

Escape of spring frost and disease through phenological variations in oak populations along elevation gradients

Cécile Françoise Dantec^{1,2}, Hugo Ducasse^{3,4}, Xavier Capdevielle^{1,2}, Olivier Fabreguettes^{1,2}, Sylvain Delzon^{1,2} and Marie-Laure Desprez-Loustau^{1,2*}

¹INRA, UMR 1202 BIOGECO, F-33610 Cestas, France; ²Université de Bordeaux, UMR 1202 BIOGECO, F-33410 Talence, France; ³IRD, UMR 5290 MIVEGEC, 34394 Montpellier Cedex 05, France; and ⁴Université Montpellier 2, UMR 5290 MIVEGEC, 34095 Montpellier Cedex 05, France

Summary

1. The timing of tree flushing follows strong phenotypic and genetic clines across environmental gradients. It may be seen as an adaptive response to abiotic (escape of spring frost and maximizing growing season length) and biotic (escape of pest and disease) hazards. However, few studies have investigated jointly both types of hazards.

2. We assessed exposure to both abiotic (spring frost) and biotic (powdery mildew) hazards within and between sessile oak populations along elevation gradients, during the flushing period in several years. For each population and phenological phenotype (early- vs. late-flushing trees), we estimated safety margins, defined as the time period separating budburst from the hazard occurrence (spore emission, spring frost).

3. We observed that powdery mildew phenology (initiation of spore release in spring) was less responsive to the elevation gradient than oak phenology (budburst) and that it was not correlated with tree phenology within populations. The spring frost and disease safety margins varied considerably between oak populations as a function of elevation and within populations in relation to tree phenological phenotype. For both hazards, safety margins decreased significantly with increasing elevation. The safety margin for spring frost was mostly positive (i.e. escape), whereas the safety margin for powdery mildew was mostly negative (i.e. exposure), leading to infection. The abiotic and biotic hazards interact in opposite directions with phenology, especially at low elevations (< 500 m) where early flushing enabled trees to escape disease while late flushing provided a higher safety margin against late frost.

4. *Synthesis.* The observed patterns suggest that oak populations are better adapted to escape spring frost than pathogen exposure all along the elevation gradient. The combination of the biotic and abiotic selective pressures may have contributed to the maintenance of phenological diversity within low-elevation tree populations. As tree and pathogen respond differently to environmental cues, climate change is likely to affect the phenological (a)synchrony between host and parasite, both within and between populations.

Key-words: abiotic and biotic hazards, flushing, phenology, plant–climate interactions, powdery mildew, *Quercus petraea*, resistance, synchrony

Introduction

The timing of spring life-history events is crucial to the fitness of trees in regions with seasons (Fenner 1998; Bennie *et al.* 2010). In particular, the timing of flushing determines

growing season length and is, thus, a major determinant of growth (Dickson 1989) and survival (Morin, Viner & Chuine 2008). Many phenological studies essentially address the question: ‘Why now, rather than earlier or later?’ (Fenner 1998), particularly in the context of climate change. There is wide agreement that environmental stresses, such as cold and drought in seasonal environments, are a strong selective force on the timing of life-history events in plants (Pau *et al.*

*Correspondence author. E-mail: loustau@bordeaux.inra.fr

2011). The timing of flushing follows strong geographic patterns of variation associated with elevation, latitude or longitude (Conover, Duffy & Hice 2009; Kremer, Potts & Delzon 2014). Moreover, the timing of flushing may differ considerably between trees from the same population (Baliuckas *et al.* 2005; Kremer *et al.* 2010; Salmela *et al.* 2013). Phenotypic variation in leaf phenology can occur as a result of phenotypic plasticity (Kramer 1995; Vitasse *et al.* 2010), which enables a single genotype to express different phenotypes in response to environmental variations (Fordyce 2006; Whitman & Agrawal 2009). Genetic variation is also involved (Ducousso, Guyon & Kremer 1996; Jensen & Hansen 2008), at the individual and/or population level, and this variation is maintained by heredity (Wagner, Booth & Bagheri-Chaichian 1997; Elzinga *et al.* 2007; Donnelly *et al.* 2012).

The timing of flushing is widely considered to reflect an evolutionary trade-off in response to two opposite abiotic forces: adjustment to the likelihood of frost occurrence, that is escape of freezing temperatures, to decrease the risk of frost damage to actively growing parts of the plant, and adjustment to the duration of the growing season, to maximize carbon assimilation and biomass production (Lockhart 1983; Leinonen & Hänninen 2002; Gömöry & Paule 2011). However, the timing of flushing in temperate trees may also be seen as an adaptive response to biotic interactions since susceptibility to herbivores and pathogens changes with ontogeny (Rathcke & Lacey 1985; Fenner 1998; Tikkanen & Julkunen-Tiitto 2003; Ghelardini & Santini 2009). Interactions with pollinators, herbivores or pathogens (for instance, escape of disease) may modulate or negate patterns of selection controlled by the physical environment (Elzinga *et al.* 2007; Pau *et al.* 2011). Moreover, a major difference between abiotic and biotic constraints is that biotic agents, such as insects and pathogens, can adapt to the host traits involved in interactions, including phenology. Co-evolutionary processes would therefore be expected to lead to phenological synchrony between parasites and their hosts (Combes 2001; van Asch & Visser 2007).

The relative contributions of biotic and abiotic selective forces to phenology may depend on local conditions, particularly along environmental gradients (Pau *et al.* 2011). At high latitudes or elevations, strong directional selection due to freezing temperatures is expected to occur on the timing of leaf flushing (Alberto *et al.* 2011; Pau *et al.* 2011) while pest and disease may exert a lower pressure, due to host density and climatic factors being less favourable for the development of large insect and pathogen populations than at lower latitudes or elevations (Busby *et al.* 2014). Abiotic and biotic constraints frequently act in opposite directions on local populations, because the main physical constraints linked to low temperatures act upstream from flushing, whereas interactions with many insects and pathogens exert their effects after flushing has occurred. Most investigations to date have dealt with the relationship of either biotic or abiotic factors to phenology (but see Lobo *et al.* 2003). There is therefore a need for experimental investigations considering both types of factors simultaneously (Elzinga *et al.* 2007; Pau *et al.* 2011).

The aim of the study was to quantify differences in exposure to both abiotic (spring frost) and biotic (powdery mildew) hazards between and within populations of sessile oak (*Quercus petraea* L.), during the period of flushing, along elevation gradients. Previous studies along the same transects in the Pyrenees showed considerable variability in flushing phenotype between populations (Vitasse *et al.* 2009a,b), due to both phenotypic plasticity (Vitasse *et al.* 2010) and genetic population differentiation (Vitasse *et al.* 2009c; Alberto *et al.* 2011). Considerable phenotypic variation for leaf flushing was also observed within populations, associated with the maintenance of genetic diversity for this trait at local scale (Alberto *et al.* 2011). This local genetic differentiation for flushing was shown to decrease with increasing elevation (Alberto *et al.* 2011). In order to assess the temporal escape of abiotic and biotic hazards by trees, we estimated safety margins, defined as the time period separating budburst from the period of hazard occurrence (spore emission, spring frost). The hypothesis that safety margins remain positive (i.e. stress escape) and constant along the elevation gradient if populations are locally adapted to their environment was tested. We then determined whether exposure to spring frosts and powdery mildew differed within populations, as a function of variations in individual tree phenology. We tested whether powdery mildew and spring frost escape favours similar or opposite phenological phenotypes (late- vs. early-flushing trees). Finally, we assessed whether differences in the duration of exposure to powdery mildew had a noticeable effect on tree infection rates.

