EXPERIMENTAL MANIPULATIONS TO PREDICT FUTURE PLANT PHENOLOGY

EDITED BY: Yongshuo Fu, Yann Vitasse and Janet Prevey

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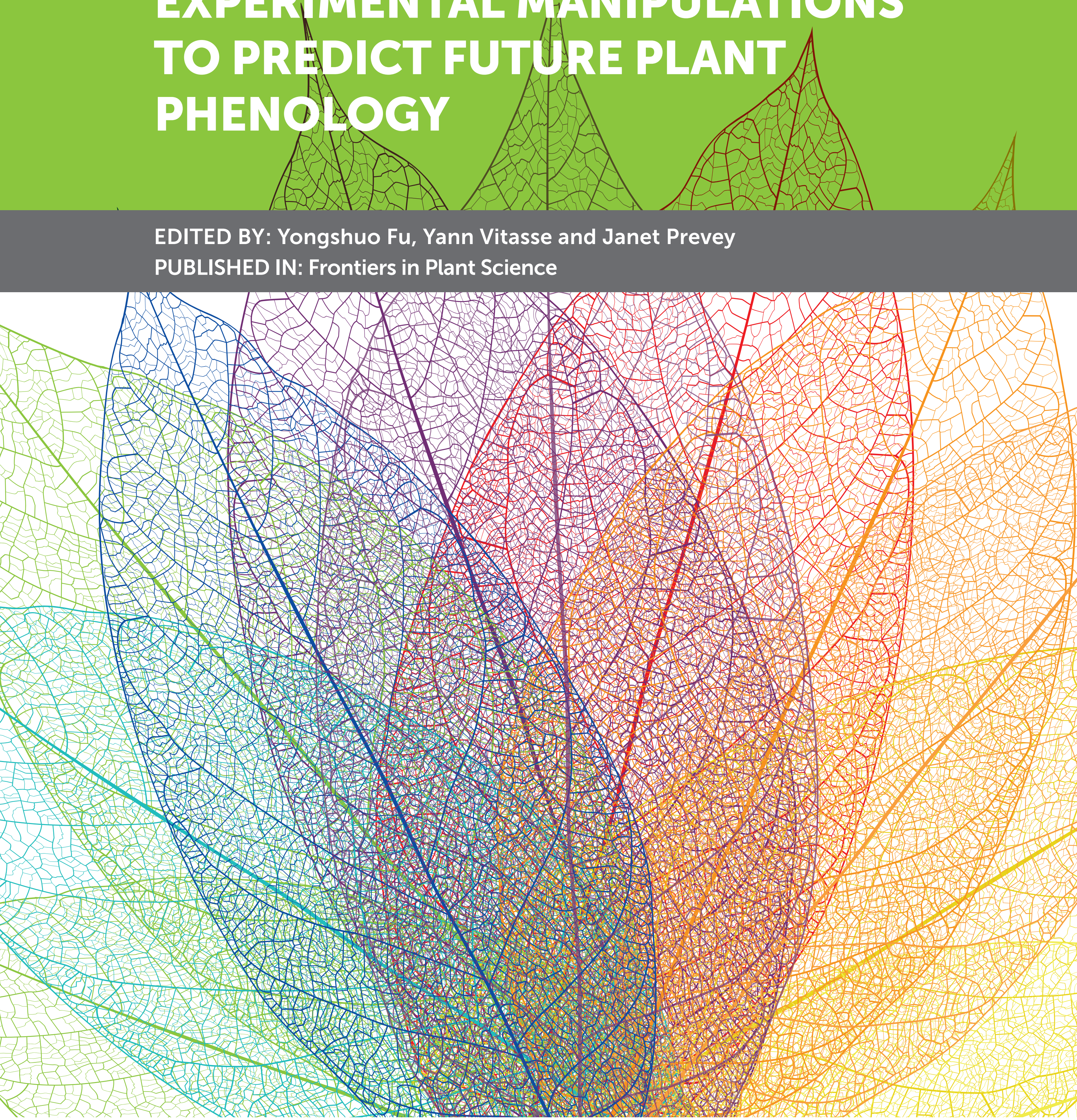

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EXPERIMENTAL MANIPULATIONS TO PREDICT FUTURE PLANT PHENOLOGY

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EDITORIAL
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Editorial: Experimental Manipulations to Predict Future Plant Phenology

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Keywords: plant phenology, climate change, vegetation, mechanistic understanding, photoperiod, temperature, warming

Editorial on the Research Topic

Experimental Manipulations to Predict Future Plant Phenology

Plant phenology is one of the most visible biological indicators of ongoing climate change. Shifting phenological events, such as earlier leaf-out and flowering in spring and overall delayed leaf senescence in fall, have been observed around the world over the past few decades, and these changes have the potential to substantially affect plant fitness and distribution (Chuine, 2010), plant-animal interactions (Rudolf, 2019), as well as regional and global-scale carbon, water, and energy balances (Richardson et al., 2013). However, how plant phenology will continue to shift with future climate change remains uncertain. For example, as temperatures rise, plants may not experience winter conditions that are cold enough to satisfy a complete dormancy release, making leaf-out less sensitive to warmer temperatures in spring (Fu et al., 2015). For summer and fall phenophases, drought and hot spells may advance or delay leaf senescence and can alter nutrient reallocation. In order to adequately predict the future dynamics of vegetation–climate systems, it is therefore essential to understand the driving factors of plant phenology and how they may change under a warmer climate. To date, most of the published literature consists of observational studies of plant phenological responses to environmental variation over time, and models are generally calibrated using long-term series of observations. However, future predictions require running simulations beyond the range of historical conditions, and seasonal changes in temperature, photoperiod, and soil moisture are often highly correlated (Flynn and Wolkovich, 2018; Ettinger et al., 2020). This further complicates the acquisition of accurate parameters that reflect the physiological functioning of plant development in response to environmental cues. Experimental studies are therefore key and urgently needed to isolate the different drivers and to examine how they separately and interactively influence changes in plant phenology.

The articles presented here address several of the outstanding gaps in knowledge of plant phenology using experimental manipulations. These studies were conducted in a wide range of ecosystems, from the coldest locations on the planet in Arctic tundra, to ecosystems with extreme seasonal fluctuations on the Tibetan Plateau, to some of the driest regions in semi-arid deserts. These studies also utilized a diversity of manipulative methods to examine plant responses to environmental cues, including passive open-top chambers, active electrical heaters, climate-controlled growth chambers, as well as making use of natural climatic and micro-climatic gradients as experimental treatments to test phenological differences.

Four of the manuscripts in this Research Topic used manipulative experiments to parse the effects of potential environmental and biological drivers on plant phenology by isolating these

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driving variables, while controlling for others, in climate-controlled growing chambers or open-top warming chambers. Wang et al. placed twigs of six shrub and tree species in growing chambers to examine the interactive effects of chilling, forcing, and photoperiod on the timing of flowering. They found that increases in chilling and forcing temperatures advanced phenology for most of the species, while longer daylength significantly advanced phenology for only two of the species, especially under low chilling conditions. Malyshev et al. Similarly, found that experimental warm spells advanced leaf-out timing of European beech and birch seedlings when applied after substantial exposure to chilling temperatures, whereas warm spells deepened dormancy and therefore delayed leaf-out when they occurred in fall for European beech. Hu et al. found that increasing winter temperature with passive warming using open-top chambers in an alpine meadow on the Tibetan Plateau advanced the timing of flowering and fruiting for most plant species. Here again, changes in the timing of reproductive phenology varied by plant species. Not only abiotic drivers influence plant phenology; phenological shifts might also be influenced by plant-animal interactions. Ulrich et al. used innovative climate-controlled chambers in the iDiv Ecotron experimental setup to test effects of invertebrate density on plant phenology. They found that a greater density of invertebrates advanced the phenology of one plant species but delayed the phenology of another, in what might be one of the first studies to experimentally examine the effects of a biotic driver on changes in plant phenology.

Three studies in this collection illustrate how plant compositional changes, in combination with experimental manipulations, ultimately influence community-level shifts in phenology. In a long-term, large-scale warming experiment conducted across eight tundra sites, May et al. found that warming shifted the length of the growing season in some sites and not others, and these differences depended on plant species composition and growth forms at each site. In another high Arctic tundra location, Chisholm et al. examined plant phenology along topographical variations in permafrost melt and observed that phenology can be delayed in permafrost depressions compared to stable ground. These changes are due to both shifts in plant species composition to later-blooming species in permafrost depressions, as well as to later timing of phenological events within species. Meng et al. conducted a reciprocal transplant study in the Tibetan plateau and found that the temperature sensitivity of community-level flowering was strongly influenced by changes in the composition of flowering functional groups in transplanted plots, as well as by direct responses to changes in temperature along the climatic gradient.

To date, there have been few experimental studies of plant phenology in desert ecosystems. Here, Howell et al. and Bao et al. examine the experimental effects of warmer temperatures and changes in precipitation on plant phenology in desert ecosystems in North America and China. Howell et al. found that warmer temperatures advanced the timing of flowering and senescence of the early-growing invasive grass *Bromus tectorum*, and led to an overall shortening of the growing season for the grass. Bao et al. found that water addition treatments advanced leaf-out and delayed leaf senescence of the dominant shrub *Nitraria tangutorum,* indicating that increased precipitation may lengthen the growing season of this shrub in desert ecosystems.

The final three studies highlight innovative methods to measure plant phenological changes in response to historical and simulated climate change. Davis et al. describe and test a machine-learning method that accurately identifies buds, flowers, and fruits on digitized herbarium specimens, and can thus allow for large-scale phenological analyses of vast natural history collections increasingly available online. Menzel et al. illustrate a simple methodology and introduce an easy-to-use Shiny application (available at www.baysics.de) to measure how chilling and forcing temperatures influence phenology of cut twigs that can be implemented by citizen scientists and students. Finally, Frei et al. test how open-top chambers in combination with active heating can accurately simulate temperature changes for larger plants and trees. This quantitative assessment indicates that warming experiments involving taller vegetation should incorporate both passive and active warming to achieve consistent temperature differences between treatments.

Results from this collection of articles show that phenological responses can vary greatly among species, ecosystems, and even microsites. This suggests that future plant phenological changes in response to climate change may be more nuanced than generalized advances of phenological events per degree temperature increase across entire vegetation types. Identifying phenological responses to environmental drivers through manipulative experiments is crucial for a comprehensive understanding of the response of species and ecosystems to ongoing climate change and for better predictions of future phenological shifts.

AUTHOR CONTRIBUTIONS

JP drafted the first version of the editorial. YV and YF made extensive edits, additions, and revisions. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Divergent Responses of Community Reproductive and Vegetative Phenology to Warming and Cooling: Asymmetry Versus Symmetry

Fandong Meng[1,2†], Lirong Zhang[1†], Haishan Niu[2], Ji Suonan[1], Zhenhua Zhang[3], Qi Wang[1,2], Bowen Li[1,2], Wangwang Lv[1,2], Shiping Wang[1,4], Jichuang Duan[5], Peipei Liu[1,2], Wangmu Renzeng[1,2], Lili Jiang[1], Caiyun Luo[3], Tsechoe Dorji[1,4], Zhezhen Wang[6] and Mingyuan Du[7]*

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Few studies have focused on the response of plant community phenology to temperature change using manipulative experiments. A lack of understanding of whether responses of community reproductive and vegetative phenological sequences to warming and cooling are asymmetrical or symmetrical limits our capacity to predict responses under warming and cooling. A reciprocal transplant experiment was conducted for 3 years to evaluate response patterns of the temperature sensitivities of community phenological sequences to warming (transferred downward) and cooling (transferred upward) along four elevations on the Tibetan Plateau. We found that the temperature sensitivities of flowering stages had asymmetric responses to warming and cooling, whereas symmetric responses to warming and cooling were observed for the vegetative phenological sequences. Our findings showed that coverage changes of flowering functional groups (FFGs; i.e., early-spring FFG, mid-summer FFG, and late-autumn FFG) and their compensation effects combined with required accumulated soil temperatureto codetermined the asymmetric and symmetric responses of community phenological sequences to warming and cooling. These results suggest that coverage change in FFGs on warming and cooling processes can be a primary driver of community phenological variation and may lead to inaccurate phenlogical estimation at large scale, such as based on remote sensing.

Keywords: community phenological sequences, reciprocal transplant experiment, plant–climate interactions, temperature sensitivities, Tibetan Plateau

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INTRODUCTION

Climate warming causes a series of ecosystem responses (Walther et al., 2002), including changes in plant phenology (Li et al., 2016; Körner, 2016). Phenological changes would have an important effect on the carbon cycle and ecosystem productivity (Wolkovich and Cleland, 2014). Long-term *in situ* or remote sensing observations and manipulative warming experiments are the main methods used in phenological studies at present (Arft et al., 1999; Walker et al., 2006; Cleland et al., 2007; Morisette et al., 2009; Pieper et al., 2011; Li et al., 2016). Some studies have found that phenological temperature sensitivities are mismatched at community and species levels (Steltzer and Post, 2009; Wolkovich et al., 2014; Meng et al., 2016; Meng et al., 2017). This mismatch may be caused by divergent responses of different species to warming (Steltzer and Post, 2009; Wolkovich et al., 2014). However, these studies only consider that warming is the principal factor, ignoring daily or annual frequent temperature fluctuations (Menzel et al., 2011; Kosaka and Xie, 2013). Ignoring cooling effects may lead to a biased prediction of phenology under the background of global warming. Several studies have found that warming and cooling exert different influences on plant phenology (Menzel et al., 2011; Wang et al., 2014a; Meng et al., 2016; Meng et al., 2017). For example, spring temperature variance reduces the temperature sensitivity of early phenophases (Wang et al., 2014c). This may be caused by a higher temperature sensitivity of phenology to cooling (8.4°C) than to warming (-2.1°C), whereas the opposite pattern has also been found for some species (Wang et al., 2014a). Moreover, some studies find that global annual mean temperature has frequently fluctuated (IPCC, 2007). For example, although the Tibetan Plateau is warming across the years, the annual average surface temperature at the Haibei Alpine Meadow Ecosystem Research Station (HBAMERS) at the Tibetan Plateau is 22 out of 44 years (from 1957 to 2000) lower than average, suggesting that warming and cooling spells have frequently occurred in this region (Li et al., 2004). However, the prevailing focus has been on the effect of warming on phenology, and few studies have distinguished differences in phenological responses to warming and cooling (Wang et al., 2014a; Meng et al., 2016; Li et al., 2016; Signarbieux et al., 2017). In particular, one study found a significant difference in the response of flowering to warming and cooling, which indicates an asymmetrical response, whereas a symmetrical response would be indicated by an equal magnitude of the response of phenology to warming and cooling on phenology (Wang et al., 2014a). However, there is little evidence to date on asymmetrical or symmetrical responses to warming and cooling for community phenological sequences.

Many studies have shown that phenological temperature sensitivity is species-specific, even under similar environmental conditions (Diez et al., 2012; Iler et al., 2013; Wang et al., 2014a, Wang et al., 2014b). Hence, phenological changes at the species level are difficult to match with changes at the community level due to divergent responses by different species (Steltzer and Post, 2009). However, few studies have focused on the compensatory effects of different species to community phenological sequences based on field observations. For example, individual species' responses may be mutually offsetting if data on advanced and delayed phenophases of individual species are pooled (Meng et al., 2016; Meng et al., 2017). Especially, climate change is the main driver of biodiversity changes (Gill et al., 1998; Sala et al., 2000; Augspurger et al., 2005; Chuine, 2010; Wolf et al., 2017). Changes in community composition induced by temperature change would affect the responses of community phenological sequences to temperature change (Cleland et al., 2006; Cleland et al., 2007). In particular, community composition may have contrasting reactions to warming and cooling (Meng et al., 2017; Meng et al., 2018). Therefore, we hypothesized that changes in the coverage of flowering functional groups [FFGs; i.e., early-spring FFG (ESF), mid-summer FFG (MSF), and late-autumn FFG (LAF)] could be associated with the response patterns (i.e., asymmetry vs. symmetry) of community phenological sequences to warming and cooling.

In this study, we used a reciprocal transplant experiment to accomplish warming and cooling at four elevation gradients (i.e., 3,200, 3,400, 3,600, and 3,800 m a.s.l.) (Wang et al., 2014a). Our previous reports focused on changes in FFG coverage affecting community phenological sequences to warming and cooling (Meng et al., 2017). Here, we explore their response patterns to warming and cooling to test whether changes in temperature and FFG coverage codetermine asymmetric or symmetric responses of community phenological sequences to warming and cooling. Our objectives are to answer the following questions: (1) Are the responses of temperature sensitivities of FFG coverage and community phenological sequences to warming and cooling asymmetrical or symmetrical? (2) What are the main factors affecting their response patterns in the alpine region?

MATERIALS AND METHODS

Design of the Reciprocal Transplant Experiment

The study site is located at HBAMERS (37°37′ N, 101°12′ E). Our experiment was carried out along four elevation gradients (i.e., 3,200, 3,400, 3,600, and 3,800 m a.s.l.), digging out 12 plots from each elevation (i.e., 48 plots in total) before soil thawing in May 2007. Each plot was 1 m × 1 m × 0.4 m (the depths at 3,600 and 3,800 m were 0.3 m due to their shallower soil layer; **Figure 1**). Then nine randomly selected plots at each elevation were reciprocally averagely transplanted to the other three elevations (i.e., three plots per elevation; **Figure 1**). For example, 9 of 12 plots from 3,200 m were randomly transplanted to 3,400, 3,600 and 3,800 m (i.e., three plots each elevation), leaving three plots in the original elevation (**Figure 1**); the other three elevations also had a similar distribution. Therefore, 12 plots of 3,200 m had three plots from 3,200 m, three plots from 3,400 m, three plots from 3,600 m, and three plots from 3,800 m (**Figure 1**). The spacing distance between each plot was 1 m. Grazing was excluded by fencing the plots at each elevation.

Community Phenological Sequences, Coverage, and Meteorological Factor Monitoring

To observe community phenological sequences and coverage, a quadrant of 1 m × 1 m was used with 100 cross points (10 cm × 10 cm) for each plot. Phenological variation and coverage of each species were recorded under each cross point. The observation intervals of community phenological sequences were 3–5 days during each full growing season from 2008 to 2010. The observation of coverage was conducted once in late August or early September in each of the 3 years. Seven

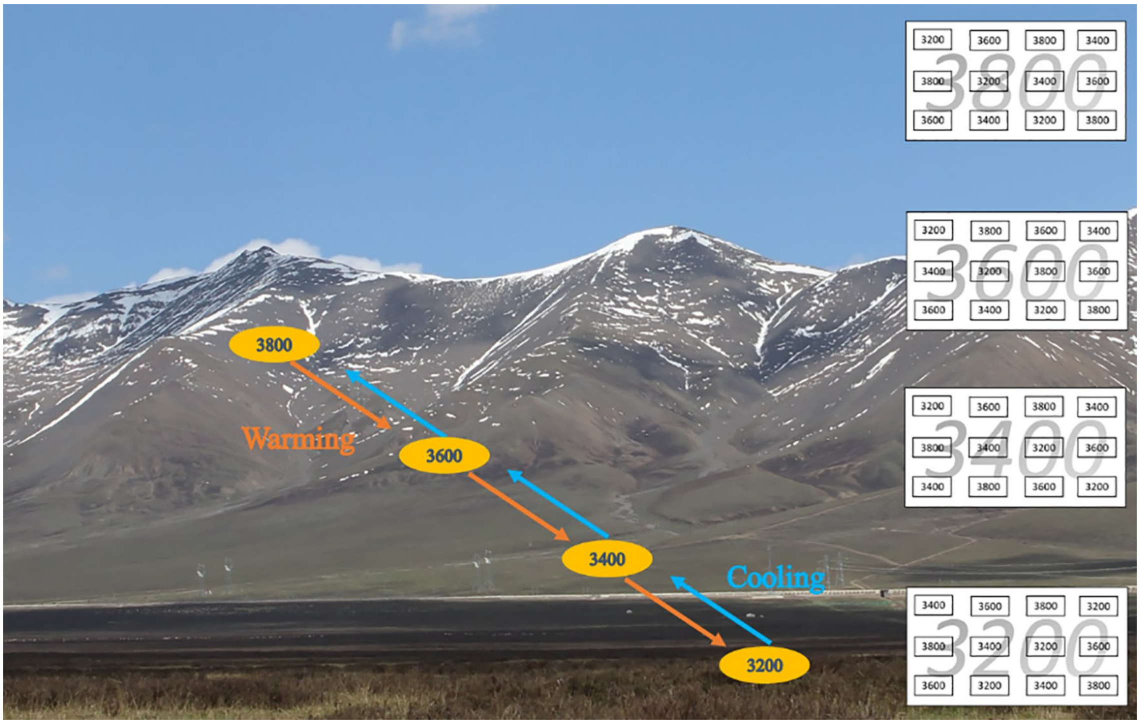

FIGURE 1 | Reciprocal transplant experiment diagram and landscape. Each elevation has 12 plots (three plots per elevation). Small rectangles with black numbers indicate plots transferred from their elevations, and large rectangles with gray numbers indicate the elevation gradient. Red arrows indicate downward transfer (i.e., warming), and blue arrows indicate upward transfer (i.e., cooling).

phenological sequences were monitored. The timings of community phenological sequences were the date on which the corresponding phenophase occurred for 15% of individuals, irrespective of plant species (Meng et al., 2017). The phenophases observed were onset of leaf out (OLO), first flower bud (FB), first flowering (FF), first fruiting set (FFS), postfruiting vegetation (OPFV), and first leaf coloring (FLC). The end of complete leaf coloring (CLC) was the date on which CLC occurred for 95% of individuals (Meng et al., 2016; Meng et al., 2017). All observed species were divided into three FFGs based on their flowering times (i.e., ESF, MSF, LAF; as detailed in Meng et al., 2017). Here, FFGs are defined as collections of organisms based on similar flowering behavioral or environmental responses. Plant species were classified into three FFGs (i.e., ESF, MSF, and LAF) based on their life history (Meng et al., 2016). Coverages of different FFGs were calculated for each plot in mid-August of each year based on the frequency of individuals of each FFG, irrespective of plant species, at the cross points of the 100 grid cells (Meng et al., 2016; Meng et al., 2017). Mean coverages of ESF and MSF across the natural elevation gradients (i.e., no translocation) were about 30% and 70%, respectively (**Table S1**, Meng et al., 2016). Meanwhile, coverages of dominant species were less than 50%, and there were more than 40 species in the natural community (**Table S1**, Meng et al., 2016).

Soil temperatures at 5-cm depth and moisture at 20-cm depth were monitored at an interval of 1 min by HOBO (Onset Computer Corporation, Cape Cod, Massachusetts, USA) weather stations at each site (Wang et al., 2014a). We compared Pearson's correlation coefficients of community phenological sequences between air and soil temperature and found that soil temperature was a better predictor than air temperature (**Table S2**), as had been found in our previous study at species level (Wang et al., 2014a). Therefore, we chose soil temperature as our predictor variable.

Data Analysis

We used a slope method to calculate the sensitivities of phenology and coverage (i.e., a coefficient based on $y = a*x + b$), where y and x are the differences between receptor and donor sites in community phenological sequences and soil temperature changes, respectively. Transfer downward and upward signified warming and cooling, respectively. Positive and negative values of the temperature sensitivities of community phenological sequences (CPS) represent delay and advance in days per 1°C change, respectively.

A general linear model was used to test the effects of treatments and their interactions on the coverage of different FFGs and the differences in phenological events in SPSS version 23. Analysis of covariance (ANCOVA) [in the form of lm(CPD~Ts*grp)] was used to test the slope heterogeneity between warming and cooling. Here, CPD signifies the differences in date of CPS between transferred and control sites; Ts represents soil temperature differences between transferred and control sites;

and grp represents the categorical variables warming and cooling. All functions and packages used were from R 3.3.3 (R Core Team, 2017). To assess the effects of coverage change on CPS, partial correlations between changes in CPS and coverage changes were calculated, setting temperature changes as the control variable. The required accumulated soil temperature (RCST) of CPS was defined as the sum of daily soil mean temperature above 0°C from 1 November of the previous year to a certain phenophase (Wang et al., 2014a). Chilling requirement (CR) was defined as the sum of daily soil mean temperature below 0°C from 1 November of the previous year to a certain phenophase.

RESULTS

Asymmetric and Symmetric Responses of the Temperature Sensitivities of Different FFGs to Warming and Cooling

Coverages of ESF and MSF were significantly affected by year, donor sites, receptor sites, and most of their interactions (**Table 1**). However, coverage of the LAF was only affected by donor site and interaction between year and donor site (**Table 1**). The temperature sensitivities of FFG coverage for ESF and MSF were -10.42 and 10.66% °C^{-1} under warming and 4.35 and -3.93% °C^{-1} under cooling, respectively (**Table S3** and **Figure 2**). When warming and cooling data were pooled, they were -3.35 for ESF and 3.39% °C^{-1} for MSF (**Table S3** and **Figure 2**). Furthermore, the regression models of LAF were nonsignificant under warming and cooling and with pooled warming and cooling data (**Table S3** and **Figure 2**). Based on the results of the ANCOVA test, slope values had significant differences between warming and cooling for ESF and MSF, but there were nonsignificant differences for LAF (**Table 2**). Thus, our results indicate that the temperature sensitivities of coverage of ESF and MSF had asymmetric responses to warming and cooling (**Table 2**).

Asymmetric and Symmetric Responses of Temperature Sensitivities of CPS to Warming and Cooling

Onset of the CPS was significantly affected by year (from 2008 to 2010), donor sites, receptor sites, phenophases, and their interactions ($p < 0.001$, **Table 3**). The temperature sensitivities were -5.8, -7.6, -6.7, -8.3, -4.1, -1.3, and 3.1°C under warming

TABLE 1 | Analysis of variance of coverage of different flowering functional groups based on general linear models over 3 years.

Source	ESF			MSF			LAF		
	SS	df	P	SS	df	P	SS	df	P
Year (Y)	1,121.318	2	<0.001	988.157	2	<0.001	15.551	2	0.128
Donor (D)	3,230.945	3	<0.001	2,600.765	3	<0.001	41.657	3	0.015
Receptor (R)	1,089.35	3	<0.001	1,103.025	3	<0.001	16.368	3	0.225
Y * D	1,135.346	4	<0.001	883.559	4	0.002	76.049	4	0.001
Y * R	1,132.716	4	<0.001	1,215.588	4	<0.001	22.261	4	0.207
D * R	745.921	5	0.004	714.522	5	0.014	1.368	5	0.996
Y * D * R	213.169	2	0.066	194.682	2	0.123	0.855	2	0.889
Error	1,780.778	48		2,137.235	48		174.143	48	

ESF, early-spring flowering functional group; MSF, mid-summer flowering functional group; LAF, late-autumn flowering functional group.

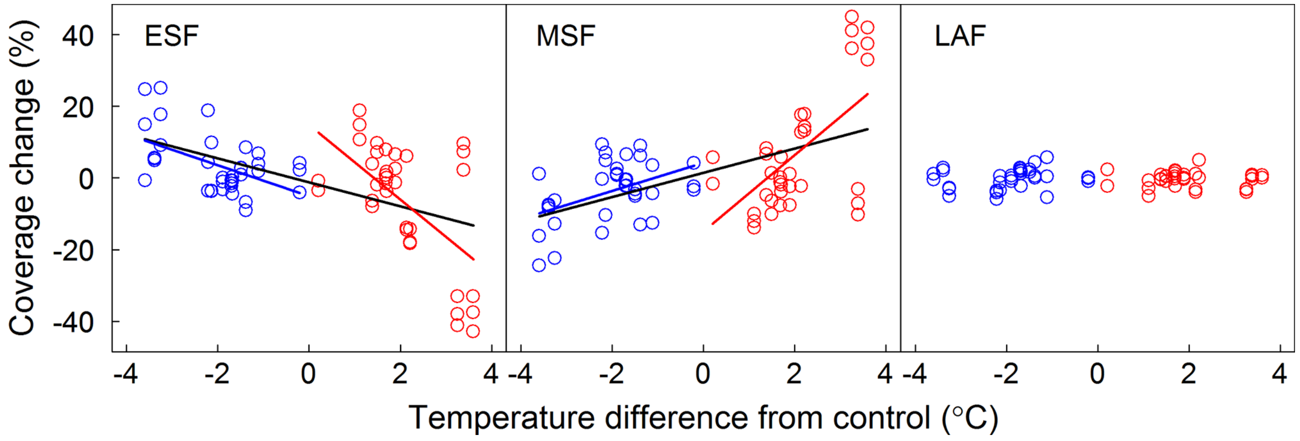

FIGURE 2 | Relationships between coverage changes of FFGs and the annual temperature difference between receptor site and donor site. Linear regression equations for warming, cooling, and pooled warming and cooling data are indicated by red, blue, and black lines, respectively. ESF, early-spring flowering functional group; MSF, mid-summer flowering functional group; LAF, late-autumn flowering functional group.

TABLE 2 | Slope heterogeneity of temperature sensitivities of different flowering functional groups and community level between warming and cooling.

	OLO	FB	FF	FFS	OPFV	FLC	CLC	ESF	MSF	LAF
PS or C	0.454	**0.014**	**0.009**	0.349	0.831	0.428	0.534	**0.026**	**0.016**	0.291

PS and C signify phenophases and coverage changes, respectively. P value in the ANCOVA method is the interaction effect in the test of the linear model. OLO, onset of leaf-out; FB, first bud/boot-set; FF, first flowering; FFS, first fruit set for forbs or seeding-set for graminoids; OPFV, onset of postfruiting vegetation; FLC, first leaf coloring; CLC, the date of complete leaf coloring; ESF, early-spring flowering functional group; MSF, mid-summer flowering functional group; LAF, late-autumn flowering functional group. Bolded text means 0.05 level.

TABLE 3 | Analysis of variance of timing differences in community phenological sequences based on general linear models over 3 years.

Source	SS	df	Sig.
Year (Y)	963.954	2	<0.001
Donor (D)	29,563.690	3	<0.001
Receptor (R)	23,278.409	3	<0.001
Phenophase (P)	1,844.144	6	<0.001
Y * D	1,351.883	5	<0.001
Y * R	572.058	5	<0.001
Y * P	892.939	12	<0.001
D * R	416.331	5	<0.001
D * P	21,423.539	18	<0.001
R * P	14,686.850	18	<0.001
Y * D * R	583.069	6	<0.001
Y * D * P	1,789.599	30	<0.001
Y * R * P	2,448.979	30	<0.001
D * R * P	1,277.900	30	<0.001
Y * D * R * P	1,080.838	36	<0.001
Error	262.000	420	

and 5.9, 10.3, 9.1, 7.8, 5.7, 3.5, and -3.0°C under cooling for OLO, FB, FF, FFS, OPFV, FLC, and CLC, respectively (**Table S3** and **Figure 3**). When warming and cooling data were pooled, the temperature sensitivities were -8.5, -8.5, -8.5, -8.6, -5.6, -3.7, and 4.3°C for OLO, FB, FF, FFS, OPFV, FLC, and CLC, respectively (**Table S3** and **Figure 3**).

Slope heterogeneities showed nonsignificant differences between warming and cooling for OLO, FFS, OPFV, and CLC, but there were significant differences for FB and FF based on the ANCOVA test (**Table 2**). Thus, our results indicate that all temperature sensitivities of vegetative phenophases and FFS had symmetric responses to warming and cooling, whereas the temperature sensitivities of flowering phenophases (e.g., FB and FF, **Table 2**) showed asymmetric responses to warming and cooling.

Effects of Biotic and Abiotic Factors on Symmetric and Asymmetric Responses of the Temperature Sensitivities of CPS

Nearly all coverage changes of ESF under warming and cooling and MSF under warming had significantly positive or negative correlations with CPS (**Table S4** and **Figure 4**). Only OLO, OPFV, and CLC had significantly negative correlations with MSF under warming. However, correlations between the other CPS and coverage changes under warming and cooling were nonsignificant (**Table S4** and **Figure 4**).

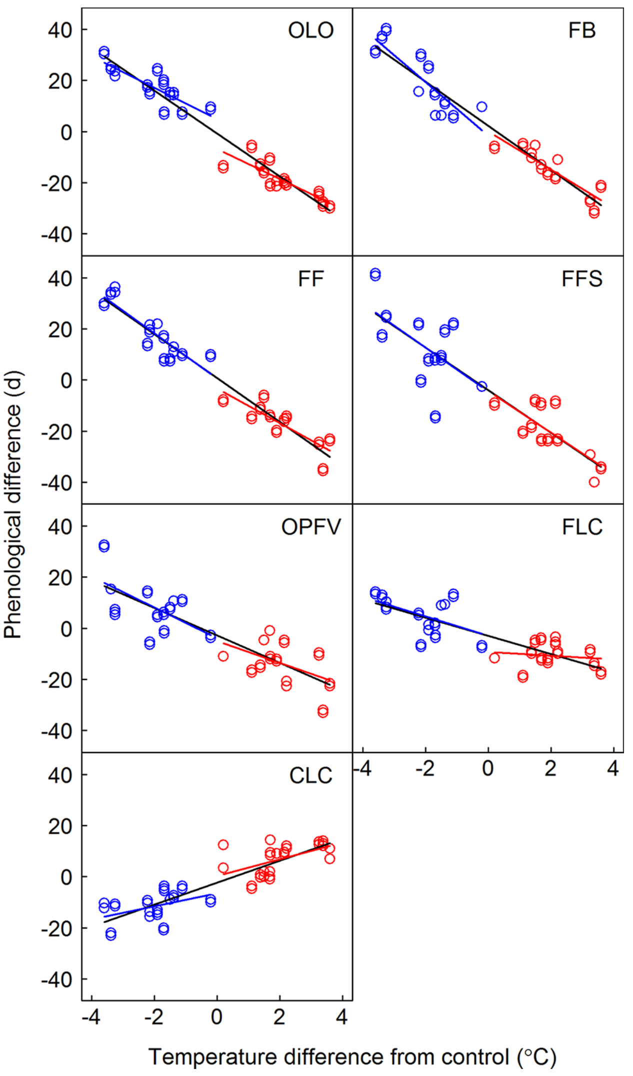

FIGURE 3 | Relationship between the differences in timing of phenological sequences and temperature differences between receptor site and donor site. Linear regression equations for warming, cooling, and pooled warming and cooling data are indicated by red, blue, and black lines, respectively. OLO, onset of leaf-out; FB, first bud/boot-set; FF, first flowering; FFS, first fruit set for forbs or seeding set for graminoids; OPFV, onset of postfruiting vegetation; FLC, first leaf coloring; CLC, the date of complete leaf coloring.

Seven phenological sequences have the same chilling accumulations (i.e., CR) after transfer (**Figure 5**). The temperature sensitivities of RCST of three early community phenophases (i.e., OLO, FB, and FF) and linear regressions of FFS were nonsignificant under warming and cooling. However, the temperature sensitivities of CR for all seven CPS and RCST in

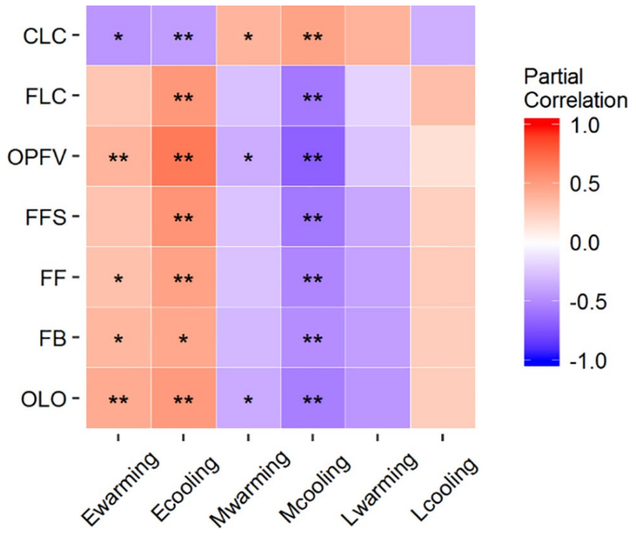

FIGURE 4 | Partial correlations between coverage changes of FFGs and differences in CPS. **P* < 0.05 and ***P* < 0.01. OLO, onset of leaf-out; FB, first bud/boot set; FF, first flowering; FFS, first fruit set for forbs or seeding set for graminoids; OPFV, onset of postfruiting vegetation; FLC, first leaf coloring; and CLC, the date of complete leaf coloring. Ewarming and Ecooling, Mwarming and Mcooling, and Lwarming and Lcooling signify coverage changes of ESF, MSF, and LAF under warming and cooling, respectively.

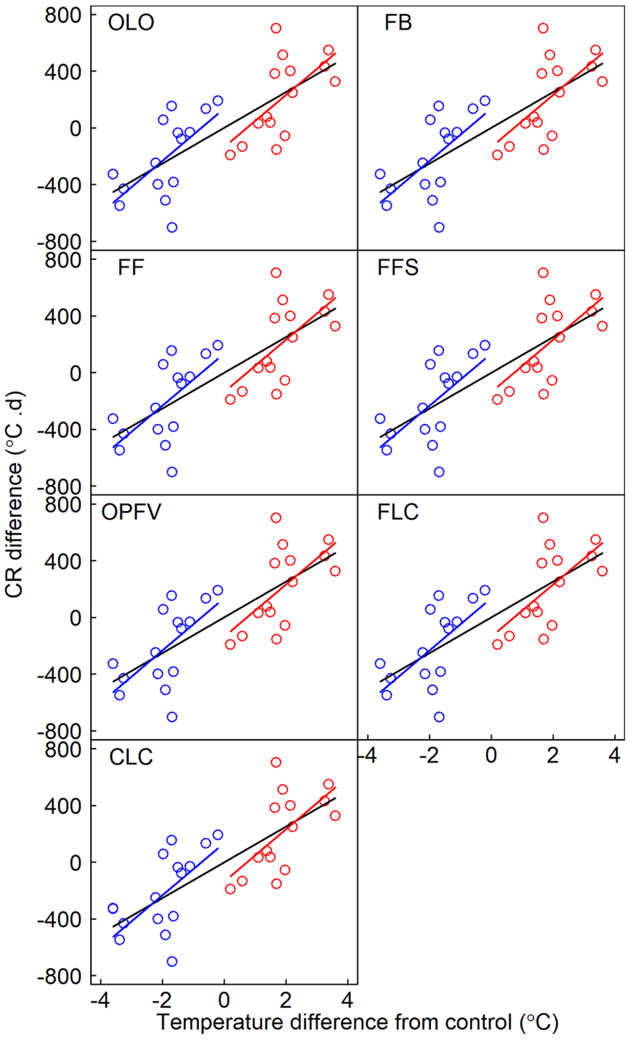

FIGURE 5 | Relationships between differences in chilling requirements (CRs) and the temperature difference between receptor site and donor site. CR is defined as the sum of daily soil mean temperature below 0°C from 1 November of the previous year to a certain phenophase. Linear regression models for warming, cooling, and pooled warming and cooling data are indicated by red, blue, and black lines, respectively. OLO, onset of leaf-out; FB, first bud/boot set; FF, first flowering; FFS, first fruit set for forbs or seeding set for graminoids; OPFV, onset of postfruiting vegetation; FLC, first leaf coloring; CLC, the date of complete leaf coloring.

late CPS (i.e., OPFV, FLC, and CLC) had symmetric responses to warming and cooling (**Tables S4** and **S5**, **Figures 5** and **6**).

DISCUSSION

Our reciprocal transplant experiment allows us to distinguish the effects of induced warming and cooling on plant phenophases. Interestingly, we found that the temperature sensitivities of change in ESF and MSF coverage and the temperature sensitivities in flowering stages (FB and FF) had asymmetric responses to warming and cooling, whereas the temperature sensitivities of vegetative phenophases and FFS had symmetric responses to warming and cooling. We propose two explanations involving biotic and abiotic factors that determine symmetric or asymmetric responses of the temperature sensitivities of CPS to warming and cooling.

Effects of Biotic Factors

Three biotic mechanisms may explain the symmetric or asymmetric responses of CPS to warming and cooling in our study. First, many studies have shown that warming significantly changes species and functional group composition (Walker et al., 2006; Chuine, 2010; Wang et al., 2012). In our study, we found that responses of coverage of ESF and MSF plant groups had contrasting trends. This may be caused by interspecific competition. For example, ESF were relatively short and MSF were relatively tall, and warming promoted tall plants. However, increased frequencies of tall plants would aggravate shelter effects on short plants. Cooling, however, would have the opposite effects. Coverage changes of different FFGs may alter the response magnitude of CPS to warming and cooling. Our previous results showed that simple correlation between FFG coverage change and differences in CPS was significant (**Table 3** in Meng et al., 2017). We further analyzed their partial correlations to eliminate the effect of temperature (**Figure 4**). Although temperature sensitivities of ESF and MSF were greater to warming than to cooling because of asymmetric responses (**Table 1**; **Figure 2**), in general, the correlation coefficients between FFG coverage changes and differences in CPS were greater under cooling than warming (**Figure 4**). Thus, ESF or MSF coverage change gave rise to symmetric responses of CPS to warming and cooling.

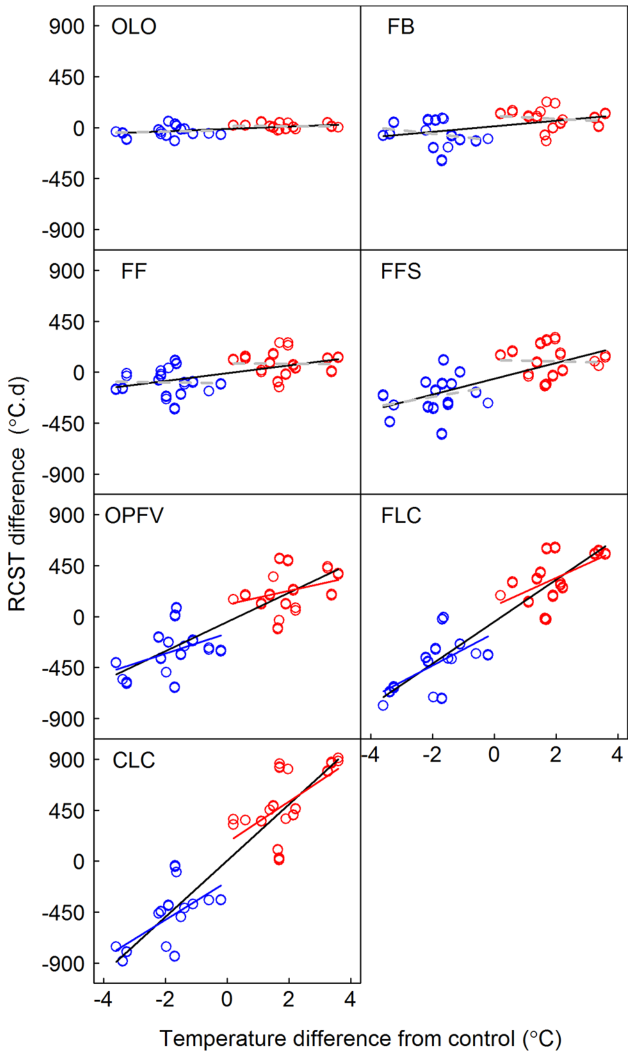

FIGURE 6 | Relationships between differences in required accumulated soil temperature (RCST) and the temperature difference between receptor site and donor site. Linear regression models for warming, cooling, and pooled warming and cooling data are indicated by red, blue, and black lines, respectively. OLO, onset of leaf-out; FB, first bud/boot set; FF, first flowering; FFS, first fruit set for forbs or seeding set for graminoids; OPFV, onset of postfruiting vegetation; FLC, first leaf coloring; CLC, the date of complete leaf coloring.

Therefore, OLO, OPFV, and CLC had symmetric responses to warming and cooling due to their significant relationships with FFG coverage change under warming and cooling (**Table 2**; **Figures 3** and **4**). FFS and FLC also had symmetric responses due to the effects of increased coverage of ESF and decreased coverage of MSF under cooling (**Table 2**; **Figures 3** and **4**).

However, our results showed that temperature sensitivities in the flowering stages had asymmetric responses to warming and cooling. Although coverage change of ESF under warming and cooling caused symmetric responses of CPS, decreased coverage of MSF under cooling significantly delayed CPS due to their significant relationships (**Figure 4**). Therefore, coverage changes of ESF and MSF caused asymmetric responses to warming and cooling in flowering stages, and flowering stages were more sensitive to cooling (**Table 2**; **Figure 3**). Such a response could prevent vulnerable reproductive tissue from being damaged due to low temperatures in spring (Körner et al., 2016; Körner and Basler, 2010; Inouye, 2008) because reproductive phenophases are the most important phases determining population dynamics (Hoffmann et al., 2010; Craine et al., 2012). These results show that changes in species richness and abundance in a community could affect the responses of CPS to warming and cooling. Therefore, ignoring changes in community composition when studying CPS would lead to inaccurate predictions.

Second, different flowering species and functional groups may have compensatory effects on CPS. In general, the vegetative stage and FFS of different species and FFGs had divergent responses to warming and cooling, with both asymmetric and symmetric responses (**Table S6**, Wang et al., 2014a). A time niche complementarity effect between species and FFGs may be associated with symmetric responses to warming and cooling (**Table S6**). This may indicate that different hierarchical levels in an ecosystem have different response characteristics and that symmetric responses at higher (e.g., community) levels are more stable compared with responses at lower (e.g., species or population) levels, which is consistent with previous phenological hierarchy theory (Li et al., 2016). We found that the temperature sensitivities in the flowering stage (FB and FF) of different flowering species and functional groups had asymmetric responses to warming and cooling (**Table S6**). Moreover, the temperature sensitivities of flowering stages for ESF and MSF were greater under cooling than under warming (6.7 vs. -5.1°C, Wang et al., 2014a). Therefore, divergent responses of species and different FFGs caused asymmetric responses to warming and cooling at the community level.

Third, pollinator availability is considered to be the most important determining factor for flowering phenology (De Jong and Klinkhamer, 1991; Mahoro, 2002; Byers, 2017). Nearly 90% of flowering plants are entomophilous (Shivanna and Tandon, 2014). Therefore, flowering could not continuously advance partly due to loss of pollinators under warming. For example, flowering advance per day would lose 0.31 pollinators under warming (Petanidou et al., 2014). However, under cooling, plants only need to postpone flowering to wait for the suitable temperature and for pollinators to come (Kudo and Ida, 2013). Thus, asymmetric responses may be attributed to avoiding a mismatch between pollinators and plant (Rafferty and Ives, 2011; Kudo and Ida, 2013). However, there were no such constraints due to symbiotic relationships for vegetative phenophases and FFS. Therefore, these phenophases showed symmetric responses to warming and cooling.

Effects of Abiotic Factors

Many studies show that chilling and heat requirements trigger the onset of plant phenology (e.g., Fu et al., 2015; Cong et al., 2017b), and they are key variables in phenological models (Chuine et al., 2000; Schwartz, 2003; Richardson et al., 2013). Our results show that chilling accumulations remained unchanged (**Figure 5**) because daily average temperature is above 0°C after OLO. Meanwhile, chilling accumulations of phenophases have symmetric responses to warming and cooling (**Figure 5**). Therefore, CRs may be not the factor leading to divergent effects on flowering and vegetative processes. However, our results showed that the temperature sensitivities of RCST in early CPS (i.e., OLO, FB, FF, and FFS) had a nonsignificant linear regression model with warming and cooling (**Figure 6**). The response mode of RCST did not match with early CPS (**Figures 3** and **6**). This may be caused by RCST in early CPS being affected by many factors, such as chilling accumulation (Fu et al., 2015). Such effects could decrease the risk of spring cold damage in order to increase plant fitness (Bagnall and Wolfe, 1978; Inouye, 2008; Post et al., 2008; Hacker et al., 2011; Haggerty and Galloway, 2011). However, we found that the temperature sensitivities of RCST in late CPS (i.e., OPFV, FLC, and CLC) had symmetric responses to warming and cooling (**Figure 6**). Their response mode matched with that of early CPS (**Figures 3** and **6**). This could explain symmetric responses of the temperature sensitivities of late CPS due to their close relationships (Franks et al., 2014). This may indicate that late CPS are mainly affected by RCST, whereas early phenophases are less strongly affected by RCST.

Implications of Symmetric and Asymmetric Responses of CPS to Temperature Change

Our results showed symmetric responses of the temperature sensitivities of community vegetative phenophases to warming and cooling spells (**Table 2**; **Figure 3**). These results suggest that cooling spells have no significant influence on the prediction of vegetative phenophases under a long-term warming trend. Although continued warming could increase heating requirements (**Figure 6**) as in other studies, we found that cooling could symmetrically decrease heating requirements for vegetative phenophases. Therefore, the responses of vegetative phenophases to long-term warming would not be diminished by short-term cooling spells. The decreased magnitude of the response of vegetative phenophases may be attributed to increased heating requirements (Fu et al., 2014; Fu et al., 2015) or increased heat requirement beyond the temperature increase (Cong et al., 2017a). However, it is noteworthy that different ecosystems may show different symmetric or asymmetric responses to warming and cooling. For example, Wolkovich et al. (2012) found that the temperature sensitivity of all pooled species observed under warming experiments is underestimated compared with long-term observations. This difference may be caused by asymmetric responses to warming and cooling due to pooling all species from different regions (**Figure 2** in Wolkovich et al., 2012). In particular, species richness and abundance would be altered under rapid warming (Richardson et al., 2013; Polgar et al., 2014; Tang et al., 2016). This would change the response pattern to warming and cooling in the future. We found that, unlike vegetative phenophases, reproductive phenophases had asymmetric responses to warming and cooling, and that they were more sensitive to cooling (**Table 2** and **Figure 3**). Therefore, pooled warming and cooling data would underestimate the responses of reproductive phenophases to warming. Hence, studies on reproductive phenophases should distinguish the effects of warming and cooling because asymmetric responses would mask the effects of warming or cooling.

CONCLUSIONS

Our results showed that coverage changes of FFGs and RCST codetermined the asymmetric (flowering stages) and symmetric responses (vegetative phenophases) of CPS to warming and cooling. Therefore, if data from warming and cooling periods under a long-term warming trend are pooled, reproductive phenophases would be underrated due to a higher effect size under cooling. Although our reciprocal transfer experiments could distinguish the different effect sizes of warming and cooling, only alpine herbaceous species were monitored, and we just conducted the experiment for 3 years. We therefore suggest that more reciprocal transfer experiments are conducted or new technologies developed to distinguish warming and cooling effects on herbaceous and woody species.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

SW designed this experiment; FM, JS, LZ, ZZ, QW, BL, WL, LJ, JD, PL, WR, CL, and MD performed this experiment; FM, HN, SW, ZW, and TD analyzed all data; FM, SW, and HN wrote this manuscript, and all persons provided some comments and suggestions for the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01310/full#supplementary-material

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The Interactive Effects of Chilling, Photoperiod, and Forcing Temperature on Flowering Phenology of Temperate Woody Plants

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The effects of winter chilling, spring forcing temperature, and photoperiod on spring phenology are well known for many European and North American species, but the environmental cues that regulate the spring phenology of East Asian species have not yet been thoroughly investigated. Here, we conducted a growth chamber experiment to test the effects of chilling (controlled by different lengths of exposure to natural chilling conditions), forcing temperature (12, 15, or 18°C) and photoperiod (14 or 10 h) on first flowering date (FFD) of six woody species (three shrubs and three trees) native to East Asia. The three-way analysis of variance (ANOVA) separately for each species showed that the effects of chilling and forcing temperature were significant for almost all species ($P < 0.05$). Averaged over all chilling and photoperiod treatments, the number of days until FFD decreased by 2.3–36.1 days when the forcing temperature increased by 3°C. More chilling days reduced the time to FFD by 0.7–26 days, when averaged over forcing and photoperiod treatments. A longer photoperiod could advance the FFD by 1.0–5.6 days, on average, but its effect was only significant for two species (including one tree and one shrub). The effects of forcing temperature and photoperiod interacted with chilling for half of the studied species, being stronger in the low chilling than high chilling treatment. These results could be explained by the theory and model of growing degree-days (GDD). Increased exposure to chilling coupled to a longer photoperiod reduced the GDD requirement for FFD, especially when plants grew under low chilling conditions. However, shrubs (except *Viburnum dilatatum*) had lower chilling and heat requirements than trees, suggesting that, by leafing out sooner, they engage in a more opportunistic life strategy to maximize their growing season, especially before canopy closure from trees' foliage. Our results confirmed the varying effects of these three cues on the flowering phenology of woody species native to East Asia. In future climate change scenarios, spring warming is likely to advance the spring phenology of those woody species, although the reduced chilling and shorter photoperiod may partly offset this spring warming effect.

Keywords: phenology, climate change, chilling, photoperiod, forcing, first flowering date, climate chamber experiment

INTRODUCTION

The changed timing of recurring biological events becomes a global concern against the background of climate warming. The earlier spring phenophases (*e.g.*, budburst date, leaf-out date) and later autumn phenophases (*e.g.*, leaf coloring date) of woody plants were observed over the past several decades in middle and high latitudes of the Northern Hemisphere (Chmielewski and Rötzer, 2001; Menzel et al., 2006; Gonsamo et al., 2013; Ge et al., 2015; Templ et al., 2017). Such climate-associated phenological change could influence carbon assimilation by modifying the length of the growing season (Keenan et al., 2014; Xia et al., 2015). Differing rates of change in phenology among interacting species result in phenological mismatches between trophic levels (*e.g.*, prey and predator, plant and their pollinators), which affect biotic interactions and community structure (Peñuelas and Filella, 2001; Burkle et al., 2013; Choi et al., 2019; Damien and Tougeron, 2019). Plant phenology also had a feedback effect on climate systems by altering the biophysical attributes of the planet's terrestrial surface and atmospheric structure and composition (Richardson et al., 2013). Therefore, in order to adequately predict the future dynamics in vegetation–climate systems and their modeling, it is essential to understand the driving factors of plant phenology.

In temperate regions, temperature is the main factor determining the budburst date of woody plants (Sparks and Menzel, 2002; Walther et al., 2002). The experimental evidence amassed to date shows that temperature exerts various influences on spring phenology in different developmental stages, and the temperature cues could be divided into winter chilling and spring forcing (Cannell and Smith, 1983; Heide, 1993; Körner and Basler, 2010; Campoy et al., 2011; Hänninen et al., 2019). Many studies found that when twigs and saplings (with dormant buds) were exposed to a longer period of chilling temperatures in natural or controlled conditions, they needed less time to budburst under the same growth-promoting conditions (Pletsers et al., 2015; Man et al., 2017; Nanninga et al., 2017). This effect could be described as the negative relationship between chilling accumulation and heat requirements of plants (Cannell and Smith, 1983). In other words, a decrease in the amount of chilling during winter could increase the demand for cumulative forcing temperatures in spring. Compared with chilling, the forcing temperature during spring is a more recognized factor driving spring phenology. Presuming the rate of plant development is positively related to temperature, higher spring temperatures would lead to faster forcing-temperature accumulation and thus an accelerated budburst (Murray et al., 1989; Hänninen, 1990). Therefore, climate warming can exert dual effects on spring phenology, because the amount of chilling may decrease due to winter warming effects (Fu et al., 2015b). Compared with chilling, the impact of spring warming seems stronger, since the empirical evidence shows an earlier onset of spring phenological events has been broadly observed across Europe (Penuelas et al., 2002; Menzel et al., 2006), North America (Cayan et al., 2001; Miller-Rushing and Primack, 2008) and East Asia (Matsumoto et al., 2003; Ho et al., 2006; Ge et al., 2015; Wang et al., 2019b).

In addition to chilling and forcing temperature, the effect of photoperiod on spring phenology has recently attracted considerable attention (Way and Montgomery, 2015). To examine whether a species is sensitive to photoperiod relied so far on controlled experiments. If the twigs or saplings of a specific species exposed to a long photoperiod need less time to attain budburst than those under a short photoperiod, this species may be designated as a photoperiod-sensitive one (Basler and Körner, 2012). According to recent work, only a small number of woody species are sensitive to photoperiod (Zohner et al., 2016), and this photoperiod sensitivity is not related to species' traits such as successional niche (early *vs.* late-successional), xylem anatomy (diffuse or ring-porous xylem) and leaf persistence (evergreen or deciduous) (Way and Montgomery, 2015). Those plant species native to low latitudes are more likely to rely on spring photoperiod as a leaf-out cue (Zohner et al., 2016). Even for the photoperiod sensitive species, many studies suggest their budburst dates only respond to photoperiod under the condition of insufficient chilling (Laube et al., 2014; Zohner et al., 2016).

As discussed above, the spring phenology of woody plants depends on at least three interacting environmental cues. But when using long-term observational data, it is difficult to identify the effect of a specific cue, because all environmental cues will covary year by year except for photoperiod (Flynn and Wolkovich, 2018). Therefore, controlled experiments offer the best way to investigate the interactive effects of chilling, photoperiod, and forcing temperature on the spring phenology of woody plants. Because controlling the environment of mature trees or shrubs in the field is logistically difficult (and very costly), most of the previous studies have carried such experiments on dormant twigs of woody plants (Primack et al., 2015). In this approach, twig cuttings are brought indoors and placed in controlled conditions, such as growth chambers, where they are monitored until they leaf out or reach the phenological stage of interest. This method has proven to be useful and realistic, since no significant differences were detected in the timing of budburst between cuttings and donor trees for three typical tree species growing under the same climatic condition (Vitasse and Basler, 2014). Currently, twig experiments focusing on one or multiple cues have been applied to woody plants in North America (Nanninga et al., 2017; Flynn and Wolkovich, 2018) and Europe (Caffarra et al., 2011; Zohner et al., 2016). In Asia, although one study did assess the effect of forcing temperature on budburst date (Wang et al., 2019a), no multispecies study has yet evaluated all three major cues through the controlled experiment approach.

To begin filling this knowledge gap, this study focused on six woody plants originating from East Asia, to investigate how different chilling, forcing temperature, and photoperiod treatments vary in their effects on spring phenology. We aimed to test two hypotheses: (1) A higher forcing temperature and increased exposure to chilling accelerate spring phenology; (2) The impact of photoperiod on spring phenology is species-specific and is dependent on the chilling conditions.

MATERIALS AND METHODS

Experimental Set-Up

Woody plant materials for the experiment were collected from Olympic Forest Park, Beijing (40° 01′ N, 116° 23′ E, 40–50 m above sea level), located 12 km north from the city center. Beijing has a typical continental monsoon climate (**Figure S1** in Data Sheet 2). Its summer months (June–August) are hot and rainy, with a mean temperature of 25.8°C and total precipitation of 364.5 mm (averaged from 1981 to 2010). Winter months (previous December to February) are cold and dry, having a mean temperature of −1.3°C and total precipitation of 9.2 mm. According to *a priori* criteria that the object plants should be widely distributed and native to East Asia, with high ornamental value, we selected six deciduous broadleaf woody plants for investigation (**Table 1**): three shrubs (winter jasmine, golden-bell, linden arrowwood) and three trees (Yoshino cherry, lilytree, wild peach). For each species, we selected three individuals as the parent plants from which to obtain the twig cuttings. Because our sampling location was a man-made forest, all the parent plants were of the same age (*ca.* 10 years old in 2017).

The chilling treatments were imposed by manipulating the duration of plants' exposure to natural chilling conditions (**Figure 1**). According to previous studies (Cannell and Smith, 1983; Laube et al., 2014; Asse et al., 2018), a temperature below 5°C was generally effective for breaking dormancy. In Beijing, the daily mean temperature usually drops to 5°C in mid-November and to 0° in mid-December, reaching its lowest level in early January (**Figure S1** in Data Sheet 2). Therefore, in the winter season of 2017, we collected twigs of the studied species on three sampling dates (14 November 2017, 19 December 2017, and 9 January 2018). The amount of chilling at the sampling dates was measured by the number of chilling days, defined as the number of days when the daily temperature was below 5°C.

$$CD(t) = \sum_{t=t_0}^{t_s} 1 \quad \text{if } T_i \leq 5 \tag{1}$$

where, CD is the number of chilling days; t_0 is the start date for chilling accumulation; t_0 was set to 1 November, following its use in previous studies (Cannell and Smith, 1983; Laube et al., 2014); t_s is

TABLE 1 | Characteristics of the six studied plant species.

Common name	Scientific name	Family	Life form
Winter jasmine	*Jasminum nudiflorum*	Oleaceae	Deciduous broadleaved shrub
Golden-bell	*Forsythia suspensa*	Oleaceae	Deciduous broadleaved shrub
Linden arrowwood	*Viburnum dilatatum*	Caprifoliaceae	Deciduous broadleaved shrub
Lilytree	*Yulania denudata*	Magnoliaceae	Deciduous broadleaved tree
Yoshino cherry	*Cerasus yedoensis*	Rosaceae	Deciduous broadleaved tree
Wild peach	*Amygdalus davidiana*	Rosaceae	Deciduous broadleaved tree

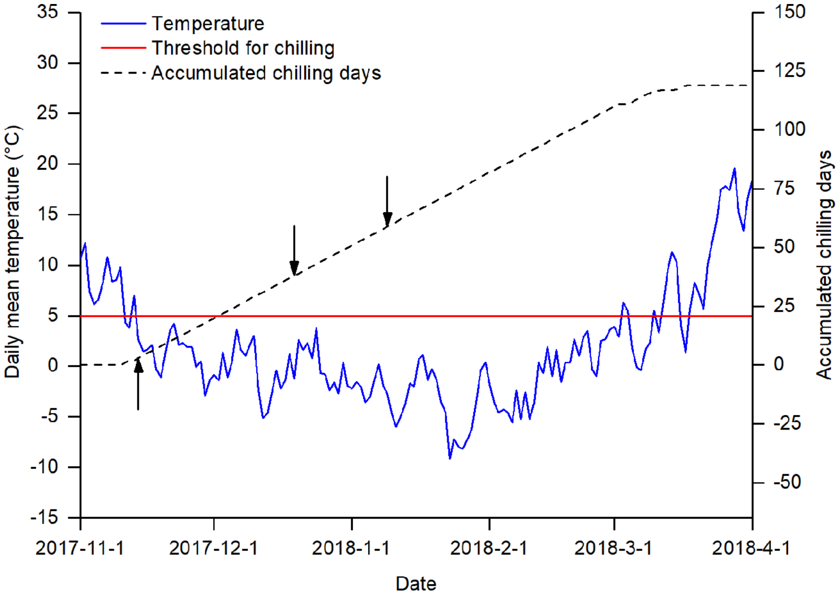

FIGURE 1 | Daily mean temperature and accumulated chilling days from 1 November 2017 to 1 April 2018. The arrows indicate the three sampling dates. The red line indicates the threshold for accumulating chilling days.

the sampling date; and T_t is the daily mean temperature (in °C) on day *t*. The daily mean temperature data for calculating the amount of chilling was obtained from the China Meteorological Data Service Center (http://data.cma.cn/site/index.html). Based on equation (1), three chilling treatments were achieved by collecting twigs at different times: low chilling (3 chilling days), intermediate chilling (38 chilling days), and high chilling (59 chilling days).

For each species, biological replicates were used in the form of one twig collected separately from three individuals. Thus, from each species on each sampling date, 18 twigs were cut (6 treatments × 3 individuals). To ensure all twigs had at least five flower buds we cut the twigs to variable lengths (usually 20–30 cm) as needed, since the cuttings' length had to be increase to accommodate those twigs with sparse buds. After cutting them, all the twigs were brought to the laboratory immediately, where they were placed into 0.25-L glass bottles filled with tap water. For each species, the three twigs from its different individuals were placed in one bottle, and six bottles were moved into different growth chambers (GXZ-500, Ningbo Jiangnan Instrument Factory, China).

