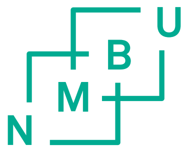

Norwegian University of Life Sciences
Faculty of Environmental Science and Technology
Department of Ecology
and Natural Resource Management

Philosophiae Doctor (PhD)
Thesis 2016:33

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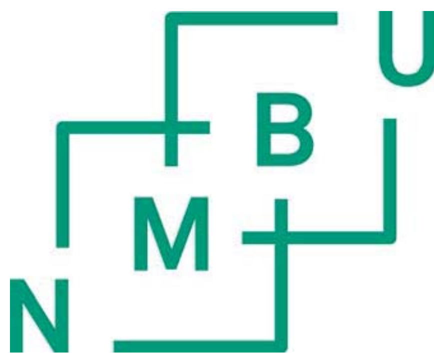

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PhD supervisors

Associate professor Line Nybakken

Department of Ecology and Natural Resource Management

Norwegian University of Life Sciences

Professor Jorunn Elisabeth Olsen

Department of Plant Sciences

Norwegian University of Life Sciences

Professor Riitta Julkunen-Tiitto

Department of Biology

University of Eastern Finland

Evaluation committee

Professor Karen K. Tanino

Department of Plant Sciences

University of Saskatchewan

Dr. Marcel A. K. Jansen

School of Biological, Earth and Environmental Sciences

University College Cork

Professor Knut Asbjørn Solhaug

Department of Ecology and Natural Resource Management

Norwegian University of Life Sciences

“Do not let anyone tell you that these people made work out of play. They simply realised that the most fun lies in seeing and studying the unknown.”

— Aldo Leopold

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Christian Bianchi Strømme, Ås 12.02.2016

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ABSTRACT

In temperate climates, phenological transitions in tree species allow survival through adverse climatic conditions in winter. Seasonal change is sensed through shifting patterns in day-length, temperature and light quality. With the ongoing rise of global temperatures, there is increased research interest in temperature effects on phenology. Based on evidence from controlled conditions, as well as indications from modelling studies of species distributions, extended growing seasons are expected mainly as a result of advanced spring bud break, and to some extent also through delayed phenological events in autumn. However, temperature effects are still poorly understood when the whole-year cycle of seasonal shifts is considered, and also in terms of interactions with day-length and light quality. The purpose of this thesis was to investigate how temperature and light conditions affect phenological transitions in two deciduous tree species present in Northern Europe. The effects of temperature and Ultraviolet-B (UV-B) radiation on phenological transitions, growth and metabolism were investigated in European aspen (*Populus tremula*) grown in field conditions. In addition, spring phenology and cold hardiness was studied in European beech (*Fagus sylvativa*) populations in Norway, which represent the species' northernmost distribution range. For Eurasian aspen, autumn temperature delayed bud formation in autumn, an effect which was stronger in male plantlets. On the other hand, UV-B had a positive effect on bud formation in autumn, counterbalancing the effect of temperature. Bud break during spring was positively affected by autumn warming, but this effect was marginal compared to the positive effect of spring temperature. For European beech, warming promoted bud break in controlled and field conditions, indicating that bud break timing at the species' northernmost distribution range is limited by temperature. For both species, it was shown that warming can be expected to extend the length of growing seasons, possibly with increased risk of frost damage.

LIST OF PAPERS

This thesis consists of the following papers that are referred to by the roman numerals (I-IV)

Paper I

Strømme CB, Julkunen-Tiitto R, Krishna U, Lavola A, Olsen JE, Nybakken L (2015) UV-B and temperature enhancement affect spring and autumn phenology in *Populus tremula.* Plant, Cell and Environment 38: 867-877

Paper II

Strømme CB, Julkunen-Tiitto R, Olsen JE, Nybakken L. Phenology, growth and metabolism of *Populus tremula* grown along a natural temperature and UV-B gradient. *Manuscript*

Paper III

Strømme CB, Julkunen-Tiitto R, Olsen JE, Nybakken L. High daytime temperature delays autumnal bud formation in *Populus tremula* under field conditions. *Manuscript*

Paper IV

Schmidt E, Strømme CB, Olsen JE, Nybakken L. Climatic effects on bud break and frost tolerance in Europe's northernmost populations of beech (*Fagus sylvatica*). *Submitted*

INTRODUCTION

"Spring is sooner recognised by plants than by men. "

- Chinese proverb

In temperate climates, colour changes in tree canopies are among the most evident signs of seasonal change perceived by humans. The emergence of flowers on cherry and peach trees is celebrated annually in China and Japan, and written records reveal that these events were held as early as the eighth century. Also during autumn across the Northern Hemisphere, the changing colours of tree foliage attract tourists with the purpose of *momijigari* in Japan ("maple hunting"), or "leaf peeping" in North America. Seasonal shifts are not only appreciated for their aesthetic value, but have for centuries also provided important information for food production and resource acquisition. Indeed, phenological knowledge has been highly valued through long parts of agricultural history, as reflected in the numerous proverbs which relate sowing, planting and harvesting to seasonal events in plants.

In woody plants, phenological events allow the survival of meristems through winter, as well as the conservation assimilates until the return of favourable conditions for growth. As shoot tips are not protected by bark, the formation of terminal buds shields both the apical meristems and the undeveloped leaves until they emerge and unfold in spring. Also during autumn, meristems gradually acquire the ability to avoid freezing within cells. Growth and survival of tree species, particularly in environments with cold winters, is thus highly dependent on bud development and the acquisition of frost tolerance occurring before the onset of freezing temperatures. As the timing of these phenological events in woody plants are highly adaptive, species and populations exhibit seasonal growth patterns that reflect local climates.

However, phenological events in plants can vary substantially between years, and there is evidence of systematic observations in Europe starting in the 18th century. Carl von Linné established a network of phenological observations in 1750, while the Marsham family recorded phenological events in a set of plants and animals from 1736 to 1925 in Norfolk, England. By combining the Marsham records with later observations and climate data, it was shown that most of the recorded phenological phenomena were significantly related to climate (Sparks & Carey 1995). Based on these relationships, warming has been predicted to yield earlier spring phenology for the tree species included in the records. Indeed, evidence

from the last three decades across Europe shows that spring phenology has advanced with warming, also for several species of trees (Menzel *et al.* 2006; Bertin 2008).

As a further rise of global temperatures is projected for the 21st century (IPCC 2013), warming effects on tree phenology have received increased research interest (Cleland *et al.* 2007; Körner & Basler 2013) and are also included in prediction models of tree distribution (see Chuine 2010 for a review). Still, the underlying mechanisms behind phenology in most tree species are not well understood, particularly in relation to warming. Temperature is not the only climatic variable sensed by plants, as growth is also adjusted to variations in day length and light quality (see Webb 2003 for a review). For phenological events in autumn, studies of temperature effects in trees are of recent origin when compared to those on the effects of day length (Kramer 1936; Vaartaja 1954; Wareing 1956; Nitsch 1957; Weiser 1970). Furthermore, tree species differ in terms of which environmental signals are involved in autumn phenology, and the same signal may involve dissimilar signalling pathways for different species (Olsen 2010).

For deciduous tree species at high latitudes, light quality has also been shown to affect autumn phenology. The proportion of red and far-red light in the solar spectrum vary throughout the year (Górski 1980), and is known to affect autumn phenology in bay willow (*Salix pentandra*) (Junttila & Kaurin 1985), hybrid aspen (*Populus tremula* x *tremuloides*) (Olsen *et al.* 1997a) and silver birch (*Betula pendula*) (Tsegay *et al.* 2005). Although less studied, ultraviolet B (UV-B) radiation has also been shown to regulate plant growth as a morphogenetic signal (Rozema *et al.* 1997; Jansen 2002; Rizzini *et al.* 2011; Jansen & Bornman 2012; Hayes *et al.* 2014). Indeed, UV-B radiation varies throughout the year it (Brown *et al.* 1994; Häder *et al.* 2007) and has been shown to affect photoperiodic sensing in *Arabidopsis thaliana* (Fehér *et al.* 2011). In this regard, there is reason to investigate whether UV-B signalling is involved in phenological transitions of tree species.

When effects on spring phenology are concerned, the relative influences of temperature and day length are debated among researchers (Chuine *et al.* 2010), and the matter is complicated further when evidence from available literature is considered. In a review on the subject, Hänninen & Tanino (2011) pointed out that for several temperate and boreal tree species, climate effects in one season affect phenology in later seasons. In particular, warm autumn temperatures may yield delayed bud break in spring, while low autumn temperatures lead to

the opposite. On the other hand, early bud break has been shown to yield faster leaf senescence in autumn (Fu *et al.* 2012). Clearly, these effects should also be considered in predictions of tree phenology under warming.

During spring, de-hardening and bud break is a result of warmer temperatures (Sarvas 1972; 1974). However, there is substantial interspecific variation in terms of thermal requirements for bud break. Some of this variation is related to the effect of chilling during autumn, which is known to lower the thermal requirement for bud break in spring (Murray *et al*. 1989). Species considered as early-flushing have a low chilling requirement, while late-flushing species such as European beech (*Fagus sylvatica*) are known to substantially delay bud break following insufficient chilling. Considering these dissimilar requirements, species can be expected to differ in terms of bud break advancement following warming. Indeed, a study of 13 temperate tree species shows that through the last three decades, the temperature requirement for bud break has increased, and the increase for late-flushing species was higher compared to early-flushing species (Fu *et al.* 2015).

Considering the available evidence, reliable predictions of warming effects on tree phenology should consider interactions between day length, temperature and light quality in a whole-year perspective. Although warming may yield longer seasons for photosynthesis and growth to occur, it may also involve increased risk of frost damage (Cannell & Smith 1986; Gu *et al.* 2008), as sub-zero temperatures occur in spring and autumn, and increase in frequency and severity with distance from the Equator and with elevation. The purpose of this thesis was to investigate the effects of temperature and different light conditions on autumn- and spring phenology in two deciduous tree species present in Norway. The main emphasis was on the dioecious Eurasian aspen (*Populus tremula*), which has a latitudinal range extending from Southern Europe and to Northern Scandinavia. Also considered in this thesis are Norwegian populations of European beech, which represent the latitudinal boundary of a tree species which is dominant on the European mainland.

Objectives

The aims were to determine

i) The effects of elevated temperature and ultraviolet B (UV-B) radiation on autumnal bud formation and spring bud break in the dioecious Eurasian aspen (Paper I, Paper II).

ii) The effects of ambient temperature and UV-B on growth, nutrient assimilation and phenolic metabolism in Eurasian aspen (Paper II)

iii) The effects of different temperature parameters on autumnal bud formation in Eurasian aspen (Paper III)

iv) Whether female and male plantlets of Eurasian aspen differ in their responsiveness to temperature and UV-B radiation (Paper I, Paper II, Paper III)

v) The effects of temperature on winter dormancy release in Norwegian populations of European beech (Paper IV)

vi) The effects of temperature on frost tolerance in vegetative buds of European beech (Paper IV)

METHODS

Field experiments using Eurasian aspen

Plant materials

In the field studies using Eurasian aspen, all plantlets originated from six different populations in Southern and Eastern Finland and were propagated *in vitro*. The original plant materials were sampled in January and February 2011 by removing shoots from one female and one male adult tree from each of the six populations, yielding a total of six female and six male clones for the field experiments. The inclusion of different populations was done with the purpose of accounting for genotypic differences in responses to experimental treatments.

Field experiments

Figure 1. Geographic locations of field experiments using Eurasian aspen performed in Finland and Norway. The enhancement experiment was held in Joensuu, Eastern Finland (location A) in 2012, while the two elevational gradient experiments were held at Fåvang, Central Norway (location B) in 2013 and 2014.

We established three different field experiments, each performed from mid-summer until late spring the consecutive year. The modulated enhancement experiment was established in Joensuu, Eastern Finland (62°60′ N, 29°75′ E) (Fig. 1) in 2012, where plantlets were exposed to different temperature and light quality treatments. These treatments were temperature enhancement, UV-B enhancement, ultraviolet A (UV-A) enhancement, and control (no

enhancement), yielding six different treatment combinations distributed among six blocks in a 6 x 6 matrix (Fig. 2). Temperature enhancement was obtained using infrared heaters while UV-B enhancement was obtained using UV-fluorescent lamps, yielding +1.4 °C and +28% increase of ambient levels, respectively. The purpose of treatments which involved UV-A enhancement was to control for possible effects of the small amount of UV-a radiation emitted from the UV-tubes. Enhancement treatments were active between 1 June and 1 October.

2012

Control	UV-B+T	UV-A	UV-B	T	UV-A+T
UV-A	T	UV-B	UV-A	UV-B+T	T
UV-A+T	UV-B+T	Control	UV-A+T	UV-A	UV-B
UV-B+T	UV-A	T	Control	UV-B	Control
T	Control	UV-A+T	UV-B	UV-A	UV-B+T
UV-B+T	UV-A+T	UV-B	T	Control	UV-A+T

Figure 2. Distribution of plot treatments in the the modulated enhancement experiment in Joensuu, Eastern Finland.

Throughout autumn 2012, the development of apical vegetative buds was recorded using a categorical scoring system based on Rohde *et al.* (2011). Throughout spring 2013, bud break was recorded using a categorical system based on Fu *et al.* (2012).

The first elevational gradient experiment was established in Fåvang, Central Norway (61°27' N, 10°11'E) (Fig.1) in 2013. At each of three elevations (237, 575 and 830 m a.s.l.), five plots containing female and male Eurasian aspen clones were established in a fenced pasture (Fig. 3). Throughout summer and autumn, plant growth was measured as stem height and basal diameter, while apical bud formation was recorded throughout autumn in the same manner as in the enhancement experiment. At the different elevations, a subset of female and male plantlets were harvested for analysis of C, N and phenolic content (Table 1). Bud break throughout spring 2014 was recorded for remaining plantlets in the same manner as during spring in the enhancement experiment.

2013

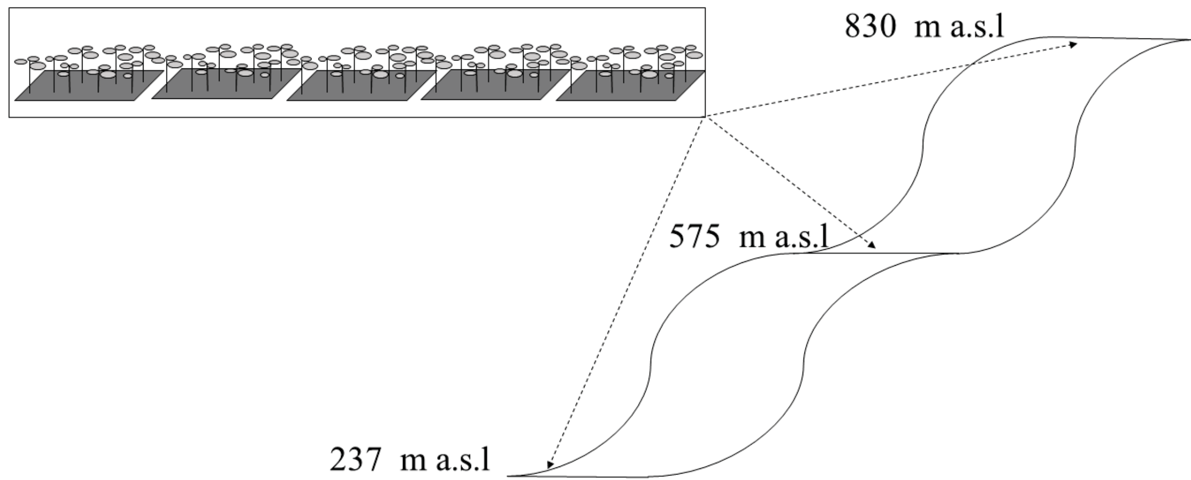

Figure 3. Design of the elevational gradient experiment established in 2013 in Fåvang, Central Norway.

2014

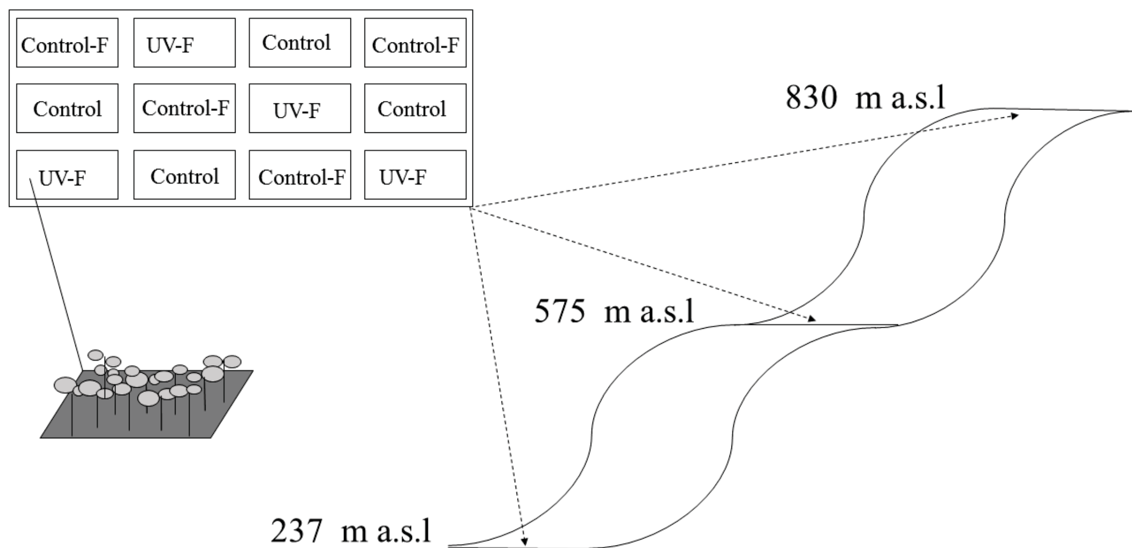

Figure 4. Design of the field experiment established in Fåvang, Central Norway, where ultraviolet-B was attenuated across different elevations in 2014. Treatments were UV-B attenuation (UV-F), UV-B transmitting cover control (Control-F) and uncovered control (Control).

In 2014, twelve new plots containing new plantlets were established at each elevation. Plots were arranged in a 4 x 3 matrix, with each of the four blocks containing three different treatments: UV-B filter, UV-B transmitting cover control and uncovered control (Fig. 4). UV-B filter treatments consisted of mounting UV-B attenuating polyester film above plots in order to reduce direct UV-B radiation. Covered control treatment consisted of mounting translucent polyethylene sheets in the same manner as for UV-B attenuation in order to control for the effect of UV-B attenuation. Plots assigned to uncovered control treatments were left uncovered in order to control for climatic effects of covering plots with sheets. Plantlet growth and bud formation was recorded throughout the growing season in the same manner as in 2013, while harvesting for C, N and phenolic content analyses was performed at 237m a.s.l. on three different dates in autumn. In addition, bud break in spring was recorded the consecutive year in the same manner as for the two previous field experiments.

Temperature data

In all three field experiments involving Eurasian aspen, temperature was logged on a ten-minute basis from mid-summer until the end of the growing season for the temperature treatments (enhancement experiment) and for the different elevations (elevational gradient experiments). Using these temperature series, we combined each recording event with daily local time points for sunrise and sunset, allowing separation of day and night temperatures. Following, we calculated mean, minimum and maximum temperatures for each interval between apical scoring dates using values from a day-time, night-time and 24-hour basis. Furthermore, we proceeded by testing and comparing the effects of the different temperature parameters on autumnal bud formation and transitions between the apical stages used.

Dormancy release and frost tolerance of European beech

In 2014, we tested temperature effects on dormancy release and frost tolerance of European beech populations in Norway (Fig. 5). We used living shoots sampled from adult trees at one-month intervals between January and March and between October and December. In the dormancy release experiments, shoots were exposed to different day length and temperature treatments in controlled conditions (Fig. 6). During each experimental period, bud break was recorded using a scoring system based on Murray *et al.* (1989).

Table 1. Gathered data from field experiments using Eurasian aspen.

Experiment	Location	Year	Data	Paper
1	Joensuu	2012	Bud development	i, iii
1	Joensuu	2013	Bud break	i
2	Fåvang	2013	Growth (height)	ii
2	Fåvang	2013	Growth (basal diameter	ii
2	Fåvang	2013	Metabolism (C and N content)	ii
2	Fåvang	2013	Metabolism (phenolic content)	ii
2	Fåvang	2013	Bud development	ii, iii
2	Fåvang	2014	Bud break	ii
3	Fåvang	2014	Growth (height)	ii
3	Fåvang	2014	Growth (basal diameter	ii
3	Fåvang	2014	Metabolism (C and N content)	ii
3	Fåvang	2014	Metabolism (phenolic content)	ii
3	Fåvang	2014	Bud development	ii, iii
3	Fåvang	2015	Bud break	ii

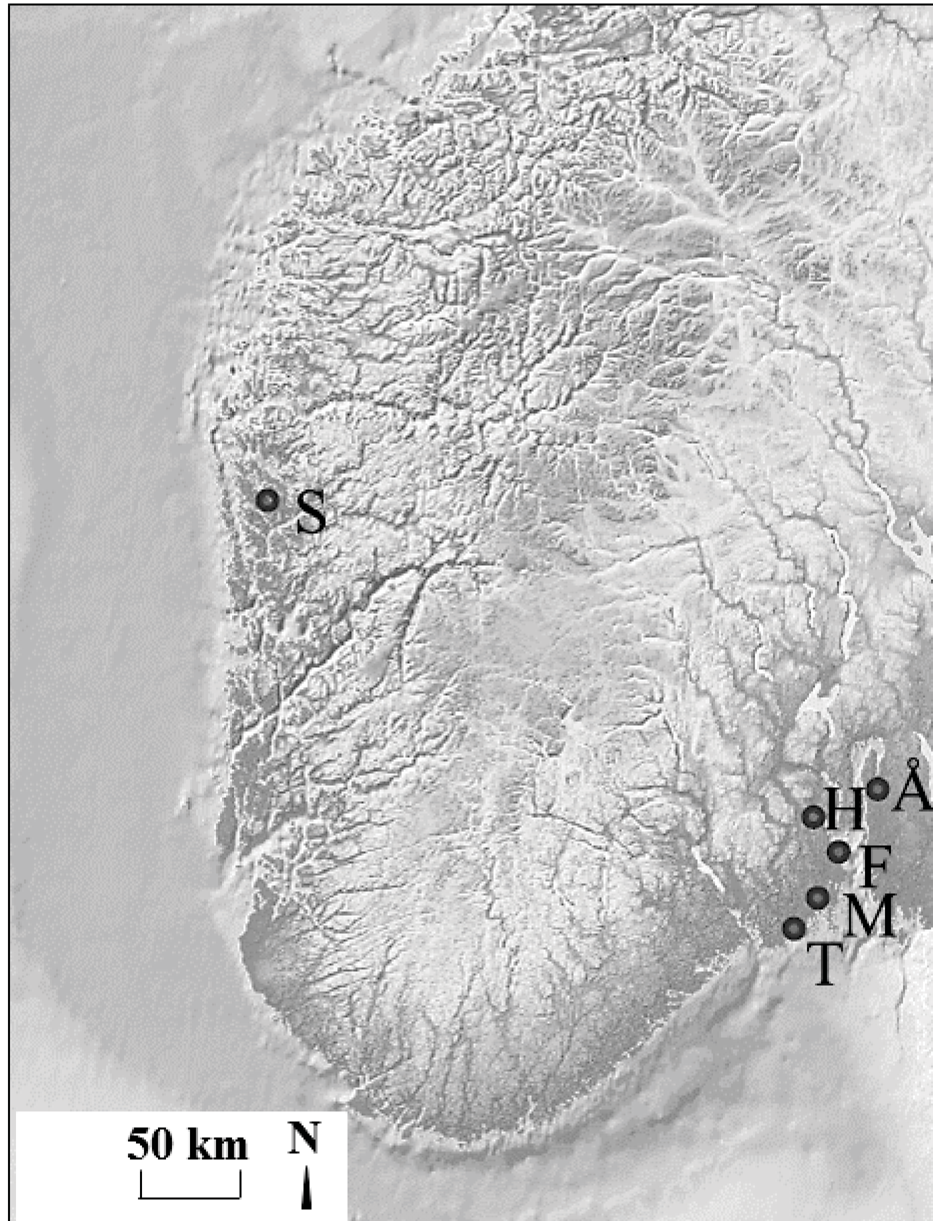

Figure 5. Locations of European beech populations sampled for the dormancy release experiments and the frost tolerance extperiments (S = Seim, Å = Ås, H = Holmestrand, F = Falkensten, M = Melsomvik, T = Tjølling).

The frost tolerance experiments were performed using shoots collected on the same dates and from the same trees as for the dormancy release experiments. The effects of different freezing temperatures on vegetative buds were tested following the same procedure as in Olsen *et al.* (1997a) (Fig. 6).

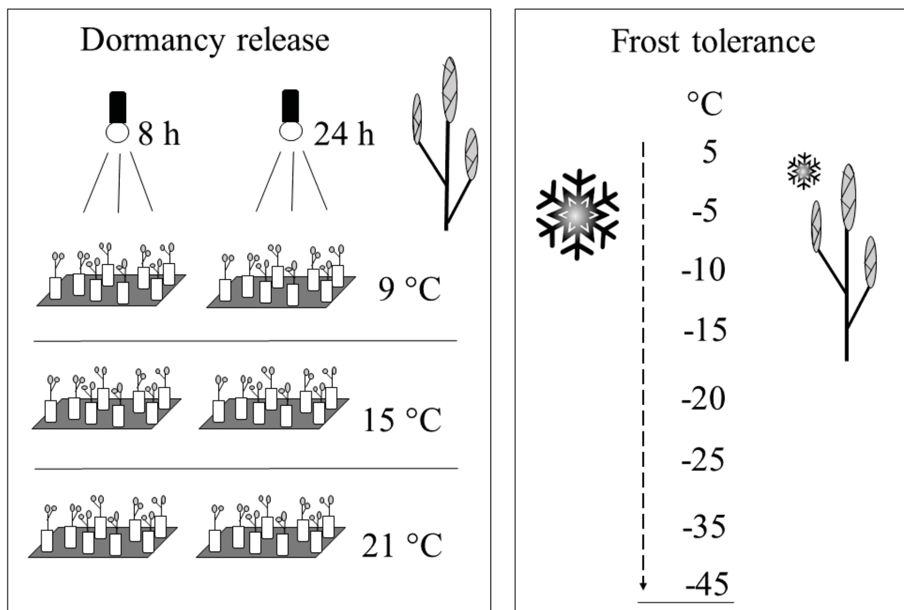

Figure 6. Experimental treatments in the dormancy release experiments and frost tolerance experiments performed in late winter and autumn 2014 using European beech shoots.

Field observations of bud break in spring

During spring in 2014 and 2015, we recorded bud break in understory saplings of European beech in four different populations in Vestfold (Fig. 7). Saplings were located within marked plots in stands which were either dominated by European beech or in mixed stands dominated both by European beech and Norway spruce (*Picea abies*). Recordings of apical stages were performed using a scoring system based on Fu *et al.* (2012).

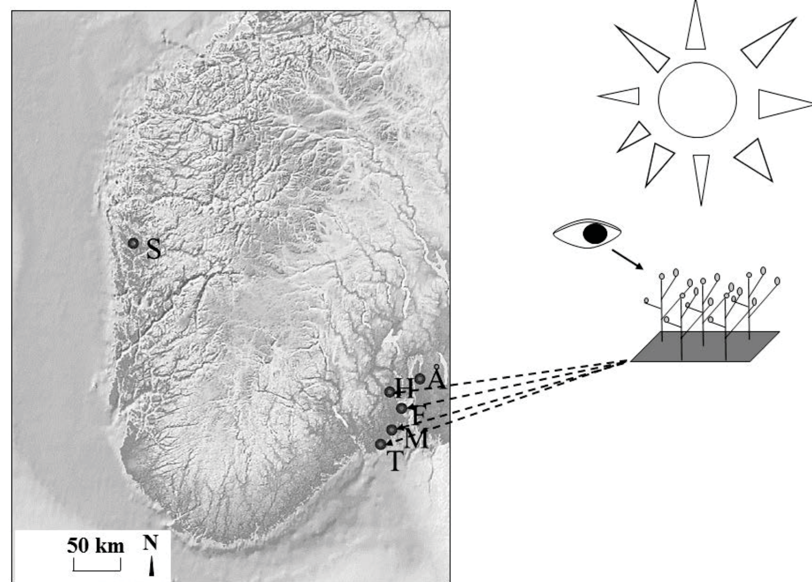

Figure 7. Locations for field observations of bud break in Vestfold populations of European beech (S = Seim, Å = Ås, H = Holmestrand, F = Falkensten, M = Melsomvik, T = Tjølling).

RESULTS AND DISCUSSION

Eurasian aspen

Temperature effects on phenology

In the field experiments using Eurasian aspen, higher autumn temperature delayed autumnal bud formation for females and males (Paper I, Paper II), yielding an extended growing season. This has been shown previously for different species and populations of *Populus* in field conditions (Rohde *et al.* 2011), where the effect of higher temperatures were attributed to a modulating effect on day-length sensing. As in our field experiments, Rohde *et al.* (2011) recorded autumnal bud set for different temperature regimes under gradually shifting day-length and light quality.

In contrast, most studies of bud formation and growth cessation in trees show that high night temperature advances bud formation under short days (see Hänninen & Tanino 2011 for a review). As those studies were performed in controlled conditions, it is possible that field studies yield dissimilar temperature effects due to a combination of climatic variables which are not reproduced in controlled environments. In our studies using Eurasian aspen,

observations were performed under fluctuating temperature, day-length and light quality, which may explain why our results differ from controlled experiments. Furthermore, we found that bud formation was best explained by maximum temperature when data from temperature treatments of the three field studies were combined (Paper III). We accounted for fluctuations of autumn temperature by calculating temperature parameters between each apical recording and tested the effects on bud formation. To our knowledge, this approach has not been used previously in studies of plant phenology, and may also have been responsible for the divergence between our results and those surveyed by Hänninen & Tanino (2011).

In the enhancement study, a temperature increase of +1.4 °C in autumn yielded advanced bud break during the consecutive spring (Paper I), indicating a carryover effect of delayed bud set on bud break. In contrast, ecophysiological models have predicted that warming should yield delayed bud break in spring due to insufficient chilling during autumn and winter (Cannell & Smith 1986; Murray *et al.* 1989). Indeed, this has been shown for a range of temperate and boreal tree species (Heide 1974; Westergaard & Eriksen 1997; Heide 2003; Søgaard *et al.* 2008; Kalcsits *et al.* 2009; Fu *et al.* 2015), which is opposite to what we observed following autumn warming. This discrepancy may be related to differing fulfilment of chilling requirement. As the modulated enhancement occurred in a boreal climate with cold winters, the chilling requirement for dormancy release was most likely sufficient. However, this may not be the case in warmer climates, where tree species are more susceptible to insufficient chilling.

In contrast to the enhancement study where temperature was only increased until 1 October, the elevational gradient yielded temperature differences on a whole-year basis. Growth and development inside vegetative buds is temperature dependent (Sarvas 1972; 1974), which explains why a 250-350 m elevation increase between locations delayed bud break with over a week (Paper II). In light of these findings, spring temperature appears to have a greater impact on bud break compared to autumn temperature in these environments, as autumn warming in the enhancement experiment yielded only 1-2 days delay in bud break. For plantlets growing at high elevation, autumn warming could thus be expected to involve only a slight increase in risk of frost damage during de-hardening and bud break. This is supported by a study involving eight deciduous tree species common to Europe, where it was shown that frost damage in buds and leaves in spring does not increase with elevation (Lenz *et al.* 2013). This was attributed to the delayed bud break and de-hardening in colder environments.

UV-B effects on phenology

Both UV-B enhancement (Paper I) and UV-B attenuation (Paper II) affected autumnal bud set in Eurasian aspen, indicating that UV-B has a signalling role for autumn phenology (Paper I). Although UV-B radiation has been shown to act as an environmental signal for plant growth, available literature does not contain evidence of UV-B affecting plant phenology. Autumn enhancement of UV-B yielded advanced bud formation (Paper I), and conversely, attenuation of ambient UV-B levels resulted in delayed bud formation at the highest elevation (Paper II). The effect of UV-B on bud formation was thus consistent across both studies, and it is possible that signalling occurs through plant hormones. Indeed, hormonal regulation of autumnal bud formation has been shown for different species in *Salicaceae* (Olsen *et al.* 1995a; b; 1997a; b; Mølmann *et al.* 2005), while UV-B has been shown to antagonise effects of gibberellic acid (GA) (Hayes *et al.* 2014) and affect concentrations of abscisic acid (ABA) in leaves (Xu *et al.* 2010). Considering that the molecular interactions involved in autumn phenology have been extensively studied in *Populus* (Ruttink *et al.* 2007), hormone profiling for plants exposed to different UV-B levels during autumnal bud formation could reveal which signalling pathways are affected.

Growth and metabolism

Increased elevation had a negative impact on plantlet size, which was smaller at higher elevations both in terms of stem height and basal diameter (Paper II). This may be related to the effect of low temperatures on bud formation (Paper I, Paper II, Paper III), resulting in shorter growing seasons for plantlets at higher elevations. Furthermore, it can be expected that temperatures at higher elevations had a negative impact on cell division and metabolism of assimilates. Indeed, it has been suggested that the latter effect is a major limitation for tree species at high elevation (Körner 1999). Interestingly, we found that leaf N concentrations diminished with increased elevation (Paper II), and it can be argued that this is an effect of lower soil N availability. However, we also found higher stem concentrations of N with increased elevation, and all plots across elevations had pre-fertilised potting compost before planting. Together, these findings suggest that low temperature promoted N translocation from leaves to stems at higher elevations, which is supported by available literature (see Cooke & Weih 2005 for a review).

In stems and leaves, individual phenolic compounds and groups of compounds both increased and decreased with elevation (Paper II), and some of these effects can be related both to

temperature affecting metabolism as well as a concentrating effect of plantlet size. Attenuation of UV-B yielded lower flavonoid content in leaves (Paper II), which is in line with evidence UV-B effects of flavonoid synthesis in boreal deciduous species (Lavola *et al.* 1997; Tegelberg & Julkunen-Tiitto 2001; Lavola *et al.* 2013; Randriamanana *et al.* 2015). In addition, this treatment yielded increased concentration of stem salicylates, an effect which has not been shown previously, according to available literature.

Sex-related differences

The magnitude of effects on bud formation differed between females and male both in relation to temperature and UV-B, which could also partially account for different responsiveness in terms of growth and metabolism. The delayed bud formation in autumn under temperature enhancement was more pronounced in male plantlets, while males were also more responsive to UV-B enhancement (Paper I), counterbalancing the effect of warming. However, we did not observe any sex related differences in bud formation in relation to UV-B attenuation, suggesting that sex-related responses occur under higher UV-B levels than those present along the natural gradient.

