THE ROLE OF TEMPERATURE IN THE PHENOLOGY OF TEMPERATE AND BOREAL TREE SPECIES

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Claudia Nanninga

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Dedication

This thesis is dedicated to my husband Alexander Potter. Thank you for your warmth, wisdom, and endless support.

Dissertation Abstract

In the current era of climate change, the phenology of trees, i.e., the timing of seasonal life-cycle events, is evolving. Due to warming springs, leaf bud break has been observed to occur earlier worldwide, even though this trend has slowed in recent years. Because the phenology of trees can be important in determining range limits, annual net primary production, and interactions with other species, it is essential to understand how climate change might impact phenological timing in the future. This dissertation describes several laboratory experiments with the goal of elucidating the effect of some of the main cues, i.e., cold winter and warm spring temperatures, on the spring phenology of boreal and temperate tree species.

With the help of others, I collected dormant twigs of temperate and boreal tree species at Cedar Creek, MN, and exposed them to different experimental conditions. In collaboration with the Technical University of Munich, Bavaria, I was also able to replicate some experiments in Germany to understand the different chilling and forcing needs of temperate and boreal species of the same genus, but different region. Dr. Sam Fahrner Ward (back then at the Entomology Department of the University of MN) and I additionally worked with tamarack seedlings and larch casebearer larvae to better understand if changes in the phenological synchrony of these interacting species could be responsible for recent larch casebearer outbreaks in MN.

I found that for both Cedar Creek and Germany, prolonged chilling throughout the winter reduced the need for forcing/the time to bud break for the large majority of species, and that there were significant differences between species within the U.S. and Germany, and across continents. If chilling were reduced in the winter due to climate

change, species with small chilling requirements could be disproportionally advantage by warmer springs, However, I also found that out of 14 species from Germany and the U.S., 7 species significantly reacted to chilling temperature and that 6 out of these 7 species broke bud faster when exposed to warmer chilling of up to 4.5ºC. In regions with very cold winter temperatures, such as MN, climate change induced winter warming could initially benefit species that prefer warmer chilling, while for species that prefer colder chilling, warmer winters could delay bud break if chilling requirements are not fully met.

I also found that warmer chilling and forcing increased phenological synchrony between larch casebearer and tamarack, but only up to 27ºC of forcing, after which larval activation slowed down. Additionally, in growth chambers of 21ºC and warmer, casebearer larvae were unable to reach adulthood. Warmer winters and springs might increase synchrony of both species, which could benefit larch casebearer due to the availability of younger, more nutritious needles with reduced defense chemicals. However, in very warm springs, survivorship to adulthood might be drastically reduced.

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Dissertation Introduction

The phenology of temperate and boreal tree species, i.e., the timing of annual life-cycle events, such as bud break and flowering, is strongly influenced by temperature and therefore sensitive to climate warming. Increases in winter and spring temperatures have already resulted in advances of spring phenology in many tree species around the world (Fu et al., 2015a; Menzel et al., 2020; Polgar et al., 2014; Yu et al., 2017). The timing of seasonal events can have strong impacts on the fitness of trees, their geographic range limits, and their ecological interactions, such as competition and parasitism, ultimately affecting net primary production and ecosystem functioning (Chuine & Beaubien, 2001; Chuine et al., 2010; Gritti et al., 2013; Harrington & Gould, 2015; Morin et al., 2007). It is important to better understand how climate moderates tree phenology to accurately determine the occurrence of life-cycle events in predictive models and to inform management decisions. This is especially relevant for species in the temperate-boreal ecotone, which often occur at the northern or southern edges of their range.

Three main abiotic cues interact with one another to influence spring phenological timing in boreal and temperate trees: winter temperatures, spring temperatures, and photoperiod. After senescence in the fall, tree buds enter a state of endo-dormancy, during which they are truly at rest and ontogenetic development is halted. Buds are hence protected from early leaf flushing and frost damage in case of a warm spell during the winter, which is particularly relevant in warmer regions (Clark et al., 2014b; Ford et al., 2016). After a period of winter chilling, usually defined as temperatures within a cold range below a specific upper threshold, buds enter a state of eco-dormancy (Hänninen, 1990, 1996; Landsberg, 1974). In this state, the ability to develop under suitable

conditions is re-established. In the spring, a period of forcing, usually defined as temperatures above a minimum threshold, eventually causes buds to break (Hänninen, 1996). It has been well established that warmer forcing reduces the time to bud break (days to bud break = DTB) and that increased chilling reduces the need for forcing (Laube et al., 2014; McKown et al., 2018). It has also been shown that a lack of chilling in the winter can delay bud break, lead to erratic bud break, or in some cases even prevent bud break (Polgar et al., 2014; Harrington & Gould, 2015; Morin et al., 2009). A longer photoperiod in the spring, the third major phenological cue, can additionally play a moderating role by reducing the need for forcing for some species (Flynn and Wolkovich, 2018; Meng et al., 2021; Wenden et al., 2020; Zohner et al., 2020).

While these basic mechanisms have been studied intensively and are well known, there are still gaps in our knowledge, which can result in imprecise model predictions with potential consequences for management decisions. One cause of uncertainty is the considerable difference between species and even genotypes regarding the required combination of phenological cues that eventually result in bud break (Hänninen, 1996; Polgar and Primack, 2011). Only few species have been studied intensively, and for most tree species, the precise amounts of forcing and chilling units that must be accumulated for bud break to occur are not known. This is complicated by the fact that all cues interact with one another to either enhance or delay bud break, depending on temperature conditions and how early spring warming occurs. Additionally, there are uncertainties around the effectiveness of temperatures, both in the upper and lower threshold levels and the relevant temperature ranges suitable for chilling and forcing accumulation (Harrington & Gould, 2015; Wenden et al., 2020). It is also still debated if temperatures

below freezing contribute to chilling accumulation. Because of these uncertainties, predictive phenological models often work with assumptions and/or generalizations, and sometimes fail to accurately predict bud break.

This dissertation reports experiments that aim to elucidate the effect of chilling and forcing on the timing of bud break in temperate and boreal species and to address existing knowledge gaps. Lately, a need for more phenological research in the form of experiments has also been expressed in the literature (Ettinger et al., 2020; Hänninen et al., 2019; Primack et al., 2015; Wolkovich et al., 2022; Zohner et al., 2017). Three observations were a basis for the experiments. First, in some studies, phenological models perform better if chilling is excluded as a parameter and only forcing and photoperiod are considered (Dantec et al., 2014). I aim to understand the importance of chilling and whether it is justified to generally exclude chilling from models for all species. Second, while worldwide spring bud break has been occurring earlier due to warmer spring temperatures, this trend has recently slowed down (Fu et al., 2019a, 2015a; Jeong et al., 2011; Piao et al., 2011; Yu et al., 2010). Several justifications for this slowed trend have been discussed, including a reduced photoperiod during the forcing phase (Fu et al., 2019b; Wenden et al., 2020), an earlier pre-season with warmer temperatures (Güsewell et al., 2017), statistical weaknesses (Ettinger et al., 2020), or reduced winter chilling due to warmer winter temperatures (e.g. Fu, Zhao, et al., 2015). Here, I aim to understand if warmer winters and therefore reduced chilling could be a major factor contributing to this trend. Third, ecological interactions are often influenced by phenological timing and established synchronies. This is especially relevant for spring-feeding insects that typically experience a narrow window of opportunity for egg

hatch or feeding and are therefore dependent on available foliage in the spring (Ward & Masters, 2007). Larch casebearer is an introduced defoliator of *Larix* spp. that was first detected to cause damage to tamarack (*Larix laricina* Du Roi) in the Lake States region in the 1920s. After a successful importation biological control in the mid 1900's, densities of larch casebearer were very low in MN for decades. However, recently there been widespread outbreaks on western and eastern *Larix* spp. (Ward & Aukema, 2019a). I aim to understand if climate change has led to a change in the phenological spring synchrony between tamarack and larch casebearer, potentially leading to exposure to younger and more nutritious foliage for larch casebearer and hence contributing to the insect's recent outbreak in MN.

In Chapter 1, I look at the effect of increased chilling time on the days to bud break in temperate and boreal tree species from MN. By collecting twigs from different species over a 2-year period throughout the winter and exposing them to spring-like temperatures in the greenhouse, I show a linear decline in bud break timing with increased ambient chilling and therefore point out the importance of chilling in reducing DTB. I am also able to show that below freezing temperatures contribute to chilling accumulation, and that there is a large difference between the chilling requirements of species.

In Chapter 2, in collaboration with the Technical University of Munich, I estimate the effect of different chilling temperatures and chilling lengths on the time to bud break in various temperate and boreal tree species across two continents (North America – U.S.A., and Europe – Germany). By collecting twigs and exposing them to 3 chilling temperatures and 2 chilling durations in the first experiment, I demonstrate a decrease in

bud break timing with increased chilling, and a significant effect of different chilling temperatures on DTB for half of the species. I also show that this effect is not always linear and that for most species, warmer chilling is more effective at reducing bud break timing than colder chilling. Additionally, in a second experiment by collecting twigs from the same individual trees throughout the winter and forcing them in the greenhouse, I again demonstrate that increased ambient chilling decreases the time to bud break/need for forcing.

In Chapter 3, I characterize the phenological relationship between tamarack and larch casebearer in collaboration with the Entomology Department of the University of MN. By experimentally exposing both species to 4 chilling and 6 forcing treatments, I establish that warmer forcing reduces the time to bud break and larval activation, but that the effect of forcing on spring activation is much stronger for larch casebearer. While warmer chilling reduces the time to bud break for tamarack, the effect is not as clear-cut for casebearer. I also demonstrate that warmer forcing increases phenological synchrony between both species up to a specific temperature threshold, after which synchrony decreases again.

Chapter 1: Increased exposure to chilling advances the time to budburst in North American tree species

Claudia Nanninga*,, Chris R. Buyarski*, Andrew M. Pretorius*, Rebecca A. Montgomery*[1]**

**Department of Forest Resources, University of Minnesota, 1530 Cleveland Ave N, St. Paul, MN 55108*

********Email: nanni003@umn.edu, Phone: 612 987 8450*

Abstract

The phenology of trees is highly susceptible to changing global temperatures. Leaf budburst advances with increasing spring temperatures but can also be delayed when warmer winters reduce chilling exposure. Results from long-term observations show that increasing temperatures have triggered advanced budburst in the past decades, but some studies also show that budburst advance has slowed recently. Here we conducted an experiment with five temperate deciduous tree species (*Acer rubrum, Larix laricina, Populus tremuloides, Quercus ellipsoidalis, Betula papyrifera)* and one invasive species (*Rhamnus cathartica*) in Minnesota, USA, to assess the impact of chilling on the timing of leaf budburst. We collected twigs over two winter seasons (2011/2012 and 2012/2013) on a biweekly basis and exposed them to spring-like temperatures of 21°C/ 16°C day and night, long day photoperiod (16 h). We found a significant relationship of advanced budbreak with increased chilling for all species tested ($p<0.001$) and significant

[1] Author Contributions: CB, CN, and RM conceived the project, RM and CB developed methodology, AP, CB and CN conducted fieldwork and performed statistical analyses, CN and CB wrote the manuscript.

differences in the timing to budburst among all species (p<0.001). *A. rubrum* responded strongly to chilling, showing a very steep linear decline in days to budburst with increased exposure to chilling. On the other end of the spectrum, *L. laricina* responded least to increases in chilling. These results suggest that rising global temperatures will likely have diverse impacts on tree species with potential implications for species interactions such as competition.

Introduction

The annual development (phenology) of temperate trees, such as budbreak, flowering, and fruiting, is an important component of the tree's fitness, realized niche, and geographic range limits (Chuine, 2010; Chuine and Beaubien, 2001; Morin et al., 2009). Because temperature plays a major role in tree phenology, rising global temperatures are predicted to have a significant influence on the fitness and distribution of tree species (Chuine and Beaubien, 2001; Cleland et al., 2007; Morin et al., 2009).

Tree budburst is a commonly studied phenophase that is activated by chemical changes within the shoot apical meristem (Rinne et al., 2001). In temperate regions, these biochemical processes are mainly stimulated by three external triggers: the length of daylight (photoperiod), chilling temperatures, and spring forcing temperatures (Basler and Körner, 2012; Körner and Basler, 2010; Laube et al., 2014; Myking and Heide, 1995). Photoperiod seems to play a subordinate role in releasing dormancy and inducing budburst, although the importance of this cue likely varies among species (Basler and Körner, 2012; Laube et al., 2014). In contrast, it is widely agreed that chilling (exposure to cold temperatures) and forcing (exposure to warm temperatures) are dominant triggers in the timing of budburst for many temperate trees (Bennie et al., 2010; Laube et al.,

2014; Myking and Heide, 1995; Polgar and Primack, 2011). After the initiation of dormancy, chilling temperatures act as a cue that supports the increase of frost-hardiness in buds. Additionally, the accumulation of chilling units activates the movement of buds out of dormancy (Hänninen, 1990, 1996; Landsberg, 1974). According to the 'sequential' chilling model, the accumulation of forcing units will be initiated only after rest completion, i.e. the fulfillment of a specific chilling requirement (Hänninen et al., 2019; Richardson et al., 1974; Sarvas, 1972), while the 'parallel' model posits that chilling and forcing units can be accumulated simultaneously (Ford et al., 2016; Harrington and Gould, 2015; Laube et al., 2014), and that the breaking of dormancy is a more dynamic process (Cannell and Smith, 1986; Landsberg, 1974). There are also several hybrid models (e.g. (Hänninen, 1990; H. Hänninen, 2016).

Common conventions exist for measuring the exposure of buds to temperature. Forcing is commonly measured in units of growing degree days (GDD's), the accumulated average daily temperature above a base threshold, that activates bud elongation and growth after dormancy release in the spring (Lechowicz, 1984; Myking and Heide, 1995). These forcing units or GDD's are also called thermal time. Chilling units are commonly measured as the number of days or hours a plant is exposed to cold temperatures below a threshold or within a temperature range as it prepares to end dormancy (Caffarra and Donnelly, 2011; Heide, 1993; Murray et al., 1989). The exact threshold and range at which chilling can be sensed by the buds has not been determined for most temperate forest tree species. While some argue that optimum chilling temperatures for temperate trees are typically above freezing (Arora et al., 2003; Harrington and Gould, 2015; Heide, 1993), much research is based on chilling ranges that

include all temperatures below a threshold (Ashby et al., 1991; Laube et al., 2014; Murray et al., 1989; Wilson et al., 2002). Additionally, it is well established that species differ in their requirement for chilling, which has been attributed to successional status (Basler and Körner, 2012; Laube et al., 2014), provenance or range (Harrington and Gould, 2015), invasiveness (Laube et al., 2014), and differences in xylem properties (Lechowicz, 1984).

Many experiments have demonstrated that a rise in temperatures leads to accelerated budburst (e.g. Bradley et al., 1999; Caffarra & Donnelly, 2011; Heide, 1993; Laube et al., 2014). However, species that require large amounts of mid-winter chilling to break bud ("high chill species") have shown a delay in budburst when winter temperatures are elevated and chilling requirements are met more slowly (Cleland et al., 2007; Heide, 2003, 1993; Murray et al., 1989; Myking and Heide, 1995). It is therefore reasonable to assume that global climate change may not result in linear advances in bud burst, but potentially in a delay for high chill species, if winter temperatures continue to increase as predicted (Morin et al., 2009; Pope et al., 2013; Schwartz and Hanes, 2010). Such species might therefore experience a reduced growing season, putting them at a competitive disadvantage (Caffarra and Donnelly, 2011; Cannell and Smith, 1986; Murray et al., 1989). Reduced chilling, especially at southern margins, can also lead to abnormal leaf development with negative effects on productivity and survival (Harrington et al., 2010; Morin et al., 2009). Long-term studies seem to support the theory of delayed budburst with reduced chilling. While the majority of such studies validate that increases in global temperature have resulted in earlier flowering (Beaubien and Freeland, 2000; Menzel, 2002; Parmesan and Yohe, 2003; Primack et al., 2009),

there is new evidence that this initial trend towards earlier budburst has slowed down in the most recent decade in Northern latitudes (Fu et al., 2019a, 2015a; Jeong et al., 2011; Piao et al., 2011; Yu et al., 2010). Even though the exact mechanisms are not known, a lack of chilling has been suggested (Fu et al., 2015a).

On the other hand, species with small mid-winter chilling requirements ("low chill species") might be better able to track climate change by leafing out earlier, potentially extending their growing season (Bennie et al., 2010; Menzel, 2002; Schwartz et al., 2006), but face an increased risk of frost damage (Cannell & Smith, 1986; Hänninen, 1996; Heide, 1993), if the onset of budburst advances at a faster rate than the beginning of the frost-free season (Schwartz et al., 2006). However, new tissue of early flushing species has been shown to be more freeze-tolerant than that of late flushers (Vitasse et al., 2014a). While some studies found increase in frost damage in the U.S. with global warming (Augspurger and Bartlett, 2003), others suggest that for most species, frost damage will not be a common phenomenon (Ford et al., 2016; Morin and Chuine, 2014). Schwartz et al., (2006) also found that, while both the date of budburst and the last frost have been earlier in North America, the relative rate of change was not the same for all areas, which complicates predictions.

The effect of chilling on tree phenology and its consequences for woody species has been studied extensively in Europe (c.f. Basler & Körner, 2012; Caffarra & Donnelly, 2011; Ghelardini et al., 2009; Hänninen, 1991; Laube et al., 2014; Murray et al., 1989; Myking & Heide, 1995), but studies in North America are less common. There are some long-term phenological studies (e.g. Augspurger & Bartlett, 2003; Beaubien & Freeland, 2000; Bradley et al., 1999), and some experimental studies (e.g. Ashby et al., 1991;

Campbell & Sugano, 1979; Norby et al., 2003), and a fast-growing body of model-fitting approaches (e.g. Clark, Melillo, et al., 2014; Clark, Salk, et al., 2014; Harrington & Gould, 2015; Morin & Chuine, 2014; A. D. Richardson et al., 2006, 2012; E. A. Richardson et al., 1974; Schwartz et al., 2006). Despite this growing amount of research, there are still gaps in our knowledge of species-specific or even genotype-specific requirements for budburst, specifically how chilling requirements compare among Northern American tree species, and how increased chilling influences the timing of budburst.

Twig experiments offer the opportunity to isolate factors that influence budburst, such as chilling or forcing, and are increasingly used by scientists (Heide, 1993; Laube et al., 2014), but we are aware of few such studies in North America (Miller-Rushing & Primack, 2008; Polgar et al., 2014). Here we conducted a twig experiment to examine the role of mid- to late winter chilling in the timing of bud burst for five native tree species from Minnesota, USA, and one invasive species. We hypothesized that increased exposure to chilling would accelerate budburst for all species and reduce the thermal time to budburst. Slower budbreak was expected at initiation of the experiment because more forcing is required under low chilling. We also hypothesized that the time to budburst would differ among species.

Methods

We conducted a greenhouse chilling experiment over two winter seasons (2011/2012 and 2012/2013) with twigs of adult tree samples (adapted from Heide 1993) from the Cedar Creek Ecosystem Science Reserve (CCESR) outside of East Bethel, Minnesota (N45°24'7.29'', W93°11'57.85''). The climate at Cedar Creek is continental,

with mean daily temperatures of -10°C in the coldest (January) and 22°C in the warmest month (July)[2]. Average annual precipitation had a range of 59.2 to 116 cm between 1984 and 2014. This experiment was conducted over two years largely because we were interested in repeatability, i.e. if the general pattern of advanced budbreak with increased chilling would be the same for both years.

Suitability of twigs in phenological experiments

The use of twig cuttings or seedlings as proxies for adult trees has been widely established (Basler and Körner, 2012; Heide, 1993; Laube et al., 2014; Polgar et al., 2014), and is an easy and inexpensive way to isolate and study drivers of leaf phenology (Primack et al., 2015). Some studies have shown that the phenological development of seedlings can differ significantly from adult trees of the same species, complicating inferences (Augspurger and Bartlett, 2003; Vitasse, 2013). In contrast, the suitability of twig cuttings from adult trees for studies of tree phenology has been questioned because of their disconnection from hormonal signals from the parent plant. While it has been demonstrated that root temperature can have an influence on the timing of budburst in *Betula papyrifera* (Hawkins and Dhar, 2012), triggers involved in dormancy and dormancy release, such as chilling, are targeted directly at the level of the bud apical meristem. Consequently, chemical changes that allow a bud to respond to forcing temperatures or daylengths are localized and integral to the bud (Arora et al., 2003; Rinne et al., 2001).

[2] Data from January 1, 1985 to December 31, 2014.

We are aware of only one study that directly compared the bud phenology of parent trees with that of their cuttings, including one species of the genus *Acer*. The authors found that the cuttings, which were kept in water under similar field conditions, reacted in a similar way phenologically to their parents, and they concluded that cuttings are better suited as surrogates in warming experiments than seedlings (Vitasse et al., 2014a,b).