In our experiment, the temperature treatments were designed to match the natural temperature variation in spring. According to Beijing's climate (**Figure S1** in Data Sheet 2), daily mean temperature generally increases from 12°C in early April to 18°C in late April. Thus, the three temperature treatments were set respectively to 12, 15, and 18°C.

In Beijing, the longest and shortest daylength within a year is 14.9 h (on the summer solstice) and 9.2 h (on the winter solstice), respectively. Thus, we chose two photoperiod treatments that approximated the maximum and minimum daylengths found under natural conditions. The long photoperiod treatment was 14 h (corresponding to the daylength in early May), while the short photoperiod treatment was 10 h (corresponding to the daylength in late January).

The treatments of photoperiod, chilling, and forcing temperature are summarized in **Figure S2** in Data Sheet 2. The growth chambers—in which the illumination, cooling, and heating systems were installed—could control the temperature and light duration automatically, according to user-defined settings. The lighting used in the growth chambers came from light-emitting diode (LED) tubes, in the form of cool white light with a photon flux density of 100 μmol m^{-2} s^{-1} (Red : Far Red = 3). To prevent the twigs and buds from drying out and failing to reach budburst, we put water-filled dishes with tissue paper into the chambers and maintained their relative humidity conditions above 70%. The twigs were re-cut and their water supply changed every second week to avoid blockages in their xylem caused by any fungi growing at the stem base.

Observations were made three times per week, for five months. For each twig, the date when at least one fresh flower had opened (sometimes several flowers opened simultaneously) was recorded as the first flowering date (FFD), which corresponded to BBCH 60 (Meier, 2001). For each species, the FFD was defined as the average of the FFD of three twigs.

Statistical Analysis

The response variable, *i.e.*, the number of days from sampling date to FFD, was analyzed in relation to three explanatory factors (categorical variables): (1) chilling days in natural conditions; (2) forcing temperature in the growth chambers, and (3) daylength in the growth chambers. For each species, the experimental data (see the **Supplementary Data Sheet**) were analyzed using a general linear model (GLM) to do a three-way analysis of variance (ANOVA) in SPSS 16.0 software (IBM Corp, Armonk, NY, USA). The mean values between treatments (or their combinations) were compared using Fisher's LSD test, at an alpha significance of 0.05.

To explain the impact of chilling accumulation and photoperiod on heat requirements of FFD, the growing degree-days (GDD) requirement was calculated as the accumulated degree-days from the sampling date to FFD on daily basis (equation 2).

$$GDD = \sum_{t=t_s}^{t_f} (T_g(t) - T_b) \tag{2}$$

where, t_s is the sampling date; t_f is the FFD; $T_g(t)$ is the forcing temperature in the growth chamber (in °C), at day *t*; and T_b is the threshold temperature for heat accumulation. Considering that 5°C was the threshold above which biological activity was generally believed to start (Cannell and Smith, 1983; Fu et al., 2015a), T_b was set to 5°C in this study.

RESULTS

Effects of Forcing Temperature on First Flowering Date

The impact of forcing temperature on the number of days until FFD was statistically significant ($P < 0.01$) for all species investigated

TABLE 2 | Analysis of variance for the effects of forcing temperature (F), chilling (C), photoperiod (P), and their interactions on the number of days until the first flowering date.

Variables	*J. nudiflorum*	*F. suspensa*	*V. dilatatum*	*Y. denudata*	*C. yedoensis*	*A. davidiana*
F	397.0**	314.9**	174.9**	100.5**	416.2**	371.6**
P	16.41**	2.794	1.073	0.362	21.41**	1.470
C	459.4**	326.5**	30.11**	1.005	131.7**	0.398
F × P	5.734**	2.417	2.833	0.814	2.700	1.174
F × C	6.373**	88.77**	0.395	0.090	58.14**	0.031
P × C	5.620**	5.465**	1.928	0.492	2.475	1.979
F × C × P	13.35**	1.348	0.587	0.362	5.437*	1.626

*The values shown are the F-statistic of the analysis of variance (ANOVA). **P < 0.01; *P < 0.05.*

(**Table 2**). The number of days until FFD occurred significantly decreased as the forcing temperature increased (**Figure 2**, **Table 3**). Averaged over all chilling (low, intermediate, and high chilling) and photoperiod (long and short photoperiod) treatments, the reduction in the number of days until FFD with increased temperature was greatest for *V. dilatatum*. The time to FFD was shortened by 57.1 days at a forcing temperature of 18°C compared with that of 12°C. Another responsive species was *C. yedoensis*, for which the difference in the number of days until FFD was 42.4 days between 12 and 18°C. Regarding *A. davidiana* and *F. suspensa*, compared with the forcing temperature of 12°C, their number of days until FFD at a forcing temperature of 18°C shortened by 32.3 days and 29.7 days on average, respectively. The flower(s) of *Y. denudata* could not open at 12°C, but at 18°C, the number of days required for FFD was 16.7 days less than that at 15°C. For *J. nudiflorum*, the effect of forcing temperature on its FFD was the weakest among the six species, as the difference between 12 and 18° C was just 10.8 days.

Furthermore, the response of FFD to forcing temperature was nonlinear (**Table 3**). At 12°C, the changed number of days until FFD per °C of warming (12 *vs.* 15°C) was stronger than that at 15°C (15 *vs.* 18°C) for all six studied species (**Table 3**). However, the extent of reduced temperature sensitivity of FFD varied among the species. The most obviously different was *A. davidiana*, for which the temperature sensitivity of FFD decreased from 9.6 days per °C warming (12 *vs.* 15°C) to 1.2 days per °C of warming (15 *vs.* 18°C). In stark contrast, the corresponding decreased temperature sensitivity was only 0.4 days/°C for *C. yedoensis*, being the weakest among the six species (**Table 3**).

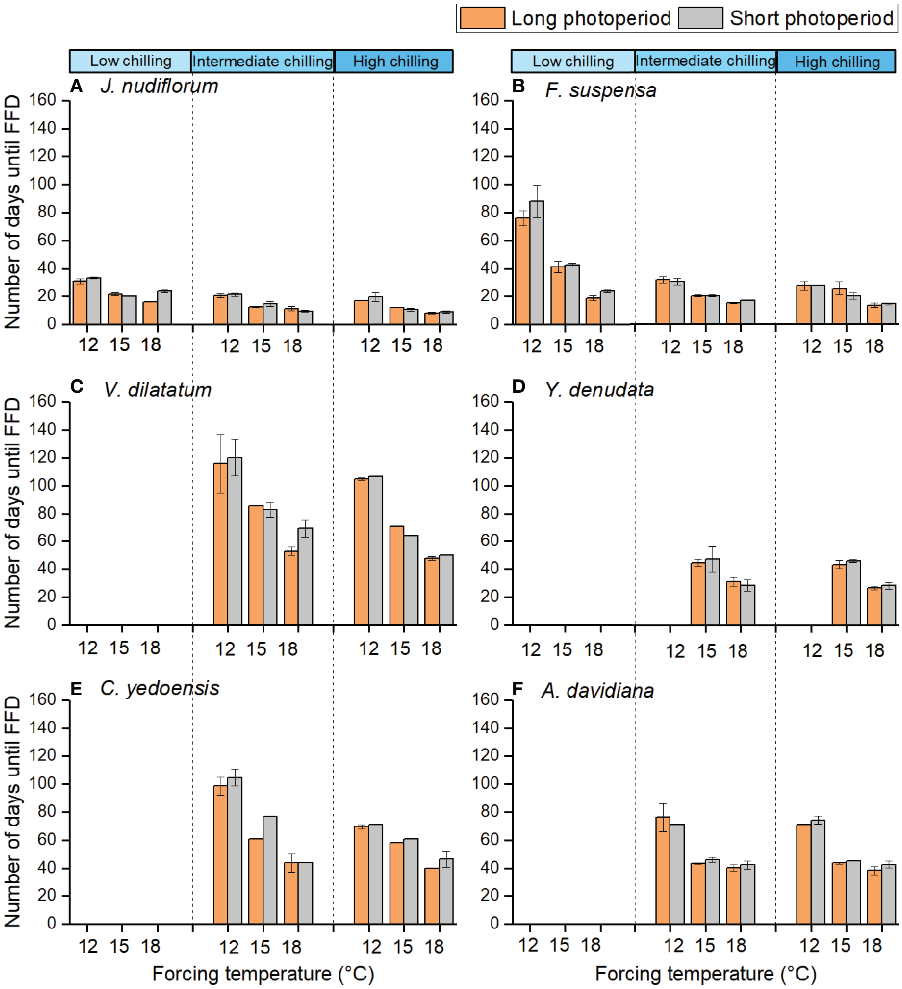

FIGURE 2 | Number of days from sampling dates to the first flowering date of six species at different chilling, forcing temperature and photoperiod conditions. The low (3 chilling days), intermediate (38 chilling days), and high (59 chilling days) indicate different chilling treatments, respectively. 12, 15, and 18°C indicate different forcing temperatures. Different colors indicate long (14h) and short (10 h) photoperiods. The flowers of the last four species could not open in low chilling condition. The error bars indicate standard deviation among three replicates (no error bar is presented when the standard deviation equals to zero). **(A)** *J. nudiflorum*, **(B)** *F. suspensa*, **(C)** *V. dilatatum*, **(D)** *Y. denudata*, **(E)** *C. yedoensis*, **(F)** *A. davidiana*.

TABLE 3 | Mean differences in the number of days until the first flowering date (FFD) among the different forcing, chilling, and photoperiod treatments.

Comparisons	*J. nudiflorum*	*F. suspensa*	*V. dilatatum*	*Y. denudata*	*C. yedoensis*	*A. davidiana*
Forcing temperature						
18–15°C	−2.3(−0.8)**	−11.2(−3.7)**	−21.0(−7.0)**	−16.7(−5.6)*	−20.7(−6.9)**	−3.6(−1.2)**
15–12°C	−8.5(−2.8)**	−18.5(−6.1)**	−36.1(−12.3)**	N	−21.8(−7.3)**	−28.7(−9.6)**
18–12°C	−10.8(−1.8)**	−29.7(−5.0)**	−57.1(−9.5)**	N	−42.4(−7.1)**	−32.3(−5.4)**
Photoperiod						
14h–10h	−1.3**	−1.6*	−2.6	−1.0	−5.6**	−1.4
Chilling						
3CD–38CD	9.2(0.26)**	26.0(0.74)**	N	N	N	N
38–59CD	2.3(0.11)**	0.8(0.04)	13.8(0.66)**	1.7(0.08)	13.8(0.66)**	0.7 (0.03)
3–59CD	11.6(0.21)**	26.8(0.48)**	N	N	N	N

For forcing temperature, the values in parentheses indicate the changes in the number of days until FFD per °C of warming. For chilling, the values in the parentheses indicate the changes in the number of days until FFD per chilling day (CD). The significance of multiple pairwise comparisons was determined with Fisher's LSD test **$P < 0.01$; *$P < 0.05$.

Effects of Chilling on First Flowering Date

Under the low chilling condition, only *J. nudiflorum* and *F. suspensa* could open flower normally, while the other four species could not under the forcing conditions (**Figure 2**), suggesting that the chilling requirements varied among species. For all species investigated, more chilling days lessened the required time to reach FFD, on average (**Table 3**), but this effect was only significant ($P < 0.01$) for four species (**Table 2**). Compared with low chilling, the intermediate and high chilling conditions led FFD occurring 9.2 and 11.6 days earlier in *J. nudiflorum* and 26.0 and 26.8 days earlier for *F. suspensa*, respectively. Concerning *V. dilatatum* and *C. yedoensis*, they required 13.8 days less until their FFD in the high chilling than intermediate chilling treatment. By contrast, for *Y. denudata* and *A. davidiana*, the difference in the number of days until FFD between intermediate chilling and high chilling conditions was small (respectively 1.7 and 0.7 days), and not significant.

Further, in the low chilling condition (3 chilling days), a one chilling-day increase could lead to 0.26- and 0.74-day advance in the FFD of *J. nudiflorum* and *F. suspensa*, respectively, but their corresponding FFD response decreased to 0.11 and 0.04 days per chilling day under intermediate chilling (38 chilling days). Thus, for these two species, if the amount of chilling was high enough, the effect of further chilling became weak. A similar phenomenon also occurred in *Y. denudata* and *A. davidiana*. Yet, in the intermediate chilling condition, further chilling was also effective for hastening FFD in *V. dilatatum* and *C. yedoensis* (**Table 3**).

Effects of Photoperiod on First Flowering Date

Averaged over all forcing and chilling treatments, the number of days until FFD under the long photoperiod was 1.0 to 5.6 days less than that under a short photoperiod (**Table 3**). Thus, a long photoperiod could promote the FFD of all six studied species, but such an effect of photoperiod was only significant ($P < 0.01$) for *J. nudiflorum* and *C. yedoensis* (**Table 2**). For *J. nudiflorum*, under the forcing temperature of 18°C coupled to a low chilling condition, a long photoperiod shortened the time required for its FFD by 8 days when compared to a short photoperiod. Long photoperiod treatment caused *C. yedoensis* to flower 16 days earlier than did a short photoperiod under the forcing temperature of 15°C and intermediate chilling condition.

Interaction Effect of Forcing Temperature, Chilling, and Photoperiod

The interaction between forcing temperature and chilling was significant ($P < 0.01$) for *J. nudiflorum*, *F. suspensa*, and *C. yedoensis* (**Table 2**). **Figure 2** shows that under the same amplitude of warming, the time to FFD of these species decreased more in low and intermediate chilling conditions than in a high chilling condition. Hence, the temperature sensitivity of FFD was stronger with lower chilling.

Another noteworthy interaction effect was that between chilling and photoperiod (**Table 2**), which was significant ($P < 0.01$) for two species (*J. nudiflorum* and *F. suspensa*). A sooner FFD under the long photoperiod was observed more frequently in low and intermediate chilling conditions than in the high chilling condition. Although other interaction effects (forcing temperature × photoperiod, forcing temperature × chilling × photoperiod) were significant in one or two species, no consistent pattern was evident.

Relationship Between Heat Requirement, Chilling, and Photoperiod

The impacts of three environmental cues could be described by the relationship between GDD requirements and chilling days under two photoperiods (**Figure 3**). The heat requirement for FFD clearly varied among species. The mean GDD requirement across all treatments was high for *V. dilatatum* (753.2) and *C. yedoensis* (603.7), followed by *A. davidiana* (496.4) and *Y. denudata* (412.0), and least in *F. suspensa* (279.2) and *J. nudiflorum* (163.3) whose GDD requirements were low. For the same chilling and photoperiod condition, the GDD requirement was similar among different forcing temperature treatments, although the difference among individuals still existed (see the error bar in **Figure 3**). Higher forcing temperatures would lead to GDD accumulating more quickly, which could explain the

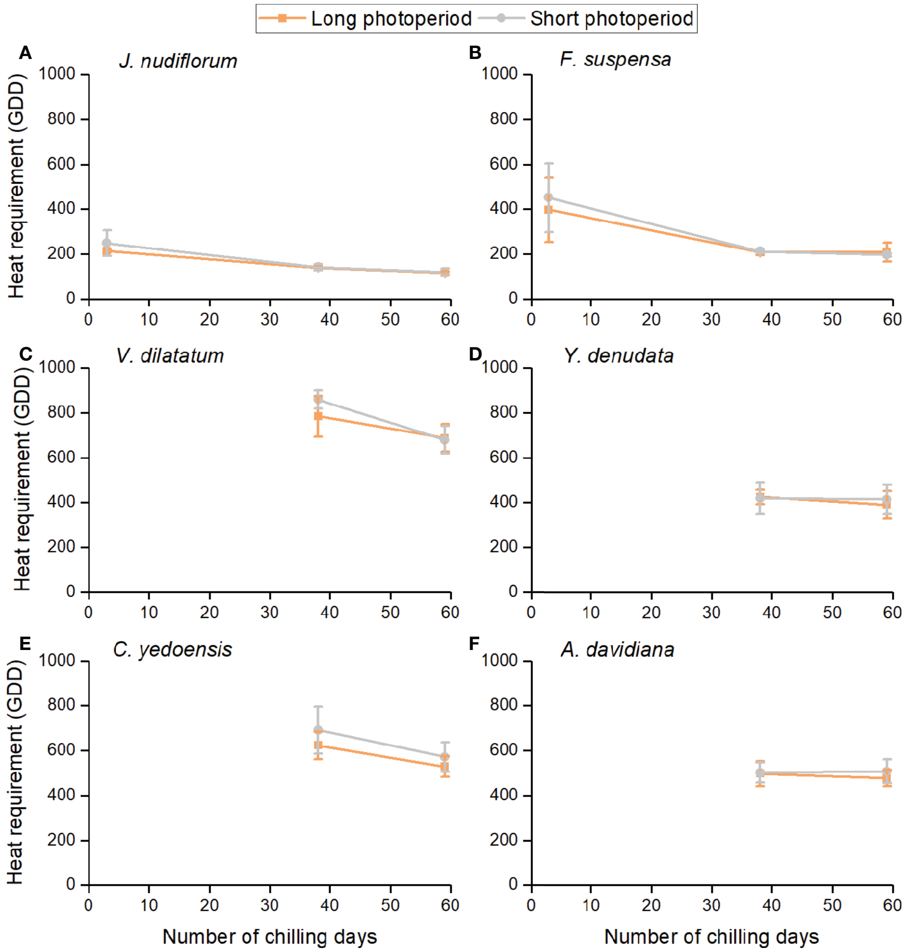

FIGURE 3 | Relationships between mean heat requirement of the first flowering date (FFD) of six species and chilling accumulation under long and short photoperiod conditions. Heat requirement was calculated as the accumulated degree days (above 5°C) from the sampling date to FFD. The error bars indicate standard deviation among 3 temperature treatments × 3 replicates. **(A)** *J. nudiflorum*, **(B)** *F. suspensa*, **(C)** *V. dilatatum*, **(D)** *Y. denudata*, **(E)** *C. yedoensis*, **(F)** *A. davidiana*.

promoting effects of forcing temperature upon FFD. The increase in chilling days reduced the GDD requirement of all species, but this effect was apparently weak for both *Y. denudate* and *A. davidiana*. For two other species (*J. nudiflorum* and *F. suspensa*), whose flowers opened in the low chilling condition, their reduced GDD requirements were mitigated by more chilling days.

Furthermore, the GDD requirements of FFD were higher under the short than long photoperiod for most experimental treatments (**Figure 3**), but the difference in the mean GDD requirement between long and short photoperiods was not statistically significant for all chilling treatments and species (*t*-test, $P > 0.05$). We noticed that the effect of photoperiod was more pronounced in the low and intermediate than high chilling condition for *J. nudiflorum, F. suspensa,* and *V. dilatatum*. For the other species, no obvious difference in the effect of photoperiod was found among chilling treatments.

DISCUSSION

Effects of Forcing Temperature

Our results indicate that increasing the forcing temperature significantly promotes the FFD of woody plants, which is consistent with findings on spring phenology of other species, such as leaf budburst in *Betula pubescens* (Caffarra et al., 2011), *Fagus sylvatica*, *Tilia cordata*, *Salix x smithiana* (Caffarra and Donnelly, 2011) and 28 woody species from two North American forests (Flynn and Wolkovich, 2018). In addition to experimental studies, the long-term observational data has also showed that interannual changes in spring phenology are negatively correlated with the temperature averaged from one to three months before these springtime events (Estrella et al., 2007; Szabó et al., 2016; Wang et al., 2018; Xu et al., 2018). Therefore, the advance in spring phenology observed over the past decades could be attributed to spring warming. Since multiple General Circulation Models (GCMs) predict the warming trends would continue under all Representative Concentration Pathway (RCP) scenarios, except RCP 2.6 (IPCC, 2013), spring phenology will likely continue to advance earlier in the future.

Furthermore, we found that the temperature sensitivity of FFD decreased as the forcing temperature was increased. Previous studies also reported similar results (Morin et al., 2010; Fu et al., 2013; Wang et al., 2019a). For example, the temperature sensitivity of leaf unfolding in oak and beech trees decreased drastically when warming exceeded +4°C (Fu et al., 2013). In our study, the mean GDD requirement for FFD in *F. suspensa* was 279.2 degree days (threshold of 5°C) and the number of days until FFD at a forcing temperature of T could thus be calculated as $279.2/(T–5)$; hence, with a higher T, the change in number of days to FFD per °C of warming decreases (**Figure 4A**). Accordingly, this reduced temperature sensitivity with greater forcing temperatures could be explained by the theory of GDD requirement. However, in our experiment, we did not consider the possible impact of temperature variation because we used a fixed (nonfluctuating) temperature treatment. In natural conditions, the temperature sensitivity of woody species' leaf and flowering phenology was weaker at locations with larger variance in local spring temperature (Wang et al., 2014) and higher occurrence frequency of freezing events (Wang Y. et al., 2019).

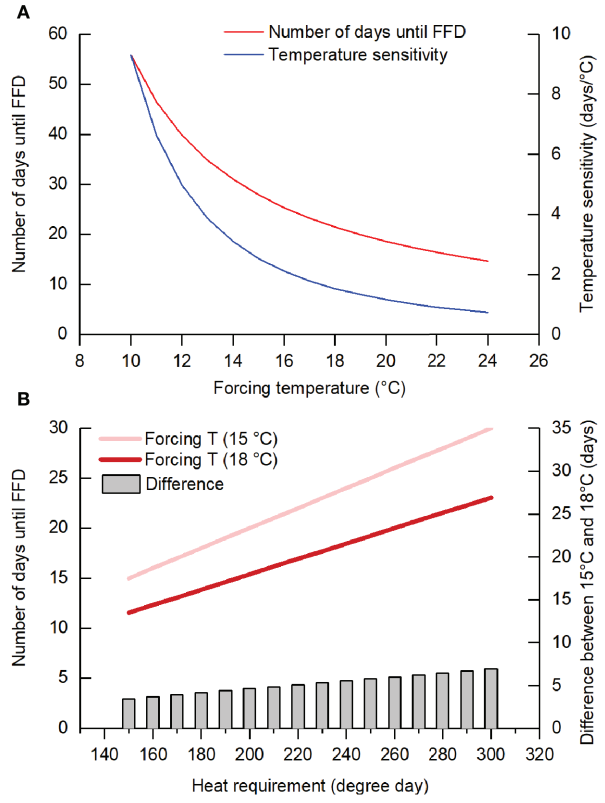

FIGURE 4 | Number of days until the first flowering date (FFD) based on the heat requirement. **(A)** When the heat requirement is constant (279.2 degree days, the threshold temperature of 5°C), temperature sensitivity (changes in the number of days until FFD per °C warming) decreases with the increase in forcing temperature. **(B)** The difference in the number of days until FFD between forcing temperature of 15 and 18°C was larger when the heat requirement became higher (with lower chilling).

Effects of Chilling

Our results suggest that increased exposure to chilling lessens the time to flowering and reduces the GDD requirements of the studied species. Such a chilling effect was also reported by other experimental studies done on leaf or flower budburst of other woody species (Okie and Blackburn, 2011; Vitasse and Basler, 2013; Pletsers et al., 2015; Man et al., 2017; Du et al., 2019). For example, in Ontario, Canada, the amount of heat required for leaf budburst of seven species decreased progressively with cumulative chilling hours (Man et al., 2017). In Ireland, a longer chilling duration resulted in earlier leaf budburst of *Betula pubescens* (German clone) and *Populus tremula* (Irish clone), and for both species less thermal time was needed to reach maximum budburst (Pletsers et al., 2015). Long-term observational data also confirms this assumption because the heat requirement of leaf-out for several European species has increased over the past 30 years, mainly due to the warming-related reductions in chilling (Fu et al., 2015a). In the model plant, hybrid aspen (*Populus tremula x tremuloides*), the physiological basis of its chilling-mediated control of bud break has been uncovered (Singh et al., 2018), and many genes and phytohormones are involved in this intricate process. Thus, the current body of evidence suggests the negative relationship between the heat requirement and amount of chilling is widespread among perennials. In natural conditions, several studies have also found that reductions in chilling days during the last several decades may have limited how much earlier leaf budburst could advance, to some extent (Fu et al., 2015b; Asse et al., 2018; Vitasse et al., 2018).

Nevertheless, the effect of chilling on FFD clearly varied among species. In the low chilling condition (three chilling days), only *J. nudiflorum* and *F. suspensa* could open their flowers normally, which suggests these species need none or very few chilling days to break their dormancy. Yet three chilling days could not break the dormancy of the other four species, since they failed to open their flowers even after a 5-month-long forcing treatment. In the intermediate chilling (38 days) condition, dormancy was released for all the six species, but the effect of further chilling was not consistent. The time to flowering for four species (*J. nudiflorum, F. suspensa, Y, denudata,* and *A. davidiana*) was very similar under intermediate and high chilling conditions. Therefore, in Beijing and other regions with cold winters, the amount of chilling is likely enough for breaking dormancy before mid-December, and the amount of chilling experienced by plants over the whole winter exceeded the chilling requirements of these species (Chmielewski and Götz, 2017). For them, the countervailing effect from reduced chilling on spring phenological advance was found to be weak. For other species (*V. dilatatum* and *C. yedoensis*), high chilling was also effective in advancing the time to flowering compared to their response to intermediate chilling. Thus, in natural conditions, for species whose spring phenology response was still sensitive to chilling in the high chilling state, the warming-related reduction in chilling days would delay the advancement of their spring phenological events.

Effects of Photoperiod

In other reported experiments, a long (short) photoperiod usually refers to daylength longer than (shorter than) 12 h, such as 12 h *vs* 8 h (Flynn and Wolkovich, 2018) and 16 h *vs.* 8 h (Zohner et al., 2016). We compared the photoperiod effect between 14 and 10 h, finding that a long photoperiod could hasten the FFD of all species albeit to differing extent and it reduced the GDD requirement of FFD in most cases. Consistent with our results from Asia, for 28 woody species from two North American forests, long photoperiod caused a 5-day advance in leaf budburst compared with that induced by short photoperiod at the community level (Flynn and Wolkovich, 2018). However, in our study, the effect of photoperiod on FFD was only significant for two of the six investigated species. One recent study found that with low chilling, only 35% of 173 temperate species relied on spring photoperiod as a cue for leaf-out (Zohner et al., 2016). Similarly, the photoperiod had little effect on both leaf-out and flowering events in 37 subtropical woody species (Song et al., 2020). Therefore, it would seem that most woody plant species do not use photoperiod as an external regulator of spring phenology.

In our experiments, though the difference between photoperiod treatments reached 4 h of light a day, the long photoperiod only advanced the FFD earlier by 1.0 to 5.6 days, an effect weaker than from forcing temperature and chilling. However, for plants grown in field conditions, the daily change in photoperiod would be constant among years at a given location. Assuming one year has an earlier FFD (t_e) and another year has a later FFD (t_l), the photoperiod was the same before $t_{e,}$ but the year with later FFD received additional sunshine hours in the timespan from t_e to t_l. We believe that such a difference in photoperiod (t_e to t_l) would be smaller than that of our photoperiod treatment lasting for five months. Therefore, the restriction of daylength on the advances in the spring phenology of woody plants was weak and difficult to detect under natural conditions.

Interaction of Multiple Cues

The ANOVA demonstrated that forcing temperature and chilling interacted to affect three species (*J. nudiflorum*, *F. suspensa*, *C. yedoensis*). The effect of forcing temperature on FFD of these species was stronger in low and intermediate chilling conditions compared to high chilling condition. Taking *F. suspensa* as an example, we presumed its GDD requirements for FFD increased from 150 to 300 degree days with a decrease in the amount of chilling. Subsequently, for different GDD requirements, we could simulate the number of days until FFD occurs at forcing temperatures of 15 and 18°C. As **Figure 4B** shows, a higher GDD requirement (in the low chilling condition) leads to more pronounced difference in the elapsed time to FFD between 15 and 18°C. Thus, the interaction between forcing temperature and chilling upon FFD documented here could be explained by the theory of GDD requirement.

The effect of photoperiod and chilling on FFD interacted for two species (*J. nudiflorum* and *F. suspensa*). As shown in **Figure 3**, the heat requirement was higher under a long than short photoperiod treatment when twigs of these species had not fully chilled yet. Similar results were also found in other species such as *Fagus sylvatica* (Zohner and Renner, 2015; Fu et al., 2019). With low chilling, 35% of 173 species leafed out later under short-day conditions than under those of long-days, but only 2% of species responded sensitively to photoperiod when exposed to high chilling (Zohner et al., 2016). All these pieces of evidence together suggest that high chilling functions to reduce the photoperiod sensitivity of woody plants. Another assumption for a photoperiod effect is that a long photoperiod could compensate for too little chilling (Myking and Heide, 1995). However, the photoperiod *Betula pubescens* experienced during a chilling treatment did not affect its leaf budburst (Caffarra et al., 2011). In *Fagus sylvatica*, its leaf primordia only reacted to light cues late in dormancy after accumulating enough warm days (Zohner and Renner, 2015). Therefore, for photoperiod-sensitive species, a long photoperiod was likely to increase the effect of forcing temperature rather than act as a substitute for chilling's effect.

Difference Among Plant Functional Types

As proposed by Körner and Basler (2010), because shrubs are shorter-lived and early successional plants, they may adopt a more risky life strategy to maximize their growing season when compared to trees (although not all tree species are long-lived, late-successional). Therefore, we expected shrubs to exhibit a lower chilling and heat requirement and to be less responsive to photoperiod. For chilling and forcing, our results generally support this hypothesis. In high chilling conditions, the mean GDD requirement—averaged over all forcing temperature and photoperiod treatments—for the FFD of *J. nudiflorum* and *F. suspensa* was only 118.1 and 203.0 degree days, respectively. Because *V. dilatatum* was the only species which flowered later than its timing of leaf-out in this study, the mean GDD requirement for its FFD was 683.9 degree days. If we consider this shrub's first spring event, the mean GDD requirement for the first leaf-out date of *V. dilatatum* was only 245.1 degree days. Relative to shrubs, the mean GDD requirement of three trees reached 402.1 to 550.2 degree days, which is higher than the GDD requirement for the first spring events of the three shrub species. Furthermore, with very low chilling (3 chilling days), shrubs (except *V. dilatatum*) could normally flower, but trees could not, suggesting the chilling requirement of shrubs was lower than trees in most cases. This difference in heat and chilling requirements between successional life-history strategies is consistent with the findings of Laube et al. (2014). However, the two species that responded significantly to photoperiod in our study consisted of one shrub and one tree;

hence, our results do not support the hypothesis that tree species are more photosensitive than shrub species.

CONCLUSIONS

In this study, we reported on a multispecies climate chamber experiment that tests the effects of forcing temperature, chilling, and photoperiod on the spring phenology of six woody species native to China. Increased forcing temperature, chilling, and daylength hastened the FFD of all species, although the effects of these cues varied and were not always significant for all species. Also, for several species, the impact of chilling interacted with forcing temperature and photoperiod, *i.e.*, the effect of forcing temperature and photoperiod tended to be stronger under a low chilling condition. These effects could be explained by the GDD theory, as more chilling exposure and a longer photoperiod reduced the GDD requirement to attain FFD. Because species responded in a complex way to multiple environmental cues, accurately predicting the FFD at the community level is challenging. In future climate change scenarios, spring warming would possibly advance the spring phenology of woody plants, but the reduced chilling and shorter photoperiod may limit the effect that spring warming has on those species having a high chilling requirement. According to our results, the impact of chilling and photoperiod on spring phenology is likely weaker than that from spring warming, because most of the woody plants are photoperiod-insensitive and the influence of chilling is generally weak at mid- to high-latitudes or at high elevations where the amount of chilling is already sufficient.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

HuaW designed this experiment. HuaW and HuiW performed this experiment. HuiW analyzed the data. HuaW and HuiW wrote this manuscript. QG and JD extensively revised the writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00443/full#supplementary-material.

DATA SHEET 1 | Supplementary data sheet.

DATA SHEET 2 | Supplementary figure.

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Warming Events Advance or Delay Spring Phenology by Affecting Bud Dormancy Depth in Trees

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The frequency of sudden, strong warming events is projected to increase in the future. The effects of such events on spring phenology of trees might depend on their timing because spring warming has generally been shown to advance spring budburst while fall and winter warming have been shown to delay spring phenology. To understand the mechanism behind timing-specific warming effects on spring phenology, I simulated warming events during fall, mid-winter and at the end of winter and quantified their effects on bud dormancy depth and subsequently on spring leaf out. The warming events were carried out in climate chambers on tree seedlings of *Betula pendula* and *Fagus sylvatica* in October, January, and February. Control seedlings were kept at photoperiod and temperature matching the daily fluctuating field conditions. Warmed seedlings were kept 10°C warmer than the control seedlings for 10 days during the respective warming periods. Warming in October increased bud dormancy depth and decreased spring leaf-out rate only for *F. sylvatica*, whereas warming in February reduced bud dormancy depth and advanced spring leaf-out rate only for *B. pendula*. Neither bud dormancy depth nor spring leaf out rate were affected by January warming. The results indicate that warming-induced changes in bud dormancy depth may explain species- and timing-specific warming effects on spring phenology. The extent to which the timing of bud dormancy phases is species-specific will influence among-species variation in future spring leaf out times.

Keywords: extreme warming events, warm pulses, dormancy induction, dormancy release, dormancy level, bud burst

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INTRODUCTION

Climate warming has often been associated with advanced spring phenology, not only via observations (Wesołowski and Rowiński, 2006; Wood et al., 2006; Richardson et al., 2010; Zohner and Renner, 2014), but also through experiments (Hole, 2014) and modeling studies (Luedeling et al., 2013; Lange et al., 2016). Recently, however, a slowing down of spring phenology advancement has been documented (Fu et al., 2015). Furthermore, temperature increases have been shown to both advance and delay budburst dates, depending on their timing (Heide, 2003; Fu et al., 2012; Luedeling et al., 2013). Antagonistic temperature affects on spring phenology may arise when the process of bud dormancy is variably affected by temperature changes, depending on the phase that the process is in when a temperature increase takes place.

Winter dormancy is an adaptation of perennial plants to survive seasonal unfavorable conditions by suspending growth and reducing their activity to a minimum. Dormancy is a state that buds of many temperate plant species develop which needs to be broken by a cold period in order for budburst to occur in spring (Sogaard et al., 2008). Dormancy depth is the level of dormancy at a particular time and is commonly estimated by exposing twig cuttings from trees to optimum growing conditions (Panchen et al., 2014; Vitasse et al., 2014). The amount of warmth required, often measured in growing degree days (GDD), also termed forcing requirements, approximates the dormancy depth. Experiments have shown that the forcing requirement increases exponentially with decreasing time spent at cool temperatures, generally accepted to be below 10°C, while being species-specific (Battey, 2000; Cesaraccio et al., 2004; Harrington et al., 2010). Furthermore, as bud dormancy depth is increased from late summer and throughout the fall, a process known as bud dormancy induction, warmer temperatures can further increase bud dormancy depth or delay its induction (Heide, 2003; Laube et al., 2014; Pagter et al., 2015). Delayed and/or deeper bud dormancy could require a longer cold period to bring the forcing requirements to the same level as in years with cooler fall temperatures. Absence of the additionally required chilling period, often driven by winter warm spells, means buds have higher bud dormancy depth in the spring and in turn open later under similar spring temperatures (Heide, 2003; Søgaard et al., 2009). Experiments showing how bud dormancy depth is affected by warming in different tree species and how such dormancy changes affect spring phenology are lacking.

In contrast to the potential delaying effect of fall warming on spring phenology (Heide, 2003), warmer temperatures can advance spring budburst if they occur after bud dormancy has largely been released following a cold period. At this time buds are in the ecodormancy phase, where cold temperatures have only a minor effect on further reducing bud dormancy depth and warm temperatures have a stronger ability to reduce bud dormancy depth by fulfilling the forcing requirements and thus advancing budburst (Kramer, 1994). There is also evidence that early flushing species, such as *Betula pendula,* require shorter chilling periods to lower their bud dormancy depth than late successional species, such as *Fagus sylvatica*, which often require a longer cold period before warm temperatures advance bud break (Murray et al., 1989; Zohner and Renner, 2014). Furthermore, in *F. sylvatica,* short photoperiod additionally prevents rapid dormancy release to a larger extent as in other tree species (Vitasse and Basler, 2013; Malyshev et al., 2018), resulting in reduced sensitivity to warming periods earlier in the year when day length is still short. It is also possible that the timing of bud dormancy induction is different in *B. pendula* compared with *F. sylvatica*, further suggesting that the same warming at a particular period may increase bud dormancy depth and hence delay budburst in one species while having a lesser effect on the other.

The effects of short-term, sudden warming events during different bud dormancy phases on changes in bud dormancy depth and subsequent spring budburst dates are unclear. Heat waves are predicted to increase in frequency and duration under future climate scenarios (Schär et al., 2004; IPCC, 2014) and future phenology models need to account for the potentially different impacts of warming during different bud dormancy stages. Furthermore, the effects of strong warming events on typically early and late flushing species need to be studied to quantify whether budburst advances or delays are likely to be more pronounced in either tree group.

I selected a common early flushing and a common late flushing tree species to study the effects of sudden strong warming events on spring phenology. I subjected tree seedlings to either fall, midwinter or end of winter warming events inside climate chambers, each time increasing the temperature by 10°C relative to ambient temperature in the field. I estimated bud dormancy depth of the seedlings prior to and after the warming events. Spring phenology after each warming event was subsequently recorded. I hypothesized that warming would delay spring phenology of seedlings in which the dormancy depth had increased following warming and vice versa.

MATERIALS AND METHODS

Plant Material

Tree seedlings were grown from a local seed source, stemming from northern Germany. One-year-old birch *Betula pendula* seedlings and two-year-old *Fagus silvatica* seedlings were grown in a local tree nursery and potted in April 2016 in 3 L pots with sandy loam soil. Seedlings were 40–70 cm tall. Fertilization was limited to horn shavings for slow release of nutrients and watered weekly. In June 2016 the trees were delivered to the test field site, located in Greifswald Germany. Direct sunlight was reduced by a net, stretched 2 m above the trees, reducing radiation by about 30%. In September the pots were placed in pre-dug holes in a sand area for additional insulation against frost. In total, 75 seedlings were used per species. Prior to each warming event, four seedlings per species were sampled destructively to estimate the starting dormancy depth. During each warming event, nine seedlings per species were placed in a warming chamber and nine seedlings per species were placed in a control chamber. Four of the nine seedlings were sampled destructively after the warming events while five seedlings were returned to the field site. Seven samplings were kept at the field site for the duration of the experiment to represent ambient budburst in the spring.

Simulation of Warming Pulses

Warming was simulated for a period of 10 days during three periods, starting on 4.10.2016, 4.1.2017, and 14.2.2017. Warming was simulated in climate chambers (Model: LT-36VL, CLF Plant Climatics GmbH, Germany). One climate chamber was programmed to keep temperature and daylight changes as close to ambient conditions as possible, adjusting its photoperiod and temperature daily (**Figure 1** and **Table 1**). In the warmed chamber temperature was maintained 10°C above the control chamber temperature. The warming magnitude was selected to reflect the biggest differences in mean January air temperatures

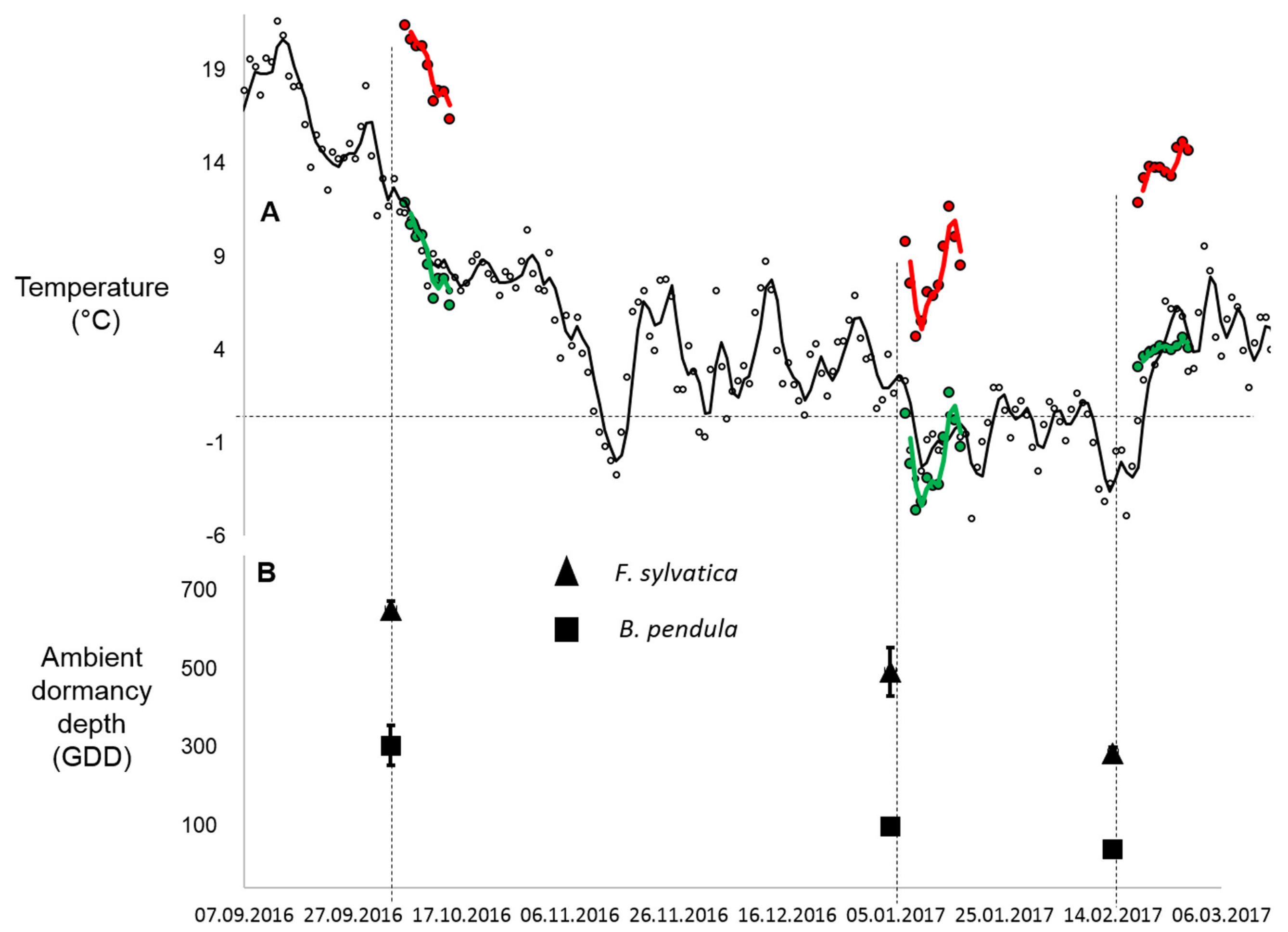

FIGURE 1 | (A) Daily mean temperatures of the warming and control chamber temperatures (warming in red; control in green) during each simulated warming pulse. Ambient temperatures at the field site where tree seedlings were overwintered are in black. Lines indicate 2-day running means. **(B)** Estimated dormancy depth of tree seedlings at the beginning of each warming pulse. Dormancy depth was estimated by calculating growing degree days (GDD) of twig cuttings prepared from ambient tree seedlings under optimum growing conditions in climate chambers. Triangles stand for *B. Pendula* and squares stand for *F. sylvatica* (*n* = 8–12 per sampling date per species). Error bars indicate standard error.

for the Mecklenburg Vorpommern region of Germany in the last 50 years (local weather data). The duration of 10 days was chosen to reflect data that warming for 6 days at 9°C during the non-growing season can be substantial enough to cause differences in plant growth (Malyshev et al., 2016). To avoid chamber bias, seedlings and chamber settings were switched between the two chambers every 2 days. The actual warming and control chamber temperatures were 19/9°C, 8/−2°C and 13/4°C for the respective warming periods. Standard deviations were similar between treatments and ambient temperature, with treatment standard deviations being between 0.4 and 0.9°C higher in the chambers compared to ambient temperatures (**Table 1**). Mean humidity inside the control and warmed chambers varied between 70 and 90%, with the differences between the control and warming chambers ranging between 1 and 5% during the warming periods. Humidity was allowed to covary with temperature manipulation. At the conclusion of warming, five trees were returned to the field site while four trees were destructively sampled to estimate bud dormancy depth of each tree after the treatment.

Effect of Warming on Dormancy Depth

Additional four tree seedlings of each species were destructively sampled before and four after each warming event to estimate the change in bud dormancy depth due to warming. Bud dormancy depth was estimated by making five twig cuttings from each of the four seedlings. Each twig cutting was approximately 8 cm in length and had at least 2 lateral buds. Seedlings had four to eight branches from which four were selected from different vertical tree sections to obtain a representative sample of the average dormancy of all lateral buds on the tree. Top portions of branches were used to create the cuttings. The terminal bud was removed and candle wax was used to prevent desiccation from the top cut surface. Removal of the terminal bud ensures that true dormancy of lateral buds can be observed without the influence of terminal buds which can prevent lateral buds from opening in the fall

TABLE 1 | Temperature means and standard deviation of temperatures in the control and warming chambers as well as the ambient temperature at the field site during each temperature manipulation period.

Warming period	Treatment	Mean temp (°C)	Standard deviation	GDD sum	Chilling day sum
4th–14th November 2016	Ambient	9.2	1.5	38	0
	Control chamber	10.0	1.9	35	0
	Warming chamber	19.1	1.7	127	0
4th–14th Janaury 2017	Ambient	−0.8	1.6	0	10
	Control chamber	−1.8	2.2	0	10
	Warming chamber	8.1	2.2	34	1
14th–24th February 2017	Ambient	3.6	3.0	5	7
	Control chamber	4.1	3.4	0	9
	Warming chamber	13.8	3.8	88	0

Daily sums of growing degree days (GDD) and chilling day sums (number of days where temperature was equal to or below 5°C) for each period are also presented.

via paradormancy (Champagnat, 1989). Using twig cuttings to describe bud dormancy changes was used instead of using whole tree seedlings due to space limitation and the method having been deemed appropriate previously (Vitasse et al., 2014; Primack et al., 2015), having been widely used to track bud dormancy changes (Ghelardini et al., 2009; Zohner and Renner, 2015; Vitra et al., 2017). The twigs were placed in deionized water in a climate chamber with a 24 h photoperiod (PAR 60–100 μmol m^{-2} s^{-1}) and a temperature of 22°C (± 2°C). The twigs were inserted into a Polyethylene foam 1 cm thick which floated in trays of water. Twigs were recut under water weekly and water was changed twice a week. Mean days to budburst for the first three twigs (out of five) per tree were recorded to estimate the time to achieve approximately 50% budburst as not all buds opened on all sampled dates for all species ($n = 12$ per treatment and species). As minor temperature differences existed during the dormancy tests, the number of days to budburst was converted to GDD, commonly used to quantify dormancy depth and calculated as:

$$GDD = \sum_{t_0}^{t_1} (T_{mean} - T_{base})$$

where t_0 is the starting day of the warming period, t_1 is the day at which budburst is observed, T_{mean} is the daily mean temperature, and T_{base} is a constant (5°C), representing a minimum temperature threshold required for stimulating budburst (Polgar and Primack, 2011; Fu et al., 2016). The warming effect on dormancy depth was calculated by dividing the dormancy depth of each twig (GDD) of each seedling following warming by the mean dormancy depth of all seedlings prior to warming (a ratio of dormancy depth after/before warming). Resulting values above 1 represented an increase in dormancy depth and values below 1 represented a decrease in dormancy depth following treatment with respect to initial dormancy depth. Dormancy depth at the start of each warming event for each species is shown in **Figure 1**. For *F. sylvatica*, only the first twig which had bud bust in each tree seedling was used for the all twigs from the October and January treatments due to high twig mortality (resulting $n = 4$ per treatment).

Phenology Monitoring

Fall leaf coloration differences were measured 2 weeks after the conclusion of the first warming event to estimate if dormancy differences were accompanied by differences in senescence rates. Six random leaves were measured per tree with a SPAD device (SPAD-502 Plus, Konica Minolta, Inc.).

Spring phenology was recorded via two methods. Firstly, percent budbreak was recorded for each tree every 2 days to estimate the date of 50% bud break. Data for *Fagus sylvatica* was lost for this first method. Secondly, the lengths of six most unfolded leaves on each tree was measured as soon as all trees from any treatment group had reached 90% complete leaf unfolding. Early flushing species often take much longer to achieve full leaf unfolding compared with late flushing species, meaning that a long delay/advancement in budburst dates may be reduced to only minor differences in the number of days with respect to completely unfolded leaves, which is most important in gaining a photosynthetic advantage. Furthermore, differences in dates of full leaf unfolding are also largely dependant on the temperature at the time of unfolding. Thus, the unfolding stage of leaves was numerically quantified by measuring the length from the emerging leaf tip to the bud. Choosing the day of measurement when the seedlings from the earliest flushing treatment group had 90% of their leaves completely unfolded approximated the maximum difference in leaf out stages among the treatment groups and made these differences better comparable between species. To make between-species comparison even more robust and better represent treatment effects on leaf unfolding, each leaf length was divided by the mean leaf length of untreated ambient tree seedlings. Seven ambient seedlings per species were measured.

Statistical Analysis

The effect of warming on leaf coloration after the first warming event was tested via an ANOVA where species and treatment (control/warming) were the influencing factors and leaf coloration (SPAD values) was the response variable.

The effect of warming on dormancy depth was evaluated by a three-way ANOVA, where the timing of warming, species and treatment (control/warming) were the influencing factors and dormancy depth ratio was the response variable. Individual tree identity was included as a random factor in the linear mixed model. Dormancy depth ratios were log-transformed to improve the homo- genetic of variances and the normality of residuals.

The effect of warming on the date of 50% budburst was analyzed for *Betula pendula* with a two-way ANOVA, where the timing of warming and treatment (control/warming) were the influencing factors and the date on which 50% budburst occurred for each tree was the response variable.

Transformation of $\wedge 0.5$ was applied for the leaf ratios to improve the homogeneity of variances and the normality of residuals. All analyses were performed using R statistical software (R Core Team, 2013). R packages lme4 and lmerTest were used for the ANOVA analyses while the package emmeans was used to compare the effects of treatments within each species and warming periods.

RESULTS

Leaf Coloration After Fall Warming

October warming delayed leaf senescence of warmed seedlings compared with the control plants, with no interaction having been detected between treatment and species (**Table 2** and **Figure 2**). Seedlings were approximately 30% more green after warming compared with control seedlings ($p = 0.04$).

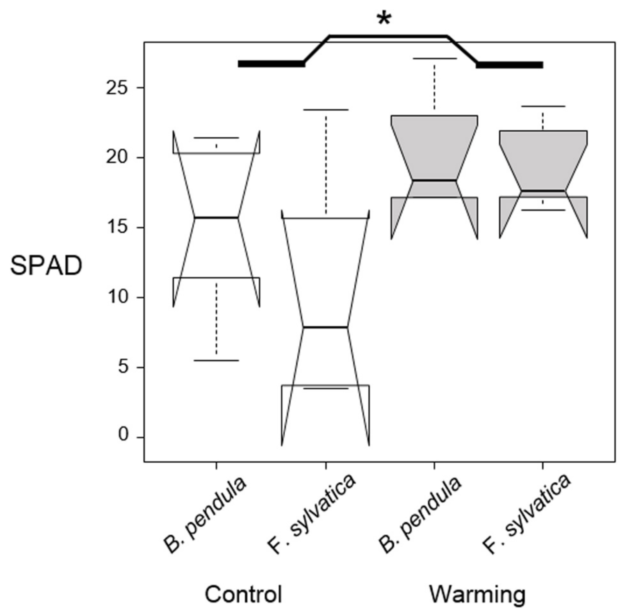

FIGURE 2 | Median SPAD values of six random leaves per tree seedling, measured 2 weeks after the end of the first warming pulse in October. No interaction between species and treatment was found. Significant difference between warmed and control plants is shown with the asterisk. Notches show the 95% confidence interval of the median and whiskers extend to a maximum of 1.5 × IQR beyond the box.

Relative Change in Bud Dormancy Depth After Warming

The effect of warming on bud dormancy depth depended on the timing of warming as well as being species-specific ($p < 0.001$ for Species*Treatment interaction and $p < 0.01$ for Timing*Treatment interaction; **Table 2** and **Figure 3A**). In October, a greater increase in dormancy depth occurred in warmed seedlings than in control seedlings only for *F. sylvatica* ($p < 0.001$). No effect of warming was detected in January for either species and in February a greater decrease in dormancy depth occurred in warmed seedlings than in control seedlings only for *B. pendula* ($p < 0.001$). In *F. sylvatica* dormancy depth was increased by approximately 3 times after fall warming compared to control, whereas the dormancy depth of *B. pendula* was decreased by approximately 2.5 times after February warming compared to control.

TABLE 2 | Influence of species (*B. pendula* and *F. sylvatica*), treatment (control/warming), and timing of warming (4th October, 4th January, and 14th February) on four response variables, as affected by increased temperature of 10°C for 10 days.

Response variable	Factor (s)	*F*-value	*p*-value
SPAD	Species	0.4	0.54
	Treatment	**5.1**	**0.04**
	Species* Treatment	0.7	0.41
Change in dormancy depth relative to ambient seedlings	Timing	2.8	0.07
	Species	**5.7**	**0.02**
	Treatment	0.2	0.63
	Species* Timing	**16.1**	**<0.001**
	Species* Treatment	**16.0**	**<0.001**
	Timing * Treatment	**7.6**	**<0.01**
	Species* Timing *Treatment	2.5	0.095
Change in spring mean leaf length relative to ambient seedlings	**Timing**	**106**	**<0.001**
	Species	0.18	0.68
	Treatment	**8.3**	**0.006**
	Species* Timing	**6.4**	**0.004**
	Species* Treatment	**4.2**	**0.05**
	Timing * Treatment	0.03	0.97
	Species* Timing *Treatment	**6.2**	**0.004**
Date of 50% budburst in spring	**Timing**	**37.0**	**<0.001**
	Treatment	**18.8**	**<0.001**
	Timing * Treatment	**19.7**	**<0.001**

SPAD response was only measured after the fall warming event. Date of 50% budburst was only measured in B. pendula in the spring. Bold text signifies significant effects of respective factors.

Effect of Warming on Day of 50% Budburst in *B. pendula*

The effect if warming on budburst dates for *B. pendula* was timing-specific ($p < 0.001$ for Timing*Treatment interaction). The date of 50% budburst in *B. pendula* was only affected by February warming, whereby warmed tree seedlings opened their buds 3 weeks earlier than the control seedlings (**Table 2** and **Figure 4**). Warming at other times had no effect on budburst dates.

Relative Change in Leaf Lengths Following Warming

There was a three-way interaction between the influence of treatment, species and timing of treatments on the relative change in leaf length in the spring ($p = 0.004$ for the Species* Timing *Treatment interaction; **Table 2** and **Figure 3B**). The effect of warming on leaf length ratios thus depended on the timing of warming as well as being species-specific. The three-way interaction resulted from warming tending to increase the leaf ratio in *B. pendula* after January warming while tending to decrease it for *F. sylvatica* (**Figure 3B**). In October, a greater decrease in leaf ratio occurred in warmed seedlings than in control seedlings only for *F. sylvatica* ($p < 0.001$). No significant

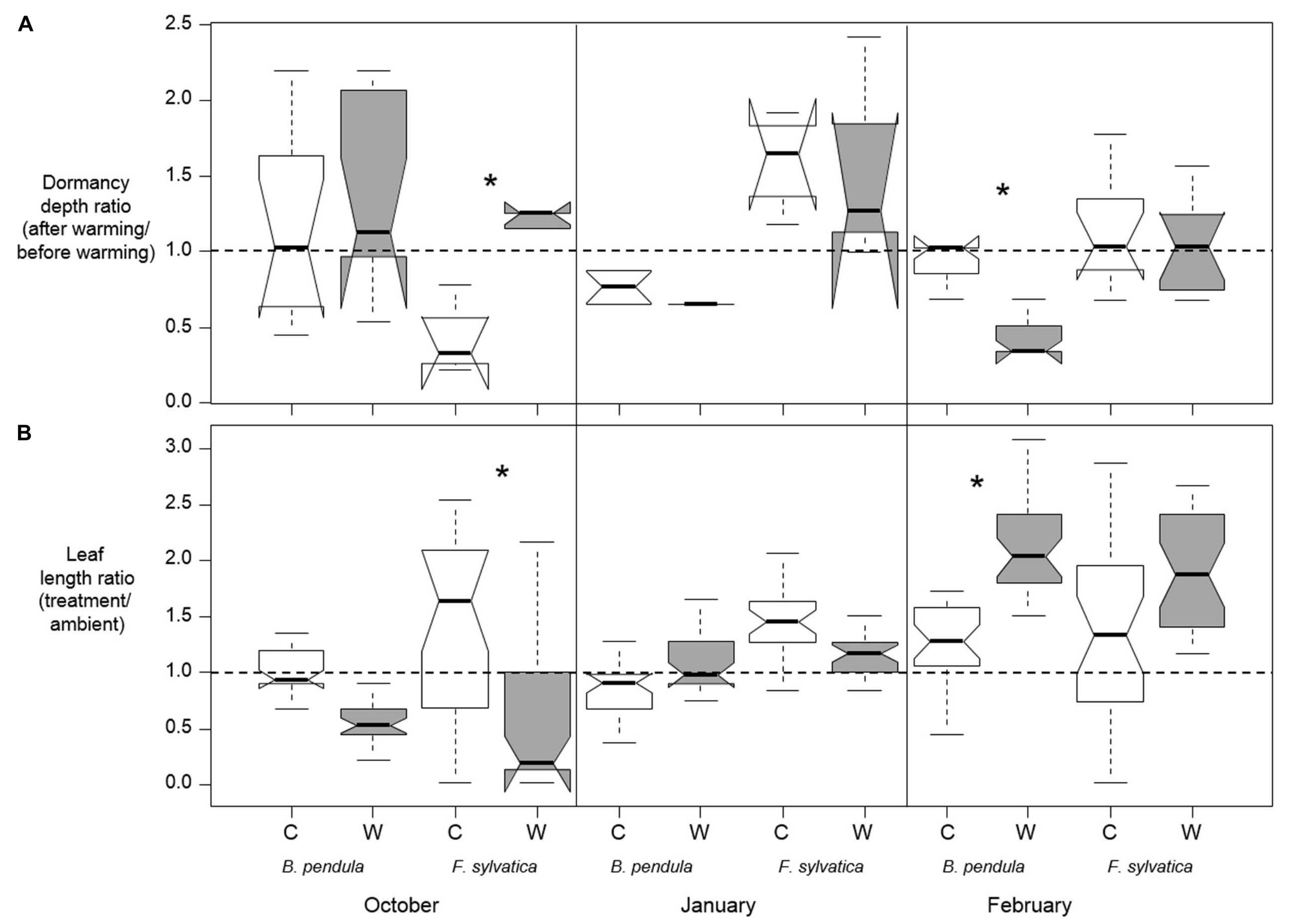

FIGURE 3 | (A) medians of bud dormancy depth ratios for each treatment, species and timing of warming (10 days of + 10°C). C stands for the control treatment and W stands for the warming treatment (see section "Materials and Methods"). Dormancy depth was estimated at the start and end of each warming pulse via twig cuttings (n = 4–12 per treatment per species per warming date), calculating the growing degree days required for bud break. Four tree seedlings were destructively sampled prior to and after each warming event. **(B)** Medians of leaf length ratios (treatment leaf length/mean leaf length of ambient tree seedlings) of each treatment and species measured in the spring following warming in October, January, and February. Leaf lengths were measured in 6 biggest leaves on each tree seedling (5 trees per treatment) when all tree seedlings of the earliest flushing treatment group had 90% completely unfolded leaves. Five tree seedlings were used per treatment (control/warming) per date per species while seven tree seedlings were kept under ambient conditions. Significant differences between treatments are shown with asterisks. Notches show the 95% confidence interval of the median and whiskers extend to a maximum of 1.5 × IQR beyond the box.

effect of warming was detected in January for either species and in February a greater increase in leaf ratio occurred in warmed seedlings than in control seedlings only for *B. pendula* ($p = 0.05$). In *F. sylvatica* leaf length decreased by approximately 6 times after fall warming compared to control, whereas leaf length *B. pendula* increased by approximately 1.6 times after warming compared to control in end of winter warming.

DISCUSSION

The antagonistic ability of warming to both delay and advance spring phenology has rarely been mechanistically explained. I have shown that the delaying effect can occur when bud dormancy is increased following warming and the advancing effect happens when warming reduces bud dormancy. Whether bud dormancy depth is increased or decreased following warming likely depends on the dormancy phase of buds, with warming during the induction phase likely increasing it and warming during the ecodormancy phase decreasing it. Furthermore, the timing of dormancy induction and ecodormancy seem to be species-specific, explaining why warming at a particular period may increase the dormancy depth in one species, but not in another.

Bud dormancy was increased by warming earlier in the season in *F. sylvatica* when the dormancy process was likely in its induction phase (**Figure 5**). The increase in bud dormancy depth has previously been tracked in several trees and found to take place in the fall, ending (reaching peak bud dormancy) between October and December (Boyer and South, 1989; Champagnat, 1989; Calmé et al., 1994). In the fall, attaining a deeper dormancy depth during the period of dormancy induction with increased temperature has been shown in poplar (Kalcsits et al., 2009) and maple (Westergaard and Eriksen, 1997). The reason behind

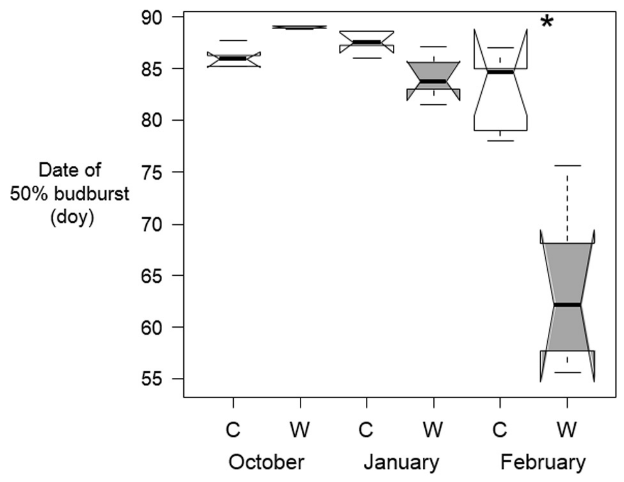

FIGURE 4 | Median dates on which 50% of buds on tree seedlings of *B. pendula* opened in the spring after being warmed by 10°C (W) and the control trees (C) during October, January, or February (*n* = 5 per treatment and sampling date). Significant differences between treatments are shown with asterisks. Notches show the 95% confidence interval of the median and whiskers extend to a maximum of 1.5 × IQR beyond the box.

warmer temperature increasing dormancy depth remains to be unknown. A warming-induced delaying effect on spring phenology during dormancy induction has also been shown, both experimentally (Heide, 2003), and retrospectively using modeling approaches explaining warming effects on spring phenology with historical climate and budburst dates (Roberts et al., 2015). Thus, warming during the fall season is likely to continue acting as an antagonist to the advancing effect of spring warming on spring phenology, albeit not to the same extent in all species. No warming effect on bud dormancy depth for *B. pendula* can potentially be explained by a different timing of bud dormancy induction in these species, although evidence for this is lacking. Another late successional species, *Quercus rubra*, has been shown to end its bud dormancy induction later than another birch species (*Betula alleghaniensis*) (Calmé et al., 1994). *B. pendula* may experience bud dormancy induction earlier in the season compared with *F. sylvatica*, already having attained full endodormancy (peak dormancy depth) before the October warming event. Regardless of the reason, future bud dormancy changes following warming in the fall are shown here to be species-specific and need to be modeled accordingly. Previous modeling approaches have suggested a stronger delaying effect of fall warming on spring phenology for *Betula pendula* compared with *Fagus sylvatica* (Roberts et al., 2015), disagreeing with my results. The discrepancy may be due to minor temperature increases in the modeling study, likely confounded with continued warming throughout the non-growing season. Experiments are thus needed that simulate a gradient of warming levels from bud dormancy induction to its release to show how projected warming will affect bud dormancy changes and in turn spring phenology in different tree species.