Male plantlets broke buds faster than females in spring following autumn warming (Paper I). In addition, timing of bud formation differed also between sexes across the elevational gradient, which was delayed for male plantlets at higher elevation (Paper II). When temperature effects on bud formation were examined more closely using data from all of the three field studies, responses varied both in relation to plantlet sex and bud development stage (Paper III). Bud formation can be dissected into several processes, and is a result of temperature and day length signalling for *Populus* (Ruttink *et al.* 2007; Rohde *et al.* 2011). As warming affects the stages of bud formation differently, considerable inter-annual variation of autumn temperatures may further complicate sex-related responses. Even so, data from our field studies suggest that warming can be expected to yield a general delay for both sexes of Eurasian aspen, while UV-B radiation has an opposite effect

Dormancy release and frost tolerance in Norwegian populations of European beech

Based on dormancy release experiments in controlled conditions and field observations of bud break, there are strong indications that bud break dates of Norwegian populations of European beech are temperature limited (Paper IV). These populations constitute the northernmost distribution range of European beech and can be expected to advance bud break in spring as a response to warming. Indeed, advanced bud break has been observed for populations at high elevation in Slovenia and in the Swiss Alps through the last decades, a trend which was not observed for low-elevation populations (Cufar *et al.* 2012; Vitasse & Basler 2013). Many deciduous species break buds solely in response to rising temperatures, early-flushing species in particular. For European beech, bud break is known for being under photoperiodic control (Heide 1993), and is inhibited as long as day length is below a minimum threshold. Once this limit is reached in spring, cell division and tissue growth within buds are dependent on warm temperatures. As for high elevation populations discussed by Vitasse & Basler (1993), the temperature dependency of bud break in Norwegian populations may be related to low temperatures when the photoperiod is sufficiently long for dormancy release.

Considering that freezing temperatures occur regularly at the northernmost distribution range of European beech, it is likely that advanced spring phenology under warming yields increased risk of freezing damage in newly emerged leaves. Whether this will constitute a major limitation for European beech in Scandinavia remains to be proven, as it has been suggested for this species that short growing seasons may be more limiting than freezing damage (Vitasse *et al.* 2014). Using sampled shoots from Norwegian populations, frost tolerance of vegetative buds was found to decline gradually from January to March, and increase in late autumn through early winter (Paper IV). Frost tolerance is known to decrease gradually through late winter and early spring with increasing temperatures (Vitasse *et al.* 2014), and the observed differences in frost tolerance between populations can be related to local temperature regimes. However, it cannot be excluded that local adaptations also had an effect, which has been shown for provenances on the European mainland (Kreyling *et al.* 2014).

CONCLUSION AND FUTURE PERSPECTIVES

Based on evidence for both species investigated in this thesis, warming can be expected to yield extended growing seasons and thus increase the susceptibility to frost damage in non-woody shoot tips and leaves. For Eurasian aspen grown under modulated temperature enhancement and temperature regimes at different elevations, temperature had a negative effect on autumnal bud formation. In addition, the observed delay in warm conditions was more pronounced in male plants. The effect of warming is opposite to previous studies in controlled conditions, and data from three field experiments show that high temperature is the temperature parameter that best explains temperature effects on autumnal bud formation. The effects of autumn warming were counterbalanced by ambient and elevated levels of UV-B radiation, which had a positive effect on bud formation. Furthermore, elevated temperature in autumn resulted in earlier bud break, but the effect of increased spring temperature was more pronounced. Thus, the carryover effect of autumn warming should not involve any substantial effect on bud break timing. For European beech, evidence from controlled and field conditions shows that timing of bud break in Norwegian populations is limited by temperature. In addition, it was possible to relate the gradual loss of frost tolerance to local temperature regimes. These populations are on the margins of the species' latitudinal distribution range, and can be expected to advance bud break dates with future warming.

Based on evidence presented here, further studies could help clarify the following questions:

- How does warming affect other tree species common to temperate climates?
- What is the role of UV-B during phenological shifts in trees during autumn, and which molecular signalling pathways are involved?
- Are interactions between UV-B and temperature a result of separate effects counterbalancing each other or signals acting in concert?
- As there is an apparent discrepancy between warming effects in natural conditions and controlled experiments, what are the underlying mechanisms?
- Do populations of European beech in Norway display adaptations to local climates?
- For natural populations of Eurasian aspen and European beech, can the likely increase in susceptibility to frost damage with extended growing seasons be verified by field observations?

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PAPER I

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Original Article

UV-B and temperature enhancement affect spring and autumn phenology in *Populus tremula*

C. B. Strømme[1], R. Julkunen-Tiitto[2], U. Krishna[2], A. Lavola[2], J. E. Olsen[3] & L. Nybakken[1]

[1]Department of Ecology and Natural Resource Management, [3]Department of Plant Sciences, CERAD, Norwegian University of Life Sciences, 1432 Ås, Norway and [2]Department of Biology, University of Eastern Finland, 20101 Joensuu, Finland

ABSTRACT

Perennial plants growing at high latitudes synchronize growth and dormancy to appropriate seasons by sensing environmental cues. Autumnal growth cessation, bud set and dormancy induction are commonly driven by the length of photoperiod and light quality, and the responses are modified by temperature. However, although ultraviolet (UV)-B radiation is well known to affect plant growth and development, information on the effects on bud phenology is scarce. We examined the separate and combined effects of enhanced temperature and UV-B on autumnal bud set and spring bud break in female and male clones of *Populus tremula* in an outdoor experiment in Joensuu, Eastern Finland. Enhancements of UV-B and temperature were modulated to +30% and +2 °C, respectively, from June to October 2012. Enhanced UV-B accelerated bud set, while increased temperature delayed it. For both UV-B and temperature, we found sex-related differences in responsiveness. Temperature increase had a stronger delaying effect on bud maturation in male compared with female clones. Also, male clones were more responsive to UV-B increase than female clones. Increasing autumnal temperature enhanced bud break in spring for both sexes, while UV-B enhanced bud break in male clones. In conclusion, we found that UV-B affected phenological shifts in *P. tremula*, and that temperature and UV-B affected genders differently.

Key-words: bud break; bud set; climate change; genders; phenology; ultraviolet radiation.

INTRODUCTION

At high latitudes, plants synchronize physiological processes to seasonal change through accurate sensing of environmental cues, namely photoperiod, light quality and temperature (Junttila 2007; Olsen 2010; Olsen & Lee 2011). This ensures growth in favourable seasons while protecting meristems during winter. Anthropogenic environmental change will yield seasons with new combinations of light quality and temperature, and there is a growing concern on possible effects on phenology and duration of the growing season (Peñuelas *et al*. 2009; Körner & Basler 2010; Hänninen & Tanino 2011; Olsen & Lee 2011). As plant performance is highly dependent on the correct timing of physiological processes to seasons, it remains uncertain how different plant species will react to environmental change.

Phenological shifts, such as bud set, dormancy induction, and acclimation to cold and drought are controlled by a complex signalling network entrained by environmental cues. For a wide range of woody species of the temperate and boreal zones, photoperiod is recognized as the main cue for autumnal growth cessation, bud set and dormancy induction (Nitsch 1957; Håbjørg 1972; Olsen *et al*. 1997a; Thomas & Vince-Prue 1997). In addition, light quality has been demonstrated to be of considerable importance particularly in northern populations of species like *Salix pentandra* and *Betula pubescens*, as well as conifers like *Picea abies*. These show an increasing requirement for far-red light in the spectrum with increasing northern latitude of origin to sustain growth (Junttila & Kaurin 1985; Clapham *et al*. 1998; Tsegay *et al*. 2005; Mølmann *et al*. 2006). Furthermore, it has been shown that far-red light advances bud break in *Betula pendula* (Linkosalo & Lechowicz 2006). Also, day extension with monochromatic blue light has been shown to delay growth cessation and bud formation (Mølmann *et al*. 2006). In these respects, that is bud set and bud break, information about the effects of the other parts of the light spectrum such as ultraviolet (UV)-radiation is scarce.

UV-B radiation varies naturally with latitude, altitude, season and time of day (Wang *et al*. 2007). In addition, concentrations of stratospheric ozone and cloudiness affect the amount of UV-B radiation reaching the ground (McKenzie *et al*. 2011). There is also geographical variation in the thickness of the ozone layer, causing differences in daily UV-B exposure levels.

The stress physiology of plants in relation to exposure to UV-A and UV-B has been extensively studied. UV-A and UV-B radiation were shown to have different effects on accumulation and concentrations of phenolic metabolites, as has been shown for *B. pendula* and *Alnus incana*, (Kotilainen *et al*. 2008; Morales *et al*. 2010). UV radiation is also recognized as an important environmental signal modulating plant growth and development (Rozema *et al*. 1997; Jansen & Bornman 2012). A UV-B receptor denoted UVR8 was recently characterized in *Arabidopsis thaliana* (Jenkins 2009; Rizzini *et al*. 2011), and blue light receptors are known to also sense UV-A.

Correspondence: C. B. Strømme. E-mail: christian.stromme@nmbu.no

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In recent years, it has been increasingly recognized that temperature also affects growth cessation and bud set in different boreal tree species. The expected increase in spring and autumn temperatures because of climate change may yield different phenological responses in tree species, (Körner & Basler 2010; Tanino *et al*. 2010; Hänninen & Tanino 2011; Vitasse & Basler 2013), as well as populations across different latitudes (Rohde *et al*. 2011a). In *Acer platanoides*, higher autumn temperatures increased the depth of dormancy (Westergaard & Eriksen 1997). Similar relationships were found for two different species of *Betula*, for *Alnus glutinosa* (Heide 2003), and for *P. abies* (Søgaard *et al*. 2008). In studied species of *Populus*, higher autumn temperatures delay growth cessation and bud set (Kalcits *et al*. 2009; Rohde *et al*. 2011a).

The underlying molecular mechanisms leading to bud set have so far been best studied in *Populus*. Signal transduction pathways involving light, ethylene and abscisic acid (ABA) control processes leading to autumnal bud set in this genus (Ruttink *et al*. 2007). Stimulation of ethylene and ABA signalling is preceded by rapid reduction in gibberellin (GA) and auxin content after transition to short days (SD) (Olsen *et al*. 1995a,b, 1997a,b). In an outdoor study of different *Populus* species and hybrids, temperature has been found to affect the onset and duration of bud set in autumn (Rohde *et al*. 2011a). Higher temperatures delay growth cessation, and also have a positive effect on bud formation after growth cessation. In addition, by transferring plants with identical genotypes to different geographic locations, it was shown that local climatic conditions affect the sensitivity to the SD signal (Rohde *et al*. 2011a). In northern ecotypes of a range of species including *Populus*, it has been demonstrated that low temperature may result in growth cessation and bud set even under long days (Mølmann *et al*. 2005; Tanino *et al*. 2010; Olsen & Lee 2011). Thus, temperature signalling could affect acclimation to future climatic conditions.

European aspen (*Populus tremula*), is a dioecious tree species belonging to *Salicaceae*, which includes several other dioecious species. Earlier studies suggest that for some species of *Populus*, males are more growth-oriented than females (Lloyd & Webb 1977), which is also shown for other tree species (Obeso 2002). *P. tremula* has a wide distribution across latitudes on the Eurasian continent (Worrell 1995). In addition to displaying adaptations to local climatic conditions (Rohde *et al*. 2011a), species of *Populus* exhibit sex-related responses to changing environmental factors. Indeed, there is concern for the vulnerability of *Populus* and *Salix* populations following climate change due to differences in performance and survival between females and males (Tognetti 2012). In *Salix myrsinifolia*, phenolic content may differ between sexes under increased temperature and concentration of CO_2 (Nybakken *et al*. 2012; Nybakken & Julkunen-Tiitto 2013). Furthermore, female and male plants perform differently with varying soil moisture such as in *Acer negundo* and *Salix arctica*, where males are more drought resistant (Dawson & Bliss 1989; Dawson & Ehleringer 1993). Males of *Populus cathayana* are more responsive to increased temperature and CO_2 concentrations in terms of vegetative growth (Zhao *et al*. 2012). Under increased temperatures and drought, higher responsiveness of females resulted in poorer performance (Xu *et al*. 2008). In *P. cathayana*, it is documented that responsiveness to light quality and photoperiod differs between sexes. While males exhibit faster leaf senescence under SDs, they do also maintain a less senescent stage than females (Zhao *et al*. 2009). Female and male plants have different responsiveness to increased UV-B radiation (Xu *et al*. 2010). Males appear to be more resistant to UV-B radiation, as biomass accumulation is less affected by increased UV-B in males than in females. However, these findings were a result of UV-B levels being almost twice as high as ambient levels. In *S. myrsinifolia*, +32% enhancement of UV-B did not yield any differences in biomass between females and males (Nybakken *et al*. 2012).

To our knowledge, no studies have investigated possible effects of UV radiation and UV temperature interactions on the timing of dormancy-related processes in perennial plant species. In addition, most studies of effects of increased temperature on phenology in trees have been performed indoors without natural fluctuations in climatic conditions. We established a multifactorial outdoor experiment where temperature, UV-B and UV-A levels were systematically increased in concert with natural fluctuations using a modulated lamp system, and we registered bud set in autumn and bud burst in spring of female and male clones of *P. tremula*. Based on recent results with *Populus* and other tree species, we expected that elevated temperature would delay bud set in autumn and result in deeper dormancy than lower temperature. We further suspected that the important environmental signals UV-B and /or UV-A would act in concert with temperature. Among the studies mentioned earlier, some have shown higher responsiveness in males to resource availability and climatic factors. We therefore also hypothesized that males would be more responsive to temperature and UV increase than females.

MATERIALS AND METHODS

Plant materials

The plantlets used in the field experiment originated from six female and six male adult aspens (about 30–40 years old) from Southern and Eastern Finland (Kaavi 62°54′ N, 28°42′ E, Liperi 62°41′ N, 29°33′ E, Loppi 60°43′ N, 24°27′ E, Pieksämäki 62°18′ N, 27°07′ E, Polvijärvi 62°52′ N, 29°19′ E and 62°49′ N, 29°20′ E, Kontiolahti 62°38′ N, 29°41′ E). One genotype was selected from each location. Because of the geographical distances between locations, it was assumed that individuals belonged to different aspen populations. The plant growth medium consisted of woody plant medium with 8.5 g L^{-1} Agar and 5 mg L^{-1} indole butyric acid. Plantlets were kept under a photosynthetic photon flux density of 70 μmol m^{-2} s^{-1} at 400–750 nm provided by fluorescent light tubes (Gro-Lux F36W, Havells Sylvania, Germany) at 23 ± 0.1 °C and 18 h photoperiod.

The plantlets were potted up using 70% commercial peat and 30% vermiculite and transferred to a greenhouse on 2 May 2012. Relative air humidity was set to 70%. The light

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conditions were enriched using 400 W high-pressure sodium lamps (GE Lighting, Cleveland, OH, USA). The photoperiod was 18 h and the temperature was set to 20 °C. With the additional warming of the lamps, temperatures varied between 20 and 23 °C, depending on time of day. The plantlets were transferred to the field site in Joensuu, Finland (62°60′ N, 29°75′ E) on 7 June in 2012 and planted on 11 June. Several plants either experienced *Venturia* shoot blight, mechanical or herbivore damage or death during the season. Thus, a total of 814 females and 838 males were recorded during bud set. After the growing season, one individual per clone per plot was harvested for other analyses. As additional plants died during winter, 667 females and 671 males were recorded during bud break in spring.

Experimental set-up

The experimental design was based on Nybakken *et al.* (2012). At the field site female and male plantlets of *P. tremula* were distributed among 36 plots arranged in a 6 × 6 matrix. A 10 cm layer of mineral soil (0.8% limed) was added to each plot. Aluminium frames (1.5 × 2.0 m) were mounted above each plot, and the spacing between the plots was 3 m in all directions. The experimental site was fenced in using a 1.5 m high metal net to prevent intrusion of large mammals, and included a 2 mm thick metal shelter set about 60 cm into the soil and 60 cm above the soil level to prevent vole intrusion.

The treatments applied were a combination of temperature and UV-B radiation enhancement continuously modulated to +2 °C and +30% increase, respectively (Figs 1–3; Supporting Information Fig. S2). The achieved levels were on average +1.35 ± 0.042 °C and +28.0 ± 0.4% of ambient temperature and UV-B levels, respectively (Figs 3–5). Thus, plants within each plot received one of six different treatments and treatment combinations [increased temperature (T), enhanced UV-B (UV-B), enhanced UV-A (UV-A), UV-B + T, UV-A + T, control with ambient temperature and

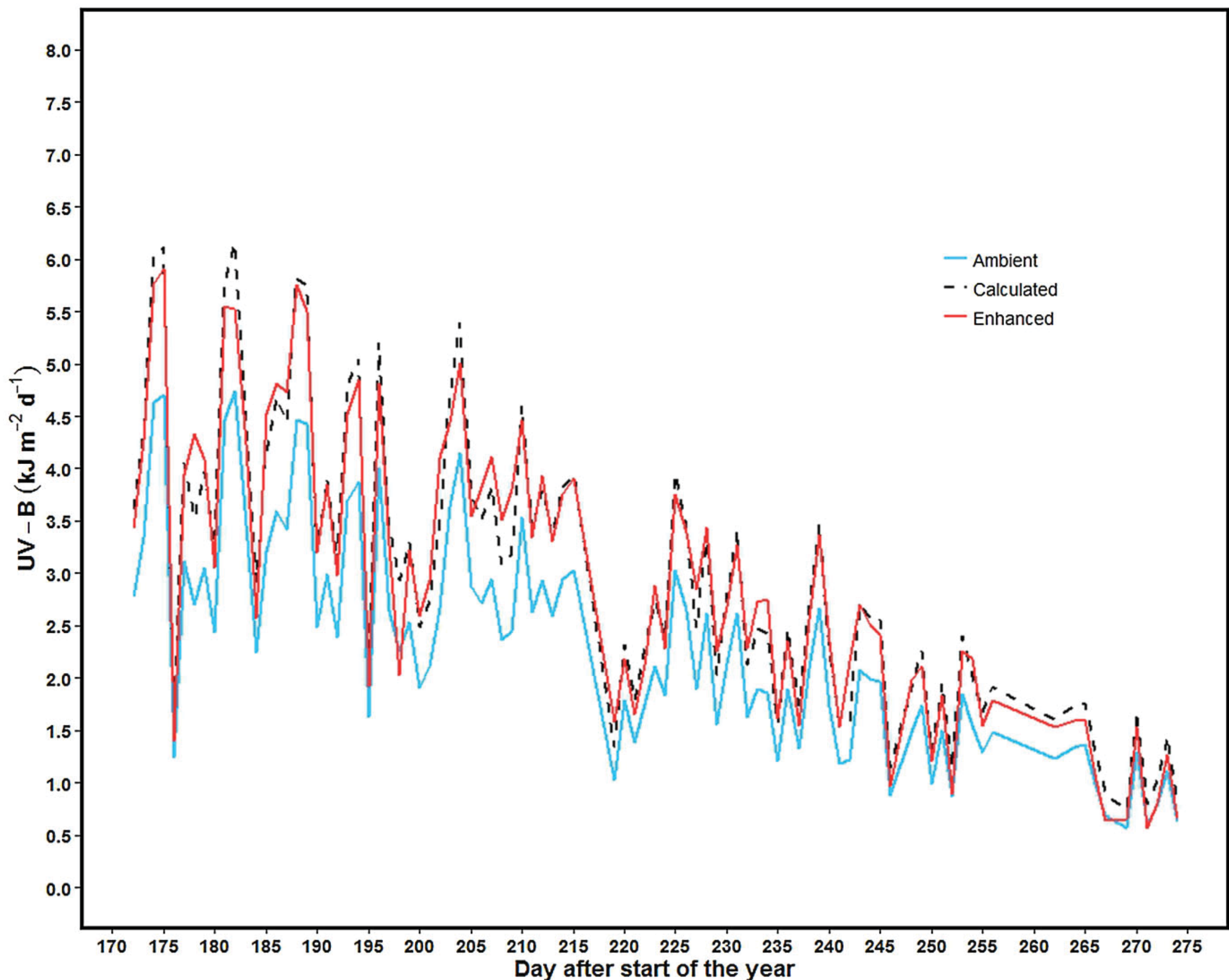

Figure 1. Performance of ultraviolet (UV)-B enhancement at the experimental site during autumn 2012, measured by four broadband UV-B sensors. Calculated values are 30% higher than ambient levels.

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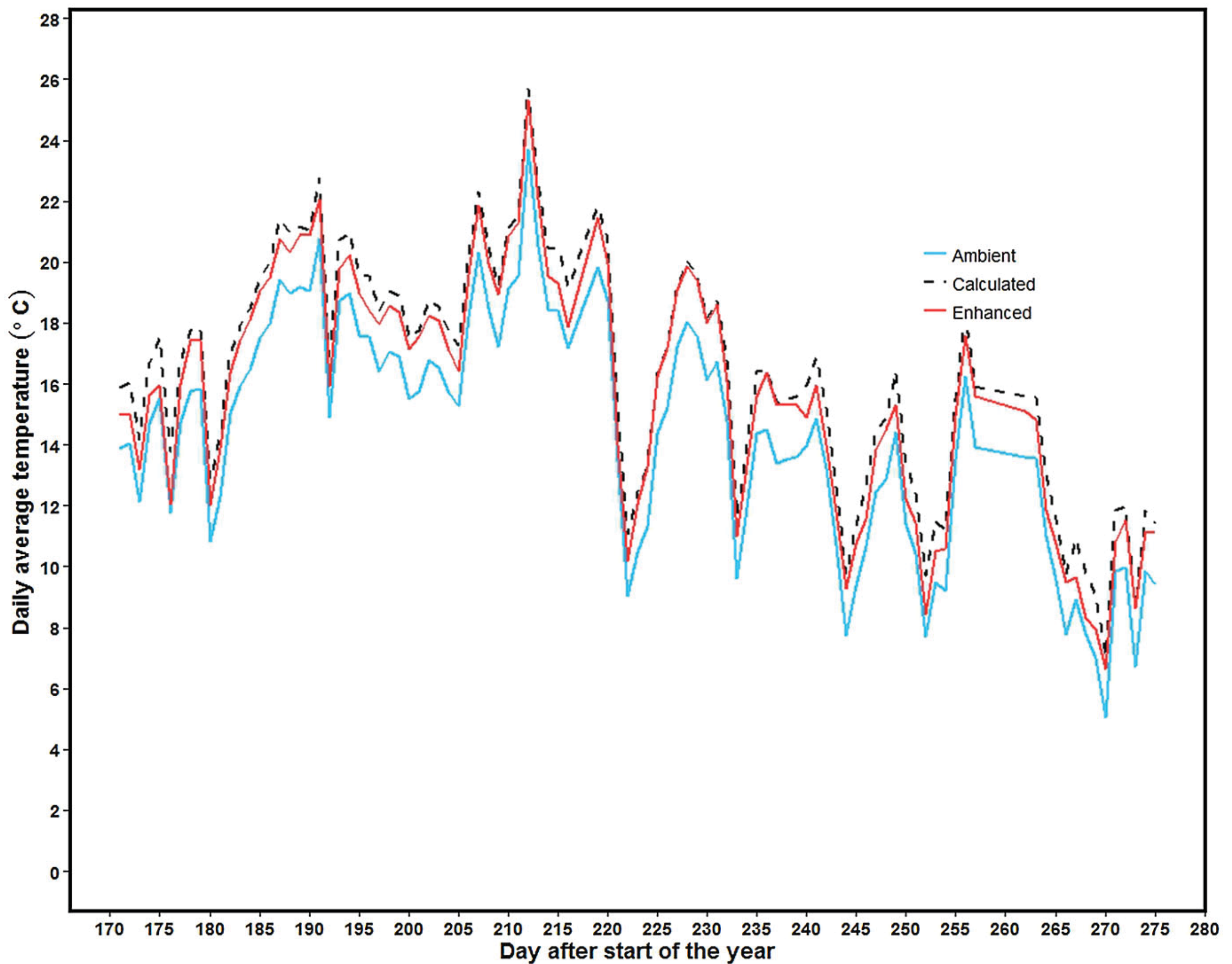

Figure 2. Performance of temperature enhancement at the experimental site during autumn 2012, that is as daily average temperature, measured by four temperature sensors. Calculated values are 2 °C higher than ambient levels.

UV radiation (C)]. On each frame, six 40 W UV-fluorescent lamps (1.2 m long, UVB-313, Q-Panel Co., Cleveland, OH, USA; Spectrum in Supporting Information Fig. S1) were mounted following a 'cosine' distribution (Björn 1990) and kept at minimum 60 cm above the plant shoot tips. The emission spectrum was obtained using an Optronic OL-756 portable UV-VIS spectroradiometer (Optronic Laboratories, Orlando, FL, USA). In the UV-B enhancement treatment, radiation below 290 nm was attenuated using cellulose diacetate filters wrapped around each lamp. UV-B-tubes emitted some UV-A and thus, plots receiving UV-B also received additional UV-A. To control for UV-A effects, six plots had UV tubes wrapped with polyester film to remove UV-B (attenuation below 315 nm). Thus, the purpose of this 'UV–A-enhanced' treatment was to evaluate the effect of the low UV-A emission provided by the UV tubes relative to the far higher UV-B emission (Supporting Information Fig. S1). In plots where UV was not enhanced, UV lamps were un-energized to obtain the same level of shading as in UV enhancement plots.

The continuous temperature enhancement was obtained using two infrared (IR) heaters (CIR 105, FRICO, Partille, Sweden) mounted along the middle length axis of the aluminium frames. In plots where temperature was not enhanced, shading was compensated for using wooden boards of the same area as the IR radiators. The aluminium frames were supported by metal posts in a manner which allowed for up- and downward adjustment, and the frames were raised every third week to keep the radiators 60 cm above the highest shoot tip. The whole system was run continuously, that is UV and temperature were increased, between 1 June 2012 and 1 October 2012.

UV-B radiation was measured using four Thies Clima sensors (Thies, Göttingen, Germany). The sensors measure radiation between 250 and 325 nm with a peak at 300 nm. Two sensors were placed above the control frames for ambient UV-B levels, and two were placed under the frames of UV-B enhancement plots for set-point values. Temperature enhancement was modulated using self-made linear temperature sensors with four PT1000 probe elements with a

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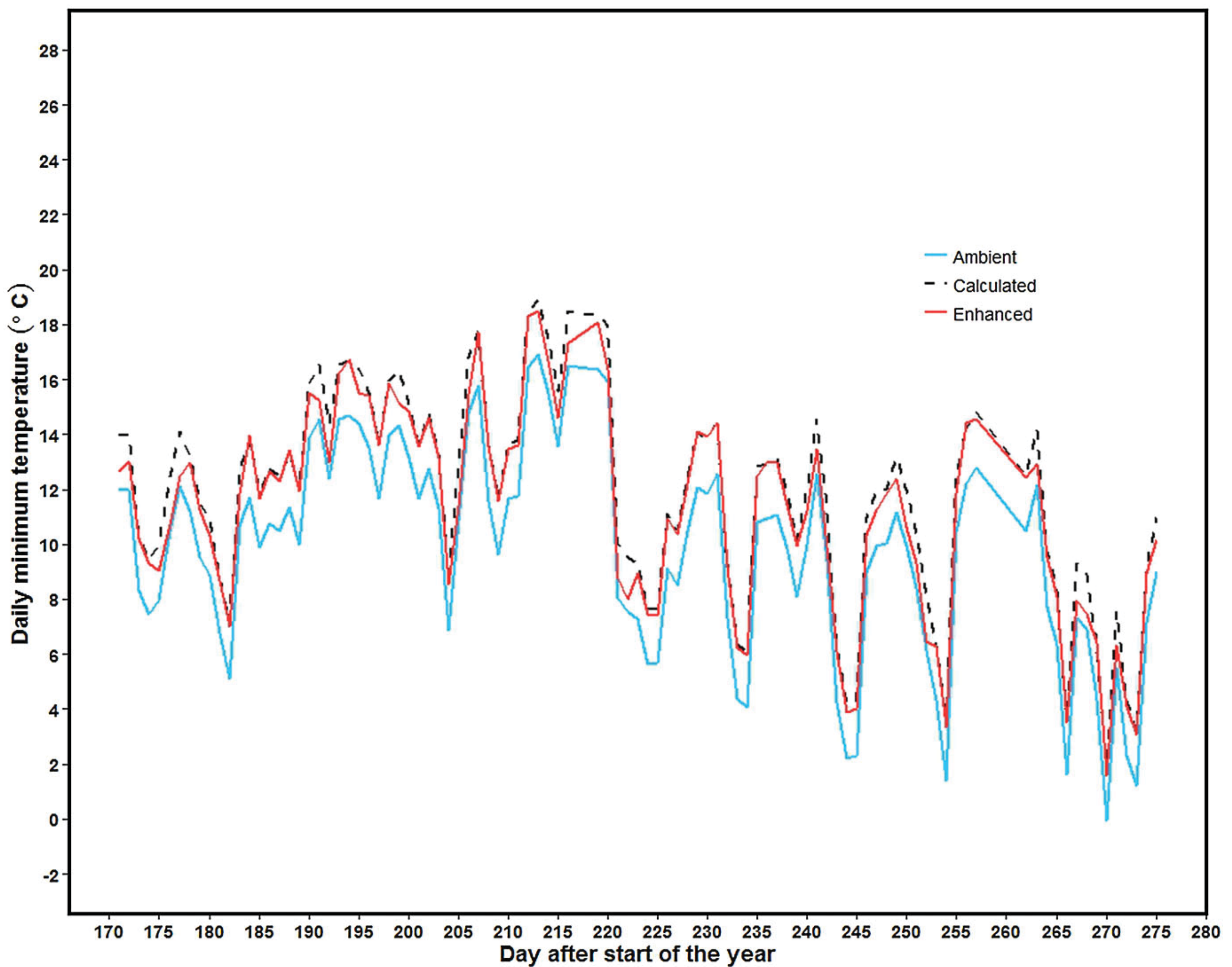

Figure 3. Performance of temperature enhancement at the experimental site during autumn 2012, that is as daily minimum temperature, measured by four temperature sensors. Calculated values are 2 °C higher than ambient levels.

connection cable for each. Two probe elements were placed above control frames, while two were placed under the temperature-enhancement frames for set-point values. Calculations of set-point values were performed using modulator software (IPC100 configuration program and e-console measuring and data saving program, Gantner Instruments GmbH, Darmstadt, Germany), which also controlled the enhancement of UV lamps and IR radiators. Soil temperature was logged in the same plots. Temperature and precipitation data for 2012 and 2013 were obtained from a meteorological station at Linnunlahti, Joensuu, less than 200 m from the experimental field (Supporting Information Figs S3 & S4).

Recording of stages of bud set and bud burst

The autumnal bud set was recorded from 16 August until 25 September 2012. Bud set was followed by assigning the shoot apex to one of three stages of bud development, based on Rohde *et al*. (2011b). The first stage includes apices between full, active growth to apices with an open bud (1), closed green bud (0.5) and brown/red closed bud (0). Scoring of apices during spring bud break was recorded from 6 May until 26 May 2013. Bud break was dissected into four stages based on Fu *et al*. (2012): closed bud (0); closed bud with visible green leaf (1); green leaf diverging from bud axis but no visible petiole (2); broken bud with at least one visible petiole (3).

Statistical analyses

The effects on bud set in 2012 and bud break in 2013 were tested using cumulative link mixed models (clmm) in R (R Core Team 2013) by applying the clmm function in the Ordinal package (Christensen 2013). In cumulative link mixed models, the response is categorical. The apical stages recorded in autumn 2012 and spring 2013 were response variables in two different models. In each model, calculations yielded coefficients, their standard errors, and *P*-values for

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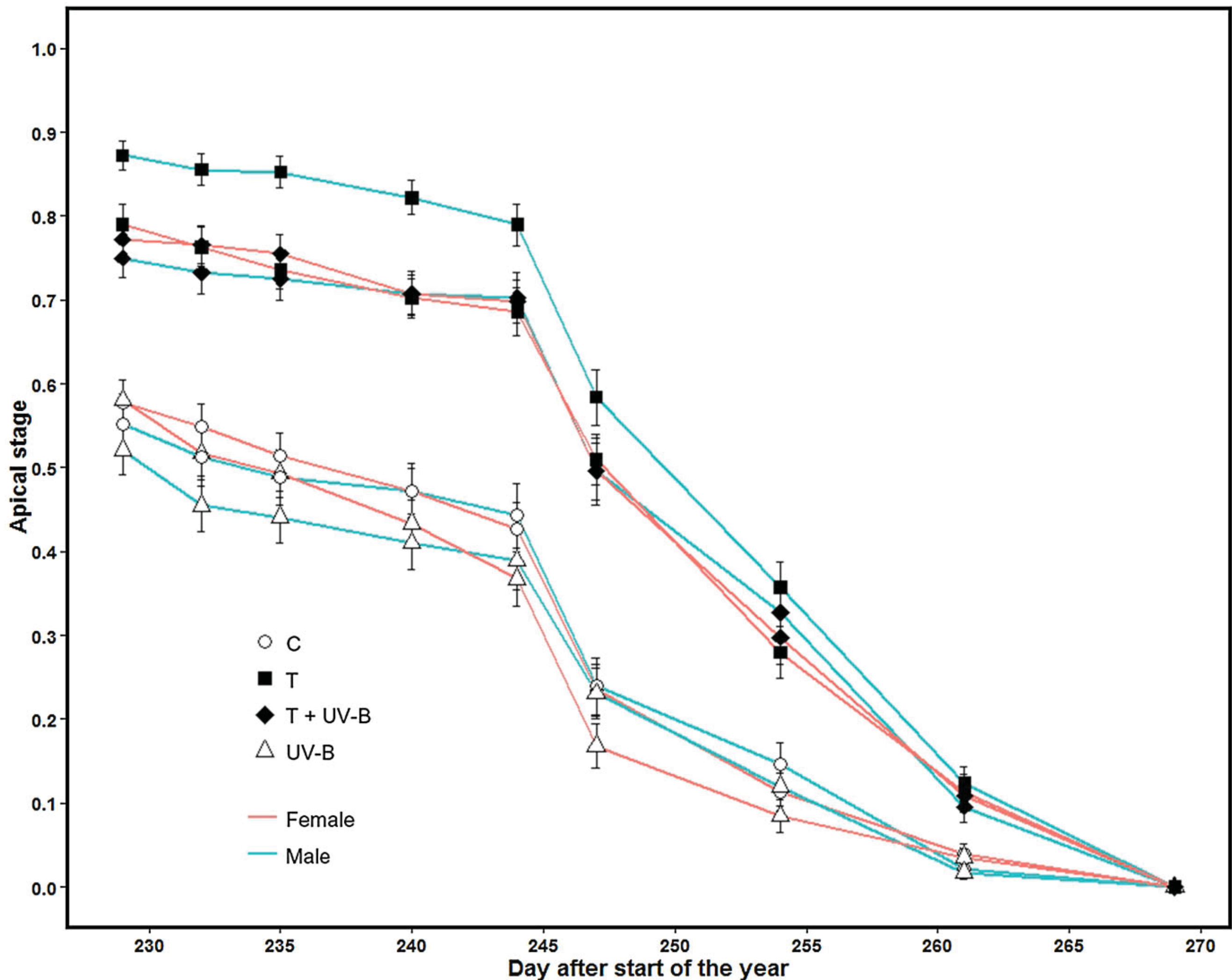

Figure 4. Average score values of apical stages (growing plants without closed buds 1; closed green bud 0.5; brown/red closed bud 0) during bud set in six female and six male clones of young plants of *Populus tremula* in autumn 2012 after exposure to enhanced temperature (T) of 1.35 ± 0.042 °C and enhanced ultraviolet (UV)-B of 28.0 ± 0.4% relative to ambient conditions between 1 June 2012 and 1 October 2012. C, control at ambient temperature and UV-B levels. The average scores ± 1 SE for treatment combinations are shown. Averages of UV-A treatments are not shown, as the effect of UVA was non-significant.

levels of the fixed factors based on their effect on the apical stage. Temperature treatment (two levels), UV treatment (three levels), sex (two levels) and day of year were fixed factors, and interactions between temperature treatment, UV treatment and sex were included. Clone identity and Plot were set as random factors.

RESULTS

Enhanced UV-B radiation significantly forced bud set in male clones ($P = 0.023$) (Table 1). This was revealed by the significant interaction between UV-B and sex.