Materials and methods

MODEL SYSTEM

Sessile oak is a broad-leaved deciduous tree species occurring over most of Europe and of high environmental, cultural and economic value. The timing of oak flushing depends mostly on temperature (Vitasse *et al.* 2009a; Laube *et al.* 2013), with only a secondary role of photoperiod (Basler & Körner 2012). Indeed, budburst follows a dormancy period encompassing an endodormancy phase in early winter which is generally released by chilling (cold) temperatures, and an ecodormancy phase in early spring which is sensitive to forcing (warm) temperatures (reviewed in Lang 1987). Powdery mildew is one of the most common fungal diseases of oaks in Europe (Mougou, Dutech & Desprez-Loustau 2008) and the main source of biotic damage in the study area (personal observations). Powdery mildew fungi are obligate biotroph parasites, infecting photosynthetic organs (Glawe 2008). In oaks, the disease is caused by a complex of several closely related species: *Erysiphe alphitoides*, *E. hypophylla*, *E. quercicola* and *Phyllostictia guttata*, with *E. alphitoides* currently highly predominant but probably of exotic origin (Mougou, Dutech & Desprez-Loustau 2008; Mougou-Hamdane *et al.* 2010). Oaks display ontogenic resistance to powdery mildew: only young expanding leaves are susceptible to the disease (Edwards & Ayres 1982). Like oaks, the fungus is affected by seasonality. Oak powdery mildew mostly overwinters as chasmothecia (sexual fruiting bodies acting as dormancy organs), which release the ascospores that form the primary inoculum in spring. The timing of ascospore release in spring was shown to be related to winter temperature, with earlier ascospore release after milder winter

conditions (Marçais *et al.* 2009). Infection results in the production of a mycelium, which produces huge numbers of conidiospores (asexual spores) at the leaf surface. Conidiospores constitute the secondary inoculum, with subsequent infection cycles during the course of the season resulting in a build-up of fungal populations. The continuous availability of susceptible leaves in an oak stand during the growing season results both from differences in flushing time between trees and the occurrence of several growth flushes in a tree during one season. Foliar infection causes a decrease in net photosynthesis and a more general change in metabolism, which may affect tree growth and survival, especially for seedlings (Hewitt & Ayres 1975; Marçais & Desprez-Loustau 2014).

STUDY AREA

The study was carried out in two valleys of the Pyrenees in southern France (from 43°15'N, 00°44'W to 42°52'N, 00°07'E) (Vitasse *et al.* 2009b). A first transect was set up in the Gave valley (Hautes Pyrénées), and a second was established in the Ossau valley (Pyrénées Atlantiques) (Fig. 1) (Table 1). These two valleys, located 30 km apart, are parallel and display an increase in elevation from north to south. The region is characterized by a temperate oceanic climate (see Table 2 and Fig. S1 for meteorological details). Mean annual temperatures decrease linearly with elevation, by about 0.43 °C for every 100 m increase in elevation (mean from 2005 to 2011). In total, 14 natural established oak populations on south-facing slopes were monitored for the different experiments, from 131 to 1630 m above sea level, in hill and mountain vegetation belts (see Table 1). The range of elevation extended over 1499 m in the Gave valley and 1355 m in the Ossau valley. We used the terms 'low elevation' (< 500 m asl), 'medium elevation' (500–1000 m asl) and 'high elevation' (> 1000 m asl) to describe populations.

METEOROLOGICAL MEASUREMENTS

Air temperature was recorded with data loggers (HOBO Pro RH/Temp, Onset Computer Corporation, Bourne, MA, USA) at each study site (14 sites). All sensors were intercalibrated in the laboratory before installation. At each site, sensors were installed 1.5 m above

the ground in an open area close to the studied population (10–100 m away from the plot, at the same elevation). Sensors were protected by a white plastic shelter, to prevent exposure to rain or direct sunlight. Data were recorded hourly, from 1 January 2005 to 31 October 2012. Several weather stations suffered short periods of technical failure. Years for which spring data were missing were not considered in the spring frost analyses.

OAK PHENOLOGY MONITORING

The timing of flushing was monitored along the two elevation gradients, for 7 years (2005–2007 and 2009–2012) on adult trees and 2 years (2011–2012) on seedlings (Table 1). In each of the 14 populations, 10–34 dominant adult trees of similar height (about 15 m) were monitored. The mean distance between studied trees within a population was 109 m in the Gave valley and 50 m in the Ossau valley. Trees were also selected for their phenology (first assessed in 2005), with the aim of capturing the widest possible variation (i.e. earliest and latest trees) for each population. We monitored 60–80 natural seedlings (20–30 cm high) protected in enclosed plots of 20 seedlings in the two low-elevation populations in the Gave valley. Bud development was monitored at 10-day intervals, in each population, from March to June. Observations were made by one or two trained observers with binoculars (magnifying power: ×10), about 15 m away from adult trees. At each date, the proportion of buds at a given phenological stage was assessed for each adult tree (Vitasse *et al.* 2009a). We considered two successive stages of interest in terms of frost and disease damage: budburst and leaf unfolding. The date of budburst was defined as the day on which 50% of the tree buds had burst and the first leaves had started to emerge (first stage of low freezing resistance) (Lenz *et al.* 2013). The date of leaf unfolding was defined as the date on which the first leaves of 50% of the buds had unfolded (beginning of the period of high susceptibility to powdery mildew) (Edwards & Ayres 1982). For seedlings, we considered only the stage of the apical bud (i.e. budburst was the date on which the apical bud burst and its first leaves began to emerge, and leaf unfolding was the date on which the first leaf of the apical bud was unfolded). The dates of the two stages were estimated, for each selected adult tree and seedling, by linear regression between two

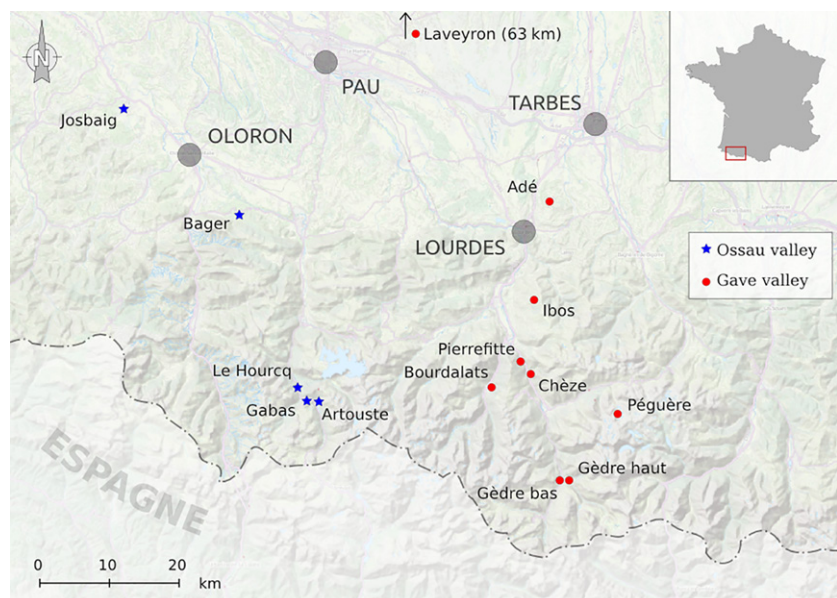


Fig. 1. Localization of the oak populations studied along the two elevation gradients in the Pyrenees. Sources: background map from Open Cycle Map Landscape.

