

Chilling outweighs photoperiod in preventing precocious spring development

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Abstract

It is well known that increased spring temperatures cause earlier onset dates of leaf unfolding and flowering. However, a temperature increase in winter may be associated with delayed development when species' chilling requirements are not fulfilled. Furthermore, photosensitivity is supposed to interfere with temperature triggers. To date, neither the relative importance nor possible interactions of these three factors have been elucidated. In this study, we present a multispecies climate chamber experiment to test the effects of chilling and photoperiod on the spring phenology of 36 woody species. Several hypotheses regarding their variation with species traits (successional strategy, floristic status, climate of their native range) were tested. Long photoperiods advanced budburst for one-third of the studied species, but magnitudes of these effects were generally minor. In contrast to prior hypotheses, photosensitive responses were not restricted to climax or oceanic species. Increased chilling length advanced budburst for almost all species; its effect greatly exceeding that of photoperiod. Moreover, we suggest that photosensitivity and chilling effects have to be rigorously disentangled, as the response to photoperiod was restricted to individuals that had not been fully chilled. The results indicate that temperature requirements and successional strategy are linked, with climax species having higher chilling and forcing requirements than pioneer species. Temperature requirements of invasive species closely matched those of native species, suggesting that high phenological concordance is a prerequisite for successful establishment. Lack of chilling not only led to a considerable delay in budburst but also caused substantial changes in the chronological order of species' budburst. The results reveal that increased winter temperatures might impact forest ecosystems more than formerly assumed. Species with lower chilling requirements, such as pioneer or invasive species, might profit from warming winters, if late spring frost events would in parallel occur earlier.

Keywords: asynchrony, budburst, climate chamber experiment, climate change, dormancy, invasive species, phenology, photosensitivity, successional strategy

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Introduction

The effects of climate change on the timing of plant life-cycle events, such as leaf-out, flowering and fruit ripening, have been reported in an impressive, still increasing body of literature (Sparks *et al.*, 2009). Changes in phenological cycles are of interest for both agriculture and forestry, and of importance for net primary production and carbon uptake (Picard *et al.*, 2005; Piao *et al.*, 2008; Richardson *et al.*, 2012). Due to vegetation-atmosphere feedbacks, phenological changes also impact climate (Menzel, 2002; Richardson *et al.*, 2013).

Terrestrial plants are the species group with highest phenological response rates to ongoing climate change (Thackeray *et al.*, 2010). In Europe, an overall advance

in spring and summer events of 2.5 days per decade (1971–2000) has been reported (Menzel *et al.*, 2006), with highly differential species-specific shifts (Menzel *et al.*, 2001; Cleland *et al.*, 2007; Willis *et al.*, 2008). As the cumulative knowledge concerning onset requirements is restricted to a limited number of species, phenological responses to climate change remain in large part unpredictable, also when based on plant functional types (Polgar & Primack, 2011).

Several factors are considered to influence phenological onset dates, with general agreement that three triggers are of major concern with respect to climate change: spring temperature, winter temperature and spring day length. First, an increase in spring (forcing) temperature leads to a general shift towards earlier spring onset (Menzel *et al.*, 2001; Chuine *et al.*, 2010; Polgar & Primack, 2011). A certain duration of cold conditions is necessary for rest completion

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(endodormancy), and the fulfilment of chilling requirements may generally decrease the need for warm (forcing) temperatures. Thus, second, warmer winter temperatures are expected to delay spring onset for species with high chilling requirements (Murray *et al.*, 1989; Heide, 1993a; Yu *et al.*, 2010; Hanninen & Tanino, 2011; Polgar & Primack, 2011). Because the effect of incomplete rest/lack of chilling has been hypothesized to cause relatively minor delays in onset dates compared to spring advances, current climate change has generally led to an advance in spring onset (Thompson & Clark, 2008). However, both modelling (Morin *et al.*, 2009) and field observation (Yu *et al.*, 2010) have shown that net delays of onset due to incomplete rest are already occurring for some species and ecosystems. This delaying effect of warm (mild) winters is expected to gain importance as temperature increases become more pronounced (Vitasse *et al.*, 2011). Third, photoperiod might also play a role: Photosensitive species (more correctly, species responsive to photoperiod) are believed to show a weaker response to spring temperature since shorter day lengths during 'earlier springs' might counterbalance the effects of higher temperature.

Effects of all three factors described above have been shown to be highly species specific (Ghelardini *et al.*, 2010; Polgar & Primack, 2011), and unpredictable interactions are expected to trigger spring phenology in future climates. While spring temperature responses have been shown to be most pronounced for early season species and events (Menzel *et al.*, 2006; Polgar & Primack, 2011), effects of chilling requirements have been suggested to be most pronounced for late successional species (Caffarra & Donnelly, 2011; Polgar & Primack, 2011; Basler & Körner, 2012). Photosensitivity has been suggested to correspond to both species origin and life strategy (Körner & Basler, 2010; Basler & Körner, 2012), with pioneer species mainly reacting to spring temperatures, and climax species additionally reacting to photoperiods (Caffarra & Donnelly, 2011). As temperature increase is a less reliable signal of spring in oceanic climates, species with oceanic distributions have been suggested to additionally rely on day length as a cue, whereas continental species are allegedly less responsive to photoperiod (Körner & Basler, 2010).

Phenology seems to facilitate plant invasions as well, as many invasive species show earlier flowering onsets and enhanced flowering periods when compared to native species (Hulme, 2011a; Wolkovich & Cleland, 2011). Invasive species also often profit from prolonged vegetation periods, but while later leaf falls in autumn have been consistently reported (Harrington *et al.*, 1989; Fridley, 2012), it remains unclear whether invasive species are also able to better track spring warming

than native species (Harrington *et al.*, 1989; Xu *et al.*, 2007; Davis *et al.*, 2010; Willis *et al.*, 2010; Hulme, 2011b) or not (Fridley, 2012).

Körner & Basler (2010) summarized their hypotheses in a picture of twigs with *Syringa vulgaris* as an exotic species only reacting to spring warming, *Carpinus betulus* as a pioneer species additionally responding to chilling and *Fagus sylvatica* as a climax species being sensitive to both chilling and photoperiod.

To date phenological research has focused on responses to photoperiod and spring temperature (Hanninen & Tanino, 2011). Previous experimental attempts to disentangle and quantify the impact of chilling and photoperiod were restricted to a limited number of species (Rollinson & Kaye, 2012), which hampers possible generalizations with respect to successional strategy, origin or species traits. In this study, we present a climate chamber experiment with a large number of woody species ($n = 36$) of different successional strategies (pioneer to climax), climatic origins (oceanic to continental) and floristic status (native, ornamental and invasive). We studied the effects of photoperiod and chilling as well as their interactions on forcing requirements till budburst. Our aim was to test the following hypotheses: (i) climax species have higher chilling and forcing requirements than pioneer species; (ii) climax species show a higher responsiveness to photoperiod than pioneer species; (iii) invasive species are less photosensitive than native species; and (iv) species with an oceanic distribution are more responsive to photoperiod than continental species. To the best of our knowledge, no experiment has previously examined the importance and possible interaction of chilling and photoperiod for such a wide range of species under controlled conditions.

