

Intraspecific variation in flowering phenology affects seed germinability in the forest herb *Primula elatior*

Lander Baeten^{1,2,*}, Bram Sercu², Dries Bonte², Margot Vanhellemont¹ & Kris Verheyen¹

¹Ghent University, Department of Forest and Water Management, Forest & Nature Lab, Geraardsbergsesteenweg 267, BE-9090 Gontrode, Belgium

²Ghent University, Department of Biology, Terrestrial Ecology Unit, K. L. Ledeganckstraat 35, BE-9000 Ghent, Belgium

*Author for correspondence: Lander.Baeten@ugent.be

Background and aims – Phenological responses to environmental cues are known to be phylogenetically conserved across species, but the adaptive nature of phenological responses to the local environment within the populations of individual species needs further exploration. In temperate forests, the canopy green up timing is expected to act selectively upon the growth and flowering phenology of understorey plants.

Methods – Here we quantified the variation in the flowering phenology of a forest herb growing in the understorey of early versus late leafing tree species and explored the effect of this variation on the germinability of its seeds. The flowering of individual plants was recorded in six populations at twelve time points in early spring. Germinability was tested in a lab germination experiment. The individual plants were visited again in a second growing season to quantify the variation in phenology between years.

Key results – Variability between plants was found within populations rather than between populations or forest stands. Phenology was consistent across growing seasons with individuals flowering either early or late in both studied years (which was not simply due to differences in plant size). Early flowering individuals had a fitness advantage in the sense that they produced higher proportions of germinable seeds.

Conclusions – We quantified phenological variation between the individuals of an understorey plant species relative to the canopy phenology and related this variation to fitness. While some interesting patterns emerged, fitness components other than seed germinability and the plant life stages following germination should be studied as well to understand the evolutionary significance of phenology in forest understorey plants.

Key words – Temperate forest, understorey herbs, tree canopy, leaf-out, local adaptation.

INTRODUCTION

The timing of seasonal events in the life cycle of plants, i.e. phenological events, are of key importance for a species' demography and its interspecific interactions (Rathcke & Lacey 1985, Forrest & Miller-Rushing 2010). Phenological traits such as bud burst or flowering time are widely studied and generally show tremendous variation among the species within communities, but also among the individuals within species (Rathcke & Lacey 1985, Fenner 1998, Diez et al. 2012). The variability within species may have important ecological consequences and is principally driven by genotype, local environmental heterogeneity (e.g. microclimatic) and stored resources in individuals, which relates to plant size and age (Forrest & Miller-Rushing 2010, Diez et al. 2012). While phenological responses to environmental cues are already known to be phylogenetically conserved across species (Davies et al. 2013), more data are needed to evalu-

ate the adaptive nature of phenological responses within the populations of individual species. If phenological variation in plants is related to fitness, the impact of environmental changes that influence timing should be evaluated in an evolutionary context.

In temperate forests, the growth and flowering of many understorey plant species occurs before the green up of the tree canopy, which is beneficial for the total photosynthetic carbon gain that can be allocated to fruit and seed production (Kudo et al. 2008). While asymmetric competition for light sets constraints on the growth of understorey plants in late spring, the growth initiation of the plants in early spring is usually constrained by low temperatures. This would lead to balancing selection, with optimal timing expected to be strongly dependent on the leafing of the trees (a 'phenological match' analogue, here of herbs relative to trees). Indeed, fairly small phenological differences between understorey

plant development and leaf-out timing of the tree canopy can bring a disproportionate benefit in the total annual irradiance received by the understorey (Augspurger et al. 2005). The onset of tree leaf development is known to be a heritable trait that varies substantially among tree species (Lechowicz 1984, Polgar & Primack 2011). To the extent that phenological responses in understorey species are adaptive, strong changes in the tree species composition and abundance in managed forests therefore potentially creates a phenological disparity, i.e. the average timing of the understorey species no longer matches the canopy green-up timing.