Table 1. Description of the oak populations studied along the elevation gradients in the Pyrenees. We show elevation (elev, rounded to the nearest 50 m asl), geographic coordinates (coordinates), mean spring temperature from the February 1 to the May 31 for the 2005–2007 and 2009–2012 periods (T_{mean} , °C), mean number of frost events ($T < 0^{\circ}\text{C}$) per year from the January 1 to the June 30 (nb_frost), minimum–maximum number of trees monitored per year for phenology over seven years (pheno_tree – we selected the first and last quartiles to represent the early- and late-flushing trees, respectively, per population and per year, for both budburst and leaf unfolding dates), number of trees studied for spore dispersal (by filter trapping in this study over 2 years (spor_filter) or by biological trapping in a previous study (Desprez-Loustau *et al.* 2010) also over 2 years (spor_biol)), number of trees studied for the scoring of infection (inf), number of seedlings studied for phenology (pheno_seedling – one enclosed plot with one filter trap for 20 seedlings) and number of years studied for spring frost calculations (frost_years, years)

Valley	Site	Elev (m asl)	Coordinates	T_{mean} (°C)	Nb_ frost	Pheno_ tree	Spor_ filter	Spor_ biol	Inf	Pheno_ seedling	Frost_ years
Gave	Laveyron	150	43°45'N, 00°13'W	11.11	26	10–28	8	1	8	60–80	7
	Ibos	400	43°15'N, 00°00'W	10.51	17	10–25	8		8	60	7
	Adé	450	43°08'N, 00°00'W	10.37	29	10–34		1			6
	Pierrefitte	650	42°56'N, 00°03'W	9.68	27	10–22					6
	Chèze	800	42°55'N, 00°02'W	8.70	36	10–25	8	1	8		7
	Bourdalats	1100	42°54'N, 00°06'W	7.19	44	10–29					7
	Gèdre Bas	1250	42°47'N, 00°01'E	7.08	59	10–28	8	1	8		7
	Gèdre Haut	1350	42°47'N, 00°02'E	5.96	62	10–27					7
	Péguère	1650	42°52'N, 00°07'E	4.69	58	10–27	8	1	8		4
	Ossau	Josbaig	250	43°15'N, 00°44'W	11.27	17	10–26			8	
Bager		400	43°07'N, 00°32'W	11.69		18			8		
Le Hourcq		850	42°54'N, 00°26'W	7.03	73	10–22			8		5
Gabas		1200	42°53'N, 00°25'W	6.40	56	10–34			8		6
Artouste		1600	42°53'N, 00°24'W	4.22	78	11–15			8		7

Table 2. Meteorological details at low (Lourdes) and high (Artigues) elevation in the Pyrenees, over a period of 50 years (1931–2011 at Lourdes, 1959–2013 at Artigues) and during the study (2005–2011) (Météo France). We show winter mean temperature ($T_{\text{mean winter}}$, °C), mean number of frost events ($T < 0^{\circ}\text{C}$) by year from March to May at Lourdes and from April to June at Artigues (spring frost events, n) and date of the last spring frost event (last spring frost event). Lourdes: 43°06'18"N, 00°03'42"W, 400 m asl; Artigues: 42°55'42"N, 00°12'18"E, 1200 m asl

	$T_{\text{mean winter}}$ (°C)		Spring frost events (n)		Last spring frost event	
	Over 50 years	2005–2011	Over 50 years	2005–2011	Over 50 years	2005–2011
Lourdes	7.0	7.9	5	4	April 2	March 14
Artigues	2.3	2.5	11	7	May 9	May 6

measurement campaigns. Finally, for each population, budburst and leaf unfolding dates were calculated as the means for the adult trees sampled. We selected the first and last quartiles as representative of the early- and late-flushing trees, respectively, per population and per year, for both budburst and leaf unfolding dates. Finally, the date of leaf senescence (i.e. the time point at which 50% of the leaves of the tree had changed colour or fallen) was monitored similarly along the two gradients, from September to December. The canopy duration was thus estimated as the difference in days between the date of leaf senescence and the date of leaf unfolding.

POWDERY MILDEW PHENOLOGY MONITORING

Phenological observations focused on spore release in spring in five Gave valley populations, assessed by passive trapping (see Table 1). First data were acquired previously (Desprez-Loustau *et al.* 2010) by using biological traps and infection counts data. In that case, young seedlings grown in a pathogen-free environment until susceptible stage were exposed to natural inoculum in each of the five stands along the altitudinal gradient in 2006 and 2007. For practical reasons, only one trap composed of 13 seedlings (approximately 200 cm² of susceptible area), changed every 10 days, could be used at each date

and altitude from early April to the end of June (see complete description in Desprez-Loustau *et al.* 2010). In order to obtain data in more years and to tackle variation in fungal phenology within stands, this previous study was extended in 2011 and 2012 with a modified trapping method. Here, we used a simple trap device constituted of Whatman® filter paper (24 cm in diameter), sprayed with Tris-EDTA buffer (dilution 4X, pH = 8) before exposure to improve trapping capacity and spore preservation, that was fixed on a horizontal wooden support, in combination with molecular specific detection of the fungus (Schweigkofler, O'Donnell & Garbelotto 2004; Garbelotto, Smith & Schweigkofler 2008). This allowed us to use several traps in each site at each exposition period. In each oak stand (see Table 1), eight traps were hanged in the canopy of trees, at a height of about 10 m above ground level. In order to take into account the possible variation in fungal phenology linked to tree phenology, four early- and four late-flushing trees, distributed throughout the stand, were selected (based on 2005–2007 and 2009–2010 assessments). One trap, fixed to a 50-cm-tall wooden stake, was also placed in each enclosed plot of seedlings used for phenology monitoring. Filters were changed at regular intervals (15 days on average), from March to September in 2011, and from March to July in 2012. At the end of the exposure period, filters were individually placed in paper bags and brought