Materials and methods

Experiment

We implemented a full factorial climate chamber experiment with combinations of three chilling and three photoperiod treatments to investigate phenological development during a 6-week forcing period.

Twigs, ca. 30 cm long, of 36 different woody species (see Tables 1 and S1) were cut within the same forest site ('Weltwald') near Freising (Bavaria, Germany) from trees of comparable microclimates (forest edge or forest roadside). Twigs were cleaned, disinfected with commercial hypochlorite solution and put into 0.1 l glass bottles filled with tap water. For each species, we kept 10 replicate twigs (originating from three donor trees) per chamber (fully randomized). To assure proper water supply, twigs were recut and water was changed every second week. The development stage of buds was recorded three times per week using a scheme following

Table 1 Effects of chilling and photoperiod on forcing sums needed until budburst. Reported are *P*-values of log-rank tests. Median C1/C3: estimates for chilling 1/chilling 3; p chill.: *P*-value of chilling effect; Median P8/P16: estimates for photoperiod 8 h/photoperiod 16 h; p photo: *P*-value of photoperiod effect; p photo C1: *P*-values of photoperiod effects within chilling 1 (low chilling); p photo C2: *P*-values of photoperiod effects within chilling 2 (intermediate chilling); ns: not significant ($P > 0.05$); bold: significant ($P < 0.05$). Rest completed C2: species with either difference C2/C3 not significant or forcing C2<C3, which identifies species for which chilling requirements should have been fully met with chilling 2 treatment. Acronyms for successional strategy (succ. strategy): p (pioneer), i.e (intermediate), c (climax); floristic status: n (native), orn (ornamental), inv (invasive). In chilling 3, only *Aesculus hippocastanum* showed reduced forcing requirements with increased photoperiod ($P = 0.008$). Numbers of twigs are given in Table S4

Species	Short name	Median C1/C3 °C days	p chill.	Median P8/P16 °C days	p photo.	p photo. C1	p photo. C2	Rest completed C2	Succ. strategy	Flor. status
<i>Abies alba</i>	Abialb	576/403	<0.001	403/403	ns	0.012	ns	x	c	n
<i>Abies homolepis</i>	Abihom	NA/403	0.117	403/374	ns	NA	ns	x	c	orn
<i>Acer negundo</i>	Aceneg	NA/212	<0.001	291/234	ns	ns	ns		i.e	inv
<i>Acer pseudoplatanus</i>	Acepe	NA/403	<0.001	539/539	ns	0.037	ns		c	n
<i>Acer saccharum</i>	Acesac	NA/389	<0.001	NA/639	ns	ns	ns		c	orn
<i>Acer tataricum</i>	Acetat	369/262	<0.001	262/285	ns	ns	ns	x	p	orn
<i>Aesculus hippocastanum</i>	Aeship	624/321	<0.001	539/479	ns	ns	0.012		i.e	inv
<i>Amorpha fruticosa</i>	Amofru	369/519	<0.001	442/539	ns	ns	ns	x	p	inv
<i>Betula pendula</i>	Betpen	300/194	<0.001	231/221	ns	ns	ns		p	n
<i>Carpinus betulus</i>	Carbet	576/194	<0.001	244/231	ns	ns	ns		c	n
<i>Cornus alba</i>	Coralb	334/262	<0.001	262/254	0.001	<0.001	ns	x	p	inv
<i>Cornus mas</i>	Cormas	300/231	<0.001	262/254	ns	ns	ns	x	p	n
<i>Corylus avellana</i>	Corave	254/194	<0.001	194/175	0.046	0.007	ns	x	p	n
<i>Fagus sylvatica</i>	Fagsyl	NA/334	<0.001	NA/442	ns	ns	<0.001		c	n
<i>Fraxinus chinensis</i>	Frachi	543/403	<0.001	466/475	ns	ns	ns	x	i.e	orn
<i>Fraxinus excelsior</i>	Fraexc	624/458	0.043	483/456	ns	ns	ns	x	i.e	n
<i>Fraxinus pennsylvanica</i>	Frapen	426/434	0.319	442/416	ns	ns	ns	x	i.e	inv
<i>Juglans ailantifolia</i>	Jugail	624/374	<0.001	442/406	ns	ns	ns		i.e	orn
<i>Juglans cinerea</i>	Jugcin	624/403	<0.001	442/442	ns	ns	ns		p	orn
<i>Juglans regia</i>	Jugreg	624/519	0.200	624/576	ns	0.016	ns	x	i.e	inv
<i>Larix decidua</i>	Lardec	300/194	<0.001	210/208	ns	ns	ns		p	n
<i>Picea abies</i>	Picabi	NA/403	<0.001	389/334	ns	NA	ns	x	c	n
<i>Pinus nigra</i>	Pinnig	543/519	<0.001	509/473	ns	ns	ns	x	p	inv
<i>Pinus strobus</i>	Pinstr	426/308	<0.001	308/321	ns	0.006	ns	x	i.e	inv
<i>Pinus sylvestris</i>	Pinsyl	600/334	<0.001	365/334	ns	ns	ns	x	p	n
<i>Pinus wallichiana</i>	Pinwal	334/285	<0.001	285/262	ns	0.001	ns	x	p	orn
<i>Populus tremula</i>	Poptre	509/308	<0.001	442/442	ns	0.021	0.039		p	n
<i>Prunus avium</i>	Pruavi	466/203	<0.001	231/257	ns	ns	ns		i.e	n
<i>Prunus serotina</i>	Pruser	509/194	<0.001	231/212	ns	ns	ns	x	i.e	inv
<i>Pseudotsuga menziesii</i>	Psemen	509/374	<0.001	374/403	ns	ns	ns		c	inv
<i>Quercus bicolor</i>	Quebic	624/308	<0.001	624/442	0.003	0.003	ns		i.e	orn
<i>Quercus robur</i>	Querob	509/374	<0.001	442/438	ns	ns	ns		i.e	n
<i>Quercus rubra</i>	Querua	NA/374	<0.001	626/560	ns	ns	ns		i.e	inv
<i>Robinia pseudoacacia</i>	Robpse	426/296	<0.001	297/297	ns	ns	ns	x	p	inv
<i>Symphoricarpos albus</i>	Symalb	426/194	<0.001	208/212	ns	ns	ns		p	inv
<i>Syringa vulgaris</i>	Syrvul	211/203	<0.001	197/194	0.023	0.026	ns	x	p	inv

BBC codes (Meier, 2001). Twigs that had not reached budburst by the end of week 6 were removed from the chambers and taken into a glasshouse with a mean temperature of 20 °C and 16 h day length. There, twig development was monitored three times per week for the next 4 weeks or until budburst to confirm whether these twigs were still alive.