Management-driven compositional changes in tree communities are widespread in temperate European forests and have strong impacts on the understorey (Van Calster et al. 2008, Verheyen et al. 2012). Tree species effects on the understorey are usually quantified in terms of understorey compositional turnover patterns, not so much by assessing the underlying demographic processes in individual herb layer species. Here we explored the variation in the flowering phenology of a forest herb [*Primula elatior* Hill (Primulaceae)] growing in the understorey of early versus late leafing tree species. We used flowering phenology as a proxy of overall plant phenology; early developing individuals (e.g. shoot elongation, leaf unfolding) also flower early (e.g. Sola & Ehrlén 2007). Our research questions were: (i) is the phenological variation within local populations of *P. elatior* lower than the variation between populations under contrasting (in terms of leaf-out phenology) canopy species?; (ii) is the timing of *P. elatior* individuals consistent across growing seasons, e.g. some individuals are consistently early in consecutive growing seasons?; (iii) does the timing of flowering have an effect on the seed germinability, an important component of fitness? While we acknowledge that our study is somewhat limited in scope, the data will illustrate interesting directions for future research on the understorey responses to changes in canopy phenology caused by forest management.

MATERIAL AND METHODS

Research sites, study species and sampling

The 34-ha Muizenbos forest is located in a flat area (10 m a.s.l.) in northern Belgium. Soils are Quaternary niveo-eolian deposits, varying from silty sand to sandy silt, supporting a natural forest plant community of rich and mesic sites (*Primulo-Fraxinetum excelsioris*). The forest forms a mosaic of forest stands with contrasting land-use history, planted tree species and past management (Verheyen & Hermy 2001). We sampled in two adjacent forest stands on similar soils (Gleysols; De Keersmaecker et al. 2004) in the ancient forest part, i.e. the stands have been at least 135 year continuously forested. While the use of adjacent stands reduced potential effects of confounding (environmental) factors, the stands were selected to principally differ in canopy tree species: (i) a stand with *Fraxinus excelsior* L. trees and a dense coppice layer of the early leafing *Corylus avellana* L. and (ii) a stand with *Populus × canadensis* Moench trees and a coppice layer of the relatively late leafing *F. excelsior*. In the first stand with *Corylus*, the first leaves emerged sixteen days earlier (6 April) than in the other stand (22 April).

The total canopy closure in summer was, however, not different between the stands: 10.0% versus 11.7% gap fraction calculated from fish-eye images ($t = -0.86$, $P = 0.48$, three images per stand). Degree days were calculated as the average of the daily minimum and maximum temperatures minus a base temperature using available air temperature data (weather station 'Herentals', c. 10 km from the forest, 1.75 m height and 15 min resolution measurements). We chose a base temperature of 4°C to calculate cumulative degree days (CDD_4) starting from 1 January, because CDD_4 was shown to be strongly related to the canopy leaf-out phenology in spring (Richardson et al. 2006). The difference in first leaf emergence of the two stands was 50 CDD_4 units.

Primula elatior is a perennial herb that widely occurs in rich and mesic ancient forests throughout western and continental Europe. It is a rosette hemicryptophyte, forming short rhizomes (very limited vegetative spread) and reproducing mainly sexually by seed. The species is early flowering (early March to mid-May) and summer green. This obligate outbreeder is pollinated by insects, seeds ripen eight to nine weeks after fertilization and seeds are shed in early August. The dormant seeds germinate from the transient seed bank in the following spring, after a period of low temperature breaking seed dormancy (Taylor & Woodell 2008).