Increased temperature, on the other hand, significantly delayed bud set in all six male and female clones ($P = 0.002$) (Table 1). There was a significant interaction between temperature and sex, as there was a stronger delaying effect on male than female clones ($P < 0.0001$). Also, there was a significant three-way interaction between UV-B, temperature and sex ($P = 0.033$). It thus appears that the effects of increased temperature were somewhat reduced when UV-B enhanced bud set at the same time (Fig. 4). As plants had either a growing apex or closed green buds at the start of the study period, the average values in each treatment were lower than 1 (Fig. 4). In the UV-A treatment, in which the UV-B emission was attenuated by polyester film, there was no significant effect on bud set of the low UV-A emission provided by the UV tubes (Table 1).

For bud break in spring, enhanced UV-B during the previous growth season had a significant forcing effect on male clones (Table 2), as there was a significant interaction between UV-B and sex ($P = 0.008$). Increased previous autumn temperature also significantly forced spring bud

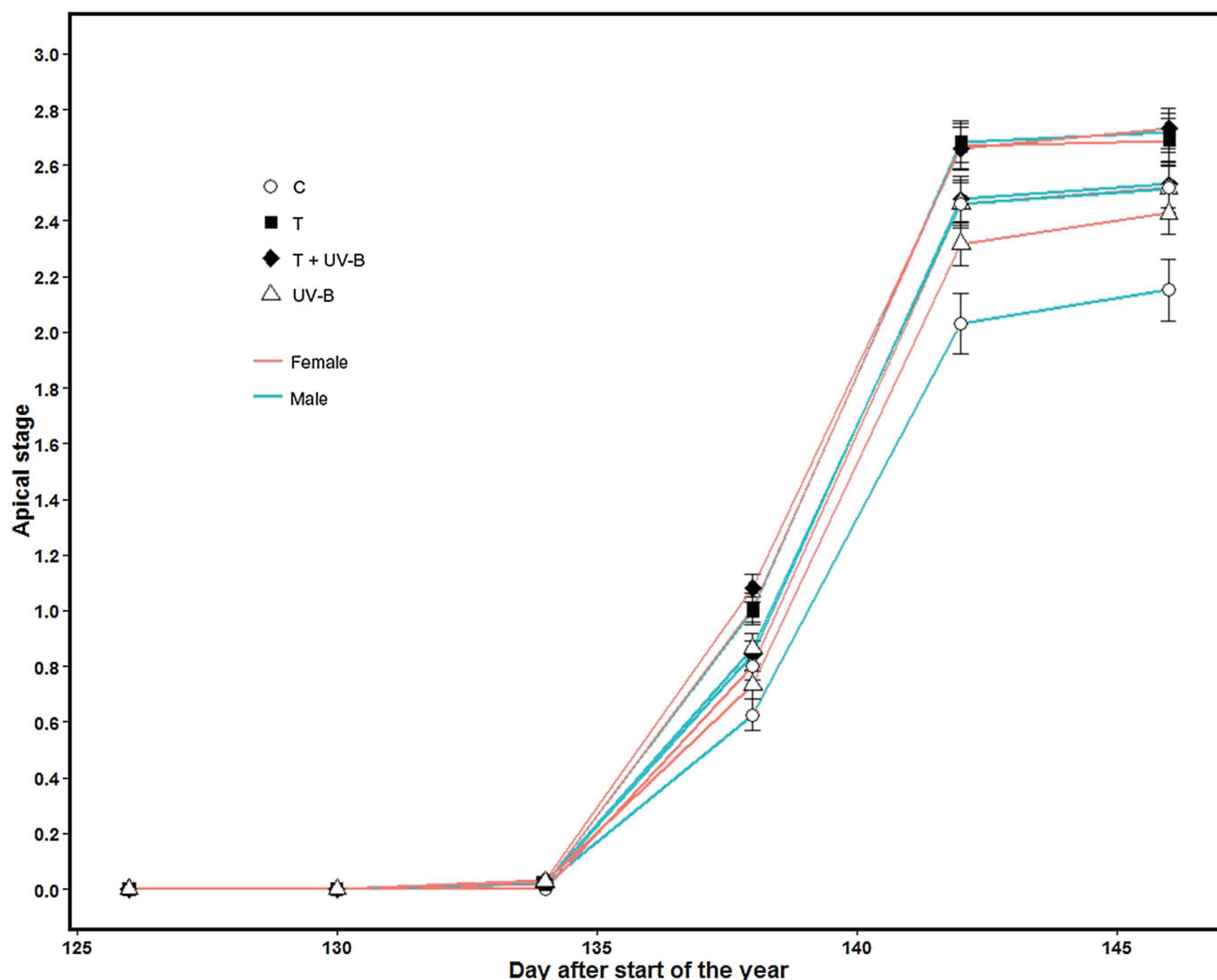

Figure 5. Average score values of apical stages (closed bud 0; closed bud with visible green leaf 1; green leaf diverging from bud axis, but no visible petiole 2; broken bud with at least one visible petiole 3) during bud set in six female or six male clones of young plants of *Populus tremula* in spring 2013 after exposure during autumn 2013 to enhanced temperature (T) of 1.35 ± 0.042 °C and enhanced ultraviolet (UV)-B of 28.0 ± 0.4% relative to ambient conditions between 1 June 2012 and 1 October 2012. C, control at ambient temperature and UV-B levels. The average scores ± 1 SE for treatment combinations are shown. Averages of UV-A treatments are not shown, as the effect of UV-A was non-significant.

break ($P = 0.044$). The significant interaction between temperature and sex was due to a stronger effect of temperature on male clones ($P = 0.002$). Female plants generally showed earlier bud break independently of treatments ($P = 0.045$), as male plants had a negative coefficient (Table 2). There was a significant three-way interaction between sex, temperature and UV-B ($P = 0.046$), which may be due to the general delay in bud break for males. Like for autumnal bud set, there was no significant after-effect of the low emission of UV-A provided by the UV tubes on spring bud burst (Table 2).

DISCUSSION

We show here that increased temperature and UV-B radiation in autumn significantly affected the autumnal bud set and spring bud break of young *P. tremula* plants. In addition, we found that the low UV-A emission from the UV tubes used for the UV-B enhancement treatment did neither affect bud set nor bud break the following spring. To our knowledge, there are no previous findings of such effects of UV-B on the phenology of tree species.

UV-B irradiance varies with latitude, altitude, time of day and season. In northern tree species in general, including *Populus*, photoperiod and light quality are known to be the primary environmental cues for the induction of apical growth cessation, bud set and dormancy acquisition through affecting a complex signalling network (Ruttink *et al*. 2007; Olsen 2010). Our data here demonstrate that UV-B promotes growth cessation and bud set. While UV radiation earlier commonly was considered a stressor, considerable knowledge on UV-B as a morphogenetic signal has emerged in recent years (Rozema *et al*. 1997; Jansen 2002; Jenkins

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Table 1. Parameter estimates, SE and z-values for covariates in the cumulative link mixed model run to investigate the effects of elevated autumn temperature (T), ultraviolet (UV)-A and UV-B enhancement on bud set in females and males of *P. tremula* during autumn 2012

Fixed effects terms	Coefficient	SE	z
T**	−1.73	0.55	−3.14
Day of year***	0.19	<0.01	69.69
UV-A	0.08	0.55	0.15
UV-B	0.05	0.55	0.09
Male	−0.01	0.74	−0.02
T × male***	−0.69	0.15	−4.75
T × UV-A	−0.09	0.78	0.11
T × UV-B	−0.07	0.78	−0.09
Male × UV-A	0.17	0.15	1.10
Male × UV-B*	0.34	0.15	2.28
T × male × UV-A	−0.23	0.21	−1.15
T × male × UV-B*	0.43	0.20	2.13

Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

2009; Jansen & Bornman 2012). Thus, although it might be speculated whether UV radiation acts via a stress-related mechanism on growth cessation and bud set, it might also be that the effect is linked to the effect as a morphogenetic signal.

Photomorphogenic effects such as reduced internode elongation and leaf expansion in response to low-fluence UV-B radiation have been found in several plant species, and particularly the phytohormone auxin has been studied in this respect (Rozema *et al.* 1997; Jansen & Bornman 2012). Even so, it remains unclear which signalling pathways could be involved in the UV-B effects on bud set. ABA is an important signal involved in bud set during SDs in *Populus* (Ruttink *et al.* 2007). In *P. cathayana*, enhanced UV-B radiation increased ABA content in leaves (Xu *et al.* 2010). Furthermore, reduction in GA content is well known to result in apical growth cessation and bud formation (Olsen *et al.* 1995a,b, 1997a,b; Mølmann *et al.* 2005). Although not yet verified, it may therefore be that UV-B radiation influenced bud set in our plants through affecting plant hormone levels such as ABA and GA.

Males were more responsive both in terms of enhanced bud set in autumn and hastened bud break in spring in response to increased temperature and UV-B. In light of the discussion earlier on possible effects of these environmental cues on hormone metabolism, it could be speculated whether hormone metabolism might be more sensitive to UV-B and temperature in males than females. Higher responsiveness in males has been found for *P. cathayana* under increased temperature and CO_2 (Zhao *et al.* 2012). In that study, males displayed an elevated height growth, total leaf number, root : shoot ratio and nitrogen uptake than females under +2 ± 0.2 °C.

Sex-related phenological responses may prove detrimental for the abundance and distribution of dioecious species in changing environments (Tognetti 2012). If the intensity of an environmental signal affects one sex differently than the other, there is a potential for undermining sexual reproduction. Also, if overall performance differs between the sexes, the sex ratio of a population may also shift. Understanding if and how sex-related responses occur in nature is of crucial importance when foreseeing possible effects of environmental change. Considering that males in our study responded more than females to a +1.35 °C increase in summer and autumn 2012, we suspect that higher warming in autumn, spring warming, or both, could amplify the differences in responsiveness between sexes.

The observed effect of temperature on bud set is in line with earlier findings in *Populus* (Kalcits *et al.* 2009; Rohde *et al.* 2011a). The rate of bud set is affected by temperature after reaching critical day length (CDL) (Junttila 2007; Rohde *et al.* 2011a). As such, higher temperature may both reduce responsiveness to the SD signal, while also accelerating bud maturation once bud scales appear. The dissection of bud set into several stages, as developed by Rohde *et al.* (2011b), comprises a stage where internode elongation has ceased, and the last, rolled-up leaves emerge. Among the described stages, this stage is closest to the time where the plant senses CDL. In our study, the first five out of seven stages described by Rohde *et al.* (2011a) were merged into one category, so the effects of temperature enhancement before and after sensing CDL cannot be separated.

As the expression of the floral integrator gene *FLOWERING LOCUS T* (*FT*) in *A. thaliana* is known to be stimulated at higher temperature (Balasubramanian *et al.* 2006), it might well be that expression of *PtFT*, which is involved in photoperiodic control of shoot elongation in *Populus*, (Böhlenius *et al.* 2006) is also affected by temperature. If so, apparently a temperature stimulation of *FT* expression resulting in delayed growth cessation and bud set is counteracted by increased UV radiation in our study. A possible explanation for the three-way interaction found between male plants, temperature and UV-B increase may be related to temperature effects on growth processes. As discussed in

Table 2. Parameter estimates, SE and z-values for covariates in the cumulative link mixed model run to investigate the effects of elevated autumn temperature (T), ultraviolet (UV)-A and UV-B enhancement on bud break in female and male *P. tremula* during spring 2013

Fixed effects terms	Coefficient	SE	z
T*	0.67	0.33	2.01
Day of year***	0.71	0.01	55.01
UV-A	−0.43	0.34	−1.27
UV-B	−0.16	0.33	−0.47
Male*	−0.69	0.34	−2.01
T × Male**	0.71	0.23	3.10
T × UV-A	0.77	0.47	1.62
T × UV-B	0.21	0.47	0.44
Male × UV-A	0.18	0.24	0.73
Male × UV-B**	0.61	0.23	2.67
T × Male × UV-A	−0.25	0.33	−0.75
T × Male × UV-B*	−0.63	0.32	−2.00

Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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Rohde *et al*. (2011a), initiation of leaf primordia is a growth-related process, and could thus be affected in a positive manner by temperature. Thus, if bud formation is induced by UV-B radiation, temperature may force further growth and maturation of buds. In *A. thaliana* and a range of other species, UV-C and stress factors like drought and nutrient deficiency accelerate flowering, which has also been shown to correlate with increased *FT* expression (Martinez *et al*. 2004). As different stresses are also known to induce bud set in woody species (Junttila 2007), it might be speculated whether *FT* expression in *P. tremula* is also affected by UV-B, but in an opposite manner than in flowering. If so, males should be more sensitive than females with respect to effects of UV-B as well as temperature.

Spring temperature is a key driver of bud break in spring, and enhanced temperature promotes bud break in species such as *Fagus sylvatica*, *Quercus robur* and *B. pendula* (Fu *et al*. 2012). In hybrid aspen (*P. tremula* × *P. tremuloides*), this is associated with increased levels of gibberellic acid (GA) (Rinne *et al*. 2011). In previous studies on autumn temperature effects on bud break, where plants were grown in controlled chambers with abrupt day length shifts, higher temperatures under SDs yielded delayed bud break in spring (Westergaard & Eriksen 1997; Søgaard *et al*. 2008; Kalcits *et al*. 2009).We found that bud break was forced by higher autumn temperature, which was contrary to our expectations and previous findings. Our study was performed in an open field, were plants experienced seasonal and diurnal shifts in climatic conditions (Figs 1–3). These conditions may provide a partial explanation of our results. It has been shown, for example that night temperature affects growth cessation, bud set and dormancy more than day temperature (Tanino *et al*. 2010). Thus, exposure to constant temperature and to diurnal temperature cycles might possibly lead to different results. We find support in our findings in a meta-analysis of phenological trend records from across Europe, which included 542 plant species. Based on all digitally available phenological records from 21 European countries, it was shown that warming in most cases advanced leafing (Menzel *et al*. (2006).

The unexpected effect of autumn warming on spring bud break could also be related to concentrations of soluble sugars. Content of soluble sugars is positively correlated with cold hardening in autumn (Junttila 2007). Thus, a delay in cold hardening may have yielded reduced sugar levels. Furthermore, bud break is correlated with low levels of soluble sugars (Lipavská *et al*. 2001), which may explain why buds receiving higher autumn temperature broke earlier the following spring. The effects of autumn temperature enhancement were clearly visible by 18 May (Fig. 5), well beyond the last events of night frost in spring 2013 (Supporting Information Fig. S2). In *S. pentandra*, GA increase is induced by increased day length (Olsen *et al*. 1997b). If bud break in *P. tremula* requires day length as an additional cue to temperature, plants would be less susceptible to frost damage following warm autumns.

Increasing autumn temperature by an average of 1.35 °C both delayed bud set in autumn 2012 and forced bud break in spring 2013. Even though enhancing autumn temperature extended the period of growth, both in autumn 2012 and spring 2013, episodes of frost did not occur during the additional days of growth. It is also uncertain whether plants would be even more affected by higher warming. Even so, episodes of frost may occur during spring in this part of Finland, which may not be unlikely in a different year. In particular, male plants may be more subjected to the risk of frost damage because of their increased responsiveness to temperature.

In conclusion, in our experiment where increased temperature and UV radiation were provided during the growth season in 2012, we found that enhanced UV-B radiation promoted autumnal bud set and subsequent spring bud break in *P. tremula*. For bud set in male plants, this counteracted the effect of increased autumn temperature, while in spring, both UV-B and temperature enhancement in autumn had a forcing effect on bud break in male plants. Our findings are in line with earlier studies on male responsiveness to environmental change, which could potentially yield sex-related differences in performance, sex ratios and survival of *Populus*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Emission spectrum of unfiltered Q-Panel UVB-313.

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Figure S2. Performance of temperature enhancement at the experimental site during autumn 2012, that is as daily average soil temperature, measured by four temperature sensors. Calculated values are 2 °C higher than ambient levels.

Figure S3. Daily average temperature for the study period in 2012–2013, measured by the meteorological station at Linnunlahti, Joensuu, less than 200 m from the experimental site of UV-B and temperature enhancement.

Figure S4. Daily precipitation for the study period, measured by the meteorological station at Linnunlahti, Joensuu, less than 200 m from the experimental site of UV-B and temperature enhancement.

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PAPER II

Phenology, growth and metabolism of *Populus tremula* grown along a natural temperature and UV-B gradient

Christian B. Strømme[1, 2], *Riitta Julkunen-Tiitto*[3], *Jorunn E. Olsen*[2, 4], *Line Nybakken*[1, 2]

[1] *Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

[2] *CERAD, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

[3] *Department of Biology, University of Eastern Finland, P.O.-Box 111, FI-20101 Joensuu, Finland*

[4] *Department of Plant Sciences, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

Corresponding author: Christian Bianchi Strømme

Email: christian.stromme@nmbu.no

ABSTRACT

Observed and projected warming has provoked research interest on tree phenology in relation to climate. While both ultraviolet-B (UV-B) radiation and warming has been shown to affect growth and development in plants, the combined effects of the two environmental factors under natural conditions has been little studied. In addition, there is concern for environmental change yielding divergent responses between sexes in dioecious tree species. Here, we present a study of the dioecious *Populus tremula* grown along an elevational gradient in central Norway where UV-B was reduced using specific screening filters, thus yielding different combinations of temperature and UV-B levels. In order to investigate the performance of *P. tremula* in the different climatic regimes, we tested for effects on growth, timing of bud formation and bud break, carbon and nitrogen contents and concentrations of phenolic compounds. Elevation had a negative effect on plantlet growth, advanced bud set, with effects differing between plant sexes. UV-B attenuation delayed bud formation and enhanced growth for males at the highest elevation, counteracting the effect of low temperature. In addition, elevation and UV-B affected concentrations of different phenolics in stems and leaves. Our data show that interactive effects of warming and other climate factors like UV-B should be considered when predicting climate change effects in plants, and add to present evidence of sex-related responses to climate change in dioecious plants.

INTRODUCTION

Different species of trees are exposed to a wide range of environments, including in areas characterised by clearly defined seasons. Increasing latitude and elevation yield shorter growing seasons and higher amplitudes of temperatures throughout the year (Körner 1999). As trees face recurring periods of climatic conditions that are unsuitable for photosynthesis and growth, adaptations to seasonality of climate allows growth-related processes to occur in favourable parts of the year, while frost tolerance and vegetative bud formation protect meristems through winter (Olsen and Lee 2011). Furthermore, translocation of macronutrients from leaves to stems of deciduous trees in autumn enhances resource use, a phenomenon that has been long known (Mitchell 1936). These phenological transitions occur in tree species that have extensive geographic distributions across latitudes and elevations, and thus across a wide range of climatic conditions. Sensing day length, temperature and light quality allows tree species to couple phenological transitions to seasonal shifts as they occur, and different provenances are adapted to local seasonal patterns.

Warming, which has been recorded worldwide over several decades, has been predicted to continue throughout the 21st century (IPCC 2014), provoking increased research efforts on warming effects on tree phenology (Steltzer & Post 2009; Körner & Basler 2010). Advanced spring phenology over a three-decade long period has been observed for several tree species across Europe (Menzel *et al*. 2006), which has been predicted as a consequence of warmer climates for boreal, temperate and Mediterranean tree species (Kramer *et al*. 2000). Furthermore, a survey of previous literature suggests that warming may both delay and accelerate phenological events in autumn, namely growth cessation and bud formation (Hänninen & Tanino 2011). This complicates general predictions of warming effects on these processes, and may be related to the diverse signalling patterns that are known to affect autumn phenology. Day length has long been recognised to affect growth cessation and bud formation in trees (Wareing 1956; Nitsch 1957; Weiser 1970). More recently, temperature has been shown to also affect growth cessation and bud formation (Kalcsits *et al.* 2009; Tanino *et al.* 2010; Hänninen & Tanino 2011; Rohde *et al.* 2011a), also in interaction with day-length (Ruttink *et al.* 2007; Søgaard *et al*. 2008; Tanino *et al*. 2010; Olsen *et al*. 2014)

Light quality has been shown to affect autumn phenology in boreal tree species, namely red, far red (FR) and blue light (Junttila & Kaurin 1985; Olsen *et al.* 1997a; Clapham *et al.* 1998; Tsegay *et al.* 2005; Mølmann *et al.* 2006; Opseth *et al.* 2015). In plants, blue, red and FR

light are involved in photoperiodic sensing through crytpochromes and phytochromes, respectively (see Webb 2003 for a review). During the last two decades, evidence of ultraviolet-B radiation (UV-B) as a growth-regulating signal for plants has emerged (Rozema *et al.* 1997; Jansen 2002; Rizzini *et al.* 2011; Jansen & Bornman 2012; Hayes *et al.* 2014). While previous research on UV-B effects on plants largely considered stress effects, signalling effects have gained increased attention following the characterisation of a UV-B specific photoreceptor, known as UV-B resistance locus 8 (UVR8), in *Arabidopsis thaliana* (Jenkins 2009). Indeed, UV-B has been shown to affect photoperiodic sensing in *A. thaliana* (Fehér *et al.* 2011), and possible effects on autumn phenology in trees should also be investigated.

The ratio of red to far red solar radiation (R:FR), varies throughout the year, having maximum levels in summer (Górski 1980). In addition, R:FR decreases with increasing canopy cover in cloudless conditions (Holmes and Smith 1977; Ballaré et al. 1987; Messier et al. 1989) UV-B irradiance also displays seasonal variation, although plant responses to this pattern are much less studied. Seasonal variation of UV-B has a higher amplitude than photosynthetically active radiation (PAR) (Brown *et al.* 1994), and the proportion of UV-B to PAR reaches maximum levels in mid-summer and minima in mid-winter (Häder *et al.* 2007). UV-B radiation varies also between geographic regions both due to varying solar zenith angle with latitude and thickness of the ozone column. Even though future UV-B levels have been projected to decrease due to ozone recovery, the recovery rate is uncertain due to climate change effects on this process (McKenzie *et al.* 2011). UV-B is also known to induce synthesis of defensive phenolic compounds in plants, including boreal tree species, such as *Betula pendula* (Lavola *et al.* 1997), *Salix myrsinifolia* (Tegelberg & Julkunen-Tiitto 2001) and *Populus tremula* (Lavola *et al.* 2013; Randriamanana *et al.* 2015). Opposite to these effects, warming has been shown to decrease concentrations of phenolic compounds, together with a positive effect on plant growth (Zvereva & Kozlov 2006; Lavola *et al.* 2013; Randriamanana *et al.* 2015).

Clearly, predictions of climate change effects on growth, performance and defence of high latitude tree species should consider interacting effects of warming and UV-B. In a recent field study of the dioecious Eurasian aspen (*Populus tremula*), we found interacting effects of increased temperature and UV-B radiation using infrared (IR) heaters and UV-B lamps, on autumnal bud formation and bud break (Strømme *et al.* 2015). On the one hand, autumn

warming delayed bud formation, while on the other hand, enhanced UV-B levels in the same period had an opposite effect. Interestingly, we found that male plants were more responsive to both treatments, which also yielded advanced bud break the following spring. Previous studies have also shown different responsiveness to environmental change between sexes in *Salicaceae*, namely in *S. myrsinifolia* (Nybakken *et al.* 2012; Nybakken & Julkunen-Tiitto 2013), *Salix arctica* (Dawson & Bliss 1989) and *Populus cathayana* (Xu *et al.* 2008; Zhao *et al.* 2009; Xu *et al.* 2010; Zhao *et al.* 2012). In this regard, there is concern for climate change effects yielding divergent performance and survival in sexes of *Salicaceae* species (Tognetti 2012).

The aim of the present study was to follow up on findings from the artificial UV-B-temperature enhancement study employing IR heating and UV-B lamps, presented in Strømme *et al*. (2015), using a different approach to test effects of ambient temperature and UV-B in natural environments on the same clones. Instead of enhancements, we used a natural temperature and UVB gradient. Since temperature decreases with elevation and UV-B increases, we established three different experimental locations along a natural temperature and UV-B gradient in a valley side in Central Norway. The experimental locations were maintained for two growing seasons, and in the second season, we employed attenuation filters mounted over plots to obtain reduced UV-B levels on subsets of new plant materials. In addition to recording phenological transitions of plants, we measured plant growth and analysed carbon (C), nitrogen (N) and phenolic contents in stems and leaves in order to evaluate plant performance and defence under the different treatments.

MATERIALS AND METHODS

Plant material

Plantlets used in the field experiments originated from six female and six male aspens located in Southern and Eastern Finland. For a thorough description of sampling locations and micropropagation of individuals see Strømme *et al*. (2015). Plantlets were potted on 4 June 2013 and on 10 June 2014 using 70% non-fertilised peat and 30% vermiculite. Prior to planting in the field, plantlets were kept in growth chambers under 230±10 µmol m^{-2} s^{-1} and a red: far-red (R:FR) ratio of 1.6±0.1 provided by 400W Philips MASTER HPI-T Plus metal halide lamps (Royal Philips, Amsterdam, Holland) and incandescent light bulbs (60 W, Osram, Munich, Germany). Photosynthetic active radiation (PAR) was measured using a LI-250 Light Meter with an attached Quantum Sensor (LI-COR, Lincoln, Nebraska, USA), while R:FR ratio

was measured using a Sky 100 radiometer with an attached 660/730 nm sensor (Skye Instruments, Llandrindod Wells, UK). The first days after potting, the plantlets were kept under a semi-translucent plastic sheet, which was gradually removed. This provided a gradual climatic shift in terms of irradiance and relative air humidity (RH). In the growth chambers, temperature and RH were 20ºC and 75%, respectively, and progressively lowered to 16ºC and 65% over seven days in 2013 and four days in 2014 to allow acclimation to lower temperature and RH. Plantlets were planted in the outdoor experimental locations on 4 July in 2013 and on 24 June in 2014due to a cooler spring in 2014.

Experimental set up

We stablished experiments at three different elevations in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley. Each location was a pasture selected with the aim of having three sites at different elevations along a natural temperature and UV gradient while keeping PAR irradiance, precipitation and soil conditions as similar as possible. Selected sites were located at 237, 575 and 830 m a.s.l. along a 3.8 km long west-east axis. Large herbivores were excluded from each location using a 2.5 m metal wire fence.

In 2013, five 180 x 120 cm plots per location were set-up by removing the uppermost 10 cm of soil, in addition to rocks in the exposed sublayer. The total removed material was replaced by 10 cm of FLORALUX peat compost (pre-limed and pre-fertilised) (Nittedal Torvindustri, Arneberg, Norway). In each plot, 40 plantlets consisting of ten clones (5 female and 5 male) were planted in five rows containing 8 plantlets each. During planting, minimum spacing between each plantlet and closest neighbouring plantlet was 20 cm. At each site, one HOBO Micro Station data-logger (Onset Computer Corporation, Cape Cod, Massachusetts, U.S.A.) was installed together with a PAR Smart Sensor (Onset Computer Corporation) and a Temperature/RH Smart Sensor (Onset Computer Corporation).

In 2014, twelve 140 x 80 cm plots were established within each location. Prior to establishment, a large tree was removed from the location at 237 m a.s.l. with the purpose of equalising total irradiance received by plantlets across the different locations (Fig. 2). As the number of plantlets per plot was reduced to 20, these were evenly distributed between three rows with the same spacing between plantlets as the previous year. At each elevation, the twelve plots were arranged in a 4x3 matrix, yielding four blocks containing each of three treatment types: UV-B attenuation, covered control and uncovered control. Plots that were assigned to UV-B

attenuation treatments were covered by Autostat CT5 polyester UV-B-attenuating filters (MacDermid Autotype Ltd., Wantage, UK) (see Supplementary figure 1 for transmittance spectrum), mounted on a 320 x 280 cm frame consisting of polypropylene (PP) tubes. Each frame was raised 1 m above the ground by four vertical PP-tubes, which were raised gradually as plantlets grew taller. As daily solar trajectories vary throughout the year, the frames were dimensioned and placed accordingly over the plots in order to provide attenuation of UV-B from direct solar radiation throughout the growing season. For this purpose, dimensions were based on recommendations on UV-B attenuation provided in Aphalo *et al.* (2012). Plots assigned to the controlled cover treatment had each a frame built in the same manner as for the UV-B attenuation treatment, but were covered by 50 µm translucent LD-polyethylene sheets instead of UV-B-filters. The purpose of this treatment was to separate the effect of UV-B attenuation from the effect of covering the plots with plastic sheets (Aphalo *et al.* 2012). The third treatment consisted of leaving the plots uncovered as a control for the effect of plastic sheet cover. Frames for covered treatment plots were installed on 7 July 2014 and kept until 1 October 2014, except for covered treatments at 237 m a.s.l., which were removed on 24 September 2014 due to damage from strong winds.

Plant measurement and recording of apical stages

Stem height and basal diameter of plantlets were measured after planting in the field and every third week throughout the growing season. Height measurements were performed by placing a ruler along the highest stem of each plantlet by allowing light contact between the tip of the ruler and the ground surface. Basal diameter was measured 1 cm from the soil surface using a digital calliper. In 2013, height and diameter were measured four and three times, respectively, while in 2014, height and diameter were measured five and four times, respectively . A three-stage system was used for scoring apices during autumnal bud formation, discerning between growing apex with score 1, green bud having closed bud scales with score 0.5, and brown/red mature bud with score 0. In situations where green closed buds broke in autumn and apices resumed growth, apices were scored as growing (Strømme et al. 2015). Some plantlets were affected by *Venturia* shoot blight or grazed upon by intruding small herbivores. Excluding these, the number of measured and scored plantlets were 175 females and 205 males in 2013 and 246 females and 419 males in 2014. We recorded bud break during spring 2014 and 2015. Bud break was dissected into four stages based on Fu et al. (2012), as reported in Strømme *et al.* (2015): closed bud (0); closed bud with visible green leaf tip (1); green leaf diverging from bud axis but no visible petiole (2); break bud with at least one visible petiole (3). During spring

2014, apices of all plantlets were recorded until all buds were fully broken. In spring 2015, apical scoring at 830 m a.s.l. was terminated before all plants had fully broken buds, as the field experiment was disassembled on 22 June 2015.

Sampling of plant material

In 2013, one plantlet from each clone was sampled from each plot at each elevation on 17 September, yielding a total of 115 harvested plantlets. In 2014, three female and three male plantlets were sampled from each of the three plot treatments at the lowermost elevation (237 m a.s.l.) on 17 September, 24 September and 7 October, yielding a total of 54 plantlets. Plantlets were sampled by cutting the stem 1 cm above the ground surface and removing the top 5 cm section of the stem. From the remaining stem, the top 10 cm section of each plantlet was cut and placed in a labelled paper bag containing silica gel, while the top- and lowermost leaf of the 10 cm stem section was placed in a separate labelled paper bag containing silica gel. The sampled plantlet parts were dried at 30 ºC in a drying oven, grinded and kept in a freezer before analyses for C, N and phenolic compounds.

Chemical analyses

Grinded samples were analysed for C and N content using an Elementar Micro Cube (Elementar Analysen, Hanau, Germany). Concentrations of phenolic compounds was analysed using high performance liquid chromatography (HPLC)

Analyses of phenolic content followed earlier published methods (Nybakken et al. 2012). Of the powdered plant material, 10 mg were weighed and extracted separately using 600 µl of cold 100% methanol. The samples were homogenized for 30 s at 5500 rpm using a Precellys 24 (Bertin Technologies, Île-de-France, France) and incubated in an ice bath for 15 min. The mixture was then centrifuged at 15,000 rpm for 3 min (Eppendorf Centrifuge 5415R, Hamburg, Germany) and the supernatant collected. The residue was re-extracted twice with incubation in the ice bath. The three consecutive supernatants were pooled together, and the extract was evaporated to dryness using an Eppendorf concentrator and stored in a -20 °C freezer until further analysis. The salicylates, phenolic acids and flavonoids were analyzed by HPLC (Agilent, Series 1100, Germany), consisting of a binary pump (G1312A), a thermostated autosampler (G1329A), a thermostated column oven (G1316A) and a diode array detector (G1315B). The phenolic metabolites were separated using a ODS Hypersil (4.6×60 mm) column (Thermo Fisher Scientific Inc, Waltham, USA). Prior to the HPLC

analyses, the dried extract was resuspended in 400 µl methanol–water (50:50). A volume of 20 µl from each sample was injected and all runs were performed at +30◦C. The phenolic metabolites were identified by comparing their retention times and UV spectra with those of standards. The mobile phase consisted of two solvents: 0.25% o-phosphoric acid and 1.5% tetrahydrofuran in Milli-Q ultrapure water (Merck Millipore, Darmstadt, Germany), and methanol 100% with a flow rate of 2 ml min^{-1}.

Climate data

In both years, temperature and irradiance was recorded on a ten-minute basis (Supplementary fig. 2 & 3). There are two gaps in the data series from 2013, as some data-loggers malfunctioned before 28 August and after 22 September. In 2014, we recorded temperature and relative humidity (RH) for three plots with different cover treatments at 237 m a.s.l. Recording occured on a five-minute basis using EL-USB-1 temperature data loggers (Lascar Electronics, Salisbury Wiltshire, UK) (Supplementary table 1; Supplementary figure 1). Each logger was raised 10 cm above ground, and measurements were performed between 9 and 31 July. UV-B irradiance at the three different elevations (Supplementary fig. 3) was calculated using the Quick TUV Calculator provided by NCAR Earth System Laboratory (http://cprm.acd.ucar.edu/Models/TUV/Interactive_TUV/) with the following settings: latitude 61.47055, longitude 4.22208, 300 DU of ozone, ground albedo (reflectivity) 0.1 and cloudless sky, while remaining conditions were kept as calculator default.

Statistical analyses

We tested the effect of elevation (three levels), plantlet sex (two levels) and day of year on stem height, stem basal diameter, apical stages and C, N and phenolic compound concentrations of stems and leaves using the R software for statistical computing (R Core Team 2015). We performed statistical tests based on procedures described in Zuur *et al. (*2009). In the analyses of data from the UV-B attenuation experiment in 2014, we included UV-B attenuation (two levels) and cover (two levels) in the models. In the model selection process, we included plant clone (random term) and plot (random term) by using the lmer function in the lme4 package (Bates *et al.* 2015) when their inclusion yielded improved models based on AIC comparison. In the opposite case, data were analysed using generalised least squares (gls) from the nlme package (Pinheiro *et al.* 2015) in R. If the effect of day of year was significant and nonlinear, we proceeded by analysing data using generalised additive models (gam) for gls models and generalised additive mixed models (gamm) for lmer models, using the gamm4

package (Wood & Scheipl 2015) in R. Effects on apical stages were tested using cumulative link mixed models (clmm) in R by applying the clmm function in the Ordinal Package (Christensen 2013).

RESULTS

Autumnal bud formation occurred earlier at higher elevations (Table 1, Table 2, Fig.1), and had a significant interaction with plant sex at 575 ($P<0.001$) and at 830 m a.s.l. ($P<0.001$) in 2013. The significant interaction between elevation and plant sex in 2013 was due to male plants forming buds earlier than females at 237 m a.s.l., while the opposite was the case for the two higher elevations (Fig. 1). All plantlets at 830 m a.s.l. and most plantlets at 575 m a.s.l. had completed bud formation by day 267 (24 September), while at 237 m a.s.l. there were 25 (out of 90) female and 35 (out of 95) male plants which had not completed bud formation by that date (Fig. 1). These observations may be due to lower air temperature with increased elevation (Supplementary fig. 2).

Earlier bud formation was also observed in 2014 at 830 m a.s.l., but this effect was overridden by UV-B attenuation ($P<0.001$). This means that females and males under UV-B attenuation at 830 m a.s.l. had apical scores similar to plantlets at 237 m a.s.l. throughout the growing season (Fig. 1). Higher UV-B levels at 830 m a.s.l. may explain why UV-B attenuation delayed bud formation only at this elevation, as calculated levels of UV-B irradiance increased slightly with elevation (Supplementary fig. 3). At 237 and 575 m a.s.l., average bud scores increased between day 254 (11 September) and 259 (16 September) in 2014 due to flushing of green closed buds and briefly resumed growth, which can be related to high average temperatures in this period. Bud formation in 2014 occurred later than the previous year, which was evident around day 265 (22 September) in 2013 when most plantlets at 575 and 830 m a.s.l. had completed bud formation (Fig. 1).