Year 1:

We harvested twigs of four temperate tree species (*Acer rubrum, Larix laricina, Populus tremuloides, Quercus ellipsoidalis)* within a small sampling area (<30 acres) on three dates between February 02 and March 06 of 2012. One dormant twig per tree was harvested on each collection date (n=5 trees per species) for a total of 60 samples. Each twig had a minimum of six live buds and a length of at least 30 cm. We carefully examined all the twigs to ensure that each had a green tissue ring at the cut end, indicating they were alive and had the potential to break bud. Immediately after cutting, we put the twigs in an oversized cooler filled with snow and transported them to a greenhouse within 1.5 hours. Once in the greenhouse, we immediately placed the twigs into 19-liter buckets (five twigs per bucket), filled with around 3 l of tap water each. To minimize obstruction of the xylem and to provide fresh water, we trimmed the end of the twigs, scrubbed the buckets, and changed the water on a weekly basis.

The greenhouse was kept at temperatures approximately simulating mid to late spring conditions for Minnesota: 21°C day/16°C night. We chose a constant long and late-season photoperiod (16 h) throughout the entire period of flushing to isolate the effect of forcing and chilling, and to exclude the effect of photoperiod on budbreak (see

methods Polgar and Primack 2011). Because of large ambient temperature fluctuations, especially in the later spring, there were slight greenhouse temperature variations (on average, the temperature variation was around 1°C, but some hours in March had a small number of hours with fluctuations as high as 3.95°C). We calculated the cumulative GDD's as follows:

$$GDD = (T_{max} + T_{min})/2 - T_{base}$$

where T stands for temperature. We assumed T_{base} to be 0°C (based on Heide (1993) and used the minimum and maximum temperature settings of the greenhouse for T_{max} and T_{min}. Based on this, twigs did not experience any GDD's prior to twig collection. Chilling hours were defined as the number of hourly temperature readings below 5°C, starting November 1st when trees were assumed to be dormant (based on Murray et al. 1989). The choice of chilling threshold did not qualitatively change results. To calculate the amount of accumulated chilling hours prior to each twig collection, we used average hourly temperature data from the CCESR.

Phenological data was collected three times per week on all branches. We defined budbreak according to BBCH7[3]: beginning of bud burst – first green leaf tips just visible (Meier, 1997). We are aware of studies that show a stronger effect of chilling on apical, compared to lateral buds (e.g. Falusi & Calamassi, 1990). For this study, we assumed that all buds were in the same state of dormancy during collection, and we made the general observation that as soon as one bud broke, most other buds on the same twig were either breaking or close to breaking. In year 1, branches that failed to break bud after eight weeks were discarded. In both years, live branches were removed from the experiment as

[3] Biologische Bundesanstalt, Bundessortenamt, and Chemical Industry

soon as at least one of the buds had broken, or when the twigs were dead. Twigs were considered dead when they appeared dried out or no green tissue was present at the cut end. In both years, the experiment was terminated when outside temperatures began to regularly exceed the GDD threshold. As we were testing for the effect of chilling, we wanted to be able to control the amount and pattern of forcing temperatures.

Year 2:

In the winter of 2012/2013, we repeated the sampling method and the greenhouse treatment, but with some adjustments: first, we increased the number of donor trees (n=10 trees per species); second, two species were added (*Betula papyrifera* and the Eurasian invasive shrub *Rhamnus cathartica*); third, twigs were harvest biweekly between 19-Dec-2012 and 28-Mar-2013, for a total of eight collections; finally, twigs were not discarded automatically after eight weeks of observation. Instead, all twigs were kept until they either broke bud or were considered dead. Table 1.1 summarizes the accumulated chill hours for twigs from each collection during the two years of the project.

To explore how our results may have been affected by our definition of a chill hour (<5°), we compared the accumulated chilling hours under this definition with three alternative scenarios, in which chill hours would accumulate at a) <3°C, b) <1°, or c) between 0°C and 7°C, starting November 1st. We chose these temperature ranges arbitrarily, but loosely based on thresholds used across the literature. For example, (Caffarra and Donnelly, 2011) used a constant 3°C in their chilling experiment, and according to some models and publications, temperatures in the range between 0 and 7°C have a chilling effect on fruit trees (Arora et al., 2003; Weinberger, 1950).

To place our experiment in the context of climate change effects on our study area, we also looked at the trends of annual chilling hours between 1989 and 2017.

Statistical Analysis

The data was analyzed with JMP® 13 (JMP®, Version . SAS Institute) and all graphs were produced with the R statistical package (R 3.3.2, Crawley, 2007). All values are mean ± standard deviation. Our analysis met the model assumption of normally distributed residuals.

To test the hypothesis that the amount of chilling had an accelerating effect on the time to budburst, we performed a linear mixed-model analysis with year as a random effect. We treated year as a random effect to incorporate variation caused by abiotic factors, such as available moisture and soil temperature, and because we were interested in overall patterns of chilling hours vs. days to budbreak. The mixed-model was run with species and chill hours and their interaction as the fixed effects and the number of days to leaf budbreak as the response variable.

Chill-hours were calculated as hours <5°C accumulated in the field since November 1st (Laube et al., 2014; Murray et al., 1989; Polgar and Primack, 2011). Because of fixed temperature conditions in the greenhouse, GDD's represent a transformation of the number of days to budbreak. Results are similar regardless of response variable, so we chose to present results with days to budbreak as the response variable.

Results

We found that species ($F_{5,463}$=193.24, p<0.0001), chilling hours ($F_{1,436}$=767.8514, p<0001) and their interaction ($F_{5,436}$=38.7976, p<0.001) were all significant predictors of

leaf budburst. For all species, the number of cumulative chilling hours and the time to leaf budbreak were negatively associated, i.e. the more chilling hours the branches were exposed to, the less thermal time they required for budburst (Fig. 1.1). The impact of chilling was most pronounced during the first collections, as can be observed in the sharp decline in the time to budburst for some species, especially *A. rubrum*. After short exposure to chilling, the twigs required a much longer thermal time to break bud than after large amounts of chilling.

The disparity in budburst timing among species was largely the result of differences in the slope of the chilling hour/budbreak relationship. The largest differences occurred with low levels of chilling, and all converged towards similar numbers of days to budbreak after higher amounts of chilling (Fig. 1.1). Of all species, *A. rubrum* required the largest amount of thermal time to break bud, especially after a short exposure to chilling, and it responded most strongly to accumulations in chilling (Fig. 1.1 and 1.2). For example, an increase of chilling from 945 to 3308 hours decreased the requirement of thermal units from on average 1409.7 to only on average 370 GDD's, a 74% change (adj R^2=0.65, p<0.001, y=100.39-6.86x). On the other extreme, *L. laricina* responded fastest when exposed to thermal time among all species, even after short amounts of chilling (Fig. 1.1 and 1.2). An increase of chilling from 945 to 3308 days led to on average 67% reduced need for thermal units (351.5 vs 118.4 GDD's) (adj R^2=0.71, p<0.0001, y=24.47-0.88x), but its thermal time requirements throughout the experiment were significantly lower than those of any other species. *B. papyrifera* generally required more thermal time than *L. laricina* to break bud, but it showed the weakest response of all species to increased chilling with 37.5% earlier budbreak (634.5 vs. 396.7 GDD's), which was still

highly significant (adj R_2=0.43, p<0.001, y=42.6-2.06x). Finally, the response pattern of *R. cathartica* closely followed that of *L. laricina*, and in general the invasive species required the second to least amount of thermal time to budbreak after exposure to different amounts of chilling (adj R^2=0.8, p<0.001, y=38.31-1.14x).

Species differed in the percentage of branches that broke bud throughout the experiment. *R. cathartica* and *L. laricina* had the highest budbreak rate at 90-100% for all collections, while the budbreak rate for *A. rubrum* was as low as 20%. The fact that as early as December 19th (year 2) most twigs had the capacity to develop leaves when forced suggests that exposure to chilling had initiated the progressive release of dormancy to a point where budbreak was possible already before our experiment was implemented (Cannell and Smith, 1986; Landsberg, 1974). The low budbreak rate of *A. rubrum* for most collections might suggest that *Acer* was prone to phloem or xylem damage during collection. On the other hand, on average 48% of the discarded *A. rubrum* twigs in year 2 achieved flower budbreak prior to removal (unpublished results).

To see if changes in temperature have already started to affect our study site, we used hourly temperature data from the CCESR (Cedar Creek Ecosystem Science Reserve) and the WRCC (Western Regional Climate Center) to calculate the number of accumulated winter chill hours below 5°C between 1989 and 2017, starting November 1 (Fig. 1.3), and found that in the last 28 years, Cedar Creek has lost on average 12.7 chill hours annually. Even though this change is not statistically significant, and, due to the high scatter the adjusted R^2 value is very low (0.04), there is still an observable trend, which over time could influence the phenology of species at the site.

Our results are based on the supposition that all the tested species were capable of sensing and accumulating chilling at all temperatures below 5°C, which we used to explain the significant decline of time to budburst with increased chilling exposure. By this assumption, chill hours increase linearly throughout the coldest winter months in both years. Fig. 1.4 shows our measure of chill hours (<5°C) ($F_{9,1}$=19.7, p=0.01628) compared to three alternative models, in which buds are assumed to accumulated chilling at <3°C ($F_{9,1}$=22.92, p<0.001), <1°C ($F_{9,1}$=39.19, p<0.001), and at a range of 0-7°C (not significant). The graph depicts the accumulation of chilling hours for both years, starting with the first collection and ending with the last collection. The lines don't start at a base of zero as ambient chilling units have already been accumulated before the first collection.

If we assume that twigs could sense and accumulate chilling at <3°C or <1°C, the pattern of chilling accumulation would still be linear and closely follow that of the <5°C scenario. However, the pattern in a model where buds only responded to chilling temperatures of above freezing (0-7°C) would differ substantially. Here, chilling hours would have mainly accumulated at the beginning of the winter season from in November, but the average daily below-freezing temperatures between December and February would not have significantly contributed to the accumulation of chilling. In that model, a linear decline in days to budbreak occurs despite no change in accumulated chill days.

Discussion

Increasing global temperatures have the potential to alter numerous aspects of ecological systems. One of the strongest signals of rising temperatures has been the change in plant phenology, especially spring budbreak and flowering. Much scientific

work has focused on changing spring conditions, rather than the potential effect of warmer winters on phenology. Specifically, increasingly warm winters might influence the amount of winter chilling, potentially affecting timing of budbreak in high chill species. Our results confirm that chilling had a significant effect on the rate of budbreak for all species in our experiment, validating its crucial role in the phenology of temperate trees.

Species-specific responses to chilling

For all species in this experiment, increased chilling significantly reduced the amount of thermal time to reach budburst, and there were significant differences among the species. These differences were especially pronounced at the beginning of the experiment, but they became less strong with increased chilling.

Of all species, the low chill *L. laricina* required the least amount of thermal time to break bud, regardless of chilling exposure, and it showed the weakest decrease in time to budburst between the first and last collection. These findings are in line with Harrington & Gould (2015). Consequently, *L. laricina* will most likely experience earlier budbreak and potentially longer growing seasons with rising spring temperatures, possibly putting it at a competitive advantage over high chill species (Polgar & Primack, 2011). On the other hand, earlier budbreak might render it vulnerable to greater frost damage if advances in budbreak outpace advances in the beginning of the frost-free season (Cannell and Smith, 1986).

On the other extreme, *A. rubrum* showed the strongest response to chilling and there was a very steep reduction in thermal time to bub break between the first and last collection. While *A. rubrum* twigs were initially much slower at developing leaves than

other species, these differences became less substantial with increased chilling. The slow response of *A. rubrum* and some other species from the first collections suggests that chilling requirements were not yet fully met (Landsberg, 1974). In the future, increasing winter temperatures in Minnesota could result in unfulfilled chilling requirements for MN genotypes of high chill species, such as *A. rubrum*, potentially delaying instead of advancing budburst. As a consequence, *A. rubrum* might experience range contractions, especially in the south (see also Harrington & Gould, 2015; Morin et al., 2008, 2009; Schwartz & Hanes, 2010), and/or fail to benefit from the prolonged growing season compared to low chill species, putting it at a competitive disadvantage. However, these assumptions need to be taken with care. For example, Marchin et al., (2015) found that after year-around warming of 5°C, budburst was still advanced in *A. rubrum* with increased spring temperatures. On the other hand, Caffarra & Donnelly (2011) reported failed budburst in *Tilia* and *Salix* with unfulfilled chilling. Additionally, range limits are also strongly determined by flowering and fruit maturation (Morin et al., 2008), and *A. rubrum* is one of the earliest species in MN to produce flowers in the spring (Burns and Honkala, 1990). Moreover, for the few twigs that flowered in our experiment, they appear not to show a strong relationship between chilling and thermal time to flowering (unpublished data). It is also possible that high chill species might experience faster budbreak with increasing winter temperatures, if chilling is accumulated more efficiently at higher temperatures. However, the pattern of a steep and linear decline of time to budburst for *A. rubrum* suggests that chilling at low temperatures is at least as effective at releasing dormancy as chilling at higher temperatures.

Because chilling affected all species in our experiment differently, it is reasonable to assume that rising global temperatures will have a noticeable effect on ecological interactions among tree species (Forrest and Miller-Rushing, 2010), especially competition for resources, such as light, with probable consequences for the composition of forests and woodlands (Augspurger and Bartlett, 2003; Laube et al., 2014). It is also likely that a changing climate will alter the interactions between trees and wildlife, potentially leading to asynchronies if the phenology of wildlife is influenced by factors other than chilling (Both et al., 2010; Polgar & Primack, 2011). These asynchronies could affect food and shelter for some species, especially long-distance migratory birds that overwinter in areas close to the equator where they are detached from changing climate cues of northern latitudes (Miller-Rushing and Primack, 2008), or for insects that primarily feed on young leaves (Polgar & Primack, 2011).

Looking at a local context, we saw that the CCESR between 1989 and 2017 lost on average 12.7 hours of chilling annually (Fig. 1.3, hourly weather data from CCESR and WRCC). If this trend was to continue linearly, the trees at the study site could experience on average 419 fewer chilling hours by the middle of this century compared to 2017, even more with potential positive feedback effects of global warming. While low-chilling species, such as *L. laricina* would be less affected by this, species with higher chilling requirements, such as *A. rubrum*, might leaf out later in the season.

Possible influence of life history traits and range on phenology

To understand the different responses of species to chilling, it is useful to compare different characteristics and ranges. In common garden studies, higher-latitude species

often leaf out before lower latitude ones (Hänninen, 1996; Myking and Heide, 1995), and for example *L. laricina* has a much more northern range than *A. rubrum*.

Several studies have shown that late successional species have a more conservative life-strategy than pioneers, requiring more chilling and leafing out later (Caffarra and Donnelly, 2011; Laube et al., 2014). Our results support this, as shown in very low chilling requirements of the pioneers *L. laricina* and *R. cathartica*, followed by the pioneers *B. papyrifera* and *P. tremuloides*, and increasing need for chilling for the late successional *Q. rubra*. On the other hand, the mid-successional species *A. rubrum* requires more chilling to break bud than would be anticipated.

Another explanation could be the organization of xylem. With some exceptions (e.g. Davi et al., 2011), it has been shown that ring-porous species break bud later than diffuse-porous species (Körner and Basler, 2010; Lechowicz, 1984; Wang et al., 1992). Compared to small vessels, large ones more often get damaged by cavitation in the winter and lose their hydraulic conductivity (Sperry & Saliendra, 1994), and new vessels need to be produced in the spring before leaves can be supported (Basler & Körner, 2012; Polgar & Primack, 2011). The results of our experiment are in general consistent with this, except for the diffuse-porous *A. rubrum*, which is much slower to break bud than the all the other species, including the only ring-porous species, *Q. rubra*. As a conifer, the wood anatomy of *L. laricina* differs from that of the other species, but the chance of embolism mainly depends on vessel diameter in angiosperms and gymnosperms alike (Pittermann and Sperry, 2003), and tracheids of *Larix* are medium-small (The Wood Database). On the other hand, (Panchen et al., 2014) found that deciduous angiosperms leafed out earlier than deciduous gymnosperms.

The spring phenology of the invasive shrub *R. cathartica* closely followed that of the low-chill *L. laricina*, with only slightly larger forcing requirements to break bud at all levels of chilling. Non-native forest invaders often use phenology to create a competitive advantage over native species (Fridley, 2012). It is believed that *R. cathartica*'s success in invading forest interiors in the U.S. is partially dependent on its ability to leaf out earlier and keep leaves longer than native tree species, effectively increasing its growing season (Becker et al., 2013; Converse, 1984; Knight et al., 2007). The high nitrogen and chlorophyll levels in early-spring leaves of *R. cathartica* also enable it to utilize the short window before canopy closure in the spring to fix large amounts of carbon (Harrington et al., 1989). Our results suggest that forcing requirements might play a role in this phenological characteristic, as *R. cathartica* required comparably small amounts of thermal time to break bud, even after short exposure to chilling. Consequently, with increasing temperatures, *R. cathartica* might become more successful in invading forests in North America in the future.

Characterization of chilling

We found a significant decrease in time to budburst with increased chilling for all species in our experiment, based on a model that assumes uniform accumulation of chilling at average temperatures below 5°C. However, this assumption is likely an oversimplification for several reasons, one of which is that chilling thresholds and ranges are thought to be species-specific (Basler and Körner, 2012; Heide, 1993; Morin et al., 2009; Myking and Heide, 1995). As a consequence, it is likely that our species accumulated chilling units at different temperature ranges and that this effect was not incorporated into our calculations. To adjust for that requires knowledge of species-

specific chilling requirements at the level of the bud. However, despite the existence of a large body of research about dormancy and budbreak, the underlying key mechanisms and genes involved in the interaction of chilling and dormancy release are still not fully understood (Hänninen et al., 2019; Howe et al., 2003; Rinne et al., 1997) and Kramer 2007). While chilling is known to play a role in triggering bud-intrinsic chemical hormonal changes involving gibberellic acid, abscisic acid, cytokinins and auxins (see literature review Arora et al., 2003) and to regulate cell-to-cell signaling within the apical meristem (Rinne et al., 2001), these chemical interactions have only been tested for a fraction of temperate forest tree species. To determine species-specific chilling requirements, one must consider the stage of dormancy, environmental conditions, as well as the relative importance of different temperature ranges and durations. Compared to our very simple model that weighs chilling units at every level equally, based on the threshold temperature <5°C, many more sophisticated models are used, but beyond the scope of this paper. For example, the fruit and viniculture industries have worked with more dynamic models (for example Utah Model) that are based on response curves that assign chilling units to temperature ranges (c.f. Alburquerque et al., 2008; E. A. Richardson et al., 1974), but not enough is known about temperate tree species to apply these models in our experiment. Many predictive models of leaf-unfolding have been developed for North American temperate tree species (for example (Clark et al., 2014; Clark et al., 2014; Ford et al., 2016; C. A. Harrington & Gould, 2015; Morin et al., 2009). Such models incorporate complex calculations of interactions between the main triggers of budburst (chilling, forcing, photoperiod) and climate change, but often contain uncertainties regarding parameters, model structure and drivers (Migliavacca et al.,

2012), leading to under- or overestimations of the timing of budbreak and senescence (Richardson et al., 2012).

In this paper, we treat chilling as the main factor that is interacting with the amount of thermal time units needed to cue budburst. However, we also show that our interpretation about the role of chilling can be altered by adjusting the range of temperatures that contribute to chilling from <5°C to a range from 0-7°C (Fig. 1.4). During the majority of December, January, and February of both experimental years, the average daily and hourly temperatures were primarily below 0°C at Cedar Creek. If the trees in our experiment only sensed above-freezing temperatures, as is sometimes suggested in the literature (e.g. (Arora et al., 2003; Rinne et al., 1997) and often applied in fruit tree research (Utah Models), the bulk of chilling accumulation would have occurred before the first collection in the fall, and in the spring. If this were the case, it would raise the question why we observed a steady decline in the timing to budburst with each subsequent collection.

One plausible explanation would be that the species we tested were indeed able to sense temperatures below 0°C. Alternately, the advance in budburst could be explained by a combination of internal and external cues that are partially temperature-independent, such as photoperiod. It is also plausible that endogenous mechanisms such as the circadian clocks allow trees 'count' days (Ibáñez et al., 2010), and that there is a relationship between number of days from some developmental milestone and the rapidity of budbreak (Yanovsky and Kay, 2003). More research is needed to untangle the relative importance of various cues for temperate tree phenology.

Conclusion

Our study shows that an increase in chilling resulted in more rapid budburst after exposure to thermal time for five North American temperate tree species and an invasive shrub, and that all species reacted differently to an increase in chilling. These results can be used as a basis for predicting the response of the species used in our experiment to a change in global temperatures. For species with low chilling requirements, increasing temperatures will most likely lead to earlier budburst, potentially prolonging their growing season. High chill species, on the other hand, may not benefit from advanced budburst and a longer growing season if their winter chilling requirements are only partially met, but this prediction has to be taken with care. Our results highlight the need to assess species-specific responses to dominant cues for budburst. This includes determining the range of chilling that is actually relevant for ending dormancy. Moreover, given the widespread distributions of the study species future research should also focus on different provenances (Migliavacca et al., 2012; Morin et al., 2009). Improving our knowledge of chilling-sensitivity contributes to better model predictions about possible vulnerabilities of species to global and local temperature increases.