In each of the two stands, we established three 3×3 m plots (at least 25 m apart) and marked all the adult *P. elatior* plants that developed inflorescences (total $N = 157$). The individuals in a single plot are further referred to as a 'population' – the average number of plants per population was 26 ± 10 SD. We visited each plant on twelve time points between 10 March 2012 ($CDD_4 = 109$) and 2 May 2012 ($CDD_4 = 374$) and recorded the number of blooming flowers. During the final survey, we also counted the number of leaves (mean = 3.6 ± 1.1 SD) and measured the length of the longest leaf (mean = 19.0 cm ± 4.1 SD) as proxies of plant size (Baeten et al. 2009). In the next growing season, we visited all plants once again (12 April 2013) and recorded the number of blooming and non-blooming flowers at that time point. Although this time of sampling corresponded to the second half of the 2012 survey period, the 2013 growing season lagged behind in terms of degree days and the CDD_4 on 12 April was only 99 (electronic appendix).

Germination experiment

We collected all the seeds from all the individuals bearing seeds in early August. Due to strong herbivory on the inflorescences (most probably snails; Baeten et al. pers. obs.), seeds could only be collected for eighteen plants (only two from the *Populus-Fraxinus* stand). The seeds were air dried at room temperature for two days. For each plant, two subsamples of maximum fifty seeds (average 38 ± 9.6 SD seeds; one subsample for seven individuals) were weighed before distributing them in petri dishes lined with moist filter paper. The filter paper was moistened every week. The dishes were immediately placed in warm incubation (20°C) for six weeks, moved to cold stratification (2°C) for sixteen weeks and put back to warm stratification (Baeten et al. 2010). The number of germinated seeds was counted on several occasions until germination virtually ceased in all petri dishes.

Data analysis

The increasing proportion of the individuals' flowers that were blooming or had bloomed over time (0–100% of the flowers) was modelled with a multilevel logistic regression: $\text{logit}(p_i) = a_{k[i]} + b_{k[i]} \text{time}_i$ (model 1). The coefficients a_k and b_k were allowed to vary by individual ($k = 1 \dots N_{\text{individuals}}$) and represent the location (parallel shifts) and steepness of the sigmoid curve describing the increasing proportion p_i of emerged flowers over time. The model accounts for the temporal non-independency of individual-level observations. Only plants with four flowers or more were analysed ($N = 109$; 74 in the *Fraxinus-Corylus* and 35 in the *Populus-Fraxinus* stand), because modelling the changes in the probability of blooming over time has low relevance for plants with few flowers.

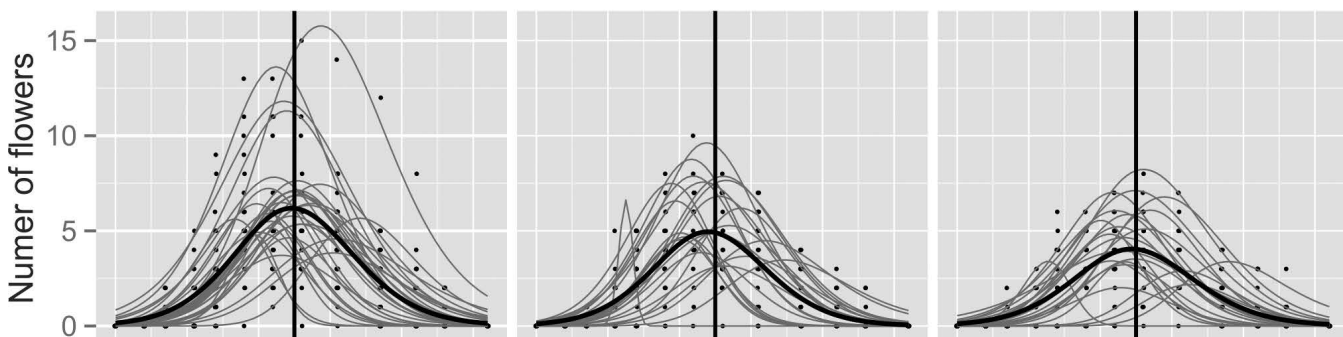
We extended the logistic model by additionally allowing the location and steepness effect to vary either by forest stand (model 2) or by population (model 3). We then compared these extended models with the previous individual-level model 1 by means of AIC and likelihood ratio tests, i.e. evaluating evidence for a difference in timing between the

forest stands and/or populations. To explain differences in timing between individual plants, we first calculated the predicted day of the year when 50% of the flowers of plant k had emerged: $T_k^{50\%} = -a_k/b_k$ (median flowering date).