In spring 2014 and 2015, bud break occurred later at 575 ($P<0.001$) and 830 m a.s.l. ($P<0.001$) compared to 237 m a.s.l. (Fig. 2), while neither plant sex nor any of the cover treatments had any significant effects.

Table 1: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of site, sex and day of year on bud formation during autumn 2013 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Fixed effect terms	Coefficient	SE	z
575m***	3.73	0.28	13.25
830m***	5.91	0.30	20.02
Male	1.13	1.24	0.91
Day of year***	0.16	0.01	29.44
575m x Male***	-1.25	0.25	-5.09
830m x Male***	-1.58	0.24	-6.49

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Table 2: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of UV-B attenuation, site and day of year on bud formation during autumn 2014 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Fixed effect terms	Coefficient	SE	z
UVB-filter	0.15	0.29	0.51
575m	0.32	0.24	1.33
830m***	1.36	0.24	5.65
Day of year***	0.13	<0.01	34.58
UVB-filter x 575m	-1.15	0.42	-0.35
UVB-filter x 830m***	-1.50	0.42	-3.61

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

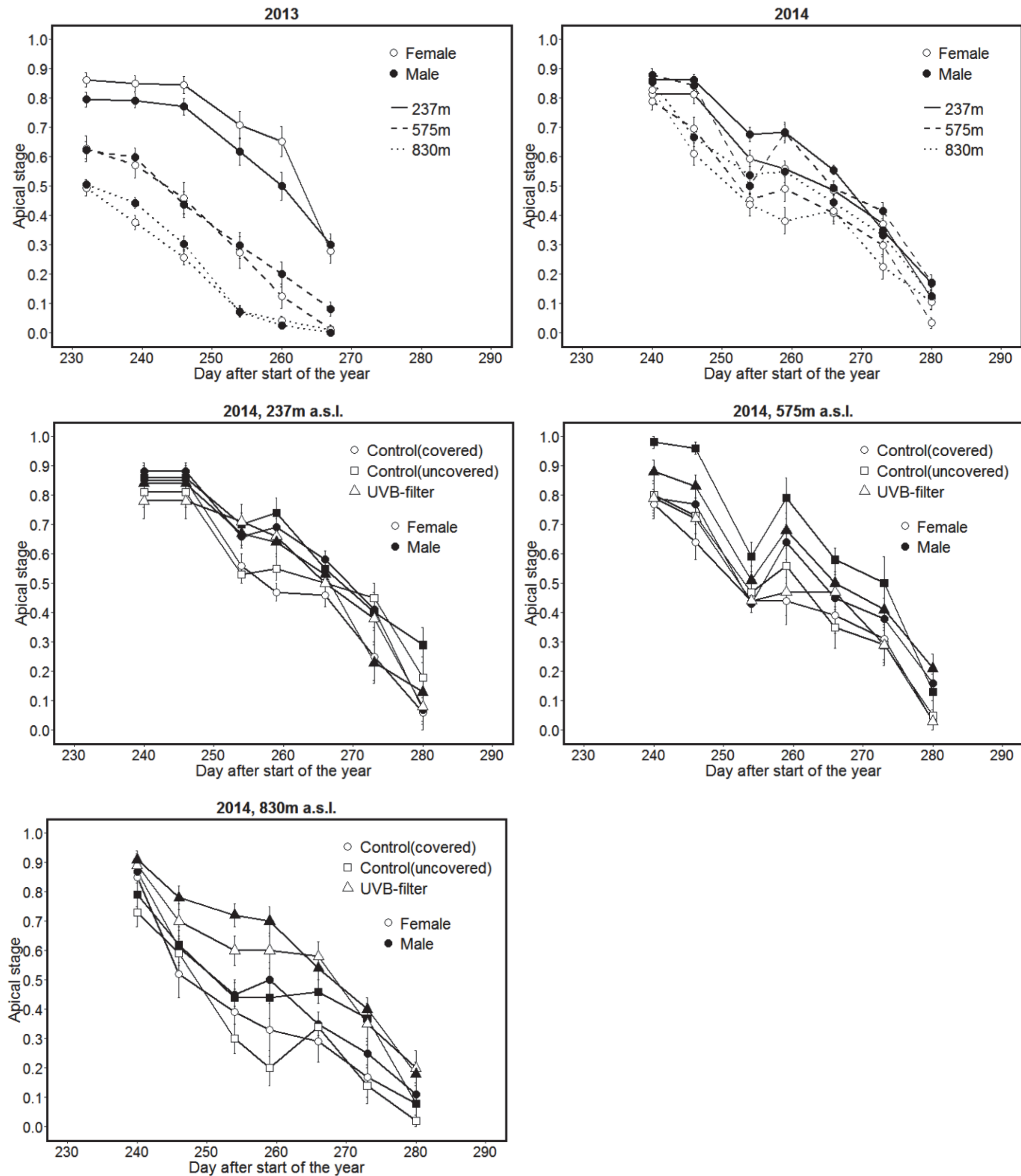

Figure 1. Average apical scores ± 1SEM of *P. tremula* grown in Fåvang, Central Norway (61°27’ N, 10°11’E) along the eastern side of the Gudbrandsdalen valley at different elevations during autumn in 2013 (175 females, 205 males) and 2014 (245 females, 419 males) (top panels), and at different elevations and treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) during autumn in 2014 (middle and bottom panels).

Table 3: Parameter estimates, SE and z-values for covariates in the cumulative link mixed models used to test the effect of site and day of year on bud break during spring in 2014 and 2015 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Year	Fixed effect terms	Coefficient	SE	t
2014				
	575m***	-2.19	0.44	-4.93
	830m***	-6.34	0.48	-13.25
	Day of year***	0.82	0.03	29.89
2015				
	575m***	-3.89	0.44	-8.88
	830m***	-10.58	0.51	-20.78
	Day of year***	0.44	0.01	37.73

Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

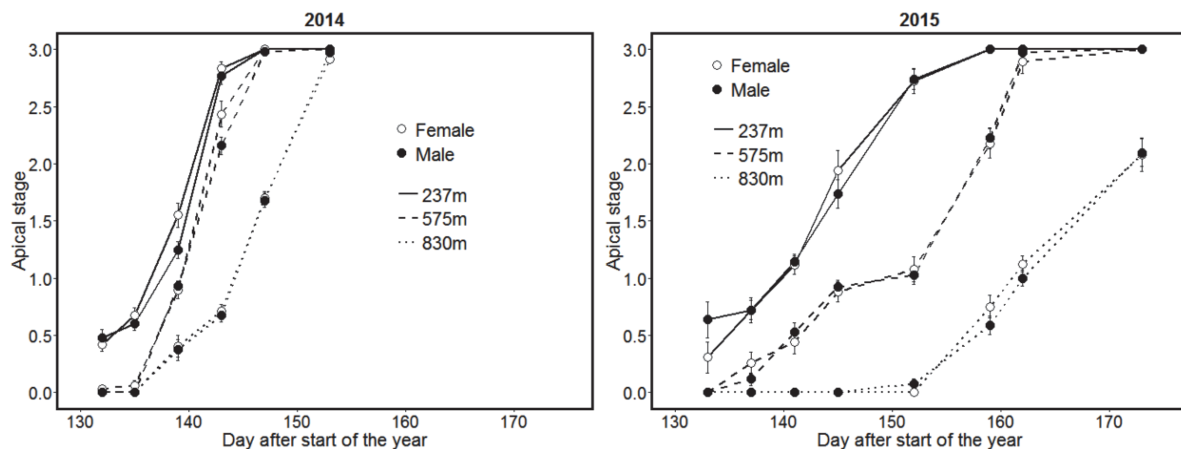

Figure 2. Average apical scores ± 1SEM of *P. tremula* grown in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley at different elevations during spring in 2014 (163 females, 188 males) and 2015 (150 females, 276 males).

Plantlets were smaller at higher elevations both in terms of stem height and basal diameter in 2013, being smallest at 830 m a.s.l. (Table 4, Fig. 3 & 4). By day 254 (11 September) 2013, plantlets at 237 m a.s.l. were 109 and 150 % taller than plantlets at 575 and 830 m a.s.l., respectively, and corresponding values for basal stem diameter were 21 and 45 %. Female plantlets were taller at 237 m a.s.l. compared to males in 2013, but these differences were less pronounced at 575 (P=0.003) and 830 (P=0.015) m a.s.l. given the significant interaction between sex and site (Table 4). Basal diameter was smaller at 575 (P<0.001) and 830 m a.s.l. (P<0.001). In 2014, males were smaller than females in terms of height (P=0.001) and basal diameter (P=0.023) at 830 m a.s.l. However, UV-B attenuation yielded increased growth of male plants at this elevation both in terms of height (P<0.001) and basal diameter (P=0.002), given the significant interactions between UV-B attenuation and sex at 830 m a.s.l. (Table 5, Fig. 3 & Fig. 4). For the same year, there was a negative effect of covering plots with either UV-B attenuation filters or translucent filter on stem height (P=0.012) and basal diameter (P<0.001). This effect yielded a 30 and 50 % increase for uncovered plantlet height at 237 and 575 m a.s.l., respectively, while stem basal diameter decreased 21, 44 and 20 % for uncovered plantlets at 237, 575 and 830 m a.s.l., respectively. UV-B attenuation in 2014 may have affected stem height and basal diameter in males by delaying bud formation at this elevation (Table 4; Fig. 3 & 4).

In 2013, sampled leaves had lowest N concentration at 830 m a.s.l. compared with 237 m a.s.l. (P=0.009). Leaves sampled at 237 m a.s.l. had 11 and 28 % higher leaf N concentration than at at 575 and 830 m a.s.l., respectively. Stem N concentration was higher at 575 (22 %) (P<0.001) and 830 m a.s.l. (70 %) (P<0.001), being higher for males at 830 m a.s.l. (P=0.047). (Table 6 & Table 8). Leaf C concentration was lower at 575 m a.s.l. compared to at 237 m a.s.l. (P=0.01), while female plants had higher stem C concentration (difference was only 1%) than males, irrespective of elevation (P=0.01) (Table 7 & Table 8. This may be related to lower C assimilation in males, as they were smaller in terms of both height and basal diameter.

Table 4: Parameter estimates, SE and t-values for covariates in the linear mixed model used to test the effect of site, sex and day of year on plantlet height and basal diameter during autumn 2013 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Analysis	Fixed effect terms	Coefficient	SE	t
Height				
	Intercept**	-13.35	4.12	-3.24
	575m***	-16.47	1.39	-11.83
	830m***	-19.08	1.38	-13.85
	Male	-6.73	4.72	-1.43
	Day of year***	0.24	0.01	23.11
	575m x Male***	3.46	1.19	2.92
	830m x Male***	2.77	1.14	2.43
Basal diameter				
	Intercept***	-4.17	5.19	-8.03
	575m***	-1.08	0.21	-5.28
	830m***	-1.74	0.20	-8.52
	Day of year***	0.05	<0.01	23.40

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

In 2014, male plantlets at 237 m a.s.l. had higher (19 %) leaf N concentration (P=0.014) and lower (15 %) stem N concentration (P=0.020) than females. UV-B attenuation yielded lower N concentration in both stems (P<0.001) and leaves (P<0.001) for both sexes (Table 9, Fig. 5), as stems and leaves had 35 and 34 % lower N concentration than control treatments, respectively. Males also had higher (3%) leaf C concentration (P<0.001) (Table 10, Fig. 5), while none of the treatments yielded any significant effects on stem C concentrations (Fig. 5).

Table 5: Parameter estimates, SE and t-values for covariates in the linear mixed model used to test the effect of site, sex, day of year, UV-B attenuation and cover on plantlet height and basal diameter during autumn 2014 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Analysis	Fixed effect terms	Coefficient	SE	t
Height				
	Intercept***	-86.46	3.22	-26.85
	575m	-1.49	2.50	-0.60
	830m***	-13.18	2.47	-5.33
	UVB-filter	0.08	3.24	0.03
	Male	3.68	2.31	1.60
	Day of year***	0.54	0.01	55.89
	Cover*	-4.95	1.85	-2.68
	UVB-filter x 575m	-2.67	4.44	-0.60
	UVB-filter x 830m	0.12	4.32	0.03
	575m x Male	-1.80	1.65	-1.09
	830m x Male**	-5.00	1.62	-3.10
	UVB-filter x Male*	-5.27	2.08	-2.53
	UVB-filter x 575m x Male	3.60	3.02	1.19
	UVB-filter x 830m x Male ***	11.75	2.87	4.10
Basal diameter				
	Intercept***	-7.85	4.20	-18.72
	575m	-0.11	0.30	-0.37
	830m***	-1.28	0.30	-4.30
	UVB-filter	0.30	0.39	0.77
	Male	0.50	0.25	2.03
	Day of year***	0.06	0.00	41.41
	Cover***	-0.97	0.22	-4.37
	UVB-filter x 575m	-0.57	0.53	-1.08
	UVB-filter x 830m	-0.24	0.52	-0.46
	575m x Male	-0.11	0.19	-0.57
	830m x Male*	-0.43	0.19	-2.22
	UVB-filter x Male**	-0.70	0.25	-2.84
	UVB-filter x 575m x Male	0.55	0.36	1.54
	UVB-filter x 830m x Male **	1.08	0.34	3.15

Significance levels: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

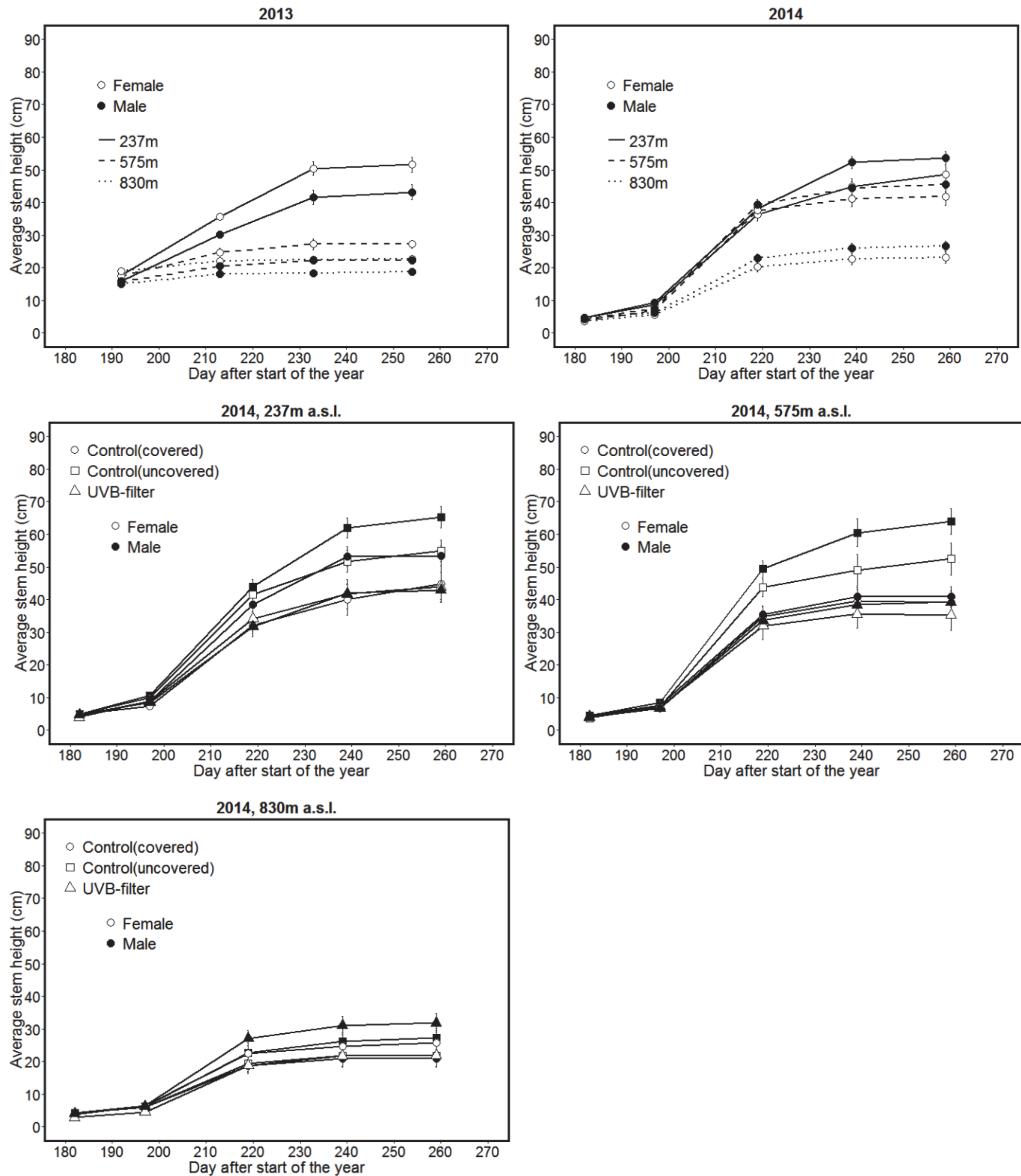

Figure 3. Average stem height ± 1SEM of *P. tremula* grown in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley at different elevations during autumn in 2013 (175 females, 205 males) and 2014 (245 females, 419 males) (top panels), and at different elevations and treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) during autumn in 2014 (middle and bottom panels).

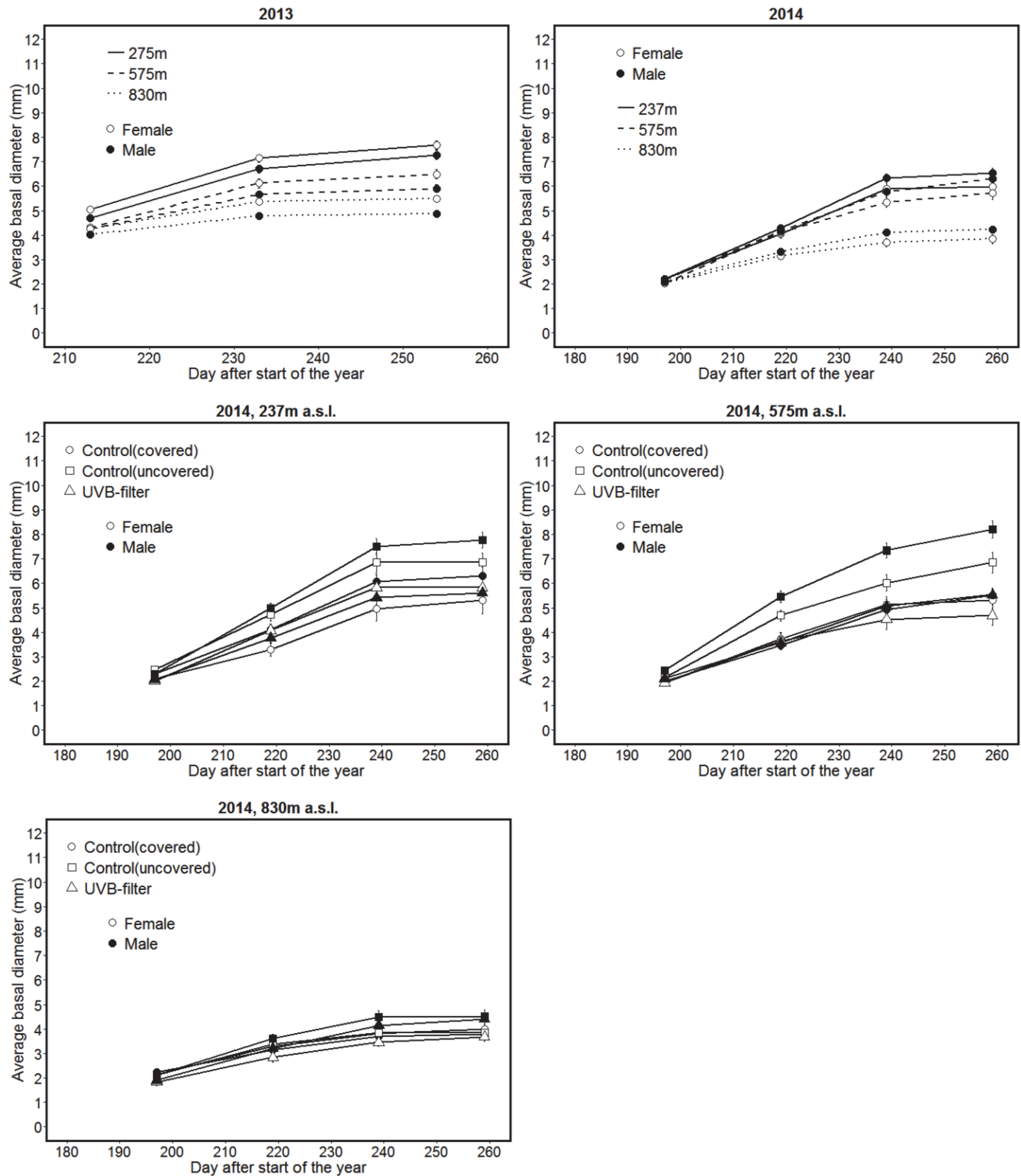

Figure 4. Average basal diameter ± 1SEM of *P. tremula* grown in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley at different elevations during autumn in 2013 (175 females, 205 males) and 2014 (245 females, 419 males) (top panels), and at different elevations and treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) during autumn in 2014 (middle and bottom panels).

Table 6: Parameter estimates, SE and t-values for covariates in the linear mixed model used to test the effect of site and sex on leaf and stem N content during autumn 2013 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

	Fixed effect terms	Coefficient	SE	t
Leaf N				
	Intercept***	2.70	0.15	15.32
	575m	-0.27	0.18	-1.50
	830m**	-0.56	0.18	-3.09
Stem N				
	Intercept***	1.22	1.76	6.95
	575m***	0.51	0.09	5.90
	830m***	0.81	0.09	9.48
	Male	0.30	0.24	1.22
	575m x Male	0.01	0.12	0.06
	830m x Male*	0.23	0.11	2.01

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Table 7: Parameter estimate, SE and t-value for covariate in the generalised least squares model used to test the effect of site and sex on leaf and stem C content during autumn 2013 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

	Fixed effect terms	Coefficient	SE	t
Leaf C				
	Intercept***	44.86	0.74	60.72
	575m*	-2.38	1.04	-2.30
	830m	-2.02	1.03	-1.96
Stem C				
	Intercept***	46.29	0.17	279.8
	Male*	-0.58	0.23	-2.55

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Table 8: C and N concentrations (%) of leaves and stems from females and males of *Populus tremula* sampled on 17 September 2013 from different elevations in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

		Leaf		*Stem*	
		C	N	C	N
830 m					
	Females	42.1±0.31	2.07±0.13	46.0±0.35	2.0±0.10
	Males	43.5±2.08	2.16±0.21	45.9±0.33	2.6±0.09
575 m					
	Females	42.8±0.51	2.57±0.08	46.6±0.24	1.7±0.08
	Males	42.2±0.40	2.30±0.09	45.5±0.21	2.0±0.09
237 m					
	Females	46.1±1.02	2.70±0.12	46.4±0.29	1.2±0.07
	Males	43.8±0.43	2.70±0.11	45.7±0.18	1.5±0.09

Table 9: Parameter estimates, SE and t-values for covariates in the generalised additive model used to test the effect of Ultraviolet B-attenuation and sex on leaf and stem N content during autumn 2014 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

	Fixed effect terms	Coefficient	SE	t
Leaf N				
	Intercept***	1.92	0.10	18.29
	UV-B filter***	-0.53	0.14	-3.80
	Male*	0.34	0.13	2.54
Stem N				
	Intercept***	1.08	0.04	25.24
	UV-B filter***	-0.25	0.06	-4.27
	Male*	-0.13	0.06	-2.40

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Table 10: Parameter estimate, SE and t-value for covariate in the generalised additive model used to test the effect of sex on leaf C content during autumn 2014 in females and males of *P. tremula* grown in the field at 237, 575 and 830 m.a.s.l. in Fåvang, Central Norway (61°27' N, 10°11'E) along the eastern side of the Gudbrandsdalen valley.

Fixed effect term	Coefficient	SE	t
Intercept***	45.94	0.19	245.93
Male***	1.51	0.26	5.73

Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

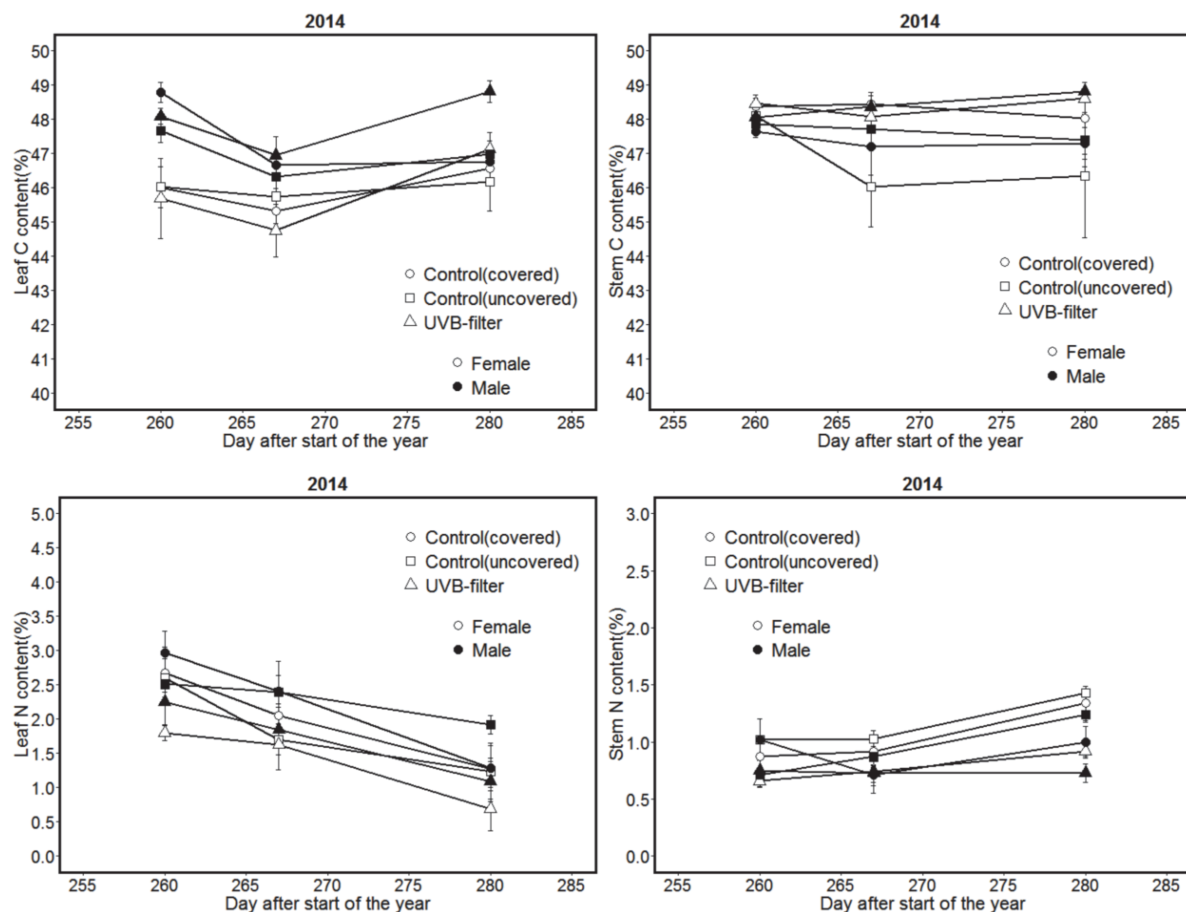

Figure 5: Percent content of C (top panels) and N (bottom panels) in leaves (left panels) and stems (right panels) from females and males of *Populus tremula* under differen treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) during autumn 2014.

Table 11: *t*-values for leaf concentrations of phenolics in females and males of *P. tremula* grown at different elevations. *t*-values were obtained using the following statistical tests in R: generalised least squares model (gls), linear mixed effect model (lmer).

	Test	830m x Male	575m x Male	830 m	575m	Male
Phenolic acid 1	lmer			-10.35***	-10.2***	
Neochlorogenic acid	lmer			-6.89***	-6.22***	
Phenolic acid 2	lmer			-7.73***	-7.77***	
Phenolic acid 3	lmer			-7.12***	-5.37***	
Chlorogenic acid 1	lmer			-2.16*	-1.06	
Phenolic acid 5	lmer	2.05*	1.67	1.39	-0.37	-0.57
Salicortin	lmer			-4.02***	-2.19*	
Quercetin arabinoglucoside	lmer			-0.61	-2.42*	
Kaempferol 3-glucuronide	lmer			4.29***	0.56	
Phenolic acid 6	lmer	-2.56*	-0.45	5.50***	0.86	0.72
Phenolic acid 7	lmer	0.55	2.33*	0.69	-0.78	-0.61
Salicylate 1	lmer			3.71**	0.09	
Tremulacin	lmer			0.23	-3.26**	
Total leaf phenolic acids	lmer			-7.14***	-6.66***	
Total leaf salicylates	lmer			0.11	-2.85***	
Total leaf phenolics	lmer			-0.34	-3.15**	

Analyses of phenolic content by HPLC allowed determination of several groups of phenolics in stems and leaves sampled in 2013 (Supplementary table 2 & 3) across elevations and at 237 m a.s.l. for the different cover treatments in 2014 (Supplementary tables 4-9). For leaves sampled in 2013, salicylates constituted the largest proportion of phenolics (67-71%), followed by flavonoids (25-28%) and phenolic acids (3-7%) (Supplementary table 2). The concentration of leaf salicylates was higher (16 %) at 237 m a.s.l. compared to 575 m a.s.l. (P<0.001), explaining why the total concentration of phenolics was higher (15 %) in leaves at this elevation (P=0.006) (Table 11). The concentration of phenolic acids decreased with elevation, being lower at 575 (73 %) (P<0.001) and 830 m a.s.l. (83 %) (P<0.001) (Table 11). However, higher concentration of phenolic acid 5 at 830 (P=0.043) and phenolic acid 7 at 575 m a.s.l. (P=0.022) were found in leaves of males, while phenolic acid 6 was present in lower concentration for males at 830 m a.s.l. (P=0.012).

Table 12: *t*-values for stem concentrations of phenolics in females and males of *P. tremula* grown at different elevations in 2013. *t*-values were obtained using the following statistical tests in R: generalised least squares model (gls), linear mixed effect model (lmer).

	Test	830m x Male	575m x Male	830m	575m	Male
Salicylate 1	lmer	0.76	2.23*	-0.69	-1.88	-0.15
Salicylate 2	lmer			4.26***	2.71**	
Chlorogenic acid derivative 1	lmer			-2.65**	-1.08	
Salicin	lmer	2.11*	1.065	-3.21**	-1.09	-2.20*
Phenolic acid 2	lmer	-2.03*	0.80	4.00***	2.52*	-0.38
Phenolic acid 3	lmer	0.53	2.54*	1.64	-0.49	-1.92
Chlorogenic acid derivative 3	lmer			3.58***	2.38*	
Salicylate 7	lmer			3.04**	3.91***	
Salicylate 9	lmer	2.67**	1.88	-3.39***	-1.66	-2.36*
Salicylate11	lmer			4.34***	3.51***	
Salicylate12	lmer			-2.28*	-2.39*	
Salicylate13	lmer					-2.22*
Salicylate14	lmer			-2.67*	-1.94	
Salicylate15	gls					5.05***
Salicortin	lmer			6.64***	3.53***	
Tremulacin	lmer			0.21	2.24*	
Total stem salicylates	lmer			5.09***	4.00***	
Total stem phenolics	lmer			4.50***	3.73***	

Table 13: *t*-values for leaf concentrations of phenolics measured on different dates (DOY) in females and males of *P. tremula* grown at 237 m a.s.l. UVB-attenuation filters (UV-F), translucent cover (both Cover treatments) and ambient UV-B levels. *t*-values were obtained using the following statistical tests in R: generalised least squares model (gls), linear mixed effect model (lmer), generalised additive model (gam), generalised additive mixed effect model (gam).

	Test	UV-F	Cover	Male	DOY
Phenolic acid 1	lmer	-3.08**			
Neochlorogenic acid	lmer		-2.74**		
Phenolic acid 2	gls			2.93**	
Phenolic acid 3	gls		-2.11*	3.59***	
Phenolic acid 4	gls				2.75**
Phenolic acid 5	lmer		-2.32*		
Salicic acid derivative	gls			-3.46**	
Phenolic acid 6	gls			-2.94**	
Quercetin arabinoglucoside isomer	gls				2.98**
Quercetin arabinoglucoside isomer	gam			6.48***	11.47***
Quercetin 3-glucuronide	gam	-4.34***			10.17***
Chlorogenic acid derivative	gls				3.89***
Phenolic acid 7	gamm			2.41*	7.25**
Kaempferol 3-glucuronide	lmer				2.79*
Salicin	gls	2.22*			
Salicortin	gls				3.15**
Total leaf phenolic acids	lmer		-2.14*		
Total leaf flavonoids	gam	-3.59***			14.45***
Total leaf salicylates	lmer			3.72***	
Total leaf phenolics	gam			3.69***	6.86**

Table 14: *t*-values for stem concentrations of phenolics measured by HPLC on different dates (DOY) in females and males of *P. tremula* grown at 237 m a.s.l. under near-ambient UV-B levels and UVB-attenuation filters (UV-F). *t*-values were obtained using the following statistical tests in R: generalised least squares model (gls), linear mixed effect model (lmer), generalised additive model (gam), generalised additive mixed effect model (gam).

	Test	UV-F x Male	UV-F	Male	DOY
Phenolic acid4	gls				2.04*
Salicylate1	gam	-2.06*	2.37*	0.56	4.28*
Salicylate14	gls				2.70**
Salicylate15	gls			-2.43*	
Salicylate16	gls				2.47*
Total stem phenolic acids	lmer			-2.19*	
Total stem salicylates	gam		2.43*		9.16**
Total stem phenolics	gam		2.30*		8.46**

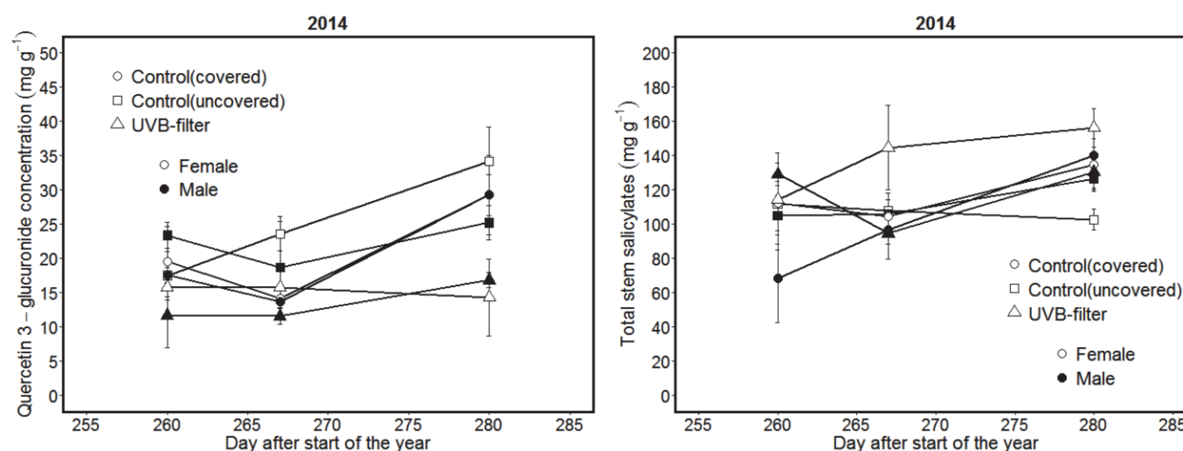

Figure 6: Concentrations of quercetin 3-glucuronide in leaves (left) and total stem salicylates (right) for females and males of *Populus tremula* growing at 237 m a.s.l. under different treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) during autumn 2014.