back to the laboratory. Upon arrival, each filter was dried at ambient air temperature and then individually placed in a plastic bag and stored at -80°C until further processing. The spores were washed off the filters, by dispensing 40 ml of Tris-EDTA buffer warmed at 65°C into each plastic bag (Schweigkofler, O'Donnell & Garbelotto 2004; Garbelotto, Smith & Schweigkofler 2008). A spore suspension was generated by backward and forward movements and friction. This suspension was poured into a Falcon[®] 50-ml tube (washings from 1 filter per tube). Tubes were stored at -20°C . We concentrated the spores on a support that could be used for DNA extraction, by filtering them with a filtration pump (Combisart[®] Systems from Sartorius Stedim Biotech). For each tube, the spores were filtered on a 47-mm-diameter sterile nitrocellulose membrane with 0.45- μm -diameter pores (Sartorius Stedim Biotech), a diameter smaller than that of the spores. The membranes were cut into two equal halves with a scalpel, and each half was placed in a 2-ml Eppendorf[®] tube with two 2-mm-diameter steel beads. The samples were lyophilized and shaken for 2 min, at 30 Hz, in a Qiagen Tissue Lyser[®]. DNA was then extracted with a kit, used according to the manufacturer's instructions (Invisorb[®] Spin Plant Mini Kit from Invitex). For the amplification of specific fragments targeting *E. alphitoides*, *E. quercicola* and *E. hypophylla*, the oligonucleotide primers ery-F (5' CTC AGT CGT GGC ATC TGC T 3') (Mougou-Hamdane *et al.* 2010) and o-micro-rev (5' CGC CTC CAG CCG AAA CC 3') (Heuser & Zimmer 2002), binding to the ITS1 region of rDNA, were used for PCR. For each sample, we used 1.5 μl of pure and 1:10-diluted DNA extracts (to dilute out potential PCR inhibitors) for PCR. A mixture of the following components was added to each DNA extract, for a final volume of 20 μl : 13.55 μl of distilled water, 2 μl of $10 \times$ PCR buffer (Life Technologies, Karlsruhe, Germany), 1.2 μl of 25 mM MgCl_2 , 0.8 μl of dNTP, 0.4 μl of each primer and 0.15 μl of *Taq* DNA polymerase (Life Technologies, Karlsruhe, Germany). PCR was performed over 45 cycles (30 s at 94°C , 40 s at 55°C , 50 s at 72°C) preceded by a primary denaturation phase (3 min at 94°C) and followed by a terminal elongation phase (7 min at 72°C). The presence or absence of DNA was determined by agarose gel electrophoresis (2% polyacrylamide gel, migration for 25 minutes at 100 V). In order to determine the *Erysiphe* species involved, two samples of PCR products were selected per tree, from 2011 and 2012, which were sequenced with the same oligonucleotide primers, by the Sanger method (dye terminators), on an Applied Biosystems[®] 3730 sequencer (Life Technologies, Karlsruhe, Germany).

Powdery mildew spores were considered to be present in a given trap filter for a given exposure period if at least one of the four PCR tests (2 membranes \times 2 dilutions) was positive for the exposed filter. For each positive filter, the date of trapping was defined as the date in the middle of the filter exposure period. In each tree canopy, the beginning of the sporulation period was defined as the date corresponding to the first positive filter. For several traps, we were unable to determine this date because some filters were absent or destroyed due to poor weather conditions.

SAFETY MARGINS

The budburst date of a tree is not informative per se of the tree ability to escape the impact of biotic and abiotic hazards in its local environment, since this also depends on the dates of hazard occurrence. In order to obtain a tree-centred relevant variable, we calculated 'safety margins' at tree level in each population and year as the time separating the budburst date from the date of hazard occurrence (spore emission, spring frost). The safety margin quantifies the stress escape,

with low or negative safety margins indicating a high probability of exposure to the biotic or abiotic stress concerned and positive safety margins indicating a high probability of escaping stress by the trees.

For abiotic hazard related to spring frost, safety margins (SM_{sf}) were calculated as follows:

$$\text{SM}_{\text{sf}} = D_{\text{bb}} - D_{\text{lsf}}$$

where D_{bb} is the budburst date of a given tree (Julian days) and D_{lsf} is the date (in Julian days) of the last spring frost event of the tree site. Choosing a temperature threshold to define a frost event is not straightforward. A standard and conservative definition of a frost event by a daily minimum temperature below 0°C was used in several studies (Alberto *et al.* 2011; Anandhi *et al.* 2013). However, when assessing risks of frost damage to plants, a more relevant threshold should refer to the temperature actually experienced by leaves and causing physiological damage. Slightly negative temperatures (around -3°C) were defined as LT50 (lethal temperatures at which 50% of the samples died) for oak leaves during flushing under controlled conditions (Liepe 1993; Lenz *et al.* 2013; Vitasse *et al.* 2014b). However, in our study, *in situ* leaf temperature is variable in different parts of the crown and for seedlings compared to trees. In particular, crown temperature may be slightly colder than the temperature recorded by the on-site meteorological sensors put at 1.50 m above soil level and below a shelter (Kollas *et al.* 2014). We therefore calculated safety margins with two values: the conservative standard value of 0°C and the -3°C value. The same were used for all trees, whatever the site and the phenological ranking (early *versus* late flushing), in the absence of any data showing within-species genetic differentiation for frost hardiness during flushing in oaks. The spring frost safety margins were calculated for 13 populations along the Gave and Ossau gradients, from 2005 to 2012 (see Table 1 for details).

For biotic hazard related to powdery mildew, safety margins (SM_{pm}) were calculated as follows:

$$\text{SM}_{\text{pm}} = D_{\text{sp}} - (D_{\text{lu}} + 20)$$

where D_{sp} is the date on which powdery mildew sporulation began (Julian days), defined for each site and year as the date for which spores were trapped in at least 75% of the traps placed in adult trees (as determined by observation or interpolation between two exposure campaigns), and D_{lu} is the leaf unfolding date (Julian days) plus 20 days, to account for the period of susceptibility of young leaves to powdery mildew (Edwards & Ayres 1982 and personal observations). Powdery mildew safety margins were calculated for six populations along the Gave gradient, in 2006–2007 and 2011–2012 (see Table 1 for details and Desprez-Loustau *et al.* (2010) for additional data for powdery mildew sporulation dates in 2006–2007).

Finally, a safety window was estimated as the difference in days between the date of the beginning of powdery mildew sporulation (D_{sp}) and the date of the last spring frost event (D_{lsf}).

ASSESSMENT OF POWDERY MILDEW INFECTION

Powdery mildew infection was assessed at the end of July, in five populations in the Gave valley (2011 and 2012) and five populations in the Ossau valley (2012) (see Table 1). For the Gave valley populations, we monitored the same trees and seedlings as were used for spore trapping. For the Ossau valley populations, we monitored four early- and four late-flushing adult trees (based on 2005–2007 and 2009–2011 assessments). For each studied oak, we

collected a single twig from each of the four cardinal points (north, south, east and west) in the canopy, at a height of about 10 m, with a pole pruner, when possible. Two leaves were randomly chosen from the first growth cycle on each shoot (second flushes were quite rare). The infection of each leaf was visually assessed, by trained observers, as the percentage of the leaf area covered by mycelium. A magnifying glass was used to facilitate this assessment if necessary. The infection percentage per tree was calculated as the mean infection level of the eight leaves. For the seedlings, infection was estimated as the percentage of the leaf area covered by mycelium, for the whole plant.