Different chilling treatments (C1–C3, low to high) were achieved by different lengths of exposure to natural (outdoor)

chilling conditions (for rough estimates of chilling conditions, see Table 2 and Figure S1). Cutting dates were 14.12.2011 (C1), 30.01.2012 (C2) and 14.03.2012 (C3), thus roughly dividing meteorological winter between winter solstice and spring equinox into three 6-week segments. First cutting date was shortly after typical leaf fall in the area (mid to end of November) to ensure that all species had entered dormancy, last cutting date was clearly before leaf-out in spring.

Table 2 Description of the three chilling treatments. Chill days (days with mean temperature below 5 °C since November 1st (according to Murray *et al.*, 1989) are achieved by outdoor temperature conditions prior to the start of the experiment (date when twigs were cut). As an accumulation of forcing units (high temperatures forcing development) also occurred in the field, the thermal time (in degree days above 0 °C since January 1st according to Heide, 1993a) at cutting dates is also given

Treatment	Start of experiment (dd.mm.yyyy)	Chill days (days)	Thermal time (°C days)	Day length at start of experiment (h : min)
Chilling 1 (low)	14.12.2011	33	0	8 : 21
Chilling 2 (intermediate)	30.01.2012	73	64	9 : 22
Chilling 3 (high)	14.03.2012	110	150	11 : 49

Climate chambers differed in photoperiod treatments, with daily photoperiods of 8, 12 and 16 h (P8, P12 and P16). Photon flux in all chambers was approximately $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ (about 10 kLux, which is roughly the amount of an overcast summer day, typical values for winter overcast days are about 1/3 of that value).

The temperature programme in the chambers for photoperiod and chilling treatments was identical, starting with 7 °C, daily raised by 0.5 °C, until 27.5 °C was reached at the end of the experiment (end of week 6). From week 2 onwards, temperature at night was reduced by 2 °C, gradually increasing to 5 °C at the end of week 6. Relative humidity was held constant at around 70%. During the second chilling experiment, an electrical failure affected all three chambers for 25 h. During this period all chambers were dark, and temperatures increased to 18 °C (target 8.5 °C). As all three chambers were equally affected, we believe within-chilling comparisons (comparison of different photoperiod treatments within chilling 2) should be largely unaffected.

Species traits

A full species list with all further information is given in Table S1. *Abies homolepis* and *Picea abies* were included only in intermediate and high chilling experiments (hence are missing for the low chilling treatment). Information on species traits was compiled from different databases. Climatic distribution of native ranges followed BIOLFLOR (Klotz *et al.*, 2002), else estimated, and was reduced to three main groups (oceanic, intermediate and continental). Taxonomy and floristic status (native, invasive and ornamental; ornamental being nonnative species which are not known to be invasive) were listed as in FloraWeb (BfN, 2013). The classification into successional strategies followed both growth form (mainly LEDA traitbase (Kleyer *et al.*, 2008)) and shade tolerance (Niinemets &

Valladares, 2006). This simple, but objective approach led to reproducible groups. These also showed consistency with respect to other traits usually linked to species' life strategies (for details see Tables S2 and S3).

Field observations

The phenological development of donor trees in the field was monitored weekly using the same methods as for the experiment. Field weather data were obtained from an adjacent meteorological station (<2 km from the field site) of the German meteorological service.

Analysis

Twigs that did not achieve budburst within 10 weeks, including the 4-week posttreatment observation period, were considered dead and removed from analysis, resulting in 2890 twigs of 3171 (91%) used for analysis. Broken down by chilling and photoperiod treatments this resulted in 89% of all twigs used for chilling 1, 90% for chilling 2 and 95% for chilling 3, 89% for the 8 h photoperiod, 86% for the 12 h and 92% for the 16 h. Results reported throughout the study refer to the number of living twigs. The reason for discarding twigs was that their deaths were most likely influenced by severe wounds from cutting. Thus, they would never have the opportunity for normal development and subsequent measurement of the primary outcomes. Nevertheless, main results were checked for bias towards discarding.

The focus of our analysis is on the budburst phenophase (BBCH7) since this is the first, also clearly defined sign of spring development, and its choice reduced the numbers of censored data (later phases were not always reached within the 6 weeks of observation in the climate chambers). The total amount of forcing until budburst was analysed as the response variable, however, the numbers of days until budburst are also shown (but not analysed). The total absolute amount of forcing is the sum of degree days, including both field and experimental conditions (starting date of accumulation: 1st January, base threshold 0 °C (Basler & Körner, 2012; Heide, 1993a)). The number of chill days was calculated as the number of days with mean temperatures ≤ 5 °C, starting November 1st (Murray *et al.*, 1989; Polgar & Primack, 2011; Basler & Körner, 2012). Please note this encompasses forcing units received in the field (see Table 2; Figure S1) and enhanced temperatures during the brief power blackout that occurred within chilling treatment 2.

Binomial generalized linear models were used to assess the impact of the treatment conditions (chilling and photoperiod) on the species' ability to reach budburst. Models with or without interaction terms were compared using Akaike's Information Criterion (AIC), which penalizes the negative likelihood function of the model by the number of parameters. Models with smaller AIC are preferred as those that will most likely have better out-of-sample validation properties. Therefore, the model with the smallest AIC value was chosen for reporting.

Due to the limited treatment period of 6 weeks, the response (forcing sums) may be right censored, in case that

budburst was not observed within the period of 6 weeks. For this type of data, forcing sums that occur before the end of observation are analysed as is, while those that occur afterwards are 'censored' as being greater than 6 weeks, but not known more precisely than that. This type of data is called right-censored survival data and requires special survival analysis. All calculations were performed in R statistical software and the Survival package (Therneau, 2013). The original use of survival analysis considers survival times and death rates, hence 'survival' in our notation is equivalent to 'no budburst', while medians are Kaplan–Meier estimates of the forcing sum at which 50% of the twigs reached budburst. Statistical significance of the treatment effect on the forcing sums was assessed using nonparametric log-rank tests, which are based on differences in survival curves (Harrington & Fleming, 1982). Assumptions for Cox proportional hazard regression models (Grambsch & Therneau, 1994) were not met, therefore several log-rank tests regarding different groups of species and different treatment hypotheses were performed. As multiple comparisons were performed, the danger of an inflation of type 1 error was present. Therefore, results with adjusted significance levels, using Holm's sequential Bonferroni method (Holm, 1979), are additionally shown. Analyses of species groups were adjusted for 10 groups (complete data set, and nine groups of species), and the four different tests were performed (overall chilling effects, overall photoperiod effects, photoperiod effect within chilling 1 and difference of chilling effect between chilling 2 and 3), yielding a total number of tests as $t = 40$.

The level of asynchrony (changes in species sequence, order of budburst) was assessed by Spearman's rank correlation. We compared the chronological rank order of median species budburst under experimental and field conditions. Differences in median ranks of species groups were tested by Kruskal–Wallis tests.