This individual-level phenology measure was then related to the individuals' number of leaves and leaf length to explore the influence of plant size on timing. To test the consistency of phenology across years, the plants' probability of being in flower by the time of sampling (12 April) in 2013 was related to their phenology in 2012 ($T_k^{50\%}$) with a logistic regression.

After exploring the variation in flowering phenology between the plants, we wondered whether the differences in timing influence the germinability of the individuals' seeds. With a multilevel logistic model (accounting for the non-independence of subsamples from the same plant) we related the proportion of germinated seeds to $T_k^{50\%}$, but also to the number of leaves and leaf length as proxies of plant size. Average seed mass was included as a covariate in all the models. All analyses were performed in R 3.1.0, using the *lme4* package for multilevel linear models and the *ggplot2* package for multi-layered graphics (Wickham 2009, Bates et al. 2013, R Core Team 2014).

A *Fraxinus* - *Corylus*



B *Populus* - *Fraxinus*

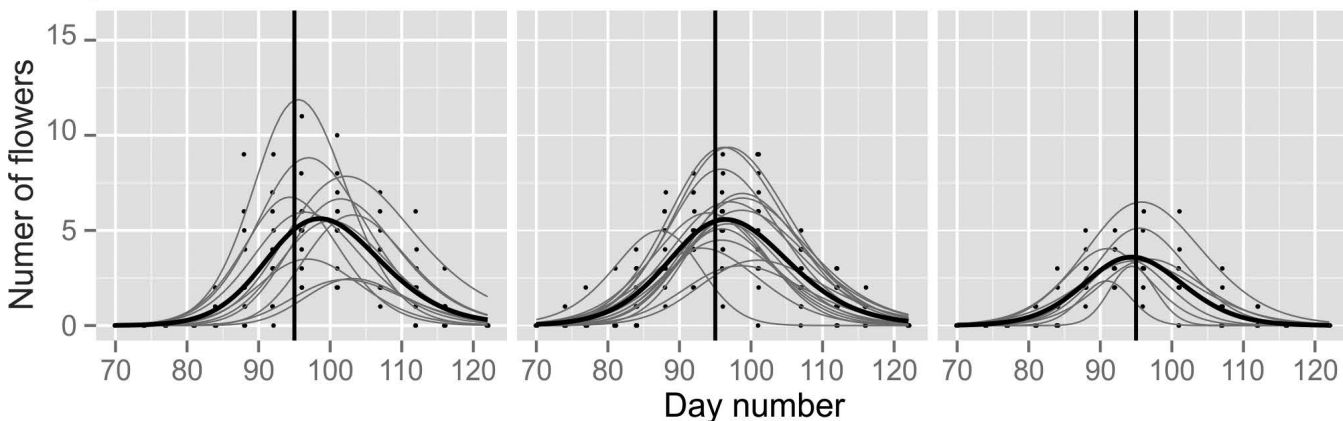


Figure 1 – Variability in flowering phenology of *Primula elatior* plants, recorded for 109 individuals growing in three populations in each of two forest stands (A, B). For each plant, the number of blooming flowers was counted on twelve time points between 10 March 2012 (day number = 70) and 2 May 2012 (day number = 123). A generalized linear model (Gaussian family, log link) was used for adding a cubic spline smoother to the data of each individual (thin grey lines). The thick black smoother describes the population-level pattern and the vertical line shows the average day of maximum flowering across all plants.