STATISTICAL ANALYSES

We used a general linear model to test the effects of elevation, phenological phenotype (early vs. late flushing) and their interaction on frost and powdery mildew safety margins (for both seedlings and adult trees) (PROC GLM, SAS). For adult trees, infection was also analysed, with a general linear model, after data log transformation, with phenological phenotype, elevation, valley x year interaction, valley x year x elevation interaction and phenological phenotype x elevation interaction as effects. We carried out Tukey's test to compare mean infection values between sites. For seedlings, infection was analysed as a binary variable (0: uninfected; 1: infected, due to the small number of seedlings infected, with low levels of damage), with a logistic model, with phenological phenotype, site x year interaction and site x year x phenological phenotype interaction as effects (PROC LOGISTIC, SAS). If interaction with phenological phenotype was found to be significant in infection and safety margin models, separate analyses were carried out by site. We investigated whether safety margin was a good predictor of damage (i.e. infection), by carrying out a general linear model analysis, by site, for both seedlings and adult trees. The distribution of residuals was analysed by Q-Q plots, the plotting of a distribution histogram, skewness, kurtosis and Shapiro-Wilk tests (PROC UNIVARIATE, SAS) and by comparisons of predicted vs. observed data plots, to check the validity of the models. The correlation between leaf unfolding and the start of powdery mildew sporulation dates within populations was analysed by calculating a Pearson's correlation coefficient from the centred-reduced values per tree (mean and standard deviation calculated for each site and year) (PROC CORR, SAS). For comparisons of sporulation start dates between seedlings and trees, we used a general linear model per year and per elevation. All statistical analyses were performed with SAS 9.2 software (SAS Institute Inc. 2011).

Table 3. Linear regressions between the timing of leaf unfolding, the timing of sporulation, canopy duration (CD), spring frost (SM_{sf}) and powdery mildew (SM_{pm}) safety margins, and elevation. We show the slope, the P -value (P), the coefficient of regression (r^2) of the linear regression and the range between the lowest and the highest elevations (in Julian days for leaf unfolding and sporulation; in days for GSL and safety margins). The timing of leaf unfolding and canopy duration were monitored in 14 populations in 7 years (2005–2007; 2009–2012), the timing of sporulation and SM_{pm} were assessed in six populations in 4 years (2006, 2007, 2011 and 2012), and SM_{sf} was calculated for 13 populations over a period of 7 years (2005–2012) with a 0 °C threshold (see Table 1 for details)

	Leaf unfolding	Sporulation	CD	SM_{sf}	SM_{pm}
Slope	0.033	0.0063	−0.045	−0.018	−0.024
Range	93 to 142	110 to 117	150 to 215	−10 to 17	−44 to −6
r^2	0.85	0.27	0.80	0.44	0.98
P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Results

PHENOLOGICAL VARIATIONS WITHIN AND BETWEEN POPULATIONS

The dates of budburst and leaf unfolding were strongly correlated (slope = 1.009, $r^2 = 0.99$, $P < 0.0001$), with a mean of 4 days between the two dates. The mean dates of leaf unfolding and sporulation at population level were significantly and positively correlated with elevation (Table 3; see Fig. 2, for example). However, the pattern of change in phenology with elevation differed considerably between oak and powdery mildew: oak had a much stronger response to elevation (leaf unfolding delayed by 3.3 days per 100 m increase in elevation) than powdery mildew (0.6 days.100 m^{−1}) (Table 3). The canopy duration was significantly negatively correlated with elevation (−4.5 days.100 m^{−1}) (Table 3).

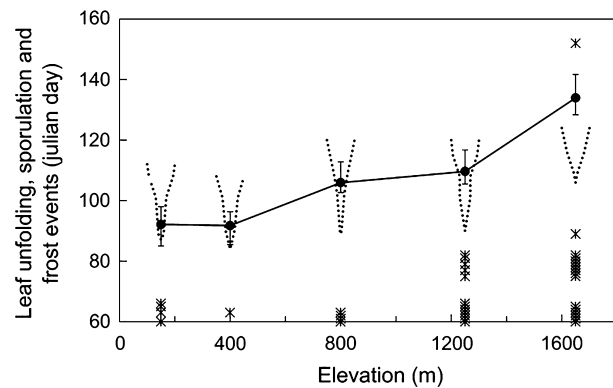


Fig. 2. Synchrony between spring frost events, powdery mildew sporulation and oak phenology. The mean date of sessile oak leaf unfolding (error bars representing the difference between the first and the last dates of leaf unfolding), dates of spring frost events (negative daily minimum temperature) and the beginning of the powdery mildew sporulation period (funnels) at five sites along the Gave elevation gradient in 2011 are represented. The funnels start on the date at which the first spores were trapped on a tree within a population. The width of the funnels then represents the numbers of spores in the oak populations, based on a semi-quantitative method and PCR results. The funnels stop when spore levels peak, these levels then remaining constant for the rest of the season. Stars: spring frost events; open circles: leaf unfolding.

In the study sites, leaf unfolding occurred 5 days later on average in seedlings than in adult trees. The variability of leaf unfolding phenology for seedlings was almost as high as that for trees (mean of 26 days between the seedlings unfolding their leaves earliest and last). For adult trees, the timing of leaf unfolding showed greater year-to-year fluctuations at high elevation than at low elevation (individual standard deviation = 9.2 and 5.8 on average, respectively; $y = 0.87 \times 0.33$, $r^2 = 0.42$, $P < 0.0001$) (see Fig. S2). This difference was not related to greater differences in winter/spring temperatures at high elevation than at low elevation (mean standard deviation = 1.0 ± 0.06 , from the beginning of February to the end of May in 2004–2012).

FROST AND POWDERY MILDEW EXPOSURE ALONG THE ELEVATION GRADIENT

Safety margins for both spring frost and powdery mildew decreased significantly with increasing elevation (Table 3) (Fig. 3). The same trend was observed for spring frost safety margins whatever the temperature threshold considered for a frost event. The altitudinal trend was slightly more pronounced for powdery mildew safety margins. In adult trees, budburst generally occurred well after the last spring frost event, whatever the elevation (see Fig. 2 as an example). The spring frost safety margin was positive for 72% of the tree-per-year observations with the 0 °C threshold and 98% with –3 °C during the study period. By contrast, the powdery mildew safety margin was mostly negative, in all populations (Fig. 3). The powdery mildew safety margin was positive for only 8% of the tree-per-year observations, these trees being located at low elevation. Safety margins varied considerably between trees within a population, for both powdery mildew and spring frost hazards ($P < 0.0001$ and $P = 0.015$ for phenology \times elevation interaction, respectively) (Fig. 3).

The duration of the safety window decreased significantly with increasing elevation by around 40 days from 100 m to 1600 m asl (see Fig. S3).