Results

The effect of chilling and photoperiod on ability to achieve budburst

The percentage of cuttings that achieved budburst in chilling 1 (low chilling) was lowest at 83% ($N = 902$), followed by chilling 2 (intermediate) at 95% ($N = 967$) and chilling 3 (high chilling) at >99% ($N = 1022$) ($P < 0.001$). Differences in budburst among photoperiod treatments did not exceed 2% (from 92%, 8 h, $N = 942$, to 94%, 16 h, $N = 972$, $P = 0.087$).

Results for single species (see Table S4) indicated that long photoperiod significantly enhanced the ability to achieve budburst for four species: *Abies alba* (P8: 85% to P16: 100%), *Acer pseudoplatanus* (P8: 64% to P16: 86%), *Fagus sylvatica* (P8: 46% to P16: 80%) and *Juglans regia* (P8: 81% to P16: 90%). Increased chilling significantly enhanced budburst ability for *Acer pseudoplatanus* (C1: 29% to C3: 100%), *Fagus sylvatica* (C1: 10% to C3: 100%), *Fraxinus excelsior* (C1: 78% to C3: 100%), *Juglans*

ailanthifolia (C1: 75% to C3: 100%), *Juglans regia* (C1: 85% to C3: 91%) and *Pinus sylvestris* (C1: 77% to C3: 100%) (for P -values see Table S4).

Treatment effects on forcing requirements

Figure 1 shows the effects of chilling and photoperiod treatments aggregated across all species (all single species survival curves are given in Figure S2). For the number of days until budburst, a clear advance in budburst with increased chilling occurs for all three chilling treatments, with medians of 15 (C3), 22 (C2) and 36 days (C1) (Fig. 1a). For the total amount of forcing (given in degree days until budburst), chilling 1 separated clearly with higher medians until budburst, whereas chilling treatments 2 and 3 differed only for late budbursts. Early onsets (until approximately 330 °C days) showed no difference in forcing requirements between chilling treatments 2 and 3 (Fig. 1b).

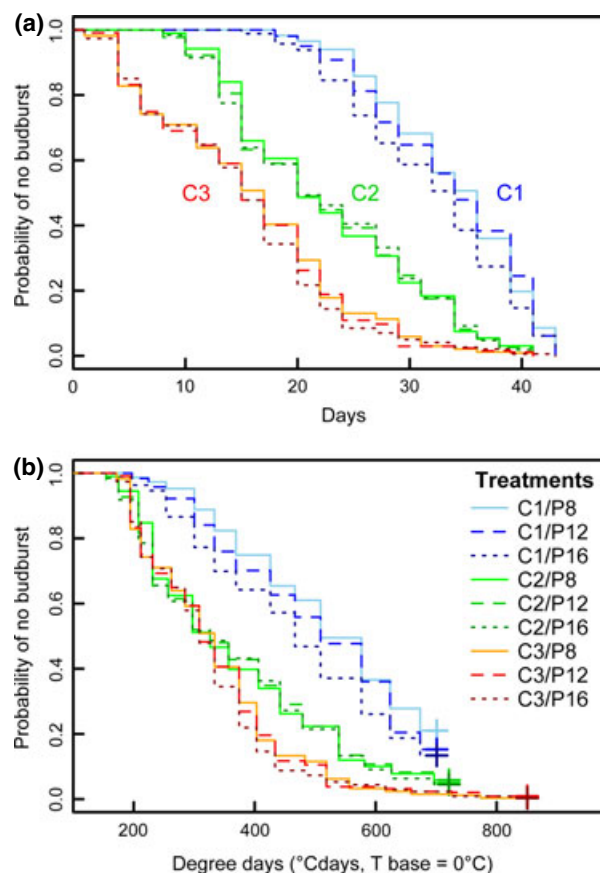


Fig. 1 Spring development ('survival') curves for all treatments, calculated for (a) the number of days in climate chamber experiment and (b) absolute degree days of forcing (field and chamber) until budburst. Treatments given as C1: low chilling, C2: intermediate chilling, C3: high chilling. Photoperiod P8: 8 h, P12: 12 h, P16: 16 h daily photoperiod.

Median forcing requirements were considerably higher for chilling 1 (509 °C days, 95% conf. intervals (CI) 509/509 °C days), than for chilling 2 (326 °C days, CI 297/357 °C days) and chilling 3 (308 °C days, CI 308/334 °C days) ($P < 0.001$). In comparison, differences in median forcing requirements according to photoperiod treatment were considerably smaller (photoperiod 16 h: 357 °C days, CI 334/369 °C days; photoperiod 12 h: 374 °C days, CI 357/374 °C days; 8 h: 374 °C days, CI 369/403 °C days) ($P = 0.018$). Even within chilling 1, where the largest differences between photoperiod treatments occurred, the difference in median forcing requirements was only 43 °C days; 466 °C days (16 h) and 509 °C days (8 h).

A reduction in forcing requirement with longer photoperiod proved to be significant within chilling 1 ($P = 0.002$), but not in chilling treatments 2 or 3 ($P > 0.05$). In contrast, a reduction in forcing requirements between chilling treatments 2 and 3 was highly significant ($P < 0.001$).

Treatment effects on forcing requirements of different groups of species

To assess whether species responses were linked to species traits, treatment effects were analysed for the different groups of species individually. The effects of chilling are shown in Table 3.

Table 3 Treatment effects on forcing requirements for different groups of species. Median C1: Kaplan–Meier estimate for chilling 1 (low); Median C2: Kaplan–Meier estimate for chilling 2 (intermediate); Median C3: Kaplan–Meier estimate for chilling 3 (high); p chilling: P -values of log-rank tests for chilling effects; p C2/C3: P -values of chi-square test for differences between chilling treatments 2 and 3; ns: not significant ($P > 0.05$); bold: significant ($P < 0.05$)

	Median C1 (°C days)	Median C2 (°C days)	Median C3 (°C days)	p chilling	p C2/C3
Successional strategy					
Pioneer	369	231	262	<0.001	ns
Intermediate	576	442	334	<0.001	<0.001
Climax	674	357	374	<0.001	<0.001
Floristic status					
Native	509	297	308	<0.001	<0.001
Invasive	509	297	308	<0.001	0.020
Ornamental	576	406	374	<0.001	<0.001
Climatic range					
Oceanic	509	326	334	<0.001	<0.001
Intermediate	509	406	334	<0.001	<0.001
Continental	426	257	285	<0.001	0.002

Regarding successional strategy, climax species showed the greatest forcing requirements for budburst (374 °C days, CI 374/374 °C days (C3), to 674 °C days (C1), CI 624/NA °C days), and the reduction in forcing requirements between chilling 2 and 3 was highly significant, equally for intermediate species. Pioneer species had both the smallest forcing requirements (262 °C days, CI 262/262 °C days (C3), 369 °C days, CI 334/369 °C days (C1)), and showed no significant differences between chilling treatments 2 and 3. The effects of photoperiod were not significant for any of the groups, but when calculated for chilling 1 separately, it proved to be significant within all groups (pioneer: $P = 0.020$, intermediate: $P = 0.040$, climax: $P = 0.018$) (survival curves are given in Figure S3).