RESULTS

Individual plants of *P. elatior* exhibited a marked variation in flowering phenology, with a nineteen day time lag between the first flowering date of the earliest plants and the plants with the latest flowering onset. Almost all (95%) of the $T_k^{50\%}$ values were between day 80 and 94, i.e. the predicted median flowering date varied by more than two weeks or 82 degree days among individuals. Variability between plants was found within populations rather than between populations or forest stands (fig. 1): model comparisons showed that the models with the location and steepness effect varying by forest stand (model 2) or population (model 3) did not improve upon the individual-level variation model (model 1) ($\Delta AIC < 3$ and likelihood ratio test $P > 0.5$ for both comparisons). The between-plant variability was partly related to plant size, with plants supporting more leaves showing earlier flower emergence (fig. 2). The length of the longest leaf, another proxy of plant size, was not related to flower phenology.

Phenology was fairly consistent between consecutive growing seasons (fig. 3): the timing in 2012 was a good predictor of the plants' probability of being in flower by the time of sampling in 2013 (steepness = -0.23, $P < 0.001$), i.e. the early flowering individuals in 2012 had a higher proportion of blooming flowers during the early 2013 recording (12 April), while late flowering individuals in 2012 had not started flowering yet by 12 April 2013. The steepness parameter of this logistic regression model expresses the decrease (multiplicative effect; $\times e^{-0.23} = \times 0.79$) in the odds of being in flower in 2013 when the 2012 median flowering date increases

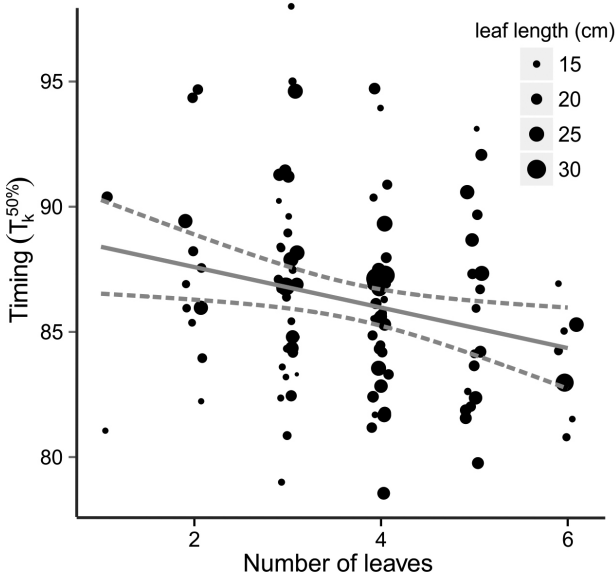


Figure 2 – Relationship between the number of leaves of *Primula elatior* individuals and flowering phenology ($N = 109$), here expressed as the predicted day of the year when 50% of the flowers had emerged (median flowering date). Points represent individual plants, with the point size proportional to the length of their longest leaf. The full line shows the fit of a linear model (slope = -0.81 days/leaf, $P = 0.01$) and the dashed lines delimit the 95% confidence interval.

es by one day (e.g. a change in probability of flowering from 0.75 to c. 0.70). Again, larger plants flowered earlier in 2013; the number of leaves was related to the probability of being in flower (steepness = 0.26, $P = 0.005$; an increase in the odds $\times 1.3$ per leaf). After accounting for variation in plant size, the effect of timing in 2012 on the 2013 flowering probability was still very important ($\Delta AIC = 49.5$, $P < 0.001$). So, independent of plant size, early plants in the first growing season were also early in the second season.

The germination percentage was related to the individuals' flowering phenology: seeds collected from earlier flowering plants showed higher germination success (fig. 4). This relationship was not due to differences in plant size between the early and late flowering individuals; the number of leaves (steepness = -0.32, $P = 0.09$) and the leaf length of the individuals (steepness = 0.10, $P = 0.40$) were not significantly related to the germination probability (multilevel logistic regression).