FROST AND POWDERY MILDEW EXPOSURE WITHIN POPULATIONS

A large variation in the date of first spore detection was observed between the traps on different adult trees within sites in a single year, particularly at low elevations in 2012 (Fig. 4). However, powdery mildew phenology was not correlated with oak phenology within populations (Pearson correlation = 0.023, $P = 0.85$) (Fig. 4). Sporulation was detected earlier (about 1 month) in seedling traps than in adult tree traps at 150 m asl in 2012 ($P = 0.0051$), but no difference was observed in 2011 ($P = 0.85$) neither at 400 m asl in 2012 ($P = 0.27$). We therefore considered that differences between traps only represented within-site variation, and this justified that the same date of inoculum exposure (calculated from data over all traps in a site) could be used for all trees. Only *E. alphitoides* was detected in all the samples studied, on the basis of its ITS sequence.

Reflecting differences in flushing dates, the powdery mildew and spring frost safety margins differed by 11–15 days on average between early- and late-flushing individuals within sites, showing a decreasing trend with elevation ($P < 0.0001$) (Fig. 3). However, difference in safety margins between early- and late-flushing individuals was positive for powdery mildew and negative for spring frost. Only early-flushing trees at low elevation had a positive or weakly negative SM_{pm} , enabling them to avoid powdery mildew spores (Figs 2 and 3). Overall, the powdery mildew safety margin was positive for 15% of the tree-per-year observations for early-flushing trees vs. only 2% for late-flushing trees, always at low elevation. The spring frost safety margin was positive for 66% of the tree-per-year observations for early-flushing trees

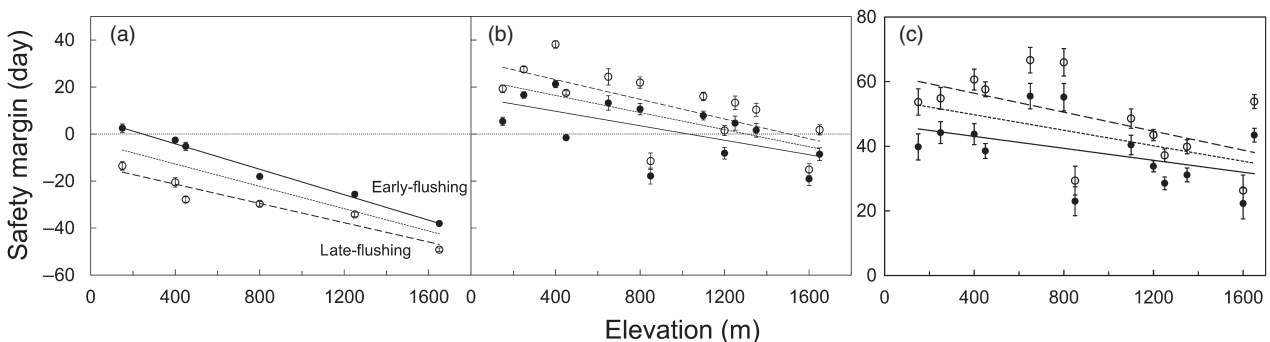


Fig. 3. Variation of powdery mildew (a) and spring frost (b, with 0 °C and c, with –3 °C) safety margins in oak populations as a function of elevation. Safety margins were calculated for early- and late-flushing sessile oak trees (mean \pm SEM). Powdery mildew safety margins were calculated for six oak populations along the Gave gradient, in 2006, 2007, 2011 and 2012; see Table 1 for details (early flushing: slope = -0.027 , $r^2 = 0.99$, $P < 0.0001$; late flushing: slope = -0.021 , $r^2 = 0.92$, $P < 0.0001$). Spring frost safety margins were calculated for 13 populations along the Gave and the Ossau gradients, from 2005 to 2012; see Table 1 for details (early flushing: slope = -0.015 , $r^2 = 0.38$, $P < 0.0001$; late flushing: slope = -0.021 , $r^2 = 0.48$, $P < 0.0001$). Closed circles: early-flushing trees; open circles: late-flushing trees; solid line: early-flushing trees; dashed line: late-flushing trees; dotted line: average of early- and late-flushing trees.

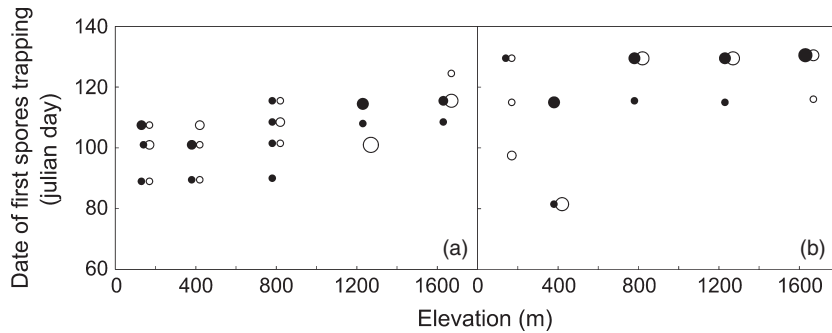


Fig. 4. Comparison of the timing of powdery mildew sporulation between oak phenological phenotypes. The dates on which the first powdery mildew spores were trapped are shown for early- and late-flushing sessile oak trees, as a function of elevation. Trapping was monitored in five oak populations along the Gave elevation gradient in 2011 (a) and 2012 (b). The size of the dots is proportional to the number of trees for which the first spores were trapped on the day concerned (from one to four trees). Closed circles: early-flushing trees; open circles: late-flushing trees.

vs. 78% for late-flushing trees with a 0 °C threshold (98% for both with a -3 °C threshold).

The spring frost safety margin was positive for both early- and late-flushing seedlings ($SM_{sf} = 27$ and 49 days, on average, at 150 and 400 m, respectively, with 0 °C threshold), whereas the powdery mildew safety margin was negative ($SM_{pm} = -9$ days and -18 days on average, at 150 m and 400 m asl, respectively). As for adult trees, powdery mildew safety margins were higher for early-flushing seedlings than late-flushing seedlings ($P < 0.0001$), whereas the opposite pattern was observed for spring frosts ($P < 0.0001$) (see Fig. S4).

POWDERY MILDEW INFECTION

Infection was lower at 200 m asl than at higher elevations, with 3% instead of 13% of the leaf area infected on average (Fig. 5; significant difference between 200 m and higher elevations in Tukey's tests). A year \times valley interaction effect on infection was found ($P < 0.0001$), but there was no effect of a valley \times year \times elevation interaction ($P = 0.17$). At low

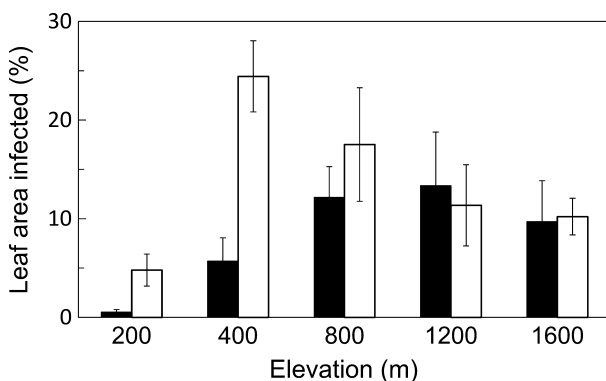


Fig. 5. Differences in the percentage leaf area infected with powdery mildew between early- and late-flushing oak trees. Infection was monitored in five sessile oak populations along the Gave gradient (2011–2012) and five sessile oak populations along the Ossau gradient (2012) (mean percentage \pm SEM). Percentages by elevation correspond to the sites of both valleys at this elevation ± 50 m. Black bars: early-flushing trees; white bars: late-flushing trees.