Bud dormancy was not affected in either species following mid-winter warming as warming took place during a period when dormancy depth was still high in both species (**Figure 5**). After attaining full dormancy, the optimal chilling temperature for fastest dormancy reduction is thought to be around 5°C or below (Murray et al., 1989; Heide, 2003; Junttila and Hanninen, 2012; Vitasse and Basler, 2013). The control treatment had 10 chilling days while the warming seedlings experienced only 1 chilling day. Trees from the warming treatment did accumulate more GDD, however (34 vs. 0 GDD), likely compensating for the reduced dormancy depth of the control seedlings. Both increased GDD and chilling days can reduce dormancy depth, with the effectiveness of chilling days being much stronger the higher the initial dormancy depth (Myking and Heide, 1995). Therefore, the seedlings' bud dormancy could have been reduced by the same amount via more chilling days in the control group and by higher accumulation of GDD in the warming treatment. Subsequently, both treatments resulted in a similar bud dormancy depth at the end of the treatment, leading to similar spring bud burst dates. The same increase in temperature in winter may therefore have milder effects on spring phenology compared with fall and end of winter or spring warming events.

Late winter warming occurred after dormancy depth has naturally already been decreased by the accumulation of chilling days at ambient field conditions, at least in *B. pendula* (**Figure 5**). Furthermore, much higher accumulation of GDD occurred in the warming treatment compared with the control (88 vs. 0.) Even though nine more chilling days had accumulated in the control treatment, likely having reduced bud dormancy, the rate at which bud dormancy is reduced via chilling during this time is much lower compared to periods when dormancy depth is much higher, as previously shown in other studies (Caffarra and Donnelly, 2010; Harrington et al., 2010; Malyshev et al., 2018). *F. sylvatica* did not react to late winter warming, potentially because its dormancy depth was still high at the initiation of the warming treatment. The additional chilling days in the control treatment were thus likely just as effective in decreasing the still high dormancy depth in *F. sylvatica* as the higher number of GDD in the warming treatment. Many studies have documented the high chilling requirements required to reduce dormancy depth in *F. sylvatica* compared with other tree species (Murray et al., 1989; Zohner and Renner, 2014; Malyshev et al., 2018), driven additionally by short photoperiod additionally reducing the rate of dormancy decrease in the species (Heide, 1993; Vitasse and Basler, 2013; Malyshev et al., 2018). Pioneer species such as *B. pendula*, which require few chilling days to release their dormancy (Heide, 2003), may therefore react more sensitively to future early spring warming periods, advancing their spring phenology to a greater extent.

In both species, fall warming delayed fall leaf coloration, yet only increased bud dormancy depth in *F. Sylvatica*. Studying and modeling dormancy induction (its timing and depth) with respect to future temperature increase may thus be more beneficial in predicting future spring phenology changes than merely monitoring senescence dates. An increased dormancy depth following warming may be the underlying cause behind the observed delayed spring phenology following delayed

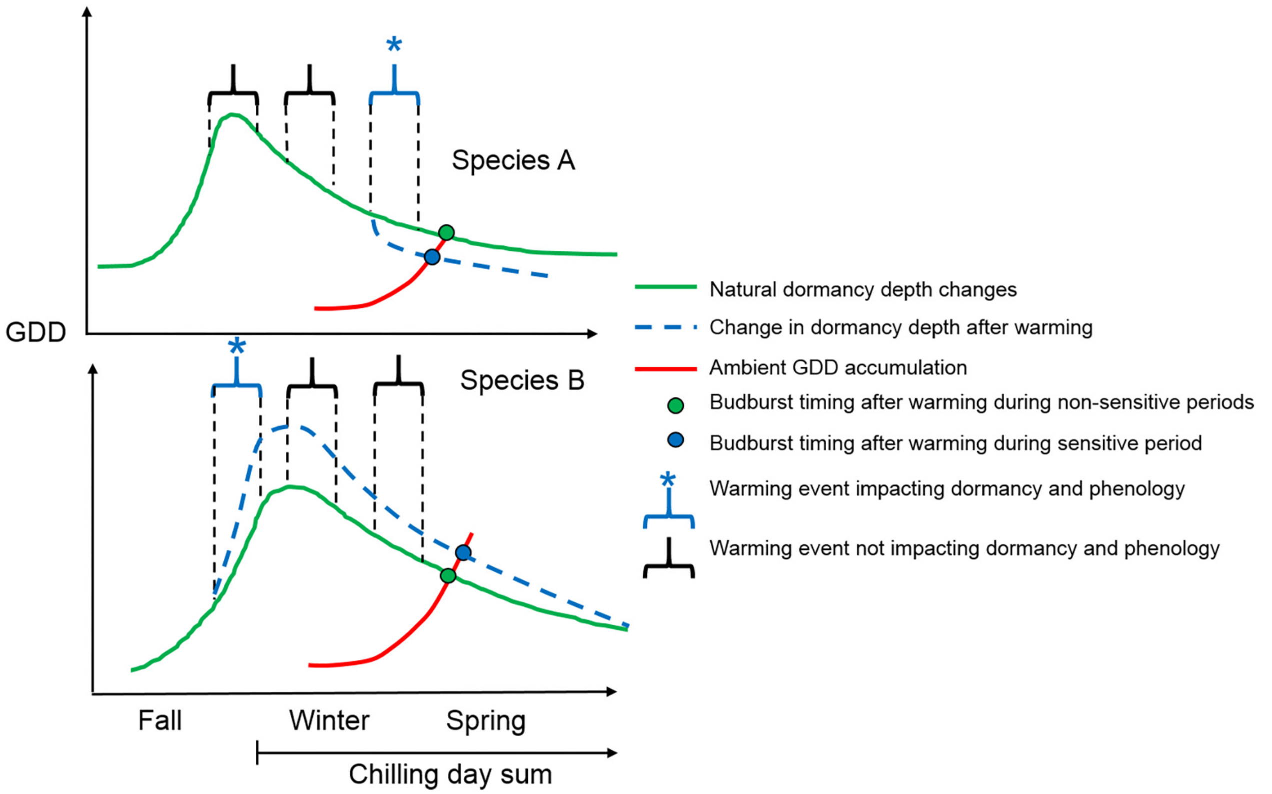

FIGURE 5 | Conceptual visualization showing how warming of the same magnitude (relative to ambient temperature) can have different effects on bud dormancy depth, depending on its timing and species. Species A represents a typically early flushing species (such as *B. pendula*) and Species B represents a typically late flushing species (such as *F. sylvatica*). The *y*-axis represents theoretical bud dormancy depths at different stages of bud dormancy induction and release phases in contrasting species (only indicative of species-specific temporal changes in dormancy depth). Species A has an earlier bud dormancy induction phase, not reacting to the first fall warming event during which the dormancy induction phase has been completed. In species B the fall warming increases bud dormancy depth (blue line), which results in a delay in spring budburst (blue point vs. green point). The spring budburst occurs when the accumulation of growing degree days (GDD – red line) from the date of maximum dormancy depth reaches the threshold GDD requirement at the end of winter (intersection of red and blue/green lines). The second warming event does not affect dormancy depth of any species as the dormancy depth is still very high and the additional GDD from warming are counterbalanced by the reduction in dormancy due to more chilling day accumulation in the absence of warming. The last warming event at the end of winter reduces bud dormancy depth in Species A (blue line), the dormancy depth of which has almost stagnated and become non-responsive to further chilling day accumulation. As a consequence, species A flushes earlier in the spring following the end of winter warming event (blue point vs. green point). Bud dormancy of Species B is still high at this point and chilling day accumulation is able to reduce dormancy depth as fast as the additional GDD from the warming event.

autumn senescence (Heide, 2003; Fu et al., 2016) and may explain the lack of phenological responses to warming in certain plant species (Cook et al., 2012). An extended growing season in the fall may have variable spring phenology knock down effects, due to its species-specific and timing –specific effects on bud dormancy. Still, milder warming, acting over months rather than days as tested here, may affect dormancy changes differently (Ex., shifting the dormancy induction timing rather than increasing dormancy depth) and needs to be further studied.

Years with strong warming spells may experience several warming spells throughout the year. Therefore, a year with a strong fall warming event may also be accompanied with winter and/or spring warming spells. Therefore, the cumulative effect of several warming spells throughout the year, including the unexplored effect of summer warming, needs to be evaluated further with future similar experiments. Effects of single extreme warming pulses on dormancy and spring phenology in species with contrasting seasonal bud dormancy patterns can be summarized, however, as seen in **Figure 5**. Nonetheless, the effects of milder and more prolonged warming (longer than 10 days) on dormancy depth and spring phenology have not been addressed here and need to be studied. Furthermore, the delaying effect of fall warming on spring phenology may be offset by the increased heat accumulation later on in the non-growing season (Fu et al., 2019). Additionally, within-species variation in warming induced bud dormancy changes has not been quantified here, although evidence shows that strong spring phenology and bud dormancy differences exist in both species studied here (Falusi and Calamassi, 1996; Junttila and Hanninen, 2012; Robson et al., 2013; Kramer et al., 2017) as well as in other species (Boyer and South, 1989). Lastly, bud dormancy depth prior and after warming events was measured in different trees here. Measuring bud dormancy depth changes in the same trees, with methods as least invasive as possible, will likely improve the correlation between the effects of temperature on bud dormancy and spring phenology.

The experiment has been carried out on tree seedlings and it is unclear if adult trees will behave the same way. Additionally, roots and buds likely experienced only mild temperature differences in the climate chambers. For mature trees, snow and leaf layer, combined with deep root growth can result in very different temperatures above and below ground (Sturm et al., 1997). Whether root temperature can affect bud dormancy is not known.

In conclusion, I have shown that differential effects of future extreme warming events on spring phenology will likely to depend on non-linear responses of bud dormancy depth to warming. Warming-induced changes in bud dormancy depth are likely to depend on the timing of warming events as well as on species-specific timing of bud dormancy induction and release. It is therefore necessary to experimentally track temporal changes in dormancy depth in different tree species from dormancy induction to its release to identify especially sensitive periods for each species.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AM carried out the experiment, analyzed the data, and wrote the manuscript.

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The Importance of Incorporating Landscape Change for Predictions of Climate-Induced Plant Phenological Shifts

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Warming in the high Arctic is occurring at the fastest rate on the planet, raising concerns over how this global change driver will influence plant community composition, the timing of vegetation phenological events, and the wildlife that rely on them. In this region, as much as 50% of near-surface permafrost is composed of thermally sensitive ground ice that when melted produces substantial changes in topography and microbiome conditions. We take advantage of natural variations in permafrost melt to conduct a space-for-time study on Ellesmere Island in northern Canada. We demonstrate that phenological timing can be delayed in thermokarst areas when compared to stable ground, and that this change is a function of shifting species composition in these vegetation communities as well as delayed timing within species. These findings suggest that a warming climate could result in an overall broadening of blooming and leafing windows at the landscape level when these delayed timings are taken into consideration with the projected advance of phenological timings in ice-poor areas. We emphasize that the impacts of geomorphic processes on key phenological drivers are essential for enhancing our understanding of community response to climate warming in the high Arctic, with implications for ecosystem functioning and trophic interactions.

Keywords: leaf phenology, flower phenology, permafrost, geomorphology, ground stability, Arctic, plant ecology

INTRODUCTION

The high Arctic is warming at twice the global average and is anticipated to have significant effects on the landscape, flora, and fauna of the region (IPCC, 2014). Warming air temperatures have already resulted in the increased thawing of permafrost and accelerated melting of ground ice (Callaghan et al., 2011; Lewkowicz and Way, 2019). It is predicted that with increased ground and air temperatures there will be large shifts in the region's flora (Elmendorf et al., 2012a) with consequences for both ecosystem functioning (Schuur and Mack, 2018) and the carbon balance (Turetsky et al., 2020). One of the key floral changes predicted is altered phenological timings, particularly advancing flowering and peak green-up dates (Arft et al., 1999; Høye et al., 2007; Oberbauer et al., 2013; Prevéy et al., 2019). The sensitivity of plant communities to phenological change is also expected to be greater in cold, high latitude sites than warmer sites (Prevéy et al., 2017). Variation in phenology can have strong influences on competitive interactions and species coexistence (Wolkovich and Cleland, 2011) and there are important concerns regarding how these altered life-cycle timings may result in trophic mismatch with insect pollinators (Høye et al., 2014; Schmidt et al., 2016) and migratory herbivores (Doiron et al., 2015).

There have only been a few long-term studies of climate-induced phenological effects in a natural high Arctic setting (e.g., Bjorkman et al., 2015; Høye and Forchhammer, 2008) due to the area's remoteness and the investment required for field collection in Arctic environments (Martin et al., 2012; Metcalfe et al., 2018). In order to overcome these challenges, studies have often utilized passive warming devices such as *in situ* experimental warming chambers to simulate the effects of climate change on plant communities (Henry and Molau, 1997; Elmendorf et al., 2012b) and plant traits (Baruah et al., 2017; Bjorkman et al., 2018). These open-top chamber (OTC) studies have demonstrated a range of effects on plants, including changes in aboveground productivity, altered species dominance, and shifting biodiversity, with strong regional variation observed in the direction of response (Elmendorf et al., 2012a). In particular, experiments utilizing OTCs have demonstrated that high Arctic phenology is especially sensitive to warming temperatures (Prevéy et al., 2017), though interestingly these responses often differ at the species- or functional group-level (Arft et al., 1999; Cooper, 2014; Prevéy et al., 2019). Previous research has found support for the correspondence between observational data and OTC studies for some plant community response variables (e.g., abundance changes; Elmendorf et al., 2015). However, this has not been the case for phenological response to warming. Wolkovich et al. (2012) demonstrated that experimental studies consistently underpredicted the response of phenology to warming, as compared to long-term studies. They suggest this may be due to artifacts introduced by the chambers themselves, as well as other proximate drivers of phenology change that are not captured through experimental warming studies.

One such neglected driver relates to the response of near-surface ground ice to climate change. Permafrost landscapes display a high degree of topographic irregularity associated with buried ground ice and the dynamics of seasonally thawed ground, generally called the active layer. The active layer is important as seasonal freeze-thaw cycles result in patterned ground features that can be highly reactive to variations in air temperature, with impacts ranging from small downward shifts in relief of a few centimeters, to ground slides and slumps measured in meters (Pollard, 2017). Up to 50% of the volume of the top 3m of ground in the high Arctic may be composed of ground ice (Pollard and French, 1980; Couture and Pollard, 1998, 2007; Liljedahl et al., 2016) mainly in the form of ice wedges. Ice wedges are a v-shaped body of ice and are a ubiquitous feature of permafrost environments, found within up to 25% of the Earth's terrestrial surface (Zhang et al., 1999). An increase in seasonal thaw can result in ice wedge melt (thermokarst), which depresses the overlying trough soil (subsidence; Liljedahl et al., 2016) and creates a highly patterned landscape of interconnected polygons with shallow troughs underlain by ice wedges (Pollard, 2017). The subsidence of ice wedges increases winter snowpack depth and the collection of surface water in an otherwise water-limited environment. This new moisture regime promotes plant growth that insulates the ground from warm summer temperatures, resulting in shallower active layers and overall colder ground temperatures throughout the growing season (Shur and Jorgenson, 2007).

A major reason for the mismatch between phenological observations and experimental warming results in the high Arctic may be the neglect of key geomorphic changes predicted to occur concurrently with climate warming. Climate change has increased rates of thermokarst in this region (Ward Jones et al., 2019) and this is predicted to continue unabated into the future (Jorgenson et al., 2015). Thermokarst is expected to drive down soil temperatures through complex hydrological interactions, resulting in the recruitment of wetland vegetation to replace traditionally polar desert habitats (Becker et al., 2016). Phenology studies employing passive warming methods generally sample from stable ground surfaces, thereby ignoring relevant factors such as changes to surface hydrology (Woo and Young, 2006), active layer depth (seasonal depth of thaw) (Jiang et al., 2012), and ground stability (Jorgenson et al., 2015). The question remains as to how geomorphological drivers may affect leafing and flowering times, and whether these results agree with previous syntheses of phenology studies in regions commonly underlain by permafrost. Given that thawed permafrost and melting ground ice result in a substantial divergence in vegetation community composition (Jorgenson et al., 2015; Becker et al., 2016), another major question is whether changes in phenology at the landscape scale are driven by changes in within-species timing or by plant community turnover resulting from the creation of thermokarst wetlands.

This study examines how geomorphologic processes act to drive phenological response in high Arctic plant communities. We adopted a space-for-time approach using both species- and plot-level measures of phenology across a thermokarst gradient to examine the influence of geomorphological change on phenology. We conducted this research at a site of naturally occurring climate-induced thermokarst on the Fosheim Peninsula, Ellesmere Island, Nunavut within the Canadian high Arctic (Pollard, 2000). This region has experienced recent widespread initialization of thermokarst activity over the past decade due to climate warming (Ward Jones et al., 2019), stressing the importance of understanding how thermokarst will impact both plant communities and their life cycles. We compared changes in a suite of phenological traits across plant communities in both undisturbed polar desert and thermokarst terrain, as well as differences across polygon features created by ice wedge degradation (elevated tops vs subsided troughs). More specifically, we examined how the development of thermokarst drives changes in community-level phenology as well as the intraspecific variability in phenology of a widespread species, *Salix arctica*. We predicted the following: (1) thermokarst troughs would experience colder ground temperatures due to the subsidence of ice wedges and associated abiotic changes (as seen in Becker et al., 2016) and (2) the phenology of vegetation in thermokarst areas would be delayed in comparison to non-thermokarst terrain due to thermokarst-driven temperature shifts

(Arft et al., 1999). As thermokarst is responsible for strongly divergent microhabitat conditions that not only directly affect phenology, but also result in large biodiversity shifts across these steep gradients (Zona et al., 2011), we further examined the contribution of species turnover and intraspecific variability to phenological responses.

MATERIALS AND METHODS

This study was conducted at a site of climate-induced thermokarst in the high Arctic on the Fosheim Peninsula of Ellesmere Island, Nunavut, Canada (79.84574°N, 85.37028°W). This region is characteristic of a polar desert environment with little precipitation, nutrient poor soils, and an extremely short growing season. The Fosheim experiences an exceedingly cold mean annual air temperature (MAAT) of −18.8°C with summer temperatures only reaching an average of 6.1°C in July (Environment Canada, 2010). These cold conditions result in deep permafrost that is over 500 m thick (Taylor, 1991), exceptionally thin average seasonal ground thaw compared to other areas of the Arctic (mean active layer of 57 cm; Couture and Pollard, 2007), and a small species pool estimated at ∼140 vascular plant species (Edlund et al., 1990). There is ∼1456.8 km^2 of ground ice in the Fosheim Peninsula alone, of which ∼700 km^2 likely consists of wedge ice (Couture and Pollard, 1998) though recent satellite based measurements have estimated that ice wedges could occur across 50% of the land cover of the peninsula, or ∼3000 km^2 (Bernard-Grand'Maison and Pollard, 2018). We selected a 200 m × 100 m study site (panel A of **Figure 1**) to be characteristic of the general landscape of the Fosheim Peninsula, with geomorphic and vegetation differences at the site representative of localized thermokarst processes predicted to increase with a warming climate as outlined in Becker et al. (2016). Panels B and C of **Figure 1** further demonstrate the differences in microtopography encountered in this region, with panel B illustrating subsidence due to ice wedge degradation (producing a polygon trough), and panel C showing the typical polygonal patterning found in these ground ice-dominated landscapes.

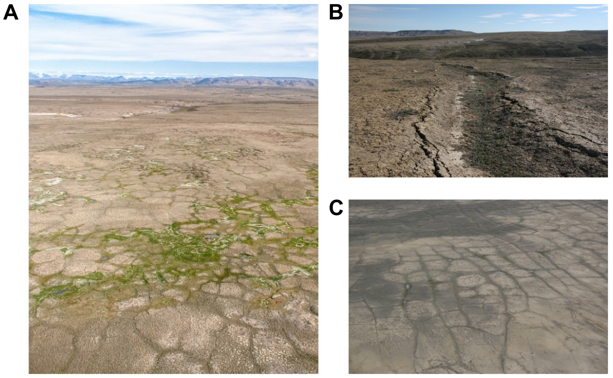

FIGURE 1 | (A) Photo depicting an area of thermokarst (ground ice melt) on the Fosheim Peninsula, Ellesmere Island, Nunavut, Canada. The photograph shows the study site during the summer season, where thermokarst and adjacent polar desert polygons were sampled. **(B)** Photo depicting a polygon trough. **(C)** This photo highlights the presence of polygon features across the landscape, showing ice wedge polygons with high relief tops and low relief troughs.

Data Collection

As thermokarst causes an abrupt disturbance in temperature regime and ground stability (Jorgenson et al., 2015), we used microtopography as a natural experiment in which to assess whether differences in abundance-weighted phenology were due more to intraspecific variability or changes in the abundance and identity of species in the community. Phenological data and ground temperature data for this study were collected in concert with abiotic and biotic data collected for a separate study during the polar summer of 2013 (see Becker et al. (2016) for further methodological details). We chose a study site containing a thermokarst wetland in order to sample an area undergoing climate-induced permafrost melt and the adjacent polar desert terrain. Both of these ground states exhibit polygonal microtopographies, with higher relief "tops" and lower relief "troughs." We established plots immediately after snowmelt using a stratified sampling approach along five transects running 200 m east to west, spaced 25 m apart north to south. We systematically sampled each polygon top and trough feature traversed by the transects, placing 0.5 m^2 plots every 5–10 m at alternating polygon tops and troughs (allowing for some spatial variation due to differences in the sizes of each polygon feature traversed). Our design is fully factorial, with plots located in either polar desert or thermokarst ground states, and top or trough polygon features. We hereafter refer to the polar desert ground state as the control state, as we are interested in the shift in phenology due to an increase in thermokarst in this region. This resulted in 80 plots in total, with 20 plots each across each of four habitat categories: control (polar desert) top, control (polar desert) trough, thermokarst top and thermokarst trough. However, we report 75 plots in our results as there were five plots found in thermokarst troughs which remained submerged with water throughout the growing season. We visually estimated community composition and relative percent cover within each plot at approximately peak biomass. All vascular vegetation was identified to the species level, with nomenclature following Saarela et al. (2013) (see **Supplementary Table S1** for species information).

We recorded hourly temperature changes through the summer season and computed total thaw degree days (TDD) using Onset HOBO data loggers buried at a depth of 10 cm (approximating maximum rooting depth) in each plot. TDD is a measure of the magnitude of warming above 0°C. To calculate TDD, we averaged temperature values to gain a daily mean temperature for each plot and summed mean temperatures for all days greater than 0°C. We chose to use TDD in our analyses, and not growing degree days (GDD, or cumulative temperature >5°C, the temperature at which plants generally experience growth), as our focal species are selected for growing in cold conditions and some of the phenophase dates observed occurred before plots exhibited GDD values greater than 0. Data loggers were installed on June 28th immediately after snowmelt and retrieved on July 20th. Some ground thaw had already occurred in order to allow for sufficient burial depth (the ground freezes to the surface every winter); as such we consider our TDD metric as the relative magnitude in temperature experienced between microtopographies for the summer, rather than the absolute TDD for the summer period.

Phenological trait measures of both flowering and leaf emergence and growth were assessed within each plot for all vascular plant species. We recorded the following phenological traits as measures: (first) initial leaf growth, (first) full leaf out, (first) flower bud (including flower heads for grasses/sedges) and (first) open flowering, using methodology detailed in the United States National Phenology Network (NPN) (Denny et al., 2014). These phenophases capture a broad range of phenological responses that may be affected by thermokarst and have different implications for interactions between trophic levels (i.e., delayed flowering time may affect pollinators more, whereas delayed leafing may preferentially impact herbivores). Phenological measures were sampled every second day in each plot during the field season. Because many species within our plots are clonal and it is difficult to differentiate individuals, we recorded plot-level phenophase measures for each species.

Statistical Analysis of Phenological Data

All statistical analyses and graphics were conducted in R version 3.6.3 (R Core Team, 2020), using the package tidyverse for data wrangling (Wickham et al., 2019) and the packages ggthemes (Arnold, 2019) and cowplot (Wilke, 2019) for graphics.

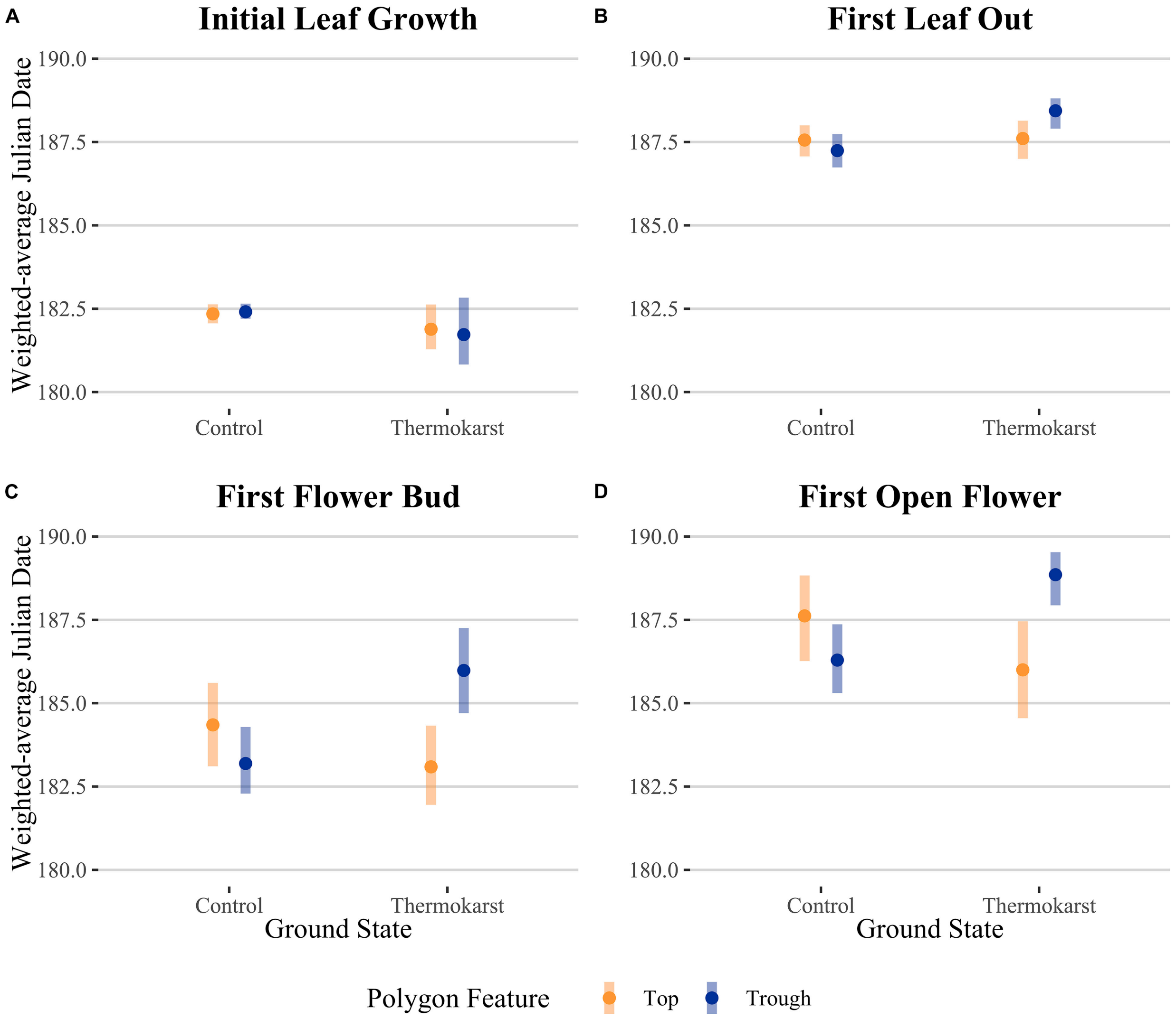

FIGURE 2 | Abundance-weighted average Julian dates for community-level **(A)** initial leaf growth, **(B)** first leaf out, **(C)** first flower bud and **(D)** first open flower across ground state (control vs thermokarst), with colors denoting polygon feature (top = orange vs trough = blue) categories. Points denote mean values, while bars represent 95% confidence intervals.

TABLE 1 | Abundance-weighted Type II ANOVA results for all four phenological timing response variables.

Response	Effect	SS	*F*	*P*
Initial leaf growth	Ground state	5.9	3.345	0.072
	Feature	0	0.018	0.895
	Interaction	0.2	0.130	0.719
First leaf out	**Ground state**	**6.0**	**4.705**	**0.034**
	Feature	0.6	0.458	0.501
	Interaction	**5.7**	**4.465**	**0.038**
First flower bud	Ground state	1.9	0.244	0.623
	Feature	18.6	2.412	0.125
	Interaction	**59.5**	**7.720**	**0.007**
First open flower	Ground state	3.7	0.569	0.454
	Feature	2.3	0.349	0.557
	Interaction	**60.0**	**9.338**	**0.003**

Significant P-values (<0.05) are in bold.

In order to compare changes in community phenological responses, we calculated weighted average Julian dates for flower bud, open flowers, initial leaf growth, and full leaf out across all species within each plot. Weighted averages were based on community relative abundances generated using the function “decostand” in the R package vegan (Oksanen et al., 2019). We compared abundance weighted phenological timing using two-way ANOVAs with type II sum of squares using the car package (Fox and Weisberg, 2019), as sample number differed based on microtopography, and compared effects of top vs trough and control vs thermokarst using the function “emmeans” in the R package emmeans (Lenth, 2020), with all pairwise comparisons corrected using a tukey *p*-value adjustment. We additionally used the package nlme (Pinheiro et al., 2020) to test for the effect of spatial variation on our results. We performed the same analyses as above using linear mixed effects models with “transect” as a random effect and assessed whether there was statistical support for the inclusion of this as a random effect in our models. As we were also interested in evaluating within-species changes in phenology, we chose one species, *S. arctica*, to compare phenology traits across microtopography using a type II ANOVA. This species was selected as it was found in 80% of censused plots at this study site (it being the only species occupying >50% of plots censused) and was the most abundant species across the site, with an average of 60% cover in occupied plots. We performed the same statistical tests as described above, assessing differences in phenophase traits of *S. arctica* (non-weighted) as predicted by ground state, polygon feature, and their interaction. We assessed importance of these predictors using both type II ANOVAs and linear mixed effects models, as described previously.

To tease apart the predominant contributors to community-averaged phenological timings, we decomposed variation in flowering and leafing dates into interspecific (species compositional turnover) and intraspecific (within-species variation) components across microtopography, following code adapted from Lepš et al. (2011). This approach is particularly suited to our study, as there are large species compositional differences in plant communities in thermokarst and polar desert habitats. Here we use “fixed” and “specific” averages to describe whether differences in mean trait variability between treatments at the plot-level are due to a change in species composition or in intraspecific trait changes. We first calculated fixed averages in phenological timings per species across the entire site, which is a single mean trait value for that species. Using only these fixed averages, we generated relative abundance weighted averages for each plot. Therefore, any observed differences in fixed averages can only be due to compositional change, or species turnover in plant communities. Specific averages used individual observations for each species within each plot to calculate a relative abundance weighted mean, and are the same averaged phenophase data that were compared in our previous analyses. The difference between fixed averages and specific averages removes the effect of species composition change, or turnover, and as such can be solely attributed to intraspecific variability in phenological timing. The response of phenophase timing to microtopography was analyzed using a two-way type II ANOVA, decomposed into the sum of squares of species turnover, intraspecific variability, and their covariation. See further details on this methodology in the **Supplementary Information** (Methods S1).

In order to assess the effects of temperature on phenology at our study site, we first tested overall differences in TDD across the summer season using a two-way type II ANOVA to account for unequal sample sizes. To test the effect of TDD on phenophases, we calculated a cumulative TDD for all days prior to the Julian date at which the phenophase trait commenced for all four phenophases tested. As we were testing relative abundance weighted means of phenophases, we rounded down the averaged number and generated a cumulative sum of average daily temperatures $>0°C$ up to that whole Julian date, such that each plot and phenophase trait was attributed a unique TDD value. We analyzed each phenophase using linear models with the plot- and phenophase-specific TDD value as a predictor. As we were interested in whether ground state and polygon feature explained any residual variation after TDD was accounted for, we performed an ANCOVA analysis with TDD as the first covariate and polygon feature, ground state and their interaction as secondary predictors. This analysis controls for the effect of a covariate TDD and tests whether there is any remaining effect of our two treatments which were not explained by temperature.

We transformed data using a square root transformation for the response community-weighted initial leaf growth dates and a ln transformation for the predictor cumulative temperature where appropriate.

RESULTS

Data loggers were successfully retrieved on July 20th after 22 days in the ground (data inclusive of Julian dates 179–200), capturing the full window between early spring (before leaf growth or flowering commenced) and summer season. Across the study site, this particular summer was characterized by a late snowmelt on June 25th, colder than average July

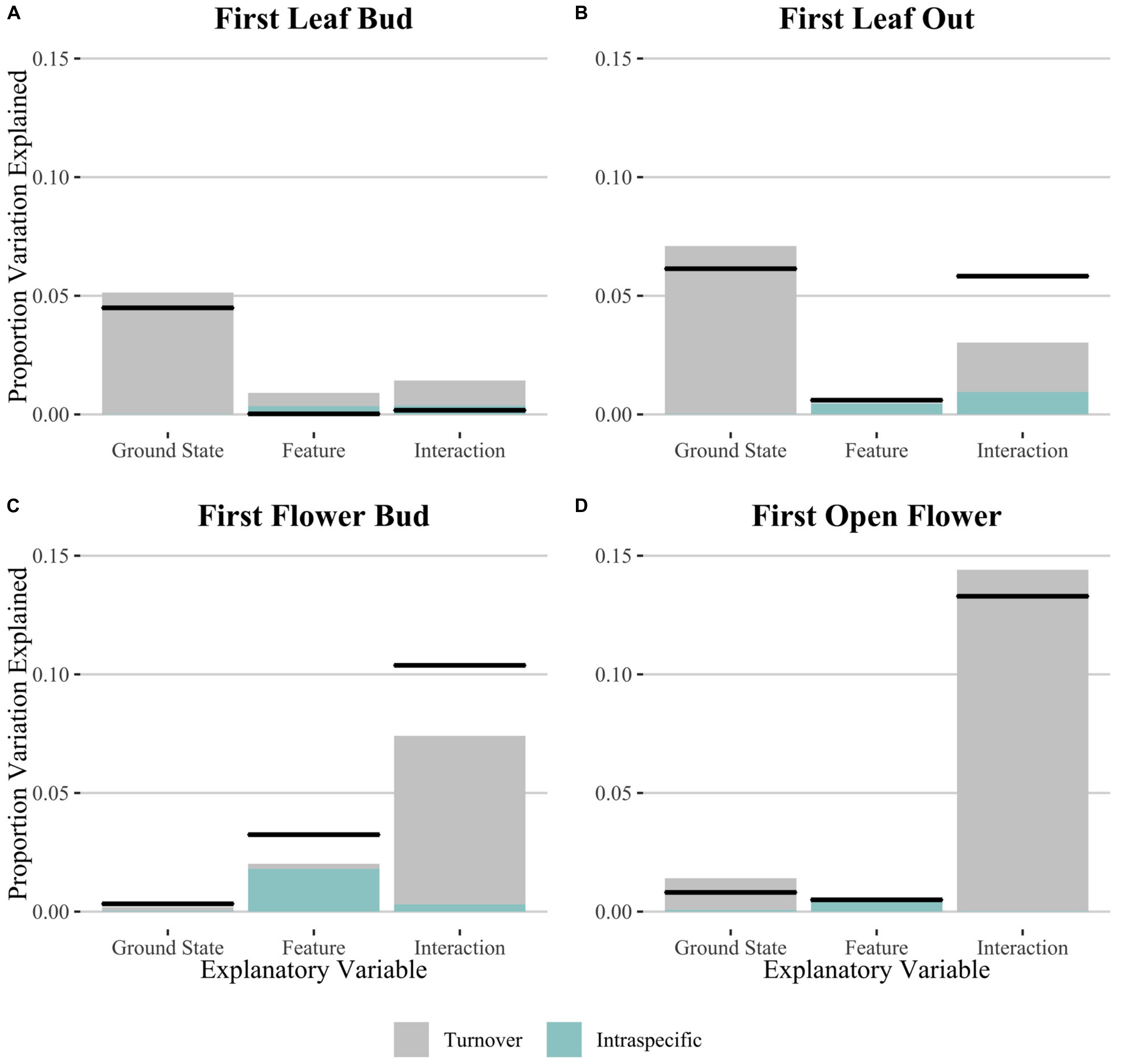

FIGURE 3 | Relative Sum of Squares obtained from variance decomposition analysis of the effect of ground state, polygon feature and their interaction on timing of phenophases: **(A)** initial leaf growth, **(B)** first leaf out, **(C)** first flower bud and **(D)** first open flower. Black lines denote total variation explained for each explanatory variable. Colored bars denote the proportional variance contribution for both species turnover (Turnover; gray) and intraspecific variability (Intraspec.; blue). See **Table 2** for associated statistics.

temperature of 4.4°C, and an early autumn snowfall on August 12th. This later snowmelt and early snowfall created a particularly short summer window for plant life to accomplish the typical lifecycle processes of inflorescence bloom, leaf growth, energy capture and storage, reproduction, and senescence. We found strong, significant differences in plot ground temperature for the different microtopographies. Thermokarst plots experienced significantly lower TDD for the study period than control areas (95.3 (SE 3.54) and 119.2 (SE 3.32), respectively; $F_{1,70}$ = 23.7, $P < 0.001$), with polygon troughs having lower TDD than polygon tops (91.5 (SE 3.54) and 122.9 (SE 3.32), respectively; $F_{1,70}$ = 41.2, $P < 0.001$) and no significant interaction effect ($F_{1,70} = 1.12$, $P = 0.294$; see panel A of **Supplementary Figure S1**).

Rather than being dominated only by *S. arctica* (as in the control plots), thermokarst plots were generally dominated by high-moisture preference species such as *Dupontia fisheri* and *Carex aquatilus*, in addition to *S. arctica* (**Supplementary Figure S2**). The entire species pool of the site was limited to 22 out of an estimated 140 indigenous to the area (see **Supplementary Table S1**) (Edlund et al., 1990), with five species unique to control plots and five species unique to thermokarst areas. Weighting phenophase traits by relative abundance, we found a significant interaction effect between polygon feature and ground state for first leaf out, first flower bud, and first open flowers timings (**Figure 2**; see **Table 1** for associated statistics). We found no significant differences between polygon tops in thermokarst or control plots for any of the phenophases. Compared to control troughs, thermokarst troughs had significantly or marginally significant delayed leaf out (1.20 days (SE 0.395), $t_{67} = -3.03$, $P = 0.018$), flower bud (2.29 days (SE 0.98), $t_{64} = -2.31$, $P = 0.099$), and open flowers (2.56 days (SE 0.94), $t_{60} = -2.74$, $P = 0.039$). Thermokarst troughs also exhibited delayed phenology as compared to thermokarst tops, with later flower bud (3.16 days (SE 1.02), $t_{64} = -3.11$, $P = 0.015$) and open flowers (2.60 days (SE 0.97), $t_{60} = -2.69$, $P = 0.045$). These significant delays (between 1.20 and 3.12 days) in three key phenological traits in plant communities found in depressed thermokarst troughs as opposed to thermokarst tops represent a 6.7–14.9% shift in timing across the growing season. The only trait to differ from these overall trends was initial leaf growth, with no significant effects of ground state, polygon feature, or their interaction.

In order to test whether spatial variation may play a role in the results we found, we performed the same analyses using linear mixed effects models including transect as a random effect (**Supplementary Table S2**). We found no statistical support for including the random effect in our models for those phenophase traits which showed a significant effect of microtopography on Julian date, with the simple linear model showing consistently lower AIC values. We then used the linear models to decompose variation due to intraspecific change and community turnover for all phenophase traits (**Figure 3** and **Table 2**). Species turnover comprised the greatest contribution to variation in phenophase explained by ground state and the interaction between ground state and polygon feature. For first leaf out and first flower bud, there was a large positive covariation between turnover and intraspecific contributions to the interaction of ground state and feature, whereas covariation in the interaction term was smaller and negative for initial leaf growth and open flowers.

We evaluated whether cumulative ground temperature up to a phenological event could explain differences in our leaf or flowering phenophase differences across our site. We found that cumulative TDD was positively correlated with first leaf bud (df = 72, $R^2 = 0.216$, $P < 0.001$), full leaf out (df = 68, $R^2 = 0.193$, $P < 0.001$), flower bud (df = 65, $R^2 = 0.505$, $P < 0.001$), and first open flowering dates (df = 61, $R^2 = 0.607$, $P < 0.001$; panel B of **Supplementary Figure S1**). We also found that the predictors ground state and polygon feature explained residual variation after TDD was accounted for in an ANCOVA (**Figure 4**; see **Table 3** for associated statistics). We found that there remained no effect of ground state or polygon feature while including TDD as a covariate for first leaf bud. The effect of site, type and their interaction were significant for first leaf out, with thermokarst troughs delayed compared to control tops (1.24 days (SE 0.35), $t_{65} = -3.50$, $P = 0.005$), thermokarst tops (1.12 days (SE 0.36), $t_{65} = -3.10$, $P = 0.017$) and control troughs (1.12 days (SE 0.34), $t_{65} = -3.27$, $P = 0.011$). For first flower bud there was a direct effect of polygon feature and the interaction of feature and ground state, with thermokarst troughs also delayed as compared to control tops (2.02 days (SE 0.68), $t_{62} = -3.00$, $P = 0.025$), thermokarst tops (2.52 days (SE 0.70), $t_{62} = -3.61$, $P = 0.004$) and control troughs (1.87 days (SE 0.67), $t_{62} = -2.78$, $P = 0.043$). For first open flowers, we found that there was no significant interaction (as found when including only the categorical predictors), but that there was a direct effect of polygon feature and ground state. For this phenophase thermokarst troughs were delayed as compared to control tops [2.28 days (SE 0.66), $t_{58} = -3.46$, $P = 0.006$] and thermokarst tops [2.11 days (SE 0.64), $t_{58} = -3.28$, $P = 0.011$], but not significantly so for control troughs.

TABLE 2 | Variance decomposition results for **(A)** initial leaf growth, **(B)** first leaf out, **(C)** first flower bud and **(D)** first open flower phenology measures.

	Turnover	Intraspecific	Covariation	Total
(A) Initial leaf growth				
Ground state	**6.7**	0	−0.8	5.9
Feature	0.7	0.5	−1.2	0
Interaction	1.4	0.5	−1.7	0.2
Residuals	66.8	50.2	8.2	125.2
Total	75.6	51.2	4.5	131.3
(B) First leaf out				
Ground state	**7.0**	0	−1	**6.0**
Feature	0	0.5	0.1	0.6
Interaction	**2.0**	0.9	2.8	**5.7**
Residuals	20.6	70.8	−5.3	86.1
Total	29.6	72.2	−3.4	98.4
(C) First flower bud				
Ground state	0.9	0.2	0.8	1.9
Feature	1.2	**10.3**	7.1	18.6
Interaction	**40.7**	1.8	17.0	**59.5**
Residuals	387.0	114.0	−7.7	493.3
Total	429.8	126.3	17.2	573.3
(D) First open flower				
Ground state	6.0	0.3	−2.6	3.7
Feature	0	2.3	0	2.3
Interaction	**65.0**	0.1	−5.1	**60.0**
Residuals	333.3	54.7	−2.2	385.8
Total	404.3	57.4	−9.9	451.8

Sum of squares were calculated using Type II ANOVAs, and are displayed for each factor in the analysis, ground State and feature, as well as their interaction, model residuals and total model variation. Sum of squares are decomposed into the contributions by species turnover, intraspecific variability and their covariation. Covariation is calculated by subtracting both species turnover and intraspecific variability contributions from the Total column, and can be negative or positive. Values are bolded if significant (P < 0.05).

In order to evaluate intraspecific variation in phenological responses across microtopography, we focused on the species *S. arctica* which was found to be widespread and abundant across both polygon feature and ground state (**Figure 5**). Both initial leaf growth and full leaf out phenophases showed significant interaction effects between ground state and feature ($F_{1,53} = 6.0$, $P = 0.018$, and $F_{1,55} = 7.1$, $P = 0.010$, respectively). However, after a pairwise comparison correction, initial leaf growth was only found to be marginally significantly delayed [1.05 days (SE 0.41)] compared to thermokarst tops ($t_{53} = -2.54$, $P = 0.065$). Leaf out was delayed 1.83 days (SE 0.69) in thermokarst troughs

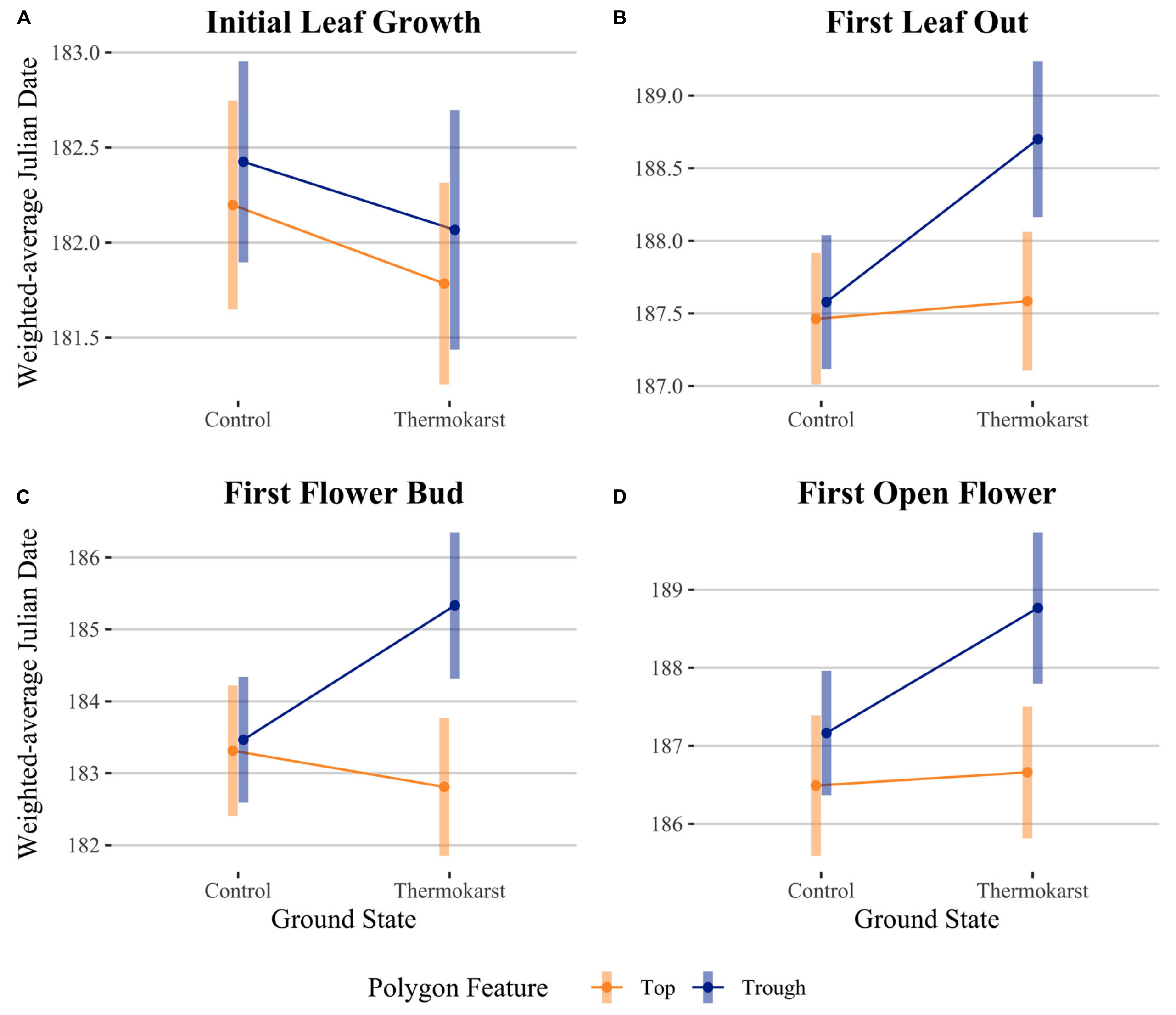

FIGURE 4 | Interaction plots for estimated marginal means comparing ground state (control vs thermokarst) and polygon feature (top = orange vs trough = blue) categories, while controlling for cumulative thaw degree days (TDD). Comparisons are **(A)** initial leaf growth (TDD = 8.0), **(B)** first leaf out (TDD = 22.6), **(C)** first flower bud (TDD = 12.0) and **(D)** first open flower (TDD = 23.1). Points denote mean values, while bars represent 95% confidence intervals.

compared to thermokarst tops (t_{55} = 2.66, P = 0.049) but presented 1.32 days (SE 0.50) earlier in thermokarst tops than control tops, though this was also found to be only marginally significant (t_{55} = 2.64, P = 0.051). We found no effect of ground state or polygon feature on first flower bud date. Polygon feature was the predominant control on first open flowering in this species, with open flowers delayed 1.01 days (SE 0.45) in troughs compared to tops ($t_{25} = -2.28$, P = 0.031; $F_{1,25}$ = 6.7, P = 0.016).

DISCUSSION

The delays we found in three key phenological traits for plant communities in thermokarst troughs demonstrate the impact of a geomorphological process that has largely been overlooked in ecology literature. Our results suggest that thermokarst, and the subsequent ground subsidence due to ice wedge degradation, decreases soil temperature and these altered abiotic conditions affect key phenophase timings. The differences we observed in plot-averaged phenophase traits are largely driven by turnover in community membership, and not intraspecific variability, which operate differently depending on microtopography. We found that ground state (thermokarst or non-thermokarst) and polygon feature (top or trough) explained residual variation in our models after accounting for temperature. This suggests that geomorphic features influence other environmental variables (such as soil moisture, active layer depth, snowpack accumulation, and spring runoff water collection; Jorgenson et al., 2015) important for predicting phenological events. Thermokarst trough communities experienced a 7–15% delay

TABLE 3 | Abundance-weighted Type II ANCOVA results for all four phenological timing response variables, including cumulative Thaw Degree Days (TDD) as a covariate.

Response	Effect	SS	*F*	*P*
Initial leaf growth	**TDD**	**27.5**	**19.553**	**<0.001**
	Ground state	2.7	1.912	0.171
	Polygon feature	1.1	0.779	0.381
	Interaction	0	0.001	0.922
First leaf out	**TDD**	**22.7**	**23.424**	**<0.001**
	Ground state	**6.2**	**6.392**	**0.013**
	Polygon feature	**5.1**	**5.229**	**0.025**
	Interaction	**4.3**	**4.415**	**0.040**
First flower bud	**TDD**	**264.6**	**73.167**	**<0.001**
	Ground state	7.4	2.049	0.157
	Polygon feature	**24.9**	**6.888**	**0.011**
	Interaction	**22.4**	**6.199**	**0.015**
First open flower	**TDD**	**205.8**	**73.125**	**<0.001**
	Ground state	**11.9**	**4.228**	**0.044**
	Polygon feature	**27.3**	**9.708**	**0.003**
	Interaction	6.9	2.454	0.122

Significant P-values (<0.05) are in bold.

in phenological timing, which is contrary to most phenological warming predictions for the Arctic. While thermokarst requires the presence of ground ice to occur, the fact that it is present in up to 50% of the near-surface ground volume of the high Arctic points to a large area of the landscape that could experience these microtopographical changes (Pollard and French, 1980). Thus, while warming in stable areas is likely to advance plant phenology, concurrent and patchy changes to the landscape via subsidence may have delaying effects on phenology and community turnover.

Arctic phenology is predominantly driven by cues which vary inter-annually (e.g., temperature, snowmelt and soil thaw), vs cues which are invariant (e.g., photoperiod; Wheeler et al., 2015), and plant phenological timing tends to be more sensitive to these variations at colder sites (Prevéy et al., 2017). As these variant drivers are impacted by thermokarst, phenology in the Arctic may be particularly sensitive to ground processes. Interestingly, the delays we found in thermokarst trough phenology (1–3 days) are similar in magnitude, but opposite in direction to the 2–5 day advancement in phenology found over two decades in an OTC warming experiment at a nearby site on Ellesmere Island, Nunavut, Canada (Bjorkman et al., 2015). We caution that the results found in our study are from a single site over a single growing season, and while there are inherent limitations with these constraints, we suggest that this study can still provide insights into the often-overlooked effect of geomorphology on vegetation in the arctic. If thermokarst-induced changes to plant communities tend to delay phenology while drier sites experience earlier phenology [or no significant change, as found in control plots in Bjorkman et al. (2015)], we may see a broadening of overall Arctic phenological windows, based on the heterogeneous response of landscape microtopography due to climate warming. Thermokarst may delay flowering such that bud break and flower development occur later in the summer growing period for some species, while earlier flowering cued by early snow melt in non-thermokarst areas may increase risk of frost damage for flowers (Wheeler et al., 2015). Thermokarst-induced conditions tend to delay snowmelt timing, an important phenological driver which we were unable to assess in this study. This may contribute to an inequality of fitness in plant communities across microtopographies, further driving differences in community-level diversity and abundance across the landscape.

Given there are such large shifts in community membership between the microtopographies at our site, we used a relative site-based measure to compare these disparate communities. Recent work suggests that this approach is a powerful way to compare phenology across studies with dissimilar plant communities (Prevéy et al., 2017), such as those created by the effects of thermokarst. Site-level measures of phenology are an important way of quantifying how resource availabilities for pollinators and herbivores in the high Arctic may shift with climate change as both resident pollinators (Elberling and Olesen, 1999; Olesen and Jordano, 2002) and herbivores (Manseau and Gauthier, 2013) tend to be generalists capable of utilizing a suite of available plant life. However, as responses to climate change are often species- and functional group specific (Iler et al., 2013; Post et al., 2016) we need to better understand how intraspecific variability and species turnover both contribute to average phenological change in plant communities. Our results suggest that phenology is largely delayed at the plot-level for thermokarst troughs because the changed abiotic conditions act as environmental filters which favor shifts in plant community membership. In certain cases, such as for the first leaf bud and first open flower phenophases, we see a negative covariance in the direct effect of ground state or feature. Here, turnover and intraspecific variability are selecting for opposing (early vs late) phenological dates, which may explain why there are few significant direct effects of polygon feature or ground state. Examining a dominant species at our site, our analysis of intraspecific variation demonstrates that *S. arctica* does shift phenophase timings in response to the changed abiotic conditions associated with ice wedge thermokarst (i.e., in general, thermokarst delays phenophase timings). However, this effect is not consistent across phenophase traits and for first leaf out can depend on the polygon feature a community is found in (e.g., thermokarst tops found an advancement in timing compared to non-thermokarst terrain).

The melting of ground ice has a multitude of effects on the ecology and vegetation of the high Arctic (Billings and Peterson, 1980). However, to our knowledge, climate-induced changes to geomorphic features have yet to be considered when projecting phenological change in a warming Arctic. The changed hydrological pattern of thermokarst results in colder and more stable ground temperatures due to increased vegetation cover, which has a dampening effect on both daily and seasonal temperature variation (Shur and Jorgenson, 2007; Becker and Pollard, 2016). The species found colonizing areas undergoing thermokarst are likely drawn from the local high Arctic species pool already present in neighboring wetland areas (Cooper et al., 2004), and these locally adapted wetland species could be timed to complete life history processes within the narrow

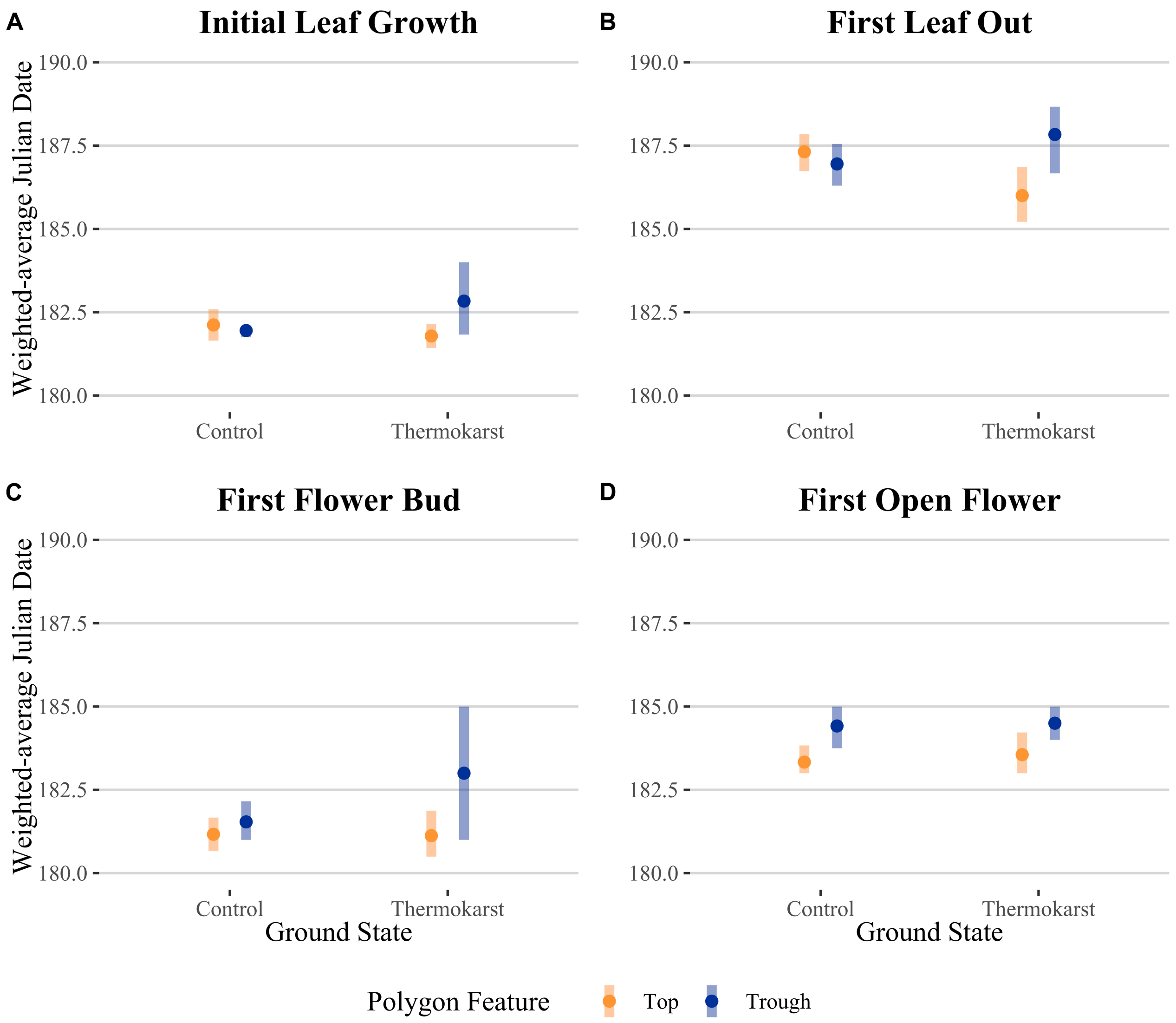

FIGURE 5 | Julian dates for *Salix arctica* phenophases **(A)** initial leaf growth, **(B)** first leaf out, **(C)** first flower bud and **(D)** first open flower across ground state (control vs thermokarst), with colors denoting polygon feature (top = orange vs trough = blue) categories. Points denote mean values, while bars represent 95% confidence intervals.

summer window despite colder summer conditions imposed by thermokarst processes (Becker and Pollard, 2016). The cooling effects of thermokarst-altered abiotic conditions might offset effects of warming air temperatures, similar to that of later snow melt (Wipf and Rixen, 2010), and are difficult or impossible to replicate using experimental warming studies. It is clear that there are multiple drivers of ecological change in the high Arctic, and an acknowledgment of the interaction between warming temperatures and geomorphological processes may alter our predictions of how climate change will impact this region.

Perhaps the greatest concern of climate change effects on high Arctic phenology is trophic mismatch, which is a lack of synchrony between the life cycle timing of consumers and their resources (Durant et al., 2007). In the case of the high Arctic, many organisms are largely transient either as resident insect pollinators with short summer life cycles, or migratory birds (McKinnon et al., 2012). Deepening snow conditions delay insect emergence (Høye and Forchhammer, 2008), and as ice wedge thermokarst leads to trough subsidence, and thereby a deeper snowpack, this suggests delayed insect emergence for colonies within troughs. Interestingly, a delayed vegetation phenology in troughs but advancement in non-thermokarst areas may lead to an overall broadening of the window for plant provisions that insects rely on. Insects that emerge late will not benefit from this, but insects that advance their timings in concordance with non-thermokarst terrain may see a net benefit in a longer period of resource availability. Many migratory birds nest in the Arctic and their reproductive timings are tuned to account for peak plant nutrition. For example, some species such as Snow Geese, *Chen caerulescens*, are only able to partially adjust their breeding phenology to compensate for availability of high-quality food and timing mismatches result in poor gosling growth (Doiron et al., 2015). Our leaf phenophase results suggest a delayed timing for peak green-up in thermokarst trough communities, which are one of the more productive wetland habitats available to these organisms in the polar desert climate. This also infers a later date for peak leaf-nitrogen, an important food source for geese (Chapin, 1980). Additionally, our results show thermokarst-induced trough community reshuffling resulted in higher biomass of *Dupontia* and *Eriophorum* species – resources preferred by growing chicks (Manseau and Gauthier, 2013). Given the prevalence of thermally sensitive ground ice in the high Arctic, the cumulative effects of shifting phenological timing on trophic mismatch should be investigated further.

CONCLUSION

The geomorphology and ecology of the high Arctic are tightly interconnected and a warming climate is predicted to affect both greatly. Given the prevalence of thermally sensitive ground ice and the resulting abiotic changes that occur due to thermokarst, it is essential that we consider this geomorphological process when predicting the effects of climate change on plant phenology. Due to the patchy but significant distribution of ground ice, at a landscape level we are likely to see two divergent phenological results as a consequence of climate change: (1) delayed phenology of communities overlaying thermokarst-affected ice wedge troughs and (2) an increased divergence in phenological timings between polygon features. We emphasize the necessity of considering geomorphological change in studies of the effects of climate change on plant communities found in areas underlain by permafrost, as these ground states underlie a large portion of the high Arctic landscape and are particularly susceptible to the effects of climate warming. Additionally, we suggest that the bridging of historically disparate natural sciences will improve on our ability to predict the effects of climate change, or at the very least, increase our understanding of the underlying intricacies of natural systems.

DATA AVAILABILITY STATEMENT

Soil temperature and phenological observation data used in this publication are available from the Figshare repository (10.6084/m9.figshare.12248312).

AUTHOR CONTRIBUTIONS

CC and MB conceived of and conducted the field work for this project. CC analyzed the data. CC and MB wrote the manuscript. All authors contributed extensively to the drafts and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Water Addition Prolonged the Length of the Growing Season of the Desert Shrub *Nitraria tangutorum* in a Temperate Desert

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Climate models often predict that more extreme precipitation events will occur in arid and semiarid regions, where plant phenology is particularly sensitive to precipitation changes. To understand how increases in precipitation affect plant phenology, this study conducted a manipulative field experiment in a desert ecosystem of northwest China. In this study, a long-term *in situ* water addition experiment was conducted in a temperate desert in northwestern China. The following five treatments were used: natural rain plus an additional 0, 25, 50, 75, and 100% of the local mean annual precipitation. A series of phenological events, including leaf unfolding (onset, 30%, 50%, and end of leaf unfolding), cessation of new branch elongation (30, 50, and 90%), and leaf coloration (80% of leaves turned yellow), of the locally dominant shrub *Nitraria tangutorum* were observed from 2012 to 2018. The results showed that on average, over the seven-year-study and in all treatments water addition treatments advanced the spring phenology (30% of leaf unfolding) by 1.29–3.00 days, but delayed the autumn phenology (80% of leaves turned yellow) by 1.18–11.82 days. Therefore, the length of the growing season was prolonged by 2.11–13.68 days, and autumn phenology contributed more than spring phenology. In addition, water addition treatments delayed the cessation of new branch elongation (90%) by 5.82–12.61 days, and nonlinear relationships were found between the leaves yellowing (80% of leaves) and the amount of watering. Linear relationships were found between the cessation of new branch elongation (90%), the length of the growing season, and amount of water addition. The two response patterns to water increase indicated that predictions of phenological events in the future should not be based on one trend only.

Keywords: desert species, growing season, foliar phenology, branch phenology, water addition

INTRODUCTION

Plant phenology, i.e., the timing of seasonal life cycle events, such as leafing out and flowering, plays a fundamental role in the function of terrestrial ecosystems (Fu et al., 2014a; Fu et al., 2015; Browning et al., 2019). The timing of phenological events is strongly controlled by the prevailing climate and has long been regarded as one of the most sensitive and accurate bio-indicators to track climate change (Badeck et al., 2004; Bertin, 2008; Gordo and Sanz, 2010). Advancing the understanding of the phenological response to climate change is therefore important for forecasts of the impact of future climate change on terrestrial ecosystems (Cleland et al., 2007; Nord and Lynch, 2009). With the climate change observed over recent years, advances of spring phenology and delays of autumn phenology have been reported worldwide such as in Europe (Menzel and Fabian, 1999; Menzel et al., 2001; Fu et al., 2014a), North America (Schwartz and Reiter, 2000; Jeong et al., 2011; Fridley, 2012), the Southern Hemisphere (Chambers et al., 2013; Ma et al., 2013), and China (Ge et al., 2014; Ge et al., 2015; Zheng et al., 2016). This variation was attributed to prevailing climate warming trends (Cleland et al., 2007; Bertin, 2008). This is true for ecosystems without chronic seasonal water stress; however, the effects of temperature on vegetation phenology may be critically modulated in arid and semiarid ecosystems by soil water availability (Moore et al., 2015). Many studies have investigated how precipitation regulates plant phenology in seasonally dry tropical forests (Hayden et al., 2010) and other water-limited ecosystems (Patrick et al., 2009; Lesica and Kittelson, 2010; Liu et al., 2015; Zhou and Jia, 2016). For example, in a water-limited ecosystem in California, the rainfall volume and timing during winter jointly influenced the timing of vegetative bud break, where high rainfall in December and March delayed bud break, while high rainfall in February advanced bud break (Mazer et al., 2015). The degree of budburst and leaf extension was shows to be a function of irrigation intensity at the Estacion Biologia de Chamela in western Mexico (Hayden et al., 2010). Based on remote sensing data, preseason precipitation (i.e., precipitation before the growing season) was found to exert a stronger influence on the starting date of the vegetation growing season (SOS) of grasslands in drier areas than in wetter areas of the Qinghai–Tibetan Plateau (Shen et al., 2011; Shen et al., 2015). Moreover, the effect of total preseason precipitation on the end date of the growing season (EOS) in dry grasslands is greater than that of temperature for Inner Mongolia, China (Liu et al., 2015; Zhou and Jia, 2016). Similar results were also reported for arid and semiarid regions of Africa (Zhang et al., 2005; Gaughan et al., 2012). Shoot elongation during the growing season is an integral component of the annual sequence of developmental events in plants (Codesido and López, 2003). Shoot elongation rates were found to be related to rainfall in the two evergreen, woody, Brazilian Cerrado species *Leandra lacunose* and *Miconia albicans* (Damascos et al., 2005).

The phenological responses of plants in desert ecosystems in particular are causing increasing concern (Ghazahfar, 1997; Ogle and Reynolds, 2004; Leeuwen et al., 2010; Kigel et al., 2013; Sakkir et al., 2014; Yan et al., 2016; Huang et al., 2018). Desert ecosystems cover approximately 30% of the land surface and are strongly controlled by water availability (Patrick et al., 2009). Precipitation and water availability likely affect desert plant phenology stronger and more directly than the phenology of ecosystems with greater precipitation (Ghazahfar, 1997; Yan et al., 2016). Several studies have explored the responses of desert plant phenology to precipitation variation in their natural conditions. For example, the onset and duration of growth in chamaephytes and therophytes are highly correlated with both the timing and abundance of precipitation, whereas phanerophytes are least affected in the gravel desert of northern Oman (Ghazahfar, 1997) and in eastern United Arab Emirates (Sakkir et al., 2014). At the regional scale, spatial shifts in the onset of the vegetation growing season are controlled by summer rainfall in the southern Sahara Desert (Yan et al., 2016). Winter precipitation explained 14.2% of the inter-annual variations of spring phenology in the desert ecosystem of northwestern China (Wu and Liu, 2013).

Deserts across northwestern China cover an area of approximately 1.3 million km^2 and are constantly expanding into neighboring ecosystems because of climatic changes and human activities (Huang et al., 2015a; Huang et al., 2015b). Future climate scenarios predict that the precipitation regimes in the desert regions of northwestern China will likely change, following an increasing trend (Gao et al., 2012; Chen, 2013; IPCC, 2014; Li et al., 2016; Wang et al., 2017). For example, based on the RCP8.5 scenario, increases in annual precipitation of 25%, 50% (Gao et al., 2012), and greater than 100% (Wang et al., 2017) of mean annual precipitation are expected in specific desert regions at the middle and end of the 21st Century compared with the end of the 20th Century (Song et al., 2020). Most of the biological processes in desert ecosystems are controlled by soil water availability, which is generally controled by rainfall events (Song et al., 2020). Precipitation increases likely impose substantial impacts on plant phenology in these deserts in response to climate change. Few studies examined the effects of water addition on reproductive phenology of six annuals on the southern fringe of the Gurbantunggut Desert in northwestern China and the results were only based on a short-time manipulative experiment (Huang et al., 2018). The results showed that water addition consistently advanced both the flowering and fruiting time of four spring ephemerals; however, their effects on two spring–summer annuals were inconsistent, where advances were found in one species, while delays were found in another (Huang et al., 2018). Since this study only focused on ephemerals, it remains unknown how the desert plant phenology of dominant perennials responds to an increase in precipitation, especially long-term precipitation increases.

To address this question, this study conducted a long-term *in situ* water addition experiment in a temperate desert of northwestern China, which is dominated by the shrub species *Nitraria tangutorum*. Five simulated future precipitation regimes (natural rain and natural rain plus an additional 25, 50, 75, and 100% of local long-term mean annual precipitation (145 mm)) were studied during the growing seasons from 2008 to 2018. A series of phenological events, including leaf unfolding (onset, 30%, 50%, and end of leaf unfolding), cessation of new branch elongation (30, 50, 90%), and leaf coloration (80% of leaves turned yellow) of *N. tangutorum* were assessed from 2012–2018. This *in situ* long-term water addition experiment provides accurate phenological information at the species level. The specific aims of this study were to: (i) clarify how increased water availability will affect phenological events (specifically, whether these will be advanced or delayed); (ii) identify how water increases affect the length of the growing season (specifically, whether it will be prolonged or shortened); (iii) determine how phenological events and the duration of the growing season respond to water addition (specifically, whether these responses will be linear or nonlinear).

MATERIALS AND METHODS

Site Description

This study was conducted at the Desert Ecosystem Water Addition Platform (106°43'E, 40°24'N, 1,050 m above sea level (a.s.l.)) which was set up in Dengkou County, Inner Mongolia, China, in 2008. The long-term mean annual precipitation is approximately 145 mm, 95% of which falls from May to September. The mean annual temperature is 7.6°C, and the mean annual potential evaporation is 2,381 mm. The vegetation is dominated by the shrub species *N. tangutorum*. Other species, such as *Artemisia ordosica*, *Psammochloa villosa*, *Agriophyllum squarrosum*, and *Corispermummon golicum*, can also be found occasionally. *N. tangutorum* has a high tolerance to drought, wind erosion, and sand burial. It is a pioneer species that is widely distributed throughout the northwestern regions of China and plays an important ecological role in the fixing of sand dunes because of its exceptional capabilities to form so-called nebkha dunes, or nebkhas (Zhang et al., 2015). Nebkhas are phytogenic mounds composed of wind-borne sediments within or around shrub canopies (Li et al., 2013). The experiment was performed in a patchy landscape with *N. tangutorum* nebkhas (**Figure 1**) interspersed on hard clay deposited by the Yellow River. The plant cover on these nebkhas was approximately 45–75%. The soil types were sandy soil and gray-brown desert soil (Zhang et al., 2015). The underground water at the experimental site was at a depth below 5 m, which does not affect plant growth. The soil chemical properties at the 0–10 cm depth are shown in **Table 1**.

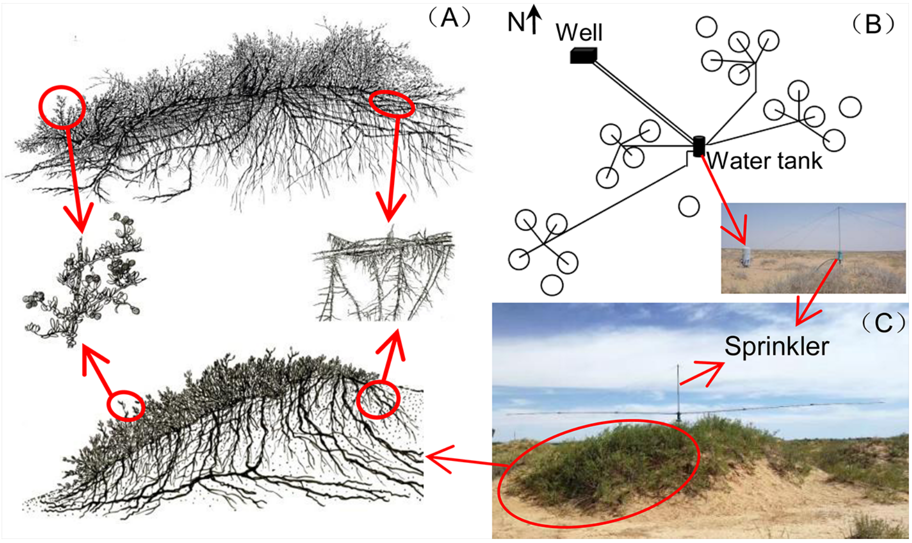

FIGURE 1 | Illustrations of *Nitraria tangutorum* nebkhas above-ground and below-ground structures, cited from Zhang et al., 2015 **(A)**; distribution of the 20 plots and the water addition system **(B)**; sprinkler on nebkhas **(C)**.

TABLE 1 | Mean (± SE) values of soil chemical properties in 0–10 cm deep soil at the end of the 2010 growing season (n = 4).

Treatment	SOC (%)	Soil N (%)	Soil C/N	Soil pH
Ctrl	0.13 ± 0.01(abc)	0.018 ± 0.001(b)	7.80 ± 0.48(a)	8.90 ± 0.11(a)
+25%	0.12 ± 0.01(bc)	0.021 ± 0.002(ab)	5.71 ± 0.71(bc)	9.02 ± 0.12(a)
+50%	0.11 ± 0.01(c)	0.024 ± 0.001(a)	4.57 ± 0.34(c)	8.92 ± 0.11(a)
+75%	0.16 ± 0.01(a)	0.026 ± 0.002(a)	5.94 ± 0.32(bc)	8.66 ± 0.07(a)
+100%	0.15 ± 0.01(ab)	0.024 ± 0.002(a)	6.48 ± 0.62(ab)	8.87 ± 0.06(a)

SOC, soil organic carbon. The same letters in parentheses within each soil property indicate no significant differences between water addition treatments, while different letters denote significant differences (P < 0.05).

Experimental Design of Water Addition Treatments

A completely random design was used with five water addition treatments: natural rain plus an additional 0% (Control), 25% (+25%), 50% (+50%), 75% (+75%), and 100% (+100%) of local long-term mean annual precipitation (145 mm). Furthermore, four replicates for each treatment were established since 2008 (113 m^2 per plot, 20 plots in total, **Figure 1B**). The water addition treatments were applied equally every month from May to September, and the additional water amounts were 0, 7.3, 14.5, 21.8, and 29.0 mm each time for the five water addition treatments, respectively. The water was pumped from a well near the plots into a water tank with water meters and then transported to each sprinkler (**Figure 1C**). The sprinklers were installed on the top of each nebkha (plot) *via* an irrigation system (**Figure 1B**). The sprinklers had two automatically rotating spraying arms (6 m in length) that could uniformly sprinkle water over the treatment area. More detailed information on the experimental design and the irrigation system can be found in our previous publication (He et al., 2019). No chemical components were added to the water, and thus, the water used here could be used to simulate natural rainfall (Song et al., 2020).

Phenology Recording

The phenology observations were conducted from 2012 to 2018 following the standard protocols of Phenological Observation Methodology in China (Wan and Liu, 1979) with minor modifications. Phenology recording was conducted by the same observer from March 2012 to May 2014 and from March 2015 to November 2018, while from May 2014 to November 2014 the recordings were conducted by another observer who had been trained for one month by the first observer. Phenological events for all shrubs in each plot (each nebkha) were recorded every other day.

A series of phenological events, including leaf unfolding (onset, 30%, 50%, and end of leaf unfolding), cessation of new branch elongation (30, 50, 90%), and leaf coloration (80% of leaves turned yellow) were recorded (**Table 2**). Precipitation, air temperature, relative humidity, and evaporation data were recorded by a standard meteorological station near the experimental plots. The soil gravimetric water content (SWC) of the 0–20 cm soil layer was measured using the oven-drying method. SWC measurements were conducted on the day before water-addition treatments and every two days after in May, July, and September in 2012, but only one day each month in 2017 (see details in **Figure 3**).

Data Processing

The observed dates of phenological events were first transformed to the day-of-year format. The length of the growing season was calculated as the difference between the days when 80% of the leaves had turned yellow and the onset of leaf unfolding. The relative change of the days (Δdays) was used to test the effects of water addition treatments on each event.

$$\Delta\,\text{days} = \frac{1}{n}\sum_{i=0}^{n}(\text{day}_{\text{treat}} - \text{day}_{\text{Ctrl}}) \qquad (1)$$

where $\text{day}_{\text{treat}}$ represents the day for a given event or the length of the growing season under water addition plots, day_{Ctrl} represents the corresponding day in control plots, and n represents the number of experimental years. Then, Δdays <0 indicates that phenological events (growing season length) were advanced (shortened) under water addition, while Δdays >0 indicates that phenological events (growing season length) were delayed (prolonged) under water addition.

TABLE 2 | Phenological events recorded in this study. All shrubs in each treatment plot (nebkha) were considered and counted.

Leaf unfolding	Onset of leaf unfolding	At least one bud in each nebkha has at least one leaf completely out of the bud, first leaves visible, but not yet at full size.
	30% of leaves unfolded	30% of buds in each nebkha have their leaves out, fully expanded.
	50% of leaves unfolded	50% of buds in each nebkha have their leaves out, fully expanded.
	End of leaf unfolding	More than 90% of buds in each nebkha have their leaves out, fully expanded.
Cessation of new branch elongation	30% of new branches ceased elongating	30% of new branches in each nebkha have their terminal withered and cease elongating
	50% of new branches ceased elongating	50% of the new branches in each nebkha have their terminal withered and cease elongating
	90% of new branches ceased elongating	Over 90% of the new branches in each nebkha have their terminal end withered and ceased elongating
Leaf coloration	80% of leaves turned yellow	Over 80% of the leaves in each nebkha show yellow color.

This study investigated whether the heat requirement (often expressed as growing degree days, GDD) and chilling day (CD) affected the inter-annual variation of the onset of leaf unfolding in the natural condition. GDD was calculated as the sum of the daily mean temperature exceeding 5°C and below 30°C from Jan 1st to the day before the onset of leaf unfolding (Fu et al., 2013; Anandhi, 2016). CD is the number of days with a daily mean temperature below 0°C from Nov 1st the previous year to the onset of leaf unfolding.

Statistical Analysis

Simple linear regression analysis was used to determine the inter-annual trends of meteorological factors and phenological events. Pearson correlation was used to analyze the relationships between phenological events and meteorological factors. Linear mixed models were used to examine the effects of water addition treatments, year, and their interactions on phenological events over the seven years (2012–2018). Water and year were used as fixed factors, while plot was used as a random factor. The dependent factor was the timing of different phenological events (Type I Sum of Squares was used). Duncan *post hoc* tests were used to determine pairwise differences for significant effects. Regression analyses were used to determine the relationships between changes in phenological events and water addition amounts or soil water content. Repeated Measurement ANOVA (RMANOVA) analysis was used to test the effects of water addition treatments, time of treatment application, and their interactions on soil water content. One-way ANOVA analyses were used to test the effects of water addition treatments on the timing of different phenological events, separately for each year. Homogeneity of variances was tested by Levene's tests. One-sample Kolmogorov-Smirnov tests were used to validate normality of the data distribution. All these above-mentioned procedures were performed in SPSS (SPSS for Windows, version 20.0, Chicago, IL, USA).

RESULTS

Meteorological Factors

Temporal variations in annual mean relative humidity, air temperature, evaporation, and annual precipitation at the study site from 1983 to 2018 are shown in **Figure SI1**. Relative humidity followed a significant increasing trend since 1983 (**Figure SI1A**). No significant temporal trends were found for air temperature (**Figure SI1B**) and evaporation (**Figure SI1C**). Annual precipitation followed increasing trends from 1983 to 1998 and from 1999 to 2018 (**Figure SI1D**).

Seasonal variations in precipitation and air temperature are shown in **Figure 2**. Mean air temperatures in winter, spring, summer, and autumn were –7.34°C, 11.39°C, 23.9 8°C, and 9.09°C, respectively. The mean annual temperature was 9.60°C for 2012–2018, and no significant increasing or decreasing trends were found (**Figures 2A–E**). Winter precipitation only occurred in three of the years from 2012–2018 and the amounts were very low, with less than 1 mm in 2014 and 2016, and less than 5 mm in 2018 (**Figure 2F**). Spring rainfall varied over 2012–2018, with the highest value in 2015 (38.6 mm, including one large rain event with 36.5 mm) and zero in 2016 (**Figure 2G**). Accumulated summer rainfall exceeded 35 mm in all seven years (2012–2018), with the highest value in 2012 (178.5 mm) and the lowest value in 2014 (38.5 mm) (**Figure 2H**). There was a large variation in autumn rainfall (2012–2018), with the highest value in 2015 (65.6 mm) and the lowest value in 2013 (7.6 mm) (**Figure 2I**). Annual precipitation in 2012, 2013, 2014, 2015, 2016, 2017, and 2018 was 213.3 mm, 59.1 mm, 95.2 mm, 147 mm, 189.2 mm, 86.0 mm, and 59.1 mm, respectively (**Figure 2J**). Based on the deviation from the long-term mean, 2012 and 2016 were "above-average" (i.e., wet) years, 2013 was an "ultra below-average" (i.e., extremely dry) year, while 2014, 2017, and 2018 were "below-average" (i.e., dry) years, and 2015 was an "average" year.

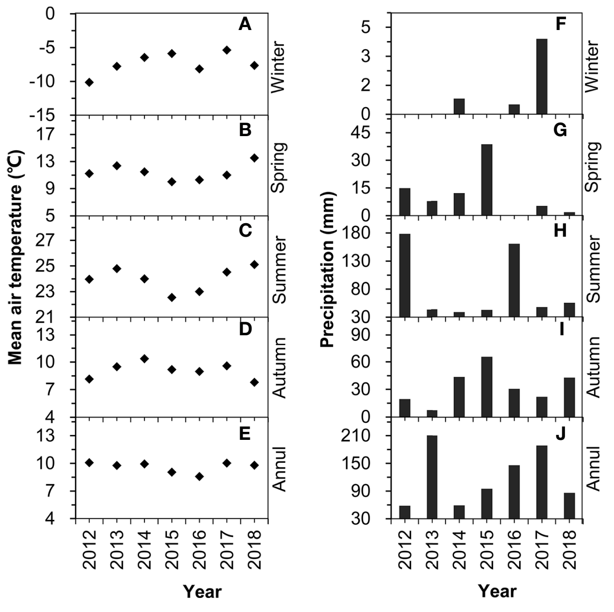

FIGURE 2 | Variations in air temperature **(A–E)** and precipitation **(F–J)** over the period of 2012–2018.

Variation Trends of Phenological Events for 2012-2018

The dates of phenological events (in days of the year) under different water addition treatments are shown in **Table SI1**. No significant advancing or delaying trends were found for almost all phenological events under all treatments for 2012–2018 (all $P > 0.05$) with only one exception (the onset of leaf unfolding) (**Table SI2**). The onset of leaf unfolding dates showed significantly earlier trends for 2012–2018 for all treatments (all $P < 0.05$, **Figure SI2**). Among all meteorological factors, only relative humidity (RH) was significantly negatively correlated with the dates of onset of leaf unfolding under all treatments (all $P < 0.05$, **Table SI3**). Almost no significant relationships were found between all other phenological events and meteorological factors (**Table SI3**). No significant effects of temperature (winter, spring, summer, autumn, and annual) were detected for any of the phenological events in this study (all $P > 0.05$, **Table SI3**).

Changes in Soil Water Content

RMANOVA analysis showed that both the amount of added water and time had significant effects on soil water content (SWC) ($P < 0.01$), and their interaction was also significant ($P < 0.01$). The SWC significantly changed after water addition treatments each month (**Figures 3A, B**). The change in magnitude depended on the amount and time of water added. Larger water addition resulted in a larger response magnitude (**Figures 3A, B**). The relative changes of soil water contents after water addition treatments were highest in spring, followed by autumn, and were lowest in summer (**Figures 3A, B**).

Changes in Spring Phenology

Linear Mixed Model analysis showed that water addition treatments had significant effects on the occurrence of 30% of leaves unfolded and the end of leaf unfolding (all $P < 0.05$, **Table 3**), and had marginally significant effects on the occurrence of 50% of leaves unfolded ($P = 0.08$, **Table 3**). Year affected all events significantly (all $P < 0.05$, **Table 2**). There was no interaction between water addition treatment and year (all $P > 0.05$, **Table 3**).

Two of the four spring events (leaf unfolding), 30% of leaves unfolded (**Figure 4A**) and the end of leaf unfolding (**Figure 4B**), showed consistent directional shifts (both advanced) in all four water addition treatment plots in all seven years except for 2014 (**Figure 4**). On average over the seven years (2012–2018), the occurrence of 30% of leaves unfolded events in +25, +50, +75, and

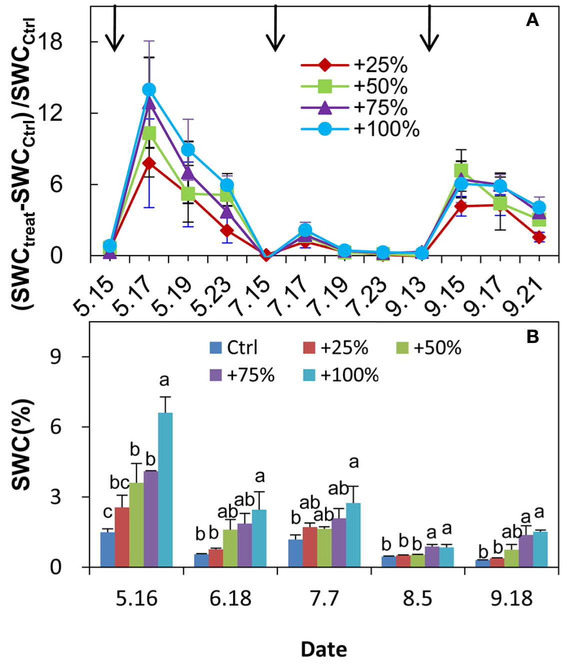

FIGURE 3 | Relative changes of 0–20 cm soil water content (SWC) compared with control (Ctrl) in +25, +50, +75, and +100% water addition treatment plots in spring (May), summer (July), and autumn (September) in 2012 **(A)**. Variation in 0–20 cm SWC during the growing season (May to September) of 2017 **(B)**. SWC_{Ctrl} represents SWC in control plots. SWC_{treat} represents the SWC in water addition treatment plots. Arrows in **(A)** represent the water addition treatment application time.

TABLE 3 | Results (*P*-values) of Linear Mixed Model (MIXMOD) analysis on the fixed effects of water addition treatments (water), year, and their interactions on the phenological events and the length of the growing season from 2012–2018.

Phenological events		**Water**	**Year**	**Water × Year**
Leaf unfolding	Onset of leaf unfolding	0.16	<0.01	0.20
	30% of leaves unfolded	0.03	<0.01	0.65
	50% of leaves unfolded	0.08	<0.01	0.73
	End of leaf unfolding	<0.01	<0.01	0.32
Cessation of new branch elongation	30% of new branches ceased elongating	0.07	<0.01	0.24
	50% of new branches ceased elongating	0.85	<0.01	0.80
	90% of new branches ceased elongating	0.01	<0.01	0.53
Leaf coloration	80% of leaves turned yellow	0.02	<0.01	0.41
The length of the growing season		0.01	<0.01	0.53

+100% treatments were advanced by 1.29, 3.43, 2.64, and 3.00 d, respectively, which was significantly different from control in the +50% treatment (*P* <0.05, **Figure 4C**). On average in six years of the study (2012–2018, excluding 2014), the occurrence of 30% of leaves unfolded events in +25, +50, +75, and +100% treatments were advanced by 1.58, 4.33, 4.08, and 3.00 d, respectively. Significant differences from the control occurred in +75 and +100% treatments (all *P* <0.05, **Figure 4E**). On average over all seven years (2012–2018), the occurrences of the end of leaf unfolding event in +25, +50, +75, and +100% treatments were advanced by 4.00, 7.00, 6.71, and 6.29 d, respectively, and significant differences from the control occurred in +50, +75, and +100% treatments (all *P* <0.05, **Figure 4D**). On average in six years of the study (2012–2018, excluding 2014), the occurrences of the end of leaf unfolding event in +25, +50, +75, and +100% treatments were advanced by 4.83, 8.92, 8.75, and 6.83 d, respectively. Significant differences from the control occurred in +25, +50, +75, and +100% treatments (all *P* <0.05, **Figure 4F**).

Changes in Autumn Phenology

Linear Mixed Model analysis showed that water addition treatments had significant effects on the cessation of new branch elongation (90%) and 80% of leaves turned yellow (all *P* <0.05, **Table 3**), while the effect on the cessation of new branch elongation (30%) was marginally significant (*P* = 0.07, **Table 3**). Year affected all events significantly (all *P* <0.05, **Table 3**). No interaction was found between water addition treatments and year (all *P* >0.05, **Table 2**).

The cessation of new branch elongation (90%) was delayed in all four water addition treatments and all seven years (2012–2018) with one exception in 2012 (**Figure 5A**). On average over the seven years (2012–2018), the cessation of new branch elongation (90%) in +50, +75, and +100% treatments were delayed by 5.82, 12.11, and 12.61 d, respectively. Significant differences from control occurred in +75 and +100% treatments (all *P* <0.05, **Figure 5C**). On average in six years of the study (2012–2018, excluding 2014), the cessation of new branch elongation (90%) in +50, +75, and +100% treatments were delayed by 6.79, 12.79, and 13.63 d, respectively. Significant differences from the control occurred in +100% treatment (*P* <0.05, **Figure 5E**).

The dates when 80% of leaves turned yellow were delayed in all four water addition treatments and in all seven years in almost all cases except for 2012 and 2014 (**Figure 5B**). On average over the seven years (2012–2018), the occurrence of 80% of leaves turning yellow was delayed in +25, +50, +75, and 100% treatments by 1.18, 5.00, 7.07, and 11.82 d, respectively. A significant difference from the control occurred in the +100% treatment (**Figure 5D**). On average over six years of the study (2012–2018, excluding 2014), the occurrence of 80% of leaves turned yellow was delayed in +25, +50, +75, and 100% treatments by 2.71, 6.92, 9.50, and 14.71 d, respectively. A significant difference from the control occurred in the +100% treatment (*P* <0.05, **Figure 5F**).

Changes in the Length of the Growing Season

Linear Mixed Model analysis showed that both water addition treatments and year had significant effects on the length of the growing season (*P* <0.05, **Table 3**), and there was no interaction between them.

The growing season was prolonged in all four water addition treatments and in all seven years (2012–2018) in all cases except for 2012 and 2014 (**Figure 6A**). On average over the seven years (2012–2018), the growing season was prolonged in +25, +50,

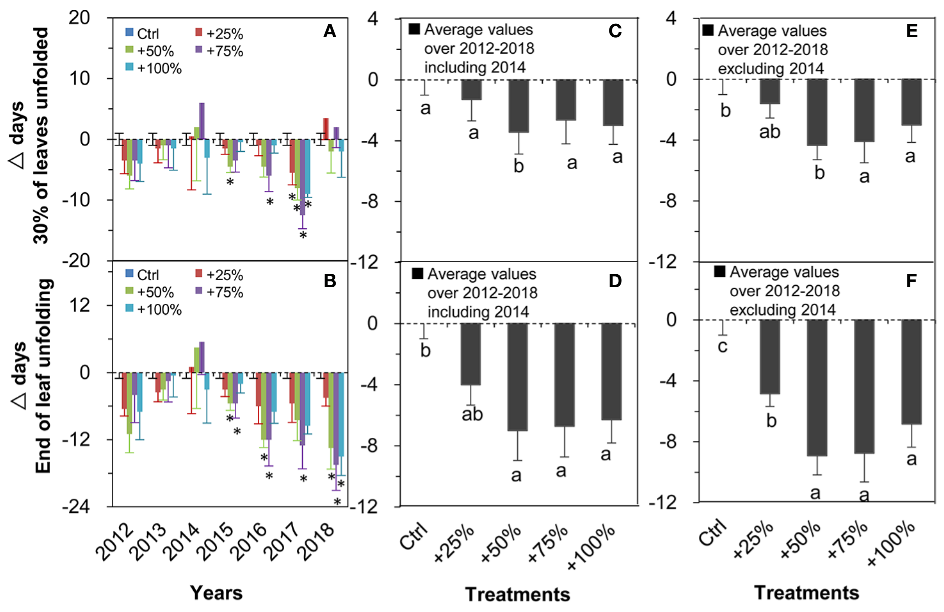

FIGURE 4 | Relative changes (△days; mean ± SE) of the two spring events of *N. tangutorum* after water addition treatments (+25% to +100%) compared with the control (Ctrl). Positive and negative values represent delayed and advanced days, respectively. **A, C, E** represent the 30% of leaves unfolded event. **B, D, F** represent the End of leaf unfolding event. * indicates significant differences at the *P* <0.05 level compared with values in control plots.

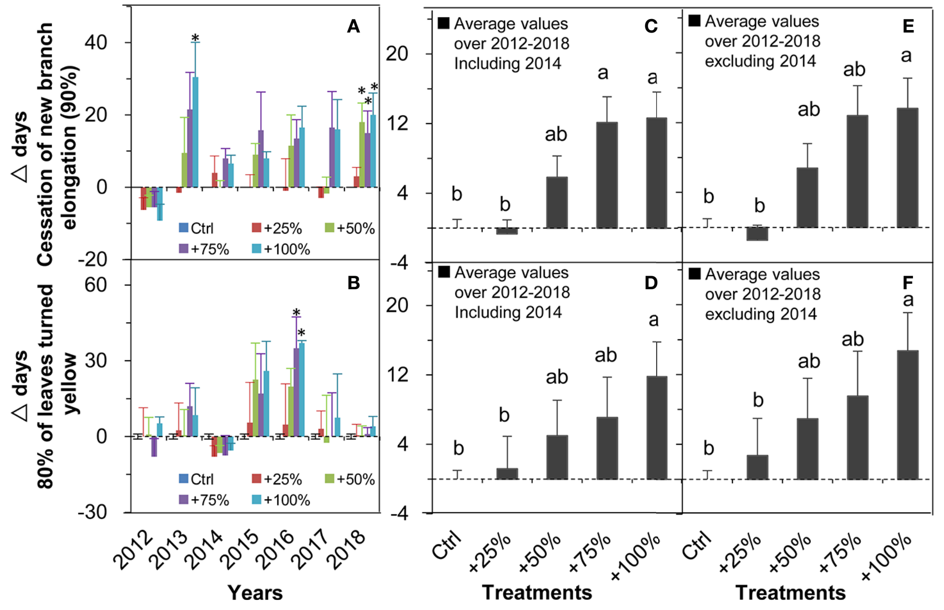

FIGURE 5 | Relative changes (△days; mean ± SE) of the two autumn events of *N. tangutorum* after water addition treatments (+25% to +100%) compared with the control (Ctrl). Positive and negative values represent delayed and advanced days, respectively. **A, C**, and **E** represent the cessation of new branch elongation. **B, D,** and **F** represent 80% of leaves turned yellow. * indicates significant differences at the *P* <0.05 level compared with values in control plots.

+75, and 100% treatments by 2.11, 7.64, 8.00, and 13.68 d, respectively, and a significant difference from control occurred in the +100% treatment (*P* <0.05, **Figure 6B**). On average in six years of the study (2012–2018, excluding 2014), the growing season was prolonged in +25, +50, +75, and 100% treatments by 4.04, 10.50, 12.08, and 16.46 d, respectively, and significant differences from the control occurred in +75 and +100% treatments (all *P* <0.05, **Figure 6C**).

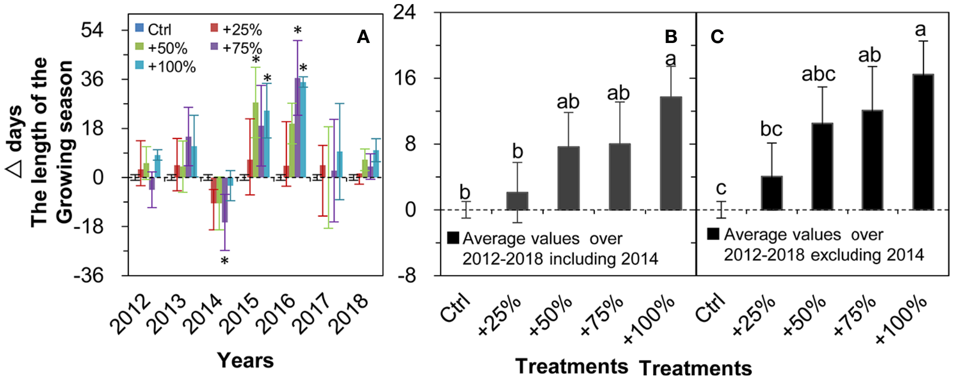

FIGURE 6 | Relative changes (△days; mean ± SE) in the growing season length of *N. tangutorum* after water addition treatments (+25% to +100%) compared with the control (Ctrl). Positive and negative values represent prolonged and shortened days, respectively. **A–C** represent the length of the growing season. * indicates significant differences at the *P* <0.05 level compared with values in control plots.

Relationships Between Phenological Events and Water Addition Amounts

Spring phenology. Significant decreases were observed in quadratic relationships between the timing of the onset of leaf unfolding and soil water content in 2012 and 2017 (all *P* <0.05, **Figure SI3A**), and between the timing of 30% of leaves unfolding (*P* <0.05, **Figure SI3B**), 50% of leaves unfolding (*P* <0.05, **Figure SI3C**), and end of leaf unfolding (*P* <0.05, **Figure SI3D**) and soil water content in 2017.

Autumn phenology. Significant positive linear relationships were found between the timing of cessation of new branch elongation (90%) and amount of water addition on average from 2012–2018 (all *P* <0.05, **Figure 7A**). Among the seven years, significant or marginally significant relationships were determined in five years including 2012 (*P* = 0.07), 2013 (*P* <0.01), 2016 (*P* = 0.02), 2017 (*P* = 0.08), and 2018 (*P* = 0.03) (**Figure 7D**). However, a significantly increased quadratic relationship between 80% of leaves turned yellow and water addition amount was determined on average over the period of 2012–2018 without an apparent threshold (all *P* <0.05, **Figure 7B**). Among the seven years, significant relationships were found in four years, such as linear relationships in 2012 (*P* <0.01) and 2016 (*P* = 0.04), and increasing quadratic relationships in 2017 (*P* <0.01) and 2018 (*P* = 0.09) (**Figure 7E**).

Growing season length. Significant positive linear relationships were found between the growing season length and amount of water addition on average from 2012–2018 (all *P* <0.05, **Figure 7C**). Among the seven years, significant relationships were found in four years, such as linear relationships in 2012 (*P* <0.01), 2014 (*P* = 0.06), and 2017 (*P* = 0.01), and an increasing quadratic relationship in 2016 (*P* = 0.08) (**Figure 7F**).

DISCUSSION

Impact of Water Addition Treatment on Spring Phenology

Han et al. (2015) conducted a two-year water addition experiment in a temperate desert steppe of northwestern China, and found that water addition treatment did not affect the timing of the green-up of dominant species but delayed the senescence time of selected species by 1.93–9.57 days in one year (Han et al., 2015). In the present study, the water addition treatment advanced spring phenology (30% of leaves unfolded, end of leaf unfolding) and delayed the autumn phenology (80% of leaves turned yellow) in the desert shrub *N. tangutorum*. This is partly consistent with the results reported by Han et al., 2015. In general, in the present study, increasing the water addition amount only affected the variation magnitudes of phenological events but not their shifting direction.

Gebauer and Ehleringer (2000) irrigated five dominant cold desert shrub species in southern Utah during different seasons. Based on stable isotope data, they found that all species derived less than 10% of the irrigation water in spring (May) and used most of the water in autumn (September). This result implies that shrubs mainly use soil water in early spring (Golluscio et al., 1998). Compared to control plots, the additionally added water could help to recharge spring soil in water addition treatment plots, and if more water was added, the deeper soil could also be recharged (Ogle and Reynolds, 2004). As a result, in the present study, it is reasonable to attribute the advanced spring phenology to the increased antecedent soil moisture condition caused by the long-term water addition treatment even though the data are not available.

Impact of Water Addition Treatment on Autumn Phenology

Autumn phenology regulates multiple aspects of ecosystem function (e.g., altering carbon/nitrogen cycling and biotic interactions), along with associated feedback to the climate system (Keenan and Richardson, 2015; Xie et al., 2018). Delayed autumn phenology lengthened the duration of the growing season of *N. tangutorum*. According to the growing season length calculation formula (time of 80% of leaves turned yellow minus time of onset of leaf unfolding), it is reasonable to conclude that autumn events contributed more to the lengthening of the growing season than the early spring event in this study. The lengthened growing season possibly implies a longer carbon

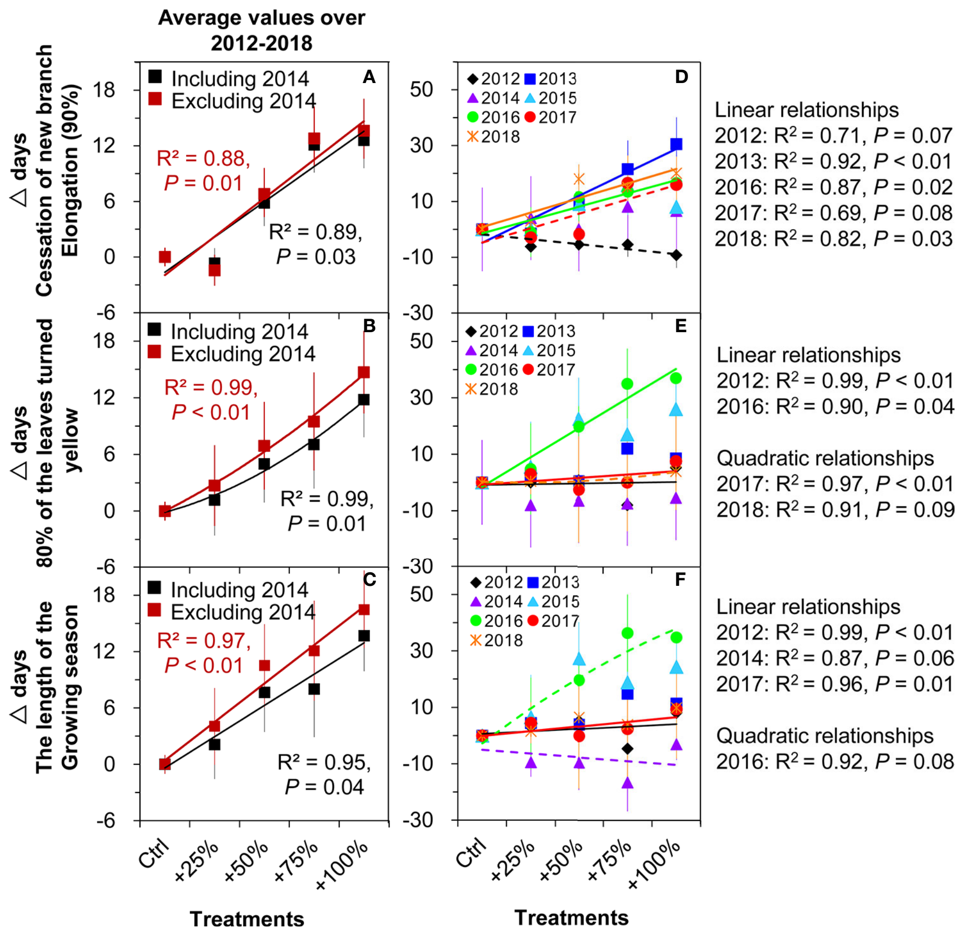

FIGURE 7 | Correlations between the amount of water addition (Ctrl), +25, +50, and +100%) and the changes (△days) of the phenological events of *N. tangutorum*. The solid lines indicate $P \leq 0.05$, and the dashed lines indicate $0.05 < P < 0.1$. **A, D** represent cessation of new branch elongation. **B, E** represent 80% of the leaves turned yellow. **C, F** represent the length of the growing season.

uptake period and increased accumulation of photosynthates (Zhang et al., 2012). This might outweigh the potential risk of frost damage because of cold spells caused by the earlier leaf unfolding of *N. tangutorum* in spring. The delayed cessation of branch elongation in the present study further confirmed this conclusion. The prolonged branch elongation stage may improve carbohydrate allocation to root production, which, in turn, would enhance water and nutrient utilization in *N. tangutorum*.

The start of autumn phenology is a highly regulated process that involves the sequential degradation of macromolecules and the extensive salvage of nutrients (Fracheboud et al., 2009). Environmental factors, such as temperature and photoperiod (Fracheboud et al., 2009), precipitation (Zhou and Jia, 2016), frost and moisture condition (rainfall patterns) (Xie et al., 2015), heat and drought stress (Xie et al., 2018), as well as spring phenology (Fu et al., 2014b; Keenan and Richardson, 2015), were found to significantly affect inter-annual variation in autumn phenology. No significant correlations were found between spring and autumn phenology in the present study. The delayed autumn phenology found in this study may be partly because the water addition treatment enhanced the activities of photosynthetic-related enzymes of *N. tangutorum* (Bao et al., 2017; He et al., 2019) and slowed the speed of chlorophyll degradation during leaf senescence (Fracheboud et al., 2009).

Effects of Year on Plant Phenology

The shifting directions under water addition treatments were consistent for almost all events except for several exceptions, such as 30% of leaves unfolded in 2014 and 2018, the end of leaf unfolding in 2014, and autumn phenology in both 2012 and 2014. GDD, CD, precipitation, and insolation have complex interactions in their effects on spring vegetation green-up phenology (Fu et al., 2012; Fu et al., 2014a; Fu et al., 2015). In 2012, 2013, 2014, 2015, 2016, 2017, and 2018 in control plots, GDD was 429.9 heat unit (HU), 392.4 HU, 303.85 HU, 269.00 HU, 195.40 HU, 241.30 HU, and 279.45 HU, respectively. In 2012, 2012, 2013, 2014, 2015, 2016, 2017, and 2018 in control plots, CD was 112, 111, 112, 107, 108, 99, and 106 d, respectively. GDD and CD in 2012 and 2014 were neither too high nor too low from 2012 cessation 2018, suggesting that heat and chilling requirements were sufficiently met to break dormancy in the two years and should not be related to the exceptions. In addition to temperature, the antecedent soil moisture plays an important role in regulating the effects of water addition on plant phenology as it may either diminish or amplify the effects of water increase on plant growth and photosynthesis (Reynolds et al., 2004). The soil water condition in the early springs of 2014 and 2018 might be dry because the soil water could not have been well recharged by the low autumn rainfalls of the previous years (Mazer et al., 2015; Moore et al., 2015; Cleverly et al., 2016) of only 7.6 mm in 2013 and 22.2 mm in 2017. Moreover, the low spring rainfall in both years (2014, 12.1 mm; 2018, 1.7 mm) further aggravated the situation. Lower water addition treatments (+25, +50, and +75%) were insufficient for the alleviation of natural drought. As a result, the delay of the spring events in +25, +50, and +75% water addition treatment plots in 2014 and 2018 can mainly be attributed to variations in the antecedent soil water availability (Ogle and Reynolds, 2004; Reynolds et al., 2004) and the complicated interactions with environmental factors (Xie et al., 2015). The advanced autumn phenology in 2012 might be related to the water stress caused by several large rainfall events during that year (Xie et al., 2015). However, the advanced autumn phenology in 2014 might be related to an extremely dry summer which resulted in heat and drought stress on plant phenology even in the water addition treatment plots (Xie et al., 2015; Xie et al., 2018). Alternatively, it might be affected by the change of the phenology observer (**Figures 4–7**, these observations have not affected the conclusion of this manuscript; therefore, the data from 2014 were included). It is reasonable to speculate that climate change projections of an earlier and longer growing season in response to the increasing precipitation in desert ecosystems remains elusive for years when plants face consecutively extreme drought and water-logging stress (Adams et al., 2015).

Responses of Phenological Events to Water Addition Treatments

Many studies have shown that water plays an important role in driving plant phenology, such as budburst (Hayden et al., 2010), green-up (Zhang et al., 2005; Liu et al., 2013), flowering (Crimmins et al., 2010; Lesica and Kittelson, 2010; Crimmins et al., 2011; Crimmins et al., 2013; Kigel et al., 2013; Sakkir et al., 2014; Huang et al., 2018), and fruiting (Lotfi and Mohamed, 2006; Galindo et al., 2014), in water-limited ecosystems. However, most of these studies are focused on natural conditions, while the relationship between water increase and phenological events based on water addition experiments has not been explored, nor has the effects of water addition on branch phenology of desert plants. The magnitude of spring phenology advance under water addition treatments might be limited by the acquisition of nutrients by roots, while root growth will be limited by the availability of photosynthates from leaves (Linares et al., 2012). In addition, leaf lifespan (autumn phenology) is associated with nutrient remobilization (especially nitrogen) and storage of photosynthates (Kozlowski and Pallardy, 1997). There are trade-offs between leaf unfolding and carbohydrate allocation to roots, as well as between leaf coloration and nutrient remobilization. That may be why non-linear patterns were found between phenology and water availability. The present study captured the nonlinear nature of plant responses to increased water availability (Ogle and Reynolds, 2004), suggesting that predictions of phenological events in the future should not be based only on linear trends.

CONCLUSION

The findings of the present study suggest that the phenological pattern of the desert shrub species *N. tugutorum* was significantly influenced by increased precipitation and soil water availability. This offers insight on the effects of other environmental factors (in addition to temperature) on phenology. However, the soil water content during non-growing seasons (late autumn, winter, and early spring) was not monitored. Given the important role of the early spring water availability, which affects early spring phenological events, the soil water content should be monitored in all seasons in the future. Since different species have different phenological responses to climate change (Xie et al., 2018), more desert species should be studied in future experiments.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FB contributed to manuscript writing, data analysis, and data collecting. ML, YC, ZX, JL, and BY contributed to the data collecting. QL and BW contributed to manuscript writing and data collecting.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01099/full#supplementary-material

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NDVI Changes Show Warming Increases the Length of the Green Season at Tundra Communities in Northern Alaska: A Fine-Scale Analysis

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A warming Arctic has been associated with increases in aboveground plant biomass, specifically shrubs, and changes in vegetation cover. However, the magnitude and direction of changes in NDVI have not been consistent across different tundra types. Here we examine the responsiveness of fine-scale NDVI values to experimental warming at eight sites in northern Alaska, United States. Warming in our eight sites ranged in duration from 2-23 seasons. Dry, wet and moist tundra communities were monitored for canopy surface temperatures and NDVI in ambient and experimentally-warmed plots at near-daily frequencies during the summer of 2017 to assess the impact of the warming treatment on the magnitude and timing of greening. Experimental warming increased canopy-level surface temperatures across all sites (+0.47 to +3.14°C), with the strongest warming effect occurring during June and July and for the southernmost sites. Green-up was accelerated by warming at six sites, and autumn senescence was delayed at five sites. Warming increased the magnitude of peak NDVI values at five sites, decreased it at one site, and at two sites it did not change. Warming resulted in earlier peak NDVI at three sites and no significant change in the other sites. Shrub and graminoid cover was positively correlated with the magnitude of peak NDVI (r=0.37 to 0.60) while cryptogam influence was mixed. The magnitude and timing of peak NDVI showed considerable variability across sites. Warming extended the duration of the summer green season at most sites due to accelerated greening in the spring and delayed senescence in the autumn. We show that in a warmer Arctic (as simulated by our experiment) the timing and total period of carbon gain may change. Our results suggest these changes are dependent on community composition and abundance of specific growth forms and therefore will likely impact net primary productivity and trophic interactions.

Keywords: Arctic shrubs, Normalized Difference Vegetation Index, climate change, seasonality, phenology, International Tundra Experiment, experimental warming

INTRODUCTION

Warming in the Arctic has been accelerating in recent decades, and the Arctic is experiencing more pronounced temperature increases compared with lower latitudes (ACIA, 2005; Serreze et al., 2009; Stocker et al., 2013). Accompanying increased temperatures are decreases in snow cover and increases in growing season length (Callaghan et al., 2011; Liston and Hiemstra, 2011). Adaptations (e.g. slow growth) to the inherently harsh growing conditions of the Arctic have caused plants to be sensitive to small variations in temperature that can be reflected in interannual variation in growth and in phenology (Callaghan et al., 1999). Measurable changes in Arctic plant growth and community dominance in response to warming have been well documented (Arft et al., 1999; Oberbauer et al., 2013; Khorsand Rosa et al., 2015; Bjorkman et al., 2015; Elmendorf et al., 2015; Bjorkman et al., 2018). However, these responses have not been uniform across the Arctic and are likely associated with local climate conditions and ecohydrology including snow and its role in growing season length and tundra plant ecophysiology (Elmendorf et al., 2012a; May et al., 2017; Prevéy et al., 2017; Jespersen et al., 2018).

In response to changes in environmental conditions over recent decades, an expansion northward and within local landscapes of shrubs and graminoids has been documented in the Arctic (Tape et al., 2006; Myers-Smith et al., 2011; Elmendorf et al., 2012b; Tape et al., 2012; Myers-Smith et al., 2015). Documenting and understanding shifts in community dominance and ecosystem function are important to accurately predict the trajectories and impact of climate change on Arctic systems. This includes how these changes are manifested in the seasonality of growth (Welker et al., 1997; Pearson et al., 2013) and the degree to which the abundance of vegetation may be expressed in spectral properties that can be measured at plot and landscape scales (Reidel et al., 2005; Raynolds et al., 2008; Gamon et al., 2013).

The immense area and remoteness of the region have made remote-sensing tools indispensable in monitoring the effects of climate change in the region (Kerr and Ostrovsky, 2003; Stow et al., 2007; Walker et al., 2012; Ju and Masek, 2016). Despite their vast area, Arctic plant communities are spatially heterogeneous at very small scales (m), due in part to differences in hydrology as result of small variation in topography (cm, Billings and Bliss, 1959; Evans et al., 1989; Walker et al., 1989; Ostendorf and Reynolds, 1998, Lara et al., 2018; Shaefer and Messier, 1995). Normalized Difference Vegetation Index (NDVI), developed by Kriegler et al. (1969), has proven to be a valuable and widely-used tool in monitoring productivity and community dominance changes across the Arctic (Reidel et al., 2005; Raynolds et al., 2008; Berner et al., 2018). Studies using remotely-sensed NDVI data have demonstrated a trend toward a greening Arctic over recent decades (Jia et al., 2003; Stow et al., 2007; Bhatt et al., 2013; Keenan and Riley, 2018), although some regions have shown browning (Phoenix and Bjerke, 2016; Zhang et al., 2017; Myers-Smith et al., 2020). In spite of the time and resource saving advantages of remotely-sensed monitoring tools at large spatial scales, the high spatial heterogeneity of Arctic tundra complicates our ability to understand and predict how specific communities respond to varying environmental conditions and across latitudinal gradients (Verbyla, 2008; Gamon et al., 2013; Guay et al., 2014; Healey et al., 2014; Reichle et al., 2018). Linking vegetation sampling with measurements of NDVI at small spatial scales should be a useful approach to improve understanding of the complexity of observed large-scale NDVI changes over time. Previous studies have focused on NDVI changes over time with regards to ambient conditions over large spatial scales (Blok et al., 2011; Gamon et al., 2013; Pattison et al., 2015; Ju and Masek, 2016) or with warming focused on a specific community type (Boelman et al., 2005). The approach that we are implementing here, however, could also be very useful when applied to ecosystem responses to experimental warming across a variety of community types and latitudes at fine temporal and spatial scales.

Species responses to experimental warming have been examined in a variety of community types across the tundra biome using traditional manual measurement approaches (Chapin and Shaver, 1985; Wookey et al., 1993; Parsons et al., 1994; Arft et al., 1999). The International Tundra Experiment (ITEX) was established in 1990 as a circumpolar network of researchers to quantify plant, community, and ecosystem process changes in the Arctic in response to experimental warming (Webber and Walker, 1991; Molau and Molgaard, 1996; Henry and Molau, 1997; Welker et al., 1997). The use of open-top chambers (OTCs) in standardized, plot-scale passive warming in tundra plant communities across polar and alpine regions has been an extremely powerful approach to understand tundra responses to both experimental and background climate warming (Arft et al., 1999; Elmendorf et al., 2012a; Elmendorf et al., 2012b; Oberbauer et al., 2013; Leffler et al., 2016; Jespersen et al., 2018). The ITEX network has shown that experimental warming causes increased growth and early phenological development in the Arctic (Wookey et al., 1993; Arft et al., 1999; Barrett et al., 2015; Bjorkman et al., 2015; Prevéy et al., 2017) as well as shifts in community dominance (Elmendorf et al., 2012a; Elmendorf et al., 2012b; Hollister et al., 2015), although the magnitude of responses varies greatly across sites. Studies of community diversity and dominance have shown that warming temperatures favor tall-statured species, such as shrubs and graminoids, and decrease cover of some cryptogams (Elmendorf et al., 2012b; Hollister et al., 2015). However, how these vegetation changes correspond to changes in timing and magnitude of NDVI at fine-scales are uncertain (Huemmrich et al., 2013; Zesati, 2017), especially as a result of experimental warming.

Here we present the results from a study of canopy-level surface temperature and NDVI across the growing season at eight ITEX sites in northern Alaska for which we had detailed plot-level measurements of plant community composition. The canopy-level surface temperature measurements allowed testing of the effectiveness of the warming treatment. The use of an LED-illuminated NDVI sensor provided high precision data allowing documentation of daily changes in NDVI independent of sky conditions. The overarching goals of this study were to: 1) test the effect of experimental warming on spring green-up and green season length, 2) identify when NDVI is near or at peak values in different tundra communities, and 3) investigate how plant community composition influences the magnitude and timing of peak NDVI values. We predicted that experimental warming increases peak NDVI values, a proxy for peak productivity, and extend the green season length at all sites. We also predicted that communities dominated by shrubs and graminoids would exhibit greater increases in peak NDVI values with warming due to increased aboveground vegetation, compared to communities with greater cryptogam dominance.

METHODS AND MATERIALS

Site Description

Eight study sites, a paired wet/moist and dry site, were arrayed across four regions of the North Slope of Alaska, USA. The regions provide a latitudinal gradient spanning Low to High Arctic from the northern foothills of the Brooks Range to the northern Alaska coast along the Arctic Ocean (Welker et al., 2005, **Figure 1**). Toolik Lake and Imnavait Creek are both inland, foothill tundra, Atqasuk is inland, positioned at the transition between foothill and coastal plain tundra, and Utqiaġvik is coastal plain tundra. Study sites at Utqiaġvik (formerly Barrow), Atqasuk, Imnavait Creek, and Toolik Lake, Alaska each consisted of a paired dry site and a wet or moist community site. Sites at Utqiaġvik, Atqasuk, and Toolik Lake

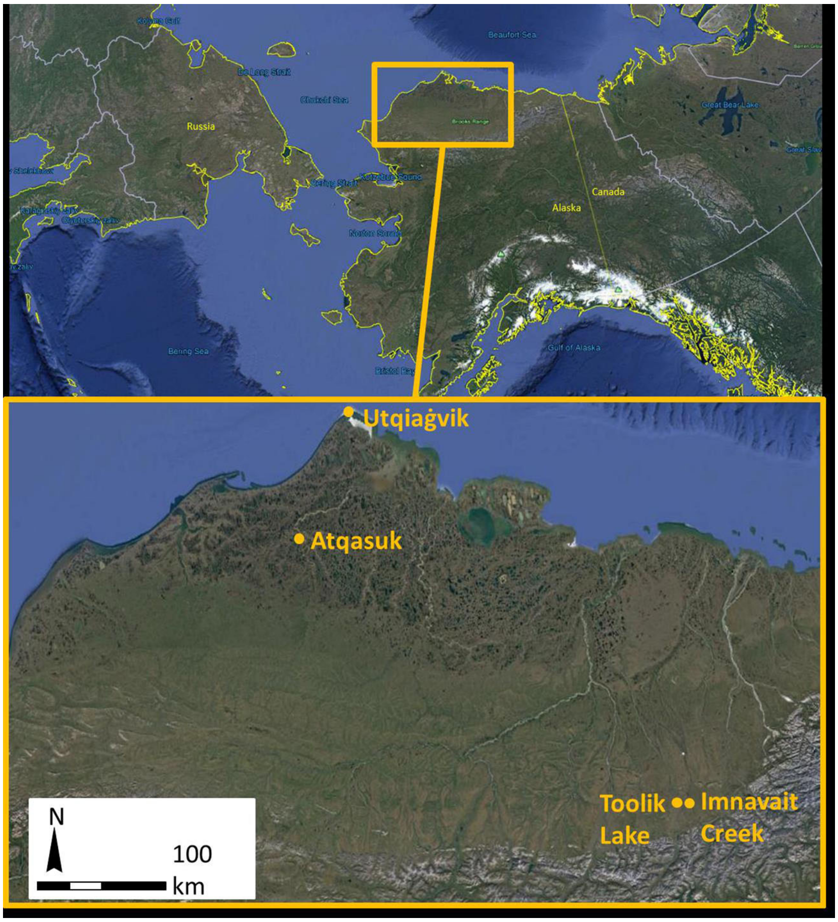

FIGURE 1 | Locations of ITEX sites on the North Slope, Alaska, United States.

were established between 1994 and 1997 (20-23 seasons of warming), and Imnavait Creek sites were established in 2016 (2 seasons of warming; Hollister et al., 2005; Walker et al., 2006). The Utqiaġvik dry site (71˚18’48.46”N, 156˚35’5.67”W) is located on an old beach ridge above a drained thaw-lake bed and is dominated by short-statured shrubs, graminoids, and lichens (**Table 1**). The Utqiaġvik wet site (71˚18’40.98”N, 156˚35’53.70”W) is located on a frequently inundated slope between a beach ridge and drained thaw-lake basin and is dominated by graminoids and bryophytes. The Atqasuk dry site (70˚27’13.81”N, 157˚24’25.37”W) is located on a well-drained ridge and is dominated by short-statured shrubs and lichens. The Atqasuk wet site (70˚27’11.33”N, 157˚23’59.61”W) is located in a frequently inundated meadow and is dominated by deciduous shrubs, graminoids, and bryophytes. The Imnavait Creek dry site (68˚36’58.37”N, 149˚18’21.49”W) is located on a well-drained slope and is dominated by short-statured shrubs and lichens. The Imnavait Creek wet site (68°36’ 56.25” N, 149˚18’ 21.17”W) is located on a slope within the head of a water track feature and is dominated by shrubs, graminoids, and bryophytes. The Toolik Lake dry site (68°37’19.04”N, 149° 35’53.71”W) is located on a well-drained ridge and is dominated by short-statured shrubs and lichen. The Toolik Lake moist site (68°37’12.33”N, 149°36’12.04”W) is located on a partially drained, gradual slope with acidic soils and dominated by graminoids (mainly *Eriophorum vaginatum* tussocks), shrubs, and bryophytes. The Imnavait Creek and Toolik Lake sites are at very similar latitudes, but Imnavait Creek is at higher elevations by almost 200 m (927 vs 736 m a.s.l.) and therefore is generally cooler. Warmed and control plots have similar species present in the communities, however, differ in abundances (Hollister et al., 2015). The number of sampled control and warmed plots, as well as dominant species varied among sites (**Table 1**). Plots were not paired and were randomly arranged across the landscape of each community type.

Treatment and Measurement

Control and warmed plots were ~1m^2 in size across all sites. Warming was achieved using hexagonal open-top chambers (OTCs) that were installed shortly after snowmelt and removed at the end of the growing season each year (Marion et al., 1997). The OTCs were constructed of Sun-Lite® HPTM fiberglass (Kalwall Corporation, Manchester, New Hampshire, USA) and installed according to the guidelines outlined in the ITEX manual (Molau and Mølgaard, 1996). Open-top chambers have been shown to warm surface air temperatures by an average of 0.6 to 2.2°C over the summer which is analogous to climate change projections (Marion et al., 1997; Hollister et al., 2006). A non-destructive point frame method was performed for vegetation community sampling according to Walker (1996). The point frame method consists of a 75 cm x 75 cm 100 point frame with measurement points every 7 cm that was leveled above the canopy. Permanent markers were used to ensure the frame was installed in the same position and orientation at each sampling. At each point a graduated ruler was lowered to the vegetation where species, live/dead status, and height were measured at the top (canopy level) and bottom (understory) contact point. All Utqiaġvik and Atqasuk sites were sampled in

TABLE 1 | Number of plots for each treatment (warmed or control) within each study site and dominant species present.

	Utqiaġvik	**Atqasuk**	**Imnavait Creek**	**Toolik Lake**
	Dry	**Dry**	**Dry**	**Dry**
Number of Plots	19	19	8	10
Year Established	1994	1996	2016	1995
Latitude	71°18’48.46" N	70°27’13.81" N	68°36’58.37" N	68°37’19.04" N
Longitude	156°35’5.67" W	157°24’25.37" W	149°18’21.49" W	149°35’53.71" W
Landscape Orientation	Drained thaw lake beach ridge	Frequently inundated meadow	Well-drained slope	Well-drained ridge
Dominant Species	*Luzula confusa*	*Ledum palustre*	*Arctostophylos alpina*	*Arctostophylos alpina*
	Luzula arctica	*Vaccinium vitis-idaea*	*Vaccinium vitis-idaea*	*Vaccinium vitis-idaea*
	Salix rotundifolia	*Cassiope tetragona*	*Cassiope tetragona*	*Cassiope tetragona*
	Vaccinium vitis-idaea	*Carex bigelowii*	*Betula nana*	*Betula nana*
	Pedicularis kaneii	*Hierchloe alpina*	*Hierochloe alpina*	*Carex bigelowii*
	Lichen	Lichen	Lichen	Lichen
	Wet	Wet	Wet	Moist
Number of Plots	19	19	8	10
Year Established	1995	1996	2016	1995
Latitude	71°18’40.98" N	70°27’11.33" N	68°36’56.25" N	68°37’12.33" N
Longitude	156°35’53.70" W	157°23’59.61" W	149°18’21.17" W	149°36’12.04" W
Landscape Orientation	Frequently inundate beach slope	Well-drained ridge	Head of a water-tract feature	Gradual acidic slope
Dominant Species	*Carex aquatilis-stans*	*Carex aquatilis*	*Eriophorum vaginatum*	*Eriophorum vaginatum*
	Dupontia fisherii	*Eriophorum angustifolium*	*Salix pulchra*	*Salix pulchra*
	Eriophorum angustifolium	*Eriophorum russeolum*	*Betula nana*	*Betula nana*
	Eriophorum russeolum	*Salix pulchra*	*Ledum palustre*	*Ledum palustre*
	Pleurocarpus moss	*Salix polaris*	*Carex bigelowii*	*Carex bigelowii*
	Sphagnum spp.	Pleurocarpus moss	*Cassiope tetragona*	*Vaccinium vitis-idaea*
		Sphagnum spp.	Pleurocarpus moss	Pleurocarpus moss
			Sphagnum spp.	Sphagnum spp.

2017, Imnavait Creek sites were sampled in 2016, and Toolik Lake sites were sampled in 2014. All sites were included for analysis despite the asynchrony of vegetation sampling because samplings are conducted independently for the larger research project of vegetation monitoring. All point frame samplings took place during peak growing season (mid- to late July; for details see Hollister et al., 2015 and Hobbie et al., 2017).

During the growing season (June-August) of 2017, canopy surface temperatures and NDVI values were recorded at each plot near solar noon (1:00-3:00 PM AKST) on an almost-daily basis (every 1-2 days). Canopy surface temperature was measured using Fluke 62 Max® infrared thermometers (Fluke Corporation, Everett, Washington, United States). As a result of a logistic issue, canopy surface temperature measurements for the month of June at the Utqiaġvik and Atqasuk sites were replaced with air temperatures recorded at a height of ~10cm using thermocouples contained in six plate Gill radiation shields at 1300 h (Campbell Scientific, Inc., Logan, Utah, USA; Robert Hollister, unpublished data). NDVI was measured using a handheld, LED-illuminated NDVI reader (GreenSeeker®, Trimble Navigation Ltd., Sunnyvale, CA, USA) at a height of approximately 50cm held perpendicular to the ground. Care was taken to ensure that the footprint of both GreenSeeker® and the infrared thermometer were similar on each plot using the GreenSeeker® LEDs and the laser on the infrared thermometer. The LED illumination of the GreenSeeker® is brighter than ambient light and thus minimizes measurement differences resulting from inconsistent sky conditions and uneven solar illumination of the surface. The GreenSeeker® uses the normalized difference between near-infrared (R_{774}) and red (R_{656}) light wavelength reflectance to calculate NDVI [NDVI = $(R_{774} - R_{656})/(R_{774} + R_{656})$].

Data Analysis

Daily mean canopy level surface temperatures for control and warmed plots were aggregated by month (June, July, and August) and combined into a full-season value in order to better compare whole month values between sites. Temperatures in each treatment were compared for each month and full season using repeated measure analysis of variance (RANOVA). The maximum seasonal NDVI value of each site was used to characterize peak NDVI, length of green season, and spring green-up. Peak NDVI was defined as the 1st day that NDVI values were above 95% of the maximum seasonal NDVI value for the season. We chose 95% of the maximum because there was a clear plateau during the summer around which NDVI values increased in spring or declined in fall. Daily changes in NDVI values have been shown to correspond to temperatures experienced within the prior 3 days (May et al., 2017), as a result, the values fluctuate a small amount throughout the season. The length of the green season was defined as the time period when NDVI values remained above 95% of the maximum seasonal NDVI value until senescence caused NDVI values to decrease below 95%. Spring green-up was defined as the time period when NDVI values were increasing between 80% and 95% of the maximum seasonal NDVI value; we recognize a longer window would be ideal, but we were logistically constrained by the need to compare across all sites and the length of the field season. Warming effects on the magnitude and the timing (day of year) of peak NDVI were compared for each site using analysis of variance (ANOVA); the length of the green season and the daily change in NDVI (calculated by average increase in NDVI value) during spring green-up were also compared for each site using ANOVA. The relationship between plant growth form percent cover and the timing and magnitude of peak NDVI was tested using a series of Pearson Correlations. All statistical tests were performed using the R statistical environment (R Core Team, 2018, Vienna, Austria).

RESULTS

Warming Effects on Surface Temperature

Full-season average canopy surface temperatures varied along the latitudinal gradient of study sites, with the effectiveness of the OTC warming greater at the southernmost sites compared to the

TABLE 2 | Average canopy surface temperature for control and experimentally-(OTC) warmed plots at all sites during the months of June, July, August, and all months combined.

Community	June			July			August			Full Season		
	Control	Warmed	p-value	Control	Warmed	p-value	Control	Warmed	p-value	Control	Warmed	p-value
Utqiaġvik												
Dry	*0.74*	*0.01*	0.966	**4.69**	**0.83**	**0.012**	4.13	0.55	0.131	3.19	0.47	0.136
Wet	***-0.05***	***0.63***	***0.007***	**4.38**	**2.51**	**<0.001**	**3.72**	**0.96**	**0.015**	**2.72**	**1.37**	**<0.001**
Atqasuk												
Dry	*5.88*	*0.75*	*0.504*	**11.08**	**1.49**	**0.016**	7.65	0.07	0.934	8.14	0.77	0.153
Wet	***3.03***	***1.39***	***0.034***	**6.56**	**1.83**	**<0.001**	5.62	1.2	0.068	**5.07**	**1.47**	**<0.001**
Imnavait Creek												
Dry	**14.63**	**3.06**	**<0.001**	**20.51**	**2.69**	**0.047**	14.62	2.22	0.285	**16.61**	**2.65**	**0.023**
Wet	**13.71**	**3.79**	**0.007**	21.58	1.85	0.354	15.01	3.15	0.101	**16.76**	**2.93**	**0.031**
Toolik Lake												
Dry	14.16	2.22	0.163	20.18	1.94	0.136	14.13	1.67	0.375	16.33	1.94	0.081
Moist	**14.63**	**1.83**	**0.005**	20.67	2.28	0.368	15.28	3.23	0.097	**16.73**	**3.14**	**0.041**

Warmed temperatures are presented as the average increase in temperature over the control plot values. Italicized values indicate shielded air temperatures collected at 10cm height in Utqiaġvik and Atqasuk in June (used a surrogate for missing surface temperatures). Bold indicates a statistically significant difference between control and warmed plot values (based on ANOVA, $p < 0.05$).

northernmost sites (**Table 2**). Warming effectiveness was variable across sites and among months during the summer. Effectiveness of OTC warming in the dry sites was lower (+0.47 to +2.65°C) compared to the wet and moist sites (+1.37 to +3.14°C). Warming treatment significantly increased June temperatures at most sites with the exception of the dry sites at Utqiaġvik (+0.01°C p=0.966), Atqasuk (+0.75°C p=0.504), and Toolik Lake (+.2.22°C p=0.163). In July, OTC warming increased canopy surface temperatures at all sites in the northernmost regions of Utqiaġvik (dry +0.83°C p=0.012 and wet +2.51°C p<0.001) and Atqasuk (dry +1.49°C p=0.016 and wet +1.83°C p<0.001). In July, the only site within the southernmost regions that showed significant increases in canopy surface temperature with OTC warming was Imnavait Creek dry site (+2.69°C p=0.047). In August, only the Utqiaġvik wet site (+0.96°C p=0.015) showed significant increases in canopy surface temperature with OTC warming.

OTC Warming Effects on NDVI

Warming treatment significantly increased the daily change in NDVI during spring green-up at all sites except Atqasuk dry and Imnavait wet (**Figure 2**). The sites that showed the largest increase were Imnavait Creek dry (+0.003 NDVI day^{-1} p=0.008) and Toolik Lake moist (+0.006 NDVI day^{-1} p<0.001). Warming treatment increased the magnitude of peak NDVI values at most sites; however, OTC warming had mixed effects on the timing of peak NDVI (**Figure 3**). Earlier peak NDVI occurred at the Atqasuk dry (-7 days p=0.012), Imnavait Creek dry (-9 days p=0.007), and Toolik Lake moist (-22 days p<0.001) sites with no significant change at the remaining sites (average differences of -2 days to +3 days).

Warming treatment increased the magnitude of peak NDVI the most at the Toolik Lake dry (+0.065 NDVI p<0.001) and moist (+0.103 NDVI p<0.001) sites, followed by the dry (+0.050 NDVI p=0.007) and wet (+0.036 NDVI p=0.029) sites at Imnavait Creek (**Figure 3**). Warming treatment at the Atqasuk sites showed mixed results with the peak NDVI at the wet site increasing (+0.029 NDVI p=0.048) while the dry site remaining unchanged (+0.006 NDVI p=0.561). The Utqiaġvik wet site peak NDVI value did not change with OTC warming (+0.005 NDVI p=0.582), and the dry site was the only site that showed a decrease in peak NDVI with OTC warming (-0.043 NDVI p=0.012).

On average, OTC warming treatment increased the length of green season, although the magnitude of the effects varied among sites (**Figure 4**). Imnavait Creek dry and Toolik Lake moist sites showed the largest increases in green season length (+13 days p<0.001 and +28 days p<0.001 respectively), while the Imnavait Creek wet and Toolik Lake dry sites showed no change (+1 day p=0.613 and +0 day p=0.822 respectively). Both the Atqasuk dry (+8 days p<0.001) and wet (+7 days p<0.001) sites increased in green season length with OTC warming. The effects of warming on green season length was mixed at the Utqiaġvik sites with the dry increasing in length (+6 days p=0.008) and the wet remained unchanged (+3 days p=0.211).

NDVI and Cover of Plant Growth Forms

In general, bryophytes and graminoids were associated with a delayed peak in NDVI and shrubs and lichens were associated with an earlier peak in NDVI (**Table 3**). The cover of bryophytes was positively correlated with the timing of peak NDVI at two sites (Utqiaġvik dry r= 0.39 p=0.032 and Atqasuk dry r=0.36

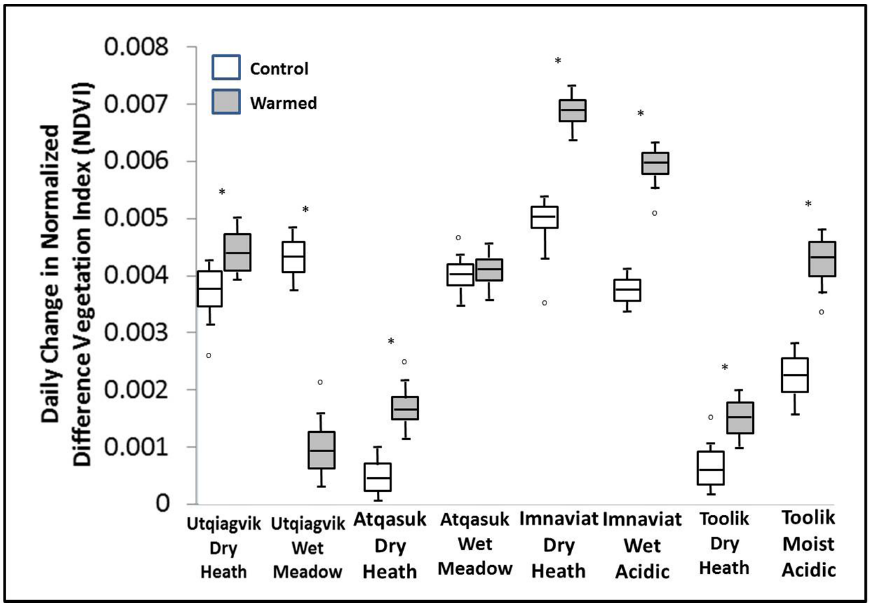

FIGURE 2 | Average daily change in NDVI during spring green-up in control (open) and warmed (filled) plots at the eight study sites. A * denotes a statistically significant difference between treatments at the site (based on ANOVA, p < 0.05).

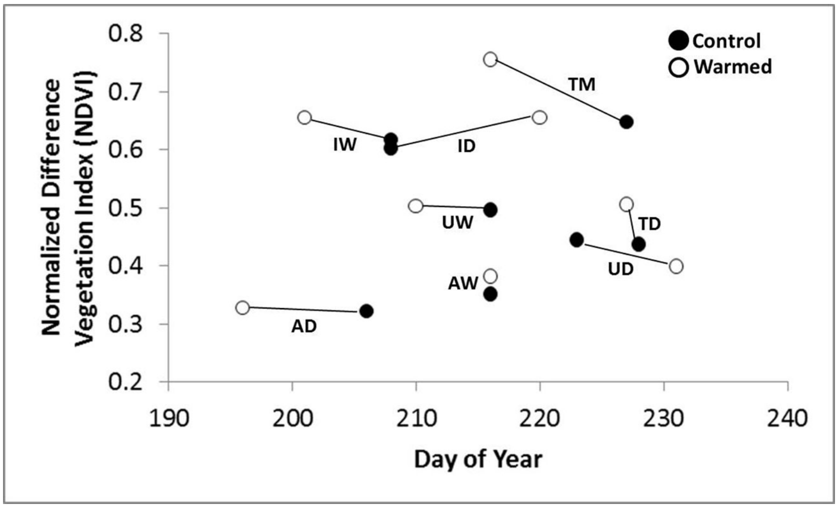

FIGURE 3 | The average peak NDVI magnitude and day of occurrence for control (filled circle) and warmed (open circle) plots at the eight study sites (UD- Utqiaġvik dry, UW- Utqiaġvik wet, AD-Atqasuk dry, AW-Atqasuk wet, ID-Imnavait Creek dry, IW-Imnavait Creek wet, TD-Toolik Lake dry, TM-Toolik Lake moist).

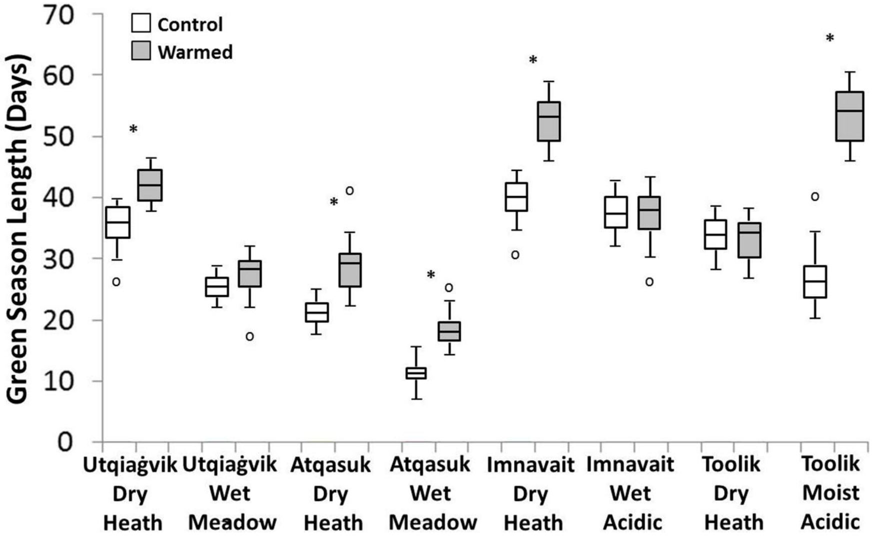

FIGURE 4 | Green season length in control (open) and warmed (filled) plots at the eight study sites. A * denotes a statistically significant difference between treatments at the site (based on ANOVA, $p < 0.05$).

p=0.047), while lichen cover was negatively correlated with the timing of peak NDVI at the Imnavait Creek dry site (r=-0.36 p=0.048). Forbs were negatively correlated with the timing of peak NDVI at the Utqiaġvik wet site (r=-0.51 p=0.010). Graminoids were positively correlated with the timing of peak NDVI at both the Atqasuk (r=0.54 p=0.005) and Imnavait Creek (r=0.45 p=0.026) wet sites. Shrubs were negatively correlated with the timing of peak NDVI; the relationship was significant at two sites for deciduous shrubs (Imnavait Creek dry r=-0.42, p=0.037 and Toolik Lake moist r=-0.54, p=0.006) and at four sites for evergreen shrubs (Utqiaġvik dry r=-0.46, p=0.023, Imnavait Creek dry r=-0.44, p0.035, Imnavait Creek wet r=-0.43, p=0.037, and Toolik Lake dry r=-0.48, p=0.026).

The relationship between plant growth form cover and magnitude of peak NDVI also varied by site, with graminoids and shrubs showing the strongest correlations (**Table 3**). Bryophytes were not correlated with the magnitude of peak NDVI at any of the study sites. Lichens were negatively correlated with the magnitude of peak NDVI at the Atqasuk dry site (r=-0.44 p=0.032). Forbs were negatively correlated with

TABLE 3 | Pearson correlations (r) between the cover of each plant growth form and the magnitude (Value) or timing (day of the year; DOY) of peak NDVI at the eight study sites.

Site	Bryophyte				Lichen				Forb				Graminoid				Deciduous Shrub				Evergreen Shrub			
			Peak NDVI				Peak NDVI				Peak NDVI				Peak NDVI				Peak NDVI				Peak NDVI	
	Cover	(SD)	Value	DOY	Cover	(SD)	Value	DOY	Cover	(SD)	Value	DOY	Cover	(SD)	Value	DOY	Cover	(SD)	Value	DOY	Cover	(SD)	Value	DOY
Utqiaġvik																								
Dry	5.0	(5.3)	0.02	**0.39**	11.9	(9.8)	-0.16	0.35	3.8	(3.7)	-0.09	0.11	22.2	(13.7)	-0.14	0.08	8.8	(3.9)	**0.58**	-0.17	19.8	(10.5)	**0.42**	**-0.46**
Wet	3.1	(3.0)	0.20	0.22	1.8	(1.0)	-0.02	-0.20	4.0	(3.5)	-0.26	**-0.51**	45.6	(12.5)	**0.54**	0.24	1.6	(4.2)	0.19	0.22	0.0	(0)	–	–
Atqasuk																								
Dry	4.0	(3.7)	-0.01	0.08	14.2	(5.8)	**-0.44**	0.23	1.2	(1.9)	**-0.50**	-0.36	14.2	(12.2)	**0.41**	0.08	1.3	(2.3)	0.20	0.20	20.9	(7.4)	0.26	-0.29
Wet	3.9	(3.1)	-0.17	**0.36**	0.0	(0)	–	–	0.0	(0.2)	0.01	-0.17	58.1	(7.1)	**0.43**	**0.54**	4.0	(3.2)	**0.60**	0.20	0.0	(0)	–	–
Imnavait Creek																								
Dry	4.0	(3.3)	0.15	0.27	12.1	(9.6)	-0.35	**-0.36**	2.2	(1.8)	0.13	0.10	17.8	(11.2)	0.24	-0.02	9.0	(6.5)	**0.37**	**-0.42**	25.1	(12.1)	**0.51**	**-0.44**
Wet	5.6	(4.3)	0.13	0.28	2.2	(1.9)	-0.04	-0.13	2.3	(0.8)	0.14	0.33	41.2	(15.6)	**0.44**	**0.45**	11.6	(7.4)	0.30	-0.31	6.7	(4.2)	**0.46**	**-0.43**
Toolik Lake																								
Dry	2.3	(1.9)	0.11	0.14	26.3	(8.3)	-0.24	-0.26	9.2	(5.4)	**0.36**	0.13	7.6	(5.8)	0.20	-0.01	12.1	(8.1)	0.24	-0.25	17.2	(10.1)	**0.52**	**-0.48**
Moist	6.7	(4.1)	0.24	0.18	1.2	(0.9)	-0.02	-0.11	0.9	(0.6)	0.20	0.19	14.0	(9.7)	**0.39**	0.26	42.0	(18.1)	**0.38**	**-0.54**	8.7	(4.9)	**0.38**	-0.24

The average plot cover (Cover; measured at the top of the canopy) and the standard deviation (SD) among plots are provided. Bold indicates a statistically significant Pearson correlation value ($p < 0.05$).

the magnitude of peak NDVI at the Atqasuk dry site (r=-0.50 p=0.013) and positively correlated at the Toolik Lake dry site (r=0.36 p=0.048). Graminoids were positively correlated with the magnitude of peak NDVI at five of the study sites (Utqiaġvik dry r=0.54 p=0.006, Atqasuk dry r=0.41 p=0.038, Atqasuk wet r=0.43 p=0.029, Imnavait Creek wet r=0.44 p=0.34, and Toolik Lake moist r=0.39 p=0.036). Deciduous shrubs were positively correlated with the magnitude of peak NDVI at four of the study sites (Utqiaġvik dry r=0.58 p=0.002, Atqasuk wet r=0.60 p=0.002, Imnavait Creek dry r=0.37 p=0.039, and Toolik Lake moist r=0.38 p=0.037). Evergreen shrubs were positively correlated with the magnitude of peak NDVI at five of the study sites (Utqiaġvik dry r=0.42 p=0.040, Imnavait Creek dry r=0.51 p=0.012, Imnavait Creek wet r=0.46 p=0.028, Toolik Lake dry r=0.52 p=0.008, and Toolik Lake moist r=0.38 p=0.045).

DISCUSSION

As anticipated, experimental warming increased canopy surface temperatures across all eight study sites. However, the magnitude of the warming effect was season and site dependent, with not all sites and months warming significantly. Full-season warming values from OTCs ranged between +0.47 to +3.14°C, which were similar to those previously reported (Hollister et al., 2006; Bokhorst et al., 2013). Contributing factors to variation in experimental warming among sites include latitude, sky conditions, sun angle, and wind conditions, with the southernmost sites demonstrating the most pronounced warming (Marion et al., 1997; Bokhorst et al., 2013). Higher sun angle and resulting increased solar radiation may also explain the increased warming effect during June and July at most sites. Late in the growing season, specifically after the first sunset, OTC effects decrease as the daily incoming solar radiation begins to decrease.

Near-daily measures of plot level NDVI showed experimental warming increased peak NDVI at most sites, but as might be expected given the differences in the effectiveness of OTC warming, the warming response of peak NDVI varied by community type and across the latitudinal gradient. The southernmost study sites demonstrated the largest increases in peak NDVI despite the difference in duration of warming treatment between Toolik Lake (23 seasons) and Imnavait Creek (two seasons). However, the differences in the NDVI response may be a result of effects of size of the temperature increase. The enhanced NDVI values were present in all Toolik Lake and Imnavait Creek plots, suggesting that species differences (e.g. *Eriophorum vaginatum* or *Arctostaphylos alpina* dominance), and the ability of these species to respond to warming may be driving factors in NDVI increases (Boelman et al., 2003; Walker et al., 2003). Moist or wet sites were more responsive to warming than their complimentary dry site from the same region. Differences in warming responses among these community types may be a result of community attributes, such as differences in productivity (La Puma et al., 2007), aboveground biomass (Boelman et al., 2003; Hudson and Henry, 2009; Hollister et al., 2015), and duration of warming treatments. Warmer temperatures may also contribute to increased soil moisture loss which is more likely to be important in dry communities. Studies in alpine regions have reported delays in phenological events and shortened growing season in response to warming-induced drying (Jonas et al., 2008; Dorji et al., 2013).

Peak NDVI was generally positively correlated with graminoid and shrub (both deciduous and evergreen) cover. The influence of graminoids and shrubs were greater in wet or moist communities due to the inherently high graminoid and shrub cover in these ecosystems compared to the more sparsely vegetated dry tundra (Welker et al., 1997; Welker et al., 1999; Hollister et al., 2015). The positive influence of graminoids and shrubs (i.e. relatively taller-statured plants) was in contrast to the marginal effects of lichen and bryophyte growth forms. Lichens and bryophytes may have their signal marginalized by the color differences inherent in each of the species. This disparity of growth form influence on peak NDVI supports the findings of previous studies that found positive relationships between vegetation biomass and NDVI values (Boelman et al., 2003; Riedel et al., 2005; Raynolds et al., 2008; Berner et al., 2018). Increased graminoid and deciduous shrub cover also generally shifted the timing of peak NDVI to later in the growing season, as these growth forms leaf out later and continue to increase aboveground biomass as the season progresses. Evergreen shrubs were associated with earlier peak NDVI as well, and this result reflects their leaf longevity (Karlsson, 1992).

Accelerated spring green-up and delayed senescence of tundra resulting in increased season length in response to warming was a postulated outcome of climate change proposed in a conceptual model early in the ITEX program (Welker et al., 1997) and are consistent with previous findings (Arft et al., 1999; Aerts et al., 2006; May et al., 2017). Increases in spring phenological progression and delayed senescence as result of increased temperature have been well documented and could lengthen the green season in the Arctic (Wookey et al., 1993; Oberbauer et al., 1998; Arft et al., 1999; Starr et al., 2000; Marchand et al., 2004; Oberbauer et al., 2013; Khorsand Rosa et al., 2015; Prevéy et al., 2017). The ability of individual species to take advantage of warming temperatures and other resulting environmental conditions (e.g. precipitation rates, soil moisture, more snow) may be a major component driving documented community composition shifts (Arft et al., 1999; Walker et al., 2006; Hollister et al., 2015) and ecosystem function changes (Oechel et al., 1992; Boelman et al., 2003; Schimel and Bennett, 2004; Welker et al., 2005; La Puma et al., 2007; Leffler et al., 2016; Jespersen et al., 2018). Lengthening of the green season will likely favor some species over others and ultimately result in changes in phenology, species composition and possibly trace gas feedbacks (Bjorkman et al., 2015; Khorsand Rosa et al., 2015; Kelsey et al., 2016). These changes are likely to impact the timing and magnitude of forage availability which will have cascading implications on food-web dynamics (Welker et al., 2005; Post et al., 2009; Myers-Smith et al., 2011; Richert et al., 2019).

The responsiveness of timing of peak NDVI to warming differed by site and species composition. Peak NDVI occurred earlier in response to warming in wet and moist communities, dominated by graminoids and deciduous shrubs, with the greatest shifts in the southernmost sites. This finding suggests that variation in the species that constitute different growth forms may cause communities to respond at different rates to warming. Variations in community dominance and ground cover have been previously shown to alter community NDVI values (Raynolds et al., 2008; Pattison et al., 2015). The Atqasuk and Toolik Lake dry sites are dominated by lichens and are more sparsely vegetated, and both sites showed peak NDVI was delayed with warming. Alternatively, the Utqiaġvik and Imnaviat Creek dry sites have a greater cover of evergreen shrubs and these sites showed peak NDVI was earlier with warming. Therefore, it is reasonable to conclude that the dominant vegetation growth form and cover extent have an effect on the responsiveness of a community type to warming, and any dominance shifts will likely impact NDVI directly and also influence how responsive NDVI is to changing temperatures in the future.

The variability of the magnitude and timing of peak NDVI across our study region shows that similar communities do not respond uniformly across a region. This response pattern begins to shed some light on why some regions green more quickly than others (Jia et al., 2003; Stow et al., 2007; Bhatt et al., 2013). Differences in the most southerly sites (Toolik Lake and Imnavait Creek) may also be a result of differences in warming treatment duration, where Toolik Lake has likely had ample time for community composition changes while Imnavait Creek vegetation is responding to novel warming. Our findings also highlight the value of coupling traditional visually-assessed Arctic plant community changes with small-scale remotely-sensed observations. The synthesis of these two methodologies will allow future studies to thoroughly investigate community-scale changes and their impact on broader landscape-scale patterns, with insight into mechanisms that drive change such as browning in regions of the Arctic (Verbyla, 2008; Phoenix and Bjerke, 2016). Understanding variations in community change mechanisms across latitudinal gradients is critical for the ability to accurately scale up landscape cover change predictions. It should be noted that the shorter duration of warming at Imnavait Creek (2 vs. ≥ 20 years) may have played a role in the magnitude of NDVI differences between treatments. Community changes associated with warming would likely impact NDVI values through the differences in growth form cover that we report here and by previous studies (Arft et al., 1999; Elmendorf et al., 2012a, Elmendorf et al., 2012b, Bjorkman et al., 2015).

Our findings also highlight the value of incorporation of ground-based remotely-sensed measurements at sites across the Arctic where detailed vegetation dynamics are also monitored. This study is the first that investigates the effects of warming on daily NDVI changes and demonstrates the complexity of scaling across heterogeneous tundra communities. Our results suggest the non-uniformity of warming effects on the magnitude and timing of peak NDVI across northern Alaska. The fact that peak NDVI was not markedly increased at most sites may be misleading given that spring greening was hastened and green season lengthened. These changes suggest that while peak NDVI may not increase markedly, the lengthening of the green season in response to warming is likely to result in greater productivity. Collectively, it appears that in a warmer Arctic (as simulated in our experiment) there may be a host of organismal and ecosystem process changes, including the period of carbon gain and greater net primary productivity as well as shifts in the timing of vegetation phenology that may have consequences for foraging ecology of ungulates and other trophic interactions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JM led data collection and analysis and co-wrote manuscript with RH and KB. RH and KB co-wrote manuscript and revisions. KB and JH assisted in data collection on the project. RH, CT, JW, WG, and SO contributed data for this manuscript, advised in revisions, and secured funding for the project.

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A New Method for Counting Reproductive Structures in Digitized Herbarium Specimens Using Mask R-CNN

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Phenology—the timing of life-history events—is a key trait for understanding responses of organisms to climate. The digitization and online mobilization of herbarium specimens is rapidly advancing our understanding of plant phenological response to climate and climatic change. The current practice of manually harvesting data from individual specimens, however, greatly restricts our ability to scale-up data collection. Recent investigations have demonstrated that machine-learning approaches can facilitate this effort. However, present attempts have focused largely on simplistic binary coding of reproductive phenology (e.g., presence/absence of flowers). Here, we use crowd-sourced phenological data of buds, flowers, and fruits from >3,000 specimens of six common wildflower species of the eastern United States (*Anemone canadensis* L., *A. hepatica* L., *A. quinquefolia* L., *Trillium erectum* L., *T. grandiflorum* (Michx.) Salisb., and *T. undulatum* Wild.) to train models using Mask R-CNN to segment and count phenological features. A single global model was able to automate the binary coding of each of the three reproductive stages with >87% accuracy. We also successfully estimated the relative abundance of each reproductive structure on a specimen with ≥90% accuracy. Precise counting of features was also successful, but accuracy varied with phenological stage and taxon. Specifically, counting flowers was significantly less accurate than buds or fruits likely due to their morphological variability on pressed specimens. Moreover, our Mask R-CNN model provided more reliable data than non-expert crowd-sourcers but not botanical experts, highlighting the importance of high-quality human training data. Finally, we also demonstrated the transferability of our model to automated phenophase detection and counting of the three *Trillium* species, which have large and conspicuously-shaped reproductive organs. These results highlight the promise of our two-phase crowd-sourcing and machine-learning pipeline to segment and count reproductive features of herbarium specimens, thus providing high-quality data with which to investigate plant responses to ongoing climatic change.

Keywords: automated regional segmentation, deep learning, digitized herbarium specimen, plant phenology, regional convolutional neural network, reproductive structures, visual data classification

INTRODUCTION

Climate change is a potent selective force that is shifting the geographic ranges of genotypes, altering population dynamics of individual species, and reorganizing entire assemblages in all environments. A key functional trait in this regard is phenology: the timing of life-history events, such as the onset of flowering or migration. The use of museum specimens has invigorated and enriched the investigation of phenological responses to climatic change, and is one of several research directions that has brought a renewed sense of purpose and timeliness to natural history collections (Davis et al., 2015; Willis et al., 2017; Meineke et al., 2018; Meineke et al., 2019; Hedrick et al., 2020). Herbarium specimens greatly expand the historical depth, spatial scale, and species diversity of phenological observations relative to those available from field observations (Wolkovich et al., 2014). In many cases, herbarium specimens provide the only means of assessing phenological responses to climatic changes occurring over decades to centuries (Davis et al., 2015). However, a great challenge in using these specimens is accessing and rapidly assessing phenological state(s) of the world's estimated 393 million herbarium specimens (Thiers, 2017; Sweeney et al., 2018).

The ongoing digitization and online mobilization of herbarium specimens has facilitated their broad access with significant economies of scale (Sweeney et al., 2018; Nelson and Ellis, 2019; Hedrick et al., 2020) and accelerated advances in scientific investigations, including phenological assessment efforts that were underway prior to mass digitization (Primack et al., 2004; Miller-Rushing et al., 2006; Davis et al., 2015). A new vision of digitization, Digitization 2.0 (*sensu* Hedrick et al., 2020), has also sparked the integration and development of new scholarly disciplines and lines of inquiry not possible previously. Whereas Digitization 1.0 refers to the generation of digitized products from physical specimens, Digitization 2.0 is the use of natural history collections to answer scientific questions using only their digitized representation, rather than the physical specimen itself.

In recent years, scientists have used these digitized herbarium specimens in novel ways (e.g., Meineke et al., 2018; Meineke et al., 2019; Hedrick et al., 2020) and greatly increased the pace at which key phenological trait data can be harvested from tens of thousands of specimens. The platform *CrowdCurio–Thoreau's Field Notes* (Willis et al., 2017) was one of the first attempts to move beyond the standard practice of coding phenology of herbarium specimens using binary (presence/absence) coding (e.g., specimen A has flowers, specimen B has fruits: Primack et al., 2004; Miller-Rushing et al., 2006). Many of these efforts have also focused largely on flowering, ignoring other key phenophases. Rather, users of *CrowdCurio* use a crowd-sourcing pipeline to score and quantify all phenophase features—bud, flowers, and fruits—for each specimen processed. This pipeline has facilitated the first development of ratio-based approaches to quantitatively assess the early, peak, and terminal phenophases from herbarium specimens and determine phenological changes within and between seasons (Williams et al., 2017; Love et al., 2019). The recent large-scale deployment of the *CrowdCurio* pipeline on the crowdsourcing website Amazon Mechanical Turk has demonstrated the power and scale of such fine-grained phenophasing to understand latitudinal variation in phenological responses (Park et al., 2019).

Despite the great promise of crowd-sourcing for phenophase detection, it is still time-consuming and can become cost-prohibitive to process entire collections spanning whole continents. Machine-learning approaches have the potential to open up new opportunities for phenological investigation in the era of Digitization 2.0 (Pearson et al., 2020). Recent efforts (Lorieul et al., 2019) have demonstrated that fully automated machine-learning methods—and deep learning approaches based on convolutional neural networks in particular—can determine the presence of a fruit or flower in a specimen with >90% accuracy. Convolutional neural networks were proven effective at predicting all phenophases of a specimen, based on classification of nine phenological categories. These predictions, estimated from proportions of buds, flowers and fruits, reach an accuracy (true positive rate) >43%, which is equivalent to the capability of human experts (Lorieul et al., 2019). This large-scale automated phenophase estimation, based on an annotation method developed by Pearson (2019), was tested on species belonging to a particularly difficult taxon (i.e., the Asteraceae family), for which visual analysis of numerous and tiny reproductive structures is known to be visually challenging. This work demonstrated the potential of deep learning technologies to estimate fine-grained phenophases, but further improvements are needed to support ecological investigation of diverse taxa.

Although Pearson (2019) successfully determined reproductive status (i.e., fertile *vs.* sterile specimens), neither the precise location (i.e., image segment) nor the number of phenofeatures present on a specimen was quantified (Lorieul et al., 2019). A quantitative machine-learning approach would have the value and impact that *CrowdCurio* has already achieved, but could be scaled-up in speed and cost-effectiveness. A recent proof-of-concept study (Goëau et al., 2020) used human-scored data to train and test a model using instance segmentation with Mask R-CNN (Masked Region-based Convolutional Neural Network: He et al., 2017) to locate and count phenological features of *Streptanthus tortuosus* Kellogg (Brassicaceae). This assessment clarified several determinants of model success for identifying and counting phenological features, including: the type of masking applied to human annotations; and the size and type of reproductive features identified (e.g., flowering buds, flowers, immature and mature fruits). Moreover, the model was more successful identifying and counting flowers than fruits, and was applied only to a single species with relatively little human-scored training data (21 herbarium specimens). The transferability of this model to other, more distantly related species was not examined.

Here, we leverage extensive data gathered using our crowd-sourcing platform *CrowdCurio* to develop and evaluate an instance segmentation approach using Mask R-CNN to train and test a model to identify and count phenological features of a larger number of species. Specifically, we investigated digitized specimens from six common spring-flowering herbs of the eastern United States: *Anemone canadensis*, *A. hepatica*, *A. quinquefolia*, *Trillium erectum*, *T. grandiflorum*, and *T. undulatum*. As with any feature detection model, accurate human-collected data are required to train, test, and refine these models. We thus gathered phenological data from these species using *CrowdCurio* to provide expert annotation data of buds, flowers, and fruits to train and test our models. Phenological data previously collected by non-expert citizen scientists was used to further evaluate the performance of these models (Park et al., 2019). Our goals were to: (1) determine how reliably we could localize and count these features; (2) determine the accuracy in automated scoring of different phenological features; and (iii) assess the transferability of models trained on one species to other, distantly related ones.

MATERIALS AND METHODS

Dataset

Our experiments are based on a subset of the data used in Park et al. (2018, 2019) comprising six species in two genera of common spring-flowering herbs, *Anemone* and *Trillium*. This subset includes 3073 specimens of: *Anemone canadensis* (*N* = 108), *A. hepatica* (*N* = 524), *A. quinquefolia* (*N* = 686), *Trillium erectum* (*N* = 862), *T. grandiflorum* (*N* = 226), and *T. undulatum* (*N* = 667). Each specimen (herbarium sheet) was previously examined using the *CrowdCurio–Thoreau's Field Notes* platform by, on average, three citizen-scientists. For the purposes of this study, these specimens were additionally scored by expert botanists to provide the most accurate training and testing data possible. Annotators added markers in the center of each visible reproductive structure (bud, flower, or fruit), and determined its type, number, and spatial location. For our experiments, we randomly split this dataset into two parts: one (*N* = 2457) for training the deep-learning models and one for testing them (*i.e.*, for evaluating their predictive performance; *N* = 615).

Apart from the comparative experiment described in *Machine-Learning vs. Crowd-Sourcing* section, only the annotations of experts were used to train and test the deep-learning models. We also only used the annotations of one of the experts for each specimen (selected in a pre-defined order). The final dataset contains 7,909 reproductive structures (6,321 in the training set and 1,588 in the test set) with the following imbalanced distribution: 492 buds (6.2%), 6,119 flowers (77.4%), and 1,298 fruits (16.4%). Fruits were counted without any knowledge of seeds.

Deep-Learning Framework

Several deep-learning methods have been developed in recent years to count objects in images. One family of methods can be qualified as density-oriented methods (Wang et al., 2015; Zhang et al., 2015; Boominathan et al., 2016). They are usually based on U-Net architectures (Ronneberger et al., 2015) that are trained on annotations of object centers (indicated by dots) and predict density maps that are integrated to obtain counts. U-Net-based methods were developed originally for counting crowds and have been extended recently to counting cells (Falk et al., 2019) and animals (Arteta et al., 2016). The drawback of these methods is that they are better suited for cases where the density of objects in the image is high. This is not true in our case; the examined herbarium specimens averaged <3 objects per specimen, even fewer if we consider buds, flowers, and fruits separately.

Another deep-learning method is "direct counting" (*a.k.a.* "glancing"), which trains the model with the true count on the global image (e.g., Seguí et al., 2015). The main drawback of direct counting is that it cannot predict a count value that has no representative image in the training set. That is, the network is not really counting but only inferring the counts from the global content of the image. In preliminary experiments (not reported here), we found that direct-count methods tended to systematically under-estimate the true counts and have an unacceptably high variance.

The alternative method that we used in this study is to equate counting with object-detection; the counts of the object of interest are then equal to the sum up the number of detected objects. To detect buds, flowers, and fruits, we used Mask R-CNN, which is among the best-performing methods for instance segmentation tasks in computer vision (He et al., 2017). We used Facebook's implementation of Mask R-CNN (Massa and Girshick, 2018) using the PyTorch framework (Paszke et al., 2019) with a ResNet-50 architecture (He et al., 2016) as the backbone CNN and the Feature Pyramid Networks (Lin et al., 2017) for instance segmentation. To adapt this architecture to the data in our study (see previous section), we had to address the following methodological issues:

1. *Mask computation*. The training data expected by Mask R-CNN must consist of all the objects of interest visible in the training images, each object being detected individually and associated with a segmented region (encoded in the form of a binary mask). However, the data available for our study did not fully meet these conditions as the objects were detected only by dot markers (roughly in the centre of the reproductive structure). From these dot markers, we generated dodecagons, such as the ones illustrated in **Figure 1**, which best covered the reproductive structures. To adapt the size of the dodecagons to buds, flowers, and fruits, we manually segmented five of each (selected at

FIGURE 1 | Example of a specimen of the training set containing six reproductive structures (flowers) marked by dodecagons.

random from each genus) and calculated the average radius of the circle enclosing each structure.

2. *Input image size*. Images were resized to 1,024 pixels (long edge) × 600 pixels (short edge). This guaranteed a sufficient number of pixels for the smallest dodecagons while maintaining a reasonable training time (5–10 h per model) on a computer comparable to a mid-tier consumer device (i.e., recent GPUs with ±12 GB of RAM).
3. *Anchor size*. Anchors are the raw rectangular regions of interest used by Mask R-CNN to select the candidate bounding boxes for mask detection. We designated their size so as to guarantee that all dodecagons had their entire area covered.

Figure 2 Illustrates four example detections using Mask R-CNN: one with a perfectly predicted count, and three with over- or under-estimated counts. For each example, we show (a part of) the original image, the ground-truth masks (computed from expert botanist input), and the automated detections computed by the deep-learning framework.

We then trained a set of models corresponding to three distinct scenarios to be evaluated:

1. *One model per species*. In this scenario, we trained one Mask R-CNN model for each species (i.e., six models in total) to detect its buds, flowers, and fruits.
2. *One single model for all species*. In this scenario, we trained a single Mask R-CNN for all species and all types of reproductive structures (buds, flowers, fruits).
3. *Cross-species models*. Last, we assessed the transferability of models trained on some species to other ones. We trained three models on only two *Trillium* species: i.e., one on *T. erectum* and *T. grandiflorum*, one on *T. erectum* and *T. undulatum*, and one on *T. undulatum* and *T. grandiflorum*. Each of these three models were then tested on the *Trillium* species not included in the training set.

Evaluation Metrics and Statistics

We evaluated the accuracy of the models in four ways:

1. *Counting error*. The counting error $e_{i,k}$ for a specimen i and a given type of reproductive structure $k \in \{bud, flower, fruit\}$ was defined as the difference between the true count and the predicted count:

$$e_{i,k} = \hat{c}_{i,k} - c_{i,k} \tag{1}$$

where $c_{i,k}$ is the true count of reproductive structures of type k in specimen i and k, $\hat{c}_{i,k}$ is the predicted count. Note that the counting error can be positive or negative. A detailed description of the distribution of the counting error is provided using letter-value plots (Heike et al., 2017), which provide a more comprehensive view of the statistics through a larger number of quantiles.

2. *Mean Absolute Error (MAE)*. The MAE measures the overall error by averaging the absolute value of the counting error of each specimen and each type of reproductive structure:

$$MAE = \frac{1}{N}\sum_i \sum_k \left|e_{i,k}\right| \tag{2}$$

3. *Coefficient of determination (R^2)*. This statistic measures the amount of variance explained or accounted by the model:

$$R^2 = 1 - \frac{\sum_i (c_i - \hat{c}_i)^2}{\sum_i (c_i - \bar{c})^2} \tag{3}$$

where i indexes the observations and ranges from 1 to the

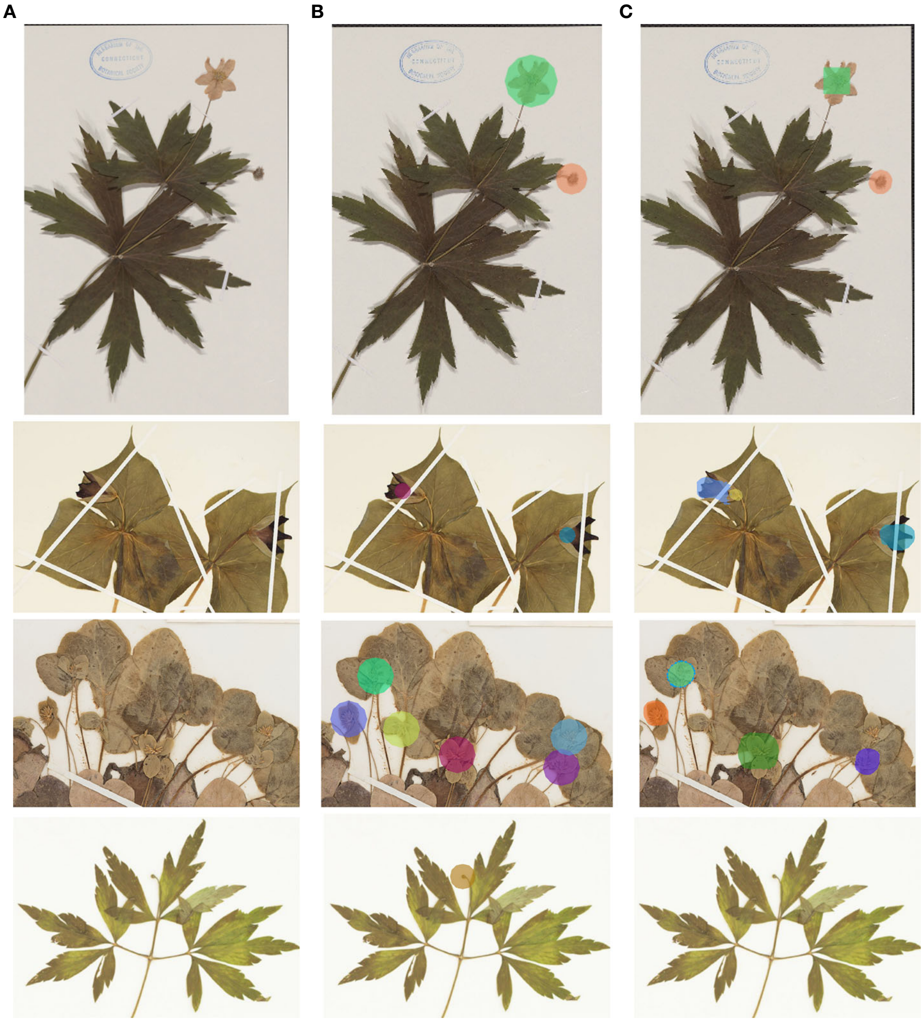

FIGURE 2 | Examples of detection (colors do not have a particular meaning)—**(A)** original image; **(B)**: ground-truth markers; **(C)**: automatically detected masks. The first row corresponds to a typical case with a perfect count. The second row corresponds to a case of over-estimated counts (one of the flowers was detected as two flowers). The last two rows correspond to under-estimated.

total number of observations, c_i is the observed count, $\hat{c}_i$ is the predicted count, and c is the mean of the observed counts.

4. *Predicted counts box-plots*. A detailed description of the distribution of the predicted counts as a function of the true counts is provided using box-plots indicating median value, quartiles, variability outside quartiles, and outliers.

Machine-Learning vs. Crowd-Sourcing

We compared the counts predicted by Mask R-CNN with those obtained when the reproductive structures on herbarium specimens were counted by crowd-sourcers (Park et al., 2019). The comparison was done on the intersection of the test sets of both studies (i.e., on 544 specimens, equal to 88% of the test set of previous experiments). These 544 specimens were annotated by 483 different annotators using Amazon Mechanical Turk. On average, each specimen was annotated by 2.5 different crowd-sourcers.

RESULTS

A Single Model vs. Species-Specific Models

The R^2 value for the separate training model for each species and the single model for all species was 0.70 and 0.71, respectively. Thus, the single model for all species provides marginally better results while being simpler to implement and more scalable. As shown in **Figure 3**, the main problem of single species training models is that they tend to over-predict the number of reproductive structures (number of positive errors >than number of negative errors; **Figure 3**). The

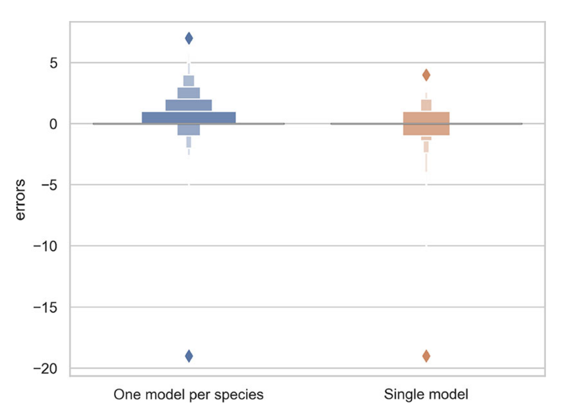

FIGURE 3 | Letter-value plot of the counting error for the two training strategies: one model per species vs. one single model for all species.

extreme outlier in **Figure 3** with a very high negative error resulted from a species being assessed by the model that had been misidentified in the collection.

The predictions of the single species training models were very accurate for ≤3 reproductive structures, whereas the single model for all species had high accuracy when ≤4 reproductive structures were present (**Figure 4**). The variance of the predicted counts was higher for specimens with more reproductive structures but the median predicted count equalled the actual count for ≤7 reproductive structures and the counting error (interquartile distance) was usually <1 structure. Specimens with >8 reproductive structures had larger errors but only accounted for 4.2% of the specimens examined.

Distinguishing Reproductive Structures

Counting Results

The overall numbers of detected reproductive structures and their relative proportions were very close to their actual values (**Table 1** and **Figure 5**). The Mean Absolute Error (MAE) was also quite low for all types of reproductive structures, but this is due in large part to the fact that the median number of structures per phase and specimen is low. The median number of fruits and buds, in particular, is much lower than the median number of flowers. The R^2 values (**Table 1**) and the box plots of the predicted counts (**Figure 6**) provide a more relevant comparison of the predictive performance for each type of structure. Flowers are the best detected structures (R^2 = 0.76), followed by fruits (R^2 = 0.33) and buds (R^2 = 0.12). The lower performance for buds is due to several factors: (i) the lower number of samples in the training set—90.25% of specimens had no buds and 98.05% had <3 buds, (ii) their smaller size and (iii), their visual appearance that is less distinctive than flowers or fruits. Fruits are affected by the same factors but to a lesser extent.

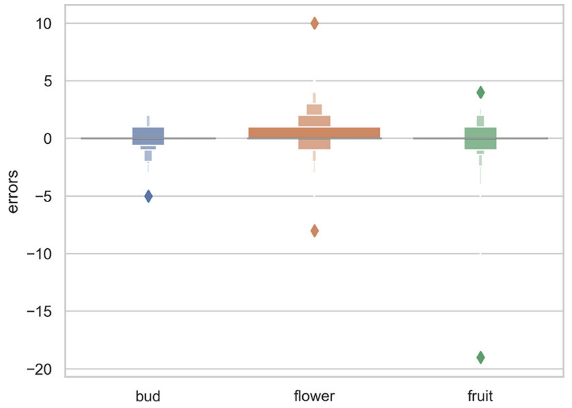

FIGURE 5 | Letter-value plot of the counting error for each type of reproductive structure.

Occurrence and Dominance of Reproductive Structures

Although the model was not developed or trained to directly detect presence or absence of each reproductive structure, we

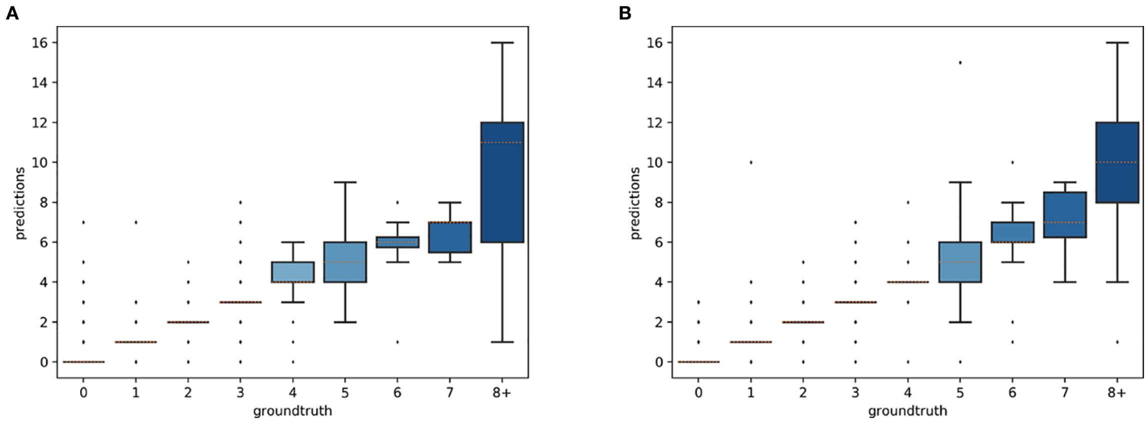

FIGURE 4 | Box-plots of the predicted vs. expected counts for the two training strategies: **(A)** separate training models for each species, **(B)** single training model for all species.

TABLE 1 | Predicted and true counts (percent of specimens in parentheses) of buds, flowers, and fruits for all specimens pooled.

	Buds	Flowers	Fruits	All
True number of structures	107 (6.7)	1,241 (78.1)	240 (15.1)	1,588
Predicted number of structures	109 (6.1)	1,431 (80.0)	248 (13.9)	1,788
MAE	0.20	0.51	0.27	0.33
*R*2	0.12	0.76	0.33	0.71

were able to extrapolate the presence of each feature and which feature was most frequent on a specimen (**Table 2**). The detection accuracy of buds, flowers, and fruits was >87% and the accuracy of determining relative abundance of a certain organ category (e.g., number of flowers >number buds or fruits) was >90% (**Table 2**). Confidence in this strong result should be tempered by the actual frequency of occurrence and dominance. Observed relative presences of buds, flowers, and fruits, and dominance of fruits *vs.* flowers all are quite disparate. Error rates (false negatives and positives) for these all are non-zero, but are lower in all presence and dominance categories (**Table 2**).

Species-Specific Models

Overall, the reproductive structures were detected more accurately for *Trillium* species than *Anemone* species (**Figures 7** and **8**). At the species-specific level, the R^2 score was lowest for *A. canadensis* (0.01) which is the species with the least number of training samples (108 specimens). The R^2 score was better for the other species and increased with the number of training samples: R^2 = 0.51 for *T. grandiflorum*, R^2 = 0.64 for *A. hepatica*, R^2 = 0.76 for *T. undulatum*, R^2 = 0.85 for *A. quinquefolia* and R^2 = 0.89 for *T. erectum*. Counting errors rarely exceeded ±2, and the few strong outliers corresponded to very difficult cases or annotation errors. The median value of predicted counts was correct in almost all cases (**Figure 7**); exceptions were for *T. grandiflorum*specimens with four structures and *A. hepatica* with seven, both corresponding to instances involving a small number of specimens with large numbers of reproductive structures.

Model Transferability

The aim of this experiment was to assess whether reproductive structures on one species could be estimated using a model trained on a different, related species. Unsurprisingly, estimation was less accurate when the target species was not represented in the training set (**Figures 9–11**). However, it is still possible to count the reproductive structures of a target species based on a model trained on different species of the same genus (i.e., without any specimen of the target species in the training data). The R^2 score was higher for *T. erectum* (R^2 = 0.72; **Figure 9**) and *T. undulatum* (R^2 = 0.66; **Figure 10**), which are morphologically more similar to one another than either is to *T. grandiflorum* (R^2 = 0.02; **Figure 11**). Figures only show the results for *Trillium* but similar conclusions were obtained for *Anemone* (R_2 scores respectively equal to 0.75 for *A. quinquefolia*, 0.39 for *A. hepatica* and −0.39 for *A. canadensis*).

Machine-Learning vs. Crowd-Sourcing

On average, the deep learning model had a significantly lower (P <0.001) MAE and better R^2 score than any individual crowd-sourcer, but still an order of magnitude larger than the MAE of botanical experts (**Tables 3** and **4**). Interestingly, we can observe that crowd-sourcers have a much harder time detecting buds than the Mask R-CNN model. The MAE obtained by averaging the counts of the different crowd-sourcers was only marginally higher than the MAE from Mask R-CNN (P = 0.3). Note that a counts averaging strategy could also be used for the deep learning approach, *i.e.*, by averaging the scoring of several deep learning models. This technique is referred to as an *ensemble* of models in

TABLE 2 | Accuracy of detection and relative dominance of buds, flowers, and fruits (data pooled for all species).

Observed	Buds	Flowers	Fruits	Flowers ≥ Buds	Fruits ≥ Flowers
	9.75	**82.92**	**20.00**	**96.09**	**21.13**
True positives (correctly detected)	51.66	97.25	78.86	98.98	76.15
True negatives (correctly undetected)	91.89	49.52	89.83	8.33	95.65
False positives	8.10	50.47	10.16	91.66	3.71
False negatives	48.33	2.74	21.13	1.01	23.84
Overall Accuracy	87.97	89.11	87.64	95.44	92.03

Values are percentages.

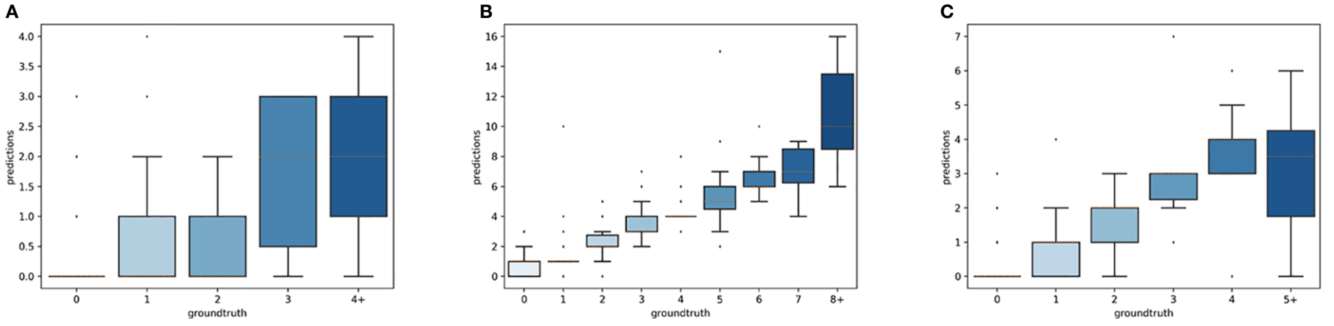

FIGURE 6 | Box-plots of the predicted vs. expected counts for each type of reproductive structure. From left to right: **(A)** buds, **(B)** flowers, **(C)** fruits.

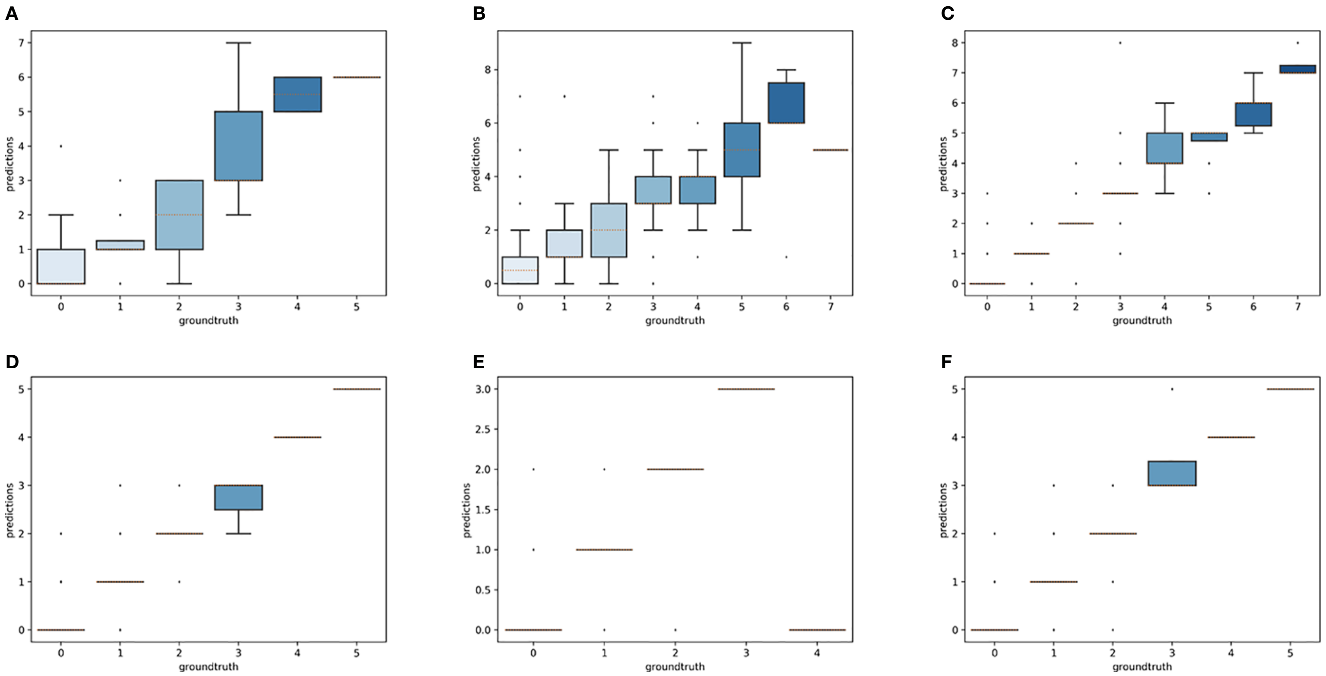

FIGURE 7 | Boxplot of the predicted counts vs. expected counts for each species. **(A)** *Anemone canadensis*; **(B)** *A. hepatica*; **(C)** *A. quinquefolia*; **(D)** *Trillium erectum*; **(E)** *T. grandiflorum*; **(F)** *T. undulatum*.

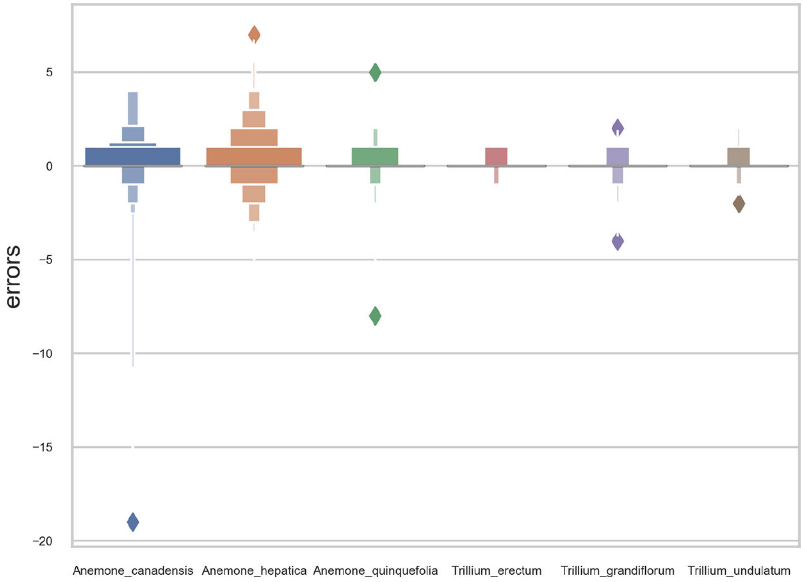

FIGURE 8 | Letter-value plot of the counting error for each species.

the machine learning community and is known to bring very significant improvements. The most simple yet very efficient method to build an ensemble is to train the same model several times but with a different random initialization of the parameters. Such strategy could be implemented in future work.

DISCUSSION

Mask R-CNN models trained with human-annotated trait data were efficient and produced robust results. Our models worked well for both identifying and counting phenological features, but

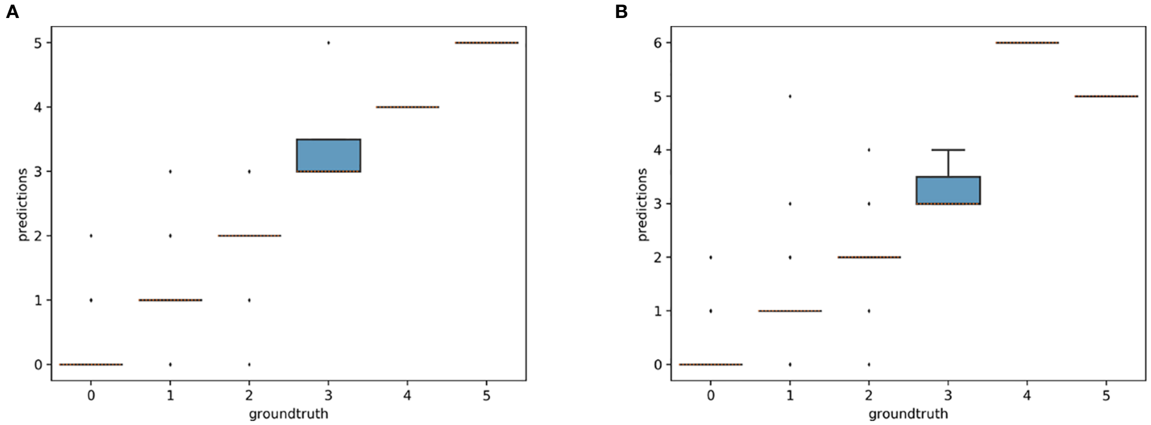

FIGURE 9 | Box-plots of the predicted counts *vs.* expected counts for *Trillium erectum*. **(A)** Model trained on *T. erectum* data; **(B)** model trained on *T. undulatum* and *T. grandiflorum*.

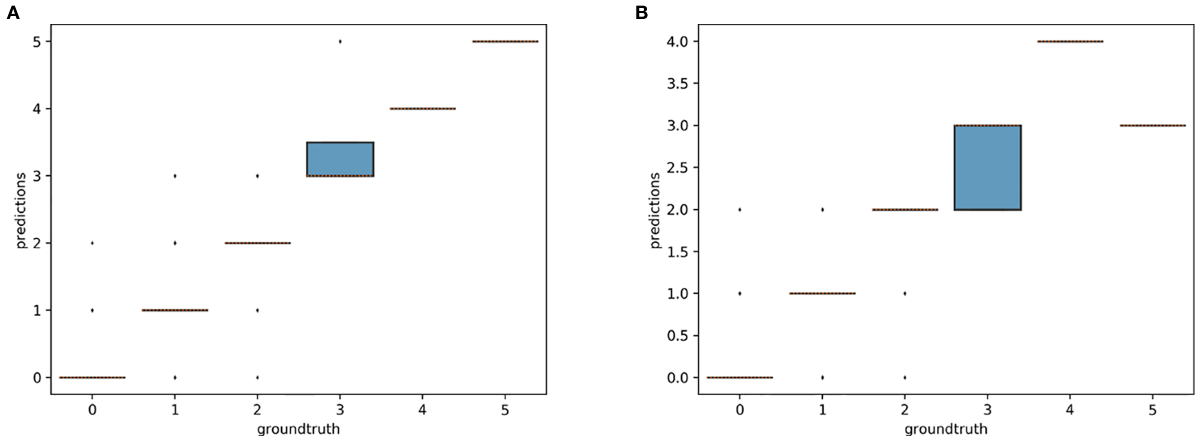

FIGURE 10 | Box-plots of predicted counts *vs.* observed counts for *Trillium undulatum*. **(A)** Model trained on *T. undulatum* data; **(B)** model trained on *T. erectum* and *T. grandiflorum*.

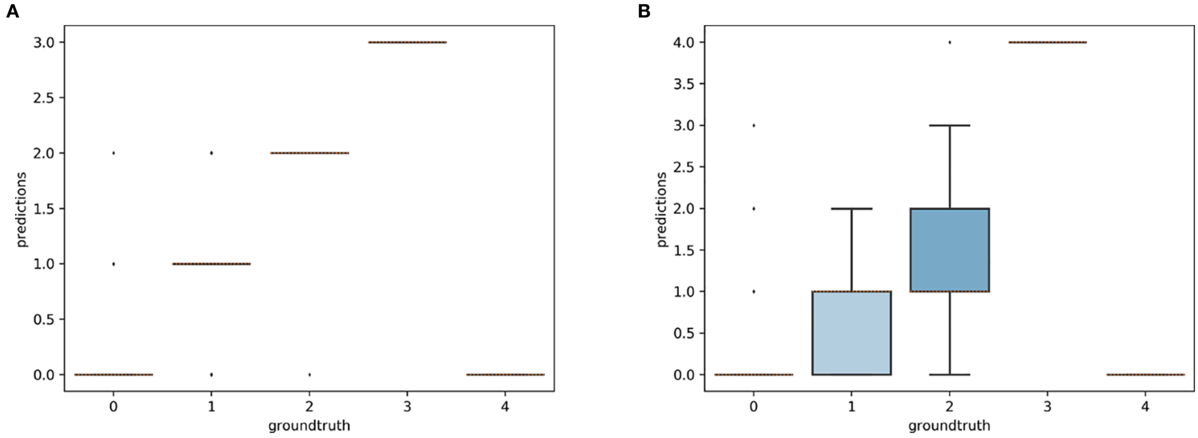

FIGURE 11 | Box-plots of predicted counts *vs.* expected counts for *Trillium grandiflorum*. **(A)** Model trained on *T. grandiflorum* data; **(B)**: model trained on *T. erectum* and *T. undulatum.*

TABLE 3 | Comparison of the counting error resulting from crowd-sourcing, deep learning and expert annotation—performance is measured by the Mean Absolute Error (MAE).

	Buds	Flowers	Fruits	All
Experts	0.009	0.027	0.073	0.036
Crowd-sourcing (isolated annotator)	0.526	0.487	0.314	0.442
Crowd-sourcing (average over all annotators)	0.418	0.405	0.243	0.355
Deep learning (model trained on all species)	**0.201**	**0.507**	**0.266**	**0.325**

TABLE 4 | Comparison of the counting error resulting from crowd-sourcing, deep learning and expert annotation—performance is measured by R2 score.

	Buds	Flowers	Fruits	All
Experts	0.989	0.996	0.961	0.990
Crowd-sourcing (isolated annotator)	–2.969	0.758	0.306	0.555
Crowd-sourcing (average over all annotators)	–1.527	0.828	0.401	0.686
Deep learning (model trained on all species)	**0.141**	**0.750**	**0.329**	**0.707**

accuracy differed for buds, flowers, and fruits. Automated counts using Mask R-CNN models were more accurate than counts made by crowd-sourcers but not those of botanical experts. Finally, the Mask R-CNN model could be transferred to other species after being trained with data from reasonably close phylogenetic relatives, with relatively small impacts on counting accuracy.

Point Masking With Minor Modification Is Efficient and Produces Robust Results

Recent efforts by Goëau et al. (2020) to segment and count reproductive structures used training data collected by botanical experts from 21 herbarium specimens of a single species (*S. tortuosus*). In our work, we applied Mask R-CNN to segment and count reproductive structures of six species, belonging to two different genera; accurate training data were derived from both botanical experts and crowd-sourcers using the *CrowdCurio* interface (Willis et al., 2017). Although Goëau et al. (2020) found that training data from point masks, like those generated from *CrowdCurio*, produced less accurate results than those derived from fully masked training data, obtaining the latter is time intensive and difficult to scale to large numbers of specimens. Whereas Goëau et al. (2020) produced three type of training data, "point masks" (produced from a 3 × 3-pixel box around a manual point marker); (ii) "partial masks" (extensions of point masks to include partial segmentation using the Otsu segmentation method (Otsu, 1979); and (iii) manually produced "full masks" of each reproductive structure, we only used modified partial masks (derived from point markers) with Mask R-CNN. These modified partial masks were scaled to the size of reproductive structures for each species and yielded high accuracy and efficiency for phenophase detection and counting. The scaling of our modified partial masks combined with the approximately circular shapes of the reproductive structures we studied likely led to the success of our approach. Our two-step workflow integrating expert-scored and crowd-sourced citizen science data with automated machine-learning models also is less time-intensive and more scalable than a workflow requiring detailed polygon masks of structures for training.

Feature Detection and Counting Accuracy Is High Across All Phenological Features

Lorieul et al. (2019) were the first to apply machine-learning to detect phenophases and developed a presence-absence model that could identify reproductive specimens with ≈96% accuracy. Their model was less accurate in detecting flowers or fruits (≈85 and ≈80% accuracy, respectively), and they did not consider buds. In contrast, we used Mask R-CNN to accurately identify the presence of each of the three reproductive stages (buds, flowers, or fruits) with ≥87% accuracy (**Table 2**). Moreover, a single globally-trained model was more efficient and had greater accuracy than multiple species-specific models (**Figures 7** and **8**). This points towards the possibility of developing a more streamlined workflow to accurately score phenophases of many different species simultaneously.

We also successfully estimated the relative abundance of each reproductive structure on a specimen with ≥90% accuracy (**Table 2**). Herbarium specimens can vary greatly in phenological state. Because different reproductive organs can co-exist at various times through plant development (and may not all be represented simultaneously on herbarium sheets), simply quantifying presence or absence of phenological structures limits inference about phenological state. In this regard, the Mask R-CNN model performed better on *Trillium*—with its large flowers and fruits, generally borne singly, and suspended on an elongate stalk—than on *Anemone*—with its small clusters of flowers on shorter stalks that are often pressed against a background of clustered leaves. The combination of smaller flowers, more complex morphology, and background "noise" on *Anemone* specimens (e.g., overlapping structures) likely made both model training and phenophase detection more prone to error. This result supports the recent hypotheses that successful application of machine-learning to phenophase assessment will be dependent on species-specific morphological details (Goëau et al., 2020). Along these lines, plant morphological trait databases could help facilitate the identification of suitable taxa to be analyzed with machine-learning methods.

Precise quantification of different reproductive structures, as demonstrated here, allows the determination of finer-scale phenophases (e.g., early flowering, peak flowering, peak fruiting). For this exercise, the lowest mean absolute error (MAE) was for bud counts, most likely due to the morphological consistency of buds and their rarity on specimens (**Table 1**). In contrast, MAE for counting flowers was significantly worse than for buds or fruits. We attribute this result to the greater number of flowers, ontogenetic variability in floral morphology, and variation in appearance of dried, pressed specimens.

Variation in appearance of reproductive features among dried and pressed specimens of a single species also could add complexity to automated detection of phenological features and merits further investigation. Perhaps more consequentially, large variation in the number of reproductive organs resulted in unbalanced datasets (**Table 1**). Numerous data augmentation approaches can be implemented to improve comparisons and model selection for such data sets (e.g., Tyagi and Mittal, 2020), but these approaches have been used more frequently in classification or semantic segmentation (Chan et al., 2019) than in instance segmentation approaches such as we used here. Developing data augmentation approaches for instance segmentation would be a useful direction for future research. But even if collectors collect more flowering than non-flowering specimens, estimating the quantity of buds, flowers and fruits on any specimen is more informative than recording only their presence or absence.

Botanical Experts Perform Better Than the Model

When considered in aggregate, the MAE for segmenting and counting all three phenophases using Mask R-CNN was lower than that of crowd-sourcers but still an order of magnitude higher than that of botanical experts (**Tables 2** and **3**). This result reinforces the suggestion that abundant and reliable expert data are essential for properly training and testing machine learning models (Brodrick et al., 2019). Additionally, it was evident in some cases that the precise detection of the phenological feature was quite inaccurate (**Figure 2**).

Machines Can Apply Learning From One Species to Another, but Success Is Variable

For the first time to our knowledge, we have demonstrated that training data from related taxa can be used to detect and count phenological features of a species not represented in the training set (**Figures 9–11**). We limit our discussion of transferability here to species of *Trillium* owing to the ease of detecting and counting phenological features in this genus. Though in some cases species-specific models were highly transferable, model transferability varied greatly. For example, training on *Trillium undulatum* and testing on *T. erectum* (and vice-versa) was more accurate than when Mask R-CNN models trained with data from either of these species was applied to *T. grandiflorum*. *T. undulatum* and *T. erectum* are more similar morphologically than either is to *T. grandiflorum*, suggesting that morphological similarity may be a better guide for transferability success than phylogenetic relatedness (see Farmer and Schilling, 2002, for phylogenetic relationships of *Trillium*). This conclusion implies that transferability may be particularly challenging for clades that exhibit high morphological diversity and disparity among close relatives. The relationship between phylogenetic relatedness, morphological diversity, and model transferability should be investigated in future studies. The assessment of the sizes of the reproductive structures that could be captured by this type of approach should also be analyzed, to facilitate transferability.

Future Directions

The presence of reproductive structures has been determined only infrequently during large-scale digitization and transcription efforts by the natural-history museums that generate this content. However, interest is growing rapidly in using herbarium specimens for investigating historical changes in phenology and other ecological traits and processes. Our results have demonstrated success in automating the collection of large amounts of ecologically-relevant data from herbarium specimens. Together with controlled vocabularies and ontologies that are being developed to standardize these efforts (Yost et al., 2018), our two-stage workflow has promise for automating and harvesting phenological data from images in large virtual herbaria. In the long term, we would like to use the *CrowdCurio* workflow to generate reliable human-annotated data to further refine automated models for detecting phenological responses to climatic change from herbarium specimens across diverse clades and geographies. Finally, our results documenting transferability of machine-learning models from one species to another are preliminary, but promising. Although our universal model trained on all taxa performed better than our individual, species-specific models, there may be better ways to guide these efforts. For example, a hierarchy of individual models could yield more accurate results. These hierarchies might be phylogenetically organized (e.g., taxonomically by order, family, genus), leveraging information about shared morphologies common to related taxa and further governed by a set of rules that parse new specimens for phenophase detection based on their known taxonomic affinities (e.g., by genera). Similar approaches are already being applied today by corporations like Tesla Motors. Their automated driving suite uses different models for vehicle path prediction versus vehicle detection (Karpathy et al., 2014; Tesla, 2019).

DATA AVAILABILITY STATEMENT

The images used and the datasets generated for this study are available from the Environmental Data Initiative doi: 10.6073/pasta/4d2e92ec343d716eb6ee3ee7cadec5ef.

AUTHOR CONTRIBUTIONS

CD conceived the idea for the study. CD, DT, IB, and DP ran a pilot feasibility study to motivate the current project. DP and GL generated, organized, and assembled expert and non-expert crowd-sourced data to train the Mask R-CNN model. JX re-coded *CrowdCurio* for these experiments. JC, AJ, and PB conducted the analyses. CD, AJ, PB, JC, DP, and AE interpreted the results. CD wrote the first draft of the *Abstract*, *Introduction*, and *Discussion*. JC, AJ, and PB wrote the first draft of the *Methods* and *Results*. All authors contributed to the article and approved the submitted version.

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Responses of Plant Reproductive Phenology to Winter-Biased Warming in an Alpine Meadow

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Climate warming is often seasonally asymmetric with a higher temperature increase toward winters than summers. However, the effect of winter-biased warming on plant reproductive phenology has been seldom investigated under natural field conditions. The goal of this study was to determine the effects of winter-biased warming on plant reproductive phenologies. In an alpine meadow of Tibetan Plateau, we deployed six large (15 m × 15 m × 2.5 m height) open top chambers (three warmed chambers and three non-warmed chambers) to achieve winter-biased warming (i.e., a small increase in annual mean temperature with a greater increase towards winter than summer). We investigated three phenophases (onset and offset times and duration) for both the flowering and fruiting phenologies of 11 common species in 2017 and 8 species in 2018. According to the vernalization theory, we hypothesized that mild winter-biased warming would delay flowering and fruiting phenologies. The data indicated that the phenological responses to warming were species-specific (including positive, neutral, and negative responses), and the number of plant species advancing flowering (by averagely 4.5 days) and fruiting onset times (by averagely 3.6 days) was higher than those delaying the times. These changes were inconsistent with the vernalization hypothesis (i.e. plants need to achieve a threshold of chilling before flowering) alone, but can be partly explained by the accumulated temperature hypothesis (i.e. plants need to achieve a threshold of accumulative temperature before flowering) and/or the overtopping hypothesis (i.e. plants need to reach community canopy layer before flowering). The interspecific difference in the response of reproductive phenology could be attributed to the variation in plant traits including plant height growth, the biomass ratio of root to shoot, and seed mass. These results indicate that a mild winter-biased warming may trigger significant change in plant reproductive phenology in an alpine meadow.

Keywords: experimental warming, flowering phenology, fruiting phenology, plant trait, root to shoot ratio, seed size, plant height, alpine meadow

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INTRODUCTION

Global mean surface temperature has increased by 0.8–1.2°C from 1950 to 2017 with a rate of 0.1–0.3°C per decade (IPCC, 2018), and it is predicted to increase (by either 0.3–1.7°C according to RCP 2.6 (Representative Concentration Pathway), and 2.6–4.8°C according to RCP 8.5) by the end of the 21st century (IPCC, 2014). Moreover, the magnitude of climate warming is heterogeneous both spatially and temporally, and is generally greater at higher latitudes (e.g. the arctic regions; Meredith et al., 2019) and higher altitudes (Hock et al., 2019). The magnitude of climate warming is also greater in winter than summer and at night than during the day (IPCC, 2007; IPCC, 2014). Typically, the Tibetan Plateau has experienced a greater than global average increase (0.16°C per decade; IPCC, 2018) in mean annual temperature (0.25°C per decade), with 0.30°C per decade in winter, greater than that of summer (0.20°C per decade) since the 1960s (Liu and Chen, 2000).

Numerous studies have shown that climate warming affects almost all aspects of biological systems. One of the most extensive studied aspect is plant phenology (Arft et al., 1999; Dunne et al., 2003; Sherry et al., 2007; Post et al., 2008; Dorji et al., 2013; Meng et al., 2019; Jabis et al., 2020), since warming-induced changes in plant phenology may affect species interactions, ecosystem nutrient cycling, and energy flow (CaraDonna et al., 2014). Climate warming is often simulated by manipulative experiments using open top chambers or infrared heaters in different terrestrial biomes (Marion et al., 1997; Arft et al., 1999; Sherry et al., 2007; Post et al., 2008; Dorji et al., 2013). These warming experiments usually elevate annual mean temperature by 1.2–5°C, which is generally greater than the predicted temperature changes at a century scale (e.g., Marion et al., 1997; Post et al., 2008; Dorji et al., 2013). However, it is often suggested that long-term and mild warming experiments are more likely to provide data reflective of more realistic conditions and results. Importantly, artificial warming often archives a higher temperature increase in summers than in winters because infrared heaters are usually turned off or less effective in winters (Kimball, 2005; Zhou et al., 2019), and because open top chambers often result in higher temperature increases in summers than winters (Marion et al., 1997; Post et al., 2008; Dorji et al., 2013).

Although studies have substantially addressed plant responses to climate warming in the growing (summer) season, winter warming is a key driver of plant performance in terrestrial ecosystems, especially in cold regions (Williams et al., 2015; Shen et al., 2016). For example, long-term remote sensing data from Tibetan Plateau show that an increase in winter temperatures would substantially advance the start date of vegetation greening (Zhang et al., 2013; Shen et al., 2016). It is surprising, therefore, that a mild winter-biased warming has been scarcely employed to examine phenological responses, especially because it is predicted to be a realistic future condition.

Winter-biased warming may have different effects on plant phenology as suggested by three different hypotheses. For example, winter warming is predicted to decrease the strength of chilling to delay the timing of leafing, flowering, and fruiting in many species (i.e. the vernalization hypothesis; Körner and Basler, 2010). The vernalization hypothesis is supported by the delay of the spring greening of vegetation in Tibetan grasslands, as recorded by remote sensing images (Yu et al., 2010). Winter warming is also predicted to enhance accumulative temperature to reach an earlier threshold for flowering, and therefore it may advance plant reproductive phenology (i.e. the accumulated temperature hypothesis; Harrington and Gould, 2015). Winter warming may also facilitate plant growth by accelerating litter decomposition (Bernier et al., 1981) and hence advance flowering phenology by allowing plants to achieve an earlier canopy layer, as suggested by the overtopping hypothesis positing that plants tend to flower when they reach their canopy layer (Wesselingh et al., 1997; Jacquemyn et al., 2010). The overtopping hypothesis has been supported by many studies (Pfeifer et al., 2006; Zhou et al., 2019). Thus, predictions based on different mechanisms are not consistent about whether winter warming will advance or delay plant reproductive phenology.

Moreover, it is not clear whether all co-occurring species will show the same response to warming (Sherry et al., 2007; Dorji et al., 2013). This is often explained by species-specific functional traits (Dorji et al., 2013; Zhou et al., 2019). For example, warming may affect growth in plant height differentially (Baruah et al., 2017), such that the time at which a plant reaches canopy spread and flowering or fruiting differs among species according to the overtopping hypothesis. Moreover, according to the seed size-time hypothesis, which proposes that perennial species with large seeds require a longer time to develop mature fruits, large seeded-species will tend to flower and fruit earlier and hence have a longer development time (Bolmgren and Cowan, 2008; Du and Qi, 2010). Consequently, if warming induced an advance of flowering or fruiting onset time in one species, it would advance the species' flowering or fruiting offset time (if seed size is unchanged by warming). In addition, plants often differ in root depth and root/shoot mass ratio (R/S), which may mediate the response of plant phenology to warming. The plants with shallow roots or lower R/S may be more sensitive (relative to the species with deeper roots or higher R/S) to warming as a consequence of soil moisture deficits (Passioura, 1983; Dorji et al., 2013), and hence the species with contrasting root depths and R/S may differ in their phenological responses to warming.

To fully understand the effect of winter-biased mild warming on plant phenology, we investigated the onset and offset times of reproductive phenology (flowering and fruiting) for 11 herbaceous species growing in both (artificially) warmed and non-warmed open top chambers for two consecutive years in a Tibetan meadow. The objectives of this study were 1) to determine whether the phenological response to artificial warming is consistent with the vernalization hypothesis, the accumulated temperature hypothesis, or the overtopping hypothesis, and 2) to test whether interspecific differences in the growth rates of plant height, root/shoot mass ratio, and seed size accounted for any of the variation in the phenological responses among the study species.

MATERIALS AND METHODS

Study Site

This research was conducted in Hongyuan County, Sichuan province, China (32°48'N, 102°33'E), which is in the eastern Tibetan Plateau with an altitude of 3,500 m a.s.l. (**Figure S1A**). The climate is characterized by long, cold winter, short spring and autumn, and a cool mild summer. According to data collected at Hongyuan County Climate Station (5 km away from the study site) from 1970 to 2016, annual mean temperature is 1.7°C, with maximum and minimum monthly means of 11.1°C and −9.3°C observed in July and January. Mean annual precipitation is 756 mm (including 73 mm snow precipitation), over 80% of which falls during the growing season from May to September (Cao et al., 2018). Relative to 1970–2000, mean annual temperature has increased by 0.97°C during 2001–2016, with a higher increase in non-growing seasons (October to April, 1.04°C) than growing seasons (May to September, 0.88°C).

The pasture has been intensively grazed by livestock (e.g. yak *Bos grunniens*) for decades. The studied meadow is dominated by an assemblage of forbs (*Saussurea nigrescens*, *Polygonum viviparum*, *Potentilla anserine*, *Trollius farreri*, *Thalictrum alpinum*, and *Anemone trullifolia* var. *linearis*), sedges (*Kobresia setchwanensis* and *Carex* spp.), and grasses (*Deschampsia caespitosa*, *Festuca ovina* and *Elymus nutans*). Vegetation coverage of the meadow is more than 90%, and average plant height is ~30 cm (Wu et al., 2011). Owing to the diverse plant species (Xiang et al., 2009), the arthropod species, like pollinators (Hu et al., 2019), herbivores (Xi et al., 2013), as well as dung decomposers (Wu et al., 2011) are diverse in the meadow.

Experimental Design

In October of 2014, six 15 × 15 × 2.5 m (height) open top chambers (OTCs) were randomly deployed in a fenced (non-grazed area) flat area of about 1.0 ha. The sides of all OTCs were covered with thin (less than 0.1 mm) steel screen with a mesh size of 0.2 × 0.2 mm. Three of the OTCs were additionally covered with transparent tempered glass (δ8). The roof of these three OTCs was discontinuously covered by 0.15 × 0.3 m (width) transparent glass strips, with a 0.6 m space between neighboring strips (**Figures S1B, C**). Each OTC was sunk 1 m into the soil, and along the OTC sides, steel screen (with a mesh size of 0.6 × 0.6 mm) was also sunk 1 m into the soil to prevent rodents from entering (**Figure S1B**). In mid-July of 2018, the transparency of the transparent tempered glass was on average (94.4%, N = 45) under full light conditions, slightly lower than that of the steel screen (97.9%, N = 45). We refer to the three OTCs with transparent tempered glass as warmed chambers and the other three as ambient, control, non-warmed chambers (**Figure S1D**).

Microclimate Measurements

In each chamber, HOBO temperature sensors (HOBO PRO, Onset Computer Corporation, USA) were used to monitor air temperature (T_i) at 30 cm above ground level. The HOBO MX2301A sensors (Onset Computer Corporation, USA) were deployed in a pair of chambers (one for warmed and the other for non-warmed) to monitor air relative humidity (M_i) at 30 cm above ground level. The air vapor pressure deficit (VPD) was calculated using the following equation (Richard et al., 1998).

$$VPD = 0.611 \quad exp\left(\frac{17.27 \times Ti}{Ti + 237.3}\right) \times (1 - \frac{Mi}{100})$$

Any abnormal microclimate values due to sensor malfunctions were removed from the data set. Soil temperature and moisture (at 5 cm) were monitored (using Watchdog2000, Spectrum Technologies, Inc., USA) for a pair of chambers (one for warmed and the other for non-warmed) since 2015. Data were sampled at 1-hour intervals.

Phenological Measurements

During the growing season of 2017 and 2018, eleven common species were chosen for phenological monitoring. They consisted of eleven species (*A. trullifolia* var. *linearis*, *T. alpinum*, *T. farreri*, *Anemone rivularis*, *Delphinium caeruleum*, *Anaphalis flavescens*, *S. nigrescens*, *P. viviparum*, *Potentilla discolor*, *Halenia elliptica*, and *Gentianopsis paludosa*). In each study year, we randomly selected and tagged 10–30 individuals for each species in each chamber (if available) before the occurrence of flower buds. For each tagged individual, the flowers or capitula were weekly counted and each of them was given a phenological score following Price and Waser (1998). Six phenological stages were recorded, including unopened buds (stage 1), open flowers (stage 2, stamens are visible), old flowers (stage 3, petals or stamens are withering), initialed fruit (stage 4, petals abscised but ovaries unexpanded), expanding fruit (stage 5, enlarged fruit), and dehisced fruit (stage 6). "Stage 6" was recorded when fruits dehisced (*T. farreri*, *D. caeruleum*, *P. discolor*, *H. elliptica*, and *G. paludosa*), and fallen (*A. trullifolia* var. *linearis*, *T. alpinum*, *A. rivularis*, and *P. viviparum*), or pappuses became fluffy (*A. flavescens* and *S. nigrescens*). For each census, an unweighted phenological score was calculated by averaging the stages present on each individual (Price and Waser, 1998; Dunne et al., 2003; Sherry et al., 2007). To reduce the variability among individual observations of phenological stages, we derived phenological variables by fitting linear regression to the sequence of phenological scores for each observed individual as a function of the day of the year for each species and for each year. Regressions were performed only with individuals for which at least four phenological scores were observed throughout the reproductive period. The 1430 individual regressions showed statistically significant fits (average r^2 = 0.93 ± 0.002 [1SE]; maximum r^2 = 1, minimum r^2 = 0.54; $P < 0.05$).

Using the regression equations, we calculated the following phenological variables for each plant: flowering onset time (stage 2), flowering offset time (stage 3.5), fruiting onset time (stage 4), and fruiting offset time (stage 6). "Duration of flowering" refers to the estimated time to progress from stage 2 to stage 3.5, and "duration of fruiting" refers to the number of days for an individual to progress from stage 4 to stage 6. Because plant abundance varied among years and chambers, only 8 species were available for analysis in 2018.

Plant Traits

Typical plant traits assumed to be relevant to plant phenology were measured for this study, including plant height, root/shoot ratio, and (individual) seed mass. Plant height at the onset time of flowering (Hf), the distance from ground-surface to stem tips, were recorded when the first open flower was observed for each tagged individual (except for *A. trullifolia* var. *linearis*, whose Hf was missed in 2018). The other traits including R/S and seed mass were measured outside chambers. More than 20 fruiting plants were randomly selected in the field and then soaked in water to remove the residual soil. Each plant was dissected into belowground parts (roots) and aboveground parts (shoots). Both roots and shoots were weighted after drying for 72 h at 75°C. In addition, one mature fruit was sampled from each plant to cunt and weigh viable seeds. Seed mass was calculated as the total seed mass divided by sound seed number. Finally, root/shoot mass ratios (R/S) and seed mass were averaged for each species (except for *T. alpinum*, whose seed mass was not measured).

Statistical Analysis

A series of generalized linear mixed models (GLMMs) was used to test the effects of warming (non-warmed vs. warmed) on reproductive phenologies and plant height for each species. In each model, warming (eight species were investigated in both study years), year, and species were set as fixed factors, and "OTC" was set as a random factor, with "individual" nested within "OTC". Moreover, GLMMs were used to test the effects of warming on the six reproductive phenologies for each species, where warming and year were set as fixed factors, and "OTC" was set as a random factor, with "individual" nested within "OTC". GLMMs were performed using the package "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017), respectively.

We also determined whether a cross-species relationship between phenological changes and species traits (including Hf, R/S and seed size) existed using linear regressions (by "*lm*"). The phenological changes, as well as changes in Hf were quantified as the relative change intensity (Ri) for each species following the protocols of Armas et al. (2004), i.e., calculated as $(P_w - P_n)/(P_w + P_n)$, where P_w and P_n were the observed phenologies/Hf in the warmed and non-warmed treatments, respectively. The index Ri had defined limits $[-1,1]$. Negative values indicated an advance in the phenological events or lower height. All observed dates were converted to Julian dates (days since Jan. 1st). The relationship between phenological changes and plant traits in 2017 were analyzed because not all species were observed in 2018. All analyses were conducted using R 3.5.3 (R Core Team, 2019).

RESULTS

Microclimate Conditions

Measurements over a span of four years showed that the mean annual temperature was 0.3–0.5°C higher at 30 cm aboveground, 0.2–0.5°C at the 5 cm soil depth in the warmed OTCs than in the non-warmed OTCs (**Table S1**, **Figures S2 and S3**). The mean temperature was 0.4–0.6°C higher at 30 cm above the soil surface, and 0.8–1.1°C higher at the 5 cm soil depth in warmed chambers than non-warmed chambers in the non-growing season. During the growing season, the increased temperature was 0.03–0.47°C and −0.2–0.8°C higher at the soil surface and at the 5 cm soil depth in the warmed chambers, respectively (**Table S1**). Temperature increase was more pronounced in winter than in summer, and it was statistically significant at night (18:00–8:00) but not during the daytime (**Figures S4 and S5**). The vapor pressure deficit was 2.6–3.7% higher in the warmed than the non-warmed OTCs in 2018 (**Figure S6**). In addition, the soil moisture was 2–3% VWC (percent in volume water content) higher at the 5 cm soil depth in the non-warmed than in the warmed OTCs (**Figure S7**).

Phenological Response to the Warming

Warming had a significant effect on the onset time of flowering and fruiting (**Table 1**, **Table S3**). Warming advanced flowering onset time for most species in both study years (significantly in 8 out of 11 species; with two exceptions in 2017 and one in 2018), with an average of 4.5 days (**Figures 1A, C**, **Tables S2 and S3**), but warming delayed the onset time of flowering and fruiting for *A. rivularis*, and *P. viviparum* in both 2017 and 2018 (**Figures 1A, C**, **Tables S2 and S3**). In contrast, experimental warming advanced fruiting onset time in 9 out of 11 species (significantly in 6 species) in 2017 and 5 out of 8 species in 2018, with an average of 3.6 days (**Figures 2A, C**, **Tables S2 and S3**), but delayed fruiting for *P. viviparum* in both 2017 and 2018.

The effect of warming was also significant on the offset time of flowering, but was not significant on fruiting offset time (**Table 1**). Warming advanced flowering offset time by averagely 3.6 days for all the species, with the exceptions of *A. rivularis* and *P. viviparum*, which were either unchanged or significantly delayed in both 2017 and 2018 (**Figures 1B, D**, **Tables S2 and S3**). The advance of fruiting offset time was less conspicuous than other phenologies. It was significant for 5 out of 11 species in 2017 and 2 out of 8 species in 2018 (**Figures 2B, D**, **Tables S2 and S3**).

The warming effect was not statistically significant on the durations of flowering and fruiting for most plant species (**Table 1**, **Table S3**). Warming extended but not significantly the flowering and fruiting durations in both 2017 and 2018 (averagely 1.7 days for flowering duration and 2.2 days for fruiting duration) (**Figure 3**, **Table S3**). However, the magnitudes of the changes of the durations was both species- and year-specific.

Relationships Between Plant Traits and Reproductive Phenophases

Because all of the phenophases were highly correlated with each other in both non-warmed and warmed chambers (Pearson's correlation: $r^2 \geq 0.94$, $P < 0.001$ and $r^2 \geq 0.93$, $P < 0.001$, respectively), only the relationship between flowering onset time and plant traits were explored. Plant height at flowering onset time was greater for most of the study species in warmed than in non-warmed chambers (**Figure 4**). The Ri of flowering

TABLE 1 | Summary of the GLMMs analysis of variance of the six phenological events (onset, offset, duration of flowering and fruiting) for two years.

	Source	Numerator DF	Denominator DF	*F*	*P*
Flowering onset time	Warming (W)	1	1256	91.0448	<0.0001***
	Year (Y)	1	1259.2	127.1054	<0.0001***
	Species (S)	7	1257.2	3298.0034	<0.0001***
	W: Y	1	1252.6	0.7007	0.402
	W: S	7	1255	13.6422	<0.0001***
	Y: S	7	1254.1	27.7809	<0.0001***
	W: Y: S	7	1254	1.5603	0.143
Flowering offset time	Warming (W)	1	1251.7	51.5803	<0.0001***
	Year (Y)	1	1252.2	82.2772	<0.0001***
	Species (S)	7	1252.1	3553.4587	<0.0001***
	W: Y	1	1248.1	1.9975	0.158
	W: S	7	1250.5	19.4363	<0.0001***
	Y: S	7	1248	13.6761	<0.0001***
	W: Y: S	7	1249.4	4.8524	<0.0001***
Fruiting onset time	Warming (W)	1	1251.2	31.7359	<0.0001***
	Year (Y)	1	1249	54.551	<0.0001***
	Species (S)	7	1251.1	2980.1729	<0.0001***
	W: Y	1	1248.1	2.1629	0.142
	W: S	7	1250	17.9819	<0.0001***
	Y: S	7	1247.5	18.0017	<0.0001***
	W: Y: S	7	1249	5.8225	<0.0001***
Fruiting offset time	Warming (W)	1	1253.6	0.6824	0.409
	Year (Y)	1	1252.6	3.6645	0.056.
	Species (S)	7	1253.1	1065.1179	<0.0001***
	W: Y	1	1252.7	1.7263	0.189
	W: S	7	1253.2	9.3641	<0.0001***
	Y: S	7	1254	41.4065	<0.0001***
	W: Y: S	7	1252.6	6.3516	<0.0001***
Flowering duration	Warming (W)	1	1253	23.8427	<0.0001***
	Year (Y)	1	1235.4	23.7434	<0.0001***
	Species (S)	7	1227.7	182.9706	<0.0001***
	W: Y	1	1253	0.4602	0.498
	W: S	7	1253.2	1.4248	0.191
	Y: S	7	1259	61.7383	<0.0001***
	W: Y: S	7	1252.6	3.7029	0.0006***
Fruiting duration	Warming (W)	1	1253	23.8427	<0.0001***
	Year (Y)	1	1235.4	23.7434	<0.0001***
	Species (S)	7	1227.7	182.9706	<0.0001***
	W: Y	1	1253	0.4602	0.498
	W: S	7	1253.2	1.4248	0.191
	Y: S	7	1259	61.7383	<0.0001***
	W: Y: S	7	1252.6	3.7029	0.0006***

*The eight species (Thalictrum alpinum; ANTR, Anemone trullifolia var. linearis; TRFA, Trollius farreri; PODI, Potentilla discolor; POVI, Polygonum viviparum; ANFL, Anaphalis flavescens; SANI, Saussurea nigrescens and DECA, Delphinium caeruleum) observed in consecutive years were included.*****P < 0.001.*

onset time was positively correlated with the Ri of Hf ($r^2 = 0.75$, $P = 0.008$), and with R/S ($r^2 = 0.71$, $P = 0.015$; **Figures 5A, B**). Individual seed mass was positively correlated with the Ri of flowering onset time ($r^2 = 0.79$, $P = 0.006$; **Figure 5C**). However, the relationship between individual seed mass and the duration of fruit development was not significant (non-warmed: $r^2 = -0.18$, $P = 0.62$; warmed: $r^2 = -0.35$, $P = 0.32$).

DISCUSSION

The data presented here show that our experimental setting resulted in a mild winter-biased warming, with a slight increase in annual mean temperature and a higher increase in winter than in summer. This warming effect has not been commonly observed in previous studies (but Suonan et al., 2016) and stand in contrast to many other open top chambers (OTCs) studies, which often achieve a summer-biased warming. One explanation for the difference reported here and in other studies may be due to the fact that wind is usually much stronger in the winter compared to the summer. Results similar to those reported here have been reported in the same study region. The results reported here for a mild winter-biased warming are consistent with the prediction of IPCC (IPCC, 2007), and thus deserves further investigation. Winter-biased warming significantly changed plant flowering and fruiting phenologies, especially onset and offset times. Because flowering onset time is positively associated with flowering offset time and the onset and offset time of fruiting, flowering onset time will be focused for the following discussion.

Although this study shows that the phenological response to warming is species-specific, most of the species in this study

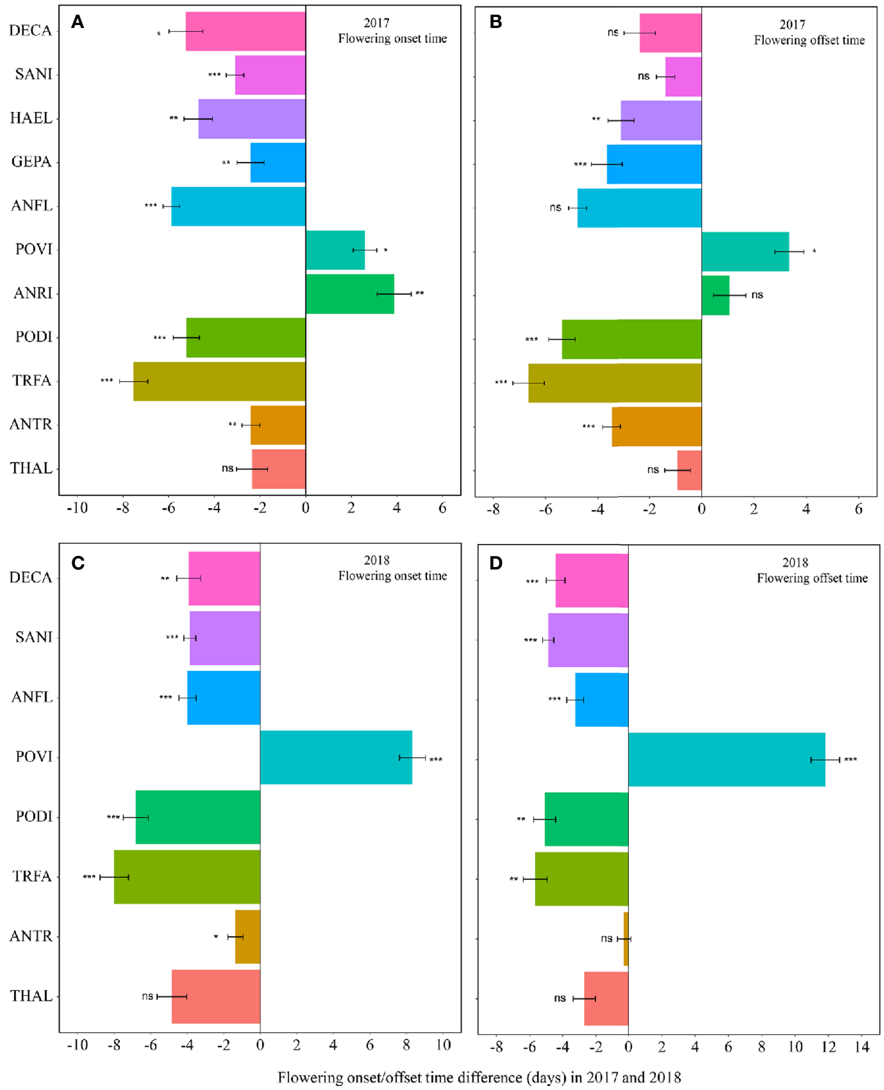

FIGURE 1 | Changes in the flowering onset time (**A**, 2017; **C**, 2018), and flowering offset time (**B**, 2017; **D**, 2018) (in days) between the non-warmed and warmed chambers from 2017 to 2018. Species are listed in the order from the earliest flowering species *Thalictrum alpinum* to the latest flowering species *Delphinium caeruleum*. A negative value indicates the warming-induced advance in flowering onset time and offset time, while a positive value indicates the warming-induced delay in the phenologies. Bars indicate the mean ± SE for each bar. The difference in each phenology is determined by generalized linear mixed models (GLMMs). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significant. THAL, *Thalictrum alpinum*; ANTR, *Anemone trullifolia* var. *linearis*; TRFA, *Trollius farreri*; PODI, *Potentilla discolor*; ANRI, *Anemone rivularis*; POVI, *Polygonum viviparum*; ANFL, *Anaphalis flavescens*; GEPA, *Gentianopsis paludosa*; HAEL, *Halenia elliptica*; SANI, *Saussurea nigrescens* and DECA, *Delphinium caeruleum*.

advanced but not delayed their flowering, a phenomenology that is similar to the findings of other experimental warming studies performed in steppes, prairies, and alpine meadows (Arft et al., 1999; Sherry et al., 2007; Post et al., 2008; Dorji et al., 2013; Jabis et al., 2020). Moreover, other experimental warming studies in the northeast of Tibetan Plateau (Haibei Alpine Meadow) have shown an advance in the onset time of flowering. In particular, using infrared heaters simulating a winter warming, Suonan et al. (2016) reported that flowering onset time is advanced on average by 12.6 days in an alpine meadow, a duration considerably greater than that observed in our study (4–5 days at most). This difference may be attributed to the difference in the temperature increase reported by Suonan et al. (2016) and in our study (i.e., > 1.5°C and < 1°C, respectively).

The advance of flowering in our study indicates that winter warming expedites flower differentiation and development. Therefore, the vernalization hypothesis (i.e., warming decreases the strength of vernalization and delays plant leafing, flowering, and fruiting; see Körner and Basler, 2010) alone cannot explain our data. Instead, the advance in flowering onset time is more

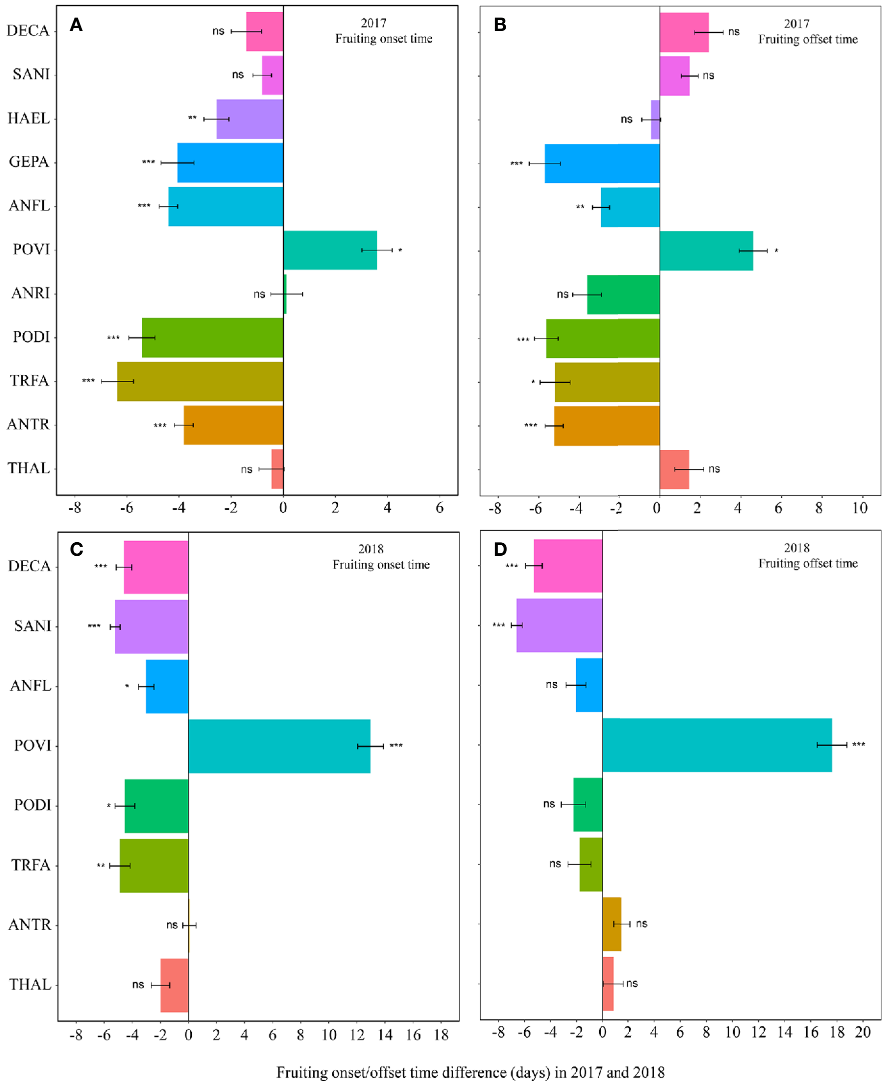

FIGURE 2 | Changes in the fruiting onset time (**A**, 2017; **C**, 2018), and fruiting offset time (**B**, 2017; **D**, 2018) (in days) between the non-warmed and warmed chambers from 2017 to 2018. Species are listed in the order from the earliest flowering species *Thalictrum alpinum* to the latest flowering species *Delphinium caeruleum*. A negative value indicates the warming-induced advance in fruiting onset time and offset time, while a positive value indicates the warming-induced delay in the phenologies. Bars indicate the mean ± SE for each bar. The difference in each phenology is determined by generalized linear mixed models (GLMMs). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, non-significant. The abbreviation of species names is same as **Figure 1**.

consistent with the accumulative temperature hypothesis (i.e., plants will flower when the accumulative temperatures reach a threshold; see Harrington and Gould, 2015). Nevertheless, it is possible that both hypotheses work as an explanation for the shift in flowering phenology such that the latter has an overriding influence, leading to an advance of flowering.

An additional observation is that plant height at the onset time of flowering is greater in warmed OTCs than in non-warmed OTCs, indicating that plants do not necessarily flower after reaching a specific height threshold. It is possible that plants only flower when they reach their maximum (physiologically optimal) height compared to other conspecifics (as suggested by the larger plant height at flowering onset time in the warmed chambers and the positive relationship between height at flowering onset time and flowering). If true, this supports the overtopping hypothesis. Achieving a maximum height may provide an advantage in attracting pollinators, because pollinators are generally scare (Peng et al., 2018). Because both the vernalization and accumulative temperature hypotheses are insufficient to explain a winter-biased warming-induced advance of flowering phenology, the overtopping hypothesis must be considered a potential candidate mechanism underlying the winter-biased warming-induced change in flowering phenology.

Although phenological advance under warming conditions is common across different ecosystems, exceptions have been observed (Price and Waser, 1998; Yu et al., 2010), i.e., not all

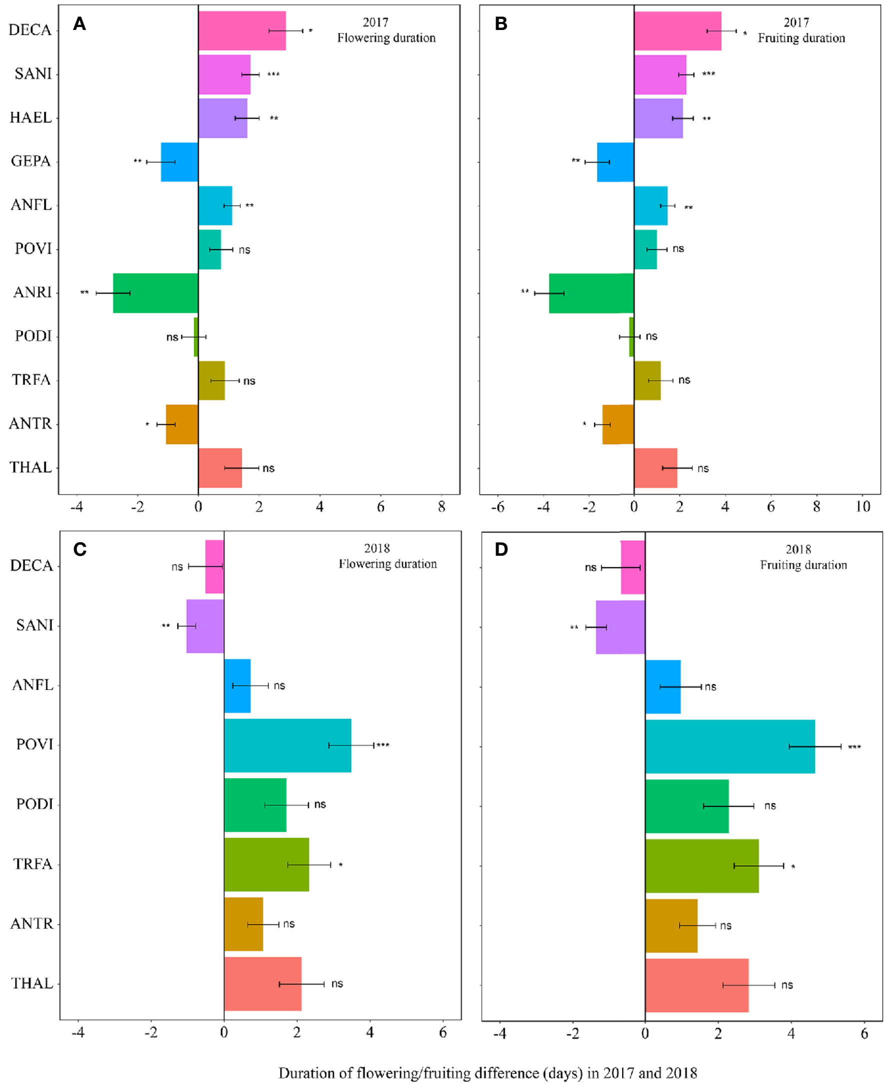

FIGURE 3 | Changes in the flowering duration (**A**, 2017; **C**, 2018), and fruiting duration (**B**, 2017; **D**, 2018) (in days) between the non-warmed and warmed chambers from 2017 to 2018. Species are listed in the order from the earliest flowering species *Thalictrum alpinum* to the latest flowering species *Delphinium caeruleum*. A negative value indicates the duration of flowering or fruiting shortened in warmed chambers, while a positive value indicates the phenologies extended in warmed chambers. Bars indicate the mean ± SE for each bar. The difference in each phenology is determined by generalized linear mixed models (GLMMs). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant. The abbreviation of species names is same as **Figure 1**.

species show the same direction and magnitude of changes in flowering time. The significant correlation between plant functional traits and changes in the onset time of flowering indicates that the response of flowering phenology to warming might be mediated by the former. One functional trait is growth in height. If warmed plants advance their shoot growth and flowering, they may avoid shading by neighbors, such that a small change in height at flowering onset time is sufficient to achieve "overtopping" success (Wesselingh et al., 1997; Jacquemyn et al., 2010). In contrast, if warmed plants delay their shoot growth and flowering, they must grow more in height to achieve competitive equality (i.e., a greater height at flowering onset time). This is implied by the positive relationship between the changes in flowering onset time and plant height at flowering onset time. The second functional trait is the R/S ratio. Plants with a lower R/S value are often more sensitive to lower soil moistures (see **Figure S7**), resulting in a slight drought that likely facilitates flowering (e.g. Yang et al., 2014). This is perhaps the reason why the species with lower R/S values advanced more in flowering than those with higher R/S. In addition, species with small seeds advance in flowering more than large-seeded species (**Figure 5C**). This positive relationship is unexpected but reasonable. Probably because the large-seeded species are taller at flowering onset time in warmed chambers (N = 10, $r^2 = 0.33$,

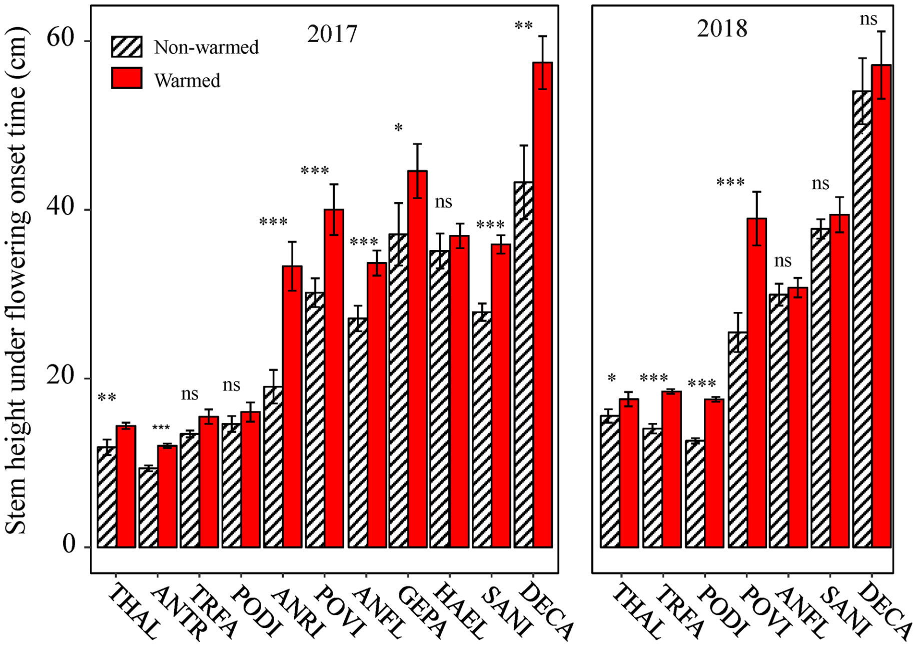

FIGURE 4 | The effects of warming on plant height at flowering onset time for the study species from 2017 to 2018. Bars indicate the mean ± SE for each bar. Species are listed in the order form the earliest to latest flowering species. The difference in plant height is determined by generalized linear mixed models (GLMMs). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant. The abbreviation of species names is same as **Figure 1**.

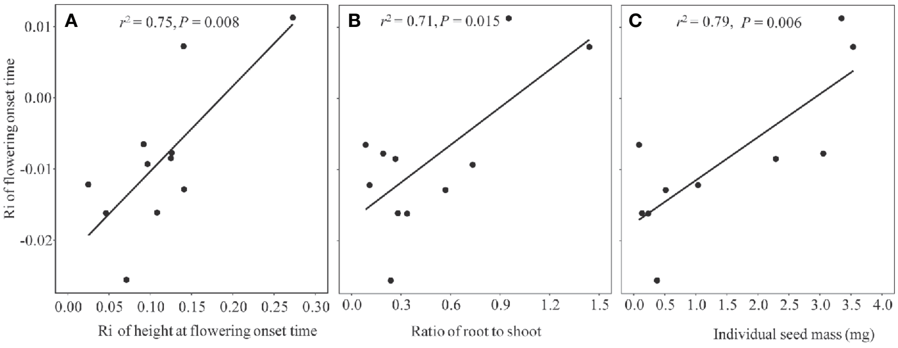

FIGURE 5 | The regression relationship between Ri index of flowering onset time and functional traits in 2017, in which more species were investigated. **(A)** Ri index of plant height at the flowering onset time, **(B)** Biomass ratio of root to shoot; **(C)** Individual seed mass (mg).

$P < 0.05$), they could have enough energy to produce large seeds in short periods of time (thus allowing for a smaller advance or even delay in flowering onset time). However, no significant relationship between the seed size and fruiting period was found, inconsistent with the seed size-time hypothesis. This inconsistency may be attributed to grazing exclusion in the chambers. Plants are normally grazed by cattle and preventing grazing may change plant vegetative growth (Diaz et al., 2007), and reproductive phenologies (Li et al., 2019), thereby disrupting the size-time relationship.

Similar to the onset and offset times, the responses of the duration of flowering and fruiting are also diverse among the study species. It seems that the durations could be lengthened, unchanged, or shortened in both study year. This is consistent with the observation that the effects of climate change on the duration of reproduction are diverse (Price and Waser, 1998; Sherry et al., 2007; Post et al., 2008; Jiang et al., 2016). The mechanisms underlying the diverse response are unknown. Because flowering and fruiting durations are crucial to the performance of pollinators and herbivorous seed predators

(Brody, 1997; Gervasi and Schiestl, 2017), the changed duration may induce change in higher trophic communities.

It is worthwhile to note that two common species *P. viviparum* and *A. rivularis* delayed their flowering. The delay can be explained by the vernalization hypothesis but not the accumulated temperature hypothesis. Moreover, *P. viviparum*, as a clonal species with bulbils, might have allocated its energy to asexual production first and then to sexual production in favorable environments. The flowering delay in the species *A. rivularis* can be explained by the change in flowering plant height, as indicated by the positive relationship between the changes in flowering height and flowering onset time.

CONCLUSION

In summary, winter-biased warming significantly changed species reproductive phenologies with most species advancing their flowering, as has been observed in previous warming studies using infrared heaters and open top chambers. These changes are inconsistent with the vernalization hypothesis, but can be partly explained by the accumulated temperature hypothesis and/or the overtopping hypothesis. This observation suggests to us that both summer-biased and winter-biased warming may result in similar changes in phenology, or that phenological changes are subject to temperature increases regardless of the season. Our data also show that not all species have the same responses (or magnitude) of change, which is also widely recorded in previous studies. We have provided a preliminary mechanistic explanation for species-specific differences, i.e., different functional traits mediate different phenological responses to warming. Even a mild warming may trigger significant changes in plant phenology, and species-specific traits can affect different reproductive phenological responses in a future much warmed world.

DATA AVAILABILITY STATEMENT

Plant phenological and traits data for this study are included in Supplementary Material.

AUTHOR CONTRIBUTIONS

SS started the project, design research, and did major revision to the manuscript. XH and WZ performed research. XH analyzed data and drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.534703/full#supplementary-material

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Chilling and Forcing From Cut Twigs—How to Simplify Phenological Experiments for Citizen Science

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Low-cost phenological experiments with cut twigs are increasingly used to study bud development in response to spring warming and photoperiod. However, a broader variety of species needs to be tackled and in particular the influence of insufficient winter chilling deserves more attention. Therefore, we investigated if and how chilling requirements can be efficiently investigated by cut twigs and how this low-tech approach could be successfully implemented as a citizen science or school project. We conducted an experiment on bud burst and leaf development of *Corylus avellana* L. twigs, with natural chilling outdoors on a shrub (S) and another chilling treatment as cut twigs in containers (C), and subsequent forcing indoors. Subsampling of the number of cutting dates and number of twigs was used to infer minimum required sample sizes. Apart from insufficiently chilled twigs, ~80% of the twigs (both S and C) reached leaf out. For multiple definitions of chilling and forcing, a negative exponential relationship was revealed between chilling and amount of forcing needed to reach certain developmental stages. At least 5 out of 15 cutting dates or alternatively half of the 10 twig repetitions, but especially those mirroring low chilling conditions, were needed to describe the chilling-forcing relationship with some degree of robustness. In addition, for cutting dates with long chilling, i.e., from January onwards, freshly cut twigs (S) required significantly more forcing to reach bud burst than twigs from containers (C), although the effect was small. In general, chilling conditions of mature shrubs were well captured by cut twigs, therefore opening the possibility of chilling through refrigeration. We conclude that experimental protocols as outlined here are feasible for citizen scientists, school projects, and science education, and would have the potential to advance the research field if carried out on a large scale. We provide an easy-to-use Shiny simulation app to enable citizen scientists to build up a bud development model based on their own experimental data and then simulate future phenological development with winter and/or spring warming. This may encourage them to further study other aspects of climate change and the impacts of climate change.

Keywords: climate change, phenology, bud burst, school project, science education, Shiny App

INTRODUCTION

Bud burst and new leaves in spring fascinate nature lovers on all continents. Their observations of spring phenology through citizen science networks are also widely used to track biological impacts of climate change around the world (Menzel, 2002; Beaubien and Hamann, 2011; Kobori et al., 2016). Yet, the environmental factors and plant physiological processes that control these spring development processes are still not fully understood. For temperate species, the interplay of cold winter temperatures that break (endo-)dormancy and warm spring temperatures forcing budburst is considered the main driver of the timing of budburst (Murray et al., 1989; Laube et al., 2014a; Polgar et al., 2014; Zohner et al., 2016). Additional cues associated with spring phenological events for some species include autumn temperature (Heide, 2003; Sparks et al., 2020), day length (Heide, 1993a; Heide, 1993b; Körner and Basler, 2010; Basler and Körner, 2012; Laube et al., 2014a), light quality (Brelsford and Robson, 2018), air humidity (Düring, 1979; Laube et al., 2014b; Zipf and Primack, 2017), or nutrient availability (Jochner et al., 2013a). Under natural *in situ* conditions it is difficult to disentangle these multiple and often co-varying environmental factors (e.g., Piao et al., 2019; Menzel et al., 2020).

In order to correctly predict budburst dates under climate change, the combined effects of cold winter temperatures (chilling requirements) and subsequent warm spring temperatures (heat sum requirements) must be considered (Chuine et al., 2016). This is an evolutionary adaptation to prevent budburst in autumn or mid-winter during unseasonal warm spells. But under climate warming, chilling requirements may not be satisfied in the future, thus preventing plants from timely budburst at the beginning of the growing season (e.g., Fu et al., 2015). Long-term *in situ* observations from phenology networks are, however, insufficient to disentangle the influence and relative importance of these two factors for different species and different geographic regions. Especially the underlying processes of chilling, i.e., the temperature response of buds during dormancy and dormancy break, are difficult to be derived from observations alone (Luedeling and Brown, 2011; Blümel and Chmielewski, 2012; Jochner et al., 2013b; Primack et al., 2015; Chuine et al., 2016).

One way to determine the effects of chilling and forcing is to experiment with woody plants under controlled conditions. Experiments with adult tree individuals under controlled (growth chamber) environments are technically challenging, however buds on twigs have been shown to serve as model units for tree individuals (Lebon et al., 2005; Basler and Körner, 2012; Laube et al., 2014a; Polgar et al., 2014; Primack et al., 2015), since excised twigs put in water may develop as they would on adult trees under normal conditions (Sønsteby and Heide, 2014; Vitasse and Basler, 2014). In contrast, tree seedlings or saplings are less desirable model units as they are known to differ in their budburst dates from adult trees (Augspurger, 2008; Vitasse et al., 2014). So far the influences of varying day length, humidity, forcing temperature and exposure time to natural chilling conditions, and freezing have been studied in factorial designed twig experiments (Laube et al., 2014a; Primack et al., 2015; Vitra et al., 2017). Mehlenbacher (1991) studied the chilling requirements of hazelnut inflorescences and vegetative buds by cutting shoots in the field at weekly intervals and forcing them in a warm greenhouse; rest completion was assumed when >50% of the buds started to develop after 3 to 4 weeks. However, horticultural studies have also exposed twigs to artificial chilling conditions for varying time periods after harvest (Gariglio et al., 2006; Jones et al., 2013; Sønsteby and Heide, 2014).

This low-tech, low-cost solution allows testing of many species. Consequently, phenological experiments on cut twigs have become more and more popular. So far, phenology experiments under controlled conditions have revealed that the influence of cold winter chilling and warm spring forcing temperatures is highly species-specific (Murray et al., 1989; Menzel et al., 2006; Laube et al., 2014a; Polgar et al., 2014; Zohner et al., 2016) and can also vary significantly among cultivars or genotypes within a species (Mehlenbacher, 1991; Gariglio et al., 2006; Sønsteby and Heide, 2014). Chilling requirements of trees may also differ among geographic regions (e.g., Mehlenbacher, 1991), leading to unexpected delays to phenology under climate warming, when chilling requirements are high (e.g., Heide, 2003; Ford et al., 2016). However, experimental scientific work was only able to capture a rather small sample of mainly northern temperate tree species, and broad inferences on regional or global phenological responses to climate change are not possible. A wider variety of species needs to be tested for solid generalizations, e.g., with respect to phylogeny or ecological strategies. Ideally, we should have experimental data of similar geographic and species coverage as we have observational data from citizen science networks for spring phenology.

Although chilling and forcing experiments are usually conducted by trained researchers in complex factorial experimental designs and are often aided by controlled environment chambers, the experiments are in principle straight-forward and could be conducted by citizen scientists at home who could thus easily help to fill some of these knowledge gaps by testing any woody species of personal preference (Primack et al., 2015). Data recording only needs a pen and paper, which is fast, easily standardized, and due to the beauty of the process highly self-rewarding. Installing and observing twig experiments during wintertime may be attractive for citizen scientists and pupils living in temperate regions, since these harbingers of spring bring attractive greenness into winterly homes. Moreover, cutting the St. Barbara twigs on December 4th has been an old tradition dating back to medieval times (e.g., Gemminger, 1874; Michels, 2004). Nevertheless, the sustainable success of citizen science experiments may depend largely on their attractiveness, a clever set-up of the experiments and data management as well as on the participants' motivation and long-term involvement.

For a successful large scale program of citizen science experiments, some research is required to develop standardized experimental protocols that can be carried out easily and yield robust results. Methods of applying chilling temperatures vary considerably, e.g., exposure of twigs to chill conditions under natural conditions and varying harvest dates, or application of chilling temperatures to cut twigs in freezers or refrigerators. It yet seems unclear which method is best suited to describe the chilling-forcing relationship properly. It remains especially unclear how often twigs need to be harvested during the dormant period, e.g., three (Laube et al., 2014a; Zohner et al., 2016), four (Polgar et al., 2014), or eight times (Murray et al., 1989) during winter, or as often as either weekly or bi-weekly (Chuine et al., 2016; Nanninga et al., 2017).

The objective of this study, therefore, was twofold. We first asked if the chilling requirements of woody species, in our case of *Corylus avellana* L., can be assessed *via* the excised twig method, using indoor and outdoor environments in lieu of growth chamber-based designs. Second, we investigated how sampling and experimental settings should be designed in order to describe the chilling-forcing relationship properly. We further tested if the twig method was a suitable tool to involve citizen scientists, reported minimum requirements, successes and drawbacks, and proposed a simulation tool to link experimental findings to potential climate change impacts.

MATERIAL AND METHODS

Experiment on Cut Twigs

The experiment was performed at the edge of a small town in southern Germany (~ 48.468° N 11.932° E), ~ 55 km northeast of Munich. In a garden adjacent to a forested area a 5 m tall hazel (*C. avellana* L.) shrub was selected. This hazel shrub had been regularly pruned in previous years; thus, uniform 2- to 3-year-old shoots branched at a height of 2 m and could be easily pruned again. The chilling-forcing experiment consisted of two chilling treatments outdoors, 1) natural chilling on the shrub (S) as well as 2) chilling of twigs that had been cut once on the first day of the experiment and then were stored outdoors in containers (C) fixed to the shrub (**Figure 1**). Treatment S represents the main experiment, while C was meant to explore the possibility of different experimental manipulations of chilling, e.g., in refrigerators, if cutting at the beginning of the trial does not have a major influence on twig development.

For both chilling treatments (S, C), spring forcing conditions were simulated by taking twigs indoors after different periods of chilling (**Figure 1**). More specifically, twigs were moved indoors to stop accumulation of chilling and to begin accumulation of heat sums (forcing) on 15 dates, beginning in 21 November 2015 until 14 March 2016 (see **Table 1**). As it would be anticipated for a citizen science study, the intervals were not always equal, which did not affect the analysis. Initial intervals were approximately 1 week until the end of January, subsequently intervals varied from 6 to 13 days.

In order to implement the chilling treatment C, on 21 November 2015, the first day of the experiment, a few randomly selected branches were cut off, and 100 twigs of 50 cm length were harvested. These twigs were placed in three green 3-L plastic containers, perforated at the bottom to avoid waterlogging, but filled with moss to prevent rapid drying. All containers with the branch supplies for the subsequent forcing experiment were thereafter attached to the hazel with metal wires at a height of 2.5 m, so that they hung outdoors in positions comparable to those of the intact branches of treatment S acquiring natural chilling (**Figure 1**). On each of the cutting dates 2 to 15 (see **Table 1**), five twigs were taken out of this prepared storage, re-cut by ~15 cm at their base, cleaned, and placed in labeled 0.1 L glass bottles filled with tap water.

On cutting dates 1 to 15, ten 30 cm long twigs of the chilling treatment S were freshly cut from the hazel shrub, cleaned, and placed in labeled 0.1 L glass bottles filled with tap water. All bottles (S and C) were placed inside a residential house on the

FIGURE 1 | Container filled with twigs harvested on 21 November 2015 (left) attached to the *Corylus avellana* L. shrub outside (treatment "C") as well as twigs in 0.1 L glass bottles filled with tap water on the windowsill inside (right).

TABLE 1 | Summary of dates when natural outdoor chilling was terminated and twigs were brought to indoor forcing conditions.

Cutting	Date	Days since start	Difference in days to last cutting date
1	2015-11-21	0	
2	2015-11-28	7	7
3	2015-12-05	14	7
4	2015-12-12	21	7
5	2015-12-19	28	7
6	2015-12-27	36	8
7	2016-01-04	44	8
8	2016-01-10	50	6
9	2016-01-17	57	7
*	2016-01-20	60	
10	2016-01-25	65	8
11	2016-02-05	76	11
12	2016-02-14	85	9
13	2016-02-24	95	10
14	2016-03-08	108	13
15	2016-03-14	114	6
*	2016-03-22	122	

*denotes days when only phenological observations were made.

south-facing inner windowsill of the kitchen. Twigs were re-cut at the lower end on each cutting date and the water was exchanged regularly on every other cutting date.

Phenological and Meteorological Data

Vitality assessment (i.e., twig status dead or alive) and phenological observations according to the Biologische Bundesanstalt Bundessortenamt und Chemische Industrie (BBCH) coding system (Meier, 2001) were done periodically throughout the experiment until 22 March 2016 (on all cutting and two additional dates, see **Table 1**). We observed the BBCH stages of bud development (principle growth stage 0) and leaf development (principle growth stage 1), i.e., 0 winter dormancy, 1 beginning of bud swelling, 3 end of bud swelling, 7 beginning of bud breaking, 9 bud shows green tips, 10 first true leaf emerged or mouse-ear stage, 11 first leaves unfolded, and 12 two first leaves. Mortality assessment was performed by carefully scratching the bark with a fingernail according to Seidel and Menzel (2016). The twigs were classified as dead when the cambium underneath was no longer green. This recording as well as the installation of the experiment (see *Experiment on Cut Twigs*) was primarily done by a 9-year-old schoolchild and her family under the supervision of the first author.

At the beginning of the experiment, two calibrated air temperature and humidity loggers (HOBO Pro v2, Onset Computer Corporation, Southern MA, USA) were placed both inside on the kitchen windowsill and outside fixed at a height of 2.5 m in the hazel shrub, where they measured these variables every 10 min (10 min temperature) in a shaded position.

Statistical Analyses

Each twig of the experiment was assigned a unique ID, which referred to the chilling treatment of the twig (twig from shrub S, twig from container C), the week or date it was taken inside to start forcing according to the predefined cutting scheme (cutting date of S or date of transfer of C, respectively) and a consecutive number. A few missing BBCH stages in the course of the observations and one missing HOBO logger day were imputed linearly. We determined for each cutting date the final BBCH stage of a twig, which was either reached when it was identified as dead, when it was removed from the experiment with leaf out (BBCH>=12), or at the end of the observations (22 March 2016). Chilling and forcing accumulation began 21 November 2015, the start of the experiment.

Chilling was determined in three ways: sum of chilling days based on the 10 min temperature recordings (temperature < 5°C, fractional days possible), sum of days with daily mean temperature < 5°C, and number of days outside. Forcing was equally determined in three ways: degree days based on the 10 min temperature recordings (temperature ≥ 5°C, fractional degree days possible), degree days based on daily mean temperature ≥ 5°C, and number of days inside. We also tested a threshold of 0°C and obtained similar results, thus only the results with a 5°C threshold were presented here. Chilling and forcing amounts were calculated from the start of the experiment until the date on which the twig was classified as dead, or having reached BBCH stage of 7, 9, 11, or leaf out.

The relationship between chilling and forcing was assumed to be exponential, as in Murray et al. (1989):

$$forcing = a + b * \exp(c * chilling) + \epsilon$$

where a, b, and c are estimated parameters, and ϵ are errors assumed to be normally distributed. The associated fits were plotted along the chilling *vs.* forcing plots.

In order to compare forcing requirements of the two chilling treatments, fresh cut twigs (treatment S) and container stored twigs (C), we calculated an ANOVA-style model with forcing depending on chilling, treatment (S or C), and their interaction. Chilling was inserted as a factorial (dummy) variable to be able to test differences for each chilling value, which corresponds to the week of cuttings. Since error variances were not identical for all chilling values, we used a GLS (generalized least squares) model, in which parameters were added to model different error variance (one for each chilling value). *Post-hoc* tests were done separately for chilling and treatment, by holding the other variable constant. P-values were adjusted for multiple testing. Some data were discarded when the number of twigs for a cutting date (chilling days) was too small (e.g., BBCH 9 or 11 and less than 25 chilling days).

In order to develop an optimal experimental protocol for implementation by citizen scientists, we studied the effect of reducing the number of observations. These sensitivity tests were performed by subsampling from the experiment and simulating the respective chilling-forcing relationships. First, we studied the effect of less frequent cutting dates. From the initial 15 cutting dates, we selected 8, 7, 5, and 4 dates, because they could be evenly distributed with uniform sampling periods (i.e., every 2, 3, or 4 weeks). For each reduced cutting numbers, we also adjusted the starting week (for 7 cuttings, starting week 1 and 2; for 5 and 4 cuttings, starting week 1, 2, and 3). The effect of the less frequent observations was compared visually using the exponential fits.

Second, we evaluated the effects of fewer branches within treatment S. At each cutting date, 10 twigs were cut from the shrubs, and we tested the effect of having only 2 to 9 twigs instead. For each number of fewer twigs (2-9), we took random samples of the 10 twigs at each cutting date, and fit the exponential curve to this subsample. This procedure was repeated 1,000 times, and the different exponential curves were plotted along the original fit to the full data set for comparison.

All computations were performed using R statistical software, version 3.6.2 (R Core Team, 2020), additionally using packages data.table (Dowle and Srinivasan, 2019), emmeans (Lenth, 2020), ggplot2 (Wickham, 2016), and the tidyverse collection (Wickham et al., 2019).

RESULTS

Meteorological Conditions

The inside HOBO logger indicated that temperature and humidity conditions were fairly stable, with the daily mean temperature fluctuating around 22.5°C and relative humidity ranging between 40 and 50% (**Figure 2**). The outside weather conditions were characterized by alternating chilling and forcing conditions. The daily mean air temperatures in December, February, and March were mostly above 0°C outdoors, whereas a longer cold spell occurred in mid-January. Relative humidity was mostly above 90% until the end of January, and gradually decreased from February onwards (**Figure 2**).

Twig Development and Survival

Not all twigs reached the last BBCH developmental stage recorded (**Figure 3**). The percentage of twigs that fully developed beyond BBCH 12 depended on the cutting date (S) or the date on which the twigs were brought inside from the containers (C), respectively. About 40% of the twigs from cutting dates 1 and 2 (S) and cutting date 2 (C) in November with almost no chilling failed to develop and were later classified as dead. For all other cutting dates, less than 20% of the twigs did not develop by the end of the experiment. Of the twigs brought inside from week 3 to 12, a higher percentage of those previously stored in the containers reached full leaf out. Twigs that were cut at one of the last cutting dates in March (13 to 15) usually had already been close to BBCH 11, whereas twigs from the containers were somewhat delayed in their development outdoors. The development of those twigs could be observed till stage BBCH 12 and beyond, since no twigs had to be removed any more as fully leafed twigs due to space restrictions.

Analysis and Simplification of Chilling-Forcing Relationships

For all treatments (S, C), BBCH stages and definitions of chilling and forcing, an inverse relationship was clearly shown between acquired chilling and amount of forcing required to reach certain development stages (**Figure 4**). For all stages (dead, BBCH 7, 9, 11, leaf out) this reciprocal relationship was almost identical regardless of the definition of chilling and forcing used (5 or 0°C threshold, based on 10 min or daily temperature values, as the number of days or daily sums). Even the simplest approach, defining the days outside as chilling days and the days inside as forcing days, led to similar relationships.

The later the developmental stage (BBCH 7, 9, 11), the higher the forcing accumulated up to that point in time for a given cutting date/chilling. Considerably higher forcing sums for leaf out and dead twigs can, however, be distorted by the fact that the

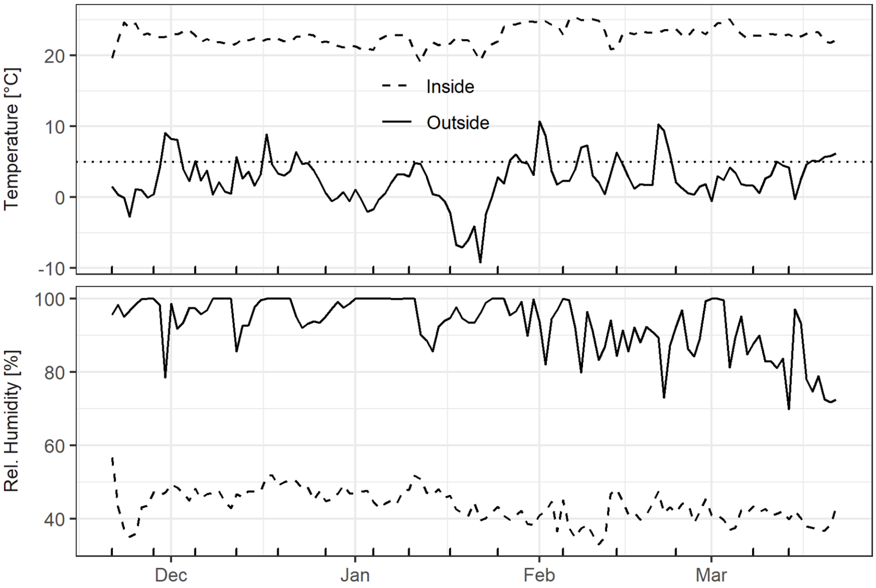


FIGURE 2 | Daily mean air temperature and air humidity outside at a height of 2.5 m in the hazel shrub as well as inside on the south-facing windowsill. Dotted line in the top panel represents the 5°C threshold used for chilling/forcing and the x-axis ticks at the bottom indicate the cutting dates.

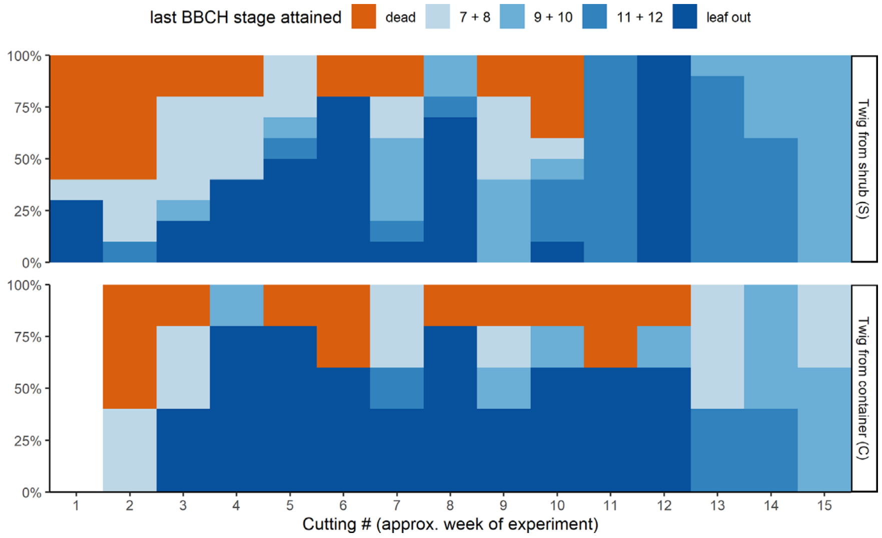


FIGURE 3 | Last developmental stages attained of the ten freshly cut twigs from the shrub (S, top) and of the five twigs taken from the container storage (C, bottom) for the different cuttings.

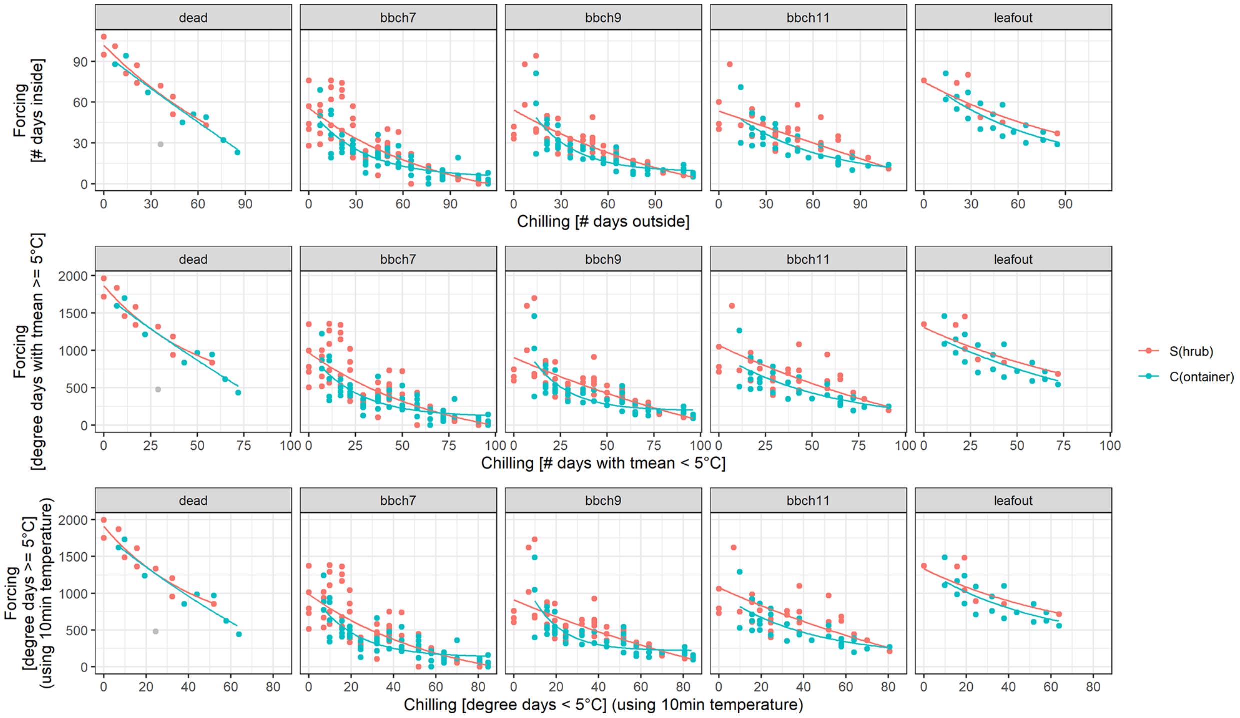


FIGURE 4 | Chilling-forcing relationships for the three different chilling and forcing definitions as well as for the two twig treatments (S freshly cut from the shrub, C previously stored cut in containers outside). Lines are the fitted exponential functions (see *Material and Methods* for equation). The gray point in the panel "dead" was considered an outlier and was excluded from the fitting.

final decision to remove a twig as fully developed or dead obviously took some time. For the later cutting dates (longer chilling), the apparent chilling-forcing relationship was fairly linear, whereas earlier cutting dates with less chilling led to the typical negative exponential function.

For median chilling/cutting dates, the required forcing for twigs previously stored in containers (C) was slightly less than for freshly cut twigs (S). However, formal testing for significant differences revealed that not all of these differences were statistically significant (**Figure 5**). In case of BBCH 7, cutting dates with long chilling in particular led to significant differences. All twigs that were cut on day 44 or later of the experiment, i.e., from January onwards, required significantly more forcing for twigs freshly cut (S) to reach BBCH 9 than for C. The same result was observed for BBCH 11, although fewer cutting dates exhibited these significant differences. Within the treatments (S, C), the forcing sums of up to about five consecutive cutting dates were not statistically significant from each other.

Simulations of the chilling-forcing relationships based on days outside and inside suggested that at least 5 to 8 cutting dates may be needed to derive stable relationships comparable to the results of the full set of 15 cutting dates from late November to early March. It was particularly important that initial sampling dates representing low levels of chilling support a solid representation and should therefore not be missed or discarded. The fewer cutting dates there were, the greater the influence of the first recordings in November on the overall chilling-forcing relationship was (**Figure 6**).

An alternative way to reduce the workload for the citizen scientists would be to reduce the number of twigs that are harvested and observed in the house. **Figure 7** displays the results of BBCH 7 simulations based on treatment S with 10 twigs at each cutting date. The exponential fit of the chilling-forcing relationship is quite stable for 9 to 7 repetitions. For 6 and 5 repetitions, the fits deteriorate on the side with little chilling which is particularly important for estimating impacts of future climate change with reduced chilling in connection with winter warming. With less than 5 repetitions, there is a considerable variation in fits.

DISCUSSION

Practicability for Citizen Science/School Projects

This study demonstrated that a phenology experiment could be successfully conducted in the home of a citizen scientist. Several points addressed in the paper may facilitate the implementation of twig experiments. Twig experiments do not require complicated and expensive equipment, but only a pair of pruning shears, if applicable plastic containers for outdoors, glass bottles, permanent markers, paper, and pencil. Manipulation of chilling and forcing temperatures can easily be achieved by staggered cutting dates and different indoor conditions (heated room, unheated room, and refrigerator). In our study the indoor air temperature fluctuated only slightly, and these fairly constant indoor conditions can be assumed for houses with central heating. In our study the outside chilling conditions were also (by chance) quite stable during winter. Consequently, the chilling-forcing relationship was described equally well by the number of days inside (in lieu of calculating forcing degree days) *versus* number of days outside (in lieu of using exact chilling degree days). However, in other years, strongly fluctuating outside conditions may be possible, so that recording of temperatures

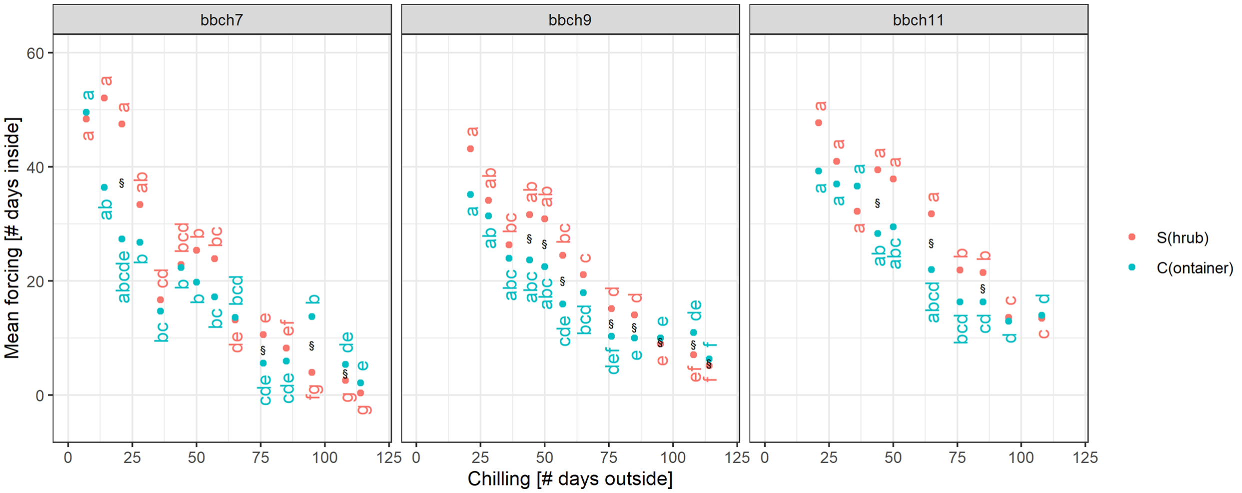


FIGURE 5 | Significance of differences in chilling and forcing at three developmental stages. Dots indicated the mean forcing (days inside) and mean chilling (days outside), § indicates a significant difference in forcing between twigs freshly cut from the shrub (S) and taken from the containers (C) at the single cutting dates. Small letters indicate significant differences of forcing between the cutting dates (or chilling days outside), separately for twigs from shrub (S) or containers (C). Mean estimates and tests based on an ANOVA-style model (see *Material and Methods* for more details), significance based on p-value < 0.05, and p-values adjusted for multiple testing.

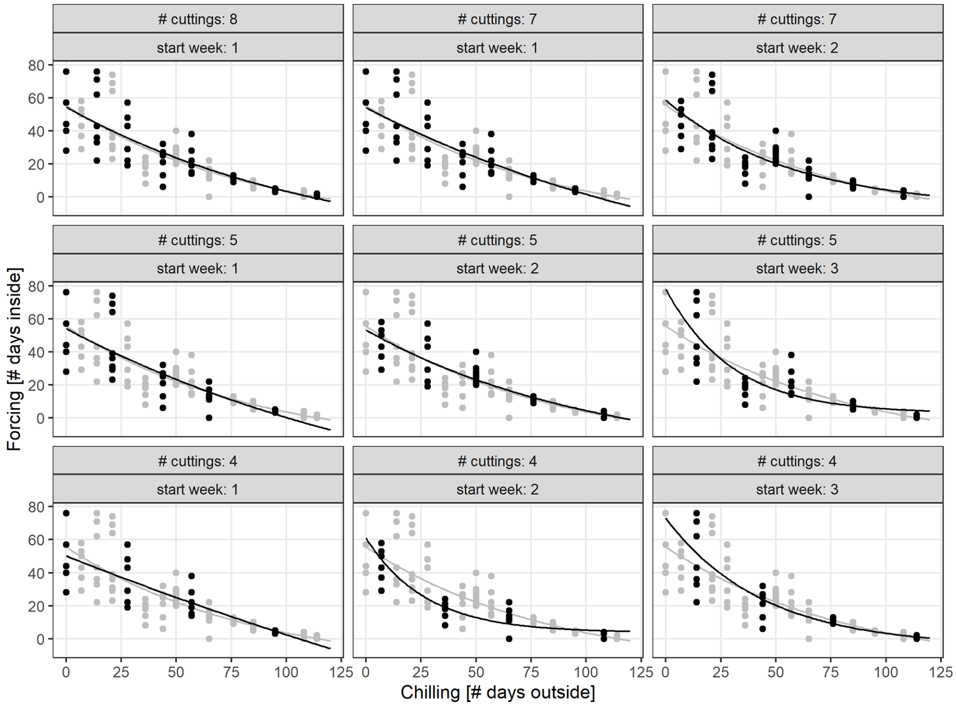


FIGURE 6 | Simulation of Biologische Bundesanstalt Bundessortenamt und Chemische Industrie (BBCH) 7 chilling-forcing relationships for less intensive monitoring (less cuttings/observations and different starting dates; indicated in panel titles). The full dataset (gray) is for twigs from shrub (S), the black dots are the simulated smaller datasets.

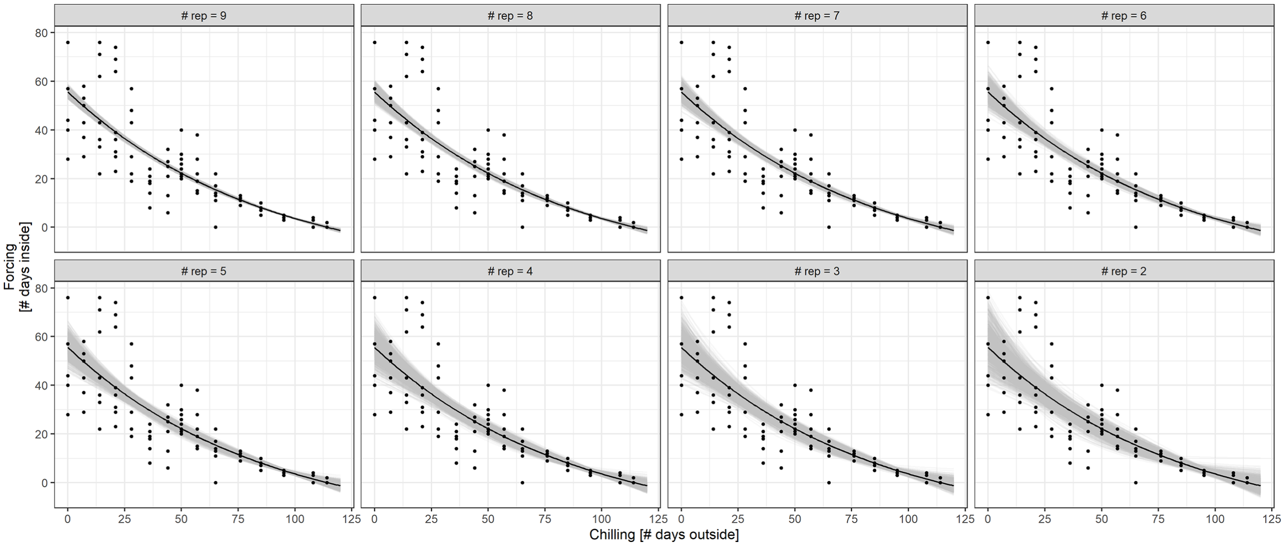


FIGURE 7 | Simulation of BBCH 7 chilling-forcing relationships for less repetitions (fewer twigs cut) in treatment S. The full dataset with the associated exponential fit is shown in black (10 twigs/points per chilling value; overplotting since multiple points with same values). The gray lines indicate fits to random subsamples with 9 to 2 repetitions instead of 10 (1,000 random subsamples per repetition value).

can be useful and is therefore recommended. We were able to measure this reference data, but it has to be assumed that in large scale citizen science networks this won't be always the case. Then, gridded daily temperature records could be used, and indoor temperatures could be assumed constant, based on an average of several readings. Alternatively, nearby weather station data or interpolated data products could be used, which are usually freely available from national or regional weather services. Although these may not account for local microenvironments, they still represent the major weather situation and would stabilize the growing-chilling relationship, thus allowing accurate inferences on the magnitude of chilling requirements for a particular species and region.

Unfortunately some issues may also impede a successful implementation. Studying different species or treatments takes up quite a bit of space, which may not be available in many households. In our case, 15 times putting 15 twigs (10 from the shrub and 5 from the containers) in the house resulted in more than 140 twigs, which had to be observed simultaneously in late January and early February. In our trial space restrictions led to twigs beyond BBCH 12 being removed and classified as full leaf out. Other interested citizen scientists could not participate due to lack of space in their apartments. Therefore, carrying out these projects in schools with more spacious classrooms and a larger floor space may be an alternative. However, schools being closed during the obligatory winter holidays (Christmas, New Year's Eve) would result in an unintentional interruption of observations and supervision of the experiments. Furthermore, a reduction of the heating during these school holidays can lead to an inhomogeneity in the forcing conditions applied. Citizen science projects could be implemented in other locations (i.e., non-school or home settings), too, such as senior or community centers.

The choice of appropriate species may depend on the availability of specimen in the vicinity. Species with earlier budburst allow welcome activities during the dark and dormant winter season. Unfortunately some of these species produce allergenic pollen (e.g., the genera *Corylus*, *Alnus*, and *Betula*), but their release could be avoided by removing male catkins. In case of severe allergic predispositions, these species should be avoided.

Suitability in Science Education

The twig experiment conducted in our study was not accompanied by formal educational surveys. Nevertheless, we assume that these experiments are well suited for science education in the context of climate change, as they deal with a relevant question in a project-driven, vivid, and interactive way. A twig experiment can be easily integrated into everyday life and literally can be carried out "in the own backyard" or home in this case. These hands-on activities motivate participants, and promote the acquisition of knowledge and scientific core competencies. The approach described is thus a textbook example of "inquiry-based learning (IBL)" which enables participants to learn about climate change impacts in their immediate surroundings (Brumann et al., 2019). In our study, observations of the participating student (motivation, work input) and a later interview supported this positive assessment. Especially the "hands-on" installation of the experiments and the cutting of twigs were welcome physical activities.

The interplay of hands-on and minds-on activities and the degree of self-activity can be varied in order to increase and harness the potential of such citizen scientists' experiments. Participants could formulate and test their own hypotheses, e.g., on environmental drivers of bud burst and leaf development. They could formulate their own ideas, try out new experimental settings or incorporate other species in future trials. Consequently, depending on their specific prerequisites or needs during their learning process, participants can complete a typical scientific research cycle in whole or in part, e.g., by developing research questions and hypotheses, selecting and applying appropriate methods, and analyzing, interpreting, and presenting the results (Huber, 2009; Reitinger, 2013; Pedaste et al., 2015). In this way, they will acquire considerable competence in scientific methods and experiments.

In school projects, the teachers provide the pupils with appropriate assistance. These adequate measures of guidance are important for the success of inquiry-based learning processes (see Lazonder and Harmsen, 2016). In general, all citizen scientists must be supported in documenting their observations and in their analyses. Therefore, an easily understandable observation manual with many illustrations as well as data entry tables (printed or computer-based) for noting down observations should be provided. In the medium term, an internet platform on which the observations can be systematically uploaded and evaluated can also support participants in their analyses. However, it is not required that all participants carry out statistical analyses. The above-mentioned inverse relationship between the acquired chilling and the amount of forcing required to reach certain development stages can be shown without formal analyses as the results can be understood by looking at simple graphs of the results. Especially younger students can also work with the simplest approach described above and define the days outside as chilling days and the days inside as forcing days.

Our study design required a high level of motivation over a considerably long period of time for school children. Thus, the question of how frequently and when twigs have to be harvested or how many twigs are needed per cutting date is of central importance. Savings at this point would reduce not only the workload, but also the space required (see *Practicability for Citizen Science/School Projects*). We have shown that the cutting dates (chilling treatments) can be reduced from weekly to bi-weekly, but especially the early cutting dates were necessary to describe the chilling-forcing relationship as accurately as possible. Similarly, halving the number of repetitions/twigs would still yield robust relationships as long as low chilling data are sufficiently represented. However, both ways of reducing the workload imply at the same time that the experiment would still last almost 4 months and that additional motivational incentives might be needed to ensure that participants carefully follow the regular phenological observations and twig care.

Finally, measures to adapt the twig experiment to the specific prerequisites and needs of the participants suggest positive motivational effects in line with the self-determination theory

(SDT) addressing the factors autonomy, competence, and relatedness (Van den Broeck et al., 2010; Thomas and Müller, 2014; Dettweiler et al., 2015). Following a given experimental setup and a fixed observation scheme may not suggest a high degree of autonomy, but reworking the experimental setup and formulating new hypotheses to be tested allow for autonomous decisions. Relatedness can be strengthened if the experiment is conducted together with family, friends or in a school context. The experience of competence can result from the sense of achievement of having made one's own scientific discoveries. However, positive effects are not only to be expected in terms of *perception* of competence, but also in terms of actual development of competences, such as scientific knowledge in plant phenology, dendrology, and micrometeorology, as well as expertise in methodological skills in the sense of the scientific (propaedeutic) method (conducting experiments, collecting and analyzing data, presenting results).

This exemplary experience of the nature of science (McComas, 1998) can in turn contribute to a positive development of the participants' epistemological beliefs, i.e., their individual views on the genesis, ontology, meaning, justification and validity of knowledge in science (Priemer, 2006). This is particularly beneficial in the context of climate change, where "fake news" is not uncommon and more understanding of science and scientific work is needed.

Chilling Experiments With Twigs

For different definitions of chilling and forcing as well as for the two treatments (S, C) the well-known negative exponential relationship as proposed e.g., by Murray et al. (1989) and many others afterwards, could be confirmed; however, in our described prototype citizen science experiment, only for one donor plant and only for 1 year. Therefore, all technical results have to be assessed with caution. The related research question was whether chilling requirements of plant species can be appropriately studied by cut and experimentally manipulated twigs. Previous studies showed that twigs can be used as a proxy for adult trees' phenological behavior, i.e., budburst timing (Vitasse and Basler, 2014). Vitasse and Basler (2014) compared the thermal time, i.e., the accumulation of degree days above 5°C since Mar 1st, to reach budburst for cut twigs put in water bottles directly positioned underneath the corresponding trees. In their study, the bud and leaf development of donor trees (adults and saplings) and their corresponding cuttings were well in parallel, with a slighter (mostly non-significant) delay of cuttings. However, using cut twigs needs to be evaluated as an experimental technique not only for studying forcing conditions, but also for investigating chilling requirements.

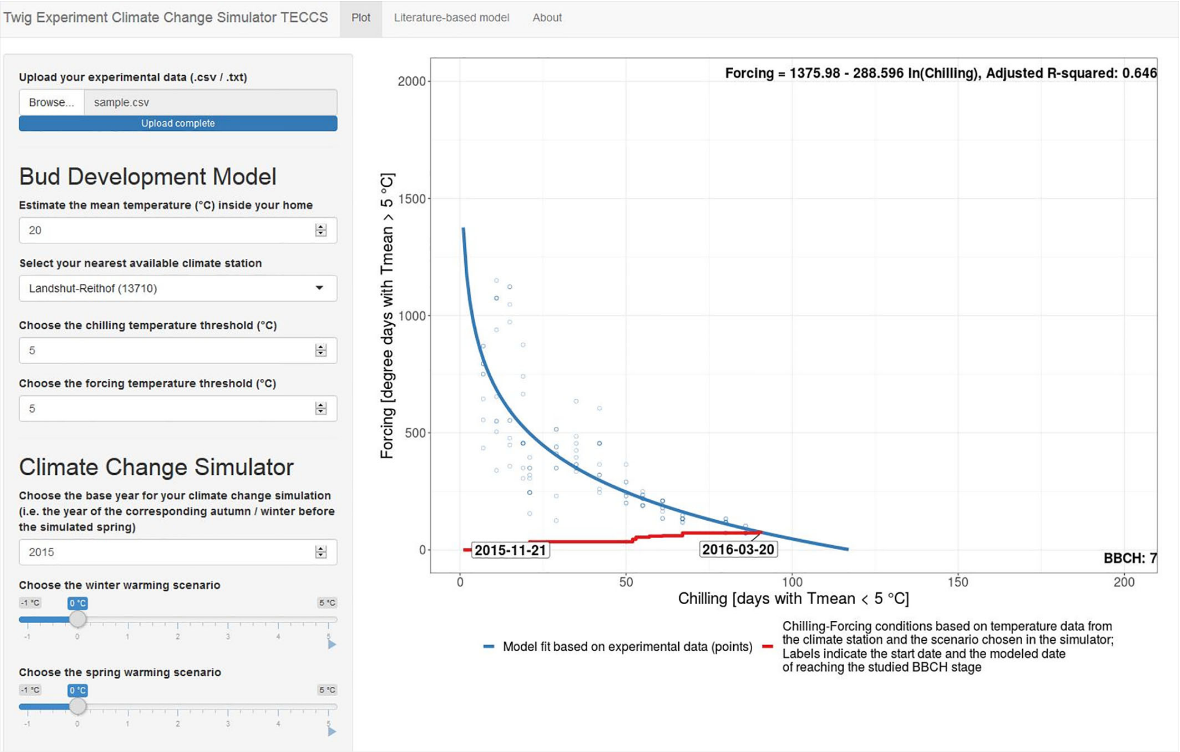
Vegetative buds of 6 out of 10 twigs cut on November 21st and 28th did not develop and were later classified as dead. This mortality rate seems high, however according to Mehlenbacher (1991) vegetative buds of hazel cultivars need 365–480 to 1,395–1,550 h of accumulated chilling (between 0 and 7°C) for >50% of the buds to develop. His conclusion was that the chilling requirement of vegetative buds is a major consideration in determining the area of cultivar adaptation. Therefore it is most likely that the chilling accumulated until the end of November in our experiment was not sufficient to complete rest. Equally for later cutting dates, some twigs did not develop, but more investigations are needed to verify whether this is related to the individual donor tree or is a general feature to be possibly overcome by more frequent recuts at the twig base or additives to the water.

We demonstrated that the chilling requirement of buds of a mature shrub was well captured by cut twigs stored outside in containers as the chilling-forcing relationships were similar between the two treatments. This finding allows proceeding with different experimental manipulations of chilling, e.g., in refrigerators. Interestingly, for identical chilling temperatures outside and forcing temperatures inside, twigs previously stored in the containers (C) needed less forcing days inside than the freshly cut twigs (S). The effect has to be related to the cutting as such, e.g., cut and interruption of transport, e.g., of dormancy promoting signals mediating the temperature response (Singh et al., 2016) or of water, if enhanced drying supported acquired chilling as Laube et al. (2014b) suggested. These differences were statistically significant, but small in magnitude for several, but not all of the cutting dates. Thus, more scientific chilling experiments are needed to get further insight into these slight differences in chilling of cut twigs *versus* intact branches. In addition, before making a broad recommendation for the use of cut twigs in citizen science experiments, trials should be conducted with more plant species.

OUTLOOK

We find that phenology experimentation with cut twigs could be a useful tool for education and outreach as Primack et al. (2015) proposed. Such trials also have potential to advance the field through large scale citizen science networks and secondary school projects by studying chilling (and forcing) requirements. These activities could contribute not only to education in phenology, dendrology, and weather, but also help to raise awareness of climate change and its impacts on nature.

For this, we developed the easy-to-use simulation tool TECCS (twig experiment climate change simulator) to study potential effects of winter and/or spring warming on budburst dates (**Figure 8**). This Shiny App allows citizen scientists 1) to upload her/his twig observation data in predefined formats (.txt/.csv, corresponding templates provided in **Supplementary Material**), 2) to easily import corresponding outdoor temperature data from the nearest climate station and to estimate forcing conditions by indicating the mean indoor room temperature, 3) to fit a chilling (CD) forcing (GDD) model on the uploaded data based on the equation GDDcrit = a +b *ln CD, 4) to vary CD and GDD thresholds from the default 5°C threshold, and 5) to simulate future bud development for a chosen base year and winter and spring warming scenarios between −1 and +5°C. Once the temperature-based chilling-forcing relationships have been estimated on their own experimental data, the TECCS simulation



Upload your experimental data (.csv / .txt): sample.csv

Estimate the mean temperature (°C) inside your home: 20

Select your nearest available climate station: Landshut-Reithof (13710)

Choose the chilling temperature threshold (°C): 5

Choose the forcing temperature threshold (°C): 5

Choose the base year for your climate change simulation (i.e. the year of the corresponding autumn / winter before the simulated spring): 2015

Choose the winter warming scenario: 0 °C

Choose the spring warming scenario: 0 °C

FIGURE 8 | Shiny App Interface of the twig experiment climate change simulator (TECCS). The Shiny App is available at www.baysics.de as well as https://github.com/yuan-oekoklima/TECCS.git for the source code. Instructions on the twig experiment for TECCS are provided in **Supplementary Material**.

tool thus allows the citizen scientists to autonomously analyze in terms of model building and simulations to answer important questions, such as "what is the likely effect of a 1°C winter warming and a 2°C spring warming on the timing of bud development?". The TECCS Shiny App (version 1 for Bavarian climate station data) is available at www.baysics.de as well as the source code available at https://github.com/yuan-oekoklima/TECCS.git.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AM designed the study, and assisted the installation and running of the experiment conducted by our citizen scientist family. MM performed the statistical analyses. YY contributed the Shiny app, and UO the didactical part. AM wrote the first draft of the manuscript, which all authors critically checked and improved by distinct contributions. All authors contributed to the article and approved the submitted version.

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Invertebrate Decline Leads to Shifts in Plant Species Abundance and Phenology

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Climate and land-use change lead to decreasing invertebrate biomass and alter invertebrate communities. These biotic changes may affect plant species abundance and phenology. Using 24 controlled experimental units in the iDiv Ecotron, we assessed the effects of invertebrate decline on an artificial grassland community formed by 12 herbaceous plant species. More specifically, we used Malaise traps and sweep nets to collect invertebrates from a local tall oatgrass meadow and included them in our Ecotron units at two different invertebrate densities: 100% (no invertebrate decline) and 25% (invertebrate decline of 75%). Another eight EcoUnits received no fauna and served as a control. Plant species abundance and flowering phenology was observed weekly over a period of 18 weeks. Our results showed that invertebrate densities affected the abundance and phenology of plant species. We observed a distinct species abundance shift with respect to the invertebrate treatment. Notably, this shift included a reduction in the abundance of the dominant plant species, *Trifolium pratense*, when invertebrates were present. Additionally, we found that the species shifted their flowering phenology as a response to the different invertebrate treatments, e.g. with decreasing invertebrate biomass *Lotus corniculatus* showed a later peak flowering time. We demonstrated that in addition to already well-studied abiotic drivers, biotic components may also drive phenological changes in plant communities. This study clearly suggests that invertebrate decline may contribute to already observed mismatches between plants and animals, with potential negative consequences for ecosystem services like food provision and pollination success. This deterioration of ecosystem function could enhance the loss of insects and plant biodiversity.

Keywords: flowering phenology, global change, iDiv Ecotron, insect decline, biotic interaction, global change experiment, peak flowering, trophic cascading

INTRODUCTION

Global warming and land-use changes alter ecosystems worldwide (Estes et al., 2011; Rasmann et al., 2014; Giling et al., 2019). Insect species go extinct (Dirzo et al., 2014; Sánchez-Bayo and Wyckhuys, 2019; Seibold et al., 2019), and the insect biomass decreases dramatically (Hallmann et al., 2017; Seibold et al., 2019). As a consequence, invertebrate community composition changes, as some invertebrates shift their distributions, causing alterations in co-occurrence patterns (Rasmann et al., 2014). Some future scenarios predict an increase in herbivory and herbivore pest outbreaks because of reduced top-down regulation due to missing key predators resulting from rising temperatures, nitrogen deposition, and habitat loss (Coley, 1998; Voigt et al., 2007; de Sassi and Tylianakis, 2012; Sánchez-Bayo and Wyckhuys, 2019; Seibold et al., 2019). Further, higher trophic levels may be more affected by environmental change than lower trophic levels (Coley, 1998; Voigt et al., 2007; de Sassi and Tylianakis, 2012). Thus, herbivorous invertebrates may benefit from both, a warmer climate favoring their developmental times and lower predator pressure, which may subsequently favor pest outbreaks (Coley, 1998).

In addition to altering insect community dynamics, climate and land-use changes also shift plant species abundances and flowering phenology. Alpine grasslands, for example, alter their plant community structure showing an increase in grass abundance due to rising temperatures (Liu et al., 2018). Grassland species from warm and temperate regions are also susceptible to anthropogenic land-use changes, such as fertilization, grazing and clipping (Borer et al., 2014; Hautier et al., 2014; Shi et al., 2015). In terms of their phenology, changes in first and last flowering day, flowering duration or peak flowering are all associated with an increase in temperatures (Menzel et al., 2006; Bock et al., 2014; Bucher et al., 2018; König et al., 2018; Bucher and Römermann, 2020). CaraDonna et al. (2014) documented temperature-driven shifts in plant communities over 39 years and stated that species-specific changes in phenology can alter temporal co-occurrence patterns. Previous findings revealed that some plant species advance or prolong their flowering period in response to changing climatic conditions or land use changes, whereas other species do not respond at all (Bock et al., 2014; CaraDonna et al., 2014; Moore and Lauenroth, 2017; Bucher et al., 2018; Bucher and Römermann, 2020).

However, climate change and land use do not only lead to phenological changes in plants, they also affect invertebrate phenology (Rathcke and Lacey, 1985; Root et al., 2003; Bartomeus et al., 2011; Burkle et al., 2013; Ovaskainen et al., 2013) and biotic interactions. In responses to rising temperatures, some bee species exhibited a larger shift in phenology than plants (Burkle et al., 2013), whereas certain solitary spring bees did not advance their phenology as much as their host plants (Kehrberger and Holzschuh, 2019). Biotic changes themselves, such as the loss of plant diversity (Wolf et al., 2017) and invertebrate biomass (Hallmann et al., 2017; Seibold et al., 2019), affect ecological relationships, e.g. plant-pollinator or competitive interactions which are related to plant fitness (Rathcke and Lacey, 1985; Visser and Both, 2005; Parmesan, 2007; Kehrberger and Holzschuh, 2019). As those plant-pollinator interaction networks seem to be less resilient to future changes, mismatches in biotic interactions are likely (Burkle et al., 2013).

Notably, multitrophic interactions, such as the relationship between plants and invertebrates, affect plant species abundance and phenology: For example, herbivore pressure is positively correlated with the number of flowers produced by a plant individual (Strauss et al., 1996). Poveda et al. (2003) reported shorter flowering durations as a response to increased herbivory and Trunschke and Stöcklin (2017) found an extension of flowering duration when pollinators were excluded. These findings provide empirical evidence for biotic interactions altering plant phenology. Consequently, these alterations may not only lead to mismatches in plant-insect interactions due to species loss and shifts in phenology but may also cause losses of ecosystem functions such as flower availability. However, despite this evidence for biotic interactions changing plant species abundance and phenology (Strauss et al., 1996; Poveda et al., 2003; Trunschke and Stöcklin, 2017; Kehrberger and Holzschuh, 2019), there have been few studies exploring potential invertebrate density effects on plant abundance and phenology.

This study aims at addressing this gap and identifying the link between invertebrate decline and plant species abundance and phenology. More specifically, we established 12-species grassland communities in 24 controlled Ecotron chambers (Eisenhauer and Türke, 2018) and with three different treatments simulating a decrease in invertebrate density by 0%, 75% and 100%. We used this experiment to answer the following questions: 1) Does a decrease in invertebrate density affect plant species composition? 2) Does a decrease in invertebrate density affect flower phenology? This research leads to a better understanding of the effects of changing invertebrate density on plant species composition and phenology in the future and evaluates the indirect effects that changes in land use may have on biodiversity.

MATERIALS AND METHODS

Experimental Setup

The experiment was carried out at the iDiv Ecotron (Eisenhauer and Türke, 2018) at the research station of the Helmholtz-Centre for Environmental Research (UFZ) in Bad Lauchstädt, Germany (51° 22' 60N, 11° 50' 60E, 118 m a.s.l.). It is located in the Central German dry area (Querfurter Platte) with a mean annual temperature of 8.9°C (1896-2013) as well as a mean annual precipitation of 489 mm (1896-2013) (Schädler et al., 2019; Siebert et al., 2019). Here, we used 24 identical experimental units (EcoUnits) with controlled environmental conditions such as light, air, and soil temperature, and irrigation (Eisenhauer and Türke, 2018). The EcoUnits further allowed us to observe the vegetation *via* two HD-IP-video cameras per EcoUnit which provided pictures taken at two different angles. Taken together, they captured at a minimum 50% of each EcoUnit. The cameras took one picture every day at 18:00 CEST with a resolution of 2688*1520 (4085760 pixels). Outdoor seasonal changes regarding the day length and temperature were mimicked. One EcoUnit combined 1.2 m^3 of standardized soil mixture (see below) and a usable air space of about 2 m^3. The soil surface was a square of 1.5 m^2. The outer dimensions of one EcoUnit was 1.55 m × 1.55 m × 3.20 m (L × W × H). For the belowground part, the internal dimensions was 1.24 m × 1.24 m × 0.80 m (L × W × H) and for the aboveground part it was 1.46 m × 1.46 m × 1.50 m (L × W × H). Prior to the experiment, the EcoUnits were filled with sieved (15 mm mesh size) top soil (80%) and sand (20%) mixture purchased by commercial suppliers (LAV Technische Dienste GmbH & Co.KG, Erdwerk Kulkwitz). Approximately 20 kg of soil from the sites where invertebrate sampling was carried out (see below) was added to each EcoUnit to inoculate soil organisms, such as soil microorganisms, microfauna (e.g. nematodes), and mesofauna (e.g. Collembola and mites), to establish a similar soil invertebrate community in the EcoUnits. This grassland site was formerly used as an arable field, where the last crop cultivation happened in 2012. The soil of the Querfurter Platte is a Haplic Chernozem, which has a high fertility and was developed on carbonatic loess substrate containing 70% silt and 20% clay. Values for pH ranged from 5.8 to 7.5, the total carbon content varied between 1.71% and 2.09% and total nitrogen ranged from 0.15% to 0.18%, in the upper 15 cm (Schädler et al., 2019; Siebert et al., 2019). Abiotic conditions of all EcoUnits were optimized to provide suitable growth conditions for the target plants: day time ranged from 5:00 to 21:00 with transitions from 0% illumination at 4:00 to 100% at 6:00 and 100% illumination at 20:00 to 0% at 22:00. The average air temperature at 30 cm above the ground level during the daytime was 24°C and changed to 19°C on average at night. Due to extreme hot weather which overheated the building the maximum temperature reached 28°C in the afternoon for a period of 10 days from May 20th. The average soil temperature at 9 cm below soil surface was 18°C. The irrigation volume was 6 l of de-ionized water per day per EcoUnit including an overflow at the edges. The same amount of viable seeds for each of 12 selected plant species (**Supplementary Table 1**, three grasses, nine herbs) belonging to a tall oatgrass meadow (Arrhenatherion elatioris) was directly sown into EcoUnits, equalling a total of 1,320 seeds (n = 1,000 viable seeds per m^2 of plant growth area). We chose species that are insect pollinated (except for the grasses which are predominantly pollinated by wind) and which are known to flower in the first year after sowing based on experience from a biodiversity experiment, the so called Jena Experiment (Weisser et al., 2017) located in 70 km distance to the Ecotron. The seed material was provided by Rieger Hofmann GmbH, Blaufelden-Raboldshausen, Germany, and was chosen from origin area No. 2 "Mitteldeutsches Tief- und Hügelland" after the rules of the Association of German Wild Seeds Producers. The species-specific numbers of viable seeds were calculated based on thousand grain weight and adjusted to germination rates, which were assessed in the laboratory beforehand. Therefore, 30 seeds of a single species were sown in a tray filled with the same soil that we used for the experiment with two replicates per species (n = 60 seeds). The seeds were not scarified prior to seeding. Germlings were counted and removed daily for a period of 22 days. Using germination rates, the required amount of seeds referring to an equal number of 110 viable seeds per species were mixed and applied regularly in EcoUnits. The seeds were sown on April 19th 2018. The experiment ended on November 15th 2018. This analysis includes data from April 26th to August 20th 2018, as there was a mid-term harvest after August 20th 2018. Thus, we had a study period of 18 weeks.

To control for potential effects of soil nutrients on plant abundance and phenology, the plant-available nutrients were examined in the soil solution. Soil solution was sampled using suction cups with a diameter of 20 mm, a length of 50 mm, a bubble point 0.89 bar, and an average pore size of 1 μm (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany) four times during the study period of 18 weeks. The sampling bottles were continuously evacuated to a negative pressure of -20 kPa. Cumulative soil water was sampled fortnightly and processed immediately for measuring the concentrations of dissolved inorganic nitrogen species (NO_3^- and NH_4^+), phosphate (PO_4^{3-}), and potassium (K^+). Measurements for NO_3^- and PO_4^{3-} were performed on an ion chromatography system DX-500 (Thermo Fisher Scientific GmbH, Dreieich, Germany), while NH_4^+ and K^+ were quantified on an ion chromatography system ICS-5000 (Thermo Fisher Scientific GmbH, Dreieich, Germany). The soil nutrient analyses revealed no difference across the treatments and over time (**Supplementary Table 2**).

To assess the effect of declining invertebrate densities on plant species abundance and phenology, three different invertebrate treatments (100%, 25%, 0%) were established with eight replicate EcoUnits each. Invertebrates were caught on adjacent oatgrass meadows of the research station in Bad Lauchstädt using Malaise traps (tall end height: 1.7 m, short end height: 0.9 m, width: 1.15 m, length: 1.88 m) with a catching height of 1 m to capture all plant visiting invertebrates, and sweep nets to catch the invertebrates directly from the vegetation. For both catching methods, we applied different catching efficiencies corresponding to two different invertebrate densities: 100% and 25%. The 100%-treatment simulated a situation without any invertebrate decline with respect to current local conditions, while the 25%-treatment corresponded to a 75% decline of the current local conditions. In addition, we added a 0%-treatment, in which no invertebrates were added. To assess the true area-specific biomass of invertebrates at the sampling site, we took suction samples using cages of the dimensions of the aboveground part of an EcoUnit (1.5 m * 1.5 m). The invertebrates, which were caught with Malaise traps and sweep nets, were introduced into the EcoUnits of the corresponding treatments 5 weeks after seed sowing when plant leaves were fully developed. To simulate natural species turnover, invertebrates were removed and replaced with newly collected specimens after eight and 13 weeks, respectively (**Figure 1**). To remove the invertebrates from the units, a modified commercial vacuum cleaner (Bosch Industriestaubsauger GAS 25) was used following a standardized procedure that defined a specific time frame of equal length for the extraction from one segment (four per EcoUnit). In parallel, invertebrates were caught in adjacent meadows as described above and introduced during the next period (**Figure 1**). Notably, this suction of invertebrates was applied to all EcoUnits to keep the disturbance levels constant across the treatments. After 18 weeks of the experiment, a third

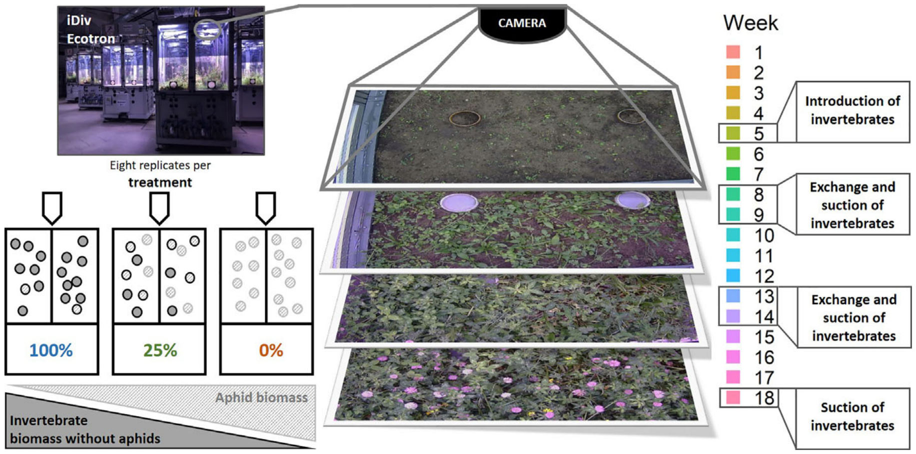

FIGURE 1 | Set up of the Ecotron experiment to assess the effect of a loss of invertebrate biomass on plant species composition and phenology of an experimental 12-species grassland community. The photo in the top-left corner shows the iDiv Ecotron (Eisenhauer and Türke, 2018). In each EcoUnit, cameras were installed which took daily pictures of the vegetation (middle). Different treatments were applied to simulate changes in invertebrate biomass (100%, 25%, and 0%) with eight replicates each (bottom left). A decrease in invertebrate biomass negatively correlated with aphid biomass (bottom left and **Supplementary Figure 1**). As indicated in the timeline (right), invertebrate introduction took place after 5 weeks, invertebrate exchange after 8 and 13 weeks. The last suction marked the end of our experiment.

invertebrate sampling was applied in all EcoUnits. Thus, we had three sampling dates, where the invertebrates were identified and weighed. As we used the biomass to analyze the invertebrate treatment (see below), we will refer to it by using invertebrate biomass instead of invertebrate density.

Even though the establishment of the invertebrate treatments was successful, we observed aphid infestations (Aphidina species), which increased in severity from the 100%- to the 0%-treatment and from week 5 to week 18 (**Figure 1**, **Supplementary Figure 1**). As aphids appeared to be a substantial driver of the treatment and to support the interpretation of our results, we assessed patterns in aphid biomass and diversity between the treatments. Analyses and results are provided in the **Appendixes S1** and **S2**. Aphid biomass was significantly different across the treatments after the first sampling event (**Supplementary Figure 1**). Total invertebrate biomass and invertebrate biomass excluding aphids showed the highest values after the last sampling. We found that aphids represented a high proportion of the total invertebrate biomass in the 25%- and 0%-treatment. A significant difference between treatments was detected for the proportion of aphids in the total biomass. Regarding the invertebrate diversity, the Shannon diversity revealed significant differences between the 100%- and 0%-treatment after the first sampling (**Supplementary Figure 2**, for statistical procedure with respect to the treatment see supplementary). Soil invertebrates were also present in the invertebrate sampling (cf. **Supplementary Figure 2**) but were not analyzed separately.

Changes in Plant Species Abundance and Phenology

Based on standardized camera pictures, we estimated plant species abundance and phenology every week. Using the cameras was necessary, because we could not open the EcoUnits as invertebrates could have escaped or been transferred from one EcoUnit to another. We took the pictures every Thursday, as the picture series started on a Thursday, and only switched to Wednesdays when the pictures taken on a Thursday were blurred. As the mid-term harvest started the day after August 20th, the last day of data sampling was a Monday. For each picture of an EcoUnit, we performed vegetation relevés using the Schmidt-Scale (1974, cited in Pfadenhauer, 1997) for plant species abundance estimations with one additional class for very low abundances: 0, 0.5, 1, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, and 100%. For each picture and species, we also estimated the percentage of flowers in a population using the same scale to capture the first flowering day and the peak flowering. That is, on the population level we estimated the proportion for the vegetative stage, the flower buds, the flowers and the end of flowering, so that taken together we described the phenological stages of the population for 100% for every week. Of the 12 plant species sown, we could only record seven species (**Table 1**). *Bellis perennis* L. and *Knautia arvensis* (L.) Coult. did not flower, grew only very occasionally underneath the plant cover, and were therefore not visible in the camera pictures. It was not possible to identify the grass species from the pictures even though at least some individuals flowered.

Statistical Analyses

Changes in Plant Species Abundance

To explore the general effects of week and the treatment on the vegetation, we conducted a principal component analysis (PCA) on the scaled and centered data of plant species abundance per treatment and week (as captured by the different pictures) using the “vegan” package (Oksanen et al., 2019) in R (R Core Team, 2018). Prior to the PCA, we checked that axis length was <3 performing a detrended correspondence analysis (DCA) following the procedure described in Leyer and Wesche (2007).

TABLE 1 | Overview of observed plant species with corresponding abbreviation, family, life from, pollination syndrome, flowering time, and the information whether the species flowered during the experiment.

Species	Abbreviation	Family	Life form	Pollination syndrome	Flowering time	Flowered
Centaurea jacea L. s. l.	Cen_jac	Asteraceae	Hemicryptophyte	Insects	Jun–Nov	yes
Lotus corniculatus L.	Lot_cor	Fabaceae	Hemicryptophyte	Insects	Jun–Aug	yes
Medicago lupulina L.	Med_lup	Fabaceae	Hemicryptophyte	Insects, self-pollination	May–Oct	yes
Plantago lanceolata L.	Pla_lan	Plantaginaceae	Hemicryptophyte	Wind, insects, self-pollination	May–Oct	yes
Scorzoneroides autumnalis (L.) Moench	Sco_aut	Asteraceae	Hemicryptophyte	Insects	Jul–Sep	yes
Trifolium pratense L.	Tri_pra	Fabaceae	Hemicryptophyte	Insects	Jun–Sep	yes
Achillea millefolium L.	Ach_mil	Asteraceae	Hemicryptophyte	Insects	Jun–Oct	no

Information based on Klotz et al. (2002) and our personal observations.

With the "envfit"-function, we correlated the variables week and treatment with the PCA axes. To better visualize temporal changes in species composition per treatment, centroids were calculated as mean values grouped by week and treatment for the first and second principle components.

To analyze the effect of week and treatment on changes in species abundance, we used boosted regression trees (BRTs) using the R package "gbm" (Greenwell et al., 2019) and the modified functions provided by Elith et al. (2008). This is a machine learning approach based on regression trees, where data transformation or elimination of outliers is not needed (see Elith et al., 2008 for further details). We ran a model for each species separately and for every plant functional group, including treatment as a factor. We used the following parameter settings: A Gaussian error distribution, as we dealt with proportional data, a tree complexity of 2, a bagging fraction of 0.5, and a learning rate of 0.01. The models were fitted with the "gbm.step"-function and assessed with the cross-validation correlation (cv). The cv is also used to show withheld portions of the data (Elith et al., 2008).

Changes in Plant Species Phenology

We assessed general patterns in flowering phenology using multivariate statistics and conducted a DCA as the length of the gradient was >3, as described above. With the "envfit"-function, we correlated week and treatment (in %) with the axes. We calculated the centroids as the mean grouped by week and treatment for the first and second DCA-axis.

To analyses effects of invertebrate biomass on plant species phenology, we used BRTs similar to the procedure we followed for the abundance. For each species, the percentage of flowers was included as the response variable, week, and treatment were explanatory variables. We used the same parameter settings that were applied for the BRTs of the abundance analyses (see above).

For the graphical presentation of all plots, we used the package "ggplot2" (Wickham, 2016).

RESULTS

Changes in Plant Species Abundance

The PCA revealed that temporal changes in plant species composition differed between treatments and that week appeared to be more important than treatment as indicated by the longer vector in **Figure 2A**. However, the analysis also showed a separation along the PC2-axis (**Figure 2B**) which was correlated with the invertebrate treatment gradient. When we compared changes in species abundances across weeks and treatments, we found that according to the relative importance values given by the species-wise BRT models, week explained from 66.7% in *Medicago lupulina* to 94.8% in *Lotus corniculatus* (**Supplementary Figure 3A**). However, treatment explained from 5.2% in *L. corniculatus* to 33.3% in *M. lupulina*. The values for the cross-validation correlation ranged from 0.49 for *Scorzoneroides autumnalis* to 0.78 for *T. pratense*. When we included aphid biomass from the three sampling dates (**Supplementary Figure 1A**) as an additional independent variable, the cross-validation correlation was higher, that is from 0.74 for *Achillea millefolium* to 0.91 for *T. pratense*, and the relative importance of aphid biomass (ranging from 34.4% in *A. millefolium* to 56.6% in *S. autumnalis*) was similar to the relative importance of week (ranging from 32.8% in *S. autumnalis* to 54.8% in *A. millefolium*) across all plant species (**Supplementary Figure 3B**). However, treatment was still important. The most abundant species in all treatments was *T. pratense*, yet its abundance increased from the 100%- to the 0%-treatment (**Figures 2C–E**). Furthermore, this species decreased earlier in the 100%-treatment (week 10) as compared with the other treatments (week 13).

The abundance of plant functional groups over time and per treatment is given in **Figure 3**. The cv that derived from BRTs ranged from 0.67 for forbs to 0.71 and 0.74 for grasses and legumes, respectively. The abundances of forbs over time showed similar patterns across the treatments. The BRT models revealed that the treatments' relative importance was 9.8% (**Figure 3A**). Legume abundance, however, decreased earlier in the 100%-treatment as compared to the 25%- and 0%-treatment (**Figure 3B**). Here, the BRT showed that treatment had a relative importance of 19.3%. For the grasses the trend was *vice versa*: The grass abundance increased in the 100%-treatment and stayed relatively low in the 25%- and 0%-treatment (**Figure 3C**). The BRT revealed a relative importance of 42.6% for treatment. For the functional groups the cv increased as well when including aphid biomass as a third explanatory variable, with 0.73 for legumes, 0.86 for forbs, and 0.91 for grasses (**Supplementary Figure 4B**). The differences in relative importance between week and aphid biomass was similar for forbs and grasses. Forbs had a relative importance value of 46.4% for week and 44% for aphid biomass. Grasses showed 36.6% for week and 43% for aphid biomass. However, the relative importance of 1% for aphid biomass was the lowest compared with all BRTs.

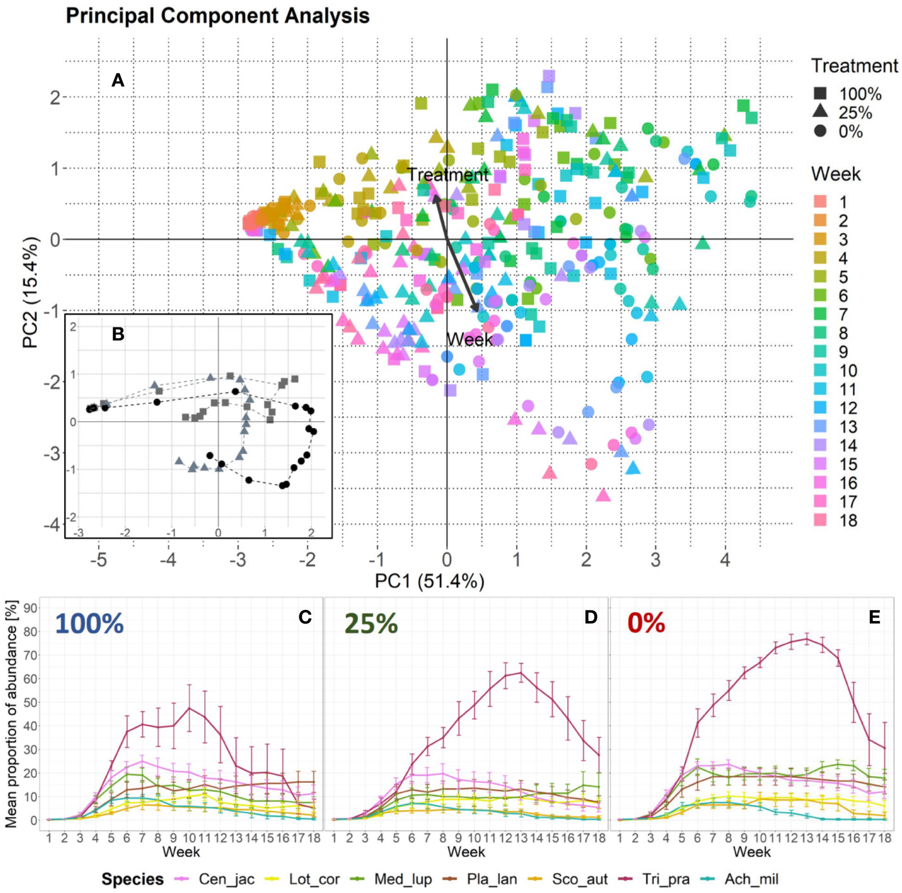

FIGURE 2 | Changes in plant species abundance over time per treatment. **(A)** Principal component analysis based on species abundance data. One data point represents one EcoUnit of the iDiv Ecotron observed in 1 week (n = 431). The community development over time (18 weeks) is indicated by a color gradient representing the week. Eigenvalues are given in percent and represent the explained variance according to the axes. The treatments are represented by filled symbols (square = 100%, triangle = 25%, circle = 0%). Variables week and treatment are post-hoc correlated ($p < 0.001$). Arrows are enlarged in scale by the factor two to fit the scale of the plot. Their lengths show differences in explained variance relative to each other. **(B)** PCA-centroids per week and treatment. The dashed lines connect the symbols that represent the plant community abundance development over time. **(C, D)** Plant species-specific changes in abundance over time as given by mean proportion of percentage values with standard error. Each invertebrate treatment is represented by a panel: **(C)** 100%-treatment, **(D)** 25%-treatment, and **(E)** 0%-treatment. The seven plant species are color-coded. Species abbreviations are listed in **Table 1**.

Changes in Plant Species Phenology

The DCA revealed that week mainly drove flowering phenology and that treatment had a marginal influence (**Figure 4A**). The DCA-centroids per week and treatment showed that the data points for the 100%-treatment developed along the treatment gradient (**Figure 4B**). On the community level, the peak flowering tended to show a higher dispersion in the 100%-treatment (week 11 to 18), that converged in the 25%- (week 11 to 17) and the 0%-treatment (week 11 to 15; cf. **Figure 5**). However, the coefficient of variation did not significantly differ between treatments, even though a higher variation was detected for the 100%-treatment compared to the 25%- and 0%-treatment (**Supplementary Figures 5A, B**).

The BRT models showed that the relative importance of week was higher than treatment in every species. In addition, treatment also influenced flowering phenology patterns over time, even though its importance differed among species. It was highest for *C. jacea* (24.6%) and lowest for *S. autumnalis* (4.3%). The cross-validation correlation showed that on the species-level between 41% in *C. jacea* and 80% in *T. pratense* of the variation was explained by variables week and treatment (**Figure 5** and **Supplementary Figure 3C**). When we included aphid biomass as an explanatory variable, the cross-validation correlation was higher (ranging between 0.63 in *S. autumnalis* and 0.86 in *T. pratense*), but nevertheless, treatment still had an influence (**Supplementary Figure 3D**). However, plant phenology responses to the treatment were species-specific (**Figure 5**). C. *jacea* tended to flower earlier in the 25%- and 0%-treatment than in the 100%-treatment. However, as there was only one flowering observation for this species in the 100%-treatment a significance test could not be applied (**Supplementary Figure 5D**).

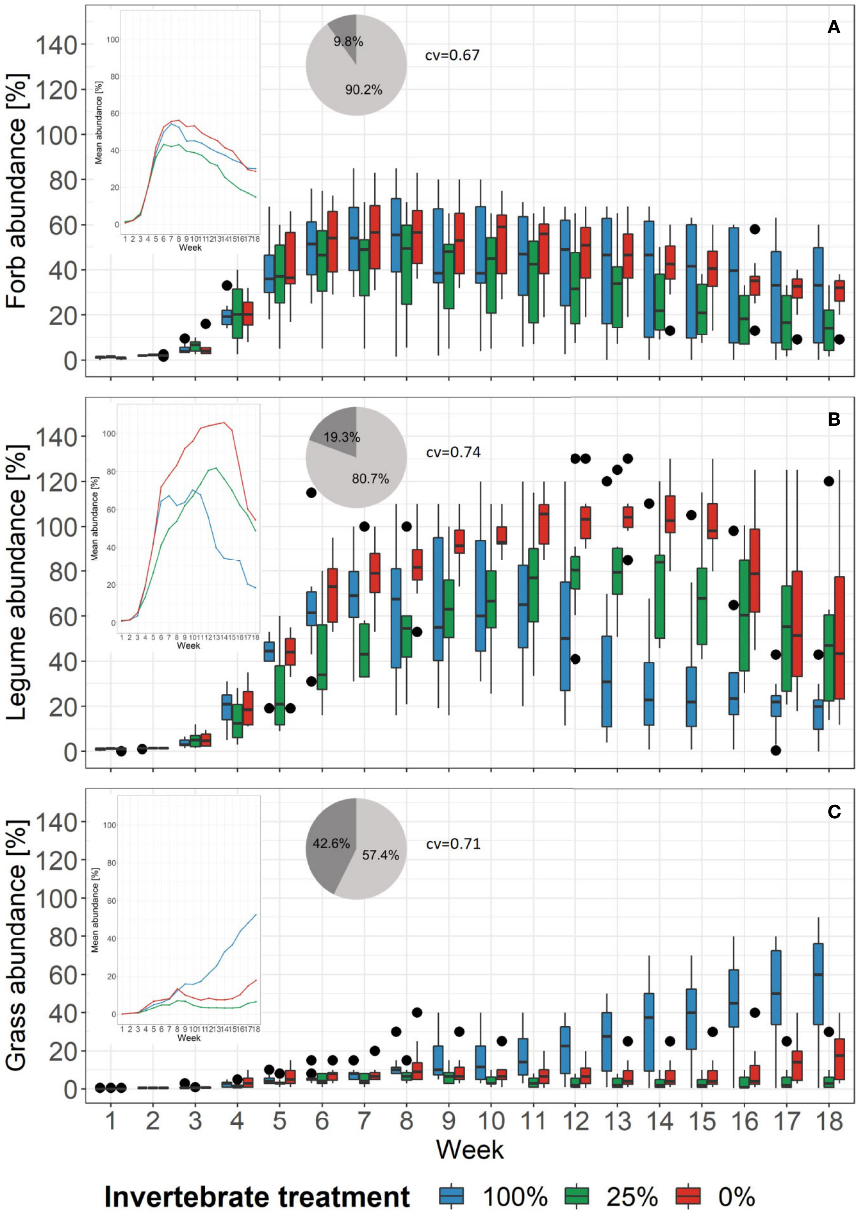

FIGURE 3 | Changes of the proportion of abundance of plant functional groups in each treatment over time. Black dots mark outliers. Small windows give the mean proportion of abundance of the same functional group as lines. Pie charts show the relative importance based on boosted regression trees of the variables week (light grey) and treatment (dark grey). Right beside it, the cross-validation correlation of the models is given (cv). See **Supplementary Figure 8** for partial dependence plots. **(A)** Forb abundance, **(B)** Legume abundance, **(C)** Grass abundance.

For *M. lupulina*, we found a delayed first flowering in the 100%-treatment compared to the 0%-treatment (**Supplementary Figure 5D**). This species tended to reach average peak flowering in week 15 in the 0%-treatment, after 17 weeks in the 25%-treatment while it did not reach peak flowering in the 100%-treatment (**Figure 5**). This relationship was opposite in *L. corniculatus*: on average, peak flowering took place earlier (in week 11) in the 100%-treatment and was delayed in the other treatments (**Figure 5**). Nevertheless, across all species peak flowering time only differed significantly across treatments for *L. corniculatus* which reached peak flowering earlier in the 100%-treatment compared to the 0%-treatment, and *P. lanceolata* which showed a later peak flowering for the 100%-treatment compared to the 25%-treatment (**Supplementary Figure 5C**). The species *S. autumnalis* did not respond to the treatment. There was also no difference in peak flowering time of *T. pratense* with respect to the treatment, however, we detected a higher proportion of flowers in the 25%- and 0%- than in the 100%-treatment (**Figure 5**).

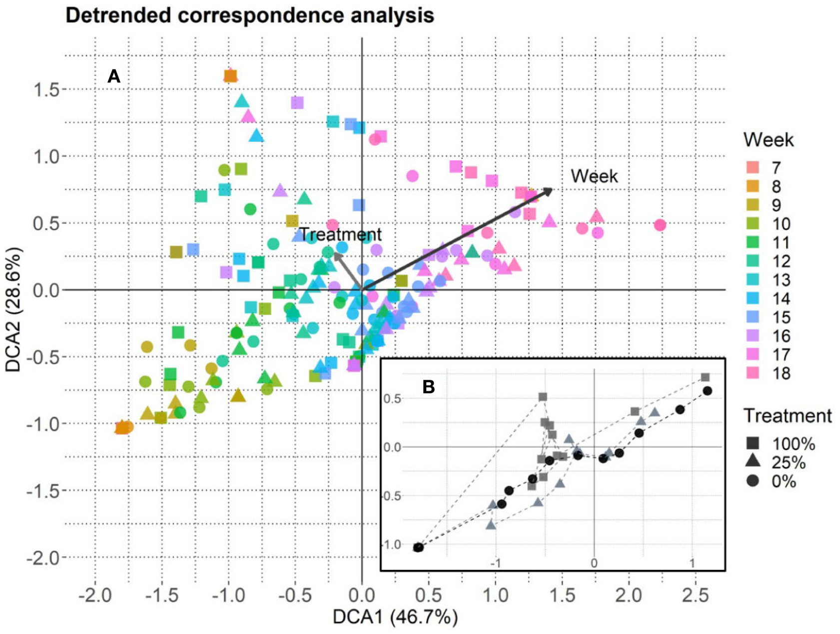

FIGURE 4 | Detrended correspondence analysis based on species-specific flowering data, axis lengths: DCA1 = 4.03 and DCA2 = 2.63. **(A)** One data point represents one EcoUnit observed in 1 week (n = 228). The community development over time (18 weeks, starting from week 7 when plant species started to flower) is given with a color gradient for week. Eigenvalues are given in percent and represent the explained variance according to the axes. The treatments are represented by filled symbols (square = 100%, triangle = 25%, circle = 0%). Variables week and treatment are post-hoc correlated ($p < 0.001$). Arrows are enlarged in scale by the factor two to fit the scale of the plot. Their lengths show differences in explained variance relative to each other. **(B)** DCA-centroids per week and treatment. The dashed lines connect the symbols that represent the plant community flowering development over time.

DISCUSSION

The results of this experiment demonstrated that in addition to the widely studied abiotic drivers like climate variables (Menzel et al., 2006; Bock et al., 2014; Bucher et al., 2018; König et al., 2018), biotic components are able to drive the abundance and phenological changes of plant species. We found that a decline of invertebrate biomass led to species-specific changes in plant species abundances over time in experimental plant communities, and to species-specific responses in the proportion of flowers. The effect of the three invertebrate treatments was apparent. However, the changes detected may not only result from decreased invertebrate densities, but may also be attributed to the changed composition regarding the ratio of predators and aphids.

Changes in Plant Species Abundance

Changes in plant species composition during the course of the experiment suggested that the plots became more similar to their initial state towards the end of the project ("circular movement"), which was mainly driven by proceeding experimental time, as plant species developed and disappeared due to their life cycles. However, the invertebrate treatment also influenced the plant species abundance, which was mainly driven by the dominance of *T. pratense* among all EcoUnits. This is also a dominant species in semi-natural mesophilic grasslands due to its ability to efficiently use limiting resources (Roscher et al., 2008). The abundances of this species decreased earlier in time for the 100%-treatment, and other species, such as *P. lanceolata*, became dominant during the last weeks of the experiment. These community changes could be a predator-mediated effect: invertebrate predators can have an indirect positive effect on plant species abundance, as their presence reduces herbivores and thus the feeding pressure on the plants (Messina, 1981; Schmitz et al., 1997; Carson and Root, 1999; Schmitz et al., 2004; Preisser and Bolnick, 2008). The shifts in plant functional groups, such as the suppression of grasses in the 25%- and 0%-treatment compared to the 100%-treatment, could have been mediated by the abundance of carnivorous invertebrates which controlled the number of herbivores or led to their behavioral changes (Werner and Peacor, 2003). Schmitz et al. (1997) demonstrated that under a low predation risk, generalist grasshoppers predominantly feed on nutritious grasses and shift their feeding to less nutritious herbs in response to rising predation risk. As grasshoppers occurred in low numbers and the grass cover constantly increased in the 100%-treatment whilst in the other treatments it remained at lower levels, we could assume that the plant consumption was more uniformly distributed across the plant species due to intact interaction networks in the 100%-treatment. Changes in the plant community composition may also be a response to changes in the soil invertebrate community. The high relative number of Collembola species, which we observed at the beginning of the experiment and which decreased over time but remained relatively high for the 100%-treatment

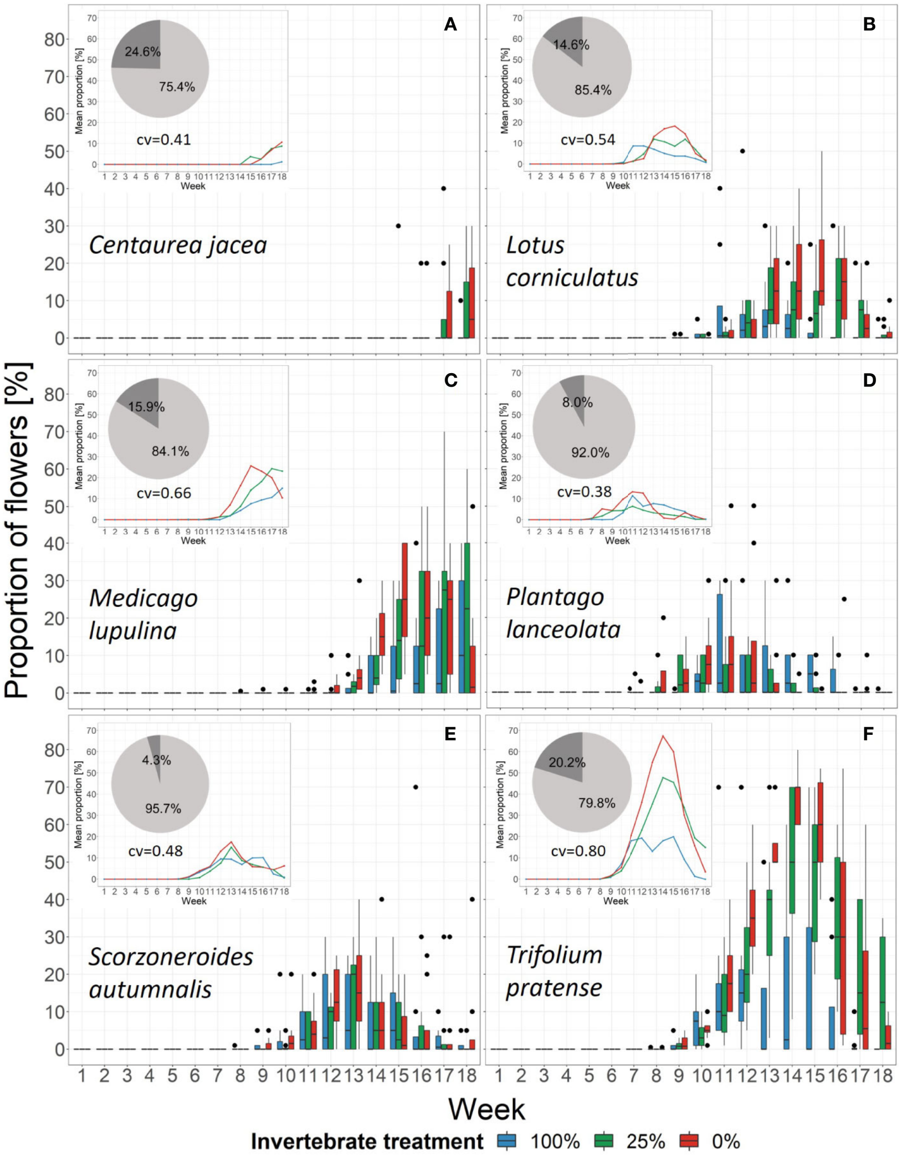

FIGURE 5 | Changes in the proportion of flowers of the six plant species in each treatment over time. Black dots mark outliers. Small windows give the mean proportion of the same species within the community as lines. Pie charts show the relative importance based on boosted regression trees of the variables week (light grey) and treatment (dark grey). Underneath the pie chart the cross-validation of the models is given (cv). See **Supplementary Figure 7** for partial dependence plots. **(A)** *Centaurea jacea*, **(B)** *Lotus corniculatus*, **(C)** *Medicago lupulina*, **(D)** *Plantago lanceolata*, **(E)** *Scorzoneroides autumnalis*, **(F)** *Trifolium pratense*.

(**Supplementary Figure 2**), indicated a change in this community may modify plant species abundance, as these decomposers are known to affect, e.g. plant growth (Partsch et al., 2006; Eisenhauer et al., 2011).

Changes in Flowering Phenology

Regarding the flowering phenology, the EcoUnits did not diverge with respect to the invertebrate treatment as shown in the DCA, yet species-specific changes within each treatment revealed strong differences. *Trifolium pratense* was not only the most dominant species, but also showed highest percentages of flowers together with *L. corniculatus*. This intense flowering may reflect a response to stress caused by higher herbivore pressure. For other herbaceous plant species, Strauss et al. (1996); Strauss (1997), and Poveda et al. (2003) suggested that they tended to increase the number of flowers per individual as a reaction to leaf herbivory, and that herbivore-induced foliar damage tended to delay flowering. In our experiment, we assumed that there was higher herbivore pressure in EcoUnits with more aphids (and lower invertebrate biomass and species diversity). We found that peak flowering of the plant species community tended to be more condensed for the 25%- and 0%-treatment where the herbivore pressure was higher as compared with the 100%-treatment where peak flowering tended to be more dispersed. This trend was however, not statistically significant. Another possible explanation for the species-specific flowering patterns could be that certain plant species, e.g. *C. jacea* or *L. corniculatus*, are not self-compatible and depend on invertebrates as pollinators, whereas others do not. The herb *P. lanceolata* is zoochorous and anemochorous, as well as self-compatible (cf. **Table 1**) and may be more resilient in terms of its flowering pattern to changes in the invertebrate community as pollinators are not obligatory for its reproduction (Clifford, 1962; Friedman and Barrett, 2009). A similar relationship was hypothesized for the first flowering days in trees in a global meta-analyses (König et al., 2018) and for herbs along elevational gradients (Bucher and Römermann, 2020). For *S. autumnalis*, there was no clear pattern. This species is also insect pollinated, but as the curves of the 25%- and 0%-treatment were relatively similar, it could be possible that this perennial plant preferred to spare its resources for the next flowering period. Plant species which depend on insect pollination and are not pollinated are expected to extend their flowering period to enhance pollination success (Alonso, 2004; Castro et al., 2008; Aronne et al., 2015). We speculate that the best compromise for the plant species in this study was to invest in a higher number of flowers to increase pollination success, but not in flowering duration. This potential trade-off could be a subject of future studies. Compared to the herbivores, we only had a low amount of pollinators in the invertebrate communities. In the study by Veits et al. (2019), it was shown that plants reacted to insect sounds by an increase in nectar content and were thus able to sense their pollinators. If plants were aware of the presence of pollinators, the differing peak flowering times across the species in the 100%-treatment could be seen as a response to the occurrence of pollinators, while their absence in the 0%-treatment may have resulted in a convergence of community peak flowering. This phenological complementarity has positive effects in nature as it provides food for pollinators over a longer time period and reduces pollinator competition (Stiles, 1975; Lobo et al., 2003). A reduction of invertebrate densities may lead to a shorter community flowering period which may result in a mismatch of biotic interactions (Goulson et al., 2015; Schenk et al., 2018). As we did not analyze mismatches in more detail, further studies are needed to investigate the degree of changes in biotic mismatches with changing invertebrate densities.

Effects of Abiotic Factors on Plant Species Abundance and Phenology

Previous studies showed that higher legume abundances and concomitant higher nitrogen rhizodeposition can result in differing soil nutrient conditions (Jensen, 1996; Fustec et al., 2010), eventually leading to species-specific responses: It has been shown that a reduction in the availability of nutrients promotes flowering in *Arabidopsis thaliana* (L.) HEYNH. and *Pharbitis nil* (L.) ROTH (Shinozaki et al., 1988; Kolář and Seňková, 2008; Wada et al., 2009; Wada and Takeno, 2010; Cho et al., 2017). In our experiment, the soil nutrient analyses did not significantly differ across treatment and over time and could thus not explain differences in the flowering pattern. Thus, the earlier peak flowering in *L. corniculatus* in the 100%-treatment could not be an effect of lower availability of nutrients. The response of *M. lupulina* to the invertebrate treatment was the reverse of the response of *L. corniculatus*. This species showed a very dispersed flowering pattern across the treatments with the peak flowering appearing first in the 0%-treatment, followed by the 25%- and the 100%-treatment. Turkington and Cavers (1979) reported a clear edaphic effect regarding the flowering of *M. lupulina* as this species delays its flowering with lower pH values. Maybe the pH changed throughout the experiment and this abiotic factor could explain the delay in first flowering of this species.

Caveats of the Study and Implications for Future Studies

A combination of different effects such as herbivore pressure, higher amount of available nutrients or the presence of pollinators, resulted from the invertebrate treatments and could have been responsible for the observed patterns in plant species abundance and phenology. The treatment of a reduced invertebrate biomass resulted in a loss of predators and thus in an increase of aphid biomass in the 25%- but also and especially in the 0%-treatment, where invertebrates appeared even though they were not introduced. Hence, the reduction of invertebrate biomass in our treatment led to significant changes in the invertebrate community represented by a concomitant reduction of invertebrate diversity (**Supplementary Figures 1** and **2**). Thus, we need to consider that the experimental treatments led not only to changes in the invertebrate biomass but also to a reduction of predator species, which worked as natural pest controls. This pattern, however, might actually reflect the consequences of the globally observed invertebrate decline: other studies have shown that an increase in temperature alters invertebrate communities in a way that herbivorous insects are favored as their developmental times decrease when the top-down regulations are reduced (Coley, 1998; de Sassi and Tylianakis, 2012; Rasmann et al., 2014) and the sensitivity of organisms to the effects of climate change increases with trophic rank (Voigt et al., 2007). Consequently, pest outbreaks are more likely in a warmer future (de Sassi and Tylianakis, 2012). Altered top-down forcing regimes associated with missing high trophic level consumers (Estes et al., 2011) might have caused the patterns in plant species abundance and phenology that we could observe in our experiment, because responses to changes in interaction networks happen relatively fast (Burkle et al., 2013). Thus, further research is needed to disentangle the effects of invertebrate densities and trophic structure on plant species communities. Expanding the study by considering also soil invertebrate communities would shed light on an underrepresented but highly influential field of interaction research (Wardle et al., 2004; Eisenhauer and Türke, 2018). In addition, soil nutrient conditions potentially explain shifts in flowering time. Another approach to explain the changes in abundance and phenology patterns may be the analysis of plant functional traits, e.g. if the shorter flowering period in the 0%-treatment leads to a resource allocation within the plant leaves as a trade-off effect. Simonsen and Stinchcombe (2014) could show that higher levels of insect damage increased leaf nitrogen for *M. lupulina*. This study gave a first impression on how a reduced invertebrate density could influence ecosystem functions with respect to plant species abundance in combination with flower availability. The research on plant functional traits could enlarge the knowledge about how plants adapt to declining invertebrate densities within and between species and how this may shape ecosystem functions.

CONCLUSION

Our results showed that changes in invertebrate communities significantly affected the abundance and phenology of plant species in a species-specific way. We observed distinct shifts in species abundance and flowering phenology as a response to the different invertebrate treatments. The shifts in plant species abundances and phenology as a response to abiotic conditions such as rising temperatures may be promoted by changing biotic components like the already observed invertebrate decline. These changes may contribute to mismatches of interactions between invertebrates and plants. Consequences could be a reduced pollination that may result in both, a lack of energy provision for pollinators and a lower reproduction success in plants. A higher abundance of herbivores in response to reduced top-down control by predators leads to more damage on plant tissue, and pollinators were shown to visit damaged plants less frequently (Strauss et al., 1996; Strauss, 1997). Thus, the decline of invertebrates may lead to a further loss of plant species. Future research is required exploring the underlying mechanisms, such as changes in mutualistic and antagonistic interactions between invertebrates and plants to disentangle the specific drivers that caused the patterns in plant species abundance and phenology we observed in our experiment. The results of this study highlight the effects of an under-appreciated driver of plant abundance and phenology with considerable impacts on ecosystem functions, namely changes in invertebrate communities.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

The Ecotron experiment was conceived, established, and maintained by NE, MT, AS, and AG. The idea of this study was developed by CR and SFB. The observations of plant species abundance and phenology was conducted by JU. The analyses were performed by JU. The text was written by JU. CR and SFB contributed significantly to statistical as well as graphical design ideas and to the text. AS provided the table for the overview of the invertebrates that were introduced to the EcoUnits and sucked out, as well as identified afterwards. ML provided soil nutrient data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.542125/full#supplementary-material

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Experimental Warming Changes Phenology and Shortens Growing Season of the Dominant Invasive Plant *Bromus tectorum* (Cheatgrass)

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Bromus tectorum (cheatgrass) has successfully invaded and established throughout the western United States. *Bromus tectorum* grows early in the season and this early growth allows *B. tectorum* to outcompete native species, which has led to dramatic shifts in ecosystem function and plant community composition after *B. tectorum* invades. If the phenology of native species is unable to track changing climate as effectively as *B. tectorum*'s phenology then climate change may facilitate further invasion. To better understand how *B. tectorum* phenology will respond to future climate, we tracked the timing of *B. tectorum* germination, flowering, and senescence over a decade in three *in situ* climate manipulation experiments with treatments that increased temperatures (2°C and 4°C above ambient), altered precipitation regimes, or applied a combination of each. Linear mixed-effects models were used to analyze treatment effects on the timing of germination, flowering, senescence, and on the length of the vegetative growing season (time from germination to flowering) in each experiment. Altered precipitation treatments were only applied in early years of the study and neither precipitation treatments nor the treatments' legacies significantly affected *B. tectorum* phenology. The timing of germination did not significantly vary between any warming treatments and their respective ambient plots. However, plots that were warmed had advances in the timing of *B. tectorum* flowering and senescence, as well as shorter vegetative growing seasons. The phenological advances caused by warming increased with increasing degrees of experimental warming. The greatest differences between warmed and ambient plots were seen in the length of the vegetative growing season, which was shortened by approximately 12 and 7 days in the +4°C and +2°C warming levels, respectively. The effects of experimental warming were small compared to the effects of interannual climate variation, suggesting that interactive controls and the timing of multiple climatic factors are important in determining *B. tectorum* phenology. Taken together, these results help elucidate how *B. tectorum* phenology may respond to future climate, increasing our predictive capacity for estimating when to time *B. tectorum* control efforts and how to more effectively manage this exotic annual grass.

Keywords: *Bromus tectorum*, climate change, dryland, invasive plants, phenology, phenophase, soil moisture, soil temperature

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INTRODUCTION

Plant community structure and function are governed in part by the timing of key life cycle events (i.e., phenology; Rathcke and Lacey, 1985; Chuine and Beaubien, 2001; Wolkovich and Cleland, 2011; Godoy and Levine, 2014). For example, plant phenology regulates growing season length, which determines the timing of seed production and dispersal, and can ultimately influence the competitive and facultative interactions among plants (e.g., Moeller, 2004). Phenological timing in plants is largely cued to climate conditions, particularly temperature and precipitation (Cleland et al., 2007). Temperature has been linked to the timing of phenophases for numerous systems and across multiple plant growth forms (Partanen et al., 1998; Sherry et al., 2007; Hülber et al., 2010; Wolkovich et al., 2012). Similarly, precipitation patterns are an important driver of plant phenology. Soil moisture, in particular, can stimulate germination and senescence can be delayed in wetter soils (Link et al., 1990; Adondakis and Venable, 2004; Estiarte and Peñuelas, 2015). In drylands, the timing and magnitude of precipitation events are important for determining growing season phenophases, including flowering, seed production, and senescence (Beatley, 1974; Prevéy and Seastedt, 2014). However, the effects of precipitation patterns on phenology are difficult to decipher because precipitation is more variable than, and oftentimes interacts with, temperature (Cleland et al., 2007). Due to these strong climatic controls over phenology, climate change is having large effects on the timing of green up, flowering, and senescence for plants in drylands and around the world (Kimball et al., 2010; Wolkovich et al., 2012).

Future climate scenarios for most drylands predict an increased severity and frequency of drought, greater variability in precipitation, and warmer temperatures (Cayan et al., 2010; IPCC, 2014; Reidmiller et al., 2018). Additionally, warmer temperatures will likely change the form of precipitation, as cooler months will experience more rain instead of snow (Barnett et al., 2005). Such changes to climate could alter species ranges, create new temporal niches in plant communities, and differentially affect multiple plant species. For example, winter annuals that grow early in the season are often limited by cold winter temperatures and may be more likely to germinate in response to winter rains resulting from increased temperatures. This earlier germination could infer a competitive advantage over plants that initiate growth later in the season, provided the temperatures are sufficiently high to meet germination requirements, but not too low to induce mortality of the seedlings (Beatley, 1974; Kimball et al., 2010; Wolkovich and Cleland, 2011). Additionally, invasive annual plants often employ more flexible resource acquisition strategies that can facilitate rapid phenological responses (Funk, 2013; Wolkovich and Cleland, 2014; Winkler et al., 2018). Given this, invasive plants may be better adapted to tolerate climate change than co-occurring native annuals and perennials, especially those existing at range edges or experiencing novel climate regimes (Seastedt et al., 2008; Willis et al., 2010; Wolkovich and Cleland, 2014; Ashbacher and Cleland, 2015; Phillips, 2019). Further, invasive annual plants may germinate rapidly, increase growth rates, or advance flower and seed production in response to earlier spring temperatures, unseasonably early rains, and nutrient pulses (Esque et al., 2010; Willis et al., 2010). Often, invasive plants are better suited to exploit temporal niches than native plants and this can allow invasives to exploit resources at times when natives are inactive, which can lead to subsequent shifts in community composition, vegetation cover, nutrient cycling, and fire regimes (D'Antonio and Vitousek, 1992; Bradley et al., 2010; Willis et al., 2010; Dickens and Allen, 2014). Together, these traits that allow rapid concerted responses to changing climate have led to predictions of increased invasive species' ranges and abundance under future climate scenarios (Bradley et al., 2010), however, data assessing climate change effects on the timing and success of exotic plants are relatively rare.

Bromus tectorum (cheatgrass) is one of the most destructive invasive annual plants in North American dryland ecosystems (Knapp, 1996). Areas where *B. tectorum* has naturalized have seen subsequent alterations to fire regimes, native plant composition and survival, and hydrological and nutrient cycles (D'Antonio and Vitousek, 1992; Rimer and Evans, 2006; Keeley and Brennan, 2012). *Bromus tectorum* is a native of central Eurasia and was introduced to the western United States in the late 1800s: the exotic grass is highly invasive, and it spread rapidly across the West. This species now covers an estimated 22.7 million hectares in the United States (Duncan et al., 2004) and an estimated one-third of the Great Basin Desert (Bradley et al., 2018). Invaded areas include at least 20 million hectares where *B. tectoru*m is dominant, or nearly so (Novak and Mack, 2001). Additionally, *B. tectorum* is continuing to spread at an estimated rate of 14% (Duncan et al., 2004), moving into high elevation areas after disturbance (Mealor et al., 2012), with millions of hectares considered highly likely to experience future invasion (Pellant and Hall, 1994). It has been suggested that *B. tectoru*m is able to successfully invade and establish because it has more competitive life history traits than native plants, traits that include high specific leaf area, high nitrogen-use efficiency, faster relative growth rates, and higher and faster rates of seed production (Hulbert, 1955; Harris, 1967; Wainwright et al., 2012). Using these traits, *B. tectorum* has been shown to respond to disturbances including fire, nutrient deposition, and climate change via an increased capacity to tune growth strategies (including phenology) to extant conditions, thereby increasing invasiveness (He et al., 2011; Liu et al., 2013; Peeler and Smithwick, 2018; Mesa and Dlugosch, 2020; Williamson et al., 2020). Once established, *B. tectorum* can limit water availability and the species' relatively early phenology can further exacerbate negative impacts on native plants (Harris, 1967; Melgoza et al., 1990; Eliason and Allen, 1997; Booth et al., 2003). *Bromus tectorum's* early and flexible phenology may synergize with future climate conditions and increase its competitive advantage over native plants, as *B. tectorum* may be able to take advantage of earlier growing seasons and early season rain events that native plants cannot utilize (Abatzoglou and Kolden, 2011; Bradley et al., 2016).

Understanding how climate change will affect *B. tectorum* phenology would further our ability to identify locations that are more susceptible to invasion, including identification of invasion hotspots that could benefit from increased control efforts (Bishop et al., 2019). Interpreting the climate controls over *B. tectorum* phenology would also improve spatial-temporal forecasts of how *B. tectorum* abundance and life cycle timing affect changes in competitiveness and fuel loads into the future (Compagnoni and Adler, 2014; Underwood et al., 2019). Further, successful control of *B. tectorum* and promotion of native vegetation is contingent upon *B. tectorum* eradication *prior* to seed production and before native plants become active (Lehnhoff et al., 2019). Accordingly, successful management relies on predicting the timing of *B. tectorum* phenophases and how invasion will be shaped by future climate (Garbowski et al., 2019). However, in spite of the utility of an improved understanding of *B. tectorum* phenological responses to climate change and of the many projections of potential effects on *B. tectorum* success, our knowledge of the controls and magnitude of these effects on *B. tectorum* phenology remains limited. This relatively poor understanding stems from both a lack of long-term observations of *B. tectorum* phenology, and from a low number of climate manipulation experiments in the ecosystems where *B. tectorum* exists (Aronson and McNulty, 2009).

To address this important unknown, we followed three stages of *B. tectorum* phenology for a decade in *in situ* climate manipulation experiments at two sites on the Colorado Plateau, United States. We used infrared lamps to actively warm plants and soils and hand watering to alter precipitation regimes in a full factorial design. We tracked the phenology of *B. tectorum* germination, flowering, and senescence weekly throughout the growing season to test the following hypotheses: 1. Warming treatments will advance *B. tectorum* germination, flowering, and senescence and will shorten the length of the vegetative growing season. 2. altered precipitation or the legacy of altered precipitation will delay phenology and extend *B. tectorum*'s growing season, and 3. background climate conditions will determine the strength of treatment effects (i.e., the magnitude of treatment effects will vary by year depending on that year's weather). Our research builds upon a previous study that assessed the first three years of *B. tectorum* phenology under ambient and warmed conditions (Zelikova et al., 2013) and allows us to explore whether treatment patterns persisted throughout a decade, as well as to elucidate how interannual variability controls this invasive species' phenological patterns.

MATERIALS AND METHODS

Study Location

Bromus tectorum phenology was assessed in three complementary climate manipulation experiments, which have been described previously (Reed et al., 2012; Zelikova et al., 2012, 2013; Wertin et al., 2015, 2017; Winkler et al., 2019). The three studies were set up in two cool desert ecosystems of the Colorado Plateau, United States. Two of the experiments were installed at the same site near Castle Valley, Utah (36.675 N, -109.416W, 1310 m elevation). The soils at the Castle Valley site are classified as sandy loam, calcareous, Rizno series. Soil texture at this site is 61% sand, 25% silt, and 14% clay [as assessed by the Soils Lab at Brigham Young University using a hydrometer (Day, 1965)]. Vegetation cover at the Castle Valley site is dominated by the perennial C_4 grass *Pleuraphis jamesii*, the perennial C_3 grass *Achnatherum hymenoides*, the C_4 shrub *Atriplex confertifolia*, and the invasive annual C_3 grass *B. tectorum*. Both experiments at the Castle Valley site have a west aspect and the slope ranges from 10°–13°. Prior to establishing the experiments, the Castle Valley site had experienced limited to no anthropogenic activity.

The second site is approximately 35 km from the Castle Valley site and is near Moab, Utah (38.31 N, -109.28 W, 1227 m elevation). Soils at the Moab site are sandy loam, Sheppard series, with a thin petrocalcic layer at a depth of 0.5 m. The soil texture at the site is 92% sand, 2% silt, and 5% clay as assessed by the texture-by-feel method (Salley et al., 2018). The vegetation cover is dominated by the perennial C_3 grass *Achnatherum hymenoides* and the invasive annual C_3 grass *B. tectorum*. The Moab site has a slope of 4° with a southern aspect. The site was periodically grazed between 1900 and 2008, and grazing was excluded prior to establishing the experimental plots. Both the Castle Valley and Moab sites have a 1.5 m tall electric fence and 0.5 m tall mesh fence around their perimeters to exclude grazing of cattle and wildlife.

Climate Manipulation Treatments

The three complementary climate manipulation experiments experienced one of two levels of warming and one of two types of precipitation treatments (**Table 1**). One experiment at the Castle Valley site began in 2005 and for most years was warmed to 4 °C above ambient: this experiment will be subsequently referred to as "CV4". The second experiment at the Castle Valley site began in the Fall of 2008 and increased plot temperatures 2 °C above ambient: this experiment will be subsequently referred to as "CV2". The experiment at the Moab site also began in the Fall of 2008 and increased plot temperatures 2 °C above ambient. This experiment will be subsequently referred to as "M2".

The 20 plots comprising the CV4 experiment were originally selected to have similar biocrust cover (Zelikova et al., 2012) and were set up in five blocks to account for downslope spatial variation within the site. Within each block, warming, altered precipitation, warming + altered precipitation, and ambient treatments were randomly assigned to each plot in a full factorial design (n = 5 plots per treatment for a total of 20 plots). In January 2009, temperature treatments in the CV4 plots were increased from 2°C to 4°C above ambient to better capture the range of expected future temperatures. The CV4 plots were subjected to altered precipitation treatments that were intended to mimic small, frequent monsoonal precipitation events. Altered precipitation treatments began in the CV4 plots in the summer of 2006 and continued until 2012 when they were discontinued due to an opportunity to assess biocrust recovery with and without warming (see Reed et al., 2012 and Zelikova et al., 2012 for additional details). For the CV4 plots, water was applied in 1.2 mm events 5 times every two weeks. Altered precipitation treatments were applied approximately between June 15 and September 15 each year.

In the fall of 2008, the CV2 plots were constructed within the same large enclosure as the CV4 plots and the M2 plots were

TABLE 1 | Experiment names, site location, year treatments began, degree of warming above ambient, and type of altered precipitation treatment are described for each of the three experiments.

Experiment name	Location	Year of initiation	Warming level	Precipitation treatment
CV4	Castle Valley, UT	2005	4 °C	Small frequent
CV2	Castle Valley, UT	2008	2 °C	Large infrequent
M2	Moab, UT	2008	2 °C	None

installed near Moab, Utah. The 20 CV2 plots were selected to have similar vegetation cover and, as with CV4, the treatments—warming, altered precipitation, warming + altered precipitation, and untreated ambient—were randomly assigned to five blocks to account for downslope spatial variation (n = 5 plots per treatment for a total of 20 plots). In contrast to the small, frequent monsoonal precipitation treatments in the CV4 experiment, CV2 had a larger, less frequent altered precipitation treatment which was designed to mimic monsoonal events based on a 30-year average (**Table 1**). Altered precipitation treatments in the CV2 plots began in the summer of 2009 and continued until 2012 when treatments ended because plants showed no responses to the treatment (Wertin et al., 2015). Altered precipitation treatments were applied with sprayers each year between approximately June 15 and September 15. The M2 plots were selected for similar vegetation cover, assigned to five blocks to account for cross-site spatial variation, and plots within each block were randomly assigned warming or control treatments (n = 5 plots in each treatment for a total of 10 plots). No watering treatments were applied to the M2 site.

Two Kalglo MRM 2408 infrared heaters (Kalglo Electronics Co., Inc., Bath, PA, United States) were placed 1.3 m above the soil surface to apply warming treatments at each site. We chose infrared heaters to experimentally warm plots because they have been shown to have a high degree of manipulative accuracy and minimally disturb soil surfaces, which is important for our study area where fragile soil surface biota strongly regulate function (Kimball, 2005; Aronson and McNulty, 2009; Reed et al., 2012). All lamps were oriented in a north-south direction to minimize shading and the warming design followed Harte et al. (1995). All plots that did not receive warming treatments were fitted with "dummy" heaters with the same dimensions and orientation. Wertin et al. (2015) describe how experimental temperatures were achieved using soil temperature sensors at 5 cm depths and how increasing the temperatures at these depths by 3.57°C and 1.58°C correspond to elevated soil surface temperatures of 4 °C and 2 °C, respectively. Campbell Scientific CR1000 data loggers (Campbell Scientific Inc., Logan, UT, United States) were programmed to constantly monitor 5 cm deep soil temperatures and toggled lamps on and off in order to maintain 5 cm deep soil temperature differences of +3.57°C in the +4°C warming plots and +1.58°C in the +2°C warming plots relative to their ambient controls. The infrared lamps used for warming have been tested and shown not to emit any visible light that would affect plant phenology (Kimball, 2005).

All plots at each site were rectangles that measured 2.5 × 2 m and were oriented such that the long (2.5 m) side ran east to west. All plots were edged with vinyl plastic flashing to prevent overland water flow and minimize roots from growing into or out of the plots. In both CV experiments, where soils are shallow (Whitney et al., 2017), flashing was buried to a depth of 15 cm. In the M2 experiment, where soils are deeper, the flashing was buried to a depth of 30 cm. At the center of both sites, meteorological stations were installed with 1-min measurement intervals and reported hourly averages. Precipitation was measured with Texas Instruments TE525WS tipping bucket rain gauges (Texas Electronics Inc., Dallas, TX, United States).

In each plot, soil temperature and moisture were measured every 15 min and averaged hourly at three soil depths. Soil microclimate probes were placed at 2, 5, and 10 cm depths in the CV2 and CV4 plots and at 5, 10, and 20 cm in the M2 plots (sensor depths varied between sites due to soil depth differences). For soil temperature, three-tipped thermopiles were constructed from 24-gauge Type-E thermocouple wires (Omega Engineering Inc., Norwalk, CT, United States). To assess soil volumetric water content, a combination of CS616 water content reflectometer and Decagon EC-5 soil moisture probes were used (Campbell Scientific, Logan, UT, United States and Decagon Devices, Pullman, WA, United States). CS616 water content reflectometers were installed at 2 cm depths in all plots at CV2 and CV4 and at 5 cm depths in all plots at the M2 site. EC-5 soil moisture probes were installed at 5 and 10 cm depths in all plots at both CV sites and at depths of 10 and 20 cm in the all plots at the M2 site. Foliar temperatures were measured to determine if plants in the experiments received higher levels of warming than what was recorded in the soils. This was achieved using Apogee SI-121 infrared radiometer sensors placed 15–80 cm above 1 focal plant canopy in randomly assigned warming and ambient plots in both CV experiments, depending on the canopy size (Apogee Instruments, Inc., Logan, UT, United States). Plants in the CV4 and CV2 experiments experienced temperatures that were on average 5 °C and 1.6°C higher in the warmed plots than ambient plots, respectively.

Phenology Measurements

Phenology measurements began in 2009 at all sites and followed a modified field observation protocol based on Wein and West (1971). Plots were scored based on the timing and duration of germination, flowering, and senescence phenophases and the Julian day when the transition from one phenophase to another occurred was recorded. Transitions from one phenophase to the next were defined as follows: germination was recorded as the first sign of germination in the plot, flowering was recorded as the first sign of flower budburst in the plot, senescence was recorded when all plants had fully senesced in the plot, and vegetative growing season was calculated as the number of days between the first sign of germination and the first sign of flowering in a plot. In all years, phenophases were scored on a weekly basis but the timing of initiation and termination of weekly surveys differed among years. In 2009 and 2011, surveys started in April and February, respectively, and ended when all plants were fully senesced in June. In 2010, surveys began in February and continued for the year and in 2015–2019 surveys occurred weekly throughout the year, thus these years captured all necessary phenological stages. Phenology was only inconsistently measured from 2012–2014. Due to the shortened measurement times in 2009 and the inconsistent measurements in 2012–2014, only data from 2010, 2011, and 2015–2019 were used in this analysis. During fall 2017, *B. tectorum* germinated at the M2 site, but plants senesced shortly after in early winter and new plants germinated the following spring. These germination and senescence events were excluded from statistical analyses since they were outliers that only occurred once during the experiment.

Statistical Analyses

Mean daily soil volumetric water content was aggregated from hourly measurements that began December 1, 2008 and ended December 12, 2019. Data were collected at 10 cm depth from the 3 Decagon EC-5 probes in each plot (Decagon Devices, Pullman, WA, United States) (n = 20, 20, 10 in the CV4, CV2, and M2 experiments, respectively). In order to zero each sensor and account for sensor drift, the minimum volumetric water content value for each sensor in each year was subtracted from all other volumetric water content values for each sensor in that year. In addition, all soil moisture data collected in frozen soils ($\leq$0°C) were discarded due to erratic probe behavior.

Soil moisture data were modeled using linear mixed-effects models using restricted maximum-likelihood (Harrison et al., 2018). A first-order continuous time covariate nested within each experimental plot was included to account for strong temporal autocorrelation between daily soil moisture (Pinheiro and Bates, 2002). Plot effects were in turn nested within blocks, while blocks were nested within year to account for spatial heterogeneity across experiments, as well as strong annual variability in climate. Continuous time covariates were scaled and centered to improve model convergence. Altered precipitation treatments did not significantly affect phenology in our experiments but warming treatments did. Therefore, this analysis of soil moisture focused only on the effects warming treatments in order to explore mechanisms for warming treatment effects on *B. tectorum* phenology. Significance of warming treatment effects was assessed using treatment-group pairwise comparisons of estimated marginal means adjusted for multiple comparisons using Tukey's method (Lenth, 2016). Separate models with identical terms were constructed for each experiment. All soil moisture data were analyzed using R version 3.6.3 and utilized the packages 'nlme' and 'emmeans' (Lenth, 2020; Pinheiro et al., 2020; RCore Team, 2020).

Bromus tectorum phenological response to warming and watering treatments was analyzed using linear mixed-effects models with a restricted maximum likelihood approach (Zuur et al., 2006; Bolker et al., 2009; Nakagawa and Schielzeth, 2013). Models were designed to test the treatment effects of warming, altered precipitation, and their interaction on the timing of *B. tectorum* phenology. Separate models were constructed for each experiment because sample size and both warming and altered precipitation treatments differed across experiments. All models included random intercepts for the 5 blocks in each experiment to account for spatial variation. Each model also included random intercepts for year, as well as random slopes for warming treatments in each year. This allowed accounting for year-to-year climate variability in *B. tectorum* phenology, as well as examination of the interaction between warming treatment effects and yearly weather. Two models were fitted for each phenology variable (germination, flowering, senescence, and vegetative growing season) at each experiment (CV4, CV2, and M2) for a total of 24 candidate models. The first of the two models had additive fixed effects of warming and altered precipitation and the second model had interacting warming and altered precipitation effects in addition to the additive warming and altered precipitation effects. For each phenology variable at each site, the final models were selected with a likelihood ratio test (Bates, 2010). Twelve final models were selected, one for the timing of germination, flowering, senescence, and total number of vegetative days (time from germination to the initiation of flowering) in each of the 3 experiments. No models with interacting warming and altered precipitation were selected and so treatment effects were tested using Type-II ANOVA on each candidate model (Kuznetsova et al., 2017; Luke, 2017). The contribution of the interaction between each year and warming treatment was quantified using conditional modes of the random effects along with their 95% confidence intervals (Fox et al., 2015). Linear mixed-effects model analysis and ANOVA analysis on the models utilized the 'lme4' package (Bates et al., 2015).

We further explored aspects of interannual variation by analyzing relationships of yearly changes in soil moisture and temperature on the timing of *B. tectorum* phenology. Mean annual soil moisture data were collected at 10 cm depth from the 3 Decagon EC-5 probes in each plot (Decagon Devices, Pullman, WA, United States) (n = 20, 20, 10 in the CV4, CV2, and M2 experiments, respectively). In order to zero each sensor and account for sensor drift, the minimum volumetric water content value for each sensor in each year was subtracted from all other volumetric water content values for each sensor in that year. In addition, all soil moisture data collected in frozen soils ($\leq$0°C) were discarded due to erratic probe behavior. Mean annual soil temperature data were collected at 10 cm depth from 3 three-tipped thermopiles in each plot. Mean annual soil moisture and temperature were aggregated from hourly measurements in each year when phenology measurements were recorded (2011, 2016–2019 for germination and vegetative growing season and 2010, 2011, 2015–2019 for flowering and senescence).

In total, twelve linear mixed effects models were constructed to explore microclimate effects (soil moisture and temperature) on each of the four phenology variables (germination, flowering, senescence, and vegetative growing season) in each of the three experiments (CV4, CV2, and M2). All models included additive fixed effects of mean annual soil moisture and mean annual soil temperature. Each model included year and block as random intercepts to account for other aspects of yearly variation beyond changes in soil moisture and temperature and spatial variation across each experiment. Marginal r^2 values were calculated to show how much variation in phenological timing was explained by the fixed effects of the models (10 cm soil moisture and soil temperature). Conditional r^2 values were calculated to show how much variation in phenological timing was explained by other aspects of annual variation and spatial variation across each experiment. Marginal and conditional r^2 values were determined with the r.squaredGLMM function in the 'car' package (Nakagawa and Schielzeth, 2013; Fox and Weisberg, 2019). The significance of mean annual soil moisture and temperature were determined with Type-II ANOVAs on each of the 12 models (Kuznetsova et al., 2017).

RESULTS

In the CV4 and CV2 experiments, all final models had additive warming and altered precipitation fixed effects and no final candidate models included warming and altered precipitation treatment interactions. Final models for M2 included warming as the only fixed effect, because no altered precipitation treatments were applied in this experiment. Model results can be found in **Table 2**.

From 2009 to 2019, experimental warming increased 10 cm deep soil temperatures above ambient soil temperatures an average of 2.73°C in the CV4 plots, 1.34°C in the CV2 plots, and 1.25°C in the M2 plots (**Supplementary Figure 1**). These increased temperatures are similar yet muted compared with the warming differences observed at 5 cm depths (Zelikova et al., 2013; Wertin et al., 2015, 2017). From 2009–2019 there were no differences in mean volumetric water content between warmed and ambient plots for any of the experiments when averaged across each year (**Figure 1**). Wertin et al. (2015) describe, in detail, the effects of the altered precipitation treatments on both soil moisture and temperature at 5 cm depths. Briefly, watering treatments did not significantly affect soil temperatures in either the altered precipitation plots or the warming + altered precipitation plots. Additionally, only the small-frequent altered precipitation treatments of the CV4 sites significantly increased soil moisture during the months when plots were watered and the large-frequent watering at the CV2 sites did not show significant measurable effects on soil moisture at the 5 cm measurement depth.

Across the years, *B. tectorum* phenology did not respond to altered precipitation treatments nor the legacy of any precipitation treatment. Accordingly, data from the altered precipitation plots were binned with data from the ambient plots and data from the warming + altered precipitation treatments were binned with data from the warming treatments allowing us to look explicitly at the effects of warming on *B. tectorum* phenology. Warming did not affect the timing of *B. tectorum* germination in any of the experiments. In contrast, both flowering and senescence significantly advanced with warming in all 3 experiments (**Figure 2** and **Table 2**). For the CV4 and CV2 experiments, of all the phenophases analyzed, warming had the largest effects on the number of vegetative days, followed by flowering phenology, and lastly the timing of senescence (**Table 2**). These patterns shifted in the M2 experiment, which showed the largest warming effects on vegetative days, followed by senescence, then flowering (**Table 2**). Despite no significant germination responses to warming treatments, our models for each experiment suggest that, across years, mean germination dates were delayed in warmed plots relative to controls (**Figures 3G,H,I** and **Table 2**). These small and non-significant delays in conjunction with large significant warming-induced advances in flowering led to decreases in vegetative days representing the largest treatment effects (**Figure 3** and **Table 2**).

When estimating the effects of interannual variation on the warming treatments, the conditional modes of the random effects showed that, in the earlier years (2010 and 2011), flowering and senescence were not consistently different between warming and ambient treatments, but flowering and senescence consistently advanced after 2014. With only one year of Fall germination recorded in the early years, it is difficult to compare earlier and later year patterns, but modeled conditional modes of the random effects show mixed earlier and later germination timing with respect to the means, and no obvious directional trend over time (**Figures 3G,H,I**).

Linear mixed-effects models examining the relationships between mean annual microclimate (10 cm soil moisture and temperature) and *B. tectorum* phenological timing only found a significant relationship between soil moisture and the timing of senescence in the CV4 experiment. In the CV4 experiment, senescence was estimated to occur 1.06 days earlier with every 1% increase in mean annual volumetric water content. All other relationships between soil moisture and phenological timing were not significant. On the other hand, all but two phenophases were significantly related to the mean annual soil temperatures. Warmer temperatures were associated with significant advances in the timing of flowering by 2.98–3.32 days per degree Celsius increase. Senescence also significantly advanced with warmer temperatures as an increase of 1 °C led to an advance of 1.28, 0.75, and 3.51 days in the CV4, CV2, and M2 experiments, respectively. Additionally, the vegetative growing season (time from germination to flowering) was significantly related to soil temperatures: warmer temperatures led to shorter growing seasons by 4.76–5.82 days per degree Celsius increase. Germination was the only phenophase that was delayed in warmer conditions and was estimated to occur 1.27 days later per degree Celsius increase. Marginal r^2 values were <0.1 for all phenophases at all sites except for flowering where marginal r^2 values were 0.21, 0.16, and 0.17 in the CV4, CV2, and M2 experiments, respectively. Conditional r^2 values were always greater than 0.65 (**Table 3**).

DISCUSSION

We predicted that warming would advance *B. tectorum's* phenological stages and that altered precipitation treatments would cause phenological delays. We found some support for our warming predictions: although we saw no significant warming

TABLE 2 | Results from linear mixed effects models.

Experiment	Phenology variable	Warming effect estimate (DOY)	Standard error	Observations	Degrees of freedom	F value	Warming Pr (>F)
CV4	Germination	3.11	1.52	99	4.01	3.36	0.140
CV4	**Flowering**	**−7.78**	**2.01**	**140**	**6.00**	**12.90**	**0.006**
CV4	**Senescence**	**−3.03**	**0.98**	**140**	**6.00**	**8.19**	**0.029**
CV4	**Vegetative Growing Season**	**−12.12**	**2.19**	**99**	**5.56**	27.4	**0.002**
CV2	Germination	2.50	1.50	100	4.00	2.43	0.194
CV2	**Flowering**	**−5.86**	**1.35**	**140**	**6.00**	**16.89**	**0.006**
CV2	**Senescence**	**−1.03**	**0.44**	**140**	**18.52**	**5.24**	**0.034**
CV2	**Vegetative Growing Season**	**−9.24**	**2.37**	**100**	**4.00**	**12.50**	**0.024**
M2	Germination	1.75	2.06	40	7.43	7.30	0.458
M2	**Flowering**	**−3.86**	**1.32**	**70**	**7**	**6.22**	**0.035**
M2	**Senescence**	**−5