Also for stems sampled in 2013, salicylates constituted the largest proportion of the concentration of combined phenolics (91-94%), followed by phenolic acids (5-8%) and flavonoids (0.3-0.4%) (Supplementary table 3). Concentration of stem salicylates increased with elevation, being higher at 575 (21 %) (P<0.001) and 830 m a.s.l. (28 %) (P<0.001)

(Table 12), while salicin and salicylate 9 had higher concentrations in males than females at 830 m a.s.l. (*P*=0.038 and *P*=0.009, respectively). At 575 m a.s.l., salicylate 1 was found in higher concentrations in males (*P*=0.028). Lower concentrations of phenolic acid 3 were measured for males at 830 m a.s.l. (*P*=0.045).

In leaves sampled at 237 m a.s.l. in 2014, salicylates constituted the largest proportion of the phenolic compounds (71-85%), followed by flavonoids (10-22%) and phenolic acids (2-7%) (Supplementary tables 4-6). UV-B attenuation yielded lower concentrations (36 %) of leaf flavonoids (*P*<0.001) due to the effects on concentrations of quercetin 3-glucuronide (*P*<0.001) (Table 13, Fig. 6), which had 55 % lower concentrations under UV-B attenuation and was the most abundant phenolic compound found in leaves for both years. An opposite effect was found for salicin, which increased (54 %) with UV-B attenuation (*P*=0.031), while a negative effect of cover treatments was found for total phenolic acid content (*P*=0.038), which was 21 % higher for uncovered plots. Males had higher concentrations (24 %) of salicylates than females (*P*<0.001), resulting in higher (21 %) total phenolics (*P*<0.001). In stems sampled in 2014, salicylates constituted the largest proportion of phenolics (91-96%), followed by phenolic acids (3-10%) and flavonoids (0.3-0.8%) (Supplementary tables 7-9). Salicylate concentration increased (4 %) under UV-B attenuation (*P*=0.045) and with sampling date (*P*=0.004) (Table 14, Fig. 6), yielding higher (4 %) total phenolic content (*P*=0.026). In addition, stems of females had higher concentrations of salicylate 1 under UV-B attenuation (*P*=0.045). Stems of females also had higher concentration (21 %) of phenolic acids, irrespective of treatments (*P*=0.034).

DISCUSSION

In 2013, the length of the growing season decreased with increased elevation due to earlier bud formation, an effect which was significantly more pronounced in female plants (*P*<0.001) (Table 1; Figure 3). Evidence suggests these observations are mostly due to lower air temperature with increased elevation (Fig. 1), as high temperature has previously been reported to delay autumnal bud formation in *Populus* (Rohde *et al.* 2011a), with a stronger response for males in *P. tremula* (Strømme *et al.* 2015). Plantlet size decreased with elevation, which can be related both to lower temperatures limiting growth-related processes in plants as well as yielding a shorter growing season. In our study, female plantlets were taller than males in 2013 (Fig. 5), but height differences between sexes were less pronounced with increased elevation (Table 4; Fig. 5). For young plantlets of *P. tremula*, warming has

been shown to yield a stronger growth response in females than males (Randriamanana *et al.* 2015), and higher temperatures at low elevations may explain why size differences between females and males decreased with elevation in 2013. Male height may also have been positively affected by delayed bud formation and thus prolonged apical growth (Table 4; Fig. 6). Another study involving *P. tremula* showed that enhanced UV-B levels in combination with warming yielded reduced stem height and basal diameter (Randriamanana *et al.* 2015). Our results are consistent with an effect of UV-B on these growth parameters as we found that UV-B attenuation increased stem and diameter growth for males at the highest elevation (Table 5; Fig. 5 & 6). The effect could well be due to the male delay in bud formation under UV-B attenuation at this elevation (Table 2; Fig. 3).

Interestingly, male plants were larger than female plants in 2014, both in terms of stem height and basal diameter (Fig. 5 & 6), which is opposite of the results from 2013. This divergence may be related to effects on phenology varying with climate. Warming has been shown to modify the CDL sensitivity of bud formation in *Populus* (Rohde *et al.* 2011a), while high temperature has been found to delay the appearance of closed green buds more strongly in males than females of *P. tremula* (Paper III). The contrasting sex-related effects between 2013 and 2014 could thus be related to inter-annual variation in temperature before and after CDL sensing, resulting in different warming effects on bud formation.

The observed delay in bud formation under UV-B attenuation at the highest elevation site is in line with findings from a previous field study, where a modulated increase of UV-B, conversely, promoted bud formation in males of young *P. tremula* plantlets (Strømme *et al.* 2015). The observed effects on bud phenology suggest that UV-B acts as an environmental signal affecting also autumn phenology in concert with temperature. Indeed, several studies do show that UV-B acts as a regulatory signal for plant growth (Rozema *et al.* 1997; Jansen 2002; Jenkins 2009; Rizzini *et al.* 2011; Jansen & Bornman 2012; Hayes *et al.* 2014). On basis of evidence from previous studies it might be speculated that the delay in bud formation could be related to regulation of the growth hormone gibberellic acid (GA) by UV-B. Down-regulation of GA is known to yield apical bud formation in *Salix pentandra* and *P. tremula*. (Olsen *et al.* 1995a, b, 1997a, b, Mølmann *et al.* 2005). A recent study shows that UV-B detection in *Arabidopsis thaliana* antagonises shade-avoidance responses mediated by auxin and GA (Hayes *et al.* 2014). If GA levels in *P. tremula* are affected by UV-B through a similar signalling pathway, it is possible that UV-B attenuation in our study prevented UV-B

from affecting GA levels. Increased levels of abscisic acid (ABA) in the apical domain is also associated with autumnal bud formation in *Populus* during short days (Ruttink *et al.* 2007), and UV-B has been shown to increase ABA levels in leaves of *P. cathayana* (Xu *et al.* 2010). It is therefore also possible that bud formation in *P. tremula* is affected by UV-B through increased ABA levels.

Considering that bud formation occurred later in males in 2013, and that UV-B attenuation delayed bud formation in 2014, it is noteworthy that none of these effects were detected in the subsequent bud break data during spring 2014 and 2015. The delaying effect of increased elevation on bud break in both years can be related to thermal requirements, as warming has been shown to advance bud break in deciduous tree species (Fu *et al.* 2012). Also previous year autumn warming has been shown to yield earlier bud break in *P. tremula*, with a significantly stronger effect on male plantlets (Strømme *et al.* 2015). In this regards, earlier bud break with lower elevation could have been positively affected by higher autumn temperatures. Still, this effect cannot be disentangled from the effect of winter and spring temperatures across different elevations. One should also keep in mind that in this study and in Strømme *et al*. (2015), we used juvenile plantlets growing closer to the ground surface than adult trees.

Leaves and stems were sampled across the three different elevations at one event during autumn 2013, and analyses revealed that partitioning of N between leaves and stems differed with increased elevation (Table 6; Fig. 7). We measured increased stem N content with increased elevation, while leaf N content decreased, suggesting a higher N storage at high elevation. In *Populus*, N cycling and storage have been thoroughly characterised, which is mediated by vegetative storage proteins (Cooke & Weih 2005) and occurs in autumn with shorter days and lower temperatures (Thomas & Stoddart 1980). As we sampled plant material across elevations only in one event, we are unable to account for the combined effects of day length and temperature on N cycling in our study. However, warming has been reported to yield slightly higher N content in leaves of *P. tremula* (Randriamanana *et al.* 2015), which could explain the lower leaf N content found in our study at higher elevations were temperatures were lower. For 2014, when plantlets were sampled only at 237 m a.s.l., the higher leaf N content and lower stem N content in males may be related to delayed N translocation with delayed phenology. The measured decrease of N content under UV-B

attenuation is in line with findings from a previous UV-B enhancement study, where increased levels UV-B yielded higher N content in leaves (Xu *et al.* 2010).

Analyses of defensive metabolite concentrations of stems and leaves reveal that individual compounds and groups of compounds both increased and decreased with increased elevation. For leaves, elevation had a mainly negative effect on phenolic acids and salicortin. Enhanced temperature has been shown to decrease content of phenolic acids and salicortin in leaves of *S. myrsinifolia* (Veteli *et al.* 2002), suggesting that temperature was not responsible for reduced concentrations with increased elevation. However, a recent study of *Vitis vinifera* showed that cool temperature decreased concentrations of phenolic acids in leaves through cold stress (Król *et al.* 2015). It is therefore possible that cold stress at high elevation reduced leaf phenolic acid concentrations in our study. Interestingly, a positive effect of elevation was found for salicortin concentrations in stems. This may be a result of a concentrating effect, as plantlets were smaller in size with increased elevation. Attenuation of UV-B in 2014 yielded lower concentrations of leaf flavonoids, mainly through lower concentrations of quercetin 3-glucuronide. Different flavonoids provide protection against UV-B in higher plants (e.g. Li *et al.* 1993; Reuber *et al.* 1996), and enhanced UV-B has been shown to increase concentrations in leaves of *B. pendula* (Lavola *et al.* 1997), *S. myrsinifolia* (Tegelberg & Julkunen-Tiitto 2001) and *P. tremula* (Lavola *et al.* 2013; Randriamanana *et al.* 2015). Phenolic acid 1 was similarly affected by UV-B attenuation, although this treatment did not affect the total concentration of phenolic acids. Phenolic acids may have a role in protecting against UV-B (Sheahan, 1996; Lavola et al., 1997), and concentrations have been shown to increase with UV-B enhancement in *B. pendula* (Lavola *et al.* 1997). The total concentration of leaf salicylates was not affected by UV-B attenuation, but the significant increase for salicin with this treatment is in line with a study involving *S. myrsinifolia* where enhanced levels of UV-B yielded lower concentrations of salicin (Tegelberg & Julkunen-Tiitto 2001). Furthermore, the total salicylate consentration in stems increased under UV-B attenuation, an effect which according to our knowledge has not been reported previously.

In conclusion, we found that growth of *P. tremula* declined with increased elevation for both years, which is likely a result of lower growth rates and earlier bud formation with lower ambient temperatures. In terms of these responses, females and males were differently affected across elevations, showing divergent responsiveness between sexes. Concentrations of N in leaves and stems suggest that translocation of N from leaves to stems occurred earlier

at higher elevation. Furthermore, at the highest elevation plantlet growth and bud set were affected by UV-B attenuation, which had a positive effect on male plantlet size and delayed bud formation in autumn. Thus, ambient UV-B levels affect bud formation at the elevation where calculated UV-B radiation was highest. In spring, plantlets delayed bud break with increased elevation, which is most likely an effect of lower temperatures during dormancy release of vegetative buds. Also, phenolic compounds in leaves and stems varied across elevations, most notably for total salicylate content in stems, which increased with elevation. In addition, UV-B attenuation yielded significantly lower contents of flavonoids in leaves, and had a positive effect on stem salicylates. Our data shows that females and males were affected differently by ambient temperature and UV-B in terms of growth and autumn phenology, and further research on the molecular background for these responses could add to present knowledge on how climate affects phenology in trees.

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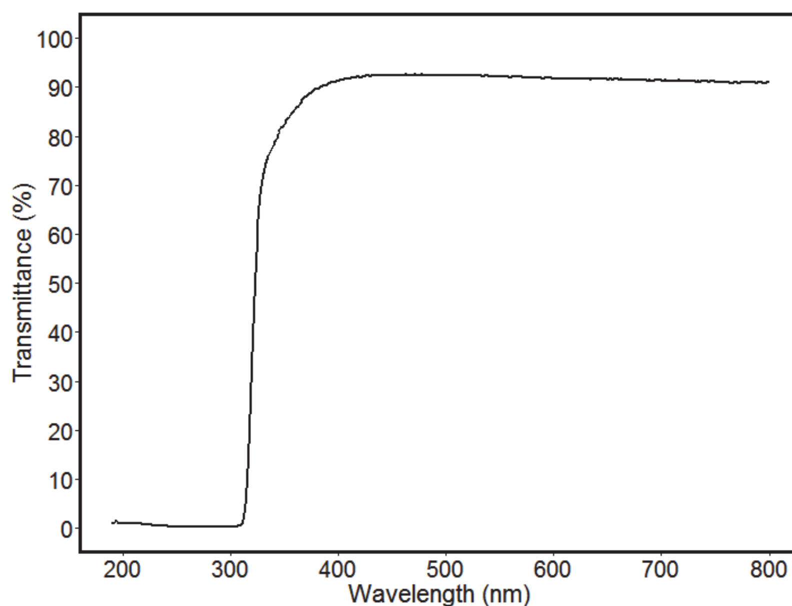
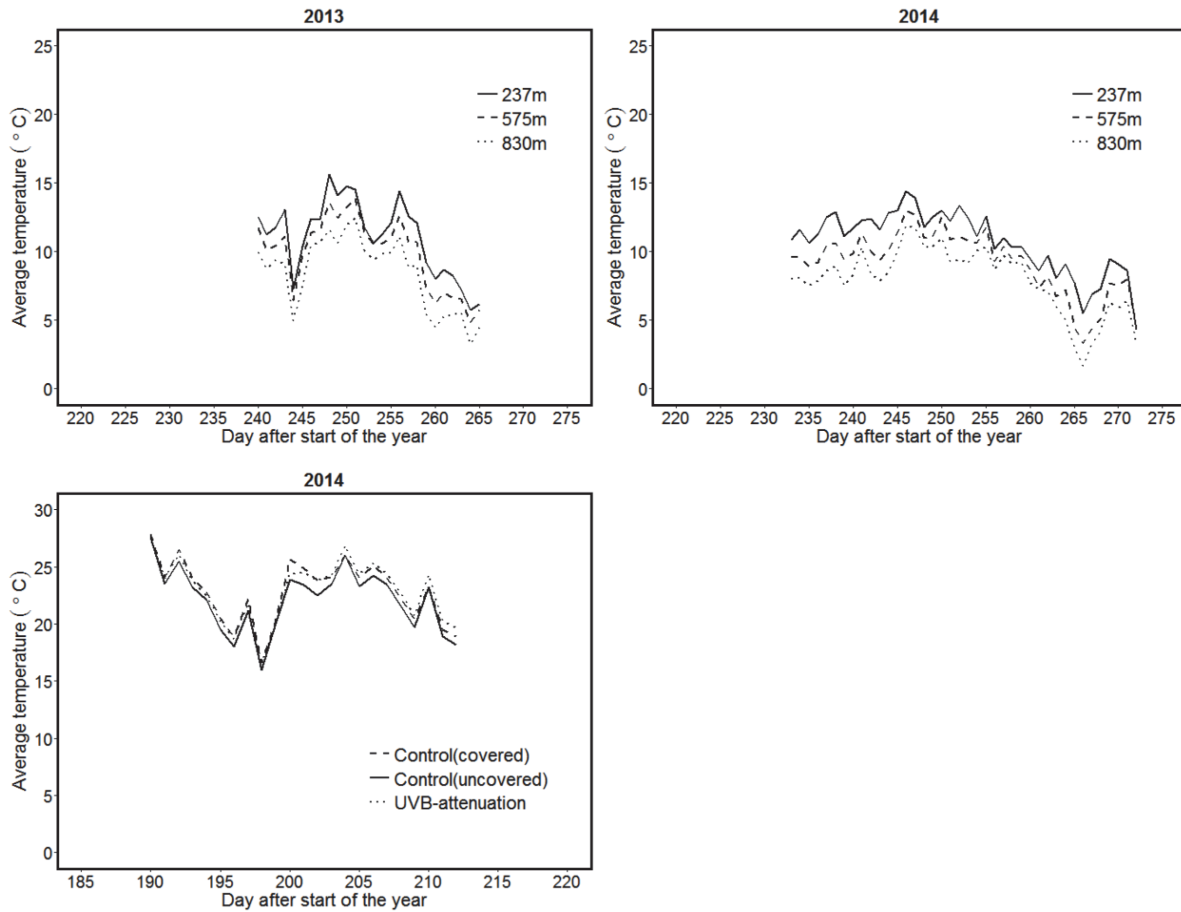
SUPPLEMENTARY MATERIALS

Supplementary table 1: Average July temperature and relative humidity (RH) ±1SEM for cover treatments applied in 2014.

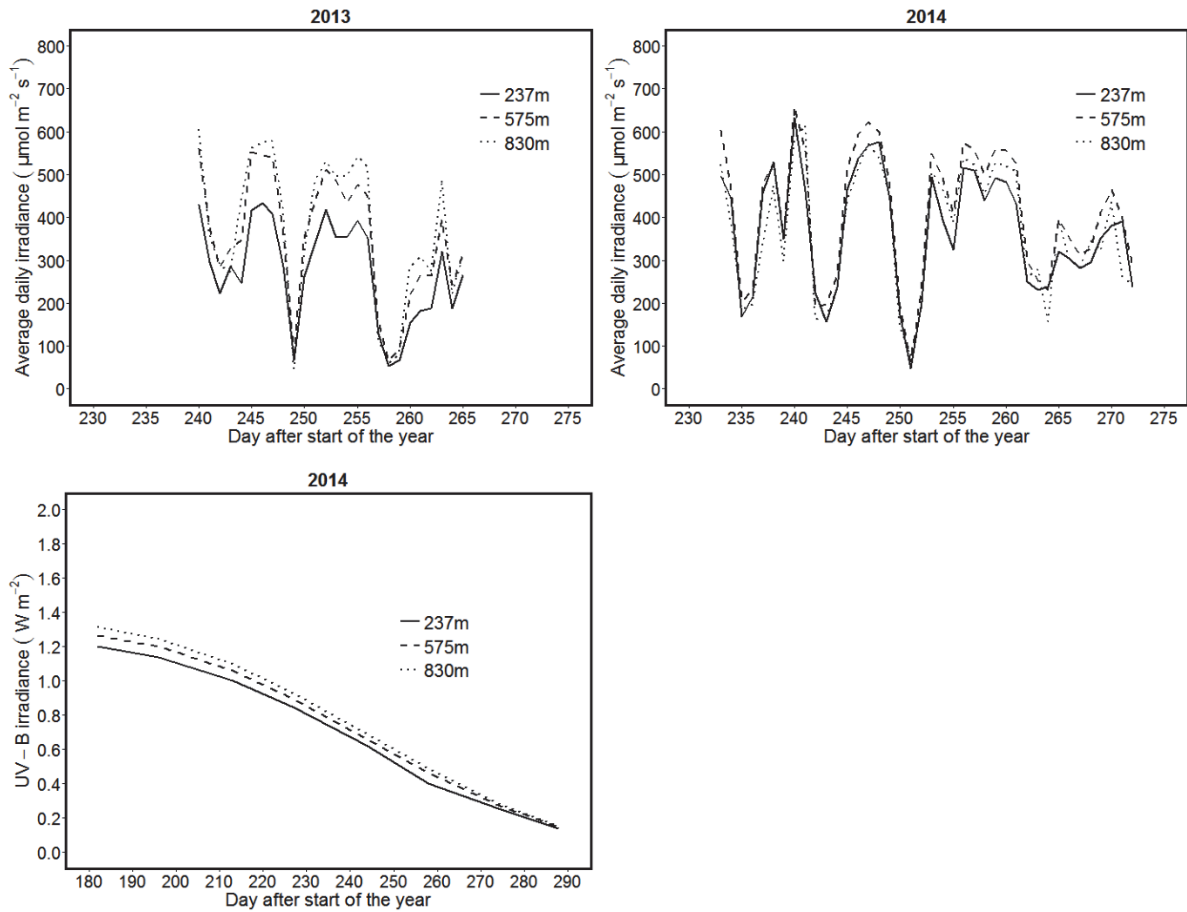
Treatment	°C	RH
UVB-attenuation	22.8±0.12	67±0.3
Control (covered)	22.7±0.13	68±0.3
Control (uncovered)	22.0±0.13	71±0.3



Supplementrary figure 1: Transmittance of the UV-B attenuation filter used in this study as measured with a spectrophotometer equipped with an integrating sphere (Shimadzu UV-2501 PC, Kyoto, Japan).



Supplementary figure 2: Daily average temperatures for different elevations (top panels) during autumn 2013 (top left) and 2014 (top right) and average daily near-ground temperature for treatments (UV-B attenuation, UV-B transmitting cover control, uncovered control) in July 2014 measured at 237 m a.s.l (bottom).



Supplementary figure 3. Daily average irradiance for different elevations (top panels) during autumn 2013 (left) and 2014 (right) and calculated UV-B irradiance (bottom) for the different locations using using the Quick TUV Calculator of the NCAR Earth System Laboratory.

Supplementary table 2: Average leaf concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown at different elevations.

	237 m		575 m		830 m	
	Female	Male	Female	Male	Female	Male
Phenolic acid 1	0.47±0.04	0.46±0.06	0.20±0.03	0.18±0.03	0.20±0.04	0.17±0.02
Neochlorogenic acid	4.08±0.45	3.86±0.39	2.50±0.22	2.34±0.28	2.37±0.28	2.13±0.12
Phenolic acid2	0.51±0.06	0.30±0.06	0.16±0.03	0.08±0.02	0.17±0.06	0.08±0.01
Phenolic acid3	0.70±0.14	0.67±0.13	0.33±0.08	0.23±0.08	0.18±0.03	0.12±0.02
Chlorogenic acid1	2.70±0.46	3.14±0.25	2.36±0.49	2.75±0.25	2.15±0.37	2.53±0.19
Phenolic acid4	0.20±0.04	0.28±0.03	0.21±0.06	0.17±0.02	0.15±0.03	0.41±0.24
Phenolic acid5	0.16±0.02	0.12±0.02	0.14±0.02	0.15±0.02	0.17±0.02	0.18±0.02
Salicortin	1.07±0.34	0.62±0.10	0.72±0.28	0.36±0.09	0.40±0.09	0.32±0.04
Quercetin arabinoglucoside	4.33±0.33	4.06±0.20	3.46±0.32	3.61±0.34	3.82±0.17	4.24±0.26
Quercetin 3-glucuronide	16.79±1.28	13.86±1.01	13.70±1.07	13.15±0.86	15.1±0.78	14.98±0.59
Kaempferol 3-glucuronide	1.39±0.18	1.19±0.08	1.34±0.15	1.27±0.11	1.62±0.14	1.63±0.09
Phenolic acid6	3.25±0.31	3.56±0.22	3.38±0.25	3.63±0.26	4.66±0.21	4.15±0.24
Phenolic acid7	0.14±0.03	0.10±0.02	0.11±0.02	0.16±0.03	0.15±0.04	0.13±0.03
Salicylate	0.71±0.08	0.73±0.06	0.74±0.08	0.72±0.09	1.08±0.10	1.13±0.09
Salicin	0.62±0.06	0.67±0.07	0.55±0.07	0.63±0.13	0.72±0.09	0.72±0.09
Salicortin	9.06±0.53	9.49±0.79	9.95±0.72	10.02±0.45	8.97±0.80	9.08±0.87
Tremulacin	58.78±4.15	47.96±2.21	45.66±3.79	42.94±2.08	56.26±2.61	50.05±2.34
Total leaf phenolic acids	6.24±0.61	5.75±0.65	3.64±0.29	3.28±0.42	3.36±0.32	3.20±0.25
Total leaf flavonoids	25.74±1.87	22.66±1.26	21.87±1.70	21.65±1.34	25.18±1.00	24.99±0.98
Total leaf salicylates	70.22±4.44	59.46±2.42	57.6±4.28	54.65±2.13	67.42±3.04	61.28±2.50
Total leaf phenolics	102.18±6.23	87.86±3.85	83.1±5.83	79.57±3.37	95.95±3.73	89.46±3.19

Supplementary table 3: Average stem concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown at different elevations.

	237 m		575 m		830 m	
	Female	Male	Female	Male	Female	Male
Salicylate1	0.24±0.04	0.23±0.02	0.17±0.03	0.26±0.03	0.21±0.03	0.24±0.03
Salicylate2	0.41±0.04	0.55±0.04	0.46±0.03	0.65±0.04	0.58±0.04	0.64±0.03
Chlorogenic acid derivative1	0.21±0.03	0.33±0.09	0.15±0.05	0.27±0.10	0.10±0.04	0.14±0.04
Phenolic acid1	1.55±0.27	1.68±0.42	1.54±0.59	1.75±0.74	0.83±0.20	0.76±0.15
Chlorogenic acid derivative2	0.30±0.07	0.39±0.08	0.26±0.07	0.43±0.13	0.23±0.04	0.33±0.05
Salicin	1.44±0.50	0.4±0.08	0.99±0.37	0.51±0.19	0.37±0.03	0.28±0.04
Salicylate3	0.59±0.06	0.63±0.08	0.55±0.09	0.64±0.11	0.54±0.04	0.42±0.03
Myricetin derivative	0.26±0.08	0.22±0.05	0.25±0.06	0.16±0.05	0.25±0.03	0.16±0.03
Phenolic acid2	3.36±0.23	3.27±0.17	4.04±0.19	4.1±0.23	4.35±0.19	3.57±0.20
Phenolic acid3	0.73±0.07	0.56±0.05	0.71±0.06	0.76±0.04	0.87±0.07	0.75±0.04
Chlorogenic acid derivative3	0.33±0.04	0.33±0.03	0.37±0.04	0.41±0.04	0.44±0.04	0.42±0.05
Phenolic acid5	0.18±0.04	0.32±0.06	0.18±0.03	0.23±0.04	0.22±0.04	0.24±0.03
Salicylate4	0.11±0.03	0.10±0.03	0.18±0.07	0.13±0.04	0.23±0.09	0.25±0.07
Salicylate5	0.30±0.06	0.38±0.05	0.35±0.06	0.47±0.06	0.48±0.08	0.42±0.07
Salicylate6	0.43±0.09	1.19±0.61	0.33±0.06	0.93±0.53	1.17±0.57	0.55±0.22
Salicylate7	2.98±0.45	5.45±1.18	6.58±0.81	7.98±0.68	5.40±0.71	7.74±0.60
Salicylate8	0.71±0.16	0.80±0.11	0.73±0.09	1.02±0.12	1.03±0.15	1.03±0.10
Salicylate9	4.35±1.16	1.79±0.24	3.01±0.58	2.37±0.30	1.99±0.20	1.92±0.14
Salicylate10	1.40±0.49	0.85±0.23	0.83±0.43	0.64±0.20	0.50±0.10	0.35±0.05
Salicylate11	1.34±0.24	1.67±0.19	1.81±0.17	2.36±0.20	2.18±0.18	2.30±0.13
Salicylate12	0.60±0.18	0.60±0.21	0.17±0.07	0.19±0.07	0.31±0.10	0.10±0.04
Salicylate13	0.26±0.09	0.19±0.08	0.34±0.15	0.11±0.05	0.31±0.08	0.18±0.07
Salicylate14	0.88±0.18	0.79±0.16	0.46±0.11	0.49±0.17	0.45±0.11	0.24±0.10
Salicylate15	0.71±0.12	1.09±0.17	0.72±0.12	1.47±0.23	0.72±0.08	1.54±0.16
Salicortin	41.51±2.90	43.64±3.00	49.10±2.50	57.51±3.73	66.64±3.86	60.24±3.81
Tremulacin	19.68±1.60	16.80±1.22	21.17±2.42	22.64±2.14	20.67±2.47	16.96±1.42
p-OH-cinnamoylsalicortin	0.88±0.41	0.43±0.19	0.24±0.22	0.40±0.20	0.30±0.19	0.11±0.06
Total stem phenolic acids	6.46±0.54	6.54±0.65	7.06±0.77	7.69±1.06	6.78±0.31	5.95±0.36
Total stem flavonoids	0.26±0.08	0.22±0.05	0.25±0.06	0.16±0.05	0.25±0.03	0.16±0.03
Total stem salicylates	78.16±4.39	76.86±3.94	87.6±3.79	100.04±5.67	103.47±4.60	95.00±4.11
Total stem phenolics	84.86±4.74	83.60±4.51	94.89±4.37	107.89±6.32	110.49±4.78	101.09±4.38

Supplementary table 4: Average leaf concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under UV-B attenuation filters.

	Female			Male		
	260	267	280	260	267	280
Phenolic acid 1	0.48±0.03	0.24±0.13	0.48±0.24	0.84±0.12	0.76±0.07	0.59±0.25
Neochlorogenic acid	2.64±0.35	1.57±0.93	3.91±2.05	5.37±1.86	4.36±0.27	6.55±0.56
Phenolic acid2	0.56±0.02	0.53±0.13	0.45±0.23	0.65±0.13	0.60±0.07	0.81±0.12
Phenolic acid3	0.73±0.15	0.38±0.24	0.55±0.33	1.42±0.55	1.15±0.13	1.58±0.20
Chlorogenic acid	0.55±0.13	0.9±0.21	0.94±0.06	0.71±0.07	0.42±0.19	0.84±0.15
Chlorogenic acid1	4.70±0.52	4.12±1.26	2.57±1.46	2.08±0.87	2.45±0.73	4.23±1.08
Phenolic acid4	0.18±0.09	0.14±0.07	0.34±0.34	0.28±0.03	0.25±0.05	0.33±0.05
Myricetin glycol	0.10±0.05	0.12±0.07	0.41±0.24	0.21±0.13	0.22±0.05	0.46±0.09
Phenolic acid5	0.54±0.07	0.42±0.14	0.20±0.20	0.19±0.12	0.14±0.08	0.43±0.04
Salicortin	0.28±0.02	0.24±0.06	0.94±0.71	0.35±0.11	0.28±0.06	0.63±0.09
Salicic acid derivative	0.54±0.07	0.58±0.07	0.67±0.45	0.28±0.08	0.41±0.11	0.47±0.04
Phenolic acid6	0.27±0.03	0.17±0.10	0.16±0.16	0.09±0.04	0.09±0.05	0.21±0.03
Quercetin arabinoglucoside isomer	4.32±0.36	3.93±0.59	4.40±1.87	3.09±1.46	2.93±0.30	4.99±0.16
Quercetin arabinoglucoside isomer	0.74±0.37	0.64±0.36	0.98±0.60	1.32±0.49	1.19±0.08	2.33±0.11
Quercetin 3-glucuronide	15.81±1.91	15.83±2.43	14.37±5.62	11.72±4.7	11.60±1.16	16.84±1.11
Chlorogenic acid derivative	0.52±0.40	0.55±0.03	0.86±0.44	0.30±0.12	0.49±0.03	0.80±0.10
Phenolic acid7	0.19±0.11	0.10±0.10	0.42±0.42	0.32±0.19	0.46±0.03	0.77±0.04
Kaempferol 3-glucuronide	3.37±0.56	2.89±0.47	7.32±2.95	3.96±0.73	5.17±0.50	6.13±0.60
Phenolic acid8	0.68±0.19	0.37±0.21	1.21±0.44	0.80±0.22	0.97±0.06	1.46±0.11
Phenolic acid9	0.32±0.05	0.17±0.10	0.77±0.30	0.42±0.07	0.45±0.06	0.82±0.10
Phenolic acid10	0.34±0.11	0.16±0.10	0.55±0.26	0.22±0.08	0.24±0.03	0.51±0.09
Salicylate	2.36±0.11	2.00±0.09	1.82±0.09	1.70±0.10	1.74±0.07	1.89±0.29
Salicin	8.09±0.92	7.43±2.37	21.29±10.57	6.86±1.42	5.74±0.38	7.47±1.55
Salicortin	81.05±13.15	58.43±6.44	88.04±31.25	76.36±11.73	84.65±4.74	109.97±2.17
Tremulacin	87.14±11.74	55.25±8.22	45.23±15.77	71.43±35.17	81.56±7.92	131.65±9.26
p-OH- cinnamoylsalicortin	0.26±0.03	0.27±0.09	0.31±0.11	0.25±0.07	0.28±0.04	0.58±0.19
Total leaf phenolic acids	6.72±0.19	4.40±1.79	8.63±4.58	10.05±3.20	8.94±0.62	13.34±0.57
Total leaf flavonoids	25.00±3.21	23.75±3.30	28.65±11.24	21.08±7.59	22.04±1.10	32.19±1.33
Total leaf salicylates	179.67±24.94	124.17±13.42	158.27±13.45	157.19±38.39	174.64±12.07	252.64±11.29
Total leaf phenolics	211.38±28.22	152.31±17.05	195.54±25.99	188.31±48.90	205.61±13.12	298.15±12.23

Supplementary table 5: Average leaf concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under UV-B transmitting cover control.

	Female			Male		
	260	267	280	260	267	280
Phenolic acid 1	0.54±0.25	0.37±0.02	0.8±0.04	0.91±0.03	0.69±0.14	0.87±0.11
Neochlorogenic acid	2.33±1.91	1.98±0.37	6.56±0.28	5.50±0.16	4.10±0.95	7.41±1.30
Phenolic acid2	0.57±0.11	0.41±0.05	0.54±0.03	0.62±0.03	0.51±0.11	0.88±0.22
Phenolic acid3	0.61±0.61	0.49±0.09	0.69±0.05	1.32±0.08	0.94±0.28	1.87±0.35
Chlorogenic acid	0.7±0.34	0.63±0.13	0.98±0.11	0.56±0.06	2.04±1.56	0.61±0.33
Chlorogenic acid1	5.82±0.66	3.01±0.23	2.28±0.39	1.8±0.31	2.13±0.34	5.05±1.34
Phenolic acid4	0.14±0.14	0.09±0.09	0.28±0.28	0.25±0.02	0.21±0.03	0.46±0.19
Myricetin glycol	0.12±0.12	0.05±0.05	0.65±0.10	0.44±0.06	0.20±0.04	0.61±0.08
Phenolic acid5	0.61±0.05	0.31±0.01	0.12±0.12	0.16±0.09	0.25±0.06	0.27±0.27
Salicortin	0.31±0.10	0.17±0.02	0.86±0.02	0.48±0.09	0.22±0.06	0.80±0.16
Salicic acid derivative	0.82±0.16	0.43±0.09	0.54±0.02	0.32±0.04	0.30±0.06	0.50±0.14
Phenolic acid6	0.32±0.02	0.21±0.05	0.20±0.20	0.07±0.04	0.15±0.04	0.21±0.06
Quercetin arabinoglucoside isomer	3.96±1.51	3.06±0.57	6.71±0.79	4.12±0.42	3.21±0.41	6.64±0.92
Quercetin arabinoglucoside isomer	0.69±0.69	0.37±0.37	1.8±0.21	1.78±0.14	1.32±0.12	2.49±0.15
Quercetin 3-glucuronide	19.58±5.10	14.19±0.13	29.27±2.97	17.60±1.13	13.76±0.85	29.28±5.78
Chlorogenic acid derivative	0.46±0.29	0.45±0.03	1.08±0.33	0.47±0.10	0.27±0.06	0.52±0.07
Phenolic acid7	0.23±0.06	0.07±0.07	0.68±0.02	0.48±0.06	0.48±0.09	0.73±0.17
Kaempferol 3-glucuronide	4.40±0.37	3.93±1.28	7.43±0.16	4.28±0.14	4.38±0.80	7.19±2.11
Phenolic acid8	0.68±0.18	0.49±0.05	1.52±0.16	1.01±0.15	0.72±0.07	1.22±0.13
Phenolic acid9	0.30±0.02	0.23±0.04	0.96±0.11	0.61±0.07	0.40±0.04	0.47±0.17
Phenolic acid10	0.33±0.04	0.19±0.06	0.59±0.04	0.27±0.04	0.21±0.03	0.53±0.04
Salicylate	2.02±0.21	2.14±0.13	4.36±3.08	1.82±0.16	2.44±0.53	3.53±1.49
Salicin	7.12±0.54	6.81±2.08	5.94±1.51	6.58±0.89	5.46±0.43	5.41±0.58
Salicortin	65.96±60	61.19±9.78	112.79±14.06	85.59±7.35	79.87±2.48	88.46±7.34
Tremulacin	63.74±16.15	57.50±7.52	26.27±4.39	105.42±14.42	82.23±7.46	102.62±1.9
p-OH- cinnamoylsalicortin	0.43±0.07	0.20±0.11	0.06±0.06	0.36±0.06	0.21±0.05	0.41±0.09
Total leaf phenolic acids	6.38±3.34	4.73±0.39	12.46±1.30	10.63±0.33	8.16±1.40	14.19±2.67
Total leaf flavonoids	29.41±7.93	22.07±0.23	47.36±3.95	29.21±1.95	23.57±0.73	47.40±8.95
Total leaf salicylates	140.38±21.27	128.41±19.67	150.8±16.96	200.53±21.76	170.69±8.00	201.69±8.57
Total leaf phenolics	176.15±32.54	155.2±19.51	210.61±22.2	240.37±23.57	202.40±6.17	263.27±17.09

Supplementary table 6: Average leaf concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under ambient irradiance.

	Female			Male		
	260	267	280	260	267	280
Phenolic acid 1	0.45±0.09	0.55±0.09	0.73±0.05	1.30±0.07	0.83±0.17	0.89±0.06
Neochlorogenic acid	1.83±0.28	2.38±0.12	6.63±0.77	8.41±0.26	6.38±1.79	6.68±0.17
Phenolic acid2	0.48±0.10	0.58±0.06	0.68±0.12	1.06±0.06	0.61±0.17	0.67±0.09
Phenolic acid3	0.42±0.05	2.04±1.49	0.77±0.1	2.31±0.13	1.45±0.54	1.52±0.06
Chlorogenic acid	1.21±0.41	0.96±0.12	0.57±0.03	0.9±0.09	0.67±0.03	0.77±0.09
Chlorogenic acid1	3.48±0.94	3.74±1.89	4.52±1.2	3.67±0.42	2.95±1.27	3.78±0.66
Phenolic acid4	0.16±0.04	0.13±0.07	0.52±0.12	0.31±0.04	0.27±0.05	0.31±0.05
Myricetin glycol	0.15±0.06	0.14±0.08	0.39±0.21	0.39±0.04	0.31±0.08	0.49±0.08
Phenolic acid5	0.38±0.09	0.63±0.06	0.37±0.09	0.45±0.05	0.30±0.14	0.43±0.07
Salicortin	0.24±0.03	0.27±0.03	0.82±0.12	0.47±0.07	0.29±0.10	0.53±0.10
Salicic acid derivative	0.49±0.07	0.70±0.03	1.10±0.59	0.35±0.03	0.25±0.04	0.34±0.05
Phenolic acid6	0.18±0.04	0.31±0.03	0.25±0.02	0.21±0.08	0.09±0.05	0.15±0.03
Quercetin arabinoglucoside isomer	3.56±0.58	4.52±0.51	5.34±2.44	5.86±0.41	4.44±1.72	6.47±0.84
Quercetin arabinoglucoside isomer	0.80±0.05	0.95±0.09	1.23±0.38	2.14±0.03	1.74±0.33	2.12±0.27
Quercetin 3-glucuronide	17.54±3.54	23.64±2.53	34.15±5.01	23.40±1.89	18.7±6.70	25.23±2.54
Chlorogenic acid derivative	0.54±0.13	0.57±0.20	1.20±0.46	0.53±0.03	0.41±0.23	0.95±0.06
Phenolic acid7	0.07±0.07	0.26±0.26	0.73±0.18	0.44±0.08	0.25±0.05	0.41±0.21
Kaempferol 3-glucuronide	3.16±0.81	4.81±0.17	9.08±1.89	5.60±0.50	4.51±0.83	5.42±0.39
Phenolic acid8	0.55±0.14	0.59±0.17	0.93±0.26	1.11±0.07	0.60±0.29	1.22±0.16
Phenolic acid9	0.28±0.08	0.32±0.04	0.70±0.07	0.57±0.07	0.31±0.11	0.67±0.13
Phenolic acid10	0.21±0.06	0.31±0.06	0.56±0.06	0.28±0.04	0.13±0.06	0.32±0.05
Salicylate	2.22±0.29	2.21±0.10	2.10±0.35	2.02±0.08	1.63±0.12	1.88±0.17
Salicin	6.99±0.39	5.17±0.34	6.24±0.76	6.59±1.84	5.47±0.22	6.09±1.07
Salicortin	58.20±10.19	76.13±2.63	97.16±10.95	85.37±4.85	53.17±10.29	78.53±4.11
Tremulacin	60.73±6.67	70.17±10.64	22.90±3.88	95.99±9.12	50.90±12.65	85.10±4.31
p-OH- cinnamoylsalicortin	0.26±0.11	0.32±0.08	0.34±0.17	0.31±0.05	0.12±0.07	0.28±0.02
Total leaf phenolic acids	4.96±0.68	8.04±1.43	13.02±0.73	15.81±0.27	10.97±3.22	12.95±0.75
Total leaf flavonoids	25.74±4.97	34.62±3.02	46.01±4.57	38.47±2.62	30.27±9.93	40.93±4.14
Total leaf salicylates	129.10±16.95	154.94±13.66	153.02±12.20	191.06±15.08	111.81±23.04	172.73±9.46
Total leaf phenolics	159.79±22.46	197.60±16.17	212.05±13.81	245.33±12.79	153.04±36.17	226.59±13.10

Supplementary table 7: Average stem concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under UV-B attenuation filters.

	Female			Male		
	260	267	280	260	267	280
Salicylate1	0.3±0.08	0.38±0.06	0.49±0.10	0.36±0.03	0.26±0.06	0.34±0.03
Salicylate2	0.71±0.16	0.83±0.08	0.86±0.07	1.04±0.05	0.55±0.10	0.95±0.14
Chlorogenic acid derivative1	0.46±0.26	0.62±0.25	0.36±0.13	0.71±0.07	0.41±0.28	0.6±0.18
Phenolic acid1	2.05±1.26	2.74±0.95	3.02±0.52	2.99±0.28	1.75±1.21	3.01±1.10
Salicylate3	0.40±0.10	0.59±0.22	0.52±0.14	0.54±0.10	0.36±0.20	0.51±0.16
Salicylate4	0.55±0.13	0.90±0.21	0.94±0.06	0.71±0.07	0.42±0.19	0.84±0.15
Salicylate5	0.44±0.28	0.36±0.24	0.48±0.14	0.25±0.08	0.54±0.34	0.43±0.18
Myricetin derivative	0	0.08±0.04	0.08±0.04	0	0.06±0.06	0.22±0.05
Phenolic acid2	2.12±0.11	3.12±1.04	2.49±0.29	2.03±0.24	1.48±0.25	2.01±0.25
Phenolic acid3	0.72±0.13	0.52±0.15	0.82±0.05	0.47±0.24	0.54±0.15	0.83±0.19
Phenolic acid4	0.38±0.21	0.62±0.17	1.24±0.26	0.65±0.06	0.45±0.13	0.81±0.15
Salicylate6	0.45±0.16	0.56±0.11	0.72±0.22	0.43±0.06	0.32±0.09	0.39±0.02
Salicylate7	1.27±0.29	1.15±0.21	1.77±0.50	1.34±0.11	0.82±0.25	1.17±0.18
Salicylate8	1.21±0.90	0.67±0.18	0.90±0.28	0.50±0.10	1.07±0.76	0.44±0.05
Salicylate9	1.45±1.07	2.27±0.40	2.79±0.68	2.63±0.61	1.15±0.44	2.11±0.12
Salicylate10	2.48±0.85	2.48±0.50	3.85±0.59	2.44±0.50	1.33±0.16	2.11±0.25
Salicylate11	0.49±0.49	1.23±0.91	1.85±0.45	0.70±0.52	1.25±0.42	1.19±0.35
Salicylate12	2.86±0.16	2.85±0.79	2.68±0.28	2.35±0.18	1.75±0.23	2.74±0.36
Salicylate13	3.02±0.10	4.22±0.95	4.87±0.81	4.52±0.59	2.35±0.51	3.76±0.58
Salicylate14	1.00±0.59	0.66±0.66	1.58±0.29	0.19±0.19	0.78±0.39	0.80±0.15
Salicylate15	2.32±0.86	1.51±0.87	3.54±0.70	1.88±0.28	1.32±0.93	1.85±0.25
Salicylate16	0.96±0.96	3.66±0.62	5.56±1.40	2.32±0.20	2.16±1.44	3.24±0.28
Salicylate17	2.36±0.99	2.78±1.06	2.64±0.82	2.05±0.87	0.83±0.83	1.97±0.80
Salicortin	64.92±4.76	80.26±17.9	85.95±3.21	70.99±5.57	55.53±10.47	67.91±6.02
Tremulacin	25.3±7.52	35.48±4.77	33.98±3.71	33.80±5.44	21.62±1.76	37.55±2.4
p-OH- cinnamoylsalicortin	2.79±1.65	2.90±1.60	1.45±0.48	1.04±0.36	1.13±0.51	1.16±0.22
Total stem phenolic acids	6.09±1.79	8.19±0.82	8.42±0.86	7.37±0.45	4.97±2.09	7.73±1.74
Total stem flavonoids	0	0.08±0.04	0.08±0.04	0	0.06±0.06	0.22±0.05
Total stem salicylates	114.35±8.18	144.70±24.73	156.32±11.14	129.21±12.35	94.56±14.85	130.43±11.37
Total stem phenolics	120.44±9.97	152.96±25.32	164.81±10.68	136.58±12.76	99.58±16.70	138.37±13.08

Supplementary table 8: Average stem concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under UV-B transmitting cover control.

	Female			Male		
	260	267	280	260	267	280
Salicylate1	0.38±0.09	0.36±0.06	0.51±0.14	0.30±0.09	0.26±0.05	0.32±0.08
Salicylate2	0.90±0.26	0.83±0.05	0.87±0.09	0.79±0.21	0.67±0.07	0.85±0.14
Chlorogenic acid derivative1	0.87±0.62	0.60±0.19	0.59±0.18	0.42±0.15	0.20±0.07	0.77±0.35
Phenolic acid1	3.30±2.30	2.99±1.07	3.32±1.25	1.75±0.56	0.93±0.26	3.41±1.37
Salicylate3	0.51±0.34	0.46±0.08	0.64±0.22	0.38±0.13	0.18±0.09	0.39±0.28
Salicylate4	0.70±0.34	0.63±0.13	0.98±0.11	0.56±0.06	2.04±1.56	0.61±0.33
Salicylate5	0.33±0.20	0.61±0.30	0.32±0.05	0.29±0.14	0.14±0.02	0.14±0.04
Myricetin derivative	0.02±0.02	0.03±0.03	0.36±0.19	0.10±0.05	0.42±0.31	0.03±0.03
Phenolic acid2	1.87±0.36	1.90±0.19	2.55±0.34	1.68±0.40	1.71±0.29	1.38±0.69
Phenolic acid3	0.62±0.14	0.59±0.13	0.76±0.06	0.65±0.20	0.46±0.14	0.48±0.25
Phenolic acid4	0.66±0.26	0.51±0.22	1.18±0.35	0.61±0.21	0.40±0.06	0.40±0.22
Salicylate6	0.33±0.08	0.44±0.09	0.40±0.03	0.21±0.11	0.38±0.10	0.76±0.26
Salicylate7	1.22±0.33	1.30±0.11	0.84±0.21	0.51±0.30	0.87±0.17	1.62±0.31
Salicylate8	0.39±0.08	0.92±0.52	0.42±0.04	0.21±0.11	0.56±0.28	0.76±0.22
Salicylate9	2.47±0.20	1.32±0.56	1.89±0.28	3.03±2.32	7.80±5.91	2.92±0.48
Salicylate10	2.23±0.71	2.03±0.57	2.58±0.51	1.05±0.54	2.47±1.35	2.50±0.38
Salicylate11	2.91±2.15	1.64±0.58	1.14±0.10	0.25±0.25	0.19±0.19	0.23±0.23
Salicylate12	1.11±0.56	1.53±0.07	2.22±0.20	1.38±0.70	6.99±5.00	3.94±0.79
Salicylate13	3.78±1.26	3.35±0.39	3.59±0.39	1.72±0.94	3.17±0.72	2.93±1.47
Salicylate14	0.39±0.20	0.93±0.23	0.83±0.43	0.20±0.20	0.15±0.15	2.13±1.72
Salicylate15	2.45±0.99	1.96±0.26	1.83±0.37	0.73±0.46	1.48±0.58	2.15±0.46
Salicylate16	3.27±0.86	1.79±0.91	3.42±0.38	1.11±0.81	1.67±0.41	3.90±1.70
Salicylate17	2.10±1.63	1.58±0.33	0.89±0.48	0.42±0.42	1.14±0.57	1.42±0.72
Salicortin	60.49±9.32	60.20±4.35	79.56±9.77	26.08±19.86	40.4±17.53	78.85±1.23
Tremulacin	26.40±3.93	21.04±2.39	32.21±4.24	28.94±5.15	24.91±2.10	32.78±1.22
p-OH- cinnamoylsalicortin	0.91±0.57	2.57±1.55	0.87±0.10	0.83±0.16	1.53±0.89	1.62±0.51
Total stem phenolic acids	7.81±3.99	7.03±1.65	9.02±2.17	5.46±1.49	3.85±0.55	6.79±1.93
Total stem flavonoids	0.02±0.02	0.03±0.03	0.36±0.19	0.10±0.05	0.42±0.31	0.03±0.03
Total stem salicylates	112.36±16.35	104.34±5.44	134.96±14.80	68.24±25.58	96.58±8.04	140.22±1.71
Total stem phenolics	120.18±20.30	111.39±4.98	144.33±16.99	73.78±26.19	100.85±8.59	147.03±0.89

Supplementary table 9: Average stem concentrations as mg g^{-1} dry weight ±1SEM of phenolics in *P. tremula* females and males grown under ambient irradiance.

	Female			Male		
	260	267	280	260	267	280
Salicylate1	0.33±0.03	0.47±0.11	0.28±0.07	0.33±0.01	0.19±0.03	0.31±0.11
Salicylate2	0.82±0.18	0.76±0.06	0.70±0.10	0.75±0.04	0.57±0.10	0.65±0.10
Chlorogenic acid derivative1	0.59±0.34	0.64±0.07	0.44±0.13	0.47±0.04	0.53±0.19	0.34±0.03
Phenolic acid1	3.21±1.39	3.78±0.43	2.19±0.63	3.09±0.17	2.87±0.27	1.74±0.28
Salicylate3	0.47±0.25	0.63±0.03	0.35±0.06	0.59±0.07	0.60±0.03	0.41±0.05
Salicylate4	1.21±0.41	0.96±0.12	0.57±0.03	0.90±0.09	0.67±0.03	0.77±0.09
Salicylate5	0.27±0.07	0.33±0.13	0.18±0.03	0.56±0.30	0.21±0.07	0.16±0.09
Myricitin derivative	0.08±0.08	0.41±0.30	0.13±0.04	0.18±0.18	0.13±0.13	0.08±0.04
Phenolic acid2	1.78±0.15	2.15±0.06	1.77±0.08	1.44±0.42	1.68±0.14	2.92±0.37
Phenolic acid3	0.62±0.18	0.75±0.15	0.43±0.22	0.76±0.11	0.64±0.11	0.85±0.19
Phenolic acid4	0.57±0.23	1.06±0.35	0.62±0.10	0.61±0.20	0.50±0.13	0.73±0.17
Salicylate6	0.36±0.06	0.31±0.04	0.47±0.08	0.59±0.01	0.30±0.06	0.35±0.03
Salicylate7	1.23±0.15	0.88±0.23	0.84±0.10	1.52±0.18	0.48±0.03	0.54±0.11
Salicylate8	0.50±0.17	0.48±0.08	0.53±0.11	0.66±0.24	0.69±0.46	0.35±0.04
Salicylate9	2.89±0.82	2.32±0.21	1.59±0.13	2.96±1.34	0.94±0.31	1.37±0.56
Salicylate10	1.99±0.3	2.36±0.60	1.79±0.21	3.23±0.25	2.16±0.85	2.27±0.59
Salicylate11	0.80±0.80	0.97±0.39	1.24±0.25	2.10±1.63	1.16±0.41	0.69±0.41
Salicylate12	3.03±0.43	1.93±0.21	1.56±0.43	2.26±2.26	2.10±0.20	2.04±0.60
Salicylate13	3.26±0.80	3.45±0.60	2.82±0.92	3.68±0.32	2.96±0.24	3.03±0.30
Salicylate14	0.44±0.22	0.41±0.27	1.97±1.17	1.68±0.17	0.46±0.46	1.12±0.85
Salicylate15	1.55±0.06	2.24±0.62	2.07±0.17	1.91±0.06	0.68±0.27	1.49±0.12
Salicylate16	1.90±1.07	2.87±1.17	2.89±0.41	3.73±1.71	1.90±0.17	2.05±1.03
Salicylate17	1.19±0.60	1.12±0.14	1.73±0.52	1.88±0.60	0.59±0.59	1.40±0.85
Salicortin	58.96±13.56	65.72±5.71	54.65±3.72	54.81±1.43	58.79±10.31	76.97±2.25
Tremulacin	30.84±8.18	20.13±6.76	26.18±2.59	20.53±13.51	30.89±1.36	30.54±2.27
p-OH- cinnamoylsalicortin	0.82±0.12	0.67±0.12	0.98±0.02	1.51±0.52	0.56±0.26	0.67±0.08
Total stem phenolic acids	7.22±2.51	8.99±0.93	5.77±1.04	6.94±0.07	6.80±0.53	6.96±0.57
Total stem flavonoids	0.08±0.08	0.41±0.30	0.13±0.04	0.18±0.18	0.13±0.13	0.08±0.04
Total stem salicylates	112.02±23.63	107.96±10.09	102.78±6.23	104.94±20.07	105.99±8.42	126.55±5.40
Total stem phenolics	119.31±26.19	117.35±10.44	108.67±6.98	112.05±19.82	112.91±9.07	133.58±5.81

PAPER III

High daytime temperature delays autumnal bud formation in *Populus tremula* under field conditions

Christian B. Strømme[1], *Riitta Julkunen-Tiitto*[3], *Jorunn E. Olsen*[2], *Line Nybakken*[1]

[1] *Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

[2] *Department of Plant Sciences, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

[3] *Department of Biology, University of Eastern Finland, P.O.-Box 111, FI-20101 Joensuu, Finland*

Corresponding author: Christian Bianchi Strømme
Email: christian.stromme@nmbu.no

ABSTRACT

Warming effects on autumnal growth cessation and bud set in trees remain ambiguous due to contrasting observations between studies under controlled conditions and field experiments. In addition, potential differential effects on the sexes of dioecious species has also received scarce attention. In a data set including three separate field experiments employing either experimental warming or an elevational gradient, we tested the effect of different temperature variables on apical, vegetative bud formation and transitions between bud stages in female and male clones of Eurasian aspen. Our data shows that increased temperature delayed bud set, and bud formation was best explained by maximum day temperature. Males were significantly delayed compared to females in forming green closed buds, a process best explained by mean 24 h temperature. Bud maturation was best explained by mean daytime temperature, and males were significantly faster than females, possibly explaining why females and males did not differ in terms of overall bud formation. Overall, our data show that the delayed bud formation in Eurasian aspen under warming is best explained by maximum day temperature. The delayed bud formation in response to warming is contrary to findings from previous studies performed under controlled experimental conditions.

Key words: Climate change**,** dioecious, ecophysiology, environmental signals, field experiments, modulation, plant-climate interactions

INTRODUCTION

The ongoing rise in global temperatures has stimulated research on possible effects on plant phenology (Steltzer & Post 2009; Körner & Basler 2010). Trees in temperate and boreal climates are characterised by alternating growth and dormancy following a seasonal pattern, and higher spring and autumn temperatures are expected to extend growing seasons. In terms of spring phenology, these predictions match field observations across Europe (Menzel *et al*. 2012).

However, plants growing at high latitudes do not solely respond to temperature (Junttila 2007; Olsen 2010; Olsen & Lee 2011). This has been most evident for autumn phenology of coniferous and deciduous tree species exhibiting a free growth pattern, and was for decades thought to be largely attributed to the effects of shortening photoperiod past a certain critical day length (CDL) (Kramer 1936; Vaartaja 1954; Wareing 1956; Nitsch 1957; Weiser 1970). However, there is also evidence on modulating effects of temperature, particularly from more recent years (Hänninen & Tanino 2011; Tanino *et al.* 2010). In addition, light quality is also an important environmental signal for high-latitude populations of boreal tree species (Junttila & Kaurin 1985; Clapham *et al.* 1998; Tsegay *et al.* 2005; Mølmann *et al.* 2006; Opseth *et al.* 2015). As autumn phenology depends on interactions between photoperiod, temperature and the spectral composition of light, these interactions should also be taken into account when predicting warming effects.

Considering the available scientific literature, effects of autumn warming on tree phenology remain ambiguous, possibly due to several reasons. Firstly, northern and southern ecotypes may react differently to autumn warming (Dormling *et al*. 1968), and temperature effects on tree phenology have been found to interact with photoperiod (Søgaard *et al*. 2008; Tanino *et al*. 2010; Olsen *et al*. 2014). Secondly, the available studies often consider different aspects of autumn phenology, as this is a process consisting of several stages such as growth cessation, bud formation and development, dormancy induction and cold hardening. Even though these stages are interconnected, they involve different signalling pathways and sets of genes (Mølmann *et al.*, 2005; Ruttink *et al*. 2007). Thirdly, the majority of studies on autumn phenology in trees have been performed under indoor, controlled conditions with constant temperatures and artificial light. This implies that the relationships between temperature and autumn phenology should be further tested under natural conditions with fluctuating temperatures and gradually altering light conditions.

Some studies on autumn phenology have included moderate variation between day and night temperatures (Heide 2003; Junttila *et al.* 2003; Søgaard *et al.* 2008; Kalcsits *et al.* 2009; Tanino *et al.* 2010). A study of Norway spruce (*Picea abies*) showed that the effect of day temperature under short days on bud set and subsequent bud break was modified by night temperature in a complex way (Olsen *et al.* 2014). Based on available literature on climate effects on plant phenology, Hänninen & Tanino (2011) stated that elevated night temperature is generally considered to induce growth cessation. Even if these findings suggest that the variation between day and night temperatures affects autumn phenology, the controlled, stable conditions in which plants were kept indoors are fundamentally different from natural environments with fluctuating temperature conditions.

In a field study with poplar (*Populus* x spp.), Rohde *et al.* (2011) observed that temperature modulates the short day-induction of autumnal bud set, with warming lowering the CDL for the initiation of bud formation. We found that an enhancement of temperature (+1.3ºC) using infra-red heating lamps in the growing season, including autumn, delayed bud formation in the dioecious Eurasian aspen (*Populus tremula*), with males being more responsive to warming than females (Strømme *et al.* 2015). Different responsiveness to environmental change between sexes has been observed for several species belonging to the *Salicaceae* (Dawson & Bliss 1989; Nybakken *et al.* 2012; Zhao *et al.* 2012; Nybakken & Julkunen-Tiitto 2013). As global warming affects climatic regimes in complex manners, there is concern for sex-related differences in responses, such as shifting sex ratios in natural populations (Tognetti 2012). Since brief temperature drops to sub-zero levels are more common with increasing latitude and elevation, delayed bud set in males could thus involve lower protection in shoot tips against frost damage than for females.

In order to address the role of specific temperature parameters on autumn phenology and to test whether the sexes as well as transitions between different developmental stages are differently affected, we used data from field experiments with Eurasian aspen grown under different temperature regimes. In the modulated warming experiment reported in Strømme *et al.* (2015), warming was obtained with infrared heaters and the effect of temperature was tested using two levels (enhanced and control). In the study presented here, we combine data from the field experiment reported in Strømme *et al.* (2015) with data originating from two field experiments performed in Central Norway in 2013 and 2014. In these two latter studies, plant material of

the same origin as in Strømme *et al.* (2015) was planted along a natural elevational gradient, yielding different temperature regimes. In the three field experiments, we recorded temperatures for each temperature regime every ten minutes, allowing the extraction of different temperature parameters from the field recordings. The effect of these parameters was tested models as continuous terms in statistical models to predict observed apical stages during bud set in autumn. As temperature modulation of photoperiodic responses has been found in previous studies (Søgaard *et al*. 2008; Tanino *et al*. 2010; Olsen *et al*. 2014), we tested whether temperature effects on phenology in Eurasian aspen were best explained by maximum, minimum or average temperature during day and night or the entire 24 h cycle.

MATERIALS AND METHODS

Plant material

Plantlets used in the three field experiments originated from six female and six male aspens located in Southern and Eastern Finland. For a thorough description of sampling locations, micropropagation of individuals and growth conditions, see Strømme *et al*. (2015). Procedures for acclimation of potted plantlets differed between the experiments. In experiment 1 established in Joensuu (62°60′ N, 29°75′ E) in 2012, *in vitro* propagated plantlets were potted into 1 l pots filled with 70% non-fertilised peat and 30% vermiculite and kept in a greenhouse between 2 May and 7 June prior to planting in the field. In experiment 2 established in 2013, plantlets were potted on 4 June and planted in the field on 4 July, while in experiment 3 in 2014 plantlets were potted on 10 June and planted on 24 June.

In experiment 2 and 3, plantlets were potted using 70% non-fertilised peat and 30% vermiculite and kept in growth chambers for acclimation prior to planting in the field. Plantlets were kept under 230±10 µmol m^{-2} s^{-1} and a red:far red (R:FR) ratio of 1.6±0.1 provided by 400W Philips MASTER HPI-T Plus metal halide lamps (Royal Philips, Amsterdam, Holland) and incandescent light bulbs. Photosynthetic active radiation (PAR) was measured using a LI-250 Light Meter with an attached Quantum Sensor (LI-COR, Lincoln, Nebraska, USA), while R:FR ratio was measured using a Sky 100 radiometer with an attached 660/730 nm sensor (Skye Instruments, Llandrindod Wells, UK). The first days after potting, the plantlets were kept under a semi-translucent plastic sheet, which was gradually removed. This provided a gradual climatic shift in terms of irradiance and relative air humidity (RH). In the growth chambers, temperature and RH were 20ºC and 75%, respectively, and progressively lowered to 16ºC and

65% over seven days in 2013 and four days in 2014 to allow acclimation to lower temperature and RH.

Experimental set up

The field experiment was established in Joensuu, Eastern Finland (62°60′ N, 29°75′ E). This was a modulated enhancement experiment where temperature was increased to 1.35±0.042ºC of ambient levels using infrared heaters. There were six temperature enhancement and six control plots containing 60 female and male plantlets in each. The enhancement system was run between 1 June and 1 October 2012. For a full description of the setup for this experiment, see Strømme *et al*. (2015).

Experiments 2 and 3 were established in Fåvang, Central Norway (61°27' N, 10°11′ E) along the eastern side of the Gudbrandsdalen valley. Each location was a pasture selected with the aim of having three sites at different elevations along a natural gradient while keeping irradiance, precipitation and soil conditions as similar as possible. Selected sites were located at 237, 575 and 830 m a.s.l. Large herbivores were excluded from each location using a 2.5 m metal wire fence. In 2013, five plots per location were set-up by removing the uppermost 10 cm of soil, in addition to rocks in the exposed sublayer. The total removed material was replaced by 10 cm of FLORALUX peat compost (pre-limed and pre-fertilised) (Nittedal Torvindustri, Arneberg, Norway). In each plot, 40 plantlets consisting of six female and six male clones were planted in five rows containing 8 plantlets each. Minimum spacing between each plantlet and closest neighbouring plantlet was 20 cm.

At each site, one HOBO Micro Station data-logger (Onset Computer Corporation, Cape Cod, Massachusetts, U.S.A.) was installed together with a PAR Smart Sensor (Onset Computer Corporation) and a Temperature/RH Smart Sensor (Onset Computer Corporation).

In 2014, four new plots were established within the fenced locations established in 2013. As the number of plantlets per plot were reduced to 20, these were evenly distributed between three rows with the same spacing between plantlets as the previous year.

Recording of apical stages

In all three studies, we used the scoring system for autumn phenology described in Strømme *et al*. (2015), which is a simplified version of the scoring system developed by Rohde *et al.* (2011

b). The three-stage system used for scoring apices during autumnal bud formation discerns between three stages; growing apex with score 1, green bud having closed bud scales with score 0.5, and brown/red mature bud with score 0. In situations where green closed buds broke in autumn and apices resumed growth, apices were scored as growing. In all three experiments, some plantlets were affected by *Venturia* shoot blight, grazed upon by intruding herbivores or broken by mechanical damage and therefore not included in the apical scoring. Excluding these, the number of plantlets scored were 251 females and 241 males in 2012, 175 females and 205 males in 2013, and 85 females and 140 males in 2014.

Temperature data

In all three experiments, temperature was recorded every ten minutes. However, there are some gaps in the temperature data series for 2012 and 2013. The enhancement system was inactive between 13 and 18 September 2012. In addition, some data-loggers malfunctioned before 28 August and after 22 September 2013.

Recording of temperature yielded data series for enhanced temperature and control treatments in 2012, and for each of the three locations used in 2013 and 2014. For each year, temperature series were combined with daily local time points for sunrise and sunset, allowing separation of day and night temperatures. Following, we calculated mean, minimum and maximum temperatures for each interval between apical scoring dates. These calculations provided us with nine different temperature predictors for bud development; mean 24 hour, minimum 24 hour, maximum 24 hour, mean daytime, minimum daytime, maximum daytime, mean nighttime, minimum nighttime and maximum nighttime temperature. Since maximum temperatures on a 24-hour basis only occurred at daytime, we omitted maximum daytime temperature from the model selection process. This substitution was not done in relation to minimum temperatures, as minimum temperatures on a 24 hour basis also occurred during daytime.

Statistical analyses

All tests of effects on apical stages were performed using cumulative link mixed models (clmm) in R (R Core Team 2015) applying the clmm function in the Ordinal Package (Christensen 2013). Cumulative link mixed models are mixed models where the response variable is categorical, which is suitable for the categorical bud data collected in the field experiments. Temperature predictors were used to test their effect on overall bud development

(all three stages), appearance of closed green buds (transition from full growth to closed green bud) and bud maturation (transition from closed green bud to mature brown/red bud) while keeping the remaining model structure the same for all models. In the analyses, data from all three field experiments were pooled together. Considering that temperatures were modified differently between the warming experiment and the natural gradient experiments, namely by modulated increase (Joensuu) in 2012 and by elevation (Fåvang) in 2013 and 2014, the pooling of all data for these analyses can be done since temperature variables were used to predict the apical stages. These temperature variables varied between climatic regimes (determined by warming treatment or elevation) and between recording dates in each year. Futhermore, since there was a marginal difference in terms of latitude between experimental sites in Finland and Norway, differences in terms of photoperiod were assumed negligible. Analyses of closed green bud appearance and bud maturation were performed using subsets of the bud development data to test which temperature predictor could best explain transitions between different stages of bud development. In addition to a single temperature term, each model also included plantlet sex (two levels) and its interaction with temperature, and also plant clone (random term) and plot nested under experimental year (random term).

Models were compared and ranked based on AIC_C in R using the model.sel function in the MuMIn package (Barton 2015). Once the most parsimonious models for overall bud development, bud set and bud maturation were identified, they were further improved by removal of terms based on AIC.

RESULTS

Mean values for daily mean, maximum and minimum temperatures varied between years and experimental treatments (Table 1). Mean daytime irradiance was slightly lower at 275 m a.s.l. in 2013 as compared to higher elevations (Fig. 3). These differences were reduced in 2014, as some surrounding vegetation was cleared at 237 m a.s.l. in early 2014 (Fig. 3). As there are no available irradiance measurements from the enhancement experiment in 2012, we were unable to compare irradiance levels between this experiment and the elevational gradient experiments performed in 2013 and 2014.

Table 1. Means and 1SE of daily temperatures (ºC) for different treatments (in 2013) and elevations (as m a.s.l. in 2013 and 2014).

		Mean	**Min**	**Max**
2012	*Enhanced*	12.9±0.53	9.5±0.72	17.2±0.63
	Control	11.7±0.52	8.1±0.76	16.3±0.66
2013	*237 m*	11.1±0.54	6.5±0.62	17.5±0.81
	575 m	9.9±0.52	5.7±0.55	15.7±0.72
	830 m	8.4±0.53	4.0±0.57	14.2±0.72
2014	*237 m*	11.4± 0.35	6.1±0.54	18.8±0.65
	575 m	10.0±0.39	4.9±0.47	16.7±0.67
	830 m	8.8± 0.39	3.6±0.42	16.0±0.68

Overall bud development, bud set and bud maturation were best explained by daytime maximum, 24 hour mean and daytime mean temperature, respectively (Table 2). Overall bud development includes all three bud development stages recorded in the field studies, covering the transitions from a growing apex to a mature brown/red bud. Throughout late summer and autumn, plantlets grown in warm conditions delayed bud development ($P<0.001$) (Table 3, Fig. 1). Compared to the enhancement experiment in 2012, the elevational gradient experiments in 2013 and 2014 yielded higher differences between treatments in terms of temperature levels and bud development (Fig. 1, Fig. 2). Considering the elevational gradient experiments, bud development varied substantially between 2013 and 2014, as plantlets in 2014 had a delayed bud development compared to the year before (Fig. 1). These differences are clearly visible around day 265 (22 September), where most plantlets at 575 and 830 m a.s.l. had formed mature buds in 2013, while the corresponding average bud stage for 2014 was reached around day 280 (7 October). In 2013 and 2014, bud development was delayed at all elevations compared with the modulated warming experiment in 2012. Furthermore, several plantlets that had developed closed green buds in 2014 resumed growth through the emergence of new leaves from the buds. As a result, the apical stage scores increased at day 259 (16 September) and 266 (23 September in 2014 (Fig. 1).

Table 2: Model selection table for bud formation explained by eight different temperature variables using Cumulative Link Mixed Models (CLMMs). The remaining fixed (Experiment, Sex) and random terms (Clone, Plot) were included in all models. Models were ranked lowest to highest based on Akaike information criterion corrected for finite sample sizes (AICc).

Candidate model	Temperature variable	ΔAICc	AICc
Bud formation	Daytime maximum	0.00	11444.7
	Daytime mean	6.20	11450.9
	24 hour mean	174.96	11619.7
	Nighttime minimum	576.55	12021.3
	24 hour minimum	657.66	12102.4
	Daytime minimum	659.60	12104.3
	Nighttime mean	927.21	12371.9
	Nighttime maximum	1317.84	12762.6
Closed bud scales	24 hour mean	0.00	4886.9
	24 hour maximum	1.94	4888.9
	Daytime mean	4.63	4891.6
	Nighttime mean	92.95	4979.9
	Nighttime minimum	131.53	5018.5
	24 hour minimum	144.67	5031.6
	Daytime minimum	150.35	5037.3
	Nighttime maximum	207.45	5094.4
Bud maturation	Daytime mean	0.00	5482.2
	Daytime maximum	32.60	5514.8
	24 hour mean	148.39	5630.6
	Nighttime minimum	276.92	5759.1
	Daytime minimum	310.69	5792.9
	24 hour minimum	322.59	5804.8
	Nighttime mean	606.11	6088.3
	Nighttime maximum	762.54	6244.7

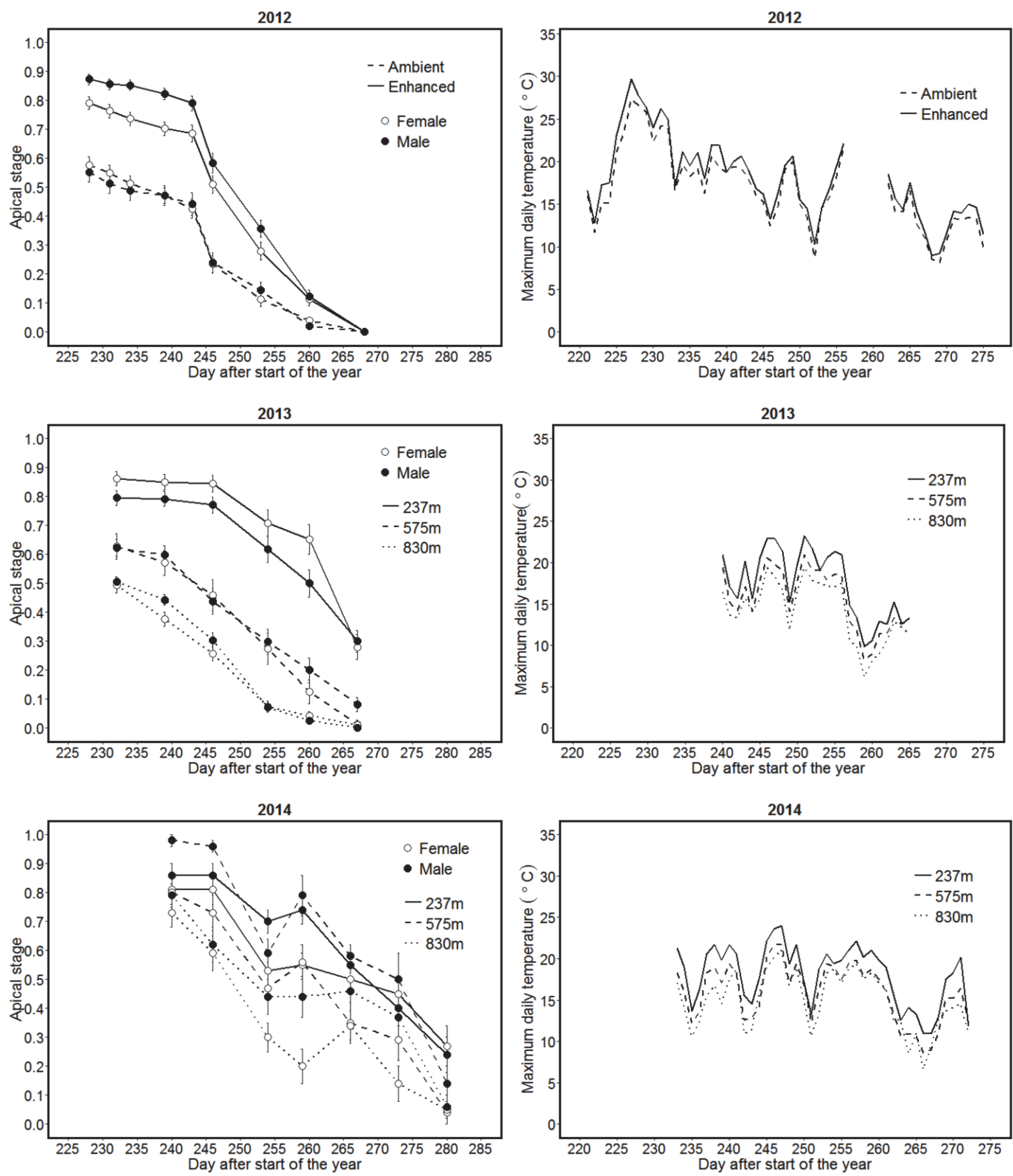


Figure 1. Average bud scores ± 1SEM under different temperature regimes for 2012 (251 females, 241 males, upper left panel), 2013 (175 females, 205 males, middle left panel) and 2014 (85 females, 140 males, lower left panel). Upper right, middle right and lower right panels show maximum daily autumn temperatures for 2012, 2013 and 2014, respectively.

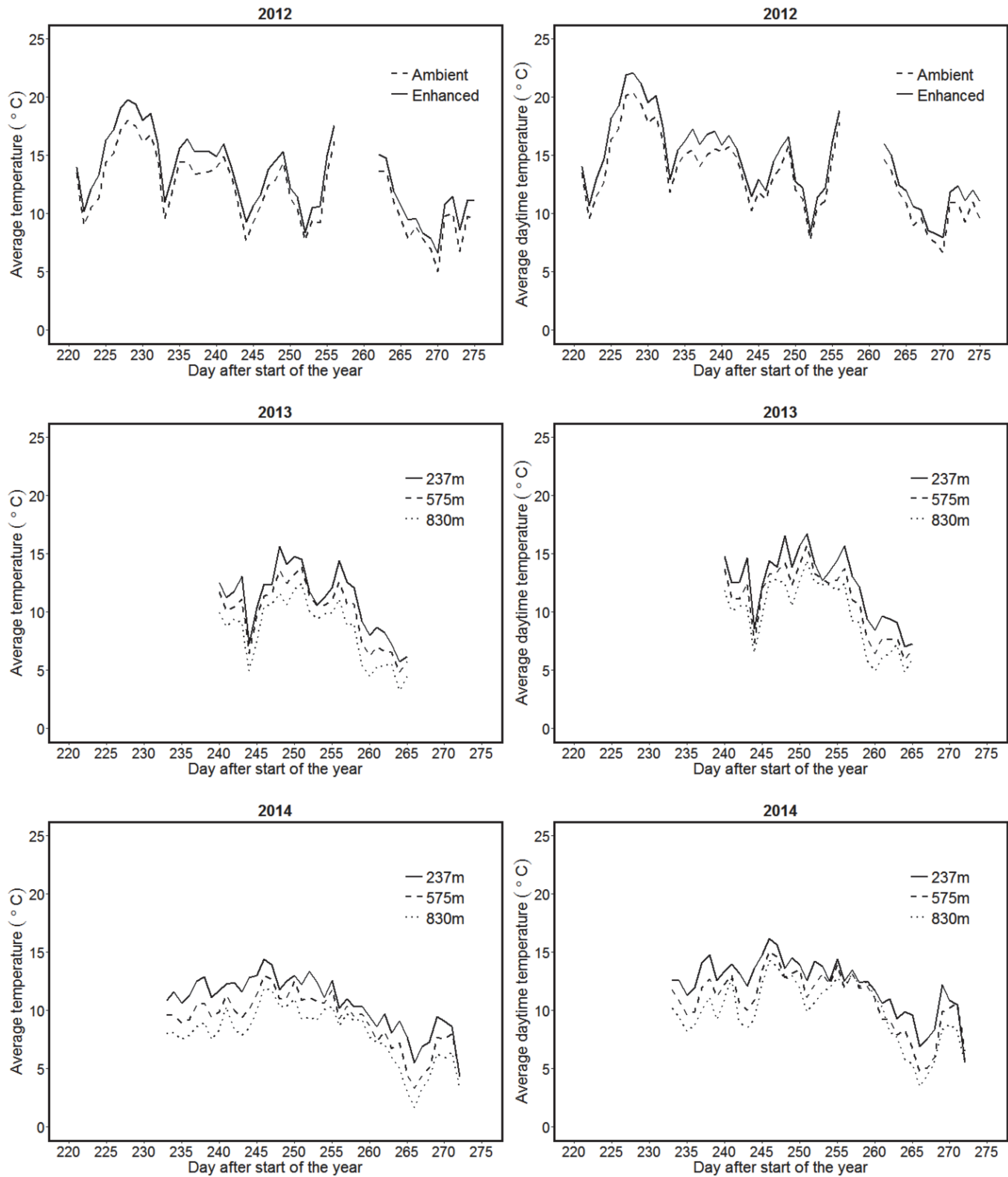


Figure 2. Mean 24 hour (left) and mean daytime (right) temperatures during autumn for 2012 (upper panels), 2013 (middle panels) and 2014 (lower panels).

Table 3: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of maximum daytime temperature on overall bud formation in females and males of *P. tremula*.

Fixed effect terms	Coefficient	SE	z
$T_{daytime\ max}$***	-0.31	0.01	35.18

Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

When testing the effects on the appearance of closed green buds, which involved the first and second bud development stage, temperature had a delaying effect which was significantly stronger in males compared to females (P=0.004) (Table 4).

Table 4: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of 24 hour mean temperature and sex on closed green bud set in females and males of *P. tremula*.

Fixed effect terms	Coefficient	SE	z
$T_{24\ hour\ mean}$***	-0.24	0.02	-10.33
Male	0.58	0.64	0.90
$T_{24\ hour\ mean}$ x Male**	-0.08	0.03	-2.92

Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

Testing the effects on bud maturation considered the second and third bud development stage. Mean daytime temperature delayed bud maturation, but this effect was significantly lower for males (P<0.001), indicating more rapid bud maturation in males than females (Table 5).

Table 5: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of 24 hour mean temperature and sex on bud maturation in females and males of *P. tremula*.

Fixed effect terms	Coefficient	SE	z
$T_{daytime\ mean}$***	-0.51	0.02	-21.02
Male**	-1.78	0.54	-3.29
$T_{daytime\ mean}$ x Male***	0.14	0.29	4.91

Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

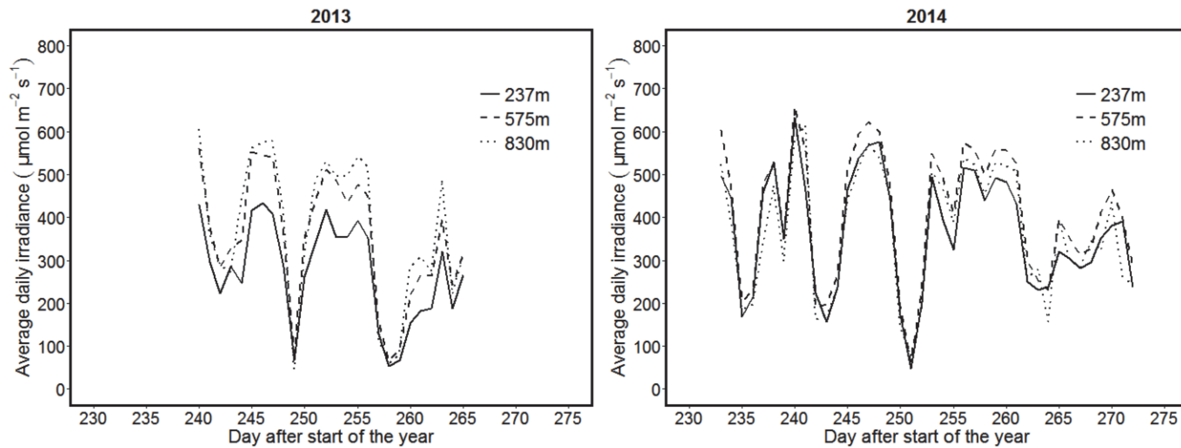


Figure 3. Mean daytime irradiance measured at each elevation during autumn of 2013 (left) and 2014 (right).

DISCUSSION

In young individuals of most high latitude tree species, autumn bud phenology in response to photoperiod and light quality is modulated by temperature (Olsen 2010; Tanino et al. 2010; Olsen & Lee 2011). Our data from field experiments, where temperatures fluctuate, show that maximum day temperature is the temperature variable best explaining overall bud formation in Eurasian aspen (Table 2 & Table 3). This is in contrast to a review by Hänninen and Tanino (2011), where high night temperature was suggested to be the most important temperature variable affecting growth cessation, based on findings from several studies under controlled conditions. Interestingly, we found that maximum night temperature was the least parsimonious temperature parameter in the statistical tests (Table 2). In some of the studies referred to, bud development was recorded (Van der Veen 1951; Heide 1974; Downs & Bevington 1981; Mølmann *et al.* 2005; Granhus *et al.* 2005; Olsen *et al*. 2014), while other studies considered growth cessation by measuring stem height (Håbjørg 1972; Dormling 1989; Kalcsits *et al*. 2009; Tanino *et al*. 2010). Since there is separation in time between growth cessation and appearance of a terminal vegetative bud, and since the duration of the different processes may be affected by environmental conditions (as further discussed below), comparison of different studies recording different aspects of autumn phenology are not necessarily straight-forward. We found that increased temperature had a delaying effect on bud formation, given the negative coefficients for temperature in the cumulative link mixed models (Tables 3, 4 and 5). A previous study has also shown that autumn warming delays bud formation in poplar, an effect that was attributed to temperature modulation of the actual CDL

for bud formation (Rohde *et al*. 2011a). Correlation coefficients for day length and temperature data from sites in 2014 were 0.72 at 237 m a.s.l., 0.64 at 575 m a.s.l. and 0.57 at 830 m a.s.l. Due to this collinearity, day length was not included in our models, which is in line with recommendations on dealing with collinearity (Zuur *et al.* (2014).

Overall bud formation for all three apical stages was best explained by maximum day temperature, while green bud appearance and bud maturation were best explained by mean 24 hour and mean day temperature, respectively (Table 4 & Table 5). As temperatures in natural conditions fluctuate, the effect of 24 h mean temperature on the appearance of green buds could be explained by a combination of high and low temperature effects. It has been shown that low night temperature induces bud set in hybrid aspen under controlled conditions (Mølmann *et al.* 2005). On the other hand, high temperature may delay this process through modulation of CDL sensing (Rohde *et al.* 2011a). The effect of average daytime temperature on bud maturation could also be related to opposing effects of high and low temperature. Still, when considering overall bud development, the process was most delayed in 2014 at 237 m a.s.l., where maximum daytime temperature was higher than any treatment in 2012 and 2013, but minimum temperature was lower than for both treatments in 2012, which was the year when plantlets formed buds the earliest in the entire study. Therefore, our data suggest that maximum temperature has a stronger effect on bud formation that low temperature.

Male plants responded more strongly to experimentally increased autumn temperatures than females (Strømme *et al*. 2015). When combining data from the elevational gradient in 2013 and 2014, we did not find a significant interaction between temperature and plant sex predicting overall bud development. However, when performing separate analyses of closed bud scale appearance and bud maturation, both models include significant interactions between temperature and plant sex. On the one hand, the appearance of closed green buds was significantly more delayed in male compared to female plants (Table 4). On the other hand, while considering bud maturation, male plants were significantly less delayed than females (Table 5). This may explain why the sexes did not respond differently to temperature in terms of overall bud formation. Furthermore, the higher responsiveness of males to temperature in 2012 described in Strømme *et al*. (2015) may thus have been a result of a strong delay in the appearance of green closed buds, yielding an overall delay for males. In the scoring system used in Strømme *et al*. (2015) and this study, the first bud stage (score 1) is a merging of five stages described by Rohde *et al.* (2011b). These five stages precede the appearance of closed

bud scales, and one of these stages is visible after the plant has sensed the CDL. It is possible that the stronger delaying effect on formation of green, closed buds in males reflects a higher susceptibility of male plants to temperature modulation of CDL sensing, as this effect was only found for the transition between the first and second stage used in this study.

In the three field experiments, bud formation, expressed as bud scores, was delayed throughout the autumn for elevated temperature treatments (Fig. 1). However, abrupt transitions in bud scores after day 244 (1 September) in all three years indicate that CDL had been sensed by the plants. The difference in latitude between Joensuu and Fåvang involves a seven-minute difference in daylength on 28 August. As both locations have similar photoperiodic conditions, the observed differences in bud set between years could largely be attributed to temperature fluctuations. Compared with the two previous years, mean 24 hour and day temperatures were somewhat higher in 2014 (Fig. 2). Indeed, bud formation was more delayed this year than in 2012 and 2013, particularly at 237 and 575 m a.s.l.

We found that temperature delays bud development in both sexes, and our findings are in line with a field study of poplar (Rohde et al. 2011). Opposite to our findings, studies under controlled conditions have found that bud development under short days is accelerated by high night temperature (Van der Veen 1951; Mølmann *et al.* 2005) and high day temperature (Granhus *et al.* 2005). This discrepancy may be related to the conditions in which temperature-mediated modulation of CDL-sensing occurs. Under constant short days in controlled conditions, high temperatures may increase the rate of bud formation induced by day-length, as temperature does for other growth-related processes. It is thus possible that temperature modulation of CDL-sensing occurs under a set of natural climatic conditions which are difficult to simulate in controlled environments. It is also possible that constant day lengths in some experimental conditions are substantially shorter than the CDL for a given species and genotype, and the short-day signal would override any possible modulating effects of temperature on CDL-sensing. Furthermore, artificial climatic regimes commonly include abrupt shifts in temperature and a light climate different from sunlight, while our field experiments were performed under fluctuating temperatures and gradual shifts in duration and spectral composition of sunlight.

As the formation of apical buds is a means of protecting the apical meristem and leaf primordia against harsh environmental conditions, delayed bud formation during autumn results in an

extended growth period, and thus, increased risk of frost damage. This could occur in warm autumns, where bud development has been delayed by high temperatures, resulting in apical meristems being more prone to frost damage. Indeed, rapid shifts to subzero temperatures in autumn are quite common at high latitudes, as recorded on 1 September 2013 (day 244) (Supporting Information Fig. S1). For some species of *Populus*, males tend to be more growth-oriented than females (Lloyd & Webb 1977). The higher probablility of *P. tremula* males to delay phenology with warming may favour aboveground branching, and thus increased light interception in following years. Even though males appear to compensate for this delay by faster bud maturation, this may involve higher susceptibility to frost damage. Bud maturation is associated with starch accumulation in the buds, whose conversion to sugars increase freezing tolerance (Rinne *et al*. 1994; Lipavská *et al*. 2000). As longer maturation periods may yield higher starch content in the buds, a shorter maturation period in males could potentially involve lower freezing tolerance than in females. During the elevational gradient experiments, we also observed that buds formed at 237 m asl. were generally larger that those at higher altitudes. This may be related to the increased number of leaf primordia during bud maturation under elevated temperature, as reported e.g. for Norway spruce (Olsen *et al.* 2014).

In conclusion, our findings show that autumnal bud set is significantly delayed with increasing temperatures, and that this is best explained by maximum day temperature. It is uncertain whether plantlets would have been similarly affected in consecutive years, as warming effects may influence phenological shifts in subsequent seasons (Hänninen & Tanino 2011). Also, the timing of phenology in juvenile plantlets may differ substantially from adult trees (Augspurger & Bartlett 2003). Even so, our study of autumn tree phenology differs from a range of previous studies in being performed outdoor under natural climate fluctuations. Our data indicate that the stages of bud development are affected differently by temperature in a manner that reduces the delay in bud formation for males. On the one hand, the appearance of closed green buds was significantly more delayed by temperature in males than females, while on the other hand, male buds also matured faster once closed bud scales appeared. Whether such plasticity of phenological transitions do reflect different growth and survival strategies under fluctuating climates remains unclear, and would require further studies along a wider range of environments. There is concern for the possible consequences of sex-related differences in climate change responses (Tognetti 2012), and further research on this topic could reveal which growth strategies are favoured under past, present and future climate change.

AUTHOR CONTRIBUTIONS

L.N., R.J.T., C.B.S. and J.E.O. planned and designed the research. R.J.T. and C.B.S. performed experiments. C.B.S. conducted fieldwork and analysed data. L.N., R.J.T., C.B.S. and J.E.O. interpreted results and wrote the manuscript.

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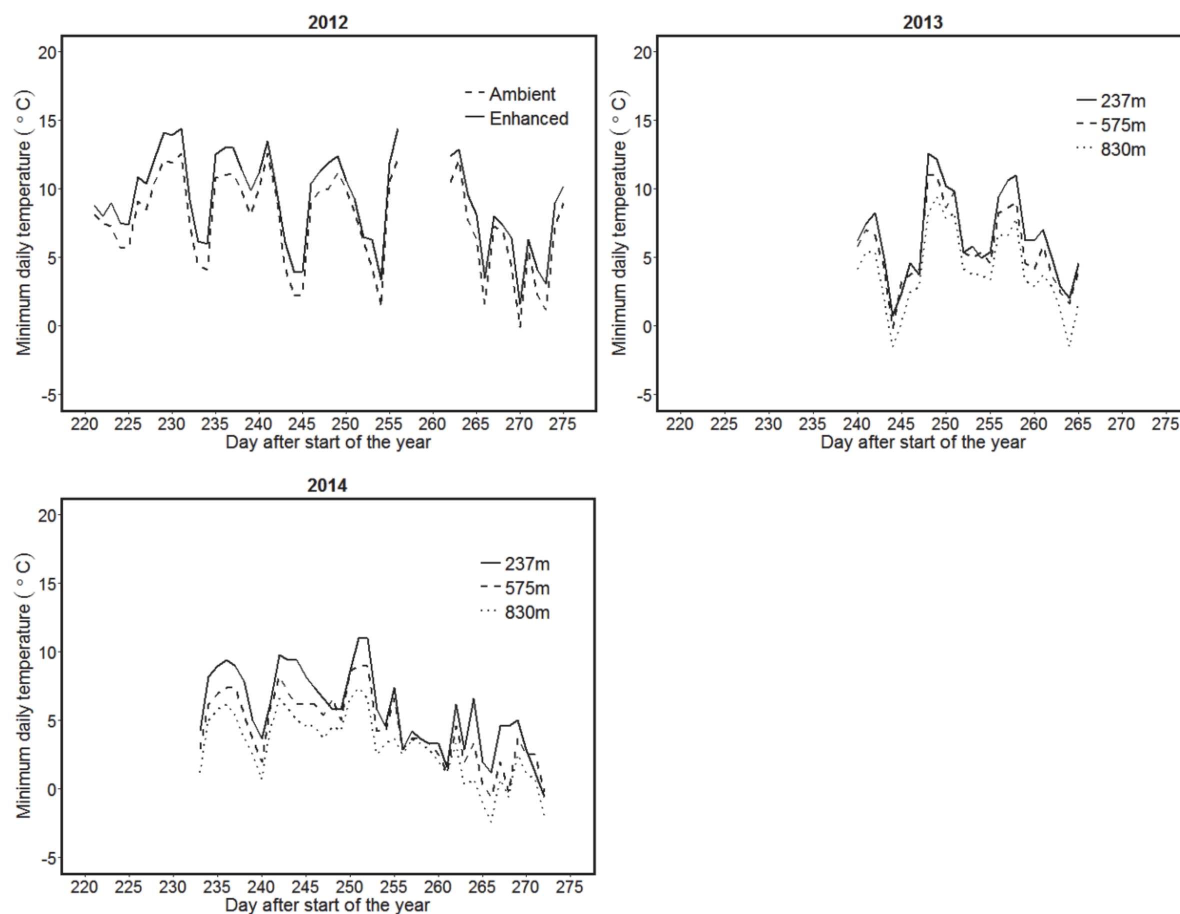
SUPPORTING INFORMATION



Supporting Information Figure S1. Mean 24 hour minimum temperatures during autumn for 2012 (upper left panel), 2013 (upper right panel) and 2014 (lower panel).

PAPER IV

Climatic effects on bud break and frost tolerance in Europe's northernmost populations of beech (*Fagus sylvatica*)

Elisabeth Schmidt[1], *Christian B. Strømme*[1], *Jorunn E. Olsen*[2], *Line Nybakken*[1]

[1] *Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

[2] *Department of Plant Sciences, Norwegian University of Life Sciences, P.O.-Box 5003, 1432 Ås, Norway*

Corresponding author: Christian Bianchi Strømme

Email: christian.stromme@nmbu.no

ABSTRACT

European beech (*Fagus sylvatica*) is among the dominant tree species in Europe, and range shifts have been observed in parts of its northernmost distribution. Despite extensive research conducted on climate effects on spring phenology, predictions of future bud break timing remains an unresolved issue due to insufficient understanding of climate effects. As warming has been shown to yield advanced bud break at high elevation populations, we investigated the effects of temperature on bud break and frost tolerance of European beech at its northernmost distribution range, involving different populations in Norway. We tested the effect of temperature and day length on bud break in controlled conditions and tested bud frost tolerance, using live material collected from wild populations. Furthermore, we observed bud break during spring in field conditions. Bud break in controlled conditions was positively affected by warming, and in the field we also observed advanced bud break in warmer sites. In addition, bud break and bud frost tolerance differed in late winter between some populations, both with sampling date and under different temperatures. The observed differences in frost tolerance between populations match differences in bud break under controlled conditions, suggesting similar effects of temperature. Our data indicate that bud break dates of European beech at its northern distribution limit are limited by temperature, similar to observations of populations at high elevation in Central Europe. This implies that warming will advance bud break at the species' northernmost distribution range, with increased risk of spring frost damage in shoot tips.

INTRODUCTION

The deciduous European beech (*Fagus sylvatica*) is among the most common tree species in Europe, being able to grow and reproduce across a wide latitudinal range from Northern Sicily to Southern Norway, as well as over different elevations (Bolte *et al.* 2007). Present populations of beech in the European mainland are concentrated in Central Europe, likely following post-glacial colonisation originating from a refugium in the Carpathians (Demesure *et al.* 1996). Thereby, European beech has over a 9000 years long period been able to colonise a wide range of geographic areas with ample climatic differences. The phenology of European beech has thus been the subject of particular interest, particularly in relation to spring bud break and the environmental factors behind this process (Vitasse & Basler 2013). Earlier spring phenology has been observed for several plant species across Europe (Menzel *et al.* 2006), and for European beech warming has been shown to promote bud break at high elevation (Vitasse & Basler 2013). As this effect has been related to the limiting effects of temperature in cold environments, it is also relevant to investigate how warming affects bud break at the species' northernmost distribution range in Norway.

A simulation study of European beech range shifts under climate change predicts further population increase in Southern Scandinavia (Saltré *et al.* 2014), and observed range expansion have been related to a high degree of phenotypic plasticity (Bolte *et al.* 2007). European beech is spreading northwards through unmanaged forests in Southern Sweden, and analyses of pollen and charcoal suggest that European beech has not yet fully reached its northern postglacial distribution limit (Björkman & Bradshaw 1996; Bradshaw & Lindbladh 2005). These studies also indicate that anthropogenic disturbance such as burning and clearing of forested areas, or cultivation of more competitive tree species, has influenced the post-glacial distribution of beech in Southern Sweden (Lindbladh *et al.*, 2000; Bradshaw & Lindbladh 2005). The species' northernmost distribution range is found in western and southeastern parts of Norway. These populations probably result from human introduction of seeds from Denmark between 500-1000 AD (Myking *et al.* 2011) and studies suggest that also the spread of these populations was favoured by similar anthropogenic disturbances as for Southern Sweden (Bjune *et al.* 2012).

The present understanding of climatic constraints on European beech relies on studies of marginal populations in North-Eastern Europe and of those at higher elevation in Central Europe. Available literature suggest that European beech favours maritime climates with

moist summers and mild winters, and is considered limited by severe heat, drought or severe frost (below -35°C) (Bolte *et al.* 2007). The latter two represent typical limitations to tree survival in cold climates. Long-term evolutionary adaptations to cold conditions ensure survival through the coldest months of the year while retaining the ability to grow and reproduce in the growing season. From the subtropics to the subarctic, tree species grow and reproduce in environments characterised by periods of adverse temperatures, thus facing a trade-off between a longest possible growing season and the avoidance of plant tissue damage during frost events (Kramer *et al.* 2010; Caffarra & Donnelly 2011). This has yielded phenological shifts at the beginning and end of the growing season. In autumn, shorter day length, lower temperature and changes in the solar spectral composition are sensed by photoreceptors in the plant, yielding a gradual induction of frost tolerance and formation of terminal buds at the shoot tips (Olsen *et al.* 1997; Olsen 2010; Tanino *et al.* 2010). These same signals make cells in the meristems dormant, which remain so until environmental conditions are favourable for growth in spring (Rinne *et al.* 2001; Welling & Palva 2006).

Avoidance of frost damage in the buds ensures survival of the enclosed leaf primordia until bud break the subsequent spring, involving physiological adaptations that make the buds able to either avoid or survive freezing (Welling & Palva 2006; Gusta & Wisniewski 2012). These mechanisms vary between different plant parts and species, and may involve osmotic regulation, production of substances that prevents ice formation and changes of cell structural composition. Acclimation to freezing is a process enabling survival at very low temperatures when not disturbed by high temperatures during the cold hardening period (Welling & Palva 2006; Olsen 2010). In With increasing autumn temperatures, full chilling is assumed to occur less often also in beech in southern areas, as a consequence of a very high chilling requirement in beech compared to other deciduous species (Vitasse & Basler 2013). Research from southern Europe indicates that the chilling requirement of beech at low elevation is only partially obtained most years (Vitasse et al. 2009). Although insufficient chilling delays bud break, photoperiod is likely to counterbalance this effect and thus yield more conserved bud break dates (Vitasse *et al.* 2013). However, warming is expected to yield advanced bud break in the cold boundary of its distribution range, where bud break in spring is limited by temperature (Vitasse *et al.* 2013). Thus, warming in these habitats may yield increased risk of frost damage.

Indeed, the time around bud break when new leaves are developing is the most frost sensitive period of the plant's growth cycle (Vitasse et al. 2014). Deciduous plant species have thus evolved traits that influence the timing of bud break in relation to the species' specific frost tolerance, and are from this characterized as early- or late-flushing species. The former group is generally opportunist/pioneer tree species that are able to start vigorous growth early in spring if temperatures are high, the latter group usually long-lived, late-successional species with a more conservative regulation of bud break (Caffarra & Donnelly 2011). European beech is considered a late-flushing species in most of its distribution range, and displays low variation in bud break dates between years compared to other broad-leaved tree species (Vitasse & Basler 2013). The explanation for this is a complex interplay of environmental factors, influencing and controlling the timing of the dormant state of the buds, but is not yet fully understood (Vitasse & Basler 2013). In Central and Southern Europe, European beech displays very low variation in bud break dates over the last three decades, despite increased spring temperatures (Vitasse *et al.* 2009; Vitasse & Basler 2013). For trees at high elevation, where the chilling degree is believed to be higher, a trend of earlier bud break time has been observed as compared to trees at lower elevation (Cufar *et al.* 2012; Vitasse & Basler 2013). Although experimental data are not available for Northern Europe, the effects of increased spring temperatures are assumed to be similar to those for high elevations, as the chilling requirement of the buds during winter is considered sufficient (Vitasse & Basler 2013). If so, larger variation in bud break dates between years can then be expected in Northern Europe, depending on the yearly variation in winter- and spring temperatures.

Indeed, a general trend of earlier bud break time for deciduous tree species in Norway, including foreign provenances of beech, has been observed over the last 40 years (Nordli *et al.* 2008). Phenological garden observations in western Norway the last four decades show that a German beech provenance (*Fagus sylvatica* 'Hartigii') on average reaches bud break one to four days earlier, with average bud break dates within one week before or after 11 May. For natural beech populations in Southern Norway, no similar trend analysis of bud break dates are known. In Ås, south-eastern Norway, Heide (1993) registered bud break dates of beech during spring in 1990 and 1991 and reported it to occur between May 5 and 8, following both a winter with mild and normal average temperatures (the period January to March 1990 was reported to be 6.5°C above the 30-year normal). It was stated that between years, bud break dates varied only with a few days, also between years with high and low average temperatures.

Predictions of future range shifts of European beech require a thorough understanding of climate effects on phenological processes, as the total effect on tree growth and fitness might be altered both positively and negatively by warming (Körner & Basler 2010). In this study, we investigated how cold hardening and bud break were affected by temperature and day length for a set of the northernmost European beech populations in Norway, both under natural and controlled conditions. In addition, we employed controlled freeze-tests to investigate frost tolerance in terminal vegetative buds. These populations at the rim of the species distribution have never been subject to any physiological or phenological studies, and controlled studies of bud break and frost tolerance in beech are scarce.

MATERIALS AND METHODS

Plant materials

Samples used in the dormancy release experiments and the frost tolerance experiments consisted of shoots collected in January, February, March, October, November and December 2014 from adult trees in six different beech populations in Norway (Table 1, Figure 1). Four of these populations are present in the region of Vestfold, where beech has its most extensive distribution in Norway. In addition, we included materials from a beech population in Ås (region of Akershus), Eastern Norway, which is most likely a result of recent introduction. We also included materials sampled from the world's northernmost beech population (Hultén 1971), located in Seim (region of Hordaland) on the western coast. This population most likely originates from human introduction prior to or during the Viking Age (Myking et al. 2011; Bjune *et al.* 2012). Shoots were sampled from south-facing branches removed with a pole pruner at heights up to 7 m. Shoots on the outermost 10 cm of the branches were used for the frost tolerance experiments, whereas shoots used in the growth chamber experiments were attached to the next 10 cm section after the first cut.

Dormancy release experiment

The experimental setup was similar to a previous study conducted in Ås during the winter seasons of 1989/90 and 1990/91 (Heide 1993), which employed samples from the Ås population included in our study. Replicate twigs (3 samples from each tree, in total 9 samples from each location) were subjected to either short day (SD) photoperiod of 8 hour light or long day (LD) photoperiod of 24 hour light, in combination with a testing temperature of 9, 15 or 21°C. The growth chambers provided no natural daylight, all having

400 W high pressure sodium (HPS) bulbs. In addition, transparent 60 W incandescent light bulbs (Osram Licht AG, Munich, Germany) were used for day length extension in all LD chambers. The SD program consisted of 8 hours of 200 µmol m^{-2} s^{-1} provided by the HPS bulbs, followed by 16 hour darkness. The LD program consisted of 24 h continuous light, first 8 h with irradiance 200 µmol m^{-2} s^{-1} provided by the HPS bulbs, followed by 16 h of 5 µmol photons m^{-2} s^{-1} provided by the incandescent bulbs only. The difference in total daily irradiance between the SD and the LD treatments were considered negligible. Combining the two different day length treatments with the three different temperature levels (9, 15 or 21°C) yielded six different treatment combinations for each sampling month. The only exceptions were January and February 2014, where the only temperature level used was 21°C. Relative humidity (RH) was kept at 65% in all experiments.

Figure 1. Map showing the locations of the European beech populations included in the study (S = Seim, Å = Ås, H = Holmestrand, F = Falkensten, M = Melsomvik, T = Tjølling).

Table 1. Geographical coordinates for the locations of the European beech populations used in the field study (all Vestfold populations) and the dormancy release- and frost tolerance experiments (all populations).

Population	**Region**	**Coordinates**
Falkensten	Vestfold	59°25'25.3"N, 10°26'11.4"E
Holmestrand	Vestfold	59°31'43.2"N, 10°11'56.7"E
Melsom	Vestfold	59°13'15.0"N, 10°21'10.4"E
Tjølling	Vestfold	59°3'45.1"N, 10°6'28.3"E
Seim	Hordaland	60°38'17.0"N, 5°12'50.9"E
Ås	Akershus	59°41'35.3"N, 10°44'53.6"E

The state of the apical buds on sampled shoots was recorded every third day using a categorical scale of 5 distinguishable bud stages (Murray et al. 1989): dormant (0), swelling started (1), fully swelled (2), green foliage visible (3), leaf fully developed (4). Sampled shoots were kept in experimental conditions for approximately 35 days. As samples from subsequent months had approximately a week overlap in the growth chambers, samples were removed from the chambers after they reached bud development stage 4.

Field observations

For the observation of spring bud break in natural environments, we established a total of 15 plots containing naturally established European beech saplings (1-2 m in height) within four different beech populations in Vestfold (approx. 20 km apart). In each location, plots were established either in semi-open stands almost entirely constituted of beech or in stands where beech and Norway spruce coexisted. All plots were 3x3 m, facing south or southwest, and had a density of beech saplings more than 1 m^{-2} (see Table 2 for details).

The development of the apical buds was recorded in spring 2014 (four dates) and spring 2015 (three dates). The scoring system used did not include bud swelling as the one used for the dormancy release experiment, but was based on Fu *et al.* (2012) and has a larger emphasis on leaf unfolding. The scoring system used for field observations had four levels: closed bud (0); closed bud with visible green leaf (1); green leaf diverging from bud axis but no visible petiole (2); broken bud with at least one visible petiole (3).

Local temperature measurements were conducted in each plot type on the Vestfold locations, using Tinytag Plus 2 temperature loggers (Intab, Stenkullen, Sweden). The loggers were placed on ground level in the centre of the plots, and recorded the temperature with 10-minute intervals in the period from April 3 to Sept 19. In addition, canopy density for each plot was estimated, taking 180° hemispherical (fisheye) canopy photographs, using a Pentax K-5II D-SLR camera (Pentax, Tokyo, Japan) with Sigma 4.5mm F2.8 EX DC HSM Circular Fisheye lens (projection distortion provided by the camera lens manufacturer). The photographs were taken from the centre of each plot at the time of minimum canopy foliage (25 April), for comparison of light conditions in the plots at the time around bud break. The photos were analyzed using Gap Light Analyzer version 2.0 (Frazer *et al.* 1999), relating each plot to its geographical position and tracking the specific solar trajectory. Using the images of canopy cover, the total amount of solar radiation (direct and diffuse) received by each plot was estimated.

Frost tolerance experiment

Shoots for the frost tolerance experiment were collected together with the samples for the dormancy release experiment. For each sampling month, 16 shoots were tested for frost tolerance during the dormancy period, each containing two to three vegetative buds. The shoots were arranged and tied in bundles and covered with moist quartz sand in open aluminium boxes (Olsen *et al.* 1997; Mølmann *et al.* 2005). Samples were split between seven different freezing temperatures treatments (-5, -10, -15, -20, -25, -35 and -45 °C) and a control treatment of 5°C. Treatments were provided by temperature test chambers (Weiss Umwelttechnik simulationsanlagen, custom made), performed in the following stepwise cooling/decooling program which differed only in target temperatures and amount of cooling/decooling:

1) 8 h at -0.5°C
2) Temperature lowered by 3°C/h until -17°C (or higher target temperature)
3) Temperature lowered by 10°C/h until -45°C (or higher target temperature)
4) Target testing temperature for 4 h
5) Temperature increased by 2°C/h until 5°C
6) Temperature maintained at 5°C for 2-3 h.

With the completion of temperature treatments, shoots were placed in moistened transparent boxes placed in natural daylight and room temperature for 5 days. This allowed the development of visible freezing damage in the buds. The relative amount of dead plant tissue in the buds was examined visually using a categorical scale of four distinguishable levels of frost tolerance (Olsen *et al.* 1997): bud completely dead (0); >50% of internal bud tissue dead (1); <50% of internal bud tissue dead (2); no visible bud damage (3).

Statistical analyses

Effects on bud development, both in the dormancy release experiment and for field observations, were tested in R (R Core Team 2014) using cumulative link mixed models (clmm) in the Ordinal package (Christensen 2015). This enabled us to use the described categories for bud break as a categorical response variable. In the dormancy release experiment, treatments performed in January and February only involved a temperature of 21 °C, and were thus combined with data from the 21°C treatment in March in order to compare the effects of sampling date under similar temperatures. Independent terms included sampling date (continuous), bud observation date (continuous), sampling location (fixed, six levels, using "Tjølling", which is the southernmost location, as dummy variable) and day length (two levels, using "short day" as dummy variable). Sample tree identity was included as a random term.

A separate model was used for testing the effect of different forcing temperatures (continuous) on buds sampled in March, while bud observation date, sampling location, day length and sample tree identity were included in the same manner as for data originating from January and February. In addition, data from October, November and December were analysed in a similar manner as the January-March data, with the exception of an added temperature term (continuous). Field observation data from 2014 and 2015 were tested using day of year (continuous), location (fixed, six levels, using "Tjølling" as dummy variable), stand type (fixed, two levels), and plot (random term).

Effects in the frost tolerance experiment were tested in two different models, one including data from January-March 2014 and another including October-December 2014. As the categories describing bud intactness represent intervals between 0 and 100% bud damage, this response was set as a continuous variable. In this respect, effects were tested using lmer in the lme4 package (Bates *et al.* 2015) in R. Independent variables included in the models

were sampling date (continuous), target temperature as Kelvin (K) for the test chamber program (continuous) and location (fixed, six levels, using "Tjølling" as dummy variable). As sampling tree identity (random term) was non-significant for both periods of the frost tolerance experiment, it was omitted from the final models, which were performed using generalised least squares (gls) in the nlme package (Pinheiro *et al.* 2015) in R.

RESULTS

Bud break in controlled conditions

In the bud break experiments, shoots sampled in January, February and March showed leaf emergence on average after 19, 14 and 9 days, respectively, under LD (Fig. 2). There was a significant effect of day length (P<0.001) (Table 2), as most buds did not even show swelling under SD (Fig. 2). Shoots sampled at Falkensten showed a significant increase in bud break with increased sampling date compared to the southernmost location at Tjølling (P<0.001). In addition, buds broke more slowly for shoots sampled at Holmestrand (P=0.033), Melsomvik (P<0.001) and Ås (P=0.002). While shoots from Tjølling harvested in January had emerging leaves (apical stage 3) on average after 19 days at 21 °C, only the shoots from Holmestrand reached a comparable level of bud break that month, which occurred one week later (Fig. 2). Shoots from Tjølling collected in March had emerging leaves on average after 11 days at 21°C, while corresponding extent of bud break was reached 12 days later for shoots from Holmestrad, Melsomvik and Ås.

When comparing different forcing temperatures using shoots sampled in March 2014, buds from Tjølling had emerging leaves after 9, 16 and 33 days at 21, 15 and 9°C, respectively, under 24 hour light (Table 3). Shoots kept under 8 hour light yielded bud swelling, but only two sampled shoots broke buds under 21°C. For all shoots sampled in March and kept at 24 hour photoperiod, buds broke significantly faster on shoots from Tjølling when compared to Falkensten (P<0.001), Holmestrand (P<0.001) and Melsomvik (P<0.001) (Table 3). However, when comparing bud break under different temperatures, bud break of shoots sampled at Falkensten were significantly more responsive to higher temperature than Tjølling (P<0.001), having emerging leaves on average after 14 days under 21°C (Table 3, Fig. 2). Shoots from Ås were significantly less affected by higher temperature than shoots from Tjølling (P<0.001) (Table 3), having a corresponding level of leaf emergence after 21 days under 21°C. Even though temperature was not recorded in the stand where the shoots were sampled, temperature data from the Ås meteorological station 4 km away show that monthly

averages for April and May (9.1ºC) are similar to the sampling location at Tjølling (8.7ºC) (Table 5; Supporting Table 1).

Table 2: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of day length, shoot sampling location, shoot sampling date and scoring day on bud break of European beech shoots sampled in January, February and March 2014.

Fixed effect terms	Coefficient	SE	z
Long day***	5.23	0.14	37.82
Falkensten***	-2.37	0.64	-3.71
Holmestrand*	-1.35	0.64	-2.13
Melsomvik***	-2.32	0.65	-3.57
Seim	-0.30	0.63	-0.48
Ås**	-2.37	0.78	-3.04
Sampling date***	0.81	0.13	6.20
Scoring day***	0.22	0.01	33.91
Falkensten x Sampling date***	0.69	0.18	-3.73
Holmestrand x Sampling date	0.34	0.18	1.86
Melsomvik x Sampling date	0.27	0.19	1.46
Seim x Sampling date	-0.27	0.18	-1.54
Ås x Sampling date	0.30	0.25	1.73

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

For shoots sampled in October, November and December, the only bud break observed was for the December samples under LD and 21°C (Supporting Fig. 1). In October and November, the number of shoots breaking buds were seven and two, respectively, while the majority of shoots sampled in December and which broke buds originated from the Seim population. This lack of response made us unable to perform converging statistical models similar to those for data collected in the previous winter, as the cumulative link mixed models failed to converge due to lack of contrasts.

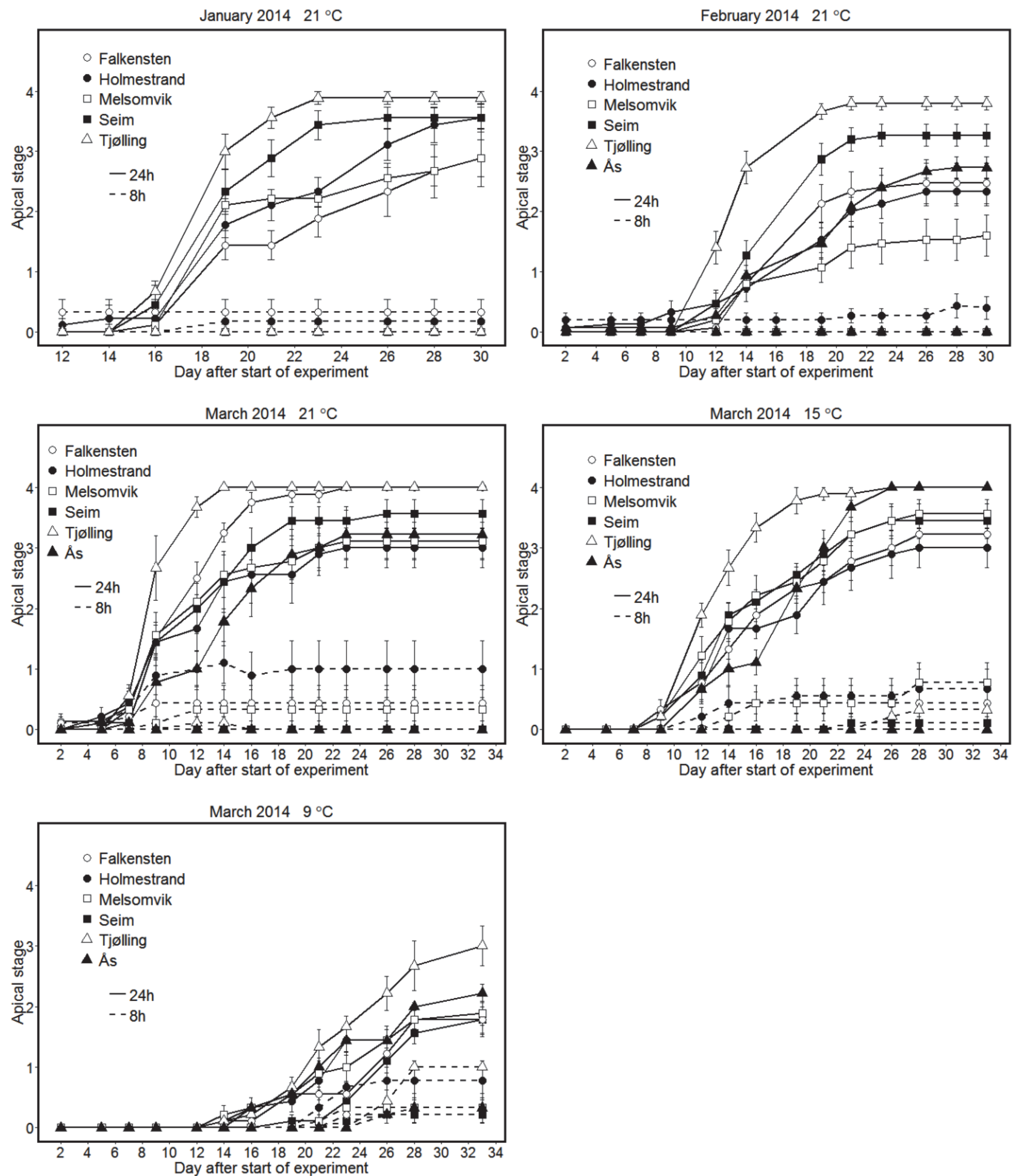


Figure 2: Average apical stages ± 1SE of European beech shoots sampled from different Norwegian populations in January (top left), February (top right) and March under different forcing temperatures (middle and bottom).

Table 3: Parameter estimates, SE and z-values for covariates in the cumulative link mixed model used to test the effect of day length, shoot sampling location, temperature and scoring day on bud break of European beech shoots sampled in March 2014.

Fixed effect terms	Coefficient	SE	z
Long day***	1.98	0.40	5.01
Temperature	0.04	0.03	1.55
Falkensten	-0.63	0.86	-0.73
Holmestrand*	2.03	0.84	2.42
Melsomvik	1.26	0.84	1.49
Seim	-1.46	0.91	-1.60
Ås	-0.10	0.91	-0.11
Scoring day***	0.26	0.01	35.91
Long day x Temperature***	0.32	0.02	14.53
Long day x Falkensten***	-2.24	0.34	-6.66
Long day x Holmestrand***	-4.08	0.32	-12.68
Long day x Melsomvik***	-2.10	0.33	-6.29
Long day x Seim	-0.21	0.43	-0.49
Long day x Ås	0.39	0.48	0.83
Temperature x Falkensten***	0.10	0.03	3.41
Temperature x Holmestrand	0.02	0.03	0.52
Temperature x Melsomvik	-0.04	0.03	-1.32
Temperature x Seim	0.01	0.03	0.22
Temperature x Ås***	-0.12	0.03	-3.99

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Bud break in field conditions

Under field conditions, understory beech trees showed delayed bud break in mixed beech and spruce stands compared to homogeneous beech stands in 2014 (P<0.001) and 2015 (P<0.001) (Table 4, Fig. 3). In 2014, mixed stands had emerging leaves on day 118 (28 April), while all the shoots from the beech-dominated stands except for Holmestrand had completed bud break on this date. The northernmost location Holmestrand had a significant delay in bud break in 2014 compared with Tjølling (P=0.019), and had a similar degree of leaf emergence as mixed stands on day 118 (28 April). These differences between stands were also observed in 2015 on day 119 (29 April), but the beech-dominated stands except Holmestrand did not complete bud break until day 126 (6 May). Considering climate, mixed beech and spruce stands had lower average and maximum temperatures throughout spring 2014 compared to homogeneous beech stands nearby, while minimum temperatures were lower (Table 5). Average temperatures at beech-dominated stands were approximately 0.5-1.0 °C higher than mixed stands, except for Holmestrand where average temperature was

similar to mixed stands. In addition, canopy transmittance measured during bud break in 2014 (25 April) was approximately 4.5-11.0 mol m^{-2} d^{-1} lower in mixed stands.

Table 4: Parameter estimates, SE and z-values for covariates in the cumulative link mixed models used to test the effect of day of year, location and stand type on bud break of European beech shoots sampled in spring 2014 and spring 2015.

Year	Fixed effect terms	Coefficient	SE	z
2014	Falkensten	0.64	1.95	0.33
	Holmestrand*	-4.42	1.88	-2.35
	Melsomvik	0.69	1.57	0.44
	Mixed stand***	-5.64	1.65	-3.43
	Day of year***	0.96	0.13	7.51
2015				
	Falkensten	0.03	1.65	0.02
	Holmestrand	-3.05	1.66	-1.84
	Melsomvik	0.98	1.40	0.70
	Mixed stand***	-5.18	1.45	-3.58
	Day of year***	0.61	0.06	10.13

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

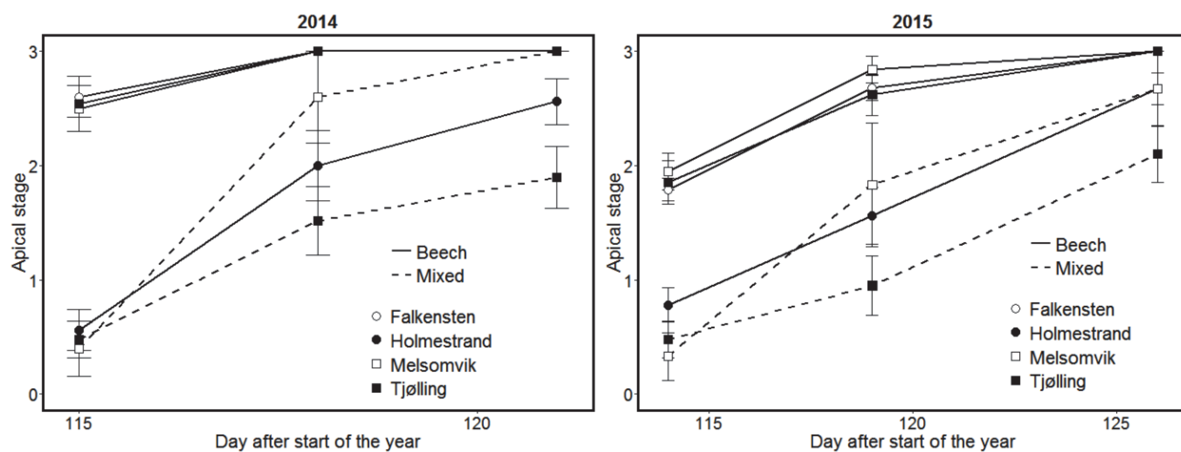


Figure 3. Average apical stage ±1SE of understory beech saplings during bud break in 2014 (left panel) and 2015 (right panel) in mixed beech and spruce stands and homogeneous beech stands.

Table 5. Measured temperatures (ºC) and canopy transmittance measured as photon flux density (mol $m^{-2}d^{-1}$) in sites during April and May 2014.

Location	Type	Average	Minimum	Maximum	PFD
Falkensten	Beech	9.88	-2.08	26.89	17.92±1.56
Holmestrand	Beech	8.63	-2.43	32.83	19.98±1.41
Melsomvik	Beech	9.24	-1.39	29.06	18.64±0.24
Melsomvik	Mixed	8.69	-0.06	17.32	13.25±0.00
Tjølling	Beech	9.75	-1.99	32.17	21.95±0.24
Tjølling	Mixed	8.77	-0.41	27.43	10.95±0.89

Cold hardiness

When exposed to freezing temperatures, shoots sampled in January, February and March showed decreasing frost tolerance with sampling date (Fig. 4). Estimated lethal temperature threshold (LT_{50}, the temperature at which 50% of the plant biomass in the buds was killed), corresponding to a score of 1.5, for all provenances sampled in January, February and March was approximately -20 to -24 °C, -14 to -22 °C, and -11 to -15 °C, respectively. The positive interaction between sampling date and temperature shows that lowering freezing temperatures yielded more bud damage with the coming of spring ($P<0.001$) (Table 6). Furthermore, freezing temperatures yielded significantly more damage on buds sampled at Holmestrand ($P=0.015$) and Melsomvik ($P=0.006$) compared to buds sampled at Tjølling. In January, estimated LT_{50} for Tjølling was approximately -24 °C, while for Holmestrand and Melsomvik this occurred at -20 °C. For February, the corresponding temperatures were approximately -22, -14 and -19 °C, while for March they were estimated to approximately -16, -14 and -12 °C.

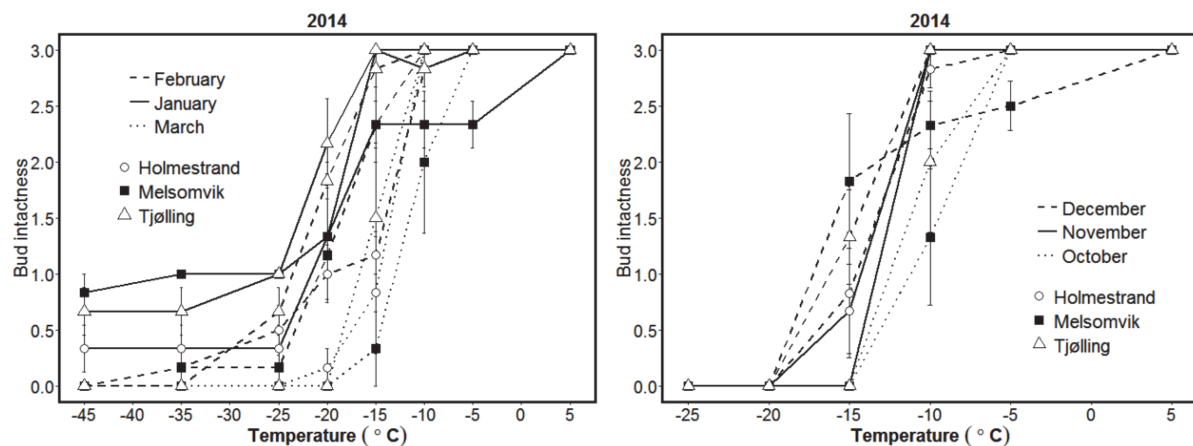


Figure 4: Average intactness ±1SE of buds exposed to freezing temperatures. Data from locations displaying significant contrasts in bud intactness are shown.

Table 6: Parameter estimates, SE and t-values for covariates in the generalised least squares model used to test the effect of freezing temperatures and sampling date on terminal buds of European beech shoots sampled in January, February and March 2014.

Fixed effect terms	Coefficient	SE	t
Intercept***	-10.35	1.22	-8.49
Falkensten	-0.08	0.09	-0.95
Holmestrand*	-0.22	0.09	-2.45
Melsomvik**	-0.24	0.09	-2.76
Seim	-0.02	0.09	-0.28
Ås	-0.11	0.10	-1.12
Temperature***	0.05	<0.01	11.29
Sampling date***	-2.44	0.55	-4.45
Temperature x Sampling date***	0.01	<0.01	3.85

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

Buds sampled in October, November and December 2014 showed increased bud intactness with increased sampling date (P<0.001) (Table 6), but were more prone to freezing damage compared to buds sampled during the previous winter in January, February, March 2014 (Fig. 4). In October, LT_{50} was approximately -10 to -12 °C for all provenances. In November, corresponding values were -13 to -14 °C, while in December these were -14 to -17 °C.

Table 7: Parameter estimates, SE t-values for covariates in the generalised least squares model used to test the effect of freezing temperatures and sampling date on terminal buds of European beech shoots sampled in October, November and December 2014.

Fixed effect terms	Coefficient	SE	t
Intercept***	-30.06	0.83	-36.25
Temperature***	0.12	<0.01	38.95
Sampling date***	0.21	0.04	5.40

Significance levels: *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

Our data suggest that timing of bud break in the wold`s northernmost native populations of European beech in Norway is mainly limited by temperature. Bud break for the Ås population has previously been reported to occur between 5 and 8 May (Heide 1993). In comparison, we observed bud break at the different field sites before 25 April in 2014 and 2015 (Fig. 2), which is in line with predictions of advanced bud break for European beech in cold environments following warming (Vitasse & Basler 2013). In addition, bud break in 2015 occurred 8 days earlier than for 2014 in the beech-dominated stands. The positive effect

of temperature on bud break was evident in the dormancy release experiment under controlled conditions, where shoots sampled in January, February and March 2014 broke buds progressively faster with sampling date. It has been suggested that for European beech growing in cold climates, bud break is more limited by temperature as compared to populations in warm climates (Wareing 1953). Indeed, advanced bud break in European beech has been observed at high elevation over the last decades in the Swiss Alps and Slovenia, a trend which has not been observed at low elevation (Cufar *et al.* 2012; Vitasse & Basler 2013).

In the dormancy release experiment, short days prevented bud break for nearly all sampled shoots (Tables 2 and 3; Figure 1). Bud break in beech is widely known for being under strong photoperiodic control, based on several studies (Vitasse and Basler 2013). However, bud break in shoots sampled in late winter showed variation between locations, both in relation to forcing temperature and sampling date. Even though the effects of chilling requirement for dormancy release and the effects of forcing temperatures are difficult to disentangle (Vitasse & Basler 2013), it is still possible to relate some of the observed differences between populations to local climates. The advancement of bud break with sampling date between January and March (Table 2; Figure 1) does also suggest that thermal accumulation had started in late winter, leading to a progressive decrease of the thermal requirement for bud break. Conversely, the low degree of bud break for shoots sampled in autumn (Supporting Fig. 1) is most likely due to unfulfilled chilling requirement (Heide 1993). Most shoots sampled at the northernmost population at Seim in December broke buds under LD and 21 ºC, while almost none of the shoots from other populations did so. It is possible that some trees in the Seim population had undergone sufficient chilling by mid-December, but as we only have climate data from Bergen 27 km further south, we are not able to relate these observations to local temperatures.

For shoots sampled between January and March, shoots from the southernmost location of Tjølling broke buds faster than remaining populations under LD treatment and at all tested temperatures (Tables 2 and 3). Interestingly, shoots from the beech-dominated stands at Falkensten showed a significantly faster bud break with advancing shoot sampling date (Table 2; Fig. 2). This may be related to the depth of bud dormancy in European beech, which is strongly affected by its chilling requirement (Falusi & Calamassi 1990; Caffara & Donelly 2011). Shoots sampled at Tjølling were collected within the mixed stands, where

mean temperatures were about one degree lower than the beech-dominated stands at Falkensten during spring 2014 (Table 5). If this difference was also consistent during winter, the buds from the Falkensten population could have received a lower degree of chilling and thus a higher thermal requirement for bud break. This could also explain the significantly stronger response of shoots from Falkensten in terms of bud break with increased temperatures in March (Table 3; Fig. 2).

Observed bud break in field conditions also suggests that temperature had a strong effect. Mixed beech and spruce stands had significantly delayed bud break compared with stands dominated almost entirely by beech, which could be related to differences in terms of average temperature between the former and the latter. This notion is further supported by the significantly delayed bud break in the beech-dominated stands at Holmestrand for 2014 (Table 4; Fig. 3), which had the lowest average temperature among the beech-dominated locations (Table 5). In a study performed in Southern Sweden where microclimates of a beech forest and an adjacent spruce forest were compared, air and surface temperatures were higher in the beech forest (Nihlgård 1969). This was attributed to the substantially higher radiation received by the ground surface in the beech forest during early spring, when canopy foliage was absent. As we observed faster bud break with higher temperatures in controlled conditions, faster bud break in beech-dominated stands may be explained by higher canopy transmittance yielding warmer microclimates.

In the frost tolerance experiment between January and March, buds displayed a maximum level of frost tolerance in January (LT_{50} between -20 and -24 °C), with a gradual decline through February (-14 to -22°C) and March (-11 to -15°C). Furthermore, buds on shoots from the southernmost mixed population at Tjølling were significantly less damaged by frost when compared to Melsomvik and Holmestrand (Table 6; Fig. 4). Considering the aforementioned differences in bud break between these populations under controlled conditions, it is possible that also these differences are due to lower temperatures at Tjølling during de-hardening compared to the beech-dominated locations at Melsomvik and Holmestrand. However, differences in frost tolerance between provenances of European beech have been related to adaptations to dissimilar climates (Visnjic & Dohrenbusch 2004; Kreyling *et al.* 2014), and it cannot be excluded that our results are also affected by adaptations to local temperatures.

In the frost tolerance tests in October, November and December, bud intactness increased with sampling date (Table 7; Fig. 4). Buds of different tree species gradually increase frost tolerance with decreasing photoperiod (Fuchigami *et al.* 1971; Junttila & Kaurin 1990; Welling *et al.* 1997; Welling *et al.* 2002), which is further augmented to a maximum by low and freezing temperatures (Weiser 1970; Greer & Warrington 1982). There were no significant differences between locations, which could be related to similar temperature regimes between locations during the hardening period. Furthermore, the low degree of frost tolerance for all populations during this period is possibly related to a particularly warm autumn (Supporting Table 1), in which buds did not obtain maximum frost tolerance due to high average temperatures in October and November.

It should be noted that our findings from the frost tolerance experiments result from a specific freezing program that differs substantially from natural environmental fluctuations, and results should thus be interpreted with caution. Cooling rates and duration of freezing exposure, resulting in varying degrees of acute and chronic stress, have a strong effect on plant tissue damage (Gusta & Wiesniwski 2012). Also, the wound at the excise surface of the twig cuttings increases exposure of interior plant tissues (Gusta *et al.* 2003), which has been shown to increase the damage risk for buds on the same shoots, also when buds are not located directly above the excise wound. Even so, our data indicate that bud dormancy and frost tolerance in European beech are similarly affected by temperature at its northernmost distribution range. Beech populations in Norway are currently limited to coastal areas with maritime climates. Even though warm autumns could be expected to limit frost tolerance in winter, it should be noted that mean winter temperatures in coastal areas of Norway are above freezing. As beech growing in Eastern Europe is unable to survive temperatures below -35ºC (Bolte *et al.* 2007), it is likely that cold winters are among the major limiting factors in continental areas of Norway. Considering projected range shifts of European beech populations with global warming, temperatures during the coldest month account for the greatest amount of variation in range expansion (Saltré *et al.* 2014). Even though it is unclear how freezing temperatures primarily limit survival of European beech at the margins of it northern distribution, it has been suggested that midwinter temperatures have a greater impact on survival through xylem embolism rather that bud damage (Kreyling *et al.* 2014). Still, provenances display greater variation in terms of buds tolerating spring frost as compared to winter frost, indicating that spring frost survival could be attributed to differences in spring phenology (Kreyling *et al.* 2014). In this regard, further studies on frost tolerance of buds

during late winter should attempt to disentangle the effects of low temperatures during cold hardening and the effects of late-winter temperatures during de-hardening.

In conclusion, our data from controlled and field conditions indicate that bud break dates in European beech is temperature limited at its northernmost distribution range. Our field observations reveal that bud break in Norwegian populations has advanced by more than 10 days compared to the observations in 1990/91, which is remarkable for a species widely known for being under strong photoperiodic control. The observed differences in temperature responses between populations during bud break matched the observed differences in bud frost tolerance during spring. This may be related to effects of local temperature regimes on chilling requirement fulfilment, cold hardening and bud break, and further studies are required to identify possible adaptations to local climates. As observed in previous studies on European beech in cold environments, we suggest that spring bud break will occur earlier for populations in Norway under warming. This may involve increased risk of frost damage in newly emerged leaves, which should be investigated through field observations both at the latitudinal and elevational margins of the species' distribution range.

AUTHOR CONTRIBUTIONS

L. N., J. E. O. & E. S. conceived the study and designed all experiments, E. S. & C. B. S. conducted materials sampling and field observations, E. S. conducted experiments, C. B. S. performed statistical analyses, C. B. S., E. S., L. N. & J. E. O. interpreted the experimental data and participated in writing the manuscript. All the authors read and approved the final manuscript.

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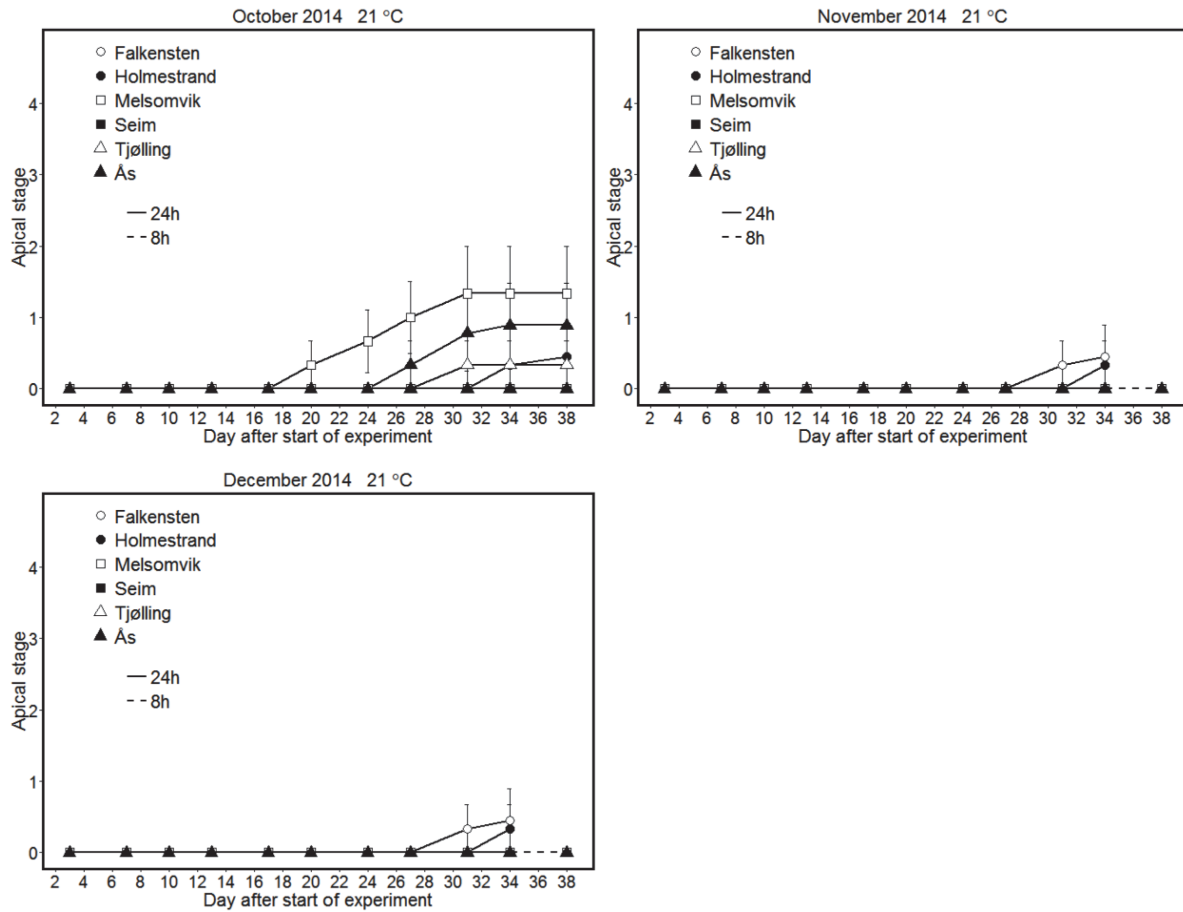
SUPPORTING INFORMATION

Supporting Table 1. Monthly means of temperatures measured at three different meteorological stations (Eklima). Melsom is the closest station to the Vestfold populations, while the meteorological station in Bergen is situated approximately 27 km from the Seim population. Values from 2013, 2014 and 30-year normals (1961-1990) are shown.

Location	**Year**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**
Melsom	2013	-4.2	-3.3	-2.4	4.1	11.9	14.7
	2014	-1.4	2.3	4.6	7.6	11.6	15.4
	Normal 1961-1990	**-3.7**	**-3.8**	**0**	**4.4**	**10.6**	**15**
Seim	2013	0.3	0.6	0.9	5.1	11.3	13.3
	2014	3.4	5.5	6	8.9	11.4	14.5
	Normal 1961-1990	**1.3**	**1.5**	**3.3**	**5.9**	**10.5**	**13.3**
Ås	2013	-5.2	-4.3	-3.5	3.6	12.1	14.3
	2014	-2.6	1.8	3.9	6.9	11.2	15
	Normal 1961-1990	**-4.8**	**-4.8**	**-0.7**	**4.1**	**10.3**	**14.8**
		Jul	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Melsom	2013	17.9	16	11.7	7.9	2.9	3.3
	2014	20.3	15.6	12.8	9.7	4.8	-0.8
	Normal 1961-1990	**16.3**	**15.2**	**11.1**	**7**	**1.7**	**-2.1**
Seim	2013	15.6	15.2	12.5	9.6	5.5	5.7
	2014	19	15.5	13.6	10.6	7.3	3.2
	Normal 1961-1990	**14.3**	**14.1**	**11.2**	**8.6**	**4.6**	**2.4**
Ås	2013	17.5	15.5	11.1	7	1.9	2.4
	2014	20	15.1	12.2	8.9	3.8	-2.6
	Normal 1961-1990	**16.1**	**14.9**	**10.6**	**6.2**	**0.4**	**-3.4**



Supporting figure 1: Average apical stages ± 1SE of European beech shoots sampled from different Norwegian populations in October (top left), November (top right) and December and kept under 21 ºC.

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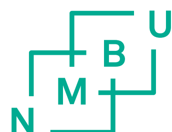

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no