elevations, late-flushing trees were much more infected than early-flushing trees ($P = 0.004$ and $P = 0.0001$ at 200 and 400 m asl, respectively), whereas infection did not vary with oak phenology at medium and high elevations ($P = 0.43$, $P = 0.88$ and $P = 0.22$ at 800, 1200 and 1600 m asl, respectively) (Fig. 5). Infection was strongly related to safety margin at low elevations ($P = 0.0001$ and $P = 0.0095$ at 150 and 400 m asl, respectively), whereas no correlation was demonstrated at medium and high elevations ($P = 0.70$, $P = 0.52$ and $P = 0.77$ at 800, 1250 and 1650 m, respectively).

Infection levels were much lower on seedlings than on adult trees (1.3% and 11% of the leaf area infected, respectively). At both sites, the infection rate was much higher among late-flushing seedlings than among early-flushing seedlings (55% versus 17% at 150 m, 33% versus 17% at 400 m), but this difference was significant only at 150 m ($P = 0.013$ and $P = 0.38$ at 150 and 400 m, respectively) (see Fig. S5). No effect of site \times year and site \times year \times phenology interactions on seedling infection was detected ($P = 0.68$ and $P = 0.53$, respectively). Seedling infection levels were correlated with safety margin at 150 m, but not at 400 m ($P = 0.0039$ and $P = 0.30$, respectively).

Discussion

Our study is one of the first to assess simultaneously the interactions of exposure to abiotic and biotic hazards with tree phenology. One striking result of this study was the considerable variation of spring frost and disease safety margins between oak populations as a function of elevation and within populations as a function of the phenological phenotype of trees (early vs. late flushing). Spring frost safety margins were generally positive, indicating an escape of spring frost in most situations. By contrast, most trees were exposed to and, indeed, infected by powdery mildew. This suggests that the local adaptation of oaks for spring frost escape is currently better than that for disease escape. For both hazards, safety margins tended to decrease along the elevation gradient. However, biotic and abiotic hazards interact in opposite directions with tree phenology. At low elevation, early flushing enabled trees to escape disease, while late flushing provided a

higher safety margin to spring frost damage. Overall, a wide safety window, resulting from both abiotic and biotic safety margins, was obtained at low elevations. These findings may account for the large within-population genetic variation observed for oak phenology, especially at low elevation (Alberto *et al.* 2011).

VARIATION IN ABIOTIC AND BIOTIC STRESS ESCAPE ALONG THE ELEVATION GRADIENT

The observed phenological patterns may be interpreted from an evolutionary perspective. The positive spring frost safety margins for most trees along the elevation gradients are consistent with an effect of past natural selection favouring phenotypes able to escape spring frost damage. Indeed, frost damage can incur a high fitness cost, especially on seedlings, and is thus expected to exert a strong selective pressure (Agrawal, Conner & Stinchcombe 2004; Alberto *et al.* 2011; Augspurger 2011; Pau *et al.* 2011). However, selection to escape late spring frosts would have been less effective at higher elevations, as expressed by smaller safety margins and observations of leaf frost damage in some years (2 out of the 9 years of monitoring, see Fig. S6). It has to be noted that an increased risk of leaf frost damage at high elevation is not always observed. Lenz *et al.* (2013) showed a constant safety margin against damage by spring freeze events in tree populations along elevational gradients. Constraints affecting the timing of flushing are likely to vary along geographic gradients and to produce different evolutionary outputs (Pau *et al.* 2011). At high latitude or elevation, fitness is more strongly correlated with the ability of fruit to ripen fully rather than with the probability of survival (Morin, Augspurger & Chuine 2007; Morin, Viner & Chuine 2008). This favours early flowering and, thus, early budburst (Franjic *et al.* 2011; S. Delzon personal observations), possibly at the expense of spring frost damage. The oak populations at 1600 m were almost at the tree line for this species (Aubert 2001; Belet 2001; Vitasse *et al.* 2012), that is the edge of the habitat in which these trees can thrive. The growing season was found to be short at such elevations. The presence of strong constraints on the timing of flushing at high elevation is consistent with the low variation in early spring phenology between trees in a given year (expressing genetic variation). However, trees displayed a high degree of plasticity, as demonstrated by the high year-to-year fluctuations in the timing of flushing (Fig. S2). This is consistent with the predictions of Pau *et al.* (2011) that trees at high elevations, which put out leaves in harsh physical conditions, are likely to be more sensitive to climate (temperature) cues.

Unlike late-frost safety margins, safety margins for powdery mildew were mostly negative, indicating an absence of escape. It can be hypothesized that selective pressure by powdery mildew on oak phenology has been lower than by frost. Fitness costs associated with foliar pathogens may be lower than with frost damage because complete leaf loss is less likely. Only high powdery mildew infection rates observed in dense oak regenerations under favourable environment and in mature trees following insect or frost defoliation can lead to

mortality (Marçais & Desprez-Loustau 2014). In addition, fitness costs of phenological maladaptation are higher for the pathogen (which needs to find susceptible hosts to complete its life cycle) than for the host, and generation times are shorter for the fungus. It is thus expected that the pathogen will always achieve a certain degree of phenological synchrony with its host, giving no chance to oaks to flush at a time when they would be safe from powdery mildew infection. The same reasoning can apply to other biotic antagonists (herbivore insects, other foliar pathogens), which exert their effects after flushing has occurred. Although safety margins for powdery mildew were increasingly negative with increasing elevation, infection did not increase above 400 m a.s.l. Both tree characteristics (lower foliage densities) and environmental conditions (shorter growing season, lower temperatures, longer leaf-wetness duration) less favourable to powdery mildew development (Jarvis, Gubler & Grove 2002) could limit disease severity at higher elevations even with good matching of oak and fungus phenologies.