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Tables

Table 1.1: Cumulative chilling hours (average hourly temperature <5 °C) for both winter seasons. Collections 1–3 in the winter 2011/2012 were paired with collections 4–6 from the winter 2012/2013 as they had comparable chilling hours.

2011/2012			2012/2013		
Collection	**Cumulative chilling hours**	**Collection dates**	**Collection**	**Cumulative chilling hours**	**Collection dates**
			1	945	19-Dec-2012
			2	1401	07-Jan-2013
			3	1665	18-Jan-2013
1	1924	02-Feb-2012	4	1977	31-Jan-2013
2	2252	16-Feb-2012	5	2313	14-Feb-2013
3	2682	06-Mar-2012	6	2649	28-Feb-2013
			7	2985	14-Mar-2013
			8	3308	28-Mar-2013

Figures

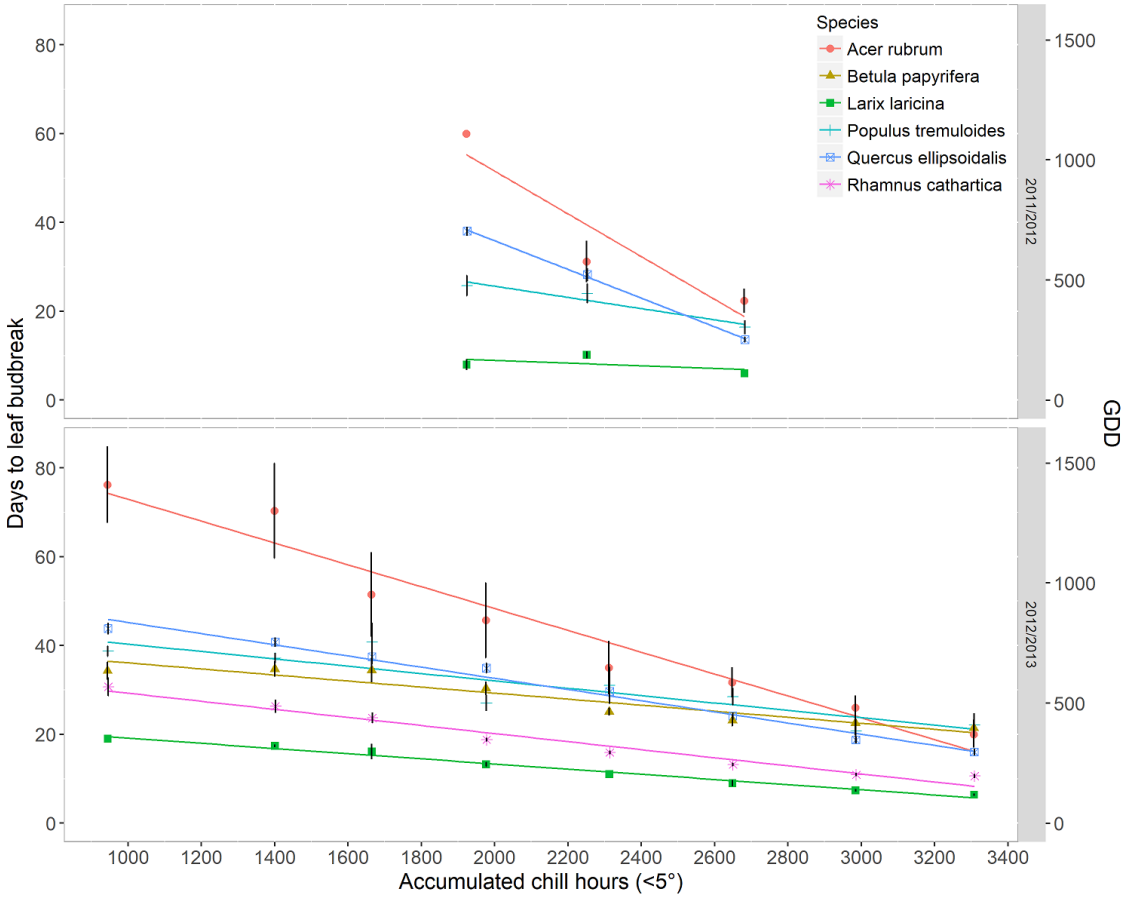

Figure 1.1: Time to leaf budbreak/thermal time (GDD's) plotted against accumulated ambient chilling hours (<5°C, starting Nov 1 according to Murray et al. 1989) for two seasons (2011/2013=Year 1, 2012/2013=Year 2) at Cedar Creek, MN. Budbreak is defined as a green leaf tip becoming visible at the end of at least one bud. Points show the average days to leaf budbreak for each species and each collection, error bars show standard errors. The first y-axis shows the number of days in the greenhouse (21°C /16°C, 16 hours photoperiod) required to break bud, the second y-axis shows thermal units (measured as GDD's). Data for both years were analyzed together.

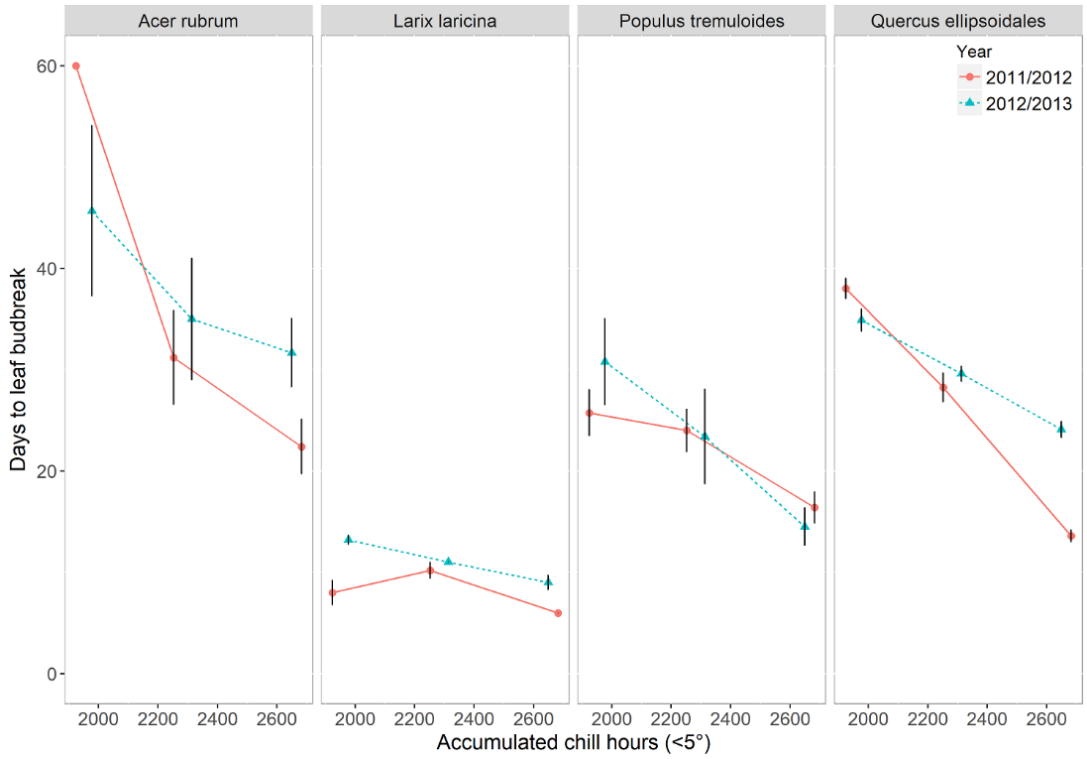

Figure 1.2: Comparison of days to budbreak with increased chilling between year 1 (2011/2012) and year 2 (2012/2013) for four overlapping species (*Acer rubrum, Quercus ellipsoidalis, Larix laricina, Populus tremuloides*). Points show the average days to leaf budbreak for each collection, bars are standard error bars. Chilling hour were accumulated starting Nov 1 of each year.

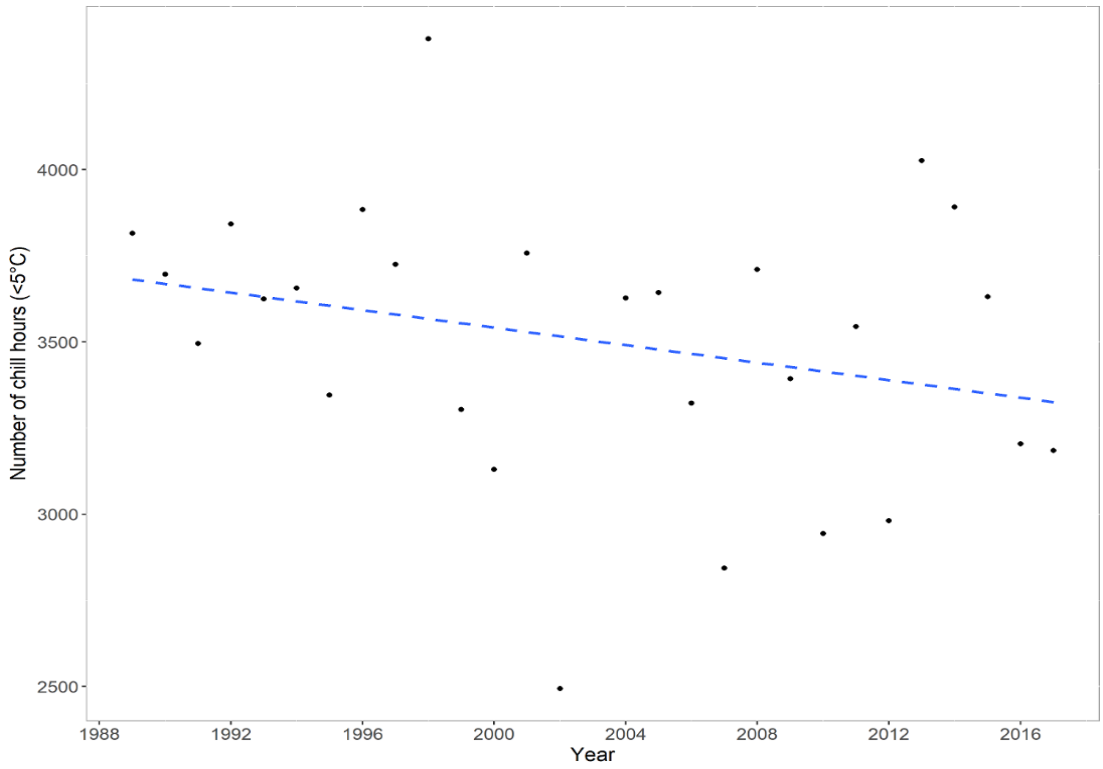

Figure 1.3: Accumulated chilling hours (<5°C, Nov 1st-May 1st), plotted by year between 1989 and 2017 at Cedar Creek, MN (data from 2003 was not available) (y=3694-12.7x, adjusted R^2=0.04, not significant). Hourly temperature data was not available for earlier years. Hourly temperature data came from the CCESR, and missing hours were complimented with data from the Carlos Avery RAWS weather station.

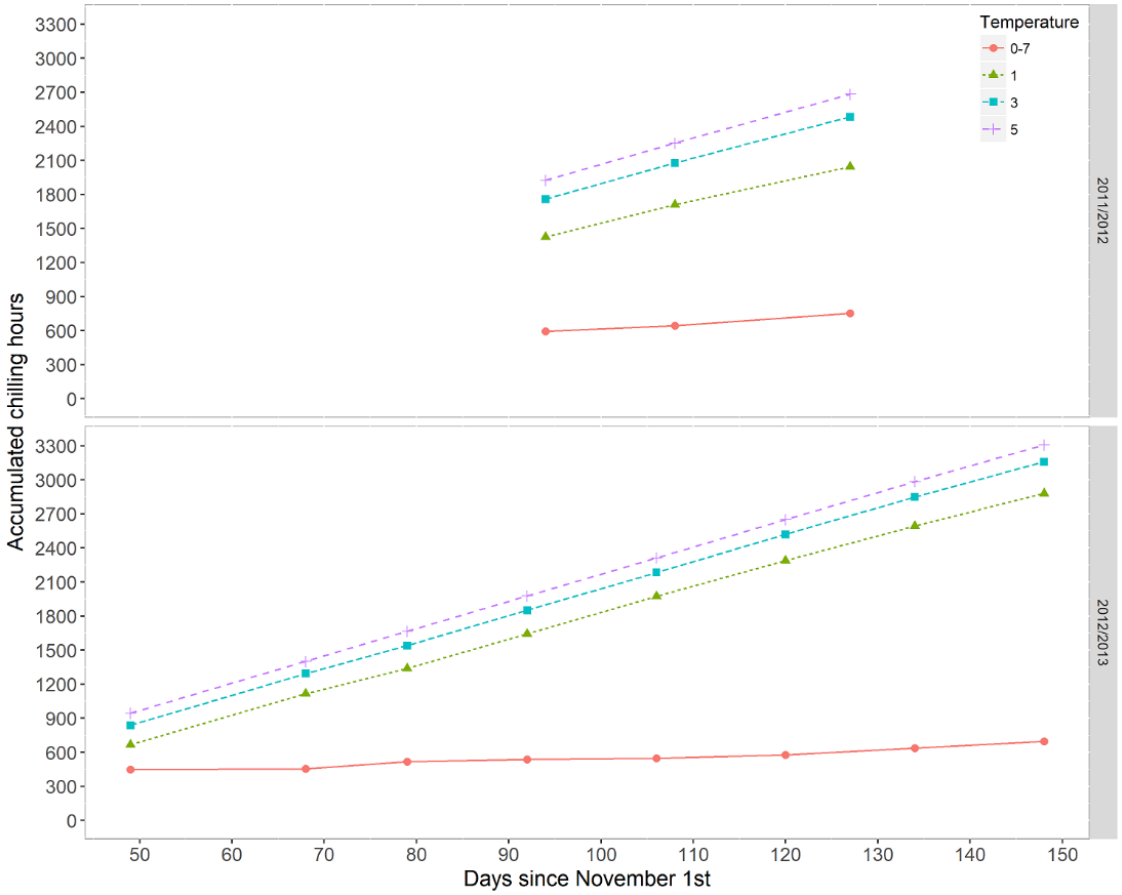

Figure 1.4: Comparison of winter chilling models with different temperature thresholds at Cedar Creek, MN (2011/2012 = Year 1, 2012/2013 = Year 2). Chilling hours were accumulated starting Nov. 1. Points show accumulated chill hours for each collection. Temperatures thresholds are <5° (y=1479.85+17.3x, adj. R^2=0.65), <3°C (y=1337.22+17.38x, R^2=0.69), <1°C (y=1068.19+17.56x, R^2=0.79), and 0-7°C (not significant). Data for both years was analyzed together.

Chapter 2: Comparison of chilling requirements of boreal and temperate tree species in Germany and North America

Claudia Nanninga*,, Rebecca A. Montgomery*, Annette Menzel***, Julia Laube******

**Department of Forest Resources, University of Minnesota, 1530 Cleveland Ave N, St. Paul, MN 55108*

****Email: nanni003@umn.edu, Phone: 612 987 8450***

**** TUM School of Life Sciences, Ecoclimatology, Technical University of Munich, Hans-Carl-von-Carlowitz Platz 2, 85354 Freising, Germany*

***** Ecological Engineering and Landscape Development, University of Applied Sciences Weihenstephan-Triesdorf*

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CN conceived and directed the study. CN wrote the initial draft of the manuscript and did the data analysis. All authors made significant contributions to the experimental design and writing of subsequent drafts. The authors declare that they complied with ethical standards and that have no conflict of interest.

Abstract

Chilling is an important cue in the spring phenology of boreal and temperate tree species. It is well established that increased chilling reduces the days to bud break (DTB), but detailed effects of different cold temperatures on chilling accumulation remain unknown for most species, and the discussion whether temperatures far below freezing contribute to chilling or not is ongoing. Depending on these detailed effects, future warmer winters could either reduce or increase chilling accumulation for different tree species and/or genotypes, resulting in delayed or advanced bud break. The consequence of changed budburst timing could be altered primary production, ecological interactions, and range limits. We investigated chilling effects on DTB experimentally, using twigs of boreal and temperate tree species in MN, U.S.A. (8 species), and Bavaria, Germany (6 species). In one experiment, we collected twigs and applied artificial chilling in cooling chambers at three different temperatures (-7/-6.5°C; 1.5/2°C; 4.5/4°C - USA/Germany) and with two different lengths (4/8 weeks). In a second experiment, we collected twigs at the same locations on three different dates and immediately placed them into forcing chambers at 21°C/16°C, 16 hours photoperiod. In both experiments, DTB was observed in the forcing chambers.

Both experiments showed that longer chilling reduced the DTB and that temperatures below freezing contributed to chilling accumulation. Varying chilling temperatures experimentally had a significant effect on DTB for seven out of the 14 species. Unexpectedly, the magnitude and direction of change with different chilling temperature was not linear, however for most species, higher chilling temperatures were more efficient than below freezing temperatures. With few exceptions, species growing

in Germany required less chilling, and boreal species broke bud before temperate species. Our study confirms the need to understand the species- and genotype-specific chilling requirements since generalizations seem inappropriate to correctly predict future leaf out timing.

Keywords: spring phenology, climate change, Germany, U.S.A, chilling, experiment, bud break

Introduction

The spring phenology of trees, i.e., the timing of leaf and flower bud break, is strongly affected by temperature. While warm or forcing spring temperatures eventually cause buds to break, most temperate and boreal tree species additionally respond to mid-winter chilling, which is usually defined as the accumulation of cold temperature units within a specific range. After reaching a certain chilling threshold, buds are moved out of a state of deep dormancy (endo-dormancy), during which development is halted, towards a state of shallow dormancy (eco-dormancy), during which the ability to develop under favorable conditions is re-instated (H. Hänninen, 2016). Long-term climate scenarios project disproportionally larger increases in winter temperatures in northern latitudes (Masson-Delmotte et al., 2021), which could alter the amount of received chilling, potentially changing the onset of spring events. The timing of bud break has shown to impact net primary production, ecosystem productivity, range limits, as well as tree distribution (Chuine et al., 2010; Gritti et al., 2013; Harrington & Gould, 2015; Morin et al., 2007), and physiology-based models have been used for decades to predict the future

phenology of different tree species to inform management decisions. To improve the predictive capacity of these models, it is essential to better characterize the role of chilling in the phenology of temperate and boreal tree species.

Chilling should be understood in the context of the two additional major phenological cues of forcing and photoperiod (day-length). The relationship between chilling and forcing is one of counterbalance: i.e., the more chilling the buds accumulate, the less forcing they require (Caffarra and Donnelly, 2011; Cook et al., 2012; Fu et al., 2019a, 2015a; Harrington and Gould, 2015; Heide, 1993; Man et al., 2021; Nanninga et al., 2017; Zhang et al., 2021). As a result, a lack of chilling during the winter months can increase the need for forcing and significantly delay bud break (Asse et al., 2018; Fu et al., 2015a, 2015b; Wang et al., 2022). Moreover, if chilling needs are not sufficiently met, bud break can be delayed, erratic (Harrington and Gould, 2015; Morin et al., 2009), or never occur at all (C. Polgar et al., 2014). Additionally, a lack of chilling can be compensated by high amounts of forcing (Bigler and Vitasse, 2019; Caffarra and Donnelly, 2011; Flynn and Wolkovich, 2018; Heide, 1993), but temperatures in the forcing range can also negate previously accumulated chilling (Rose and Cameron, 2009). Because of this 'dual effect of warming' (Morin et al., 2009) in the winter and spring, the trend towards earlier or later bud break with climate change is generally not linear (Chuine et al., 2016; Flynn and Wolkovich, 2018; Wolkovich et al., 2022). The third major cue, photoperiod, mainly acts as a buffer to reduce the risks of early bud break during periods in which late spring frosts still occur (Flynn and Wolkovich, 2018; Meng et al., 2021; Wenden et al., 2020; Zohner et al., 2020), but in some species also reduces the need for forcing (Flynn and Wolkovich, 2018; Fu et al., 2019b).

Although it is well established that increased chilling reduces the need for forcing or the days to bud break (DTB), there are still gaps in our knowledge regarding effective chilling temperatures, i.e., temperatures that are suitable for chilling accumulation. This is partly due to considerable variation among species, both in the sense of the effective temperature ranges and the quantitative thresholds for effective chilling (Harrington & Gould, 2015; Nanninga et al., 2017). Studies show that species can have mild, moderate, and strong needs for chilling (Nanninga et al., 2017; Polgar et al., 2014; Wenden et al., 2020), but the optimal species-specific chilling temperature ranges are not known for most species (Ettinger et al., 2020; Wenden et al., 2020; Zhang et al., 2021). For example, compared to Sarvas (1974) who found an optimal chilling rate at 3.5°C for *Fagus sylvatica*, Campbell & Sugano (1975) found it to be 4.4°C for *Pseudotsuga menziesii*. Especially it remains unclear if and how effectively chilling temperatures below freezing (<0°C) contribute to chilling accumulation (Chen et al., 2019; Luedeling et al., 2013; Wenden et al., 2020) or not. For example, Man et al., (2017) found that temperature below -3.4°C had no effect for boreal species in Ontario, and other studies suggest that below freezing temperatures do not contribute to chilling accumulation at all (Polgar & Primack, 2011). Contrary to that, a large body of literature found that <0°C temperatures are in fact suitable for chilling (Baumgarten et al., 2021; Ford et al., 2016; Güsewell et al., 2017; Meng et al., 2021; Nanninga et al., 2017; Zohner et al., 2020). Others suggest that above freezing temperatures are potentially more effective for chilling accumulation (Harrington et al., 2010).

Chilling requirements can also differ among similar species from separate locations (Misra et al., 2021; Zhang et al., 2021). High chilling requirements usually

occur in species or genotypes from warmer regions (Hänninen, 1996; Myking and Heide, 1995; Yang et al., 2020), such as lower altitudes (Bigler and Vitasse, 2019; Vitasse et al., 2009a, 2009b) and latitudes (Wenden et al., 2020), or generally from areas with more unpredictable weather patterns as a protective mechanism against late spring frost damage (Zohner et al., 2017). For those species or genotypes, forcing periods are generally shorter, which is related to the later fulfilment of chilling requirements to end endo-dormancy (Wenden et al., 2020). As a result, chilling is often a dominant cue for species in warm regions (Yang et al., 2020), which results in less responsiveness to climate warming for species from warmer parts of their distribution (Liu et al., 2019; Ma et al., 2018; Wenden et al., 2020). However, there is little information about species-specific differences in chilling requirement/responses linked to plant traits.

Differences in chilling requirements can also be found across continents. For example, Zohner et al., (2017) showed that temperature variability was higher in North America than in Europe, resulting in higher chilling requirements in North American species. Two recent studies from Minnesota and Ontario found that boreal species have lower chilling requirements and are therefore more responsive to chilling than temperate species (Man et al., 2021; Montgomery et al., 2020). Depending on whether or not warmer winter temperatures are more suitable for chilling accumulation, climate change could differently affect the chances of boreal and temperate species to fulfil their chilling requirements, potentially leading to phenological asynchrony and changes in competition (Montgomery et al., 2020).

While many observational studies show that warmer springs have advanced the timing of bud break around the world (Fu et al., 2015a; Menzel et al., 2020; Polgar et al.,

2014; Yu et al., 2017), others found that this trend has slowed down in the past few decades (Beil et al., 2021), possibly due to a lack of winter chilling caused by climate change (Chen et al., 2019; Fu et al., 2015b; Menzel et al., 2020; Wenden et al., 2020; Zohner et al., 2020). Whether or not a lack of chilling is indeed responsible for this trend depends on local winter temperatures and the effectiveness of these temperatures for chilling accumulation (Wang et al., 2022). Recent studies suggest that factors other than chilling might be contributing. For example, Menzel et al., (2020) demonstrated that the slowing trend of advancing bud break was still attributable to a slowing increase in spring temperatures, and Cornelius et al., (2013) showed the retarding influence of snow cover on smaller vegetation forms. Another study relates the slowing trend to delayed dormancy induction due to warmer fall temperatures (Beil et al., 2021). Additionally, Ettinger et al. (2020) found that, while chilling was the strongest cue in their meta-analysis, there was no evidence for reduced chilling for most sites, except for those with very warm average winter temperatures of >4°C. According to some studies, some species can also be categorized as 'spring-only responders', i.e., they only react to forcing in the spring and are insensitive to chilling (Cook et al., 2012; Pope et al., 2013). To confirm this, models that predict phenological spring events sometimes perform better when only pre-season warming (forcing) is considered (Dantec et al., 2014; Fu et al., 2012). However, other recent work points out the better performance of parameters describing the shift from endodormancy to ecodormancy (i.e., chilling) for accurate predictions (Chuine et al., 2016; Dantec et al., 2014; Zohner et al., 2017).

Understanding all facets of chilling and its impact on forcing requirements can help predict future bud break by improving phenological models, which is important to

inform management decisions in forestry, for urban green infrastructure, or species selection for assisted migration (Silvestro et al., 2019). Due to the interrelation of forcing and chilling, and their joint dependency on rather unknown temperature values, observational studies are limited. A need for more chilling research in the form of experiments has been expressed recently (Ettinger et al., 2020; Hänninen et al., 2019; Primack et al., 2015; Wolkovich et al., 2022; Zohner et al., 2017). However, while studies that deal with chilling are common, and while it has been well-established that increased chilling reduces the need for forcing (Laube et al., 2014; McKown et al., 2018; Nanninga et al., 2017), experiments that look at the actual chilling temperature and its effect on bud break and forcing needs are rare (but see Baumgarten et al., 2021). Additionally, to our knowledge, experimental studies that compare the impact of chilling temperatures on bud break between similar species from different continents do not exist. Here, we conducted a twig experiment to compare the effect of chilling on bud break for boreal and temperate species in Minnesota (MN), USA, and Bavaria, Germany. We tested the following hypotheses: 1. Below freezing temperatures contribute to chilling accumulation, hence reduce the time to budbreak; 2. Warmer chilling temperatures are less effective than colder chilling temperatures in reducing the days to bud break (DTB); 3. Longer chilling duration decreases the time to bud break. 4. Boreal species have lower chilling requirements than temperate species, both in the U.S. and Germany.

Methods

We conducted a chilling and greenhouse experiment across two continents with twigs of adult boreal and temperate tree species (Table 2.1). Twigs in the U.S. were collected at the Cedar Creek Ecosystem Science Reserve (CCESR) outside of East

Bethel, Minnesota (N45°24'7.29'', W93°11'57.85). Twigs in Germany were collected at Freising, Bavaria, located close to the Technical University of Munich campus (N48° 24' 1.38566", E11° 43' 4.82416"). The climate at Cedar Creek is continental, with mean temperatures of on average around -10°C and 21.5°C in the coldest (January) and warmest month (July) respectively (averaged from 1988 to 2014[4]). The average annual rainfall in Cedar Creek and Freising is around 660 mm and 792 mm respectively. Even though located at a higher latitude, the climate in Germany is influenced by the North Atlantic drift, resulting in on average milder winter temperatures in Freising compared to MN. Mean monthly temperatures in Freising range from -1.1°C in the coldest (January) and 17.9°C in the warmest (July) months (climate station Freising-Weihenstephan of the German Meteorological Service, 1981-2010).

We harvested twigs from 6 and 8 temperate and boreal tree species from Bavaria and MN respectively during three collection dates from beginning of November to mid-January in winter 2015/2016. Two species were sampled in both the U.S. and Bavaria (*Larix laricina*, *Quercus rubra*), all other samples were taken of species from the same genus (e.g. Birch - U.S.: *Betula papyrifera*, Bavaria: *Betula pendula*). We chose 10 donor trees per species in both locations from which to collect twigs. Some samples were lost while processing: Four twigs from the *Q. rubra* collection in Bavaria, and ten twigs of *P. grandidentata*, U.S., for the 4 weeks collection. The ambient temperature variations between November 1, 2015, and the end of twig collection were more extreme in

[4] Data from 01/01-01/04/2010 missing

Minnesota compared to Bavaria, ranging from a daily mean temperature of 13.3°C (11/4/2015) to -23°C (1/17/2016), compared to from 13.4°C (11/7/2015) to -7.4°C (1/15/2016) in Bavaria (Fig. 2.1).

Experiment 1: Artificial chilling in chilling chambers

To determine the effect of chilling temperatures and chilling length on timing of spring bud break, we conducted an experiment that included a combination of chilling temperature (3 levels) and time (2 levels), i.e., six combinations (U.S. and Germany respectively): ~-6.5/-7°C, ~1.5/2°C, ~4.5/4°C for a period of 4 or 8 weeks. Because it was difficult to adjust from Fahrenheit to Celsius, the temperatures in the chilling chambers varied slightly between Germany and the U.S. Chilling treatments were chosen for three reasons. First, they cover a broad range of chilling (11°C); second, temperatures far below freezing were included; and third, at chilling temperatures above the forcing threshold of 5°C (Bigler and Vitasse, 2019; Marchin et al., 2015; Vitasse and Rebetez, 2018), it becomes difficult to disentangle the effect of chilling and forcing in these kind of twig experiments. To exclude the effect of photoperiod, twigs were chilled in the dark (24/7). On November 21/27, 2015 in the U.S. and Bavaria respectively, we harvested n=10 twigs per species for each of six experimental conditions for a total of n=480 in MN (8 species) and n=360 in Bavaria (6 species). We used only twigs with a minimum of six live buds and made sure that they had a green tissue ring at the cut end, indicating they were alive and had the potential to break bud. After cutting, the twigs were placed in iced coolers and immediately transported to the University of Minnesota St Paul campus (USA) and Technical University of Munich-Freising campus (Germany).

Twigs in the warmer two treatments were placed in small glass jars with water, and twigs in the below freezing treatment were wrapped in moist tissue inside of Ziploc bags. Glass jars were checked for water on a regular basis and water was added when evaporation had occurred. We also cut the end of the twigs and exchanged the water on a weekly basis to minimize xylem clogging and potential problems with soiled water. After 4 or 8 weeks of chilling, the twigs were moved to growth chambers that were set to resemble spring-like temperatures: 21°C day/ 16°C night, 16h photoperiod.

In the growth chambers, the seedlings were scored for bud break every 2-3 days. Bud break was defined as at least 1 bud broken according to the Biologische Bundesanstalt, Bundessortenamt, and Chemical Industry (BBCH) 7: beginning of bud burst – first green leaf tips just visible (Meier, 2001). We also recorded the percentage of twigs that were able to break bud. Dead twigs and twigs with broken or dead buds were recorded and removed from the experiment and counted as unable to break buds. Potential causes for mortality were damage during cutting and handling, or twigs might have died during chilling treatments.

Experiment 2: Chilling at ambient temperature

To better understand the effect of chilling length on bud break in ambient conditions, we conducted a second experiment with the same species from both countries. We collected n=5 twigs per donor during three collections (USA/Bavaria: November 21/27, 2015, December 17/12, 2016, January 14/22, 2016) for a total of n=120 in MN (8 species) and n=90 in Bavaria (6 species). Twig collection and transportation were repeated as in the chilling chamber experiment. After arrival at the universities, the twigs

were immediately placed in small water jars and flushed in the warming chambers at 21°C/16°C, 16h photoperiod.

Because we were not able to measure when exactly the trees became dormant prior to collection, we started collections when all leaves of the donor trees had visibly senesced. Because it is possible that chilling had already accumulated in the buds before the collection, we calculated chilling hours at ambient temperature before the collection, starting November 1. We chose an upper chilling threshold of 5°C according to Murray et al., (1989). We chose November 1 as a random date for the initiation of chilling accumulation, because it is not uncommon for temperatures above 5°C to occur in October in both locations, and choosing November 1 increased the likelihood of buds being dormant before the initiation of the experiment. To calculate the amount of accumulated chilling days prior to collection, we used weather data from the CCESR (USA) and the Technical University Munich (Germany). Chilling was calculated as hours below the threshold of <5°C.

Statistical Analysis

All data were analyzed, and all graphs were produced with R statistical software (R 3.3.2, (Crawley, 2007). We examined the effect of chilling temperature, and chilling length on bud break timing and bud break percentage for all species in the experiment from Germany and the U.S. Analytical assumptions of normality and homoscedasticity for the models were examined by graphical inspection of residuals. The effects of chilling temperature, chilling time, species, and their interaction on days to bud break were analyzed using linear models. We initially ran a full model that included data from both

Germany and the U.S. However, because we found a significant difference between the data from both countries, we analyzed all the data from the U.S. and Germany separately.

For experiment 1 (artificial chilling in chilling chambers), we analyzed the full models for U.S. and Germany data using mixed effects models. The response variable, DTB, was log(x) transformed and fit as a function of chilling time (discrete) and chilling temperature (discrete) with species as random effect. To examine the response of each species to chilling time and chilling temperature on DTB, we also ran separate analyses for each species in both countries using ANOVA.

For experiment 2 (chilling at ambient temperature), the impact of chilling length on the timing of bud break was analyzed using ANCOVA. The continuous predictor was chilling hours, and the discrete predictor was species. The response variable DTB was log(x) transformed. The second order term chilling time$^{\wedge 2}$ *(i.e., chilling time squared* to account for potential curvature of the data) was not significant for either location, so we excluded it. We chose models with higher adjusted R^2: Germany data was analyzed without interactions and U.S. data included interactions.

Chilling hours were calculated as hours <5°C accumulated in the field since November 1st (Murray et al. 1989, Polgar and Primack 2011, Laube et al. 2014). Because of fixed temperature conditions in the greenhouse, accumulated days to bud break were chosen as the response variable (as opposed to GDD's, which in our case represent a transformation of the number of days to budbreak).

Results

Experiment 1: Artificial chilling in chilling chambers

In the full models for both countries, chilling temperature and chilling time significantly affected the time to bud break, but their interaction was not significant. Longer chilling time significantly decreased the DTB (USA: $F_{1,392}$=158.92, P<0.001, Germany: $F_{1,369}$=46.01, P<0.001, Fig. 2.2), but even though chilling temperature significantly affected DTB in both countries (USA: $F_{2,392}$=8.20, P<0.001, Germany: $F_{2,369}$=9.40, P<0.001, Fig. 2.2), the actual temperature effect was mixed, i.e., warmer chilling could either increase or decrease DTB. Consequently, we analyzed the effect of chilling temperature and time for each species and each country separately.

Chilling temperature significantly affected the timing to bud break for seven out of the 14 species (Table 2.1, Fig. 2.3). Of these seven species, the only species to break bud earlier at colder chilling was *A. saccharinum*. However, this trend was only apparent after 4 weeks of chilling, where in the -6.5°C treatment, twigs needed on average 32 (±1) days of forcing to break bud, while in the +4.5°C treatment, they needed on average 40 (±2) days of forcing. All other species that were significantly affected by chilling temperature broke bud faster in the 1.5/2°C and 4.5/4°C treatments compared to the -6.5/-7°C treatment (Fig. 2.3).

We found a significant difference in DTB among all species in the U.S. and Germany. In both countries, species of the *Acer* family required the longest time to break bud, while *L. laricina* required the least time (*Appendix SI*, Fig. S2.1). For 2 of the 14 species in the experiment, DTB was neither significantly impacted by chilling time nor by temperature (*P. grandidentata*, *Q. robur*), and chilling time was not significant for *B. pendula*. For 11 out of the 14 species, chilling time was significant. For example, over all chilling temperatures, time to bud break for *A. pseudoplatanus* was on average 39 (±1)

after 4 weeks, compared to 32 (±1) after 8 weeks – a difference of close to 8 days. There was also higher variability regarding bud break after 4 compared to 8 weeks of chilling (*Appendix SI*, Fig. S2.1).

Comparing the two overlapping species in Germany and the U.S., we found significant differences between country of origin for both species (Fig. 2.4). *L. laricina* from Germany was significantly faster at breaking bud under the same experimental conditions than the same species from the U.S. ($F_{3,95}$=23.12. P<0.001). *Q. rubra*, on the other hand, showed the opposite results, i.e., twigs from the U.S. required significantly less time to break bud under the same conditions ($F_{3,95}$=37.03, P<0.001). For *L. laricina* the difference between both countries was on average only 2.5 days (Ger: 10.21 (±0.48), USA: 12.70 (±0.39)), but for *Q. rubra* the difference was 9 days (Ger: 35.71 (±1.2), USA: 26.61 (±1).

All species in the experiment, in both Germany and the U.S., were able to break bud after being treated in the chilling chambers, and the bud break percentage (survival) was generally high (on average >60%, except for *L. laricina* in the 1.5/2°C treatment, where bud break percentage was ~50%) (*Appendix SI*, Fig. S2.2a). There was no detectable pattern in the rate of survival among the three chilling treatments and between chilling time.

Experiment 2: Chilling at ambient temperature

For both locations, DTB was significantly affected by the length of chilling exposure (USA: adj. R^2 = 92, Germany: adj. R^2 =0.82, Fig. 2.5). Longer exposure to chilling significantly reduced the time required to break bud (USA: $F_{1,75}$=314.50, P<0.001, Germany: $F_{1,80}$=50.73, P<0.001), and there was a significant difference between species

(USA: $F_{7,75}$=90.57, P<0.001, Germany: $F_{5,80}$=50.18, P<0.001), but the interaction among species and chilling time was only significant for the U.S. (USA: $F_{7,75}$=7.01, P<0.001). As an example, *Quercus rubra* buds from the first collection broke on average around 15 and 6 days later than buds from the third collection in Germany and the U.S. respectively. Because all species were able to break bud after the first collection, it can be assumed that chilling requirements for dormancy release were already fulfilled. At temperatures below freezing, there was still a continuous decrease in the need for forcing with increased chilling, suggesting that those temperatures were contributing to chilling accumulation. This is especially relevant for twigs from Minnesota, which were exposed to average daily temperatures of <0°C for the last 30 days of the experiment.

The timing to bud beak was significantly different for all species in Germany and the U.S. At both locations, species of the *Acer* family broke bud slowest and required on average more chilling than all other species, while *Larix laricina* broke bud fastest and required on average less chilling (Fig. 2.5). However, because of steep declines in need for chilling in some species, these difference between DTB became less pronounced after the third collection when more chilling had been accumulated. For example, while after the first collection the difference between average DTB for *A. rubrum* and *L. laricina* was 43.5 days (74.75 (±4.97 SE) for *A. rubrum* vs 31.25 (±0.75 SE) for *L. laricina*), after the third collection it was only 19.8 days (±1.58 SE) for *A. rubrum* vs 8.25 (±0.75 SE) for *L. laricina*). When directly comparing species of the same family between both locations, it was evident that the patterns of bud break were similar (Fig. 2.5). The slopes of reduced DTB with increased chilling were comparable for most species in the same families.

Bud break percentage or survival was generally high for all species (>50%), but more consistently close to 100% in Germany, except for *Q. rubra* from the third collection. All species in the experiment in both countries were able to break bud after being treated in any of the chilling treatments, which means that sufficient chilling had been accumulated within the buds to enable bud break (*Appendix SI*, Fig. S2.2b). Like with the twigs chilled in chambers, there was no detectable pattern in the rate of survival among the 3 chilling treatments.

Discussion

The timing of spring bud break can be a very important factor in the fitness of temperate and boreal tree species, partially because earlier springs allow for an earlier achievement of minimum resources that are needed to initiate reproduction (Journé et al., 2021), may influence the number of flowers as such (Meng et al., 2022) or trigger spring frost risks (Zohner et al., 2020). Chilling as a phenological cue can impact bud break timing, which can lead to ecological advantages or disadvantages. For example, if a species can benefit from warmer future winter temperatures better than others, it might be able to break bud and photosynthesize earlier, potentially outcompeting other species (see e.g Uphus et al., 2021). Understanding the response of species to chilling is therefore important when making future predictions using phenological models. In our experiments, we exposed temperate and boreal species to different chilling lengths and temperatures, and we collected twigs during different times throughout the winter, after which we forced them in heated chambers, to elucidate the effect of chilling on their timing of leaf bud break. Most species in both locations broke bud earlier after chilling with mild temperatures in comparison to colder temperatures, but even the twigs from our

coldest treatments (-6.5°C U.S./-7°C Germany) accumulated chilling and broke bud earlier after longer exposure to chilling (8 vs. 4 weeks). Additionally, boreal species in general broke bud faster in both locations than temperate species, and overall, species from Germany required less chilling than species from the U.S.

Timing of bud burst related to chilling temperature

Both our experiments confirm that chilling is an important aspect in the timing of spring leaf bud break. We show that for half of the species in our experiment, exposure to various chilling temperatures lead to significant differences in bud break timing. However, chilling temperatures were not equally effective. While for most species, warmer chilling decreased the need for forcing most, this relationship was not always linear. For example, for *Q. macrocarpa*, after eight weeks, chilling was least effective in the 4.5°C treatment, and for *A. saccharinum*, colder chilling increased the time to bud break. This is in line with Wang et al. (2022) who found a negative correlation of warmer chilling and chilling accumulation. However, all of our other species that were significantly affected by chilling temperature broke bud faster at warmer chilling temperatures (Fig. 2.3), indicating that, depending on location and current winter temperatures, climate changed induced increases in mid-winter temperatures could accelerate their bud break in the future, which confirms findings from Harrington & Gould (2015) and Man et al., (2017). Our results suggest that overly simple generalizations regarding chilling requirements must be avoided and that species-specific chilling requirements need to be considered to better inform predictive models. It also suggests that the recent slowing trend of earlier spring phenology cannot simply be explained with warmer winter chilling for all species, and that whether warmer winter

temperatures play a role depends on the region and the prevailing winter temperatures. This is also in line with Ettinger et al., (2020) who established that reduced chilling could only be detected in very warm regions.

Recent studies found that the maximum and minimum temperatures during the day (Bigler and Vitasse, 2019) and the diurnal temperature range between maximum and minimum temperatures (Huang et al., 2020) can have an important effect on the timing of bud break (Meng et al., 2019). In this study we did not account for that, and twigs were chilled at constant temperatures in the dark. Future experiments could incorporate even larger chilling ranges and differentiate between the diurnal temperature ranges.

Our results also indicate that chilling can be accumulated at below freezing temperatures, because very cold chilling (both ambient chilling and the artificial -6.5/-7°C treatments) contributed to advances in bud break after increased chilling time, showing that buds accumulated chilling units at those low temperatures. This is in line with findings from Baumgarten et al., (2021). However, contrary to those findings, for most of the species in our experiment, chilling above freezing was more effective. Additionally, in both experiments the survival of twigs at temperatures far below freezing was very high. In many phenological studies and models, a lower chilling threshold of 0°C is used, and for boreal species, the lower threshold is sometimes set to -3.4°C (Man et al., 2017). Our findings suggest that these thresholds should not be universally applied, and that all temperatures below freezing should be considered. It also supports the findings of Zohner et al., (2020), who found that in Europe, phenological models for boreal species performed better when below freezing temperatures were included, compared to models with a chilling range of 0-5°C.

Finally, our study clearly confirmed that across both countries, the length of chilling is an important factor in reducing the time to bud break for 78% of the species in experiment 1 (*artificial chilling in chilling chambers)* and for 100% of the species in experiment 2 (*chilling at ambient temperature*). Generally, the effect of chilling time was stronger for species from the U.S. (Fig. 2.2 a and b). Chilling time also had a stronger effect than chilling temperature (see also Baumgarten et al., 2021). For the three species that showed no response to chilling length in the chilling chamber experiment (*P. grandidentata*, *Q. robur*, *B. pendula*), it is possible that optimal chilling requirements were met after four weeks, and that consequently increased chilling had no added effect (Harrington et al., 2010).

Species differentiation

The magnitude of phenological trends in our experiments differed among species in both countries. While some species required large amounts of chilling to reduce the need for forcing, others broke bud quickly after short exposure to chilling. For example, our results confirm findings from previous studies that *A. rubrum* requires high, while *L. laricina* requires low amounts of chilling (Harrington and Gould, 2015; Nanninga et al., 2017). Two species, *P. grandidentata* and *Q. robur*, responded to neither chilling temperature nor length, and could therefore be classified as non-responders (Cook et al., 2012). They did, however, show decreases in the time to bud break with longer chilling under ambient chilling conditions. With future warmer winters, the bud break of non-responders is unlikely to be largely affected, while the species with high chilling requirements might experience insufficient chilling. The consequence could be delayed bud break (Man et al. 2021) or erratic formation of leaves (Chuine et al., 2016;

Harrington and Gould, 2015; Man et al., 2017). In a warming experiment in Minnesota, (Montgomery et al., 2020) found a stronger phenological response of boreal compared to temperate species in warmer plots. We also showed that, apart from *P. tremula* in Germany, boreal species broke bud on average faster than temperate species, i.e., they required less chilling or possibly less forcing. Additionally, there was a trend towards faster bud break with warmer chilling among boreal species. As a group, boreal species might therefore benefit more than temperate species from the expected warmer winters in northern latitudes in the future, which could have consequences on ecological interactions and range limits. On the other hand, if they break bud too early, they might also be at higher risk of late frost damage (Man et al., 2017; Menzel et al., 2015). However, remote sensing has shown that between 1984 and 2013, U.S. boreal forests show more heterogeneous phenological changes with climate change than temperate forests (Melaas et al., 2018), emphasizing again the importance of understanding each species' chilling and forcing preferences separately to make accurate predictions.

Many studies suggest that higher chilling requirements in some species or genotypes can be a protective mechanism. For example, species from warmer areas (lower latitudes and altitudes) with early warm springs often have high chilling requirements, which delays bud break and protects from late spring frosts (Jensen and Hansen, 2008; Salk, 2020; Usmani et al., 2020; Vitasse and Rebetez, 2018). This is confirmed by recent studies, which show that warmer winters, and hence warmer chilling temperatures, are currently advancing bud break in cold regions, while they are delaying bud break in warm regions (Man et al., 2017; Wenden et al., 2020; Yang et al., 2020). Zohner et al., (2017) also found higher chilling requirements in species from North

America compared to Europe, and suggested the reason was a protective mechanism from more unpredictable temperature patterns in North America. Our study supports these findings. Comparing the same species or species with the same genus across both countries, we found that individuals from Germany generally broke bud faster than those from the U.S., suggesting that they had lower chilling requirements or possibly lower forcing needs. This was most pronounced after 4 weeks, compared to 8 weeks of chilling (Fig. 2.2 c) in experiment 1 (*artificial chilling in chilling chambers)* and for the first 2 collections (Fig. 2.5 a) in experiment 2 (*chilling at ambient temperature*). The opposite was true, however, for *Q. rubra*, both in experiment 1 (Fig. 2.4) and experiment 2 (Fig. 2.5). Despite these differences, the patterns of bud break were very similar for species of the same family, which is especially apparent in the similar or even overlapping slopes of reduced DTB with increased chilling in experiment 2 (Fig. 2.5). This suggests that the difference in chilling requirement can be related to the different genotypes and adaptation to local climates in both countries and suggest that generalizations regarding the phenology of the same or similar species across different regions should be avoided.

When comparing the phenology of different species and/or genotypes, we are aware that chilling is not the only important cue that could impact observations. It is hence possible that, additionally to different chilling preferences, boreal species and species from Germany also responded more readily to forcing accumulation in the growth chambers, which could have contributed to the faster bud break. This study concentrates on and discusses the effect of chilling. The significantly reduced need for forcing with increased length of chilling shows that chilling influenced bud break timing, and how much the different forcing requirements impacted DTB is beyond the scope of this study.

Implications for modeling phenology

Some recent studies discuss the relevance of chilling in bud break and the necessity to better understand chilling to improve the predictability of phenological models (Ettinger et al., 2020; Flynn and Wolkovich, 2018; Hänninen et al., 2019; Zohner et al., 2017). These models are relevant as they help in predicting future range limits and can support management decisions and the prioritization of management areas (Ettinger et al., 2020). After decades of observed advances in bud break, a slow-down of this trend has been observed in recent years. Some studies relate this slow-down to reduced winter chilling (e.g. Fu, Zhao, et al., 2015), or suggest it could be a statistical artifact (Ettinger et al., 2020). Others found that it could be caused by an earlier pre-season with warmer temperatures (Güsewell et al., 2017), reducing the amount of photoperiod during the forcing period (Fu et al., 2019b; Wenden et al., 2020). Our findings confirm that winter chilling is highly relevant in determining the timing of bud break and should be considered as a factor in phenological models. However, in our experiment, warmer chilling temperatures were more effective at reducing the DTB for many of the species, suggesting that the slowing trend of advanced DTB might not be related to chilling for all species. This again highlights the importance of understanding the species- and genotype-specific responses to chilling, especially temperature ranges suitable for chilling accumulation. The effectiveness of chilling, which weighs the effect of chilling temperature on the need for thermal time, is commonly depicted as a bell-shaped curve with a peak of effectiveness calculated as one chilling unit, and a decrease in effectiveness up to an upper and lower threshold (Chuine, 2000; Sarvas, 1974). The species in our experiment do not show this pattern, with the exceptions of *L. laricina*

(USA), and *Q. robur* after four weeks of chilling. It is possible that the peaks of species were located outside of the chilling range used in this experiment. We also set the upper temperature threshold in the chilling chambers to 5°C (Richardson et al., 1974), the estimated lower threshold for forcing, because at higher temperatures it would have been impossible to distinguish the effect of chilling and forcing (H. Hänninen, 2016). We did, however, use a large range of chilling temperatures of 11°C in both countries, which suggests that maybe the bell-shaped curve is not the ideal way of calculating chilling accumulation for all species and that chilling can be accumulated at large temperature ranges. It is possible that for those species with no significant response to chilling temperature, simpler threshold models are more suitable (H. Hänninen, 2016). For the two species (*P. grandidentata*, *Q. robur*) that showed neither sensitivity to chilling time nor to chilling temperature, it might be possible to exclude chilling as a parameter from phenological models.

Conclusion

Our experiments confirmed that chilling is an important cue for the spring bud break in temperate and boreal tree species in both the U.S. and Germany. In contradiction to current models, our data clearly shows that below freezing temperatures contribute to chilling accumulation and therefore these need to be incorporated into phenological models. For most species in our experiment, increased chilling reduced the DTB, and half of the species experienced changes in the DTB when chilled at different temperatures. Because the magnitude and direction of change with changing chilling temperatures were not linear, we expect varying responses of species and genotypes to warming winter temperatures. Generally, boreal species broke bud earlier than temperate species, and

patterns of bud break were similar. Our results highlight the need for further experiments to best understand species-specific chilling requirements, both regarding temperature and time, and that generalizations should be avoided, especially in phenological models.

Tables

Table 2.1: Statistical analysis (ANOVA) of the effect of chilling time (chilltime) and chilling temperature (chilltemp) on the time to bud break in 6 species from Germany and 8 species from the U.S. Significance levels are ***highly significant, **moderately significant, *marginally significant. We did not add the direction of change as it was not always linear (see Fig. 2.3).

COUNTRY	SPECIES	TYPE boreal = B Temperate = T	CHILLTIME	CHILLTEMP	INTERACTION
USA	*Larix laricina* (LaLa)	B	<0.001***	0.11	0.57
USA	*Populus tremuloides* (PoTr)	B	<0.001***	<0.001***	0.27
USA	*Betula papyrifera* (BePa)	B	<0.001***	<0.001***	0.33
USA	*Acer saccharinum* (AcSa)	T	<0.001***	0.081	0.15
USA	*Acer rubrum* (AcRu)	T	<0.001***	0.75	0.25
USA	*Populus grandidentata* (PoGr)	T	0.31	0.87	0.57
USA	*Quercus Macrocarpa* (QuMa)	T	<0.001***	0.0083**	0.23
USA	*Quercus rubra* (*QuRu*)	T	0.0011**	0.018*	0.31
GER	*Larix laricina* (LaLa)	B	<0.001***	0.28	0.50
GER	*Populus tremula* (PoTre)	B	<0.001***	0.025**	0.59
GER	*Betula pendula* (BePe)	B	0.14	0.017**	0.85

GER	*Acer pseudoplatanus* (AcPs)	T	<0.001***	0.68	0.12
GER	*Quercus robur* (QuRo)	T	0.15	0.46	0.77
GER	*Quercus rubra* (QuRu)	T	<0.001***	0.0073**	0.059

Figures

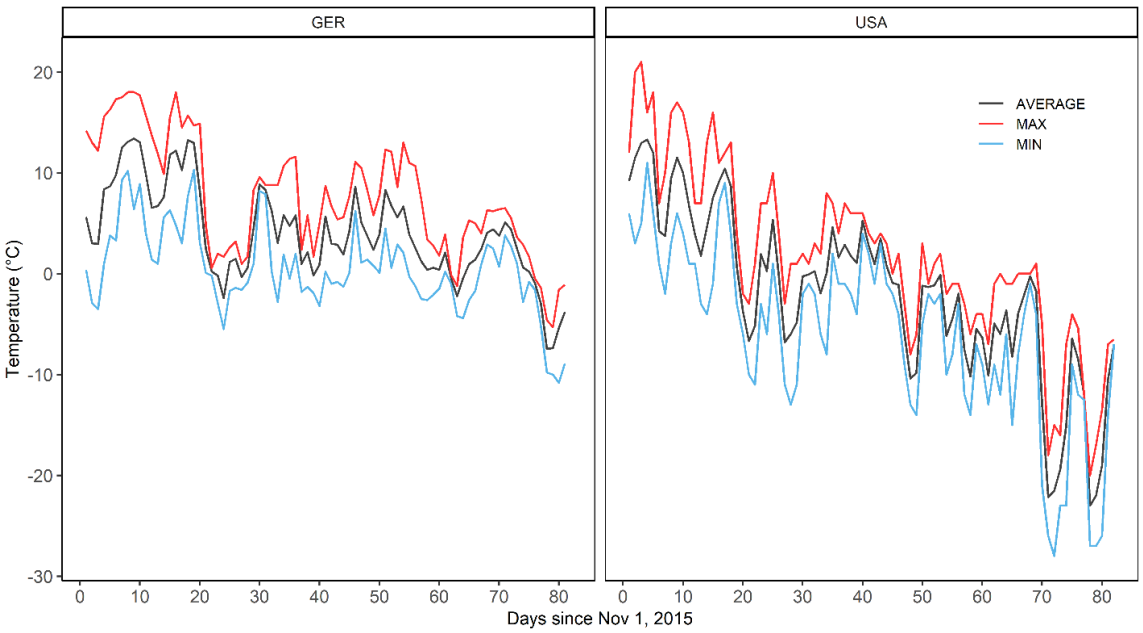

Figure 2.1: Daily ambient temperatures (average, maximum and minimum °C) in Bavaria and Minnesota starting November 1, 2015 throughout the 8 weeks of twig collection.

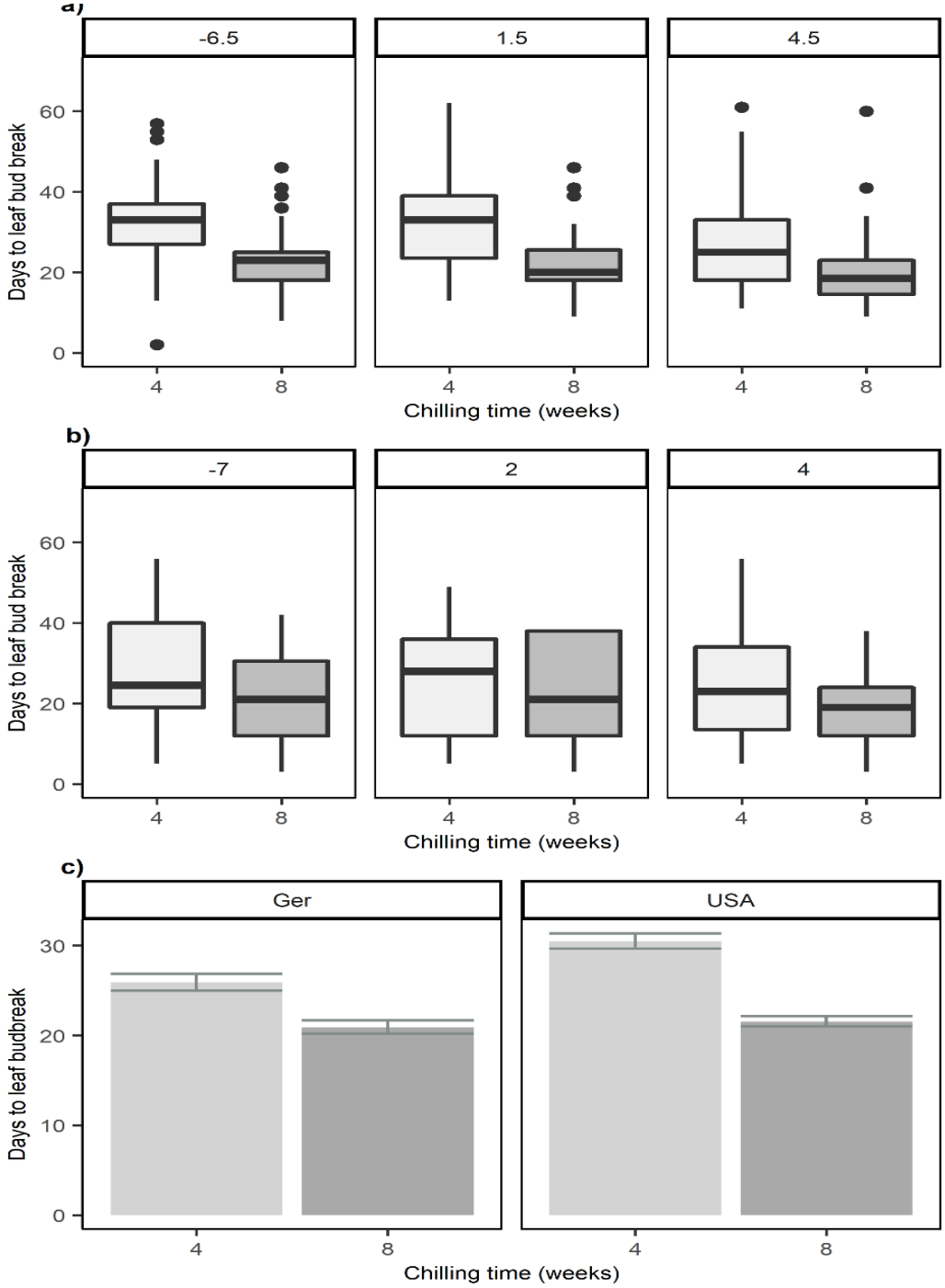

Figure 2.2: Effect of experimental chilling on the time to leaf bud break in twigs from 8 species in the a) USA and 6 species in b) Germany. Twigs were chilled at 3 different temperatures and after 4 weeks (light gray) and 8 weeks (dark gray) exposed to 21°C/16°C in growth chambers until leaf bud break. The graph shows the effect of chilling length (4 and 8 weeks) across all

chilling temperatures and species for the U.S. and Germany. The average days required to achieve bud break are shown in c).

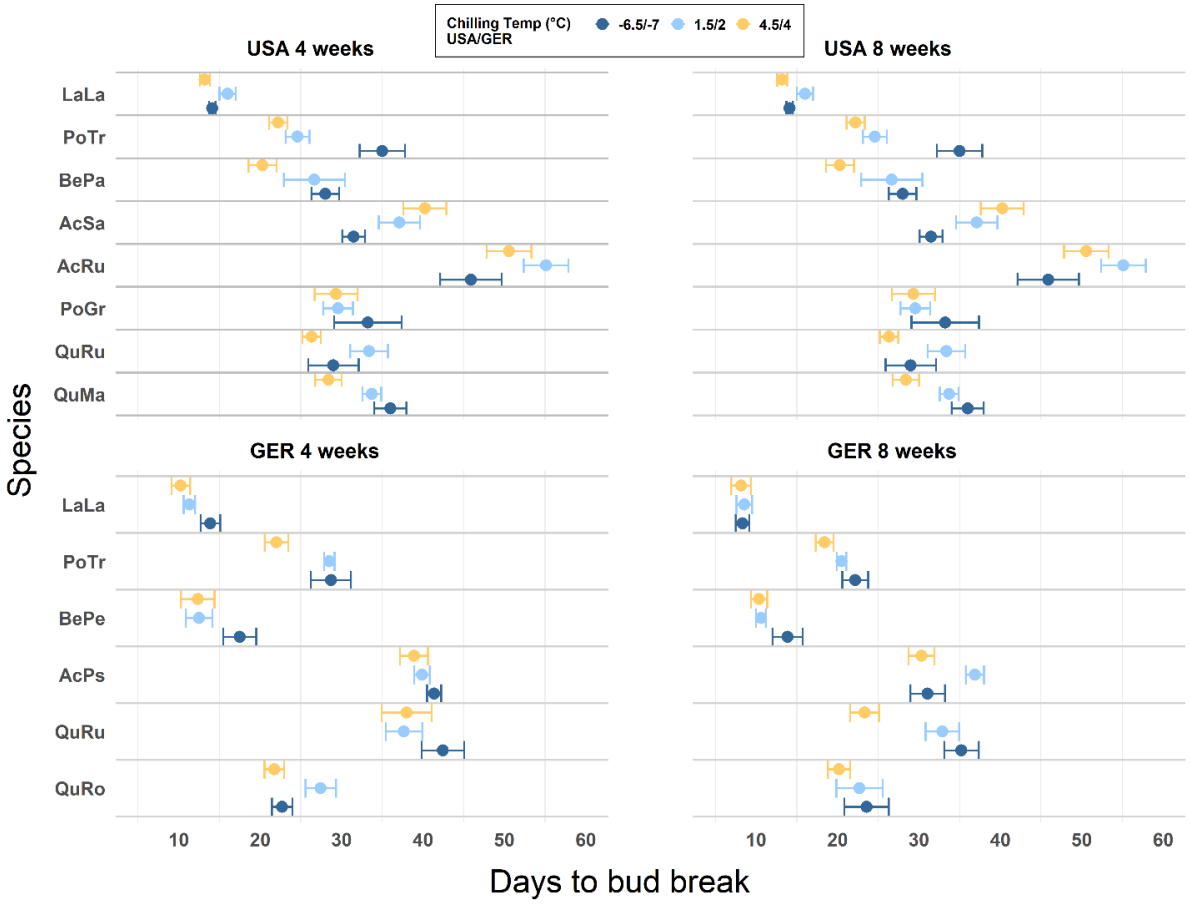

Figure 2.3: Effect of experimental chilling on the time to bud break in twigs from 8 species in the USA and 6 species in Germany. Twigs were chilled at 3 different temperatures and after 4 and 8 weeks exposed to 21°C/16°C in growth chambers until leaf buds burst. Boreal species in both the U.S. and Germany are listed first.

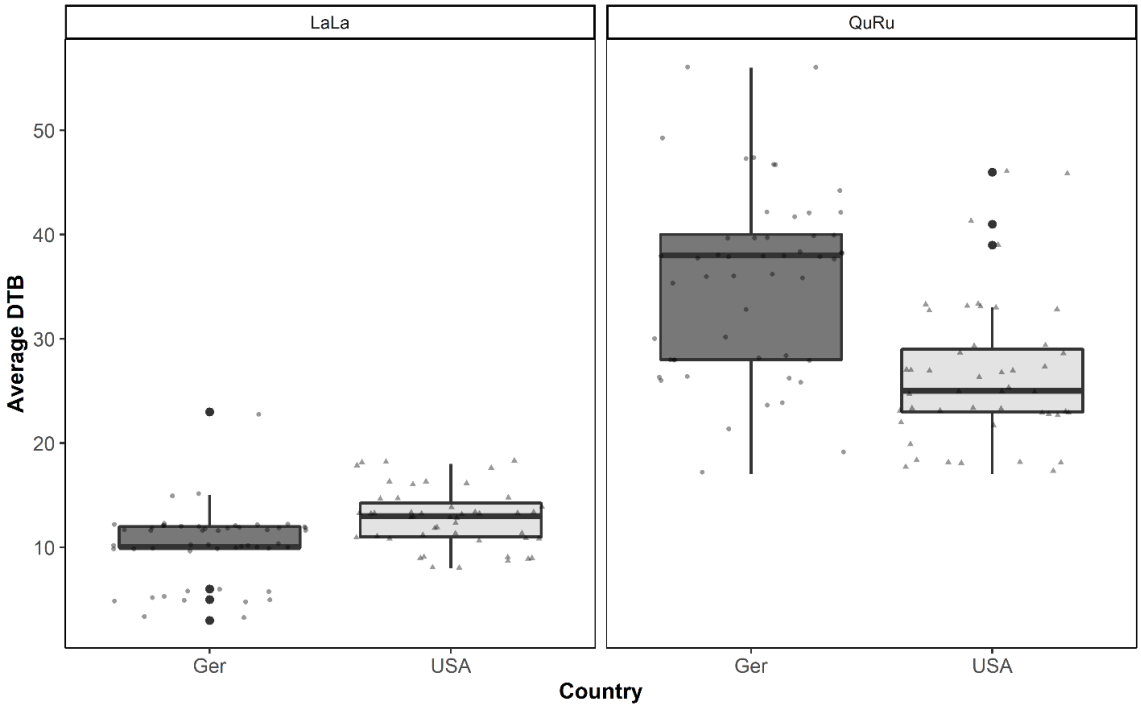

Figure 2.4 Effect of experimental chilling on the time to bud break in twigs from Larix laricina and Quercus rubra in the USA and Germany. Twigs were chilled at 3 different temperatures and after 4 and 8 weeks exposed to 21°C/16°C in growth chambers until leaf buds burst. The graph compares the days to leaf bud break over all experimental conditions for both species and both countries. There is a significant difference of DTB with chilling exposure for both Q. rubra ($F_{1,97} = 32.59$, $P<0.001$) and L. laricina ($F_{1,97} = 16.85$, $P<0.001$).

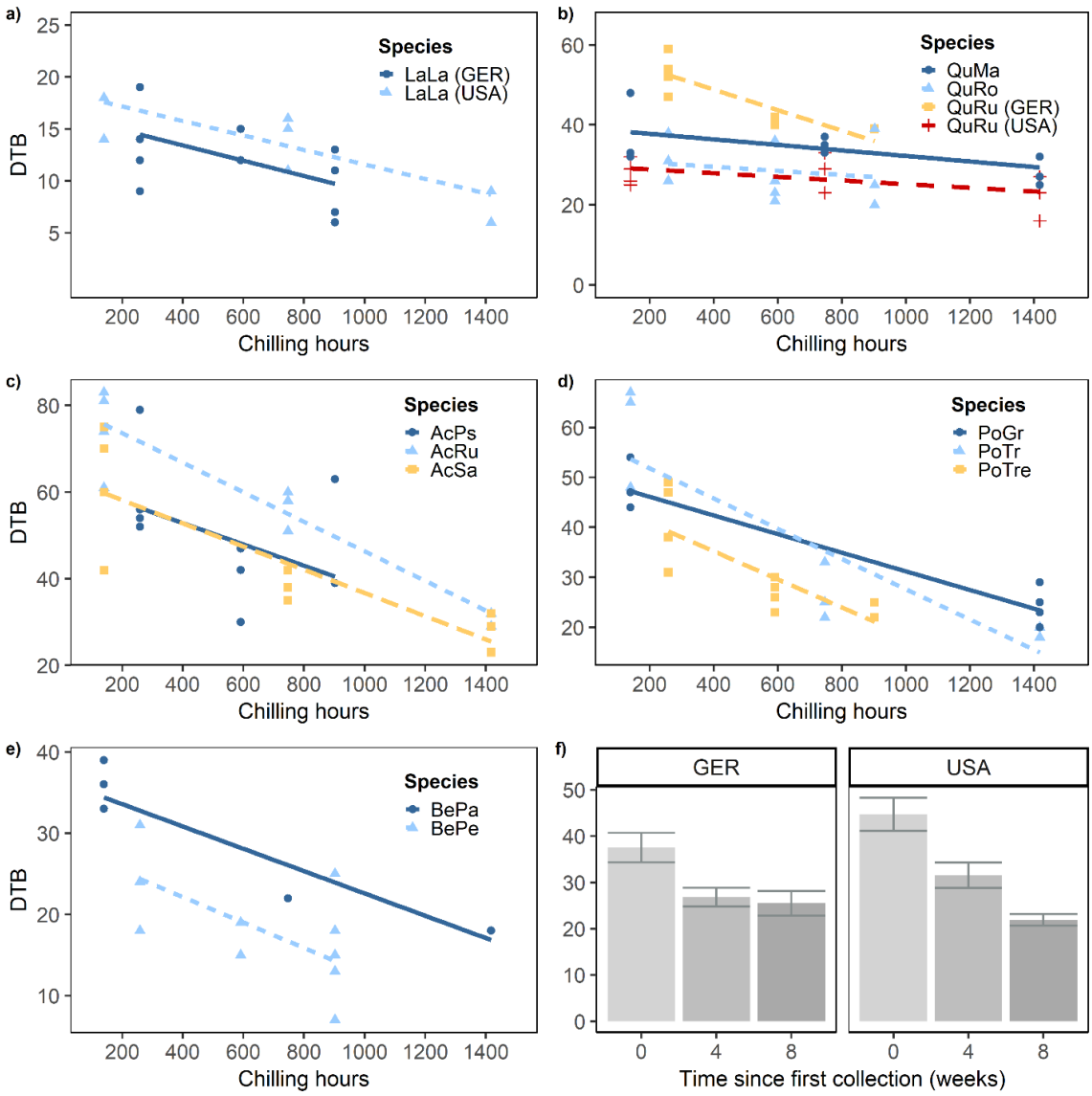

Figure 2.5: Effect of ambient chilling on the time to bud break in twigs from boreal and temperate tree species in the USA and Germany. Twigs were collected during 3 different times throughout the winter of 2015/2016 and placed in growth chambers at 21°C/16°C, 16 hours photoperiod. The graph compares the days to leaf bud break split up in individuals with the same genus. Chilling hours are counted as hours below the chilling threshold of 5C since November 01, 2015.

Chapter 3: The effects of chilling and forcing temperatures on spring synchrony between larch casebearer and tamarack

Claudia Nanninga[5*], Samuel F. Ward[6,7], Brian H Aukema[2], Rebecca A Montgomery[1]

[*] Department of Natural Resources Science and Management, University of Minnesota, St Paul, 1530 Cleveland Ave. N., MN 55108, USA; Email: nanni003@umn.edu;

Phone: (612) 987 8450; Fax: (612) 625-5212; ORCID: 0000-0003-2361-6839

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CN and SW conceived and directed the study. CN wrote the initial draft of the manuscript. All authors made significant contributions to the experimental design, data analysis, and writing of subsequent drafts. The authors declare that they complied with ethical standards and that have no conflict of interest.

[5] Department of Natural Resources Science and Management, University of Minnesota, St Paul, 1530 Cleveland Ave. N., MN 55108, USA

[6] Department of Entomology, University of Minnesota, St Paul, MN 55108, USA

[7] Current address: Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, 100 Old Hwy. 12, Mississippi State, MS 39762

Abstract

Spring phenological synchrony can be important for tree-insect interactions. Depending on the magnitude and direction of phenological shifts, overwintering insects could be affected in many ways, e.g., facing starvation or having to contend with increased chemical or physical defenses of host trees. If temperature has different influences on the phenology of trees and insects, climate change can alter spring phenological synchrony. In this experiment, we exposed tamarack seedlings and larch casebearer larvae from Minnesota, USA, to a variety of chilling and forcing temperatures and measured spring phenology (twig bud break and larval activation). We additionally measured casebearer performance on seedlings that were exposed to different forcing × chilling levels, tracking larval survivorship to adulthood. Warmer forcing enhanced larval activation and bud break, but larval development slowed down past 21°C. Higher chilling temperatures accelerated bud break, but the effect was inconclusive for larvae. There was no chilling × forcing interaction for either species. Spring activity accelerated more quickly with increases in temperature for larvae than for seedlings, resulting in increased phenological synchrony at warmer temperatures. Activation rates for overwintering larvae were highest at 27°C, while survivorship to adulthood following spring activation was highest at 21°C. At temperatures at or beyond 27°C, no larvae reached adulthood. Warmer winters and springs will likely initially increase spring synchrony between tamarack and larch casebearer, exposing larvae to younger, potentially more nutritious foliage, but extremely warm spring temperatures may decrease survivorship of larvae to adulthood.

Keywords: climate change, *Coleophora laricella*, *Larix laricina*, Lepidoptera, phenology, spring

Introducion

Tree-insect interactions are fundamental to the functioning of forest communities (Bascompte and Jordano, 2007). While many interactions are beneficial, large-scale insect outbreaks have the potential to impact the fitness and/or competitive capabilities of trees by reducing tree growth rates, inhibiting seed production, and causing widespread tree mortality (Dale et al., 2001; Kurz et al., 2008). As a result, insect outbreaks can lead to shifts in forest composition and productivity (Anderegg et al., 2015). Climate change can facilitate outbreaks if the ecological interactions between insects and their hosts are governed by temperature. For example, temperature acts as one of the main cues that influences the timing of phenological events in trees and insects, such as leaf bud break and the onset of larval activity, respectively (Chmura et al., 2019). Depending on specific responses to cues, an altered temperature regime could result in changes to phenological synchrony, i.e., the congruent timing of annual life cycle phases and hence changes in insect abundance (Lehmann et al., 2020; Schwartzberg et al., 2014; van Asch and Visser, 2007; Visser and Both, 2005).

The spring phenology of trees and insects can be complex. Bud break in trees is driven by the interaction of cold autumn/winter and warm spring temperatures, though photoperiod can influence timing for some taxa (Basler & Körner, 2012; Caffarra & Donnelly, 2011; Laube et al., 2014; Körner & Basler, 2010). Chilling, defined as the accumulation of cold units within a specific temperature range, moves tree buds from a state of deep dormancy into shallow dormancy, once sufficient chilling has occurred. In

this state of shallow dormancy, exposure to forcing, i.e., the accumulation of warm units within a specific temperature range, eventually results in bud break (H. Hänninen, 2016). Because tree species have different chilling and forcing thresholds and optimum temperature ranges for accumulating chilling and forcing units, the timing of leaf bud break often varies among species under the same climatic conditions or within the same species under different climatic conditions (Hänninen, 2016; Harrington & Gould, 2015; Laube et al., 2014; Panchen et al., 2014). For insects, diapause can mediate how the timing of their spring activity changes with temperature(van Asch and Visser, 2007). Many studies have dealt with the different sensitivities of insects to warm spring temperatures, with warmer temperatures in the spring generally accelerating larval development (Memmott et al., 2007; Schwartzberg et al., 2014; van Asch and Visser, 2007; Visser and Holleman, 2001). Whereas the effects of cold temperatures on survival (i.e., cold tolerance) have received considerable attention, less is known about how seasonal activity responds to winter chilling (i.e., the role of accumulated cold units in breaking insect dormancy or diapause). Chilling accumulation, i.e., the time spent below a low temperature threshold temperature, can shorten diapause and reduce the number of heat units required to molt into the next instar (Wipking, 1995; Hibbard & Elkinton, 2015). Conversely, some species appear to be unaffected by chilling (Hodek, 2002). It is important to understand these complex interactions of phenological cues in both trees and insects to make predictions about future climate-related phenological mismatches and potential changes in synchrony.

Spring phenological synchrony can be especially important for insects feeding on deciduous host trees (van Asch and Visser, 2007). Because defoliators depend on the

presence of suitable leaves, mistimed activation by insects can cause starvation or force larvae to search for other food sources if no foliage is available (Hunter and Elkinton, 2000; Lawrence et al., 1997), consequently benefiting the host. Additionally, if insect activation occurs considerably later than bud break, leaves might be less nutritious or better defended against herbivory (Feeny, 1970). Because defoliators can face a short window of time, during which young, nutritious foliage is available, phenological matching can influence insect distribution and abundance, and hence insect outbreaks (van Asch and Visser, 2007). This paper explores the effects of temperature on the spring phenological interactions and synchrony between the defoliator larch casebearer (Coleophora laricella Hübner; Lepidoptera: Coleophoridae) and its host tamarack (Larix laricina (Du Roi) K. Koch) in Minnesota, USA (MN).

Tamarack is a deciduous gymnosperm native to the boreal forests of North America. The spring phenological responses of tamarack to temperature cues have not been studied extensively, but it has been suggested that Larix spp. have low chilling (Harrington and Gould, 2015) and forcing requirements (Malyshev et al., 2018) and that increased exposure to chilling in the winter decreases the need for forcing (Laube et al., 2014; Nanninga et al., 2017). Warmer forcing accelerates spring bud break in tamarack (Rossi and Isabel, 2017), but it is not clear how different chilling temperatures affect chilling accumulation to reach the required threshold. Larch casebearer is a defoliator of Larix spp. that was accidentally introduced from Europe into the U.S., where it was first detected in the late 1800s. After decades of low density casebearer populations, perhaps due to the initial success of biological control, there have been recent widespread outbreaks on western and eastern Larix spp. (Ward & Aukema, 2019a) Larch casebearer

has one generation per year. Adults eclose in mid-spring and oviposit individual eggs onto larch needles. Each larva then feeds inside a needle for the first two instars before molting into a third instar and constructing a case from a hollowed larch needle. Larch casebearers overwinter as third instars, molt into fourth instars before resuming feeding in spring, and eventually pupate in their cases attached to host foliage. Research on adult eclosion and on the effect of temperature on larval activation, defined as the time point in spring when larvae molt into fourth instars, detach their cases from silken plugs, and actively wander in search of plant resources, is rare. Ward et al. (2019a) found that increased forcing temperatures and longer photophases accelerated activation up to a threshold of around 30°C but did not consider the effects of chilling.

The spring phenological synchrony between larch casebearer and tamarack could be affected by climate change due to projected increases in winter and spring temperatures in Minnesota (Intergovernmental Panel on Climate Change, 2021). Depending on the rate of chilling and forcing accumulation at different cold and warm temperatures for each species, warmer winters and springs could either delay or accelerate bud break and/or larval activation. Because a change in synchrony may have impacts on the fitness and survival of both tamarack and larch casebearer, and potentially larch casebearer outbreaks, we aimed to better understand the role of temperature in both species' spring phenology. While it is well-established that longer chilling exposure and warmer spring temperature enhance bud break in tamarack, there are gaps in our knowledge regarding the effectiveness of actual chilling temperatures for accumulating chilling. Additionally, the role of winter chilling in casebearer phenology and phenological matching between larch casebearer and tamarack is poorly understood. Here, we conducted a chilling and

forcing experiment with tamarack seedlings and larch casebearer larvae to better understand the potential role of climate change in the recent larch casebearer outbreaks in Minnesota, and to better predict future tamarack-casebearer interactions. Our specific aims were to quantify the effect of different chilling and forcing combinations on bud break timing, timing of larval activation, and moth development.

Methods

Tamarack: chilling and forcing treatments

Three-year old tamaracks were obtained from the MN State Forest Nursery (Badoura, MN) and potted in Saint Paul in May 2015. For methods on tree planting and care prior to use in assays, see (Ward et al., 2019c) Briefly, bareroot lifted seedlings were potted using Sunshine Mix #8 (Sun Gro® Horticulture, Agawam, MA) in 10.2 cm × 24.1 cm pots (TP49, Stuewe & Sons, Tangent, Oregon), watered every 2-3 days, and stored outside in a wooden, roofless cold frame on the campus of the University of Minnesota, Saint Paul (44.9886 °N, 93.1802 °W) until exposure to experimental treatments began 20 November, 2015.

To determine the effect of chilling and forcing temperatures on the incidence and timing of spring bud break, tamarack seedlings (n=48) were first assigned to a combination of chilling (4 levels) and forcing (6 levels) temperatures (i.e., n=2 trees in each of the 24 combinations). Trees were then placed into one of four dark coolers on 20 November 2015 at -7°C (C1), 1°C (C2), 4°C (C3), or 10°C (C4). Table 3.1 shows chilling and forcing temperatures and relative humidity (RH) in all treatments). After 118 days of the chilling treatment, on 16 March, 2016, seedlings were transferred to their

preassigned forcing treatments: 6.5°C (F1), 10°C (F2), 15.5°C (F3), 20.5°C (F4), 27°C (F5), or 32°C (F6). Photoperiods in each chamber were 14L:10D.

Following transfer to growth chambers, seedlings were scored for bud break every 2-3 days. Bud break was defined as at least 1 bud broken according to BBCH (Biologische Bundesanstalt, Bundessortenamt, and Chemical Industry) 7: beginning of bud burst – first green leaf tips just visible (Meier, 1997). Seedling survival and damage, i.e., dead twigs and branches, were also recorded. Because of imprecision in the chambers, the humidity ranged from 83 to 100% RH in the chilling treatments and from 55 to 91% RH in the forcing treatments (Table 3.1). To reduce the effect of humidity on seedling health and development, the pots were watered with 200 ml of water as soon as the soil started drying, usually weekly.

To test if chilling requirements had been met by 20 November, 2015, 12 additional seedlings were placed in a greenhouse with temperatures of 21°C from 6am-6pm, 16°C from 6pm-6am, and at ambient photoperiods (~9.5 hours).

Larch casebearer: chilling and forcing

Twigs with overwintering, third instar larch casebearers were collected from a site near Jacobson, MN, U.S.A. (46.9982 °N, 93.1073 °W) in early November 2015. Twigs were clipped from tamaracks and were within 2 m of the ground. Larvae were left intact on twigs until use in assays and were stored outside in a well-ventilated plastic container (46 × 31.1 × 17.8 cm) inside of the cold frame where trees were stored. On 20 November, 2015, insects were carefully removed from twigs with forceps and placed in groups of three into 96 Petri dishes (*n*=288 larvae, 9 × 50-mm polystyrene dishes, Falcon Labware,

Oxnard, CA, USA). Four petri dishes were randomly preassigned to each of the 24 chilling × forcing combinations and transferred into treatments on the same dates as the tamarack seedlings: 16 November, 2015 for chilling and 16 March 2016 for forcing. Larvae were monitored for activation (i.e., active wandering) every 2-3 days following transfer to forcing treatments and activated larvae were removed from dishes upon being detected.

We acknowledge that in the chilling treatments that were set at 10°C, twigs and larvae might have been exposed to temperatures above their lower developmental threshold of 5°C (Ward et al. 2019b). It is therefore possible that both species experienced a combination of cues with chilling and forcing occurring simultaneously (Heikki Hänninen, 2016).

Larch casebearer: moth eclosion

In a separate experiment, larvae were collected on 24 March, 2016 at the same site near Jacobson (see above), placed into nine Petri dishes in groups of ~70, and transferred into a growth chamber at 18L:6D (20 °C) to promote activation. Between 8 April and 28 April, activated larvae were caged in groups of five to six onto the flushed trees from the "tamarack: chilling and forcing" experiment in the same growth chambers and tracked until moth eclosion. Cages were constructed of low-density polyethylene tubing (diameter: 12.7 cm, thickness: 0.15 mm, ULINE®) with one side replaced with fine mesh for ventilation.

Statistical analysis

Forcing and chilling temperatures were treated as continuous predictors for all analyses. The effects of chilling temperature, forcing temperature, and their interaction on days to bud break (ln(*x*)-transformed) were analyzed using multiple regression. The effects of the same predictors plus a second order term for forcing on the incidence of larval activation (binary response) and days to larval activation (ln(*x*+1)-transformed) were analyzed using generalized linear mixed-effects models. The small constant (x+1) was added to larval activation timing because some of the larvae activated upon removal from chilling treatments (i.e. 4.8%, or 12 individuals from the warmest and 2 individuals from the 1°C chilling treatment). It is possible that these larvae had already activated prior to transfer to forcing treatments, given that they were not tracked during chilling. For the model of larval activation incidence, a logit link function and binomial error structure were used. Petri dish was fit as a random intercept in all models pertaining to larval data.

To discern the difference in the magnitude of phenological adjustments to different chilling and forcing regimes between tamarack and the larch casebearer, we directly compared the average time to spring activation and bud break between the two species. For tamarack, we averaged time to bud break for each combination of treatments. For the larvae that activated, we averaged time to activation for all larvae across both Petri dishes for all treatment combinations, producing 24 values of mean activity for each species. We then subtracted the corresponding averages to establish the difference in time (days) until activation for each treatment combination. Non-activated larvae (n = 100, or

28.8 %) and dead seedlings (n = 2, or 4.1 %) were not included in this calculation. The impact of chilling temperature, first and second order terms for forcing temperature, and the interaction of chilling with the second-order term for forcing temperature on phenological synchrony (days to casebearer activation minus days to tamarack bud break) were analyzed with multiple regression. To choose models, we first analyzed the data including all variables and interaction terms and compared the AIC of those models. We chose the models with lower AIC.

The proportion of larvae per Petri dish surviving to adulthood was logit-transformed and regressed on a term for tree chilling (while larvae from this experiment were not exposed to experimental chilling, the trees were), first and second order terms for forcing temperature, and a term for phenological asynchrony. We defined asynchrony for this analysis as the days between bud break and placement of larvae onto trees. We then used stepwise, backwards elimination of predictors to develop a final model by removing predictors until only those with $P < 0.05$ remained.

All data were analyzed and graphs produced with the R statistical software (R 3.3.2, R Core Team 2018; (Bates et al., 2015; Kuznetsova et al., 2017; Roux et al., 1997a).

Results

Tamarack: response to chilling and forcing

Time to bud break in tamarack was significantly affected by forcing and chilling temperatures, but the interaction between chilling and forcing was not significant. Warmer chilling temperatures significantly reduced the time required for bud break ($F_{1,43}=45.07$, $P<0.001$, Fig. 3.1). For example, over all forcing levels, twigs chilled at -7°C required on average 13.1 (± 1.8 SE) days to break bud, while those chilled at 4°C

and 10°C required only 7.2 (±1.1 SE) and 6.1 (±1.2 SE) days, respectively. Twig damage (i.e. death of entire twigs) and seedling mortality was only found in the coldest chilling level (-7°C). Here, an increasing temperature range between chilling and forcing levels increased damage to twigs and whole seedlings (*Appendix SI*, Fig. S3.1. shows mortality and damage of seedlings in the -7°C treatment).

The time to bud break in the tamarack seedlings decreased significantly with warmer forcing temperatures ($F_{1,43}=148.85$, $P<0.001$, Fig. 3.1). For example, over all chilling levels, twigs forced at 6.5°C required on average 15.1 (±2.2 SE) days to bud break, compared to twigs forced at 20.5°C and 32°C, which required on average 6.1 (±0.8 SE) and 3.8 (±0.8 SE) days, respectively.

All of the additional seedlings that were flushed in a greenhouse at the start of the experiment were able to break bud (data not shown).

Larch casebearer: response to chilling and forcing

Larvae exposed to warmer chilling temperatures activated significantly earlier than those at colder chilling levels ($t_{184}=-9.70$, $P<0.001$, Fig. 3.1). For example, over all forcing levels, larvae chilled at -7°C required an average of 68.5 (±7.8 SE) days to activate, while those chilled at 10°C required an average 15.5 (±3.7 SE) days.

The time to activation in larch casebearer larvae decreased nonlinearly with warmer forcing (Fig. 3.1). For example, larvae exposed to a forcing temperature of 6.5°C on average activated after 100.1 (±8.4 SE) days, while those forced at 20.5°C and 27°C activated after 14.0 (±1.4 SE) and 18.5 (±2.3 SE) days, respectively. Time to activation was only 5.8 (±3 SE) days in the warmest forcing chamber (32°C); however, only 15% of

larvae emerged at that treatment level. A linear term for forcing ($t_{184}=-6.10$, $P<0.001$) was negatively correlated with time to activation, while a second order (quadratic) term for forcing ($t_{184}=4.33$, $P<0.001$) was positively correlated with time to activation.

The proportion of casebearer larvae that activated was significantly influenced by chilling ($Z= 8.55$, $P=0.009$) and forcing ($Z = 1.07$, $P<0.001$) (Fig. 3.2). None of the larvae from the two coldest chilling levels, and only one individual from the 4°C chilling level, activated at 32°C. Incidence of larval activation was generally high, ranging from 62% to 92% from 6.5°C to 27°C of forcing respectively, above which activation dropped to 15% at 32°C of forcing.

Potential for phenological asynchrony

Chilling and forcing significantly altered the phenological synchrony (i.e. the difference in days to casebearer activation and tamarack bud break) between larvae and seedlings, but the interaction of chilling and forcing was not significant (Fig. 3.2). Tamarack always broke bud before larch casebearer activation and required less forcing (Fig. 3.1), except for in the 10°C treatment. Higher chilling temperatures significantly reduced the length of the interval between larval activation and bud break ($F_{1,18}=28.11$, $P<0.001$, Fig. 3.3). Across all forcing levels, larval activation in the -7°C chilling chamber lagged an average of 49.5 (±18.1 SE) days behind bud break compared with a 9.7 (±6.3 SE) days lag at 10°C. The difference between bud break and larval activation decreased nonlinearly with warmer forcing, thus increasing phenological synchrony. A linear term for forcing ($F_{1,18}=45.45$, $P<0.001$) was positively correlated with synchrony, while a second order term for forcing ($F_{1,18}=16.3$, $P<0.001$) was negatively correlated

with synchrony, suggesting that warmer temperatures increase synchrony up to 21°C of forcing, but beyond that decreased synchrony. Even though time to both bud break and larval activation significantly decreased with warmer forcing, the decreases were greater for larch casebearer (Fig. 3.1). Consequently, the increased spring phenological synchrony between the two species that was caused by forcing temperatures was mostly driven by the impacts of warmer forcing on the timing of larval activation.

Moth eclosion

Survival to adulthood by larvae was greatest at intermediate temperatures between 10.5°C and 21°C, whereas no moths emerged at or above temperatures of 27°C (Fig. 3.4). A variable for temperature was positively correlated with proportional activation (slope ± SE: 0.58 ± 0.10, $t = 4.90$, $P = 0.0001$) and a quadratic term was negatively correlated with activation (slope ± SE: -0.016 ± 0.003, $t = -5.19$, $P < 0.0001$), indicating decreased eclosion rates at low and high forcing temperatures. The proportion of moths that emerged at 6, 11, 16, and 21 °C were 0.02, 0.08, 0.28, and 0.33, respectively. Chilling and days of phenological mismatch did not significantly influence the percent survival to adulthood and were removed from the model.

Discussion

The potential impact of climate change on spring phenological synchrony of tamarack and larch casebearer depends on their responses to chilling and forcing. Quantifying the impacts of changing temperature regimes is therefore important for understanding the causes and consequences of the ongoing outbreaks of larch casebearer and potentially better predicting outbreaks in Minnesota and other regions invaded by larch casebearer. In Minnesota, the origin of seedlings and larvae used in this experiment, the average temperature in the winter months between 2000 and 2022 was around -8.5°C in December, -11.6°C in January, and -10.5°C in February. The average temperature in the spring was around -2°C in March, 5.5°C in April, and 12.5°C in May (NOAA National Centers for Environmental Information, 2023). We note that some of our target temperatures are not ecologically relevant for Minnesota, even with projections that indicate an increase in average winter (December to February) temperatures of 4°C (low emissions) to 6°C (high emissions) (Liess et al., 2022) and an increase in average spring (March to May) temperatures of 1.2°C (low emissions) to 3°C (high emissions) (Handler et al., 2014). We also note that temperature variability could be important, and we used fixed temperatures, so our results should be interpreted with caution.

Chilling may be an important factor in the phenological synchrony between tamarack and larch casebearer. Although previous studies have established that longer exposure to chilling in *Larix* species reduces the need for forcing (Harrington & Gould, 2015; Nanninga et al., 2017; Malyshev et al., 2018), to our knowledge, no study has looked at the rate of chilling accumulation at different temperatures. In this experiment, we found that warmer chilling temperatures significantly reduced the time to bud break.

We note that the time to bud break at the warmest chilling level of 10°C was above the forcing threshold of ~ 5°C (Ward et al., 2019a). According to the parallel model (Hänninen, 1987), at 10°C chilling and forcing units were likely accumulating simultaneously for the tamarack seedlings, which would explain the faster bud break time for that level. We did not account for accumulation of chilling under ambient conditions before the experiment. The fact that the subset of seedlings that were flushed in a greenhouse at the start of the experiment were all able to break bud suggests that minimum chilling requirements had already been met. However, the extended exposure to controlled chilling still accelerated bud break. This finding is in line with previous studies, which showed that even after endo-dormancy induction, increased chilling continues to reduce the time to bud break(Harrington and Gould, 2015; Laube et al., 2014; Nanninga et al., 2017). Given that chilling was most likely already accumulated before the initiation of the experiment, the results regarding chilling effects need to be taken with care.

Like tamarack, larch casebearer larvae showed significant decreases in time to activation with higher chilling temperatures, potentially indicative of a chilling effect. There is a lack of studies investigating responses of larch casebearer phenology to cold temperatures, but it appears that chilling effects in insects are highly species-dependent, and can advance, delay, or have no effect on timing of spring activity (Hibbard & Elkinton, 2015; Higaki & Ando, 2005; Hodek, 2002; Roux et al., 1997; Wipking, 1995; Xiao et al., 2013). As an alternative explanation, the delayed activation of casebearer larvae that were chilled at -7°C could be the result of injury from prolonged cold exposure that inhibited casebearer larval development (Turnock et al., 1983).

Additionally, as with tamarack, the warmest chilling level was above the developmental threshold of ~ 5°C (Ward et al., 2019a), i.e., it is likely that larvae were forced rather than chilled at 10°C. Thus, the effects of chilling and forcing temperatures are hard to disentangle.

Bud break in *L. laricina* seedlings occurred earlier with warmer forcing, which is in line with findings from previous studies (Harrington and Gould, 2015; Rossi and Isabel, 2017). The capacity for ontogenetic development in the bud depends on the previously accumulated chilling units and the state of dormancy (Vegis, 1964). The threshold of optimal forcing therefore changes with the state of bud development (i.e., as mediated by prior chilling), but this state of development could not be determined externally.

Warmer forcing also decreased the time to activation for larch casebearer up to ~21°C. The development rates of insects often increase with temperature up to a maximum rate, beyond which warmer temperatures correspond to slower development (Régnière et al., 2012). Our results suggest the upper developmental threshold of 4th instar larvae and/or pupae lies somewhere from 21-27 °C. While the lower humidity in the 21°C treatment (55%) could have impacted larval development, the high humidity in the 27°C (91%) and 32°C treatments (85%) did not result in decreased time to activation. Forcing also impacted the activation success of larvae, measured as the incidence of activation. The highest success rate of larval activation occurred at ~27°C, beyond which there was a rapid decline, possibly due to heat stress. The high temperature levels may have resulted in metabolic rates of larvae that outpaced acquisition of nutrients and water. Moving larvae from -7°C and 1°C chilling to 32°C forcing, resulting in a sudden

temperature increase of around 38°C and 33°C respectively, caused 100% mortality. However, Ward et al., (2019c) found that overwintering larch casebearer moved from multiple weeks of storage at colder temperatures (e.g., -22 to -27 C) immediately into 20 C had activation rates of 75-90%. The seedlings also experienced twig damage and mortality when moved from the coldest chilling to the hottest forcing level (*Appendix SI*, Fig. S3.1). Thus, both species appeared to be impacted by transfer shock when moved between temperature extremes. These extremes are not representative of shifts in ambient temperatures in MN. ch

Spring phenological synchrony can be impacted if the interval between larval and bud activation is either increased or decreased by temperature changes. Our results show that the difference in the timing of phenological spring events between tamarack and larch casebearer was not consistent over different forcing and chilling levels, and that larch casebearer had a more pronounced advancement in spring activity with warmer temperatures compared with tamarack. Thus, changes in synchrony were dominantly driven by shifts in larch casebearer phenology (Fig. 3.3). Generally, synchrony increased with warmer forcing, but was decreased when temperatures approached ~21°C, beyond which larval activation slowed down. Moreover, the percentage of larvae that molted into adult moths increased up to 21°C with very high success rates. Beyond 21°C, however, temperatures became deleterious. Survival of larch casebearer from spring activation to eclosion has not been studied in the field, but we note that caging insects onto trees at constant temperatures could have reduced survival. Additionally, more variability in phenology than reported here likely exists across the expansive range of tamarack and larch casebearer, and such variability could further alter phenological synchrony.

Taken together, our findings suggest that climate warming might increase spring phenological synchrony and moth eclosion up to a certain point, beyond which tamarack-casebearer synchrony and casebearer survivorship might decrease. The predicted temperature increases in MN of 4°C to 6°C in the winter (Liess et al., 2022) and 1.2°C to 3°C in the spring (Handler et al., 2014) by 2100 would increase spring phenological synchrony and therefore translate into initial exposure of casebearer to younger foliage. For example, in December, the current average temperatures of -8.5°C could increase to on average -2.5°C, which is warmer than the coldest chilling treatment. Additionally, in the constant temperature settings in our experiment, we were not able to account for daily and diurnal temperature fluctuations, and it is likely that both species would be exposed to hours of much warmer temperatures closer to the second coldest chilling treatment. Our study corroborates previous findings that, on average, tamarack breaks bud several weeks before larch casebearer activates (Ward et al., 2019a,d), except for the 10°C chilling treatment. For some spring-feeding lepidopterans, feeding on earlier foliage increases larval performance (Fuentealba et al., 2017), but spring feeding larch casebearers appear able to complete development on foliage from a range of age classes (Ward et al. 2019a) and changing concentrations of monoterpenes had no discernible effect on larval survival (Ward et al., 2019b). Thus, it is possible that the effect of increased synchrony on larval success might not be an important driver of casebearer population dynamics. However, the effect of other defenses, such as sesquiterpenes and diterpenes, on casebearer feeding have not been explored, and we note that trees were fertilized, which could have altered tamarack defenses (Powell and Raffa, 1999), and, in turn, masked effects (positive or negative) of changes in phenological synchrony. More

work is needed on the effects of other secondary metabolites or plant nutritional status on spring-feeding casebearer larvae as potential determinants of insect fitness. If bud break and larval activation are timed much more closely together, it is possible that casebearer larvae are exposed to very underdeveloped needles, which could reduce the food source and lead to reduced fitness or starvation. However, because larch casebearer overwinter as third instars and start feeding in the spring as fourth instars, they might be more robust than other spring feeders who overwinter in earlier developmental stages. In our experiment, we worked with young tamarack seedlings, which could exhibit different physical defenses, chemical defenses, and phenological patterns from those of adult trees (Vitasse, 2013).

Increased synchrony that results in the consumption of younger foliage could affect the ecology of tamarack forests in northern MN. If young foliage is consumed earlier in the year, the chances of carbohydrate accumulation and ultimately growth could be affected. Consecutive years of defoliation by larch casebearer can severely damage or kill tamarack trees, which are already stressed by a warmer and drier climate (Department of Natural Resources-Division of Forestry, 2013). Weakened and stressed tamaracks are likely less able to create sufficient defense mechanisms and become even more prone to infestation by more lethal insects, such as eastern larch beetle (Ward & Aukema, 2019b). Further work is needed, however, to understand the non-lethal effects of changing synchrony on both casebearers and tamarack as well as how temporal patterns of phytochemistry shift with ongoing climate change.

Tables

Table 3.1: Chilling and forcing temperatures, and relative humidity (RH) in the 24 treatment combinations. The chilling and forcing temperatures were chosen to elucidate the effect of a considerable number of temperature combinations.

Treatment	Chill Temp (°C)	RH (%)	Treatment	Forcing Temp (°C)	RH (%)
C1	-7.1± 0.01	83	*F1*	6.4 ± 0.05	90
C2	0.82± 0.02	100	*F2*	10.1 ± 0.02	83
C3	3.9± 0.04	91	*F3*	15.5 ± 0.01	60
C4	9.7± 0.00	96	*F4*	20.7 ± 0.01	55
			F5	27.2 ± 0.04	91
			F6	31.6 ± 0.03	85

Figures

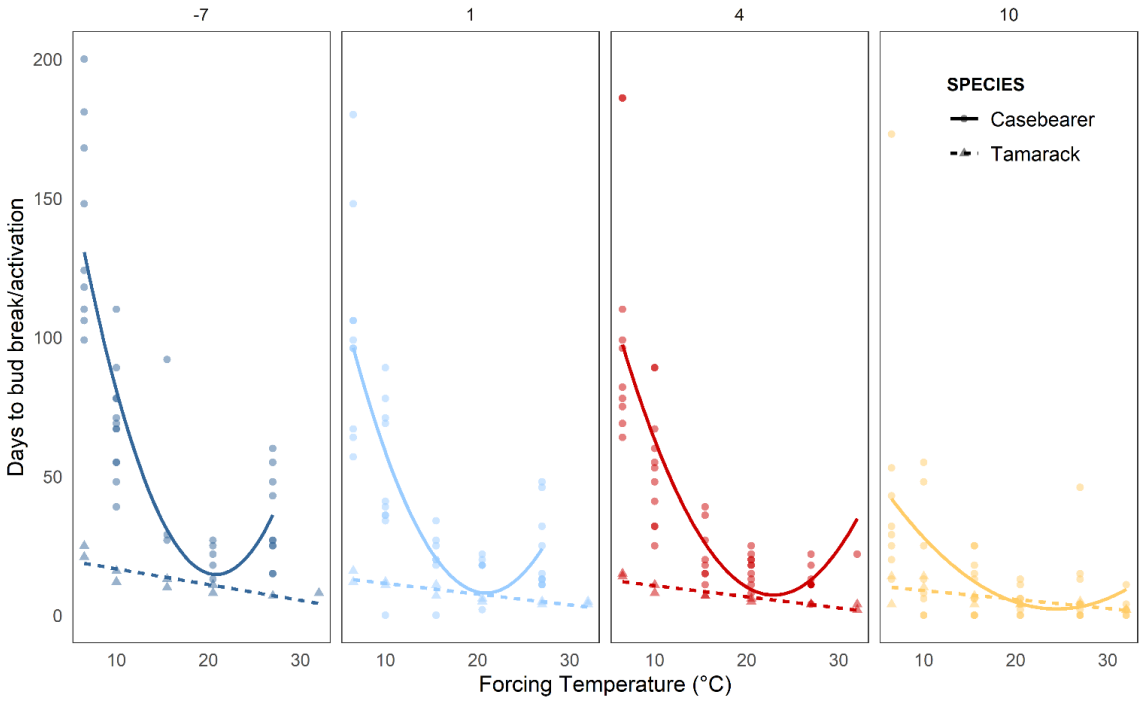

Figure 3.1: The effect of chilling and forcing temperatures on the timing of spring phenological events of tamarack seedlings and larch casebearer larvae. Seedlings and larvae were first exposed to four chilling levels (°C) in freezers for around 3 months and then transferred to growth chambers with six different forcing temperatures (°C). The dotted lines line show the effect of chilling and forcing on days to bud break (tamarack seedlings), (multiple linear regression: $F_{2,43}$= 69.9, P<0.001, R^2=0.81, *log(y)=3.01-0.05(forcing)-0.04(chilling)*); the solid lines show the effect of a linear term for forcing (mixed effects model: $F_{1,184}$ = 18.8, P < 0.0001), a linear term for chilling ($F_{1,184}$ = 18.8, P < 0.001), and a quadratic term for forcing ($F_{1,184}$ = 18.8, P < 0.001, *log(y+1)=5.99-0.26(forcing)+0.005(forcing)^2-0.10(chilling)*) on days to larval activation (larch casebearer).

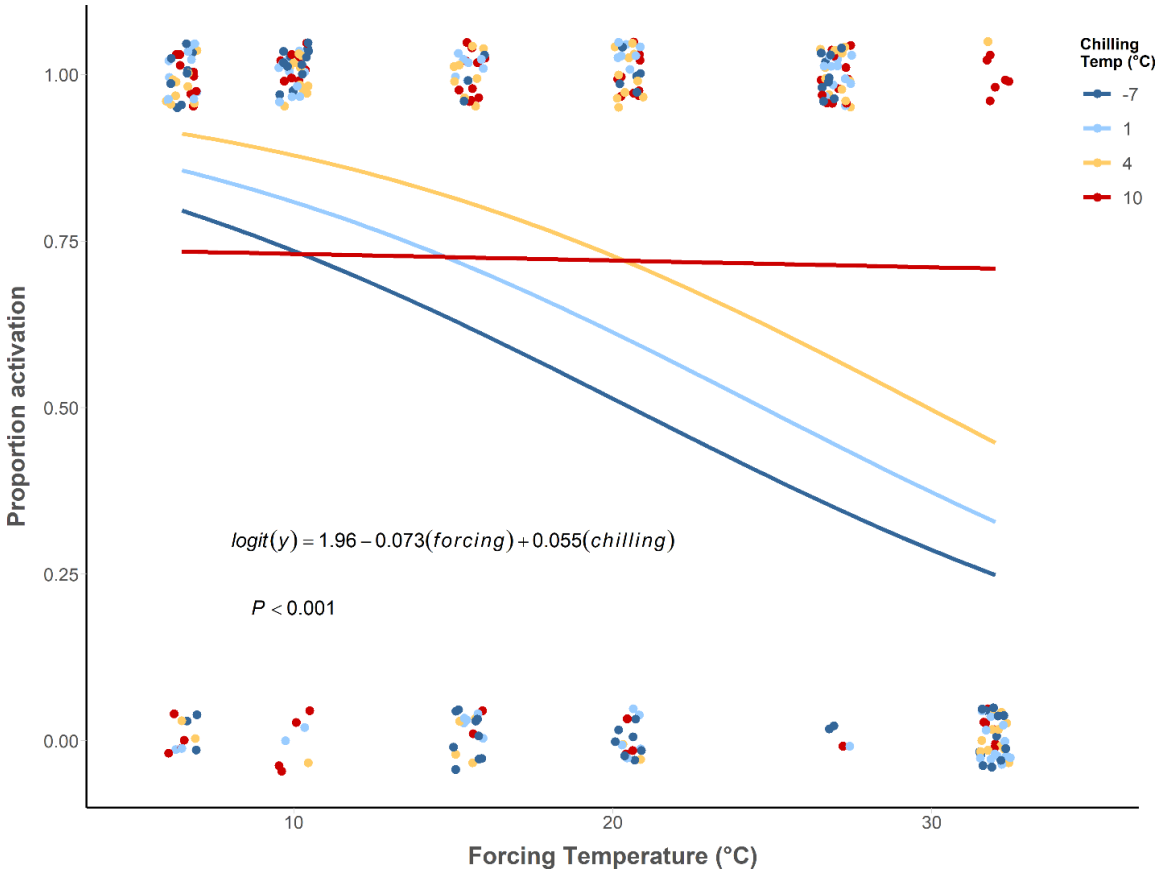

Figure 3.2: Incidence of larval activation by larch casebearer. Larvae were first exposed to four chilling levels (°C) in freezers for around 3 months and then transferred to growth chambers with six different forcing temperatures (°C). Fit lines are from a generalized linear mixed-effects model with a logit link function and a binomial error structure. Each data point (jittered) shows activation/no activation of larvae at different forcing temperatures (x axis) and chilling temperatures (lines). Even though chilling was fit as a continuous predictor in this and other models, we graphed the chilling lines as discrete categories to make displaying these data easier.

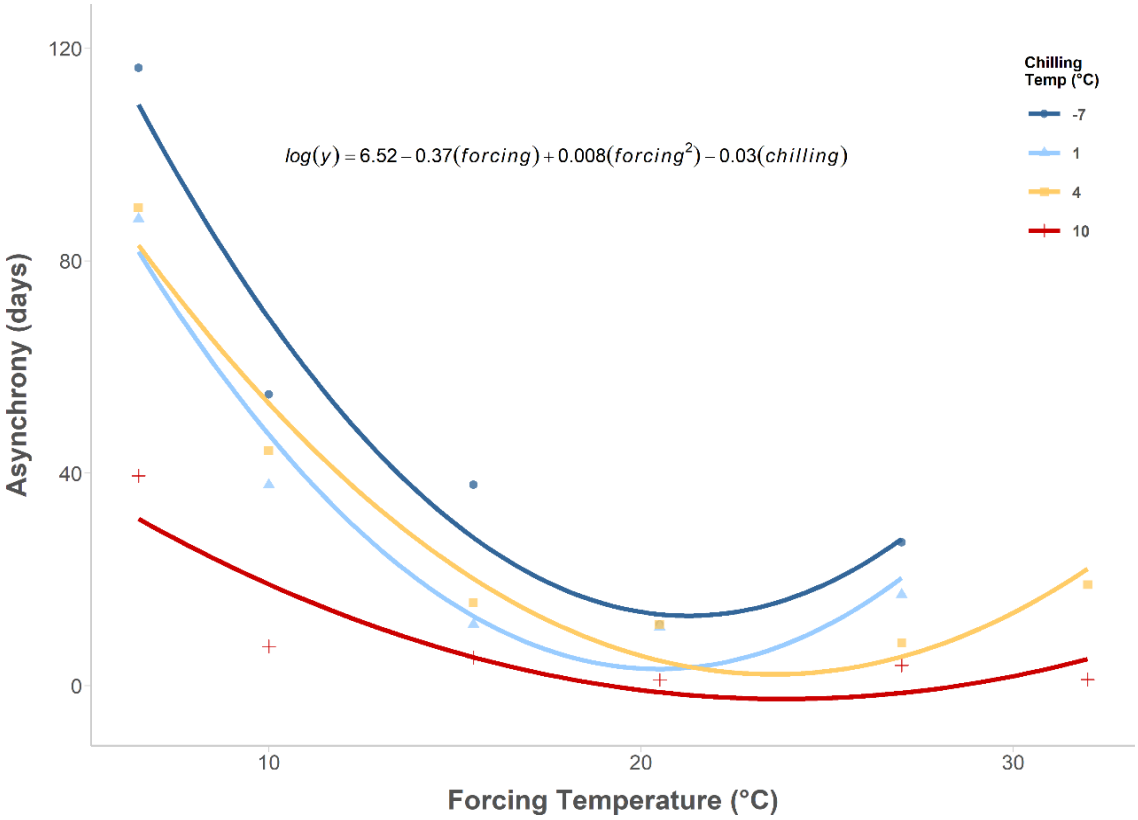

Figure 3.3: Effect of chilling and forcing on the phenological synchrony of tamarack and larch casebearer. The graph shows a linear term of chilling, a linear term of forcing and a quadratic term of forcing on **phenological synchrony** (average days to larval activation – average days to bud break), $P<0.001$, $R^2=0.83$, between tamarack and larch casebearer.

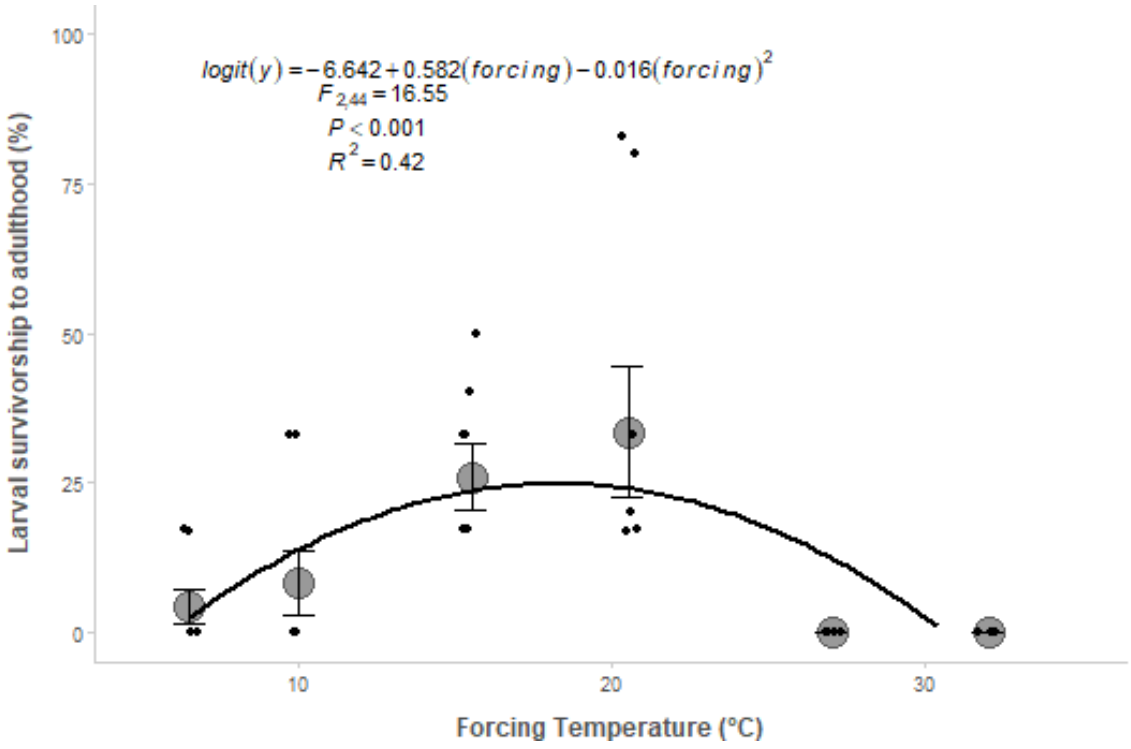

Figure 3.4: Percentage of larch casebearer larvae surviving into an adult moth stage at different forcing temperatures. Field-collected larvae were activated in a growth chamber and subsequently placed onto seedlings in each of six forcing levels. Points (jittered) show the percentage of larvae that survived into adulthood. The large circles show mean survivorship ± SE.

Dissertation Conclusion

1. **Increased ambient chilling reduced the time to bud break in temperate and boreal tree species in Minnesota and Germany.** In the experiments during which twigs were chilled at ambient temperatures and then exposed to spring-like temperatures in the greenhouse throughout the winter, the decrease in bud break timing was linear and significant for all species.
2. **Increased experimental chilling reduced the time to bud break in temperate and boreal tree species in Minnesota and Germany.** In the experiments during which twigs were chilled in cooling chambers for 4 and 8 weeks, there was a significant decrease in bud break timing for 11 out of 14 species.
3. **Chilling temperatures below freezing (0°C) were effective at contributing to chilling accumulation in all experiments.** In MN, most of the outdoors temperatures during the ambient chilling experiments were below freezing and prolonged exposure resulted in decreased DTB. Additionally, in both MN and Bavaria in the artificial chilling experiment, increased chilling from 4 to 8 weeks in the coldest chilling chamber (-6.5°C/-7°C U.S.A/Germany) resulted in reduced DTB for the species that were responsive to chilling (see 2 above). This demonstrates the effectiveness of temperatures below 0°C in contributing to chilling accumulation.
4. **Species have very specific and differing chilling and forcing requirements, both within and across countries.** We confirm that species differed significantly

in their chilling and forcing requirements. Our results suggest that species-specific chilling requirements need to be considered to better inform predictive phenological models.

5. **Differences in chilling temperatures had a significant effect on the bud break timing for half of the species in our experiment.** For species that were significantly impacted by chilling temperature, the effect was not always linear, i.e., some species preferred the temperatures in the medium range. Of the species who were significantly impacted by chilling temperature, only one preferred colder chilling. All other species reacted with reduced bud break when exposed to warmer chilling up to 4°C Germany/4.5°C U.S.A. Our results suggest that overly simple generalizations regarding chilling requirements must be avoided in predictive phenological models.
6. **Chilling is an important cue.** Trees show a decrease in the time to bud break with increased chilling, with very few exceptions. Additionally, for many species, the actual chilling temperature is relevant in bud break timing. Warmer winter temperatures could contribute to a lack of chilling accumulation and a delay in bud break timing for species in warmer regions. However, because warmer chilling is more effective for most species, warmer winters could initially have a beneficial effect for species in very cold regions, such as MN.
7. **Warmer forcing decreased the time of activation and bud break for larch casebearer and tamarack respectively.** The effect of reduced time to activation with increased forcing temperatures was much more pronounced for larch

casebearer. Additionally, while chilling decreased bud break timing for tamarack, the effect was not clear-cut for larch casebearer.

8. **Warmer forcing and chilling increased phenological spring phenology for larch casebearer and tamarack.** The increase in synchrony was mainly driven by the strong impact of warmer forcing on larch casebearer activation. Our results suggest that increasing temperatures could increase the phenological synchrony of both species further, potentially benefiting larch casebearer due to an availability of younger and more nutritious needles. However, this would only be the case for temperatures up to around 27°C. Additionally, at around 21°C, casebearer larvae did not survive to reach adulthood.

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Supplemental Information (SI): Appendix.

Appendix Figures:

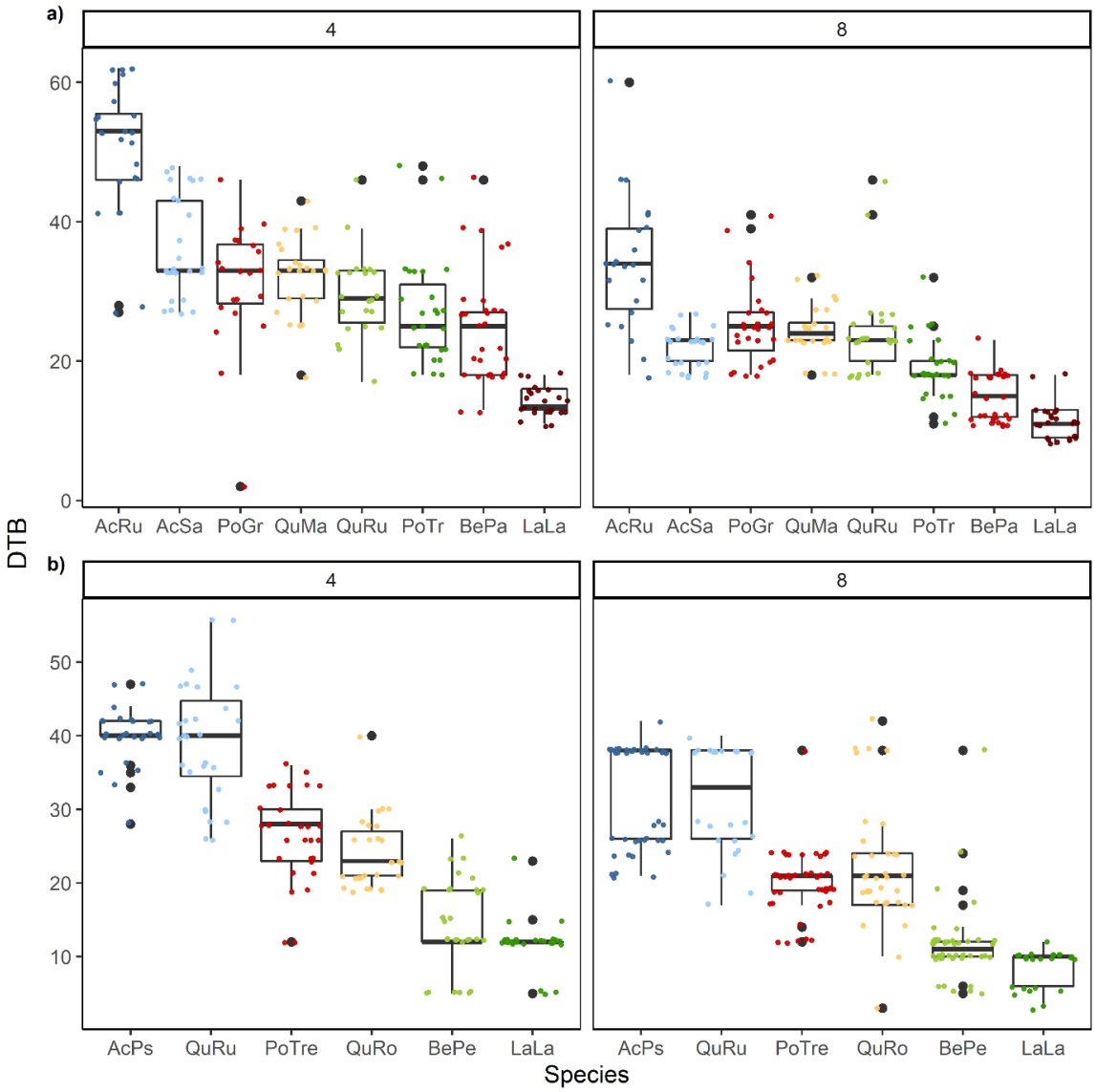

Figure S2.1: The graph shows the sequence of bud break timing in species from the a) U.S. and from b) Germany.

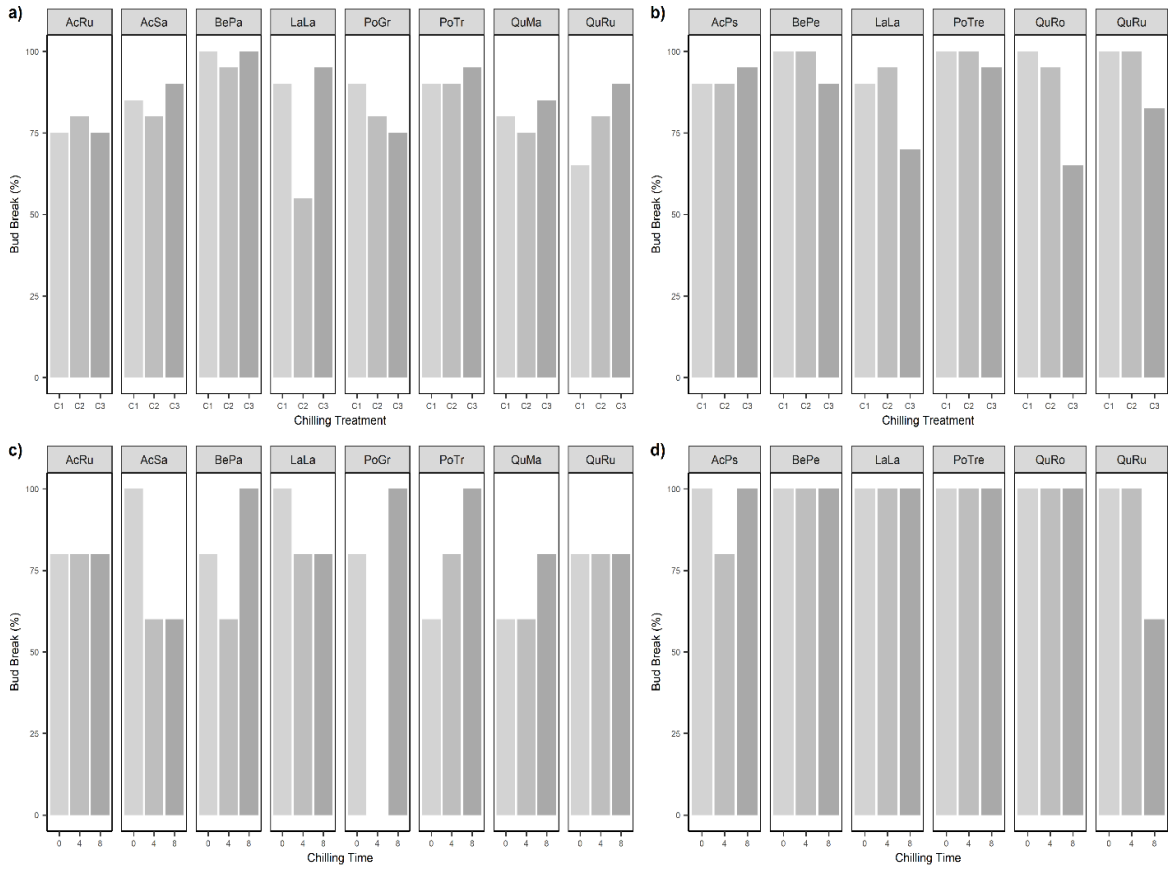

Figure S2.2: Bud break percentage (survival) of twigs from experiment 1: artificial chilling in chilling chambers (a) USA, b) Germany); and from experiment 2: chilling at ambient temperature (c) USA, d) Germany).

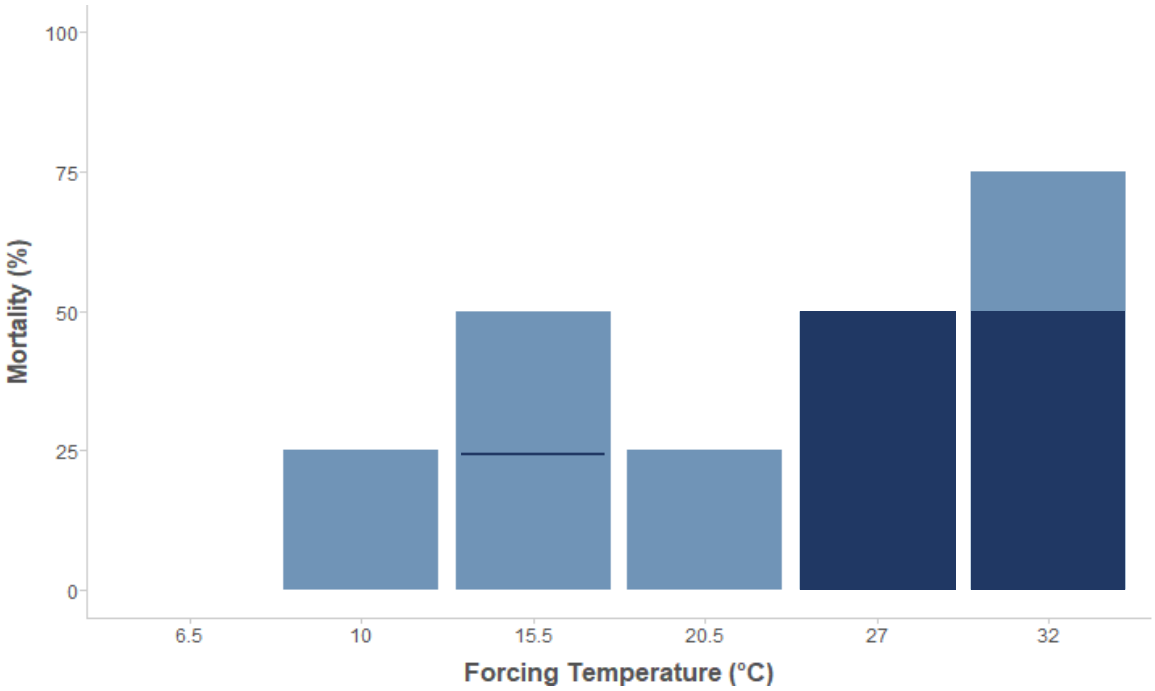

Figure S3.1: Mortality of tamarack seedlings in the -7°C chilling treatment for each forcing treatment (n=2 per treatment). Death of a whole seedling (dark blue) counts as 50% mortality, dead twigs in a seedling (light blue) count as 25% mortality. No mortality was observed in other chilling treatments.