Regarding floristic status, the median estimates of forcing requirements for native and invasive species under all treatment combinations, including all photoperiod treatments, were identical (308 °C days, native CI 285/308 °C days, invasive CI 308/308 °C days (C3) and 509 °C days, both CI 466/509 °C days (C1)), but higher for ornamental species (374 °C days, CI 374/374 °C days for C3 to 576 °C days, CI 574/624 °C days for C1). There was a statistically highly significant reduction in forcing requirements between chilling treatments 2 and 3 for native and ornamental species (both $P < 0.001$), and a lesser reduction for invasive species ($P = 0.020$) (see Table 3). A reduction in forcing requirements by longer photoperiod was statistically significant only for native species ($P = 0.021$) (survival curves are given in Figure S3).

Among groups with different climatic ranges, species with a continental distribution had the lowest forcing requirement from 285 °C days, CI 262/285 °C days (C3) to 426 °C days, CI 426/509 °C days (C1). Photoperiod had no significant effect in any group, except when calculated for chilling 1 separately when significant effects for oceanic species were found ($P = 0.020$). There was a statistically significant reduction in forcing requirements between chilling treatments 2 and 3 for all groups (survival curves are given in Figure S3).

Regardless of successional strategy, floristic status or climatic range, the reduction in forcing requirements with chilling was highly significant for all groups. In contrast, an overall reduction in forcing requirements with longer photoperiod was significant only for native species, while within chilling 1 photosensitive responses were found for pioneer, intermediate and climax as well as oceanic groups.

P -values adjusted for multiple comparisons are given in Table S5; qualitative statistical assessment of these departed only slightly from Table 3. However, all photoperiod effects on forcing requirements were non-significant after adjustments. Differences between C2

and C3 proved to be nonsignificant for invasive and pioneer species. Chilling effects remained highly significant for all groups.

Treatment effects on individual species forcing requirements

Four species showed an overall reduction in forcing requirements with longer photoperiod (*Cornus alba*, *Corylus avellana*, *Quercus bicolor* and *Syringa vulgaris*). Photoperiod effects within chilling 1 or chilling 2 were found for an additional eight species (results for all species are given in Table 1).

As Figure 2 shows, a decrease in forcing requirements with increased chilling exposure was found for almost all species. Out of the species already included in chilling 1, chilling reduced forcing requirements significantly for all but three species (*Amorpha fruticosa*, *Fraxinus pennsylvanica* and *Juglans regia*). In total, 19 species showed no further reduction in forcing requirements between chilling treatments 2 and 3, hence can be assumed to be fully chilled under chilling 2.

Calculations based on the complete data set (including the 4-week posttreatment observation period and based on 100% of twigs, which means also the nondeveloping twigs) departed only slightly: While photoperiod effects on *Acer pseudoplatanus* (climax, native) failed to achieve significance ($P = 0.062$), additional significant effects of photoperiod for *Pinus sylvestris* (pioneer, native, $P = 0.032$, C1) and *Pseudotsuga menziesii* (climax, invasive, $P = 0.050$, overall effect) were found. Chilling effects for *Fraxinus excelsior* (intermediate successional strategy, native) were only borderline significant ($P = 0.054$).

Effects of reduced chilling exposure on budburst sequence of species

As species responses to different chilling treatments differed considerably, we assessed how individual shifts affected the chronological order of species to budburst. Therefore, rank correlations of budburst under field and experimental conditions were calculated (correlation statistics given in Table S6). While

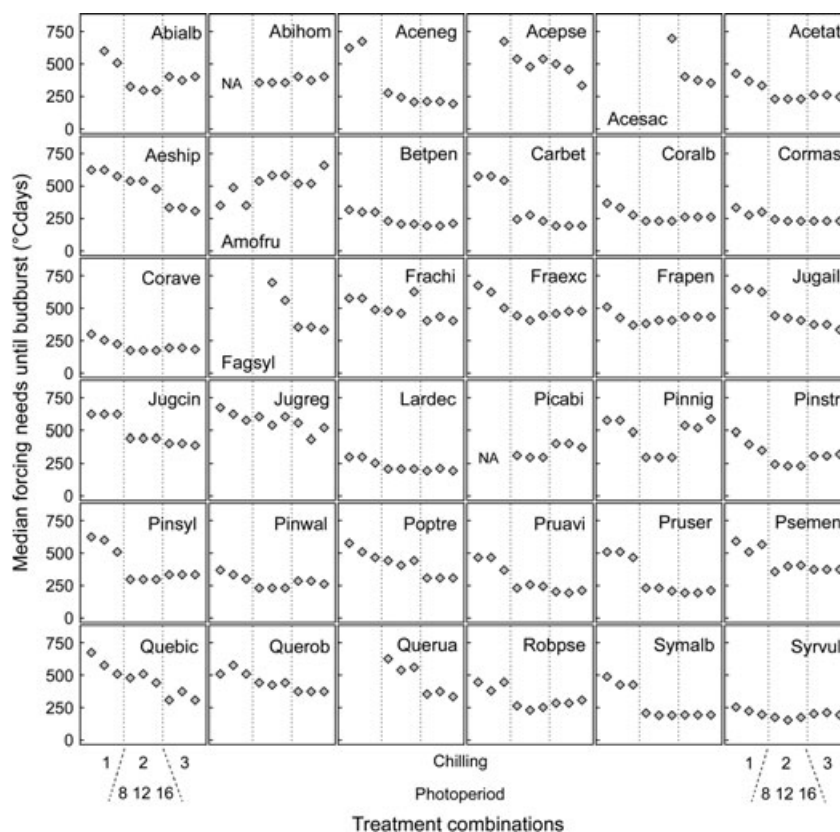


Fig. 2 Median forcing requirements until budburst for all treatments. The x-axis divides all different treatment combinations, with chilling 1, 2, 3: low, intermediate and high chilling, and photoperiods 8, 12, 16: 8 h, 12 h and 16 h photoperiod daily. Species abbreviated names are given in Table 1.

photoperiod treatments only led to minor changes in species order, chilling treatments had major impacts. Reduced chilling led to high departures of rank orders. For the lowest chilling treatment (C1), correlation coefficients (r_s) were as low as 0.51 (P8) and 0.54 (P12/P16). Overall the species order was closest to the field sequence for chilling 3 and 12 h photoperiod ($r_s = 0.89$), which also was the treatment closest to natural spring conditions in field with respect to photoperiod and chilling.

Shifts in the order of budburst were only linked to successional strategy, whereas for floristic status and climatic range no significant between-group differences were found (Kruskal–Wallis tests based on rank orders of budburst). With reduced chilling, pioneer species advanced in order of budburst, while climax species showed a relative delay. Therefore, differences in ranks between successional strategies were significant within the low and intermediate chilling treatments (C1: $P = 0.001$, C2: $P = 0.005$), but not under high chilling treatment (C3) and in field observations (see Fig. 3). Pioneer species were also quite early to budburst after long chilling (C3 and field), but differences from intermediate and climax species clearly increased with a reduction in chilling exposure.

Besides this relative advance of pioneer species, or relative delay of climax species, reduced chilling influenced the sequence of species in a rather chaotic, so far nonpredictable way (see Fig. 4).

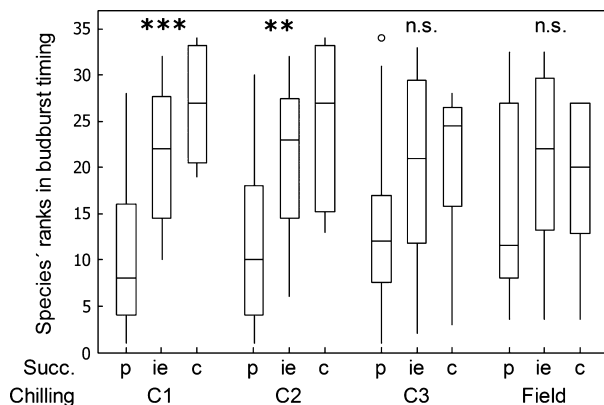


Fig. 3 Rank orders of budburst of successional strategies under different chilling and field conditions. Calculations are based on values of the 12 h photoperiod treatment. Acronyms for successional strategy (Succ.): p (pioneer), ie (intermediate), c (climax). Chilling treatments C1 (low), C2 (intermediate), C3 (high) and Field (field observations). Significance levels of between-group differences are shown, with *** indicating P -value < 0.001 , ** P -value < 0.01 . Low ranks indicate earlier budburst.

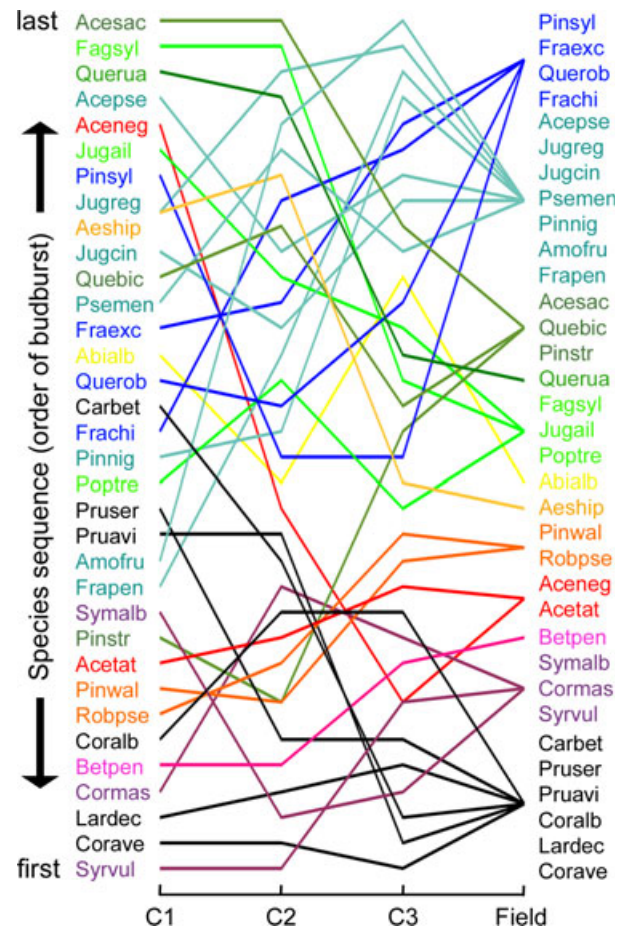


Fig. 4 Species' chronological order in budburst under different chilling and field conditions. C1: low chilling, C2: intermediate chilling, C3: high chilling, Field: field observation. Species ranks were calculated based on the 12 h photoperiod treatment. Full species names are given in the Table 1. Colours are given according to budburst timing in the field, from black (early species) to blue (late species).

Discussion

Is twig phenology a sensible proxy for tree phenology?

Studies on tree phenology face several restrictions. Experimental designs must confront either technical challenges due to the size of adult individuals or work with highly reduced sample sizes. Although inclusion of young individuals or seedlings overcomes these problems, generalization may suffer if young trees show different reactions to adult individuals (Augspurger, 2008; Vitasse, 2013). Hence, most of the current knowledge on tree phenology originates from experiments with only a few selected species, often seedlings, or from correlative studies (Rollinson & Kaye, 2012).

As far as we are aware, Heide (1993a, b) introduced the method of using twigs as proxies for trees in ecology, albeit the method has a longer history in horticulture (several examples given in Dennis, 2003 and Luedeling, 2012). But to date no study has compared the phenology of twigs or cuttings with the behaviour of donor trees [Couvillon *et al.* (1975) showed parallel development of rooted cuttings and trees]. Many side effects have to be considered, such as the cut itself, enhanced danger of fungal infections, missing connection to stem- or root-bound plant hormones, or changes in water uptake (experimental considerations are discussed in Dennis, 2003). On the other hand, it has been shown 'that dormancy release is based on processes that are intrinsic to the bud itself' (Rinne *et al.*, 2001), which suggests that those might be of subordinate importance. In experiments fixed day lengths instead of a steady day length increase, or unnatural climatic conditions are frequently used, which might lead to additional side effects. Furthermore, light conditions within chambers are homogenous, while in forests they are not.

As controls, the phenology of the donor trees under field conditions was recorded. Despite a reduced observation frequency (once a week in the field vs. three times in treatments), the difference in rank orders of species between chilling 3 and the field was rather small (rank correlation coefficient of 0.89). Six additional chill days occurred in the field in early 2012 (after the start of the chilling 3 treatment). Former studies reported that considerable effects of chilling still occur after 110 chill days, as shown for *Fagus sylvatica* (Murray *et al.*, 1989; Vitasse & Basler, 2013) and *Robinia pseudoacacia* (Murray *et al.*, 1989), and this might also be true for some of the other species used in this study. In addition, the winter of 2011/2012 was generally quite warm, and the number of chill days was rather low (see Figure S4). Some of the remaining differences in species sequence between chilling 3 and the field may thus be attributable to both differences in observation frequency and additional chilling effects. Therefore, we conclude that twig phenology is a useful proxy for tree phenology in studying differences related to traits.

General effects of chilling and photoperiod

Our data confirm that a 'residual effect of dormancy' (Campoy *et al.*, 2011), defined as a reduction in forcing requirements with increased chilling, exists for almost all species. However, there was no significant reduction in forcing requirements for *Abies homolepis*, *Fraxinus pennsylvanica* and *Picea abies*, whereas for *Juglans regia* chilling increased the ability to achieve budburst, but did not reduce forcing requirements. *Amorpha fruticosa*

showed increased forcing requirements with chilling (see Fig. 2). This might be attributable to missed chilling requirements of the species, lower chilling requirements than chilling 1, or be the result of experimental constraints. As *Abies homolepis* and *Picea abies* were missing in the low chilling treatment, requirements might have been fully met already. As forcing temperature thresholds are species specific (Polgar & Primack, 2011), the threshold used for the calculation of forcing sums (0 °C) might have been too low for some of the species. This should lead to an overestimate of forcing requirements in chilling treatments 2 and 3. Examination of Figure S5 based on median number of days till budburst suggests this possibility, not only for *Amorpha fruticosa* but also for *Abies alba*, *Fraxinus pennsylvanica*, *Picea abies* and *Pinus* species.

The experiment revealed the large magnitude of the chilling effect: median forcing requirements were reduced on average by 200 °C days (low compared to high chilling), and days until budburst advanced by 21 days. In contrast, the effects caused by photoperiod were minor, with an advance of only 20 °C days for photoperiod 16 h compared to 8 h.

Short photoperiod also did not restrict species' ability to reach budburst, as suggested by Heide (1993b). The four species which had lower budburst percentages were, in effect, able to reach budburst within 6 weeks in chilling 2 to a certain degree (*Abies alba*: 90%, *Acer pseudoplatanus*: 80%, *Fagus sylvatica*: 10%, *Juglans regia*: 70%). We thus conclude that short-day conditions did not completely prevent the development of any species.

Across all species, a reduction in forcing requirements with longer photoperiod was found to be significant only within the low chilling treatment. The number of species reacting to photoperiod declined sharply with increased chilling (low chilling: 10 species photosensitive, intermediate chilling: three species) (Table 1). None of these total of 12 photosensitive species responded to photoperiod after rest completion (see Table 1), and the species that did not respond to chilling were also insensitive to photoperiod (see Fig. 5). Hence, at least for the 36 species studied, incomplete chilling requirements seemed to be a mandatory precondition for a photosensitive response. This is in clear contrast to the previous hypothesis that 'plants become receptive to photoperiod signals' after chilling fulfilment (Körner & Basler, 2010). Yet, it has to be remembered that in chilling 3, photoperiod 8 h, twigs had already received longer photoperiods in the forest prior to the start of the experiment, which could partly confound our findings. However, results from chilling 3 are consistent with the pattern observed in chilling 1 and 2.

		Chilling			
		sensitive till high chilling		sensitive till intermediate chilling	insensitive
Photoperiod	insensitive	<i>Juglans ail.</i> <i>Betula pen.</i> PSEUDOTSUGA MEN. <i>Quercus rub.</i> ACER SAC. <i>Juglans cin.</i> <i>Acer neg.</i> <i>Prunus avi.</i> CARPINUS BET. <i>Larix dec.</i> <i>Quercus rob.</i> <i>Symphoricarpos alb.</i>	(PICEA ABI.) <i>Prunus ser.</i> <i>Pinus nig.</i> <i>Acer tat.</i> <i>Cornus mas</i> (ABIES HOM.) <i>Fraxinus pen.</i> <i>Fraxinus exc.</i> <i>Fraxinus chi.</i> <i>Pinus syl.</i> <i>Robinia pse.</i>		
	sensitive with low chilling	ACER PSE. <i>Quercus bic.</i>	<i>Pinus wal.</i> Juglans reg. Pinus str. ABIES ALB. <i>Syringa vul.</i> <i>Cornus alb.</i> <i>Corylus ave.</i>		
	sensitive with intermediate chilling	<i>Populus tre.</i> FAGUS SYL.			
	sensitive with high chilling	Aesculus hip.			

Fig. 5 Classification of 36 species according to influences of chilling and photoperiod. Colours refer to floristic status of species (black: native, red: invasive, blue: ornamental), font size to successional strategy (small: pioneer to capital: climax). Columns show reaction to chilling treatments: 'insensitive' (chilling insensitive species), 'sensitive till intermediate chilling' (species with chilling requirements fulfilled in chilling 2, hence only responding to chilling between C1 and C2) and 'sensitive till high chilling' (species also responding to chilling between C2 and C3 treatments). Rows show sensitivity to photoperiod treatments: 'insensitive' (no response to photoperiod), 'sensitive with low chilling' (sensitive to photoperiod under C1), 'sensitive with intermediate chilling' (sensitive to photoperiod under C2) and 'sensitive with high chilling' (sensitive to photoperiod under C3); (*Abies hom.*) and (*Picea abi.*): species missing within C1. Full species names are given in Table 1.

Do successional strategies or climatic origins of species matter?

As suggested in recent research (e.g. Körner & Basler, 2010), we expected climax species to show higher chilling and forcing requirements, and a higher responsiveness to photoperiod than pioneer species. For chilling and forcing, our results clearly supported this hypothesis. The advance of climax species in budburst (chilling 1 compared to chilling 3) was generally higher (difference of 300 °C days) than the advance of pioneer species (107 °C days). For climax species a reduction in forcing requirements between C2 and C3 was significant, but for pioneer species it was not. We conclude that winter rest was completed for most of the pioneer species with chilling 2 (67%, 10 of 15 species), while for many climax species further chilling was necessary, and only 38% (3 of 8) species had low chilling requirements (see Table 1). Climax species had higher forcing requirements than pioneer species; even within the high chilling treatment the median estimate of forcing for climax species was 112 °C days higher than for

pioneer species. This finding is in agreement with results of former studies (Murray *et al.*, 1989; Caffarra & Donnelly, 2011).

In contrast, for photoperiod, our results did not support the hypothesis that climax species generally are more photosensitive than pioneer species (see also Fig. 5). We found photosensitive species within all successional strategies, and photosensitivity in all successional strategies was dependent on chilling fulfilment.

For species with low chilling requirements, such as the pioneer species *Corylus avellana* or climax *Abies alba*, the effects of photoperiod only occurred within the low chilling treatment. In contrast, species with high chilling requirements, such as the climax species *Fagus sylvatica*, or the pioneer *Populus tremula*, reacted to photoperiod in intermediate chilling. Previous studies have reported contradictory conclusions with respect to the photosensitivity of species. Basler & Körner (2012) only found significant photoperiod effects for climax species, and not for pioneer species. Other studies also demonstrated photosensitivity for pioneers (Heide, 1993a; Caffarra & Donnelly, 2011).

Basler & Körner (2012) studied only one single chilling treatment (92–119 chill days, depending on altitude of origin), whereas Heide (1993a, b) and Caffarra & Donnelly (2011), who also found photosensitivity within pioneer species, worked with different chilling exposures. As stated earlier, 90–120 chill days are not sufficient to complete winter rest for all species (Murray *et al.*, 1989). Therefore, it seems likely that differences in photosensitivity between successional strategies, as reported in the past, were biased towards fully chilled pioneer species, and only partly chilled climax species. Hence, if a slightly higher percentage of climax species reacted to photoperiod, this most probably has to be attributed to higher chilling requirements, and not to higher overall photosensitivity.

The hypothesis that species with an oceanic distribution show higher photosensitivity than continental species (Körner & Basler, 2010) is also not supported by our study. Photosensitivity was found for species of all climatic ranges. Oceanic species as a group showed no significant effects of photoperiod.

Are invasive tree species more plastic in their phenological responses?

Both a lack of photosensitivity and low chilling requirements should allow species to react more flexibly to climate change than their competitors. It has been suggested that invasive species already profit from changing spring conditions due to high phenological plasticity. Within our studied species, native species as a group showed a response to photoperiod, whereas ornamental and invasive species did not. But photosensitive species were found within all groups (native: 36% (5/14) of species photosensitive, invasive: 36% (5/14) and ornamental: 25% (2/8)). The native group also had the highest percentage of species with high chilling requirements (native: 57% (8/14), invasive: 36% (5/14) and ornamental: 50% (4/8)), hence a higher photosensitivity of native species might be linked to higher chilling requirements, and not to higher overall photosensitivity.

The invasive group we used had the highest percentage of species with an early chilling fulfilment. Taxons which are known to be overrepresented in invasive species, such as the genus *Pinus* or the family *Rosaceae* (Richardson & Rejmanek, 2011), showed rather low chilling requirements in this experiment, whereas some of the underrepresented taxons (such as genus *Acer* or family *Fagaceae*) showed rather high chilling requirements. This suggests that low chilling requirements might be common among invasive species.

Although the native and invasive species we used did differ with respect to chilling requirements, they showed very similar median forcing requirements

under all nine combinations of chilling and photoperiod. As the generation times of woody species are long, *de novo* genetic adaptations in the new ranges should be of minor importance (Thackeray *et al.*, 2010). An *a priori* similarity of forcing requirements to native species might be a factor in the establishment of new ranges. Recent research on the phenology of invasive species has focused on differences that help explain their success, such as earlier leaf-out in spring (Xu *et al.*, 2007), later leaf fall (Fridley, 2012), longer growing season (Harrington *et al.*, 1989), or more flexible response to increasing temperatures (Davis *et al.*, 2010). Our results suggest that invasive species might not directly profit from increased spring temperatures, but rather indirectly from warmer winters.

Hulme (2011b) suggested that a more flexible response of invasive species to climate change relates to the high percentage of pioneer species. For our subset of species, both invasive and native pioneer species generally showed low chilling requirements, and hence, in effect, should track spring warming more easily than native climax species.

Will warming winters alter the chronology of spring events?

Asynchrony, as a temporal 'ecological mismatch' (Polgar & Primack, 2011) has been shown to increase with climate change between different guilds, while less attention has been paid to intraguild asynchrony or clear shifts in temporal overlap (Forrest *et al.*, 2010), besides the well-known fact that species differ in individual reaction to climate change (Menzel *et al.*, 2001, 2006; Cleland *et al.*, 2007; Willis *et al.*, 2008). It has been shown that high interyear variability of onset dates does not cause substantial changes in the species sequence of onsets (Wesolowski & Rowinski, 2006), and the onset dates of species are even predictable from those of co-occurring species (Linkosalo, 2000). The chronological order of phenological events between different plant species is assumed to be stable (e.g. in phenological calendars). Our results indicate that this rather stable chronosequence of leaf-out will change when species chilling requirements are not met.

The temporal niche is an important concept for both species coexistence and ecosystem stability (Loreau & de Mazancourt, 2008), hence differences in phenological responses to climate change are supposed to alter species fitness (Walther, 2004), their abundance and distribution (Willis *et al.*, 2008). Differences in plant phenological responses to climate change might not only disrupt interactions (Fitter & Fitter, 2002; Peñuelas *et al.*, 2002; Forrest & Miller-Rushing, 2010), lead to less or more hybridization opportunities (Fitter & Fitter, 2002),

or change the timing and intensity of water uptake (Polgar & Primack, 2011). Principally, it should lead to changes in springtime light availability, and our data suggest that changes might be rather drastic, e.g. *Carpinus betulus* changes from early to rather late, *Fagus sylvatica* from intermediate to second-last species to budburst.

Former studies reported that early springtime light conditions are of utmost importance for carbon budgets of shrubs, for which up to 40% of total annual carbon gain occurs before canopy closure (Harrington *et al.*, 1989; Xu *et al.*, 2007). Probably even more important, the survival of seedlings also depends critically on early spring light conditions (Augsburger, 2008). This suggests that changes in budburst sequence will also influence the species composition of future forests. According to our data, primarily pioneer species might profit from warmer winters.

Relative importance of chilling and photoperiod

With the precondition of incomplete rest, photoperiod caused significant effects for about one-third of all species tested. Under the assumption that climate change will lead to comparable spring temperatures 4 weeks earlier, and hence comparable temperatures with shorter days than at present. For Freising, the daytime difference from 1st May to 1st April is 1 : 40 h, and an advance of 6 weeks would lead to differences of 2 : 40 h. This is still considerably below the treatment differences manipulated experimentally in this study.

Albeit only being a rough estimate for effective chilling conditions, the number of chill days per year for the study site is rather variable (see Figure S4) and declined significantly during recent decades (linear trend 1972–2000: -0.70 chill d yr⁻¹). This suggests that the chilling treatments applied (reduction of 40 and 77 days) might be rather realistic scenarios for the end of the current century, albeit it has to be noted that chilling metrics used in horticulture predict rather stable or even increasing chill during warming winters for some regions (Luedeling *et al.*, 2011; Luedeling, 2012).

As shown earlier, overall chilling effects led to an advance in budburst of 200 °C days, or 21 days (low vs. high chilling treatment), while photoperiod effects were rather negligible (20 °C days, 8 h vs. 16 h photoperiod). Even if only the photosensitive species and the maximum reduction in forcing requirements (chilling treatment with largest difference) are considered, long photoperiod caused advances averaging 90 °C days. For the same species, an average reduction in forcing requirements of 194 °C days was caused by chilling treatments.

To conclude, this experiment revealed significant, but comparably small effects of extreme photoperiod

treatments only for a few species, in contrast to pronounced effects of chilling for almost all species. With respect to future climate change, chilling, and not photoperiod, is hypothesized to be of primary ecological relevance.

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Supporting Information

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

Figure S1. Temperatures/day lengths before the three cutting dates.

Figure S2. Survival curves for single species.

Figure S3. Survival curves for different groupings of species.

Figure S4. Number of chill days under field conditions (Freising 1972–2001) in comparison to the winter of the experiment (2011/2012).

Figure S5. Median number of days until budburst for all treatments.

Table S1. Species description.

Table S2. Species classification into ecological groups.

Table S3. Mean values of the assigned successional strategies.

Table S4. Significant effects of chilling and photoperiod treatments on species' ability to reach budburst.

Table S5. Treatment effects on different groups of species, *P*-values adjusted for multiple comparisons.

Table S6. Comparison of species' chronological order to budburst under experimental vs. field conditions.