VARIATION OF HAZARD ESCAPE WITHIN POPULATIONS

At low elevations, phenological differences between trees resulted in contrasting exposures to biotic and abiotic hazards. Although both early- and late-flushing trees showed mostly positive frost safety margins during the study period, it cannot be excluded that in rare late-frost events, late-flushing trees have an advantage over early-flushing trees due to their larger safety margin. Strong selection caused by rare catastrophic events has been suggested to play an important role in the evolution of natural populations (Agrawal, Conner & Stinchcombe 2004). On the other hand, only early-flushing trees escaped powdery mildew exposure. Our observations suggest that the 'spore cloud' reached all the trees at a given site at about the same time. No spatial effects could be detected on the first spore trappings within a site (spatial analysis, performed with ARCGIS 10.2 software; data not shown). Moreover, the timing of spore trapping was not related to host tree phenology. This suggests that no local phenological adaptation of powdery mildew populations occurred in response to variation within oak populations, by contrast to reports of the local adaptation of powdery mildew strains, at tree level, in terms of their ability to infect trees (Roslin, Laine & Gripenberg 2007; Desprez-Loustau *et al.* 2011). A lack of local adaptation of powdery mildew to achieve phenological synchrony with its host has already been reported over a larger spatial scale (Desprez-Loustau *et al.* 2010). This contrasts with findings for other antagonistic interactions with oaks (e.g. for caterpillars), for which adaptive deme formations have been observed at very fine spatial scales (van Asch & Visser 2007). Early- and late-flushing trees were thus exposed to different powdery mildew pressures (spore concentrations) during the flushing period, in populations growing at low elevation (< 500 m). This resulted in higher levels of infection in late-flushing trees than in early-flushing trees. At low elevation, escape of biotic (powdery mildew) and abiotic (spring frost) hazards therefore corresponds to opposite phenological

phenotypes. Such opposite selective forces may have contributed to the maintenance of local variability for the 'time of flushing' trait at low elevation (Alberto *et al.* 2011). The large difference between the last spring frost date and the sporulation date (i.e. the safety window) at low elevation makes it possible for oak populations to maintain this variability, with early-flushing trees escaping powdery mildew disease (and possibly damage due to other defoliators) and having a longer growing season, and late-flushing trees being more likely to survive late spring frost events. Seedlings in the low elevations sites displayed the same trends as adult trees, with early-flushing seedlings being at an advantage with respect to powdery mildew and late-flushing seedlings being at an advantage with respect to spring frosts. This was important to check because selective forces on tree species are strongest during the juvenile stage.

ESCAPE VERSUS OTHER STRATEGIES OF DEFENCE AGAINST BIOTIC AND ABIOTIC HAZARDS

Our study focused on phenological variations as related to escape of abiotic and biotic hazards. Escape, that is processes and mechanisms preventing encounter with the deleterious agent, is the first line of defence (Combes 2001), in particular temporal escape for sessile organisms such as trees (Vitasse, Lenz & Körner 2014). However, organisms can use a second line of defence composed of two types of mechanisms, those which prevent or limit the damage caused by the deleterious agent and those which reduce the negative consequences of damage for fitness. The former are referred to avoidance or resistance according to authors and research fields, while tolerance corresponds to the latter (see e.g. Burdon 1987; Agrawal, Conner & Stinchcombe 2004; Baucom & de Roode 2011; Vitasse, Lenz & Körner 2014, for terminology issues and definitions).

During winter, leaf tissues enclosed within buds remain frost hardy, but their vulnerability to freezing temperatures gradually increases prior to and just after budburst (Kalberer, Wisniewski & Arora 2006; Lenz *et al.* 2013). Young leaves are particularly vulnerable to frost and many pathogens and herbivores, due to their anatomical, physiological and biochemical properties (Coleman 1986). The escape of spring frost by late flushing may be a major adaptive process along the elevation gradient (Gömöry & Paule 2011). Trees have optimized the timing of budburst in relation to the probability of frost in spring, by developing complex mechanisms of chilling/thermal time requirement and photoperiod (Cannell 1997; Basler & Körner 2012; Laube *et al.* 2013). By delaying flushing, trees maintain their winter frost hardiness for as long as possible, relative to adjustments of growing season length. A frost escape strategy may prevail in *Q. petraea* since this species was shown to be among the less freezing-tolerant species during bud burst and leaf unfolding (Lenz *et al.* 2013). However, a possible genetic differentiation for frost avoidance or tolerance between populations of *Q. petraea* along elevational gradients would deserve investigation (Vitasse, Lenz & Körner 2014).

Disease escape as a defence strategy in plants (trees) has been little documented (but see Ghelardini & Santini 2009), especially compared to the large number of studies related to insect herbivory (Aide 1992; Tikkanen & Julkunen-Tiitto 2003; Lamarre *et al.* 2014). Conversely, resistance and tolerance mechanisms are thought to play a critical role in the adaptation of plants to pathogens (Bell 1981; Burdon 1987; Combes 2001) and have been well documented for powdery mildew diseases (Glawe 2008; Desprez-Loustau *et al.* 2014). In particular, plants can rely on induced resistance (Verhagen, Van Loon & Pieterse 2006; Kempel *et al.* 2011), which is expressed only in response to the biotic stressor and is thus a valuable strategy in terms of costs, against non-permanent stress factors such as diseases or pests (Agrawal 1999; Agrawal & Karban 1999). Thus, at low elevation where environmental conditions are favourable to the fungus, oak defence against powdery mildew may be based on both phenological escape and resistance. At higher elevations, oak resistance to powdery mildew would be sufficient to ensure that infection rates remain moderate, due to less favourable environmental conditions for pathogen populations.

IMPLICATIONS IN A CONTEXT OF CLIMATE CHANGE

Local patterns of adaptation resulting from past selection processes may not be suitable in the face of rapid changes in environmental conditions. Climate change is causing many trees to flush earlier in spring, exposing them to new hazards. Several studies have found that this major advance in the date of leaf unfolding may increase the frequency of spring frost damage (Kramer 1995; Hänninen 2006; Schwartz, Ahas & Aasa 2006; in Northern and Eastern Europe). However, some studies have indicated that spring frost risk is declining with climate change, because the date of the last frost is advancing more rapidly than flushing dates (Schwartz, Ahas & Aasa 2006; in Western Europe; Anandhi *et al.* 2013; Ge, Wang & Dai 2013). This pattern may also be accentuated if the timing of flushing does not advance because chilling requirements remain unmet (Fu *et al.* 2012; Laube *et al.* 2013; Dantec *et al.* 2014). Climate change will also alter the phenological relationships between interacting species, because responses to climate change may differ between species (Both *et al.* 2009; Singer & Parmesan 2010), as shown here for powdery mildew and oak. Given the complexity of climatic effects on the exposure of trees to biotic and abiotic hazards, process-based models of tree and fungus phenology (including chilling and thermal requirements) will be required to explore outputs in the future, using local climate scenarios.

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Data accessibility

Data available from Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.b02tf> (Dantec *et al.* 2015).

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Supporting Information

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

Figure S1. Meteorological data: daily minimum temperatures from January to May 2011 in the lowest and highest study sites

Figure S2. Year-to-year fluctuations of spring phenology in oak populations along the elevation gradients.

Figure S3. Safety window, i.e. difference in days between the date of the beginning of powdery mildew sporulation and the date of the last spring frost event- with 0°C and -3°C thresholds for frost (Mean ± SD over 4 years)

Figure S4. Powdery mildew (A) and spring frost (B; threshold = 0°C) safety margins of early- and late-flushing oak seedlings.

Figure S5. Differences in powdery mildew infection between early- and late-flushing oak seedlings.

Figure S6. Late-frost damage to newly-emerged leaves on an oak tree at 1600 m asl in the Pyrénées in 2007. Leaves did not develop and were shed rapidly. Late frost damage was observed two times since 2006 and damages were higher in early-flushing trees at high elevation.