DISCUSSION

The flowering phenology of *P. elatior* varied considerably between individuals (maximum nineteen days for first flower emergence and two weeks for the median flowering date) and most variation was found between plants growing within the same population. Plants growing under the canopy of dissimilar tree species, in terms of leaf-out phenology, did thus not differ in their average timing (fig. 1). Plant size partly explained phenology, which is consistent with the idea that larger (perennial) plants flower earlier because they have greater stored resources (e.g. Ollerton & Lack 1998, Diez et al. 2012), although much variation remained unexplained (fig. 2). We also found evidence for consistency in the timing

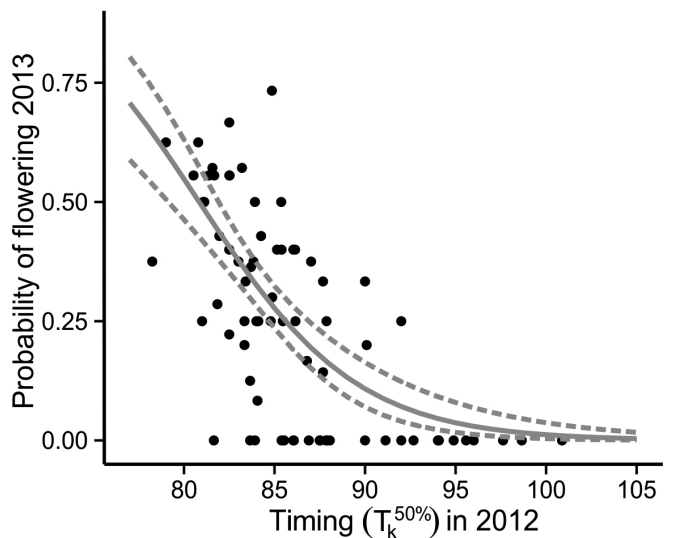


Figure 3 – Relationship between the *Primula elatior* individuals' probability of being in flower at one time point in early spring 2013 (12 April) and their flowering phenology in 2012 (expressed as the predicted day of year when 50% of the flowers had emerged, i.e. median flowering date). Points represent individual plants, the full line shows the fit of a logistic model and the dashed lines delimit the 95% confidence interval.

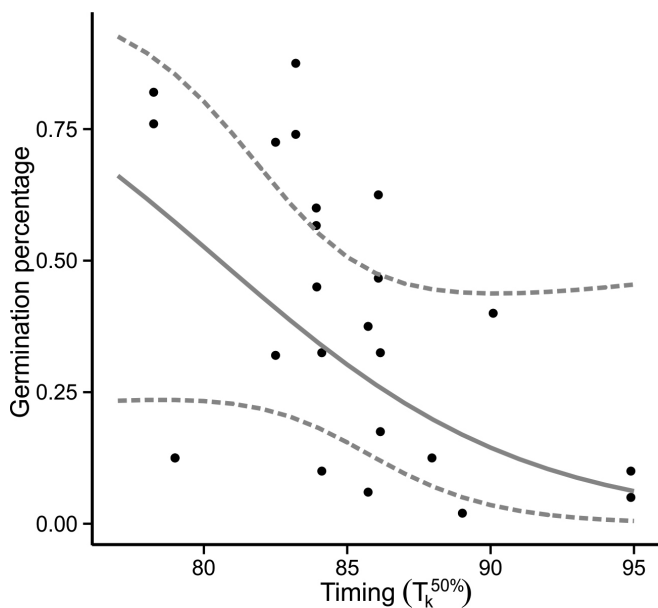


Figure 4 – Relationship between the flowering phenology (predicted day of the year when 50% of the flowers had emerged, i.e. median flowering date) of *Primula elatior* individuals and the germinability of their seeds. The full line shows the fit of a multilevel logistic model (steepness = -0.34 , $P = 0.03$) and the dashed lines delimit the 95% confidence interval. The steepness parameter expresses the decrease in the odds of germination ($\times e^{-0.34} = \times 0.71$) with increasing median flowering date, e.g. a change from 50% to 41.5% germination when the median flowering is reached one day later.

