.664597 | -7.054362 | 1050 | 27.9 ± 4.4 | 7.5 | 1589 |

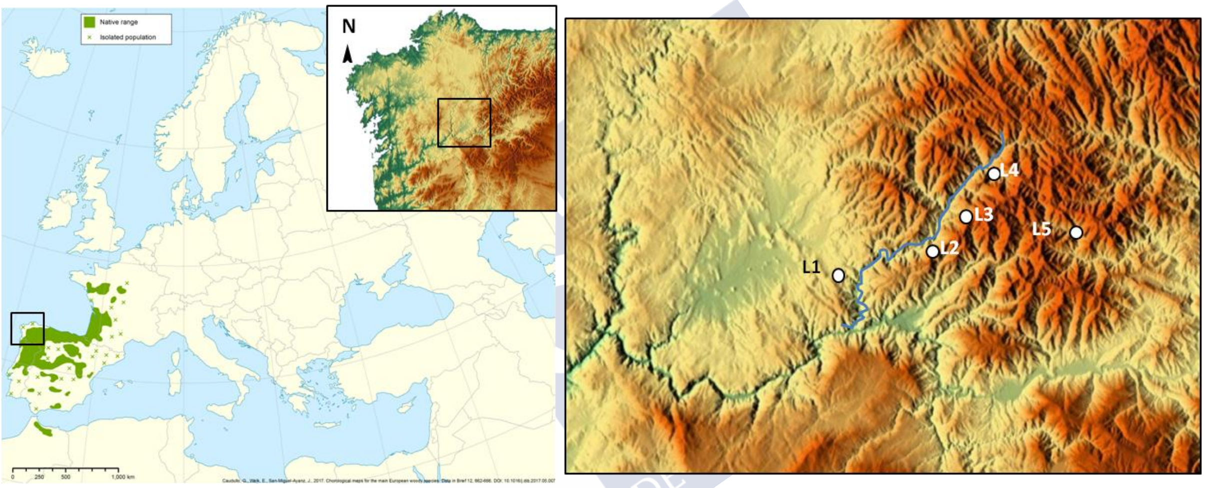

**Fig. 1** Distribution map of *Q pyrenaica* (Caudullo *et al.*, 2018). Dots represent the five stands selected for the study in the northwestern Iberian Peninsula, along the River Lor (L1-L5).

## Meteorological data

We recorded air temperature hourly at each site during the whole period for monitoring, using sensors (iButton DS1922L, San Jose, CA, USA) set at 2 m above the ground surface. Subsequently, hourly values were used to calculate daily mean, maximum, and minimum values, and the corresponding thermal amplitudes. Temperature time series were corrected to complete missing data by simple linear regression with daily records from nearby meteorological stations belonging to the weather service Meteogalicia (http://www.meteogalicia.gal). Precipitation data were also from three meteorological stations along the altitudinal gradient, which belonged to the same weather service.

## Xylem sampling and wood phenophases

At each sampling date, we extracted at least two microcores (2 mm diameter, ca. 15 mm length) at breast height (1.3 m) out of each selected tree. For this, we used a Trephor tool (Rossi *et al.*, 2006a), and followed a perpendicular direction to the slope. The samples were immersed in 50% alcohol for fixation, and preserved at 5ºC in the lab until further use.

We processed one microcore per tree, by paraffin embedding, cutting thin sections with a rotary microtome, and staining following the same protocol as in previous works (Pérez-de-Lis *et al.*, 2016b;Guada *et al.*, 2018). The preparations to be observed contained mature and developing xylem of the current year, the cambial zone and adjacent phloem, and at least one previous complete tree ring.

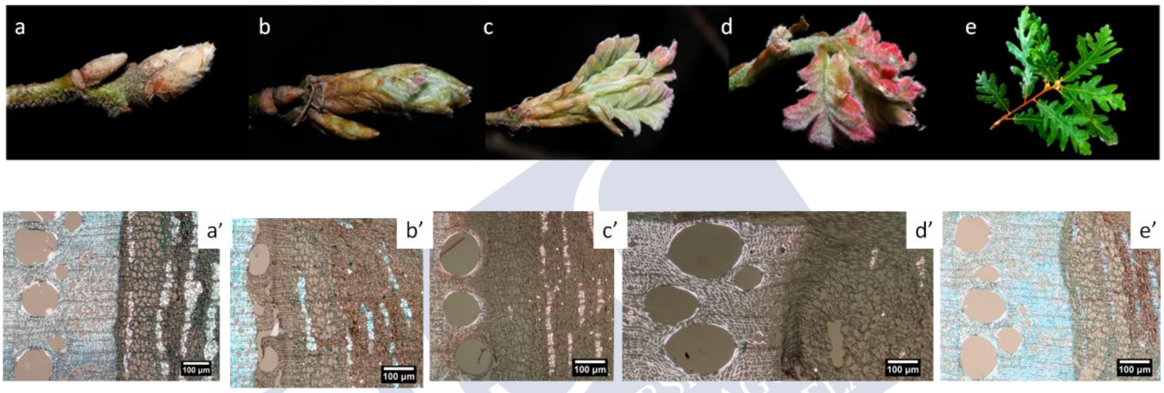

**Fig. 2** Leaf growth phenophases and transversal thin sections of earlywood development images observed under polarized white light. The upper row shows primary growth phenophases recorded: **a** Bud dormancy, **b** Budburst, **c** Leaf unfolding, **d** Small leaves, **e** Full leaf expansion. The lower row shows xylem development recorded: **a'** Dormant cambium, **b'** Vessel expansion, **c'** vessel maturation, **d'** Latewood maturation, **e'** Cessation of cell expansion.

Observation and width measurements of the wood phenophases of the 1,007 microcore cross-sections (553 tress in 2012, 454 trees in 2013) were performed on images taken with a digital camera (Canon EOS 600D, Tokyo, Japan), coupled to a transmitted light microscope (Olympus BX40, Tokyo, Japan), and equipped with a polarized filter in order to detect secondary cell wall deposition; image magnification was 0.926 µm/pixel. We measured the width zones of cell expansion and maturation along three radial lines per image (Fig. 2); and each phase of cambial activity was expressed as particular day of year (DOY), including: a) dormant-cambium, b) beginning of cell enlargement in the earlywood (bE,), c) beginning of earlywood maturation (bM), d) onset of latewood maturation (bLW), and e) cessation of cell expansion (cE). The onset of cell enlargement was considered when the most recent ring contained at least one enlarging cell; whereas the onset of earlywood vessel maturation was defined by the deposition of the secondary cell wall in cells, detected by birefringence under polarizing light; the cessation of xylem growth corresponded to the moment when no expanding cells were further detected.

### Leaf phenological observations

Trees selected for sampling were used to record leaf phenology. For this, we observed the stages at the top of the main branches using binoculars (10 × magnification), and the corresponding phase was expressed as a particular day of year (DOY).

We considered six different phenophases (Fig 2), namely: a) bud dormancy, b) budburst (BB), c) leaf unfolding (LU), d) appearance of small leaves (SL) and e) full extension (FL). Bud dormancy described the overwintering stage, while bud swelling was identified by the separation of buds from the stem, together with the exposition of areas of lighter colored tissue as a result of the initial extension of cataphylls with the separation of scales. Budburst corresponded to the presence of green-colored expanding buds with no unfolded leaves, and leaf unfolding ended as soon as the leaf blade was clearly visible, but not the petiole. Appearance of small leaves was defined as the moment when at least one leaf was completely out of the bud, and current year twigs and petiole could be visually appreciated; and full extension was recorded when leaves attained at least the 50% of their apparent final size.

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### Statistical analyses

Critical dates for xylem (bE, bM, bLW, cE) and leaf phenology (BB, LU, SL, FL) were assessed from the observed absence or presence of each of the phenological events at each sampling site and date; these observations were expressed as binary data (0 no active phenophase; 1 active phenophase). Afterwards, logistic regressions were performed using the DOY for each event as independent variable, and considering a probability of 0.5 as the most likely DOY for the detection of a given event (Rathgeber *et al.*, 2011). The effects of year, site, and their interaction were studied. Independent variables considered in the models tested were as follows: DOY + year; DOY+ site; DOY + year + site; DOY + year × site. The model providing the lowest AICc for each event was selected as the best fit. In order to identify the relationship between climate and growth, we established linear correlations between the DOYs of each phenological event and different averages of maximum, mean, minimum temperature, and thermal amplitude. The average periods were the annual average, and 30-day averages every 15 days. A more detailed analysis of the most relevant variables was performed involving running temperature means of 10 to 60 days, shifted in only one day.

Relationships between primary (leaf phenology) and secondary growth (cambial phases) at a tree scale were assessed using two different approaches. First, we selected the trees with a specific stage of wood formation (namely expansion of the first earlywood vessels), which were classified according to the phenological phase of the crown in order to know what percentage of trees had started crown development without undergoing earlywood maturation. The second approach consisted in select trees with mature earlywood vessels within each of the different phenological crown phases in order to calculate the proportion of trees that had started earlywood maturation for each leaf phenological stage. All statistical analyses were performed using the R statistical software (R Core Team, 2017).

## Results

### Temperature across sites

The mean, minimum, and maximum annual temperatures did not differ between 2012 and 2013 ($P < 0.05$). Despite being located in one of the most continental areas of the northwestern Iberian Peninsula, the study sites still have a strong oceanic influence, with a mean temperature of 11 ºC during the study years, and a range for a maximum annual temperature of 17 ºC to a minimum value of 6 ºC. The variation of mean annual temperatures across the sites averaged 2.62 ºC, from 12.15 ºC at L1 (the lowest site) to 9.5 ºC at L5 (the highest site). The minimum absolute temperature at all sites ranged from –7.5 ºC at the highest (L5) and lowest site (L1) to –5.3 at a middle elevation (L2). The coldest period, with records below 0 ºC in all the sensors, was from November to February, and late frosts still occurred from March until the end of April. The maximum absolute temperature ranged from 31 ºC to 35 ºC in August, respectively at L4 and L1. Although there were no significant differences between annual temperatures, it should be noted that the thermal amplitude in March considerably differed between the two years, being much higher in 2012 due to the absence of rainfall (Fig 3 and 4).

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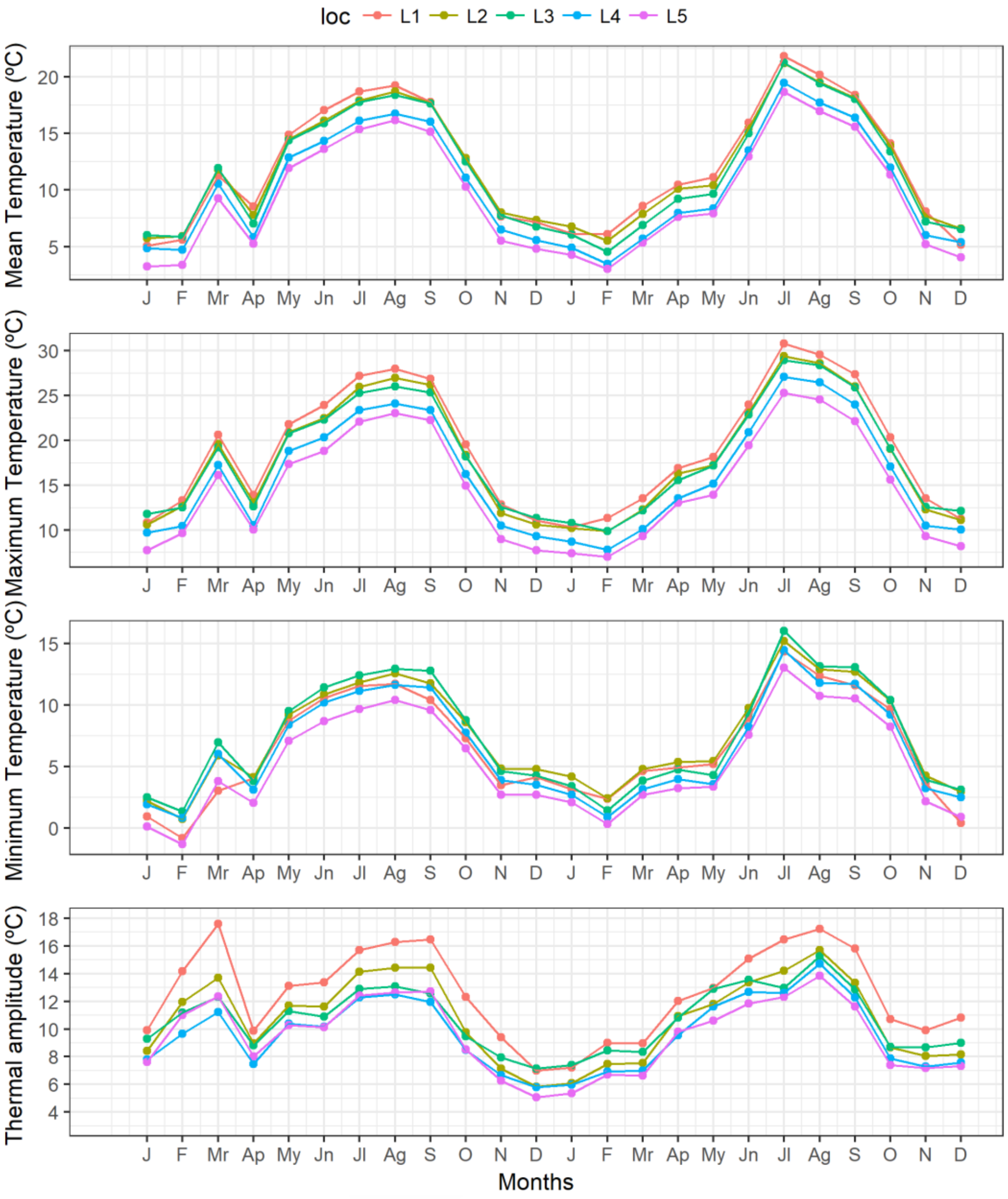

**Fig. 3** Monthly temperature in variation of mean, maximum, minimum, and thermal amplitude, at the five study sites in 2012 and 2013.

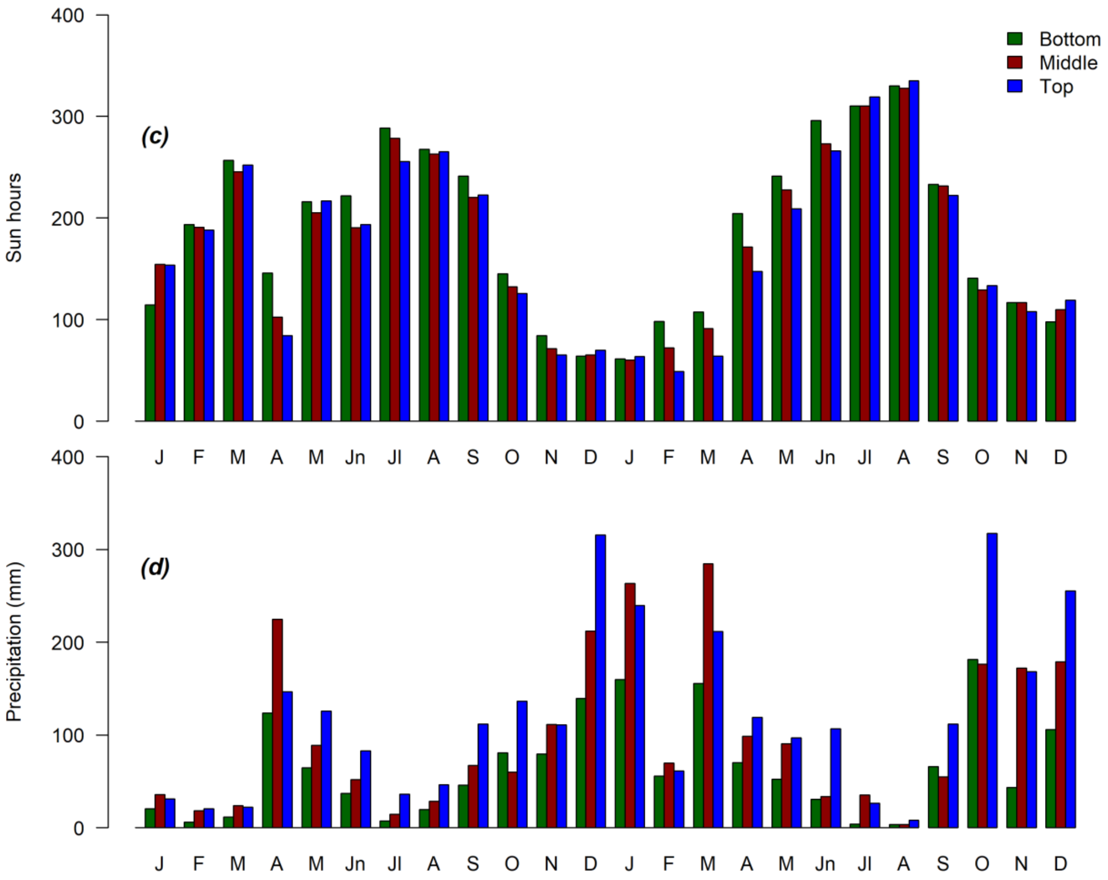

**Figure 4** Accumulated monthly sun hours and precipitation recorded in the weather station located in the bottom (433 m a.s.l.), middle (777 m a.s.l.) and top (1310 m a.s.l.) along the study gradient in the years 2012 and 1013

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## Xylem dynamics

In 2012, the cambium was already active on the first sampling date, since it had a higher number of cambium cells (6–8 cells) than that observed in November (5–6 cells), and at the beginning of sampling of 2013 (Fig. 5). The number of cambial cells increased from June to early August, reaching maximum values (10–12 cells in 201,2 and 7–9 in 2013) during July in both years. However, average number of cells in July differed between years, and was greater in 2012 (10.6 ± 1.5) than in 2013 (8.0 ± 1.4).

The cell enlargement phase, i.e., the period that spanned at least one expanding cell, was also active on the first sampling date (April 26th, 2012; 117 DOY); consequently, the comparison between years could not be performed. This phase extended until early September in 2012, but there was a re-growth during late September and early October; in contrast, it finished in late August in 2013, without an apparent re-growth later in the season.

During the active period, cell enlargement followed a bimodal pattern, with two peaks of width increment, clearly visible at most sites in 2012. The first peak corresponded to the expansion of the first earlywood vessels in spring (early

May; 132 DOY), and the second on was coupled to the summer peak of latewood formation (early July; 188 DOY). This pattern was less conspicuous in 2013, whereby only the first earlywood peak in May (128–144 DOY) was clearly marked.

Differences in ring width between years can be explained by conditions limiting cell enlargement, both during summer and the autumn pulse. Thus, a greater ring width was observed in 2,012 (1205.91 ± 57.41 μm) than in 2013 (815.21 ± 39.03 μm) together with two peaks of cell enlargement (Fig. 5).

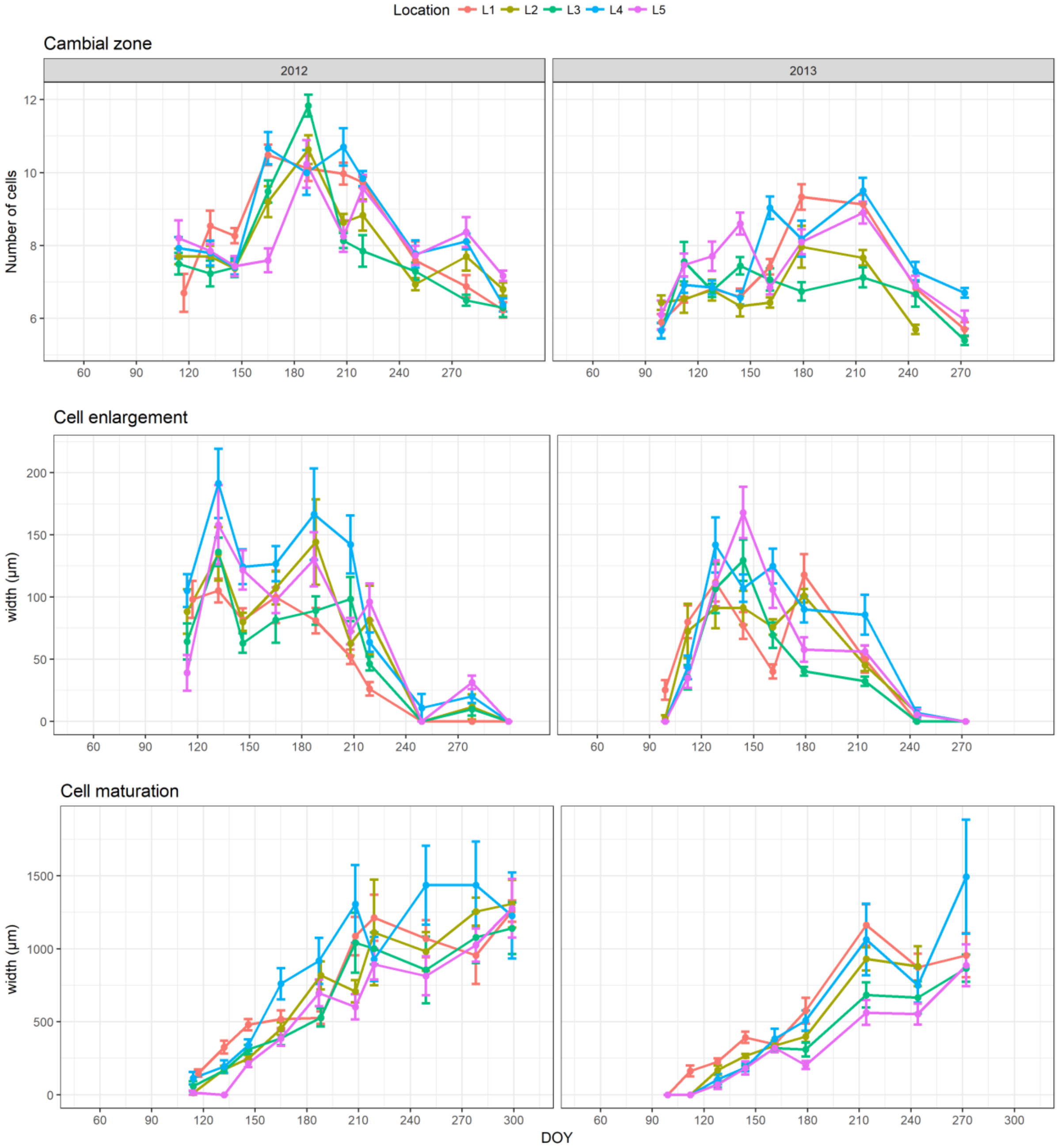

**Fig 5** Seasonal dynamics of radial growth for *Q. pyrenaica* in the 5 locations studied following the altitudinal gradient during 2012 and 21013 (N = 10 trees × 5 sites × 2 years). Standard errors are the vertical lines.

### Relationship between primary and secondary growth

We sampled a total of 96 trees showing the phenophase ‘cell enlargement’, but not having started cell maturation yet, and calculated the proportion of trees with different crown phenophases at this specific period (Fig. 6a). Out of the 37 trees sampled in 2012, only three bore leaf unfolding; in 2013, there was one unique tree at full leaf expansion out of the 59 sampled trees. The number of trees at earlier phenological phases of crown development increased to nine at budburst in 2012 and 18 in 2013, while a higher number of trees had not started budburst (25 in 2012, 38 in 2013).

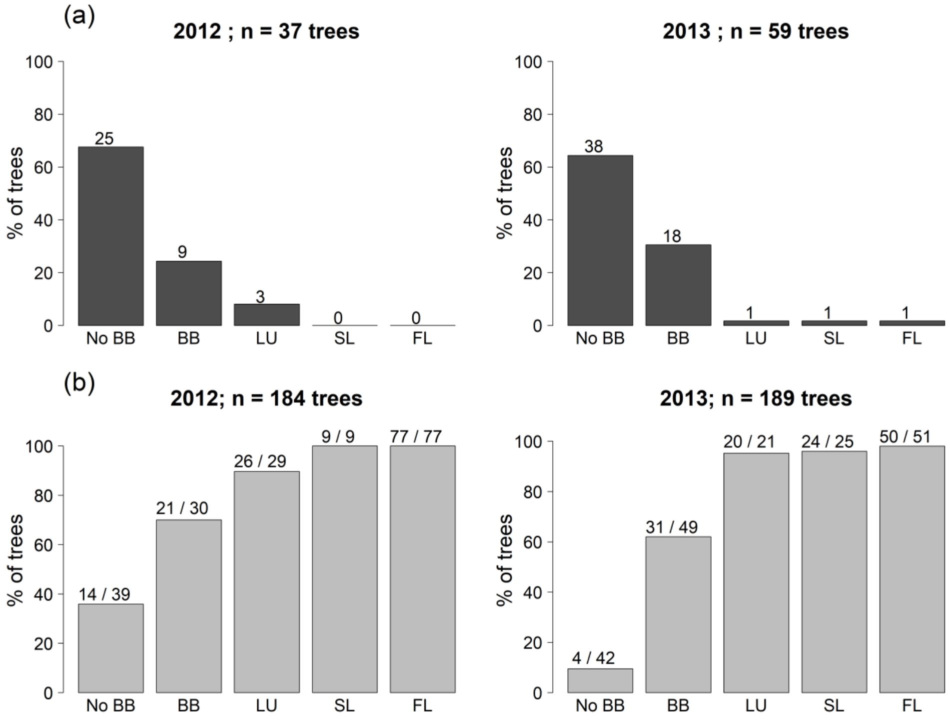

**Figure 6** Relationship of primary and secondary growth at tree scale. **(a)** Percentage of different crown phenophases at the moment of beginning of cell enlargement; number of trees per each crown phenophase are indicated above the column. **(b)** Percentage of trees with vessel maturation for each crown phenophase in spring; the number of trees with maturation out of the total amount trees for each specific crown phenophase is indicated above the column. No budburst (No BB), budburst (BB), leaf unfolding (LU), small leaves (SL), full leaf expansion (FL).

The second approach, which aimed at defining the proportion of trees in each of the different crown phenophases with already mature earlywood vessels, included a total of 373 trees sampled during the two years (Fig. 6b). There was an increasing proportion of trees with mature cells along with the successive phenophases of crown development. The trend was similar for both years, and the trees at leaf unfolding were those that had a greater proportion of mature earlywood vessels.

These results indicate that there were no expanding leaves at the beginning of vessel enlargement yet. In fact, 96 trees underwent enlargement of the first earlywood vessels in 2012 and 2013, but ca. 94% did not bear any phenological stages further than budburst (Fig. 6a); in the same period, 92% of the 50 trees showing leaf unfolding had already begun the maturation of the first row of vessels (Fig. 6b).

## Temperature and tree growth relationships

The best fit of the logistic regression for all the events of leaf phenology corresponded to the model considering the interaction between year and site (Table 2). This indicates that the effect of a specific year on the delay or advancement of crown growth varied across sites. There was also an effect of the year for xylem development, but unlike leaf phenology, it was similar at all sites because the interaction ‘Site × Year’ did not improve its factorial effect; this confirms that phenological events are site-specific, but independently of the year.

**Table 2** Generalized linear models to evaluate the effect of site and year on the occurrence of the main phenological events. AIC increments (DAICc) for each model are shown as compared to those of the model with the lowest score (the best-fitted model). Selected models are highlighted in bold. Beginning of cell enlargement (bE); beginning of maturation (bM); beginning of latewood maturation (bLW); cessation of cell expansion (cE); budburst (BB); leaf unfolding (LU); <50% of its apparent final size (SL); >50% of its apparent final size (FL); d.f. refers to degrees of freedom.

| | | Xylogenesis | | | | | Leaf phenology | |
|---|---|---|---|---|---|---|---|---|
| Fixed effects | d.f. | bM | bLW | cE | BB | LU | SL | FL |
| **DOY + Site × Year** | 11 | 0 | 2.49 | 3.32 | 0 | 0 | 0 | 0 |
| **DOY + Site + Year** | 7 | 3.69 | 0 | 0 | 7.71 | 77.42 | 40.26 | 22.91 |
| **DOY + Year** | 3 | 76.01 | 52.90 | 8.21 | 26.06 | 132.80 | 93.70 | 162.26 |
| **DOY + Site** | 6 | 76.52 | 27.96 | 11.08 | 26.15 | 79.01 | 39.88 | 45.27 |

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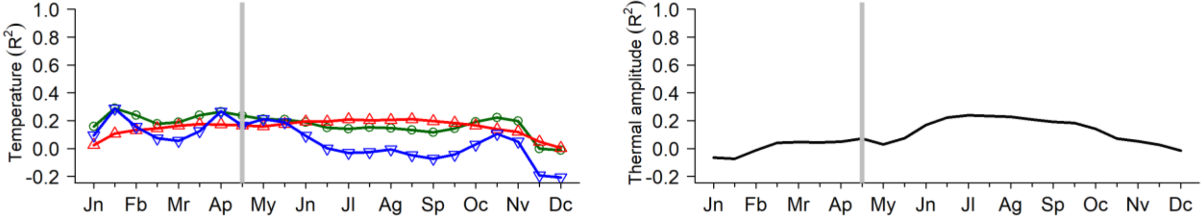
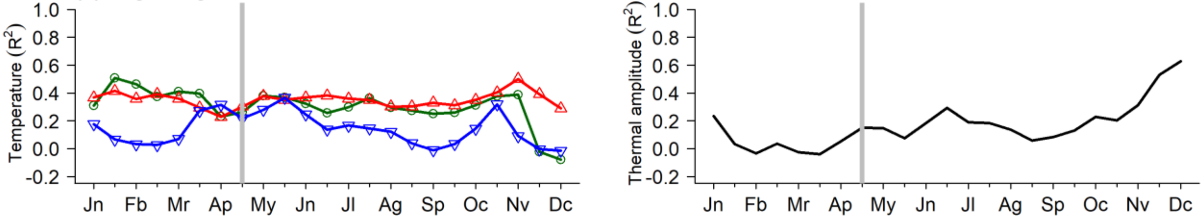
Running means of monthly temperatures every 15 days do not seem to identify a specific relevant time window for the onset of cell maturation and budburst. Coefficients of the models were rather low, ranging from –0.15 to 0.5 (Fig. 7a and b). By contrast, the cessation of cell enlargement was associated to ' Site + Year ' differences in the annual and monthly maximum temperature (hardly different from 'Site × Year'); and particularly to those in thermal amplitude, which reached *r*–squared values of 0.7–0.9 for most periods, suggesting that differences in local temperature across sites were stable throughout the year (Fig.3). These significant results were also evident when the relationships between xylem phenological events and climatic variables were established with values recorded annually (Table 3), monthly (Fig 7), or during intervals ranging from 10 to 60 days (Fig.8a) and the effect of the year for the significant results is not relevant for maximum an thermal amplitude (Fig. 8b).

**Table 3** Statistical values of the linear regression of different events expressed as day of the year (DOY) as dependent variable and the annual climate variable (thermal amplitude, mean, maximum and minimum temperature) at each site as independent variable, and year as fixed factor. The percentage of variation explained by the variable, extracted from the sum of squares, is provided, as well as the *p*-value of Type I test of the model.

| | Onset cell maturation | | Cessation cell enlargement | | Budburst | |
|---|---|---|---|---|---|---|
| | Sum Sq (%) | *p* | Sum Sq (%) | *p* | Sum Sq (%) | *p* |
| **Tmean** | 33.646 | 0.079 | **56.825** | **0.016** | 19.462 | 0.202 |
| **Tmax** | 26.608 | 0.127 | **69.484** | **0.005** | 33.478 | 0.08 |
| **Tmin** | 22.802 | 0.163 | 0.049 | 0.953 | 0.217 | 0.898 |
| **Amplitud** | 8.772 | 0.406 | **79.563** | **0.007** | 36.988 | 0.062 |


(a) Beginning budburst


(b) Beginning cell maturation

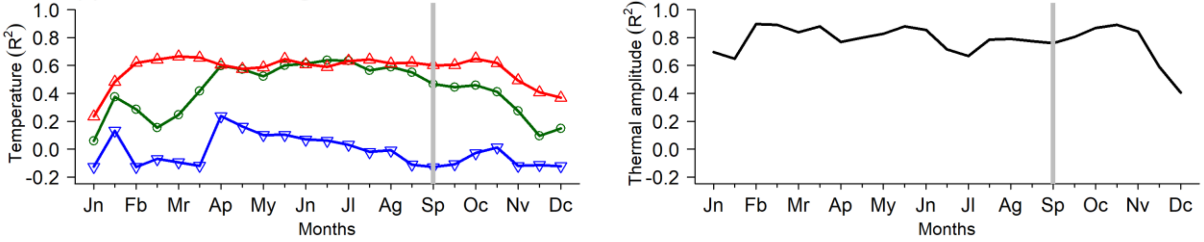

(c) Cessation cell enlargement

**Fig. 7** Adjusted R-square of the linear regression of 30-day average temperate at each site on the dates of beginning and cessation of cell maturation and budburst. Regressions were calculated for progressive periods separated in 15 days. *Line (without points)*: thermal amplitude; *blue line (triangle down)*: minimum temperature; *green line (circle)*: mean temperature; and *red line (triangle up)*: maximum temperature.

# Discussion

## Relationships between primary and secondary growth

Our results show that *Q. pyrenaica* has clear synchronicity between primary and secondary growth at specific key moments, whereby the maturation of newly built earlywood vessels is concomitant to leaf unfolding. In ring–porous species, earlywood vessels of the current year are only functional during one season, and carry 90–95% of the water flow (Ellmore & Ewers, 1986). Consequently it is essential to understand the importance of specifying the time of vessel formation with respect to leaf formation in order to understand carbon balances between previous season's storage and new assimilates (Barbaroux & Bréda, 2002).

In general, there is a some temporal link between primary and secondary growth, i.e., the timings for leaf phenological phases with respect to those of cambial cambial activity, but observations performed in a wide range of species suggests that they can be somehow different among ring-porous species (Suzuki *et al.*, 1996). Thus, Sass-Klaassen *et al.* (2011) compared *Fraxinus excelsior* and *Q. robur*, and found differences that primary and secondary growth did not follow the same synchronicity between both species. They concluded that leaf phenology cannot be taken as a global indicator of the status of vessel formation, and suggested further research to find out the species-specific consistency of the relationships between vessel development and leaf phenology. However, investigations carried out so far are scarce, and in general they are not specifically focused on relating primary and secondary growth, but just arise from studies on xylogenesis.

As regards ring-porous oaks, other authors explored cambial dynamics in *Q. robur* and *Q. pyrenaica* (Pérez-de-Lis *et al.*, 2017), coupled them to other ecophysiological traits such as leaf area index and sap flow measurements in *Q. pubescens* (Lavrič *et al.*, 2017), used acid fuchsin dye to evaluate whether the network of the current year earlywood vessels was functional in *Q. serrata* (Kudo *et al.*, 2015), or quantified leaf phenology along with the variation of dry matter content in *Q. pyrenaica* (Guada *et al.*, 2018). All these approaches showed that the expansion of earlywood vessels already started before budburst, although their maturation can take place either before or after budburst or leaf unfolding, probably depending on the species and/or site characteristics; furthermore, even genetic variability within species has been considered, as suggested by Puchałka *et al.* (2016) in the case of *Q. robur*. The precise identification of the moment when earlywood vessels become functional is critical to understand tree functioning, but it is not straightforward. In this sense, Kitin & Funada (2016) reviewed the synchronization between primary and secondary growth in ring-porous species, and underlined the importance of considering the functionality of earlywood vessels by observing the presence of

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perforations in longitudinal thin sections. Therefore, the combination of precise histological observations and objective, quantitative phenological surveys (e.g., Guada *et al.* (2018) are needed to fully understand the association between primary and secondary growth in the early season.

The comparison of the instant relationship between leaf and vessel development also contributes to understanding their potential links at growth resumption. In this sense, this study provided a wide sample range to robustly relate primary and secondary growth. Based on a sample size of 373 randomly-selected trees during the spring period in two years, it let us observe that 89% of the trees in 2012, and 95% in 2013, had already undergone cell maturation in earlywood when leaf unfolding occurred; in addition, 92% of the trees had begun the maturation process of the first vessel row when small leaves were visually appreciated, suggesting that the newly-formed vessels are supplying water for leaf expansion, but not for bud swelling yet.

On the other hand, in a similar study carried out in nearby locations of the more Atlantic species *Q. robur*, Guada *et al.* (2019) showed that between vessel maturation and leaf unfolding is not as synchronized between years as in *Q. pyrenaica*, and can be delayed even until the appearance of small leaves; this fact pinpoints that the required thermal depedences for tree growth are probably different for the two species. Nevertheless, the process of maturation of the first vessel row of the current year occurs before full leaf expansion for both oaks. However, we cannot rule out that differences in genotypic diversity due to sexual and asexual reproduction with respect to other oaks might also be involved (Valbuena-Carabana & Gil, 2017), as they result in a lower variability of *Q. pyrenaica*.

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## Relationships to temperature

*Q. pyrenaica* seems to have a better response to growth in its final stage of development than at the beginning of its growth derived by annual maximum temperature and thermal amplitude. Trees follow a clear seasonal pattern along the vegetation period, but these phenological events can be modified by internal (e.g., genotypic diversity) and external factors such as meteorological conditions. The most relevant phenological events are the beginning (breaking of dormancy, onset of cambial activity, budburst, flowering…) and the end (radial growth cessation, leaf senescence, and abscission) of the growing season.

In cold climates, differences in local temperature among areas are rather stable all year round. Therefore, mean annual temperature at the sites can be taken as an indicator to study the variability in xylem phenology of conifer species. This was tested by Rossi *et al.* (2016) across a wide range of boreal ecosystems in three continents, and concluded that mean annual temperature can be considered a predictor of growth in conifers in cold climates. However, local temperature differences in temperate forests are not stable throughout the year, and the annual mean temperature cannot be considered as an indicator to explain the beginning, cessation, and durations of xylem and leaf phenology events. In fact, it is only a short time window that explains the variation among sites, as evidenced for *Q. robur* under Atlantic conditions. Thus, weather characteristics at the moment of just before these key events can somehow lead to advancements or delays in the different events for both xylem and leaves, or even in the interaction between both processes (Guada *et al.*, 2019).

Although crown development has been reported to require a minimum temperature threshold to break dormancy (Wilkinson *et al.*, 2017), it is considered to be also induced by photoperiod (Basler & Korner, 2014). However, photoperiod differences were small in our study due to the latitudinal proximity of all sites, which let us optimize the understanding of the role of temperature along the altitudinal gradient. We found that site temperature did not appear to be the main factor affecting the onset of cell maturation or budburst of *Q. pyrenaica* in this area. Nevertheless, other studies in the northwestern Iberian Peninsula report some thermal requirements for spring phenological events of oaks. Thus, Jato *et al.* (2015) reported the role of temperature (chilling and heat-accumulation requirements) in advancing the flowering of *Quercus* species, including *Q. pyrenaica*, but different temperature regimes where the northernmost sites had a comparative shorter pollen season than the southermost sites, because the favorable temperature for the soutermost sites lengthens the pollination period, although it is not clearly indicated whether the temperature anticipates or delays the flowering event. Pérez-de-Lis *et al.* (2016b) compared *Q. pyrenaica* and *Q. robur*, and found that the onset of earlywoood formation and budburst were associated to a particular growing degree days, with *Q. pyrenaica* requiring higher thermal requirements, probably due to a greater dependence of *Q. robur* on temperature for budbreak, but on photoperiod for *Q. pyrenaica*.

If budburst in temperate forest is driven by a counterbalance between photoperiod and temperature (Basler & Korner, 2014), the absence of a temperature effect in the present work may indicate the dominant role of photoperiod on the beginning of crown and xylem development in *Q. pyrenaica*; this would be compatible with the lack of differences among most sites. However, a temperature influence cannot be neglected on xylem maturation. We cannot completely rule out an effect of temperature on growth resumption for this species. It is possible that i) the 12-15-day sampling interval did not have

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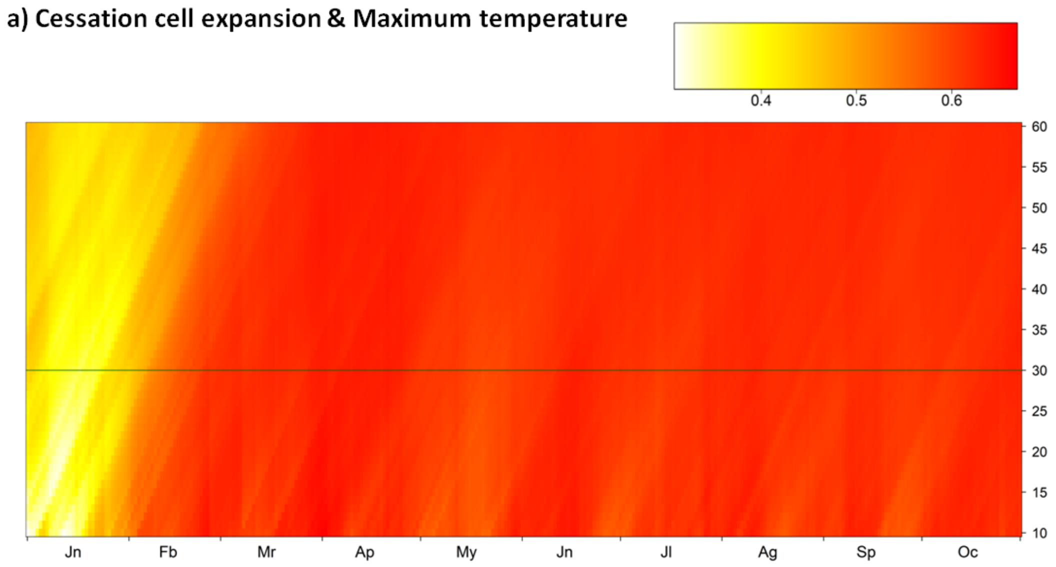
enough resolution for the detection of a temperature effect, ii) temperature differences among sites are not enough to identify these effects at our gradient, or iii) the two study years were not optimal or sufficient for detecting such temperature influence. In fact, previous dendrochronological analyses of earlywood vessels of *Q. pyrenaica* along a gradient in northwestern Iberia (Souto-Herrero *et al.*, 2018b) did show some differences in their response to spring temperature; however, the underlying mechanism for this response could not be elucidated by simply analyzing tree-ring chronologies. The complexity in understanding the effect of temperature on earlywood development was also evidenced by Pérez-de-Lis *et al.* (2016b), who hypothesized that vessel size of *Q. pyrenaica* was unrelated to vessel enlargement period, so that an earlier onset would probably not result in larger vessels, unlike *Q. robur*. Consequently, we are the opinion that the influence of temperature in determining the moment of growth resumption, if present, was probably small as compared to the photoperiod influence.

In contrast, the cessation of cell expansion in October was closely linked to temperature, especially to maximum temperature and thermal amplitude (i.e., the difference between maximum and minimum daily temperatures), whereby a warmer autumn seems to lengthen the growing season. A similar pattern of autumn cambium recovery after a growth arrest in September was also observed by Pérez-de-Lis *et al.* (2017) for *Q. pyrenaica*, and our results suggest that the maximum temperature, which also determines thermal amplitude, was the main climatic factor implied in this last pulse of growth. Under Mediterranean climates, where water stress can limit or stop summer growth, cambial reactivation has been observed to be induced by autumn precipitation in *Juniperus thurifera* and *Pinus halepensis* (Camarero *et al.*, 2010;Pacheco *et al.*, 2018). In our study, the gradient of *Q. pyrenaica* is located along the Atlantic-Mediterranean transition, and all sites under the same photoperiod. It seems that the beginning of crown and wood formation are controlled by photoperiod, whereas at the end of the season temperature gains importance in regulating wood formation at the sites of gradient where autumns become less severe for *Q. pyrenaica*. Pérez-de-Lis *et al.* (2017) describes that leaf abscission is subsequent to the cessation of cell expansion in the latewood in lower elevation sites of our same region. Therefore, given the plasticity of the cambium in response to temperature, this interval can lengthen if the environmental conditions are favorable. In a study carried out on *Pinus nigra*, Camarero *et al.* (2016) showed detrimental consequences caused by climatic anomalies at the end of tree growth. Thus, favorable autumn-winter temperatures produced a delay physiological hardening of the cambial tissues just before the abrupt drop in temperatures at the end of December, leading to frost damage in the xylem, which induced an abnormal growth in the cambium for the following year. In deciduous species, a warmer autumn may extend the growing season, but early leaf inactivity has been observed to be forced if there is an increased thermal stress during the summer (Xie *et al.*, 2015). How temperature interacts to influence wood phenological events in spring has been thoroughly studied, but its effect in autumn remains poorly known. Understanding and constructing robust process-based autumn phenology models is crucial to improve the forecast variations in the duration of spring and autumn phenology, which play an important role in regulating carbon uptake (Wu *et al.*, 2018;Lang *et al.*, 2019).



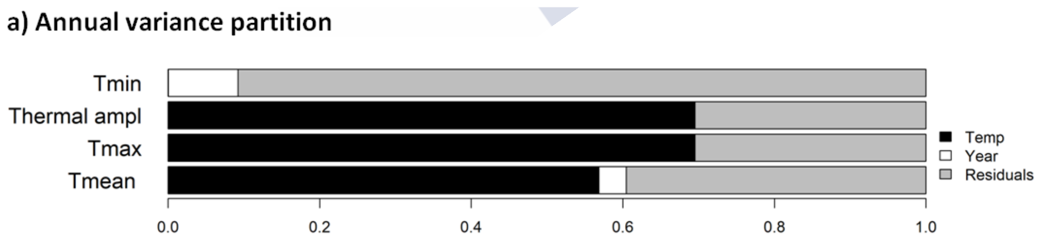


**Figure 8 (a)** Adjusted R-square of linear regression relating the dates of maximum temperature on cessation of cell expansion. Running means were calculated for periods of 10 to 60 days, shifted in one day. Horizontal line indicates the average for a 30-day period. **(b)** Variance partition of the phenological event ‘cell cessation’, with annual temperature and year as independent variables. Temperature variables considered are annual maximum, mean temperature, and thermal amplitude.

## Concluding remarks

*Q. pyrenaica*, though occupying a somehow restricted geographical area (Sánchez de Dios *et al.*, 2009), is an important species from a biogeographical point of view. It belongs to a group of species usually referred as 'sub-Mediterranean oaks', which in fact constitute the transition between the Atlantic and Mediterranean regions (Benito Garzón *et al.*, 2008), and are therefore of great importance for inferring the effects of climate change on the distribution of forests (Souto-Herrero *et al.*, 2018b). In this sense, the fact that the effect of temperature on growth is evident for wood formation, proves the importance of using xylem anatomy to understand the role of climate on growth (Fonti *et al.*, 2010;García-González *et al.*, 2016). Moreover, wood features, such as the size of earlywood vessels, clearly linked to xylogenesis, proved to be a powerful tool for elucidating the climatic control of wood formation in the study region (Souto-Herrero *et al.*, 2018b), and also highlighted the importance of temperature during both the dormant season and growth reactivation.

*Q. pyrenaica* has been reported to follow a much more conservative strategy than *Q. robur* with regard to radial growth (Pérez-de-Lis *et al.*, 2017), because its growing season is shorter (later leaf flushing). As *Q. pyrenaica* is distributed under continental Mediterranean climate, this delay in the onset of cambium resumption and budburst is an adaptive strategy aimed at preventing late spring frost, and is apparently controlled by photoperiod. In contrast, temperature gains importance in regulating the cessation of wood formation, by extending the growth period if autumns become less severe, and annual temperature values can be considered because differences in local temperature across sites were stable throughout the year. This similar leaf and cambial growth at the beginning of the season also makes the relationship between primary and secondary growth show a clear synchronicity at all sites, confirming that an appropriate maturation of first earlywood vessels is essential for crown development.

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# Anatomical tree-ring chronologies and seasonal patterns of cambial dynamics are valuable indicators of tree performance of two oak species at the Atlantic-Mediterranean boundary

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## Abstract

We analyzed the tree rings of two oak species (*Quercus robur* and *Q. pyrenaica*) at their distribution boundary towards the Mediterranean region. For this, two nearby sites with slightly different regime of water availability regime were selected for each species. We developed chronologies of radial increment (expressed as latewood width) and functional anatomical traits (size and number of earlywood vessels) for the last decades, and also monitored wood formation along two growing seasons. Studies for each species were considered as different experiments, so that only comparisons within species were initially performed.

Despite being species with contrasting requirements, as *Q. robur* is typical of mesic habitat, whereas *Q. pyrenaica* is considered sub-Mediterranean, their results were comparable. There were some differences between sites were scarce, especially for *Q. pyrenaica*, but the main factors controlling growth were identified at all sites. Earlywood characteristics were mainly related to temperature, whereas water availability responded to precipitation, regardless of the species. However, vessels of low-elevation *Q. robur* seemed to be controlled by factors affecting reserve balance, whereas those of high-elevation *Q. pyrenaica* were associated to spring conditions to resume growth. Summer water availability was linked to a wider latewood for both species. In addition, the analysis of xylogenesis carried out at all sites was essential to interpret climate responses, which evidences the existence of a cause-effect relationship. Our results suggest that the combination of anatomical traits and radial growth are a useful tool to understand the behavior of these species in boundary distribution areas.

**Keywords:** dendrochronology, xylogenesis, leaf phenology, *Quercus robur*, *Quercus pyrenaica*.

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## Introduction

Oaks (*Quercus* spp.) are dominant species in European forests, extending from the Urals to the Mediterranean Basin. Among them, nemoral oaks (*Q. petraea* (Mattuschka) Liebl., and mainly *Q. robur* L.), adapted to mild conditions, are keystone components of temperate forests (Ellenberg, 2009), but reach their southern distribution boundary towards the Mediterranean Region. Afterwards, they are progressively replaced by more drought-tolerant species, such as evergreen oaks (*Q. ilex* subsp. *ilex* L., *Q. ilex* subsp. *ballota* (Desf.) Samp.) or other xerophile species (Amaral Franco, 1990). However, transition zones are often occupied by the so-called 'sub-Mediterranean' oaks (Sánchez-de-Dios *et al.*, 2009), which are usually more xerophytic, but deciduous with marcescent habit (e.g., *Q. pyrenaica* Willd., *Q. pubescens* Willd., *Q. faginea* Lam.…).

Summer drought is one of the main factors driving the distribution of these species, and an attribute of Mediterranean climate and its associated vegetation. According to the IPCC (2013), summer drought is expected to increase, which can lead to local extinctions, as some species populations can override their ecological limitations. This is the case of the Iberian Peninsula, where prediction models anticipate the replacement of *Q. robur* and *Q. petraea* by sub-Mediterranean oaks, among which *Q. pyrenaica* is the most relevant species (Benito Garzón *et al.*, 2008;Sánchez-de-Dios *et al.*, 2009). At present, *Q. pyrenaica* dominates boundary areas and subhumid mountains of the Mediterranean region in Iberia. Its characteristics to deal with these harsh environments, such as pubescent leaves, a thick bark, and a very late flushing season confers a higher drought tolerance than nemoral oaks, as well as the avoidance of late frost.

Among these boundary areas, northwestern Iberia is one of the most abrupt transitions between the Atlantic and the Mediterranean regions. Natural forests of both *Q. robur* and *Q. pyrenaica* coexist at their distribution edge, being therefore a very appropriate territory to understand the behavior of both species under changing conditions. *Q. robur* woodlands are limited to areas with Atlantic climate, where they almost spread along the whole altitudinal range, but tend to be replaced by *Q. pyrenaica* as conditions become more Mediterranean and continental towards the inland. But Mediterranean influence is also present at low-elevation coastal oak forests, the so-called thermocolline belt (Rivas-Martínez, 1987). Thus, very mild conditions throughout the year, mainly caused by temperature rather drought, favor the presence of Mediterranean or even sub-tropical (Macaronesian) floristic elements (Izco *et al.*, 1990), but with some differences between Atlantic and Cantabrian areas (Amigo *et al.*, 2017). In

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contrast, *Q. pyrenaica* tends to occur as conditions become more xeric. As it is well adapted to continentality, tends to dominate mountain areas (Díaz González & Penas, 2017), but it also coexists in many areas in the inland (Diaz-Maroto *et al.*, 2007). Understanding the performance of these forests by inferring how they could cope with climate change, and consequently how vegetation changes could occur, is of great relevance from a biogeographic point of view. Moreover, this knowledge should be very important for conservation management, because this region suffers from a strong human pressure, and most natural forests have been destroyed by constructions, agricultural fields, or fast-growing tree plantations such as *Eucalyptus globulus* Labill. or *Pinus radiata* D. Don.

One of the most critical tree adaptations to climate change is the modification of their phenological events (Menzel *et al.*, 2006), so that a lengthening of the growing season, and particularly an advancement of the moment of reactivation after winter dormancy have been observed (Vitasse *et al.*, 2017). But an increment of spring temperature anticipating leaf unfolding can involve some risks, as trees become more vulnerable to late frost (Gu *et al.*, 2008). On the other hand, there is a close link between foliar and vascular phenology, i.e., leaf development and cambial activity (Takahashi *et al.*, 2013;Guada *et al.*, 2018), and increasing spring temperatures are also associated to an anticipation in the onset of cambial activity (Yang *et al.*, 2017;Begum *et al.*, 2018). This association is especially relevant in the case of ring-porous trees as oaks, because the formation of the first earlywood vessels is linked to leaf phenology, whereby the expansion of the first elements is concomitant or just preceding bud break, and their maturation is required for full leaf expansion (Kitin & Funada, 2016). However, Guada *et al.* (2019) recently argued that spring temperature might alter the synchronicity between primary and secondary growth.

The study of xylogenesis allows identifying the immediate responses of trees to changes along the growing season, and helps understanding how trees cope with climate change (Cuny *et al.*, 2015;Camarero *et al.*, 2016;Guada *et al.*, 2016). In contrast, dendrochronological techniques add a retrospective point of view, and both approaches can be combined to better understand tree reactions. Furthermore, they become a very powerful tool when combined with anatomical studies (Pacheco *et al.*, 2015), as quantitative wood anatomy has proven to be very valuable for the study of climate change (Fonti *et al.*, 2010). This is the case of the earlywood vessel size of oak, whose chronologies were able to identify the main factors controlling wood formation(Gea-Izquierdo *et al.*, 2012;Rita *et al.*, 2016;Souto-Herrero *et al.*, 2018b) even in areas where climate conditions are

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relatively mild (Souto-Herrero *et al.*, 2018a), or under the lack of a clear single limiting factor (Fonti & Garcia-Gonzalez, 2008).

Recent investigation carried out in northwestern Iberia pinpoint that both the monitoring xylogenesis (Pérez-de-Lis *et al.*, 2016b;Pérez-de-Lis *et al.*, 2017;Guada *et al.*, 2019) and dendrochronological wood anatomy (Souto-Herrero *et al.*, 2018b;a) are very useful tools to understand the performance of both *Q. robur* and *Q. pyrenaica*, especially if combined to achieve a better understanding of the underlying mechanisms (González-González *et al.*, 2015). The separate dendrochronological analysis of earlywood and latewood chronologies was much more successful than the single analysis of ring width (Souto-Herrero *et al.*, 2017;2018b), and only when both tree-ring compartments were analyzed together, did climate-growth relationships provide a feasible interpretation of the ongoing processes. In addition, monitoring wood dynamics allowed elucidating the role of temperature on spring reactivation (Guada *et al.*, 2019), the close relationships between leaf phenology and vessel formation (Guada *et al.*, 2018), the environmental control of vessel size and latewood growth (Pérez-de-Lis *et al.*, 2016b;Pérez-de-Lis *et al.*, 2017), or the influence carbohydrate dynamics on early growth(Pérez-de-Lis *et al.*, 2016a). All these studies evidence that both species do not follow the same strategy of wood formation, probably due to their different ecological requirements.

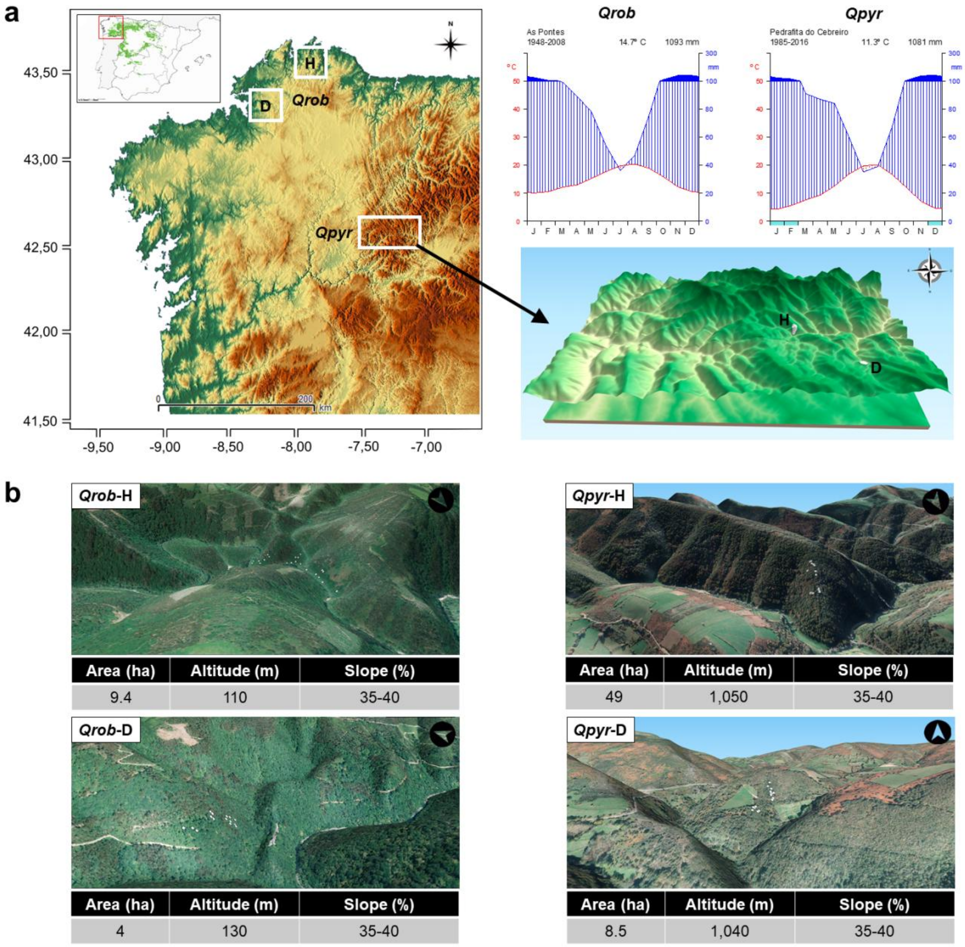
In this paper, we aim at elucidating the responses of radial growth of oaks at the transition between the Atlantic and the Mediterranean regions. Through two independent studies, we compared i) *Q. robur* at two thermophile sites of its lower distribution limit, but more humid (Cantabrian coast) or drier (Atlantic coast) summer conditions; and ii) *Q. pyrenaica* at its upper boundary, also slightly differing in water availability due to their facing within a mountain range, where the southern slope had more Mediterranean conditions. For this, we developed tree-ring width and anatomical chronologies, and monitored wood formation at the four sites. Our main objectives are i) to assess how powerful these methodologies are in order to identify the main factors driving growth performance at the boundary to the Mediterranean region, and ii) to evaluate to what extent these factors are sensitive to small differences in the regime of water availability.

## Materials and Methods

### Study area and sites

We selected two areas in the northwestern Iberian Peninsula, respectively corresponding to the lower limit of *Q. robur* (***Qrob***), and the upper limit or *Q.*

*pyrenaica* (**Qpyr**); within each area, two stands were chosen, slightly differing in their summer water regime. ***Qrob*** sites were located close to the coastline, whereas ***Qpyr*** sites occupied mountain areas inland, above 1,000 a.s.l. (Fig. 1). As both areas had different characteristics, the analyses at the ***Qrob*** and ***Qpyr*** sites were taken as two independent experiments, and all pairwise comparisons were just performed within species. In fact, although regional climatic regime is mainly Atlantic with more than 1,200 mm of annual precipitation (with a maximum during fall and winter), its seasonal distribution is very variable spatially. Mediterranean influence is associated to a remarkable summer drought towards the inland, but also at some lowland areas along the Atlantic, where maximum temperature can reach high values (Table 1).



*Qrob*-H

| Area (ha) | Altitude (m) | Slope (%) |
|---|---|---|
| 9.4 | 110 | 35-40 |

*Qpyr*-H

| Area (ha) | Altitude (m) | Slope (%) |
|---|---|---|
| 49 | 1,050 | 35-40 |

*Qrob*-D

| Area (ha) | Altitude (m) | Slope (%) |
|---|---|---|
| 4 | 130 | 35-40 |

*Qpyr*-D

| Area (ha) | Altitude (m) | Slope (%) |
|---|---|---|
| 8.5 | 1,040 | 35-40 |

**Fig 1 (a)** Sites of *Quercus robur* (**Qrob**) and *Quercus pyrenaica* (**Qpyr**) in the northwestern Iberian Peninsula at Dry (**D**) and Humid (**H**) sites, with climate diagram for a nearby weather station. **(b)** Physiographic characterization of the selected sites is provided, with sampled trees with white dots.

Source: GIS-FOREST (INIA) 2016, https://sites.google.com/site/sigforestspecies/)

**Table 1** Description of the sites included in the analysis, with their identification codes (ID), geographical location, and elevation, of *Q. robur* (*Qrob*) and *Q. pyrenaica* (*Qpyr*) in DRya and humid (H) sites. Normal annual temperature (Temp), precipitation (Prec), annual water balance (W.B.) and summer W.B. values obtained from Rodríguez-Lado *et al.* (2016) (source: digital database at http://rgis.cesga.es) and two years temperature from sensors at the study sites mean (T.mean), minimum (T.min), and maximum (T.max).

| ID | Latitude (N) | Longitude (E) | Altitude (m.a.s.l.) | Normal Values: Temp (ºC) | Prec (mm) | Annual W.B. (mm) | Summer W.B (mm) | 2012/13 years values: T.mean (ºC) | T.min (ºC) | T.max (ºC) |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Qrob*-D** | 43.416991 | -8.064395 | 125 | 13.2 | 1158 | 562.2 | -174.3 | 13.8 | 9.9 | 19.6 |
| ***Qrob*-H** | 43.676000 | -7.708347 | 125 | 13.0 | 1194 | 475.3 | -168.0 | 12.3 | 9.0 | 16.1 |
| ***Qpyr*-D** | 42.684528 | -7.094356 | 950 | 8.5 | 1428 | 724.3 | -161.1 | 10.3 | 6.5 | 16.1 |
| ***Qpyr*-H** | 42.664597 | -7.054362 | 1050 | 8.82 | 1433 | 830.0 | -146.2 | 9.5 | 5.3 | 14.8 |

***Qrob*** was studied in coastal areas (43.42-43.68 ºN, 7.71-8.06 ºW), where natural vegetation is dominated by oak (*Q. robur*) forests, with *Q. pyrenaica* is usually absent. Both sites belong to the association *Blechno spicantis-Quercetum roboris*, and specifically to its most thermophile facies (Izco *et al.*, 1990). The mild (13 ºC) and moist (*ca.* 1,200 mm) conditions throughout the year are indicated by the presence of some subtropical elements, such as Macaronesian ferns (*Culcita macrocarpa* C. Presl., *Woodwardia radicans* (L.) Sm., *Hymenophyllum tunbrigense* (L.) Sm., *Asplenium onopteris* L., or *Dryopteris guanchica* Gibby & Jermy). These two woodlands correspond to the mouths of two rivers. River Eume is facing the Atlantic coast (*Qrob*-D), which results in a drier summer due to a higher temperature and consequently evapotranspiration, whereas the river Sor flows into the Cantabrian Sea, yielding more humid summer conditions (*Qrob*-H), with mean maximum temperature *ca.* 3 ºC lower during summer (Table 1).

***Qpyr*** stands were located at the southwestern edge of the Cantabrian Mountain Range (42.64-42.70 ºN, 7.02-7.13 ºW), an area of a complex topography with peaks around 1,300 m. With a mean annual temperature below 10 ºC, summers are mild (17 ºC), and winters cold (4.7 ºC), with frost periods, frequent fogs, and thermal inversions in the valleys; precipitation is high (>1,400 mm/year), also concentrated in autumn and winter (often as snow), but irregularly distributed, with a negative water balance during summer. The selected sites constitute the upper distribution boundary of the species, still lie within the Atlantic biogeographical region (Rodríguez Guitián & Ramil Rego, 2008), and belong to the association *Linario triornithophorae-Quercetum pyrenaicae* (Rivas-Martínez, 1987), usually poor communities on acid soils. The most humid site (*Qpyr*-H) was facing north on deep schistic soils with abundant

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organic matter, and accompanied by *Fagus sylvatica* L. on the wettest depressions; this moister environment was indicated by species such as *Corylus avellana* L., *Ilex aquifolium*, *Vaccinium myrtillus* L., *Omphalodes nitida* (Willd.) Hoffmanns & Link or *Physospermum cornubiense* (L.) DC, and abundant bryophytes. In contrast, the driest site (*Qpyr*-D) was facing south on a moderate slope, and was a monospecific formation of *Q. pyrenaica* with a poor understory of *Rubus* sp. or *Pteridium aquilinum* (L) Kuhn in Kerst., with *Pyrus cordata* Desv. and *Erica arborea* L. towards the boundary of the forest; no elements evidenced a high humidity; furthermore, forest of the Mediterranean *Q. ilex* subsp. *ballota* occurred not far from this site.

### Xylem sampling and wood phenophases

We monitored wood formation at these forests during two consecutive years, from March to November in 2012, and from February to September in 2013, with a sampling interval of 12-15 days in spring, and 20 in summer. During each visit, we randomly selected ten trees per site within an area of *ca.* 1 ha, in order to have a representative sample of the whole forest. Sampled trees had a diameter at breast height (DBH) ranging 20-40 cm (average was 28.2±4.9 cm, with no significant differences between sites), and trees with polycormic stems, partially dead crowns, or evident damage were avoided.

We used a Trephor tool (Rossi *et al.*, 2006a) to extract at least two wood microcores at breast height (1.3 m), perpendicularly to the slope, which contained mature and currently developing xylem, the cambial zone and adjacent phloem, and one to a few complete tree ring. At the lab, we handled the samples following the protocol by (Pérez-de-Lis *et al.*, 2016b;Guada *et al.*, 2018) to obtain permanent slides, which includes fixation in alcohol, paraffin embedding, cutting by a rotatory microtome, and differential staining.

We took images of the microscopic slides (0.926 µm/pixel) using a digital camera (Canon EOS 600D, Tokyo, Japan), coupled to a transmitted light microscope (Olympus BX40, Tokyo, Japan, 40 × objective); a white light polarizing filter help detecting secondary cell wall deposition. On these images, we identified cambial phenological phases, counted the number of cambial cells, and measured the width of expansion and maturation zones along three radial files. Vessel enlargement was recorded when the most recent ring contained at least one enlarging vessel element; the onset of earlywood vessel maturation was defined by the deposition of secondary cell wall, detected by birefringence under polarizing light.

## Leaf phenological observations

We also recorded leaf phenology of each individual tree sampled for wood formation, by observing the top of the main branches using binoculars (10 × magnification), and it was expressed as a particular day of year (DOY).

We identified four phenophases, namely, a) budburst, b) leaf unfolding, c) appearance of small leaves, and d) full leaf extension. Budburst was characterized by expanded buds with no unfolded leaves; leaf unfolding ended as soon as the leaf blade was clearly visible, but not the petiole; appearance of small leaves was considered when at least one leaf was completely out of the bud, and the petiole could be visually appreciated; and full extension was recorded when leaves attained at least the 50% of their apparent final size.

## Dendrochronological sampling, ring width, and vessel measurements

At each site, we selected 11-26 dominant or isolated oaks for developing tree-ring chronologies, and extracted at least two 5-mm increment cores at breast height. Cores were air-dried, mounted on wooden supports, and prepared for an optimal visualization of tree rings and vessel elements. We obtained a regular cross-sectional surface with a WSL sliding microtome (Gärtner & Nievergelt, 2010), manual polishing (sandpaper P220 to P1200, FEPA Abrasives), removal of dust and tyloses by high-pressure water blast, and finally staining with black printer ink, and rubbing chalk on the surface (Souto-Herrero *et al.*, 2018b).

We measured earlywood (EW) and latewood widths (LW) to the nearest 0.001 mm, with a tree-ring measuring linear stage (Velmex TA UniSlide, Velmex Inc., Bloomfield NY, USA) and a binocular microscope (Olympus SZ60) at 20-40× magnification. Crossdating was obtained by first comparing individual curves, and then statistically verified using COFECHA (Grissino-Mayer, 2001). Further measurements of the earlywood vessels were performed on sequences of high-resolution digital images (5,184 x 3,456, 17.9 Mpx) taken with a Canon EOS 600D camera coupled to the binocular microscope and a mechanically-driven platform, and stitched (PTGui ver. 9.1.8 Pro, New House Internet Services B.V., Rotterdam, The Netherlands) into a single file per core, and saved into TIFF files. These images were processed in ImageJ (Schneider *et al.*, 2012), using the VesselJ plugin and Autovasos (for further details on the process of image analysis, see (Souto-Herrero *et al.*, 2017;2018b). We only retained those vessels larger than 10,000 µm, and separated vessels contained in the first row from the others. All raw data (ring widths and vessel measurements) from cores of the same tree were respectively averaged or pooled for each ring.

## Variable survey and tree-ring analyses

We obtained chronologies for latewood width (LW), and earlywood vessel number (NV) and size, which was expressed it as the hydraulic-weighted diameter ($D_{\mathrm{H}}$), following (Sperry *et al.*, 1994); $D_{\mathrm{H}}$ estimates the average diameter for the theoretical conductivity of a given stem (Fichot *et al.*, 2009), and is probably the most appropriate variable to relate earlywood vessels and environment (Souto-Herrero *et al.*, 2017;2018b), for being very robust to the presence of small vessels (García-González *et al.*, 2016). Vessel size was considered separately for the first vessel row ($D_{\mathrm{H}}$-r1), and for vessels outside this row ($D_{\mathrm{H}}$-nr1).

Individual series were detrended by first adjusting a 32-year cubic smoothing spline with 50% cutoff (Cook *et al.*, 1992), which was flexible enough to minimize both age trend and growth variations related to forest dynamics. After detrending, we computed growth indices by division (Fritts, 1976b), applied autoregressive modeling to remove persistence, and averaged the indices into a chronology for each variable and site using a biweight robust mean (Mosteller & Tukey, 1977). The obtained chronologies were subsequently assessed for their quality, using the most common parameters in dendrochronology, namely the mean sensitivity (*MS*), an indicator of interannual variability; the first order autocorrelation coefficient (*AR1*), which measures the influence of previous year on growth; the mean correlation between trees (*Rbt*), i.e., the mean value of all possible Pearson's cross-correlation coefficients; and the expressed population signal (*EPS*), indicating the extent to which the sample size is representative of a theoretical population with an infinite number of individuals (Wigley *et al.*, 1984).

## Comparison of series and climate-growth relationships

For each species and variable, we ordinated individual tree-ring series by varimax-rotated principal component analysis (PCA), in order to establish if there was a clear trend between both sites. The calculation was performed from the correlation matrix of all selected trees, which were pairwise compared along their entire length, and the correlation coefficient corrected for a theoretical common length (50 years). In fact, all ***Qpyr*** trees overlapped for more than this length, but the length of ***Qrob*** series was shorter (especially at ***Qrob*-H**), and consequently only series overlapping at least 25 years to all trees considered; thus, five trees were excluded (two at ***Qrob*-D**, three at ***Qrob*-H**; out of a total of 40 ***Qrob*** trees).

For both species, we selected the period 1960-2013 (53 years) for the establishment of climate-growth relationships, because it was covered by at least

10 trees per site. Monthly series of climatic variables related to temperature (mean, minimum, and maximum temperature), and water regime (total precipitation, standardized precipitation-evapotranspiration index – SPEI – at one and three months). All data were obtained from CRU TS 4.01, a gridded source at 0.5º × 0.5º of geographical resolution available from the KNMI Climate Explorer (http://climexp.knmi.nl/). The SPEI is a multiscalar drought index that combines precipitation and potential evapotranspiration (Vicente-Serrano *et al.*, 2010), which has proven to be very useful to understand the response of trees using both dendrochronological series (Peña-Gallardo *et al.*, 2018) and xylogenesis (Ren *et al.*, 2015).

Climate-growth relationships were evidenced by the computation of Pearson's correlation coefficients between the site chronologies and the monthly meteorological records. Confidence intervals for the correlation functions was achieved by the bootstrap technique (Guiot, 1991), using the correction proposed by Mason & Mimmack (1992) for the percentile method. According to own observations of cambial activity and leaf phenology (Pérez-de-Lis *et al.*, 2017;Guada *et al.*, 2018;Guada *et al.*, 2019), and to the analyses of xylogenesis presented in this paper, we considered the growing season as spanning from previous to current May in the case of earlywood, and from previous May to September for LW. In addition, we used seasonal means for the periods of dormancy (December-February), late dormancy-quiescence (February-March), or early spring (March-April, April-May, March-May) for earlywood variables; and late spring-early summer (May-July), or current summer (June-July, July-September) for latewood width.

## Results

### Xylogenesis and leaf phenology

For ***Qrob***, the dormant cambium consisted of 5–7 cells for both sites and years (Fig. 2), but increased during the growing season, reaching a maximum (9.52 ± 0.28 in 2012; 8.33 ± 0.29 in 2013) around the summer solstice (178 DOY). The pattern of cell enlargement was bimodal, with maxima at the formation of earlywood vessels, and at the development of summer latewood. In 2012, the first peak occurred earlier at ***Qrob*-D** (90-120 DOY) than at ***Qrob*-H** (120-150 DOY), whereas this pattern was less evident in 2013, when the highest values occurred in May (120-150 DOY) at both sites; but there was an averall larger width increment for the Cantabrian ***Qrob*-H** (108.83 ± 16.01 µm vs. 74.08 ± 9.13 µm). In addition, growth cessation occurred later at ***Qrob*-H**, with two trees still

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undergoing cell enlargement at the end of September 2012 (265 DOY), and even one in November 2012 (318 DOY).

The onset of cell maturation in 2012 was anticipated for ***Qrob*-D** (90-120 DOY vs. 120-150 DOY) as well, but also delayed in 2013 at this site (May, 120-150 DOY). The total maturation zone was wider in 2012 (1,452 ± 307.89 µm) than in 2013 (540.98 ± 105.63 µm) at ***Qrob*-D**, and similar in both years at ***Qrob*-H** (1,150.55 ± 139.35 µm in 2012; 1,043.70 ± 207.63 µm in 2013).

As regards leaf phenology (Fig. 3), timings from budburst to full leaf development were earlier and shorter at ***Qrob*-D** (90-122 DOY vs. 91-157 in 2012; 73-126 DOY vs. 95-143 in 2013). But when comparing these stages in trees already undergoing enlargement of the first vessels, we found all phenophases at ***Qrob*-D**, while trees at ***Qrob*-H** were mostly at budburst, or even less developed.

Dormant cambium of ***Qpyr*** was similar to ***Qrob*** (5-7 cells), but it was already active on the first sampling date in 2012 (Fig. 2b). This number of increased until reaching a maximum in June and July (DOY 150-210), with a higher average number of cells in 2012 (10.13 ± 0.44; 187 DOY) than in 2013 (9.19 ± 0.23; 214 DOY), and no differences between sites. The pattern of cell enlargement was also bimodal, coupled to earlywood vessel formation and summer latewood increment, and was more remarkable in 2012 (early May, 132 DOY; early July, 188 DOY), than in 2013, when just a single maximum occurred in May (128-144 DOY). As a result, the average width of the cell maturation zone was wider for 2012 than for 2013 (1,406.80 ± 169.06 µm vs. 887.20 ± 143.27 µm), but with no differences between sites. Cambial activity ceased at the end of summer (249 DOY, early September in 2012; 244 DOY, late August in 2013), but reactivated for an autumn (278 DOY; early October) regrowth just in 2012, for seven trees out of ten at each site.

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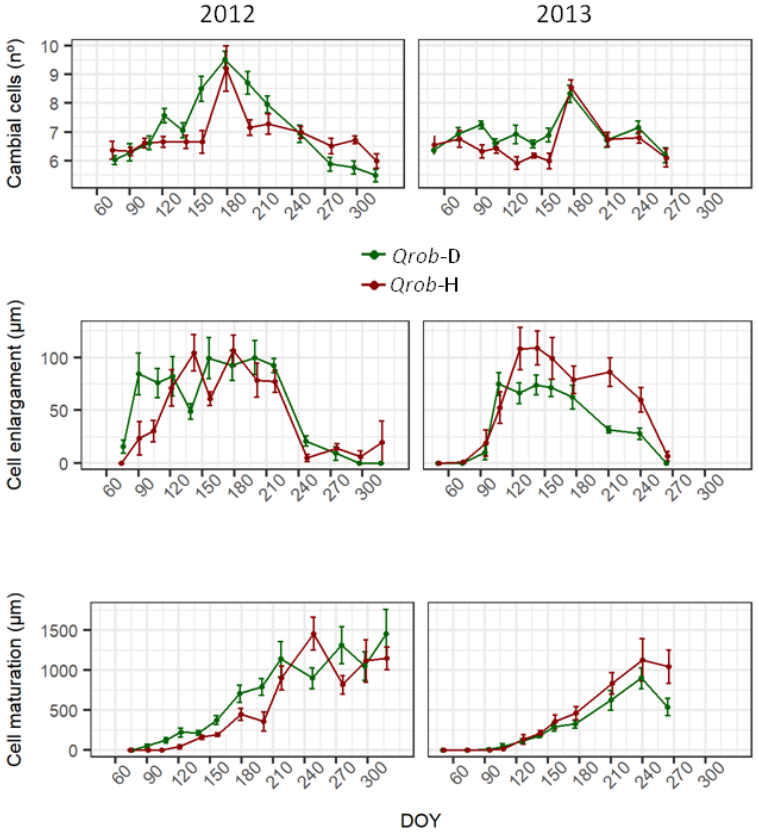
a) ***Quercus robur***



b) ***Quercus pyrenaica***

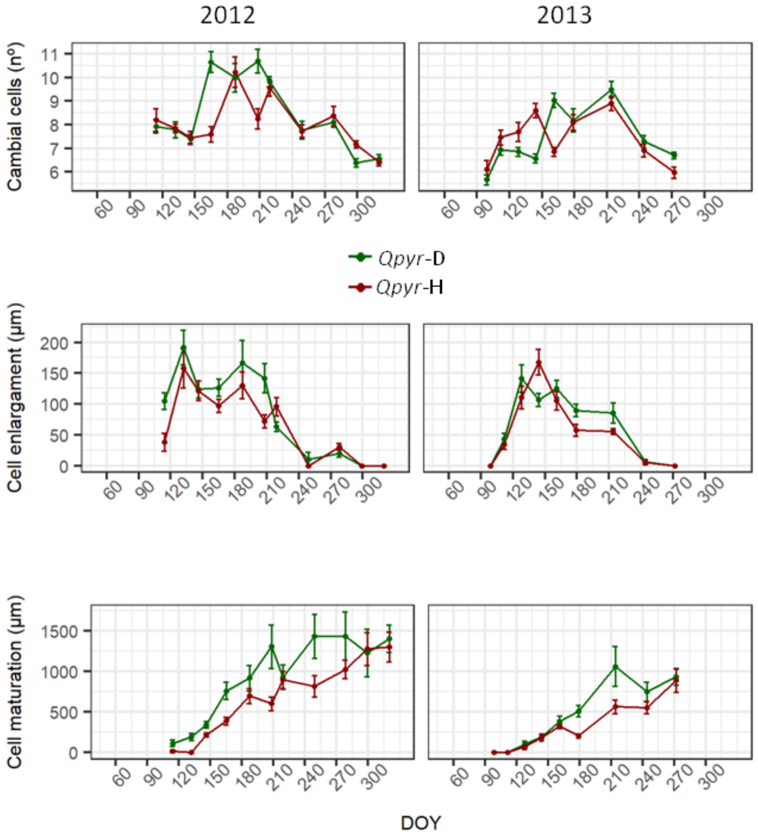


**Fig 2** Seasonal dynamics of radial growth in 2012 and 2013 **(a)** *Quercus robur* (*n*=10) at the study sites 'dry' (***Qrob*-D**) and 'humid' (***Qrob*-H**); and **(b)** *Quercus pyrenaica* (*n*=10) at the study sites 'dry' (***Qpyr*-D**) and 'humid' (***Qpyr*-H**). Vertical bars represent the standard error, DOY day of the year.

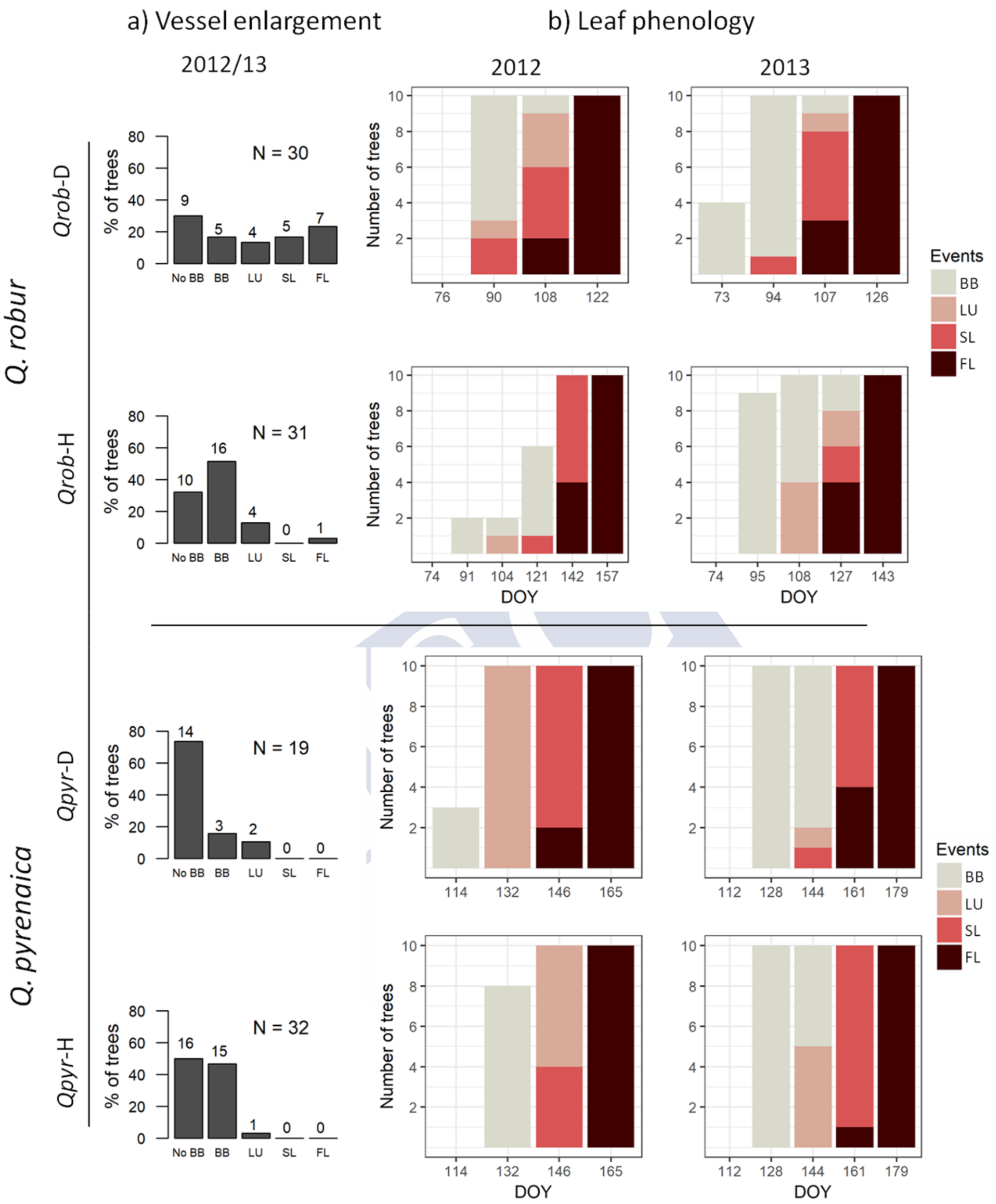


**Fig 3** Leaf phenology and secondary growth of *Q. robur* (*Qrob*) and *Q. pyrenaica* (*Qpyr*) at 'dry' (D) and 'humid' (H) sites. **(a)** Percentage of different crown phenophases at beginning of cell enlargement; number of trees per each crown phenophase are indicated above the column. No budburst (No BB), budburst (BB), leaf unfolding (LU), small leaves with <50% of their apparent final size (SL), full leaf expansion with > 50% of their apparent final size (FL). **(b)** Temporal variation of the number of trees for each leaf phenophase. Leaf phenology events are indicated in the legend, with lighter colors progressively referring to later stages (budburst, leaf unfolding, leaves with <50% of their apparent final size, and leaves with >50% of their apparent final size).

There were slight differences in leaf development, with an earlier budburst at ***Qpyr*-D** in 2012 (114-165 DOY vs. 132-165 DOY), and apparently no differences in 2013 (128-179 DOY). But most trees at ***Qpyr*-D** had not undergone budburst when we observed the enlargement of the first earlywood vessels, and neither did half of them at ***Qpyr*-H**, while the others were found to be at budbreak; but in no case were leaves expanded, confirming that vessels start forming before this stage. Furthermore, xylem maturation was found to be concomitant to leaf unfolding in 2012, and to budburst in 2013 (Fig. 2 and 3).

## Tree-ring analyses and climate-growth relationships

The comparison of individual series by PCA (Fig. 4) showed that differences among sites are not remarkable, for both ***Qrob*** and ***Qpyr***; but the common signal, i.e., the similarity among trees from both sites is considerably higher for ***Qpyr***, as expressed by the highest variance in the first eigenvector; among variables, LW and Dh-r1 are the most relevant ones. In contrast, the variables depending on vessels outside the first row (NV and Dh-nr1) have a much lower signal, with a negligible explained variance (12.1% and 15.0%, respectively) for ***Qrob***, and slightly higher for ***Qpyr*** (23.6% and 27.8%), but always lower than that of $D_{\mathrm{H}}$-r1, and especially, LW. As regards the ordination of individuals, at least for $D_{\mathrm{H}}$-r1 and LW there is in general a trend to split individuals from each contrasting site along the first eigenvector, or even along the second for ***Qpyr***. However, there is no clear separation, as there are always a few trees from every other site scattered, but it is more evident for $D_{\mathrm{H}}$-r1 than for the other variables.

The analysis of chronology quality confirms $D_{\mathrm{H}}$-r1 and LW as the most appropriate and reliable variables for further dendrochronological analysis (Table 2). Common signal can be considered optimal for LW, and high for $D_{\mathrm{H}}$-r1, for both ***Qrob*** and ***Qpyr***. For the former, signal is better for ***Qrob*-H**, where the values of EPS are even higher with a considerably lower number of trees; in contrast the quality of $D_{\mathrm{H}}$-nr1 and especially NV is poor. As regards ***Qpyr***, differences between sites are negligible (except for series length), and signal is also very high for LW, and high for $D_{\mathrm{H}}$-r1; for the other variables, the values are still acceptable, especially for $D_{\mathrm{H}}$-nr1. MS is high for LW, and low for vessel size, whereas AR1 evidences a notable persistence, and justifies the application of the autoregressive modeling.

The results of climate-growth relationships enhance the similarities among sites for both species, but also evidence the existence of some differences. As a rule, vessel size ($D_{\mathrm{H}}$-r1) is mainly related to temperature, whereas LW was linked to precipitation (Fig. 5-6); the climatic association of the variables $D_{\mathrm{H}}$-nr1 and NV was poorer.

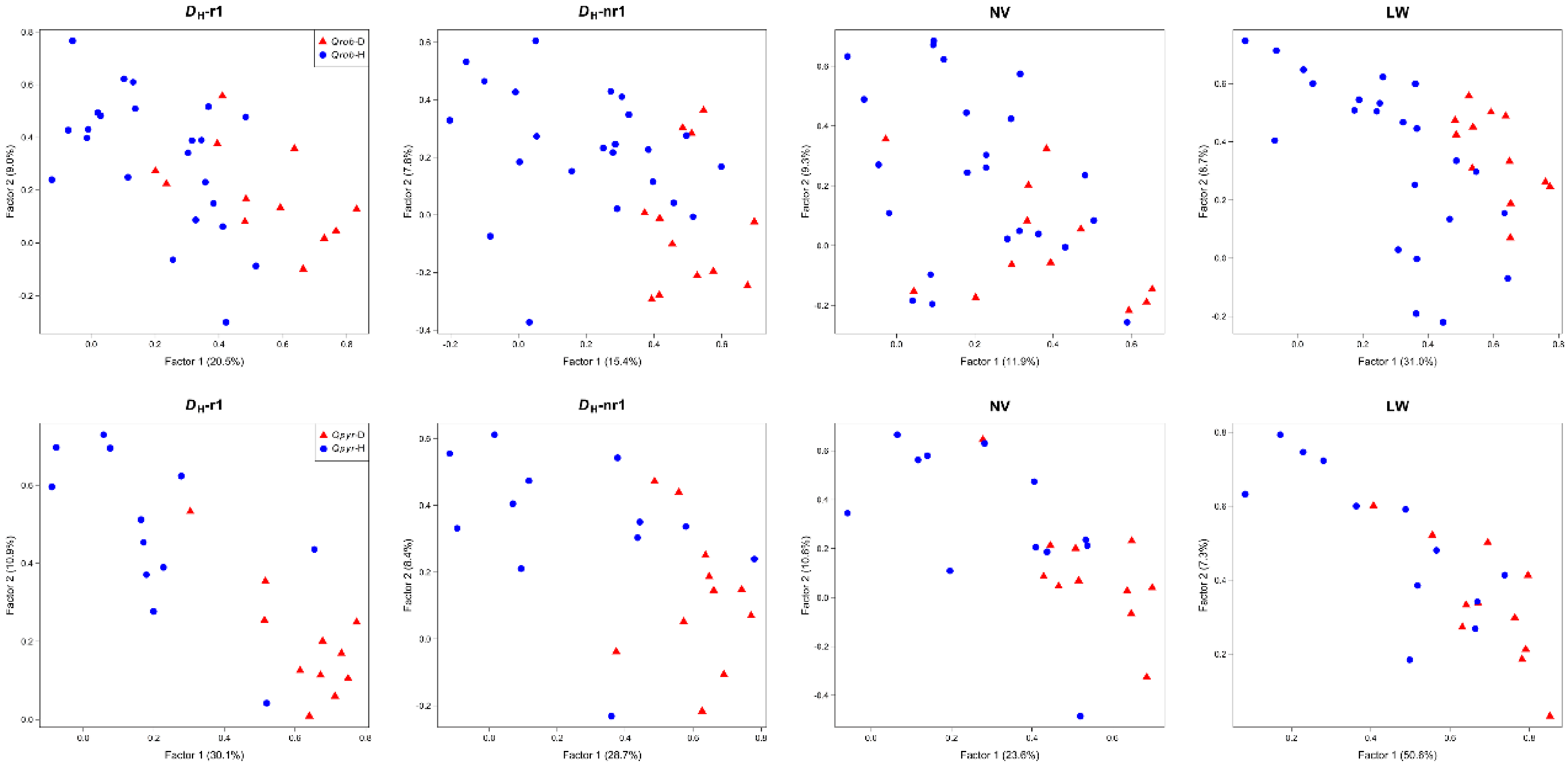


**Fig 4**. Principal component analysis (PCA) of individuals per variable at all study sites of hydraulically-weighted diameter ($D_H$) of the first row of earlywood vessels ($D_H$-r1), for vessels outside this row ($D_H$-nr1), number of vessels (NV), and latewood width (LW).

For ***Qrob***, there is a strong negative correlation between $D_H$-r1 and the temperature during dormancy and quiescence (December-February), i.e, high temperatures during this period are coupled to small vessels and vice versa. There are however some differences between sites, because the signal is maximized in early winter (December) at ***Qrob*-H**, but at the end dormancy (February) at ***Qrob*-D**.

| | *Variable* | *Start* | *End* | *Mean* | *SD* | *MS* | *AR1* | *Trees* | *Rbt* | *EPS* | *SNR* | *%Var* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Qrob*-D** | **$D_H$-r1** | 1906 | 2013 | 261.0 | 37.8 | 0.09 | 0.62 | 14 | 0.29 | 0.84 | 5.35 | 35.2 |
| | **$D_H$-nr1** | 1906 | 2013 | 206.0 | 35.4 | 0.12 | 0.51 | 14 | 0.21 | 0.77 | 3.30 | 29.1 |
| | **NV** | 1906 | 2013 | 42.3 | 16.6 | 0.20 | 0.55 | 14 | 0.09 | 0.56 | 1.25 | 20.7 |
| | **LW** | 1906 | 2013 | 2.08 | 1.28 | 0.42 | 0.64 | 14 | 0.46 | 0.92 | 10.80 | 50.80 |
| ***Qrob*-H** | **$D_H$-r1** | 1903 | 2013 | 281.8 | 40.4 | 0.07 | 0.65 | 26 | 0.14 | 0.80 | 3.92 | 22.3 |
| | **$D_H$-nr1** | 1903 | 2012 | 223.9 | 37.1 | 0.10 | 0.57 | 26 | 0.08 | 0.67 | 1.99 | 14.5 |
| | **NV** | 1903 | 2013 | 46.4 | 14.0 | 0.18 | 0.50 | 26 | 0.09 | 0.69 | 2.26 | 16.2 |
| | **LW** | 1903 | 2013 | 2.43 | 1.10 | 0.26 | 0.68 | 26 | 0.21 | 0.86 | 6.14 | 26.34 |

| | *Variable* | *Start* | *End* | *Mean* | *SD* | *MS* | *AR1* | *Trees* | *Rbt* | *EPS* | *SNR* | *%Var* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Qpyr*-D** | **$D_H$-r1** | 1942 | 2015 | 282.9 | 40.9 | 0.07 | 0.69 | 11 | 0.38 | 0.87 | 6.59 | 44.6 |
| | **$D_H$-nr1** | 1942 | 2015 | 224.3 | 32.1 | 0.09 | 0.61 | 11 | 0.35 | 0.85 | 5.78 | 41.7 |
| | **NV** | 1942 | 2015 | 48.3 | 19.4 | 0.18 | 0.63 | 11 | 0.26 | 0.79 | 3.81 | 33.7 |
| | **LW** | 1942 | 2015 | 1.41 | 0.61 | 0.35 | 0.43 | 11 | 0.55 | 0.93 | 13.23 | 59.35 |
| ***Qpyr*-H** | **$D_H$-r1** | 1893 | 2015 | 283.5 | 38.4 | 0.07 | 0.74 | 12 | 0.26 | 0.81 | 4.21 | 33.1 |
| | **$D_H$-nr1** | 1893 | 2015 | 221.0 | 34.5 | 0.11 | 0.55 | 12 | 0.18 | 0.72 | 2.52 | 26.5 |
| | **NV** | 1893 | 2015 | 43.2 | 21.1 | 0.18 | 0.61 | 12 | 0.22 | 0.77 | 3.43 | 30.3 |
| | **LW** | 1893 | 2015 | 0.92 | 0.74 | 0.38 | 0.71 | 12 | 0.50 | 0.92 | 11.78 | 54.41 |

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**Table 2.** Statistics of the chronologies obtained, and indicators of chronology quality for the common interval 1960-2015. SD: standard deviation; MS: mean sensitivity; AR1: first-order autocorrelation; Rbt: mean correlation between trees; EPS: expressed population signal; SNR: signal-to-noise ration; %Var: variance in the first eigenvector.

Furthermore, ***Qrob*-D** even records some association during March, and a stronger response to maximum temperature, whereas the results at ***Qrob*-H** are more driven by mean and minimum temperature. Responses of earlywood are not observed later in the season, except a weak positive correlation to minimum temperature for $D_H$-nr1 at ***Qrob*-H**, where NV is also highly related to temperature in previous spring, but with a low chronology quality. With regard to LW, only a slight response to temperature is observed at early summer, probably coupled to precipitation.

As opposed to temperature, precipitation is not related to vessel size in the same growing season, but there is a high correlation to LW, especially during

June and July, stronger for ***Qrob*-H**. Moreover, it appears to be a short-term effect, as it is clearer for 1-month SPEI than for a 3-month period; a few feeble correlations were also present for late winter and early spring. In the case of earlywood, there was a certain inverse link between the vessels formed later in the season and temperature in the previous season ($D_H$-nr1 to August at ***Qrob*-D**; NV to June at ***Qrob*-H**).

***Qpyr*** also exhibited an association between earlywood and temperature, as well as LW and precipitation. $D_H$-r1 shows remarkable differences between sites, with a strong link at ***Qpyr*-H**, but low at ***Qpyr*-D**; in fact, warm spring (April, cambial reactivation) temperatures seem to enhance vessel size at ***Qpyr*-H**, with a stronger effect of minimum temperature, whereas there is a weak negative association to temperature in late winter (February-March, quiescence) at ***Qpyr*-D**. Besides, $D_H$-nr1 and NV have a still significant positive correlation to temperature at the end of the previous season (September). The same response to April temperature, but weaker, is observed for LW, but in this case also for ***Qpyr*-D**; in addition, warmer conditions in September appear to stimulate growth as well.

Relationships to water availability are clear, with a strong correlation during summer (June through August-September, but maximized at the beginning of summer) at both sites, though more remarkable at ***Qpyr*-D**. A weaker negative association to conditions in previous June were also detected for both LW at ***Qpyr*-H**, and for $D_H$-nr1 at ***Qpyr*-D**.

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## Discussion

In our study, we compared two different nearby sites for each one nemoral (*Q. robur*) and one sub-Mediterranean (*Q. pyrenaica*) oak species, both growing at their distribution boundary towards more Mediterranean conditions. We obtained tree-ring chronologies of radial increment (namely LW), and anatomical features (earlywood vessel size and number, expressed as $D_H$-r1, $D_H$-nr1, and NV). In addition, we monitored leaf phenology and cambial dynamics along two growing seasons for a better understanding of the tree-ring results. However, we should not directly compare species to each other, since they were growing in different areas, and under different climatic conditions (*Q. robur* in coastal lowland, and *Q. pyrenaica* on inland mountains). In fact, we did not aim at elucidating the different mechanisms of both species, which have been already studied elsewhere (Gea-Izquierdo & Cañellas, 2014;Pérez-de-Lis *et al.*, 2016b;Pérez-de-Lis *et al.*, 2017;Souto-Herrero *et al.*, 2018b;a), but to understand i) how these species perform at their Mediterranean distribution boundaries, and ii) how the combined analysis of earlywood anatomy and radial increments contributes to understand their performance. For this, we will first examine the reliability of the results obtained and their ecological meaning, and then we will use this information to discuss their relevance for the study of tree behavior in the Atlantic-Mediterranean biogeographic boundary.

### Comparison of sites and relationships to climate

At a first step, we observed optimal quality of chronologies for further dendrochronological analyses in both earlywood and latewood. But for the former only the vessels in the first row ($D_H$-r1) can be considered reliable, and achieved values of EPS around or slightly higher than 0.85, the most standard reference in dendrochronology (Wigley *et al.*, 1984). In contrast, variables influenced by vessels formed later in the season ($D_H$-nr1 and NV) had a much lower common signal, so that their results should be interpreted more carefully. Nevertheless, their contribution to the understanding of climate-growth relationships is scarce, as only $D_H$-r1 and LW are enough to understand the main role of climate on growth. The values of common signal in the earlywood can be considered to be high, and are not far from those often observed for ring width. Previous studies reported much lower climatic signals for anatomical variables, especially for vessel size, and for several regions and species (Fonti & Garcia-Gonzalez, 2004;Kniesel *et al.*, 2015;García-González *et al.*, 2016). In fact, García-González *et al.* (2016) argued that the 'classical' dendrochronological assessment of the signal is probably not appropriate for vessel features, and several recent papers do not even report these values among their results (Matisons *et al.*, 2012;Castagneri *et al.*, 2017;Souto-Herrero *et al.*, 2018a).

The analysis of the tree-ring series (i.e, comparison of individual series and climate-growth relationships) pointed out that there is a clear climatic signal for both ***Qrob*** and ***Qpyr***, but also that there are some differences between sites. Moreover, the most important signals can be explained from a biological point of view; for this, the monitoring of xylogenesis during 2012 and 2013, combined with previous surveys in the region (Pérez-de-Lis *et al.*, 2016b;Pérez-de-Lis *et al.*, 2017;Guada *et al.*, 2018;Guada *et al.*, 2019) proved to be fundamental for the interpretation of the climatic responses. Consequently, results evidence the existence of a cause-effect relationship of meteorological conditions upon earlywood anatomical structure ($D_H$-r1) and radial increment (LW).

First, the comparison of individual trees by PCA indicated that these two variables recorded the main macroclimatic signal, as expressed by the common variance explained, but also site-related conditions. Thus, there was a trend to split trees between sites for both species, although this was more a trend than a clear separation, because most but not all individuals from the same site were grouped together. This pattern of variation is also observed when interpreting the results of xylogenesis and the correlations to climate.

For ***Qrob***, the size of the first row of earlywood vessels ($D_H$-r1) was strongly linked to temperature during the dormant season, and also probably during quiescence, whereby a warm temperature during this period was coupled to small vessels. An association between earlywood vessel size and dormancy has been observed for several regions (Fonti *et al.*, 2007;Pritzkow *et al.*, 2016), and could be due to different mechanisms. But other low-elevation woodlands in the study region (González-González *et al.*, 2014;Souto-Herrero *et al.*, 2018a), and other areas in northern Spain (Akhmetzyanov *et al.*) also showed this result, which has been commonly associated to carbohydrate balance at the beginning of the growing season, whereby a shortage of reserve as a result of a more active consumption due to high winter temperatures could result in smaller vessels (González-González *et al.*, 2015;Souto-Herrero *et al.*, 2018a). It is widely accepted that ring-porous trees restart cambial activity before bud break (Suzuki *et al.*, 1996;Kudo *et al.*, 2014;Copini *et al.*, 2016;Pérez-de-Lis *et al.*, 2016b), but vessels become functional just before leaf expansion (Guada *et al.*, 2018;Guada *et al.*, 2019), after lignification of the secondary vessel and appearance of perforations (Kudo *et al.*, 2015;Kitin & Funada, 2016), thus contributing to full leaf expansion. Consequently, initial vessel formation occurs before assimilation, and must entirely rely on previous year's storage (Barbaroux & Bréda, 2002;El Zein *et al.*, 2011).

In view of our results, the explained mechanism is plausible at these coastal ***Qrob*** sites, with the timings also in accordance with our observations of xylogenesis. Thus, the December-February signal must affect some process not directly related to vessel growth, because enlargement of these first elements occurs during March-April. Reserve balance is probably the most reasonable process, because sugar concentration was found to be linked to earlywood vessel diameter (Pérez-de-Lis *et al.*, 2016b), although other explanations such as auxin sensitivity were also proposed for a similar relation (Fonti *et al.*, 2007). However, there could still be some differences in the mechanisms at both sites, since the signal is delayed at ***Qrob*-D** (shifted from December to February, and more dependent on maximum temperature), despite exhibiting earlier onset of enlargement and maturation of the first vessel elements. Other signals in the vessels were considerably less robust, but confirm no direct effect of climate on vessel growth, except for a weak link of favorable spring conditions for vessels not in the first row; in addition, these vessels could also be somehow influenced by the prior growing season, probably through regulation of storage processes again.

As opposed to earlywood vessels, radial increment expressed as latewood width is enhanced by water availability during the growing season, especially during June and July. This result must be associated to assimilation, because a low water availability limits photosynthesis, resulting in narrow rings (Bréda *et al.*, 2006). Furthermore, the period observed for the response is in agreement with the wood formation dynamics observed, because the maximum growth rate occurred around summer solstice. It is also interesting that the strongest responses were directly observed to precipitation, whereas the role of temperature regulating water balance is much less evident in the rings. Although there were important differences in summer temperatures between both sites, this was not evidenced by the results. We hypothesize that summer drought occurs later in the season, when growth has already slowed down, and the lack of a late summer/early autumn cambial regrowth also supports this idea. In addition, due to the higher summer temperature, ***Qpyr*-D** could be more affected by drought in some years, but also compensate with higher photosynthetic rates under favorable conditions.

Climate-growth relationships differed for ***Qpyr***, especially as regards earlywood, but a similar overall pattern was observed, whereby earlywood vessels were controlled by temperature, and radial increment by precipitation. In this case, $D_H$-r1 exhibited important differences between sites, because high spring temperatures (April) were clearly associated to larger vessels at ***Qpyr*-H**. In contrast to ***Qrob***, there seems to be no relation during dormancy, because low winter temperatures probably reduce respiration rates. The positive response in April should be produced by the threshold temperature to initiate cambial activity and support the first stages of vessel enlargement, as has been observed at other mountain areas (González-González *et al.*, 2014;Souto-Herrero *et al.*, 2017). Furthermore, the fact that this signal is minimized by minimum temperature pinpoints the frost-avoidance strategy of *Q. pyrenaica*, achieved through its late flushing (Pérez-de-Lis *et al.*, 2016b). The dates obtained by xylogenesis, and the corresponding phenological events also support this idea. However, the results were different for ***Qypr*-D**, where this signal was absent, but there was a weak negative association to late winter temperature instead. These differences may be due to slightly warmer conditions for being on a sunny slope at a lower elevation, or to the lack of interspecies competition as opposed to ***Qpyr*-H**. In addition, previous autumn could also affect the formation of vessels at ***Qpyr*-H**.

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In contrast to vessel size, which appeared to be independent of water availability for ***Qpyr***, confirming previous experimental results (Fernández-de-Uña *et al.*, 2017), LW was closely linked to summer precipitation, but stronger at the driest site ***Qpyr*-D**. As happened for ***Qrob***, assimilation is probably the most relevant processed involved, and it is also around the summer solstice that the response is maximized, concomitantly to the fastest growth rate. These results are sustained by xylogenesis, whose identification of growth cessation during 2013 suggests that summer drought can be enough to inhibit radial increment. In fact, summer water availability was reported as the main factor controlling radial growth of *Q. pyrenaica* in the Iberian Peninsula (Gea-Izquierdo & Cañellas, 2014;Fernández-de-Uña *et al.*, 2017). But another relevant feature revealed by both xylogenesis and dendrochronology is the existence of a certain pulse of growth reactivation at the end of summer, as observed in 2012, and evidenced by the positive correlation between LW and temperature in September. This somehow bimodal pattern is typical plastic behavior in the Mediterranean Region (Camarero *et al.*, 2010), and has previously been reported for northwestern Iberian oaks (Pérez-de-Lis *et al.*, 2017).

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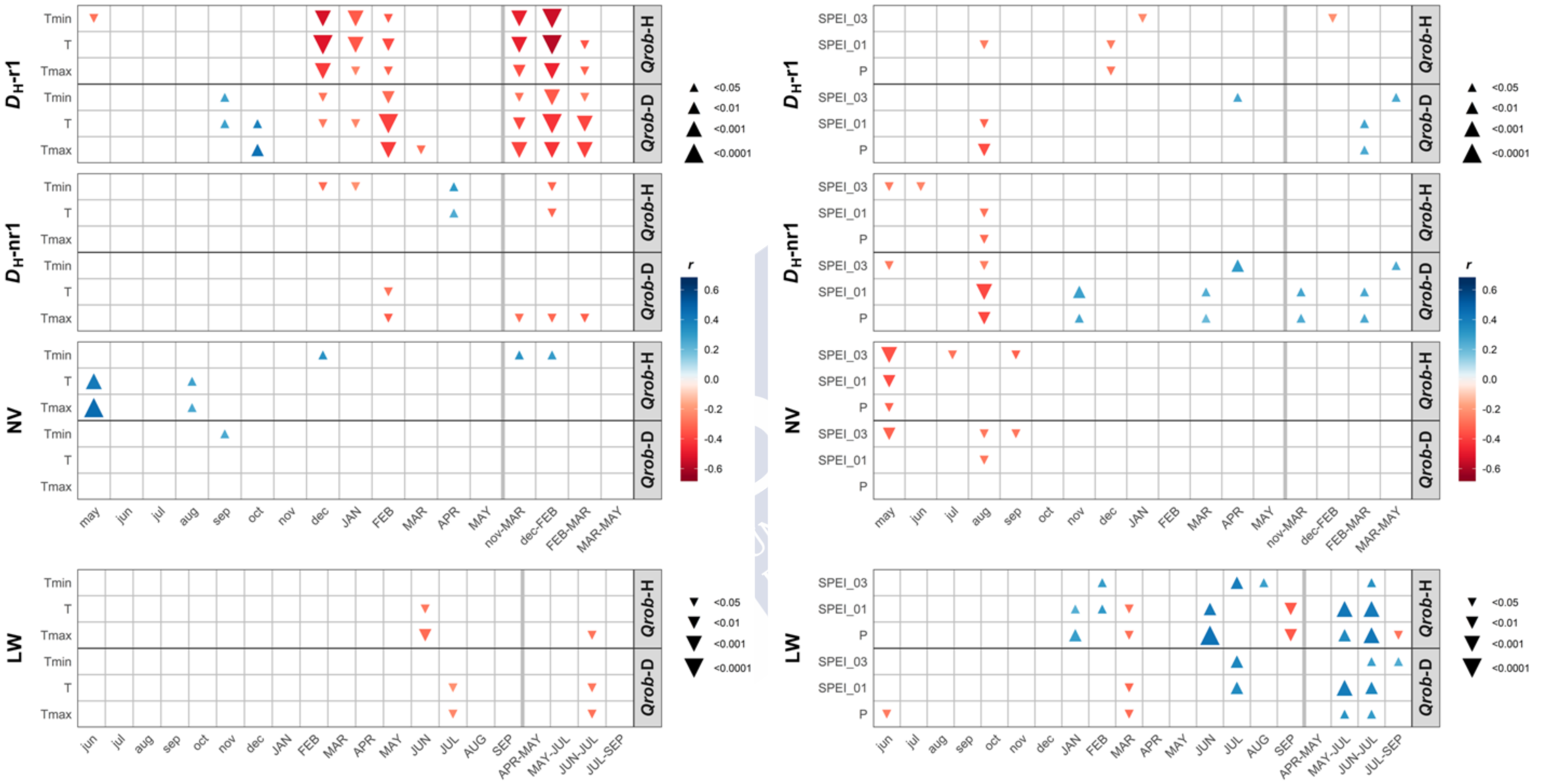

**Fig. 5**. Climate-growth relationships for *Quercus robur* (***Qrob***) at dry (***Qrob*-D**) and humid sites (***Qrob*-H**), for hydraulically-weighted diameter (***D*$_H$**) of the first row of earlywood vessels (***D*$_H$-r1**), for vessels outside this row (***D*$_H$-nr1**), number of vessels (NV), and latewood width (LW). Climatic variables are: Maximum (Tmax), minimum (Tmin), mean (T) temperature, standardized precipitation-evapotranspiration index (SPEI) at one (SPEI_1) and three months (SPEI_3), and total precipitation (P).

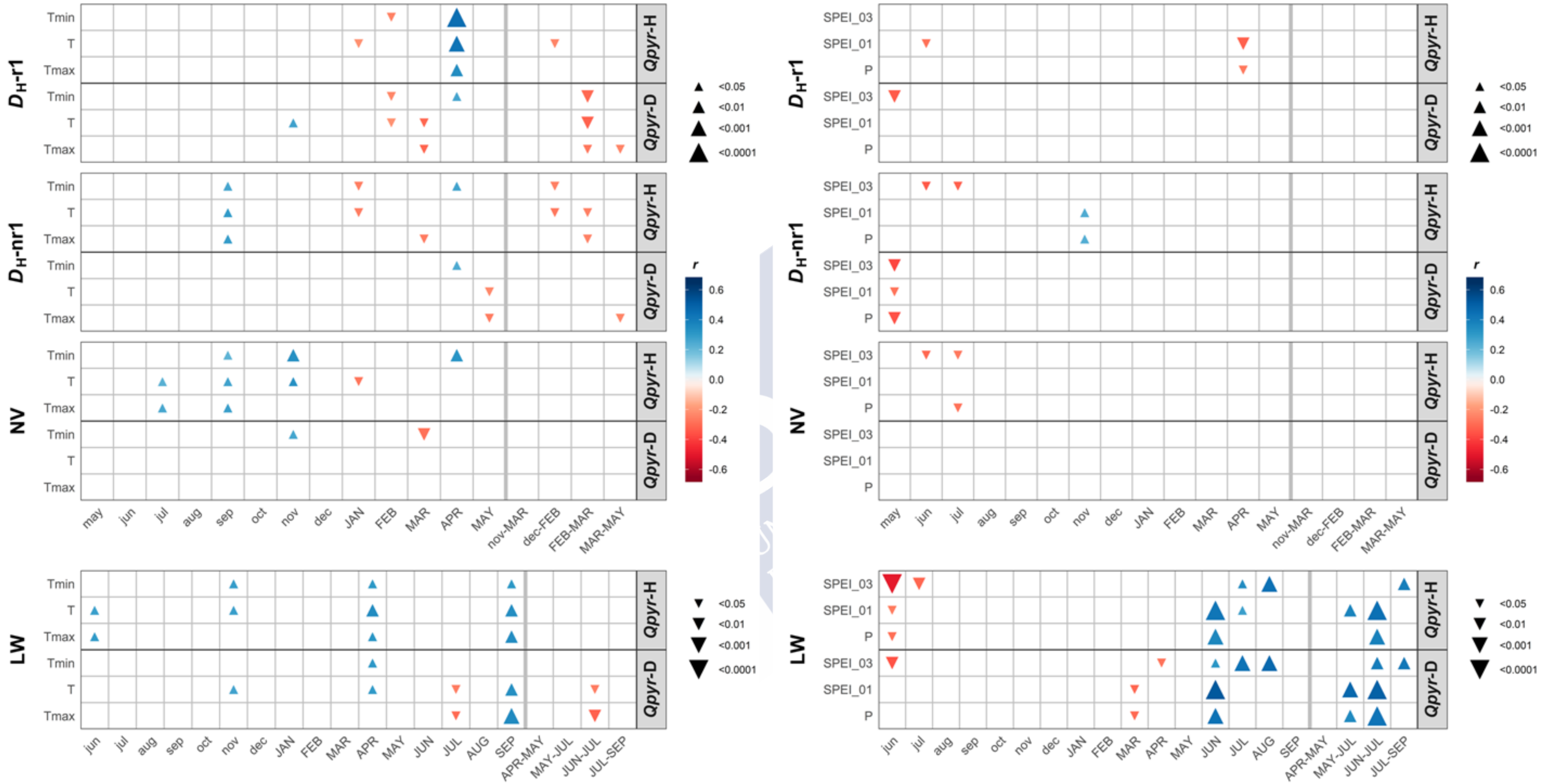

**Fig. 6**. Climate-growth relationships for *Quercus pyrenaica* (*Qpyr*) at dry (*Qpyr*-D) and humid sites (*Qpyr*-H), for hydraulically-weighted diameter ($D_H$) of the first row of earlywood vessels ($D_H$-r1), for vessels outside this row ($D_H$-nr1), number of vessels (NV), and latewood width (LW). Climatic variables are: Maximum (Tmax), minimum (Tmin), mean (T) temperature, standardized precipitation-evapotranspiration index (SPEI) at one (SPEI_1) and three months (SPEI_3), and total precipitation (P).

## Relevance for biogeographic vegetation studies

Our study involved the analysis of the influence of climate on wood formation of two species at their distribution limit between the Atlantic and the Mediterranean regions. Despite each species was typical of either one of the regions, we found common patterns that shed light on the study of the behavior of vegetation in boundary areas.

The analysis of tree rings and climate has been classically based on the 'Principle of Limiting Factors' (Fritts, 1976b), which relies on the idea that tree-ring width mainly depends on a single prevailing factor. This is often the case in many regions, such as the boreal or alpine timberlines, where summer temperature constraints growth, or semi-desertic areas, with an important limitation imposed by precipitation. But the situation is different in mesic areas of mid-latitudes, where a single prevailing factor that controls growth in most years usually lacks. Tree-ring width, the commonly-used dendrochronological variable often fails at identifying the most important climatic constraints at these areas. However, the use of earlywood vessels proved to be successful to establish climate-growth relationships under the absence of a single limiting factor (Fonti & Garcia-Gonzalez, 2008), which can even facilitate tracking the origin of the wood (Akhmetzyanov *et al.*, 2019).

In this paper, we showed that the complementary use of $D_H$-r1 and LW for the understanding the climatic responses of oak at the Atlantic-Mediterranean boundary, reinforcing what previous studies proposed for the same region (Souto-Herrero *et al.*, 2017;2018b;a). Furthermore, we did not only study the response of tree-ring chronologies to elucidate climate-growth relationships, but also employed wood formation dynamics to better calibrate the dendrochronological results. By monitoring wood formation, we ensure that our observations support the existence of cause-effect relationships, which in turn is one of the most important features that an ecological variable requires to be correctly used as an environmental proxy (Bradley, 1986).

The boundary between the Atlantic and Mediterranean regions is a complex area. Although the Mediterranean region is characterized by the existence of a prevailing limiting factor, because the long drought period can often span more than three months, transitions can be gradual, and include some characteristics of the Atlantic Region. This is probably the reason why the combination of LW (controlled by the single prevailing factor water availability) and $D_H$-r1 (an apparently powerful proxy of mesic regions) has been successful at our study boundary areas, as well as at other sites along this transition zone (Souto-Herrero *et al.*, 2018b). This idea is reinforced by the fact that LW demonstrated to be closely linked to water availability, whereas $D_H$-r1 was dependent on

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temperature, regardless of the species (*Q. robur* vs. *Q. pyrenaica*) or the geographical location (coast vs. mountain). Furthermore, the existence of very mild temperatures or high precipitation records did not prevent these variables from recording the environmental signal. In fact, earlywood features proved be very useful to identify the effect of climate of climate along geographical boundaries, at both local (Souto-Herrero *et al.*, 2018b) and continental (Martínez-Sancho *et al.*, 2017) scales.

We think that our results are relevant for the study of Iberian oaks under a scenario of climate change. In the Iberian Peninsula, the boundary between the Atlantic and the Mediterranean region spans from the Pyrenees along the southern slope of the Cantabrian Range until the northern Atlantic coast (Rivas-Martínez, 1987), where changes from coastal areas towards the mountains often occur in a short distance. These areas are therefore very sensitive to environmental changes, which can modify species balance. In fact, some models predict future changes in the distribution of rear-edge oak forests in Iberia (Benito Garzón *et al.*, 2008), and an increased growth of some species, which could lead to mortality of others (Benito-Garzón *et al.*, 2013). Marcescent sub-Mediterranean oaks as *Q. pyrenaica* have a prevailing role in all predictions (Sánchez-de-Dios *et al.*, 2009).

Xylem adjustment is one of the mechanisms that Mediterranean oaks use to cope with a dryer climate (Castagneri *et al.*, 2017) and climatic extremes (Rita *et al.*, 2016). For these reasons, the existence of a ‘multiproxy’ approach to understand the role of climate on oak growth, which combines growth performance (expressed as ring width or latewood width), and the main hydraulic properties of the ring (described by earlywood anatomical features) constitutes a fundamental tool to assess the present and future situation of Iberian oak forests.

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## Conclusion

We carried out two different studies on oaks at the edge of their distribution boundary between the Atlantic and the Mediterranean regions, using chronologies of earlywood vessel size and latewood width. Despite being different species, with different ecological requirements, and also at diverging locations (coast vs. inland), there was a common pattern in both cases, as the size of the first earlywood vessels was controlled by temperature, whereas precipitation was coupled to radial increment. We found reliable cause-effect explanations for the identified climate-growth relationships, and the analysis of cambial dynamics was in agreement with the timings of the responses observed.

In view of these results, we propose that a combined ‘multiproxy’ approach using earlywood anatomy and radial increment is a powerful indicator of the growth performance of trees at their distribution boundaries.

# Supporting Information

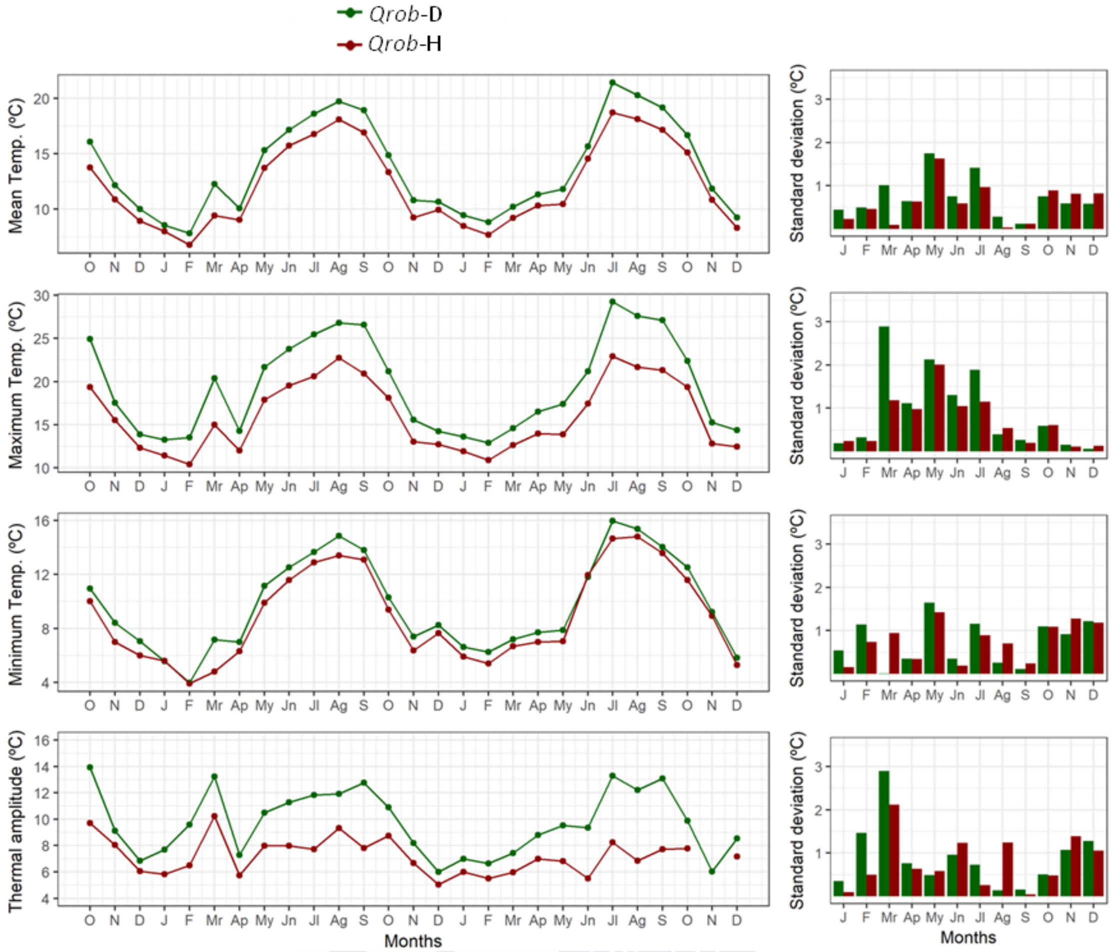

**Fig S1** Temporal variation of monthly mean, maximun, minimum temperature, and thermal amplitude at the study sites, *Qrob*-D and *Qrob*-H, from October 2011 to December 2013. The barplot represents the standard deviation per month between 2012 and 2013.

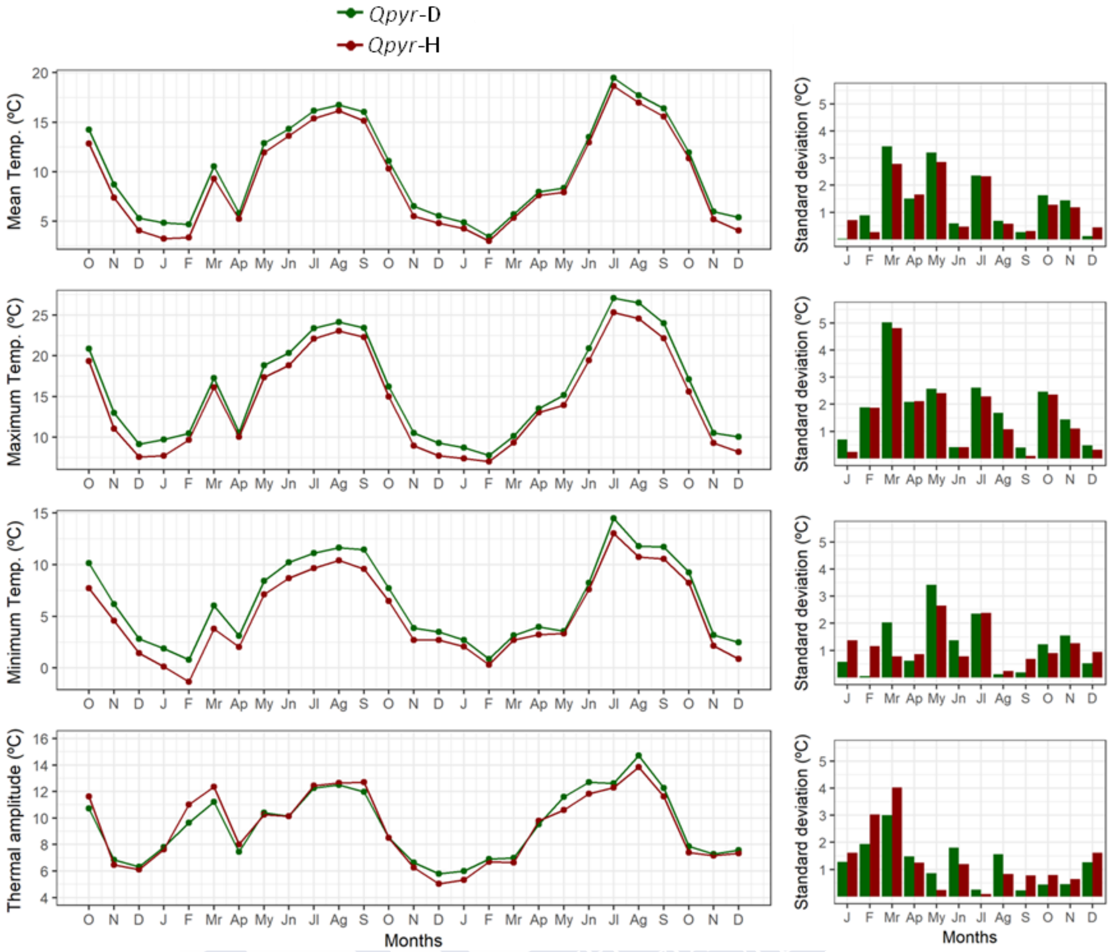

**Fig S2** Temporal variation of monthly mean, maximun, minimum temperature, and thermal amplitude at the study sites *Qpyr*-D and *Qpyr*-H from October 2011 to December 2013. The barplot represents the standard deviation per month between 2012 and 2013.

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# General Discussion

# General discussion

## Link between primary and secondary growth

The sampling design used in Chapter II and III was based on the random selection of individuals at each sampling date, and on the simultaneous recording of phenological stages of primary and secondary growth. This differs from other studies of xylogenesis, in which the same individual is usually followed throughout the year (Rossi, 2003;Oberhuber *et al.*, 2011). This novelty has allowed us to increase the number of trees per site, so that we were able to perform multiple pairwise comparison of phases, and thus to establish relationships between primary and secondary growth in a more robust way.

In ring-porous species, the comparison between primary and secondary growth aims at understanding two processes, namely i) to identify the gap between leaf and wood development in order to determine which parts of the wood (first row of vessels, earlywood, and latewood) are formed due to photosynthesis of the current year's leaves, or from the reserves accumulated during the previous year; and ii) to elucidate the synchronization between leaf formation and wood development, which will help to predict the formation of growth structures of the wood from phenological observations at a naked eye.

One of the first studies addressing these issues for quantitative anatomy time series in ring-porous species was carried out on *Quercus robur* and *Fraxinus excelsior* (Sass-Klaassen *et al.*, 2011), and found significant differences in the synchronicity between both species, which led to the conclusion that leaf phenology cannot be taken as a global indicator of the status of vessel formation. Therefore, more research was performed to improve the consistency of the species-specific relationship between vessel and leaf phenology. In a review of studies on several species and sites, Kitin & Funada (2016) observed that leaf unfolding/full leaf expansion was always preceded by cell enlargement of the first earlywood vessels as a common pattern.

Our study on this synchronicity for *Q. robur* (Chapter II) and *Q. pyrenaica* (Chapter III) in the northwestern Iberian Peninsula, which is the distribution boundary of both species, confirms that the beginning of the xylogenesis also preceded leaf unfolding in 330 out of a total of 410 trees for both species and two years (Chapter II; Fig. 2a and Chapter II; Fig. 4a). Therefore, these results suggest that current year's photosynthates were still not available to develop the first rows of vessel in the wood.

The main difference between both species we found in our study was the synchronicity between the maturation of the first row of vessels and the phenological status of the leaf. Our results show that, in *Q. robur* (Chapter II;

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Fig. 2b), the maturation of the first vessel row occurred between leaf unfolding and the detection of small leaves on the crown of 85 trees out of a total of 103; while in *Q. pyrenaica* (Chapter II; Fig. 4b), the maturation of vessels took place between budburst and leaf unfolding in 46 of 56 trees for the two years of study.

More recent works report the same pattern of vessels development, i.e, enlargement of the first vessels starts before the budburst either in the northern (Poland) (Puchałka *et al.*, 2017) or the southern distribution (Spain) of *Q. robur*, and in *Q. pyrenaica* (Pérez-de-Lis *et al.*, 2016b), although the onset of maturation differs between species and among sites in agreement with our results.

For a long time, the first erlywood vessels of ring-porous trees have been assumed to be formed before budburst in early spring, considering that they were involved in supplying water to the growing leaves and shoots. However, it was in recent years that the development of earlywood elements from the beginning of growth to maturity has been studied more in detail. It has focused on the functionality of the vessels for water transport, either from the observation of perforations in thin longitudinal sections of the vessels (Kudo *et al.*, 2015), or through direct determination of sap flow and the leaf area index (Lavrič *et al.*, 2017), showing that requirements of early leaves must be fulfilled by latewood vessels formed in previous years (Kitin & Funada, 2016) while new vessels become functional for water transport during leaf development, as evidenced by the increasing sap flow recorded upon budburst.

If the formation of new tissues in the branches and crown requires hydration to maintain the necessary turgor for extension, the consequent increase in water content can be measured by determining the dry matter content (DMC) of branches, buds, new leaves and shoots. This increase may be associated either with the development of new vessels or the functionality of those of previous years. The results of Chapter I on the DMC content of branch organs show that both processes contribute to water supply, which is in agreement with the microscopic observations on vessel anatomy made by Kudo *et al.* (2015) and Kitin & Funada (2016), confirming the usefulness of the method proposed by Palacio *et al.* (2008).

The increase in water content (decrease of DMC) at the initial phases of crown development, with less water demand before the maturation of the current year vessels, suggests the involvement of functional vessels of previous years (latewood vessels). On the other hand, the maximum values of water content (minimum value in DMC) indicate that at least the first row of current year´s vessels must be functional to continue the extension of new developing shoots, and therefore to complete the new crown. In addition, the DMC methodology allows an objective classification of bud and leaf phenophases, which avoids the subjectivity of the observer when identifying phenological phases (Chapter I).

## The effect of temperature on leaf and cambial development.

Variations in the temperature before or/and at the moment of specific phenological events can differentially affect the synchronicity between primary and secondary growth (Chapter II). All tree species adjust the timing and length of their growth to climate, with variations that depend on specific regional drivers, local adaptations, or individual plasticity (Rossi *et al.*, 2014).

Most of the xylogenesis research carried out so far focused on conifer forests of cold climates and high elevations (Rossi *et al.*, 2008;Rossi *et al.*, 2016), where the average annual temperature at each site determines the onset, cessation, and duration of the vegetative period of trees. In temperate forests, these relationships are not so clear, because the difference in temperature among sites does not remain constant throughout the year, as it usually does in cold climates.

Chapters II and III focused on assessing the effect of temperature on natural woodlands of *Q. robur* and *Q. pyrenaica*, based on a spatio-temporal study at 14 sites, which represent the three main climatic influences (Atlantic, Cantabrian, and sub-Mediterranean) in the northwest of the Iberian Peninsula. Results for *Q. robur* (Chapter II; Fig. 6a) suggest that resumption of primary growth requires a minimum threshold temperature, because budburst resulted to be more linked to minimum temperatures. By contrast, the beginning of cell enlargement probably relies on maximum temperature, because warming increases growth rate. Thus, Kudo *et al.* (2014) and Begum *et al.* (2017) showed that localized heating for six or four weeks induced respectively earlier cambial reactivation in seedlings of *Q. serrata* and poplar (*P. sieboldii* × *P. grandidentata*). In both studies, an elevated temperature (20 ± 5 ºC) anticipated xylem development, suggesting that the variation of temperature modifies the onset of cell enlargement.

The observed response of primary growth to temperature is consistent with the results reported by Wilkinson *et al.* (2017) for the south of England, who found a negative correlation between the date of budburst and the mean daily air temperature for *Q. robur* and *Q. petraea*, whereby spring warming had a considerably larger effect on budburst than winter chilling. Crown development requires a minimum threshold of temperature to break dormancy (Caffarra & Donnelly, 2011;Prislan *et al.*, 2013;Wilkinson *et al.*, 2017), although interactions between temperature and photoperiod need also to be considered (Basler & Korner, 2014).

By contrast, this temperature effects at growth resumption were not detected for *Q. pyrenaica*, neither for primary nor for secondary growth (Chapter III),

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although it cannot be ruled out that the sampling interval (12-15 days) may have been too wide to detect these effects due to the higher growth rate of *Q. pyrenaica* compared to *Q. robur* (Pérez-de-Lis *et al.*, 2017). The effect of photoperiod is likely to be more important than temperature for this sub-Mediterranean species, homogenizing the timings of the beginning of wood and leaf formation between sites, and consequently the synchronicity between primary and secondary growth (Chapter I and III). However, this synchronicity is less homogeneous for *Q. robur* than for *Q. pyrenaica* in order to allow finding variations between years and sites (Chapter II). In fact, minimum temperature from mid-March to mid-April was estimated to increase the onset of budburst of *Q. robur* by 7.2 DOYs ºC$^{-1}$, while March maximum temperature anticipated cell enlargement by 2.8 DOYs ºC$^{-1}$, so that the variations between both processes were not coupled.

Although the response of *Q. pyrenaica* to the climate (Chapter III) differs from that of *Q. robur*, no time windows related to phenological events were detected by running means of temperature for different period lengths (Chapter III; Fig 5). Also, the mean annual temperature was able to explain the differences among sites with respect to the cessation of cell expansion, as well as the maximum temperature and thermal amplitude, suggesting that a warmer autumn causes a lengthening of the *Q. pyrenaica* growing season.

Sites of *Q. pyrenaica* bear a sub-Mediterranean climatic influence, and the elevation spans between 400 and 1,000 m a.s.l.; this contrasts to *Q. robur*, which is growing under Atlantic-Cantabrian climatic influence, at 150 to 600 m a.s.l. Such climatic differences drive diverging growth strategies. Annual temperature range for *Q. robur* was 9-13 ºC among the nine sites, and daily mean temperature values below 0 ºC were only achieved during one day in February at the most continental and highest site; whereas *Q. pyrenaica* sites had an annual temperature range of 7.5-12.7 ºC, and the coldest period, below 0 ºC recorded in the sensors was from November to February, with late frosts in March and late April.

It seems that the strategy of *Q. pyrenaica* to initiate growth by adjusting to photoperiod rather than to temperature allows it to adapt to climates with late frosts by reducing its growth period. The lower thermal requirements of *Q. robur* probably make it more competitive than *Q. pyrenaica*, although this implies a higher risk of frost damage to the leaves (Vitasse *et al.*, 2014). However, cold and early springs can delay the onset of *Q. robur* growth, as occurred in 2013 with respect to 2012 (Chapter II).

Understanding the environmental control of growth resumption in the native forests can help inferring the effect of climate change. However, this seems to be

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more difficult for *Q. robur*, not only due to the timing, but also because the relationship between primary and secondary growth can be modified by external factors in different ways. For *Q. pyrenaica*, photoperiod appears to be the main climatic variable responsible for the beginning of the tree growth, while cessation of xylem growth seems to be mainly driven by thermal amplitude.

## Relevance for dendrochronological studies

The aim of this Thesis has been to understand the process of wood formation for both *Q. robur* and *Q. pyrenaica* in the northwestern Iberian Peninsula, and only Chapter IV does introduce its link to retrospective analysis using dendrochronological series. However, the available information on tree-ring series, especially involving the study of earlywood vessels, has considerably increased in recent years (González-González *et al.*, 2013;González-González *et al.*, 2015;Souto-Herrero *et al.*, 2017;Pérez-de-Lis *et al.*, 2018;Souto-Herrero *et al.*, 2018b;a), and a better understanding of the potential underlying mechanisms is necessary.

Previous works show that vessel size of the first earlywood vessel elements of ring-porous trees are strongly linked to climate, and are especially relevant to identify the conditions that constrain growth as a consequence of the dormant period (González-González *et al.*, 2015;Souto-Herrero *et al.*, 2018a). This is due to the fact that warm winter conditions, especially coupled to water saturation, appear to reduce vessel size, probably as a consequence of the regulation of the amount of available carbohydrates. It must be born in mind that oaks, as ring-porous trees, build their first vessel elements from the reserves stored in the previous growing season (Barbaroux & Bréda, 2002;Michelot *et al.*, 2012a), and before current year’s leaves become photosynthetically active. Furthermore, this signal is mostly linked to low elevation areas, where winter conditions can be considerably warm. On the other hand, vessels can also be coupled to temperatures at the moment of reactivation, which is apparently a direct effect by modulating phenological thresholds. This is the case of colder areas, where winter respiration is probably not so active, but daily temperatures are critical for bud break and cambial resumption, as happens in mountain environments (Souto-Herrero *et al.*, 2017;2018b).

In order to shed some light on the meaning of these relationships, Chapter IV combined the dendrochronological approach to the wood monitoring studies which constitute the core of this Thesis, with tree-ring and wood anatomical series. The results achieved were in accordance to those previously found, but there were two facts that confirmed the optimal combination of both techniques, because i) each trait was associated to a specific process of wood formation

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(earlywood vessel size to expansion and maturation of the vascular elements, latewood width to the rates of cell production), and ii) wood formation dynamics were in agreement with the climatic responses of the series, and allowed pinpointing the exact stage of the season when certain processes were involved.

Thus, vessel size was mainly controlled by temperature, and it was the first vessel row that recorded this signal. Results from Chapter I and Chapter II let us confirm that this vessel size, i.e, the onset of vessel maturation, was achieved before or during budbreak in order to provide enough water for full leaf expansion (Kudo *et al.*, 2015;Kitin & Funada, 2016); and also that temperature is involved in initial leaf and vessel development, as well as in their interactions (Chapter II). On the other hand, Chapter III evidenced that the responses observed for *Q. pyrenaica* occur at the moment of budbreak and vessel development. Interestingly, water availability was only linked to radial increment, i.e., to the number of cells produced. In this sense, Chapter II and III showed that the maximum rate of cambial activity occurred around summer solstice, consequently concomitant with the responses to June-July precipitation evidenced by dendrochronology. Furthermore, the early-autumn regrowth occasionally observed is also in agreement with a weak response to conditions during current September identified by tree-ring analysis.

Finally, this integration of dendrochronology (by separately analyzing earlywood vessel elements and latewood growth) with xylogenesis provided the link to associate the underlying mechanism of the relationships between tree responses and climate. This explains now why anatomical features were successful at identifying such responses in mesic areas, lacking a prevailing factor constraining growth (Fonti & Garcia-Gonzalez, 2008;Souto-Herrero *et al.*, 2018a). Such finding is of great biogeographical relevance, because anatomical series constitute a powerful tool to study oak performance along the Atlantic-Mediterranean boundary (Souto-Herrero *et al.*, 2018b), also stated in Chapter IV, or even for climate reconstruction in these areas (Souto-Herrero *et al.*, 2017). Therefore, the link provided by xylogenesis to clearly evidence the existence of a cause-effect relationships confirms one of the fundamental properties of ecological traits to be considered as proxies (Bradley, 1986).

## Methodological synergies

In this Thesis, the study of the tree growth in forests under natural conditions has been undertaken by means of complementary methodologies in order to model tree growth from a geographical down to a cellular scale. While dendrochronology in Chapter IV allows the retrospective study of the radial growth and the anatomical characteristics of the wood, xylogenesis and leaf phenology in Chapter II and III address short-term responses of the main phases of wood formation and crown development within the growing season. In addition, relating the leaf phenological events with the water or dry matter content (DMC) of crown organs in Chapter I adds valuable information on the water requirements for the phases of foliar development. Although these observations provide partial information on the growth process of the tree, their integration provides a more complete knowledge due to their complementarity.

Dendrochronology is a useful tool for determining the adaptive responses of trees to environmental variations (Fritts, 1976b;Fritts & Swetnam, 1989b), which is usually achieved by statistically relating climatic and growth variables. The development of the so-called 'tree-ring anatomy' or 'quantitative wood anatomy', i.e., the application of dendrochronological methods to annual values of anatomical features, broadened the usefulness of tree rings as promising proxies for global change studies (Fonti *et al.*, 2010) or climate reconstructions (Fonti *et al.*, 2009;Souto-Herrero *et al.*, 2017). However, a detailed description of wood formation through xylogenesis is required to understand retrospective studies by linking them to xylem formation patterns and processes. The combination of both methodologies is not common, but has already been successfully applied in conifers in order to assess the effects of drought on forest decay under continental Mediterranean climate (Pacheco *et al.*, 2015;Camarero *et al.*, 2016;Guada *et al.*, 2016). In ring-porous species, Pérez-de-Lis *et al.* (2018) combined both techniques to analyze the timing of earlywood vessels occlusion by tyloses in *Q. robur* and *Q. pyrenaica*, and so did González-González *et al.* (2015) to compare climate-growth relations of both species growing at the same site. But the results of Chapter IV, which show with precision how xylogenesis can contribute to understand the results of tree-ring analyses, highlight the need of integrating both approaches.

The combination of xylogenesis and quantitative anatomy allows a deeper insight on to the potential underlying cause-effect relationships in the climate correlations. Thus, the size of earlywood vessels of *Q. robur* was found to be conditioned by the onset of their formation, the larger vessels being the result of an earlier resumption of xylem formation (Pérez-de-Lis *et al.*, 2016b), which is a fundamental contribution to understand the variations in vessel size in dendrochronological series. Similarly, xylogenesis has shown that ring width is modulated by the predisposing effects of the size of dormant cambium, as it affects xylem production, and also by the length of the growing season (Pérez-de-Lis *et al.*, 2017), whose duration can be modified by the advancement or delays in the onset of wood formation due to several external environmental factors, such as temperature, floods, competition between individuals, outbreaks,

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or diseases (Copini *et al.*, 2016) In short, stress situations have not been assessed in detail for ring-porous species. Chapters II and III show spatial-temporal studies illustrating the effect of temperature on the growing season duration xylogenesis. They indicate that thermal amplitude in late winter (i.e., the difference between maximum and minimum daily temperatures) was the variable with the most significant effect on the beginning of growth for *Q. robur*, and in autumn for the cessation of growth for *Q. pyrenaica*.

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# Conclusions

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# Conclusions

The analyses carried out of this Ph. D. Thesis and their interpretations resulted in the following conclusions:

1) The measurement of dry matter content (DMC) in crown organs is a powerful tool to quantify primary growth of trees, from bud swelling to full leaf extension, while avoiding the subjectivity of visual inspection. These are the most difficult phenophases to be determined by visual inspection with binoculars. Consequently, an objective classification of bud and leaf phenophases according to their DMC is proposed, which avoids observer's subjectivity.

2) There is a synchronicity between primary and secondary growth, since the development of the first earlywood vessels starts before or during budburst. Although water demands for bud swelling and leaf unfolding can be provided by the previous latewood vessels, the functionality of the newly vessels is necessary for the full leaf extension. However, this synchronicity can be modulated by temperature, at least for *Q. robur*.

3) The dynamics of wood formation are similar for both species, and most of the differences correspond to the beginning of the growing season, along with their dependence on environmental conditions. The resumption of growth is earlier for *Q. robu*r, but the maximum rate of expansion is detected around the summer solstice for both species.

4) In the case of *Q. robur*, variations in temperature at late winter or at the beginning of spring (quiescence) explained the differences in the timing of leaf development and earlywood observed between years and sites. On the contrary, such relationship was not found for *Q. pyrenaica*, suggesting a more relevant role of the photoperiod at the beginning of the season, while the differences in the cessation of xylem growth seem to be due to thermal amplitude determined by the maximum temperature.

5) The retrospective analysis of tree growth by dendrochronological studies, integrating quantitative anatomy (earlywood vessels) and radial increment (width of latewood) is a very useful tool to understand climate-tree growth relationships of *Q. robur* and *Q. pyrenaica* at their distribution boundary, which are respectively linked to temperature and precipitation. Nevertheless, the contribution of xylogenesis is fundamental for a correct interpretation, as it allows identifying the potential cause-effect relationships of the observed responses.

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# Resumen

# Resumen

Los bosques proporcionan hábitats adecuados para la obtención de materias primas para la alimentación o su empleo como medicinas y materiales de construcción. También aportan múltiples beneficios al ecosistema, tales como proteger al suelo de la erosión, regular el ciclo del agua y purificar el aire. Además, son los principales reservorios de biodiversidad, tanto de plantas como de animales. En las últimas décadas, el reconocimiento de la importancia de los bosques ha aumentado debido al papel clave que desempeñan en la mitigación del cambio climático mediante la absorción y el almacenamiento de carbono. La interrelación entre los bosques y el clima se traduce en cambios en los procesos físicos, químicos y biológicos que afectan a la dinámica vital del planeta, el ciclo hidrológico y la composición atmosférica.

El incremento de la temperatura media anual en el último siglo está modificando el funcionamiento de los ecosistemas y su composición, lo que puede atribuirse en gran medida a que se superan los umbrales fisiológicos de las especies. Por ejemplo, el crecimiento de los bosques en las regiones boreales o alpinas está limitado por la temperatura, mientras que en las zonas semidesérticas el factor limitante es el régimen hídrico. Ambas son variables climáticas predominantes que con frecuencia limitan el crecimiento del bosque, pero en muchos climas templados europeos, con un moderado o bien equilibrado suministro de humedad (mésico), como en el noroeste de la Península Ibérica, el control climático del crecimiento es más complejo y una sola variable predominante no es generalmente suficiente para comprender las relaciones entre crecimiento y clima.

Desde el punto de vista biogeográfico, el noroeste ibérico tiene una posición clave para estudiar los efectos de las variaciones climáticas sobre los ecosistemas forestales templados, ya que se trata de la frontera entre las regiones atlántica y mediterránea, de modo que muchas especies alcanzan sus límites de distribución en esta área. Sin embargo, la presencia y abundancia de estas especies puede modificarse con el tiempo, particularmente como resultado de los cambios ambientales que se vienen produciendo en las últimas décadas. Este es el caso de las especies dominantes en los ecosistemas forestales de esta región, como *Quercus robur* L. y *Quercus pyrenaica* Willd., cuya respuesta adaptativa a los cambios ambientales es marcadamente diferente por su comportamiento atlántico o mediterráneo/continental, respectivamente. En consecuencia, un mejor conocimiento de las características funcionales de las especies dominantes es fundamental para predecir el impacto de las variaciones climáticas.

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Una de las respuestas más significativas del árbol a las situaciones de estrés es la del crecimiento radial, es decir, la formación de la madera. Aparte del incremento cuantitativo, las características anatómicas de la madera, como el área de los vasos conductores, proporcionan herramientas de gran interés para comprender la respuesta adaptativa de los árboles a las condiciones ambientales. Esta respuesta puede ser evaluada retrospectivamente con ayuda de técnicas dendrocronológicas, pero una de sus limitaciones es que las asociaciones entre clima y crecimiento suelen analizarse a través de la correlación estadística entre los registros meteorológicos y las variables de crecimiento, sin un conocimiento sistemático de los patrones y procesos de formación del xilema, que son fundamentales para establecer relaciones causa-efecto. Las etapas de esta formación no se pueden identificar a simple vista, a diferencia de las hojas, cuyo desarrollo fenológico es fácilmente observable y es un indicador de los procesos fisiológicos vinculados a la síntesis y movilización de carbohidratos que se están produciendo en ese momento.

Por lo tanto, para la correcta interpretación de los resultados dendrocronológicos se requiere un mejor conocimiento de los procesos de formación de la madera (xilogénesis) y de las hojas (fenología foliar) durante el período de crecimiento, así como identificar en qué medida ambos procesos están sincronizados. Para ello, en la presente Tesis Doctoral se pretende alcanzar los siguientes objetivos:

(1) Verificar un método cuantitativo para describir las fenofases del desarrollo de las hojas a partir del contenido en agua de los órganos de la corona, así como establecer su utilidad para el estudio de la dinámica de formación de madera.

(2) Describir las principales fases de la formación de la madera en un ciclo intra-anual, estableciendo sus relaciones con el estado fenológico del árbol, así como las condiciones ambientales.

(3) Identificar los principales factores climáticos que influyen en la formación del anillo anual durante toda la vida del árbol, especialmente sobre la madera temprana dada su importancia funcional.

(4) Establecer las principales diferencias de comportamiento entre las dos especies de roble dominantes (*Q. robur* y *Q. pyrenaica*) y las áreas geográficas (Cantábrico, Atlántico, continental interior) para identificar las posibles respuestas a los cambios ambientales.

Para conseguir estos objetivos se han realizado tres experimentos en bosques de robles del noroeste de la Península Ibérica, en los que la metodología de xilogénesis constituye el núcleo principal del trabajo.

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El primer experimento (**Experimento 1**) tuvo como objetivo estudiar la sincronía anual entre el crecimiento primario y secundario, así como la forma en que esta relación puede ser modificada por el clima. Consistió en el monitoreo, durante dos años consecutivos, de la fenología foliar y cambial en nueve masas forestales de *Q. robur* y cinco de *Q. pyrenaica*, ubicadas en tres gradientes de elevación bajo tres influencias climáticas: Atlántica, Cantábrica e interior continental.

El segundo experimento (**Experimento 2**) consistió en un estudio dendrocronológico de *Q. robur* y *Q. pyrenaica* en dos de las masas forestales de cada especie, seleccionadas a partir del primer experimento, y que constituyen localidades contrastadas en el límite de distribución de cada especie. Para ello, se extrajeron testigos de madera con el fin de establecer la cronología de los árboles a partir de los anillos, midiéndose también su anchura y el tamaño de los vasos conductores en la madera temprana. Estas cronologías fueron comparadas entre sí y con registros meteorológicos para establecer relaciones con el clima. Los resultados de los experimentos de xilogénesis y fenología foliar de estas localidades se utilizaron para la interpretación de los resultados.

En un tercer experimento (**Experimento 3**), se cuantificó el contenido de materia seca (DMC), es decir, la inversa del contenido en agua de varios órganos de la copa: ramitas de uno, dos y tres años, yemas, brotes del año y hojas en diferentes fases fenológicas de *Q. pyrenaica* para el período vegetativo del año 2013. El objetivo de este estudio fue comprobar la utilidad del DMC para la identificación cuantitativa de los estados fenológicos de las hojas, evitando la subjetividad del observador. Esta idea se basó en la hipótesis de que los nuevos brotes requieren tejidos hidratados para mantener la turgencia necesaria para su extensión, lo que supone una reducción del DMC. El muestreo de los órganos de la copa se llevó a cabo recolectando una rama de tres años de edad en 11 árboles, a intervalos de 10 días, a la vez que se registraba de manera visual la fenología foliar y se medía la longitud de los brotes nuevos en cada fecha de muestreo. También se procesaron dos microcores opuestos del tronco para el estudio de xilogénesis, con el fin de establecer la sincronización entre crecimiento primario y secundario.

El **Experimento 1** se realizó en el curso de tres ríos: los ríos Eume y Sor, cuyas cuencas están dominadas por bosques de *Q. robur*, y el río Lor, con abundancia de formaciones naturales de *Q. pyrenaica*. Los tres están situados en el noroeste de la Península Ibérica y representa las tres influencias climáticas dominantes en la región anteriormente mencionadas.

Los bosques naturales de las dos cuencas dominadas por *Q. robur* tienen un gradiente altitudinal de 500 m desde la cuenca alta (650 m s.n.m.) hasta donde todavía se conservan bosques naturales cerca de la desembocadura en el Océano Atlántico o el Mar Cantábrico (150 m s.n.m.). La zona presenta un clima atlántico suave, con una temperatura media anual de 9 a 13 ºC según el lugar de muestreo, y una precipitación total de 900 a 1.500 mm; las precipitaciones máximas se producen durante el otoño-invierno, y el grado de sequía estival varía en función de la elevación o posición hacia la costa. Sin embargo, aunque ambas son de régimen predominantemente atlántico, existen algunas diferencias en cuanto el régimen hídrico estival. En la costa Atlántica se presenta una mayor tendencia mediterránea durante el verano (mayor temperatura, sequía más intensa) por influencia del anticiclón de las Azores, mientras la circulación del norte suaviza las condiciones en la costa cantábrica, con una mayor disponibilidad hídrica.

Los bosques estudiados para *Q. pyrenaica* siguen un gradiente altitudinal de 400 a 1.050 m s.n.m. Los lugares presentan un clima atlántico, pero una cierta continentalidad por la posición interior y de montaña, y una distribución mediterránea de las precipitaciones, caracterizada por períodos estivales secos, mientras los inviernos son muy húmedos invierno (350-600 mm). La temperatura anual oscila de 7,5 a 12,7 ºC entre localidades. Las condiciones mediterráneas aumentan a medida que disminuye la altitud, y la mayor parte de esta área puede considerarse como de transición de la Región Atlántica (Región Eurosiberiana).

A lo largo de ambos ríos, se monitorizó la formación de madera y de las hojas en las nueve localidades (4-5) de *Q. robur* y las cinco de *Q. pyrenaica* desde principios de primavera hasta el otoño en 2012 y 2013. Los tres gradientes climáticos fueron muestreados en tres días consecutivos a intervalos de 12-15 días en primavera y de 20 días en verano, seleccionando al azar diez árboles (de 20-40 cm de diámetro) por localidad en cada fecha de muestreo, dentro de un área de aproximadamente 2 ha por bosque, y sumando un total de 3.182 árboles muestreados.

En el **Experimento 2**, el estudio de *Q. robur* se llevó a cabo comparando las localidades de baja altitud en las zonas atlántica y cantábrica, que difieren principalmente en su régimen térmico durante el verano. Para *Q. pyrenaica*, se compararon los dos sitios más altos (1.000 m), pero que presentan una orientación diferente, y aparentemente un notable contraste en su grado de influencia mediterránea.

El **Experimento 3** se analizó la especie arbórea *Q. pyrenaica*, por presentar una mayor homogeneidad fenológica a nivel individual en primavera, en una localidad seleccionada fuera de los tres gradientes anteriormente mencionados.

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Los tres experimentos descritos se presentan en la Tesis estructurados en cuatro capítulos, cada uno de los cuales sigue el formato de un artículo científico, habiendo sido ya publicados los dos primeros de ellos.

En el **Capítulo I** se describe el Experimento 3, cuyos resultados muestran una disminución del valor de DMC (el opuesto al contenido de agua) de todos los órganos durante el desarrollo de la copa del árbol hasta alcanzar un valor mínimo a principios de junio, seguido de un aumento gradual hasta los valores iniciales a finales de septiembre. El período de extensión de los nuevos brotes coincidió con el valor más bajo de DMC, y también con el comienzo de la maduración de la madera temprana en el tallo principal, lo que sugiere que la máxima hidratación del tejido se alcanza cuando los vasos de la madera temprana se vuelven funcionales para cubrir los requisitos de agua para el brote y la extensión de la hoja. Estos resultados confirman la utilidad de los valores de DMC para cuantificar con precisión la fenología del crecimiento primario desde el crecimiento de la yema hasta la extensión total de la hoja. En consecuencia, el estudio ha permitido relacionar la secuencia del desarrollo de la madera temprana con los eventos de crecimiento primario, cuantificados por el contenido de agua de los órganos de las ramas, mostrando que la expansión de la primera hilera de vasos de la madera temprana y el hinchamiento de las yemas son eventos simultáneos. Ello sugiere que el agua demandada por los brotes en desarrollo es suministrada por vasos de madera tardía, traqueidas o células vivas, ya que los nuevos vasos no alcanzaron su tamaño final hasta la rotura de yemas. El subsiguiente despliegue de las hojas coincide con la hidratación de las ramitas de un año, probablemente porque la red de vasos recién formada ya es capaz de proporcionar suficiente agua para atender la mayor demanda que supone la elongación de los brotes.

En los **Capítulos II** y **III** se desarrolla el Experimento 1, en el que se investigó el efecto de la temperatura sobre la actividad cambial y la fenología foliar del roble a través de los tres gradientes de de bosques naturales de *Q. robur* y *Q. pyrenaica* en su límite de distribución, con el fin de alcanzar una mejor comprensión de su respuesta a los cambios ambientales y el impacto en el ciclo del carbono. En ambos capítulos las fases fenológicas foliares y de madera se relacionaron con la temperatura media del aire durante períodos de 10 a 60 días a lo largo del año para identificar las ventanas temporales más relevantes para la formación de madera y la fenología foliar, así como las relaciones entre ellas.

En el **Capítulo II** se describe el estudio de las nueve localidades de *Q. robur*, distribuidas en dos gradientes altitudinales, desde la costa Atlántica (cuatro bosques) y Cantábrica (cinco) al interior, en el noroeste de Iberia. Los resultados muestran que la sincronía entre crecimiento primario y secundario no es estable entre localidades y años. Los vasos en la primera línea de madera temprana se expandieron antes de la aparición de las primeras hojas pequeñas, y posteriormente maduraron para transportar la savia que demanda el despliegue completo de las hojas. El avance o retraso de la reactivación cambial y de la rotura de yemas varió entre sitios y años, principalmente en función de la temperatura de la primavera, y las mejores relaciones entre crecimiento y clima se maximizaron con los valores de temperatura máxima y mínima. Estos resultados permiten concluir que la temperatura modifica de manera diferente el inicio de las primeras fenofases del crecimiento primario y secundario, así como la sincronización entre ellas. Sin embargo, la maduración de los primeros vasos de la madera temprana es condición necesaria para la extensión completa de las hojas.

En el **Capítulo III** se describe el estudio de las cinco localidades de *Q. pyrenaica* en un gradiente altitudinal y continental, cuyos resultados muestran una mayor sincronía entre crecimiento primario y secundario que la observada en *Q. robur* (Capítulo II). Esto confirma que una adecuada maduración de los primeros vasos de madera temprana es esencial para el desarrollo de la copa. Las diferencias de temperatura no adelantaron ni retrasaron el comienzo del crecimiento primario y secundario entre localidades, pero condicionaron el final de la actividad cambial, siendo la amplitud térmica (es decir, la diferencia entre las temperaturas máximas y mínimas diarias) la variable que mostró la mejor relación. Estos resultados sugieren que el fotoperiodo es probablemente la principal variable responsable del inicio de la actividad cambial en *Q. pyrenaica*, favoreciendo una mayor sincronía entre crecimiento primario y secundario, así como entre localidades y años, mientras que el cese del crecimiento del xilema puede ser explicado por la amplitud térmica, que en cierto modo define el grado de continentalidad.

En el **capítulo IV** se describe el estudio dendrocronológico de *Q. robur* y *Q. pyrenaica* (Experimento 2) empleando anatomía cuantitativa, en bosques ubicados en los extremos de los gradientes, y que representan las situaciones más próximas al límite de distribución de cada una de las especies. En el caso de *Q. robur* se compararon las dos localidades más termófilas, y por tanto una mayor tendencia mediterránea, pero que difieren ligeramente en su xericidad estival (puntos inferiores de los gradientes atlántico y cantábrico). Por el contrario, en *Q. pyrenaica* se compararon los dos puntos de mayor altitud, es decir, aquellos de carácter más atlántico, pero que también difieren en su régimen de disponibilidad de agua. En cada una de las localidades se obtuvieron cronologías anuales, tanto de anchura de los anillos como de las características de los

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elementos conductores. Los resultados del trabajo muestran que en ambas especies las variables anatómicas están relacionadas con la temperatura, especialmente durante la dormición o quiescencia, mientras el incremento radial depende de la disponibilidad hídrica en el momento de mayor tasa de crecimiento. Además, los estudios de xilogénesis llevados a cabo en las mismas localidades mostraron gran coherencia con los resultados dendrocronológicos, facilitando la interpretación de una relación causa-efecto en las relaciones observadas. Por tanto, este estudio confirmó la gran utilidad de combinar análisis de anatomía y crecimiento radial para comprender la respuesta de los árboles en el límite de su distribución.

Un análisis global de los resultados de los cuatro capítulos permite concluir que las diferencias encontradas en el efecto de la temperatura sobre el crecimiento de las dos especies arbóreas estudiadas permite comprender múltiples aspectos relacionados con sus límites de distribución y su capacidad de adaptación a posibles variaciones climáticas. La estrategia principal de *Q. pyrenaica* consiste en iniciar el crecimiento ajustándose más al fotoperíodo que a la temperatura, lo que le permite adaptarse a climas más continentales, con heladas tardías, al conseguir evitar estas condiciones desfavorables retrasando las primeras fases fenológicas. Por el contrario, los menores requerimientos térmicos y mayor plasticidad de *Q. robur* le confieren una mayor exposición a las heladas tardías en el momento del despliegue de la hoja, aunque esto implica un mayor riesgo de daño por congelación de las hojas; sin embargo, las primaveras frías pueden retrasar el inicio del crecimiento de *Q. robur*. Además, el período de dormición es en general más corto en *Q. robur*, lo que debería ir asociado a un menor consumo de carbohidratos por respiración, así como a mayor tiempo de acumulación por fotosíntesis. Por lo tanto, parece que la estrategia de *Q. pyrenaica* es más conservadora y más regular en cuanto a la variación interanual de sus respuestas fenológicas; sin embargo, el mayor riesgo asumido por *Q. robur* debería otorgarle ventaja competitiva en caso de que las condiciones sean favorables.

Aunque el conocimiento del control ambiental sobre el crecimiento del bosque nativo debería permitir inferir el comportamiento de las especies frente al efecto del cambio climático, en el caso de *Q. robur* el grado de dificultad es elevado porque no sólo la cronología de los eventos del crecimiento primario y secundario, sino también la sincronía entre ellos, muestran respuestas heterogéneas a la influencia de factores externos. Por el contrario, en el caso de *Q. pyrenaica* el fotoperiodo parece ser la principal variable climática responsable del inicio del crecimiento del árbol, mientras que el cese del crecimiento del

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xilema está modulado por la amplitud térmica, pero la relación no es tan fuerte como para descartar otros factores como el fotoperiodo.

Desde el punto de vista metodológico, los resultados también han permitido comprobar por primera vez que la determinación del contenido en agua o materia seca de los órganos de la copa es una técnica muy valiosa para cuantificar el crecimiento primario de los árboles, desde el hinchamiento de las yemas hasta la expansión total de las hojas, que son las fenofases más difíciles de determinar mediante inspección visual con prismáticos. Este método constituye una herramienta sencilla para comprender la dinámica de hidratación de los tejidos durante el desarrollo de las copas, y permite monitorear el contenido en agua de las yemas, ramitas y hojas en relación con el crecimiento secundario, en condiciones de campo, siempre que las ramas sean fácilmente accesibles. En consecuencia, se propone una clasificación objetiva de las fenofases de la yema y de la hoja según su contenido en materia seca, lo que evita la subjetividad del observador. Además, estos resultados, junto con las observaciones fenológicas y su comparación con las fases cambiales a lo largo de los gradientes, muestran que los nuevos vasos conductores de madera temprana deben ser funcionales para cubrir los requisitos de agua para la extensión del brote, pero no para la apertura de las yemas. Asimismo, también han mostrado la utilidad de los estudios de xilogénesis para llevar a cabo una correcta interpretación de los análisis obtenidos mediante dendrocronología y anatomía cuantitativa de la madera, y por tanto su importancia para poder establecer relaciones causa-efecto en las respuestas observadas en los árboles frente a las variables climáticas.

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Los resultados de la presente Tesis Doctoral permiten extraer las siguientes **conclusiones**:

(1) La medición del contenido de materia seca (DMC) en los órganos de la copa es una potente herramienta para cuantificar el crecimiento primario de los árboles desde el hinchamiento de las yemas hasta la extensión total de las hojas, que evita la subjetividad de la inspección visual. En consecuencia, se propone una clasificación objetiva de las fenofases de la copa desde el hinchamiento de la yema hasta el desarrollo de las hojas según su DMC, lo que evita la subjetividad del observador.

(2) Existe una sincronía entre el crecimiento primario y secundario, ya que la formación de los primeros elementos conductores de madera temprana se inicia antes o durante las primeras fases de apertura de las yemas. Aunque el agua necesaria para el hinchamiento de las yemas y despliegue foliar inicial pueda ser aportada por los vasos conductores de madera tardía formados en años anteriores, es necesaria la funcionalidad de los vasos recién formados para que

tenga lugar la extensión total de la hoja. Sin embargo, esta sincronización es susceptible de ser modificada por la temperatura, al menos para *Q. robur*.

(3) La dinámica de formación de madera a lo largo de la estación es similar en ambas especies, y la mayor parte de las diferencias se encuentran al inicio de la estación de crecimiento, tanto en lo que respecta al momento en que tiene lugar, como en su dependencia de las condiciones ambientales. El reinicio de la actividad tiene lugar antes en *Q. robur*, pero la máxima tasa de expansión se detecta en torno al solsticio de verano para ambas especies.

(4) En el caso de *Q. robur*, las diferencias de temperatura al final del invierno o inicio de la primavera (quiescencia) explicaron las diferencias en el momento de desarrollo de la hoja y la madera temprana observadas entre años y entre sitios. Por el contrario, esta relación no se encontró para *Q. pyrenaica*, sugiriendo un papel más relevante del fotoperiodo al inicio de la estación, mientras las diferencias en el cese del crecimiento del xilema sí parecen ser debidas a la amplitud térmica.

(5) El análisis retrospectivo del crecimiento por técnicas dendrocronológicas, combinando anatomía cuantitativa (vasos de madera temprana) e incremento radial (anchura de la madera tardía) es una herramienta de gran utilidad para comprender las respuestas de *Q. robur* y *Q. pyrenaica* al clima en su límite de distribución, estando ligadas a la temperatura y precipitación respectivamente. No obstante, la aportación de la xilogénesis es fundamental para su correcta interpretación, ya que permite inferir las posibles relaciones causa-efecto de las respuesta observadas.

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## List of publications

**Guada, G., García-González, I., Pérez-De-Lis, G., Vázquez-Ruiz, R.A., Montserrat-Martí, G. (2018).** Dry matter content during extension of twigs, buds and leaves reflects hydraulic status related to earlywood vessel development in *Quercus pyrenaica* Willd. *European Journal of Forest Research* **137,** 307-319.

**Guada, G., Vázquez-Ruiz, R.A., García-González, I. (2019).** Response patterns of xylem and leaf phenology to temperature at the southwestern distribution boundary of *Quercus robur*: A multi-spatial study. *Agricultural and Forest Meteorology* **269-270,** 46-56.

**Guada,G., Vázquez-Ruiz, R.A., García-González, I.** Temperature regime controls the cessation of wood formation rather than the beginning in a sub-Mediterranean ring-porous oak. *Submitted.*

**Guada,G., Sass-Klaassen, U.G.W., Souto-Herrero, M., García-González, I.** Anatomical tree-ring chronologies and seasonal patterns of cambial dynamics are valuable indicators of tree performance of two oak species at the Atlantic-Mediterranean boundary. *Submitted.*

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