of flowering across growing seasons, with individuals flowering either early or late in both studied years. This temporal relationship was not simply mediated by plant size (and therefore probably also plant age), that is, the consistently early flowering individuals were not only the relatively large individuals. While phenotypic variation in phenology in natural habitats may be largely due to environmental heterogeneity, the similar order of flowering of individuals in consecutive years at least suggests a genetic basis for flowering phenology in *P. elatior* with early and late genotypes (Rathcke & Lacey 1985), although we cannot conclusively prove this. The environmental heterogeneity is, however, unlikely to be the principal driver here because early and late flowering individuals were often only few centimetres apart and the understorey light regime should not be varying dramatically at this scale. A final important finding in this study, albeit with low sample size, is the relationship between flowering timing and seed germinability. The early flowering individuals had a fitness advantage in the sense that they produced higher proportions of germinable seeds.

There seemed to be a genetic basis for flowering phenology in *P. elatior* and phenology was linked to fitness, so early flowering genotypes might be expected to be promoted under a canopy of early leafing tree species. Yet, local adaptation to the different canopy-related light regimes was lacking here, which may be due to a combination of several factors. First, because we used adjacent stands to reduce potential confounding by environmental factors other than the light regime, gene flow might hinder local adaptation at this spa-

tial scale (Kawecki & Ebert 2004). While *P. elatior* seeds are not dispersed far, insect pollinators of this self-incompatible species can easily spread pollen to different stands (Taylor & Woodell 2008). Second, the light regime has potentially been too variable relative to the generation time of the long lived *P. elatior*. For instance, as in large parts of Western Europe, the dense coppice layer of both stands has been frequently cut for centuries (our stands were last coppiced in 1976–1978). *Primula elatior* exhibits massive flowering and reproduction after coppicing (De Keersmaeker et al. 2011) and without light being a constraint, we believe there has been no strong filtering of early versus late flowering individuals during these major population growth events. In addition, bud burst in trees varies considerably between years, so even without the coppicing, variability among years can select for larger phenotypic variation in understorey phenology (fluctuating selection). Similarly, there are likely costs of too-early flowering, notably the higher risk of frost damage. Such costs may not be manifested every year, but could select against early flowering, helping to maintain variation in flowering in the population. Third, unlike factors such as pollinator presence (Rathcke & Lacey 1985), light availability only indirectly acts selectively on flowering phenology, e.g. through its effect on vegetative traits such as shoot growth and leaf unfolding, which in turn constrain flowering (Sola & Ehrlén 2007). The main vegetative development in the vernal *P. elatior* may be simply too early in the season for the differences in canopy closure between stands to impose a significant selection pressure. Whether or not vegetative phenology varied between the stands was not studied here. For early summer bloomers the impact of canopy timing may be more important, since they suffer from severe resource limitation caused by decreasing light availability during fruit development (Kudo et al. 2008).

In sum, our study is among the first to analyse phenological variation between the individuals of an understorey plant species relative to the canopy phenology and to relate this variation to fitness. While some interesting patterns emerged, notably the link between flowering timing and seed germinability, several of the alternative explanations above are in need for further exploration. Studying species with different life histories such as (early) summer bloomers may reveal stronger effects of light variation (examples in our forest: *Geum urbanum* L. and *Polygonatum multiflorum* (L.) All.). In addition to quantifying variation of timing in the field, reciprocal transplantation experiments and common garden experiments (with additional light treatments) could lead to stronger inferences about the role of light variation in generating phenotypic variation in phenology. Furthermore, phenological traits other than flowering (e.g. traits related to vegetative growth) should be measured, i.e. traits that are more directly related to light availability on the one hand and put constraints on the species' reproduction on the other hand. Finally, fitness components other than seed germinability and the plant life stages following germination should be studied as well to fully understand the eco-evolutionary consequences of phenological variation in forest understorey plant species.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (<http://www.ingentaconnect.com/content/botbel/plecevo/supp-data>) and consist of the changes in cumulative degree days with base temperature 4°C (CCD_4) in spring 2012 and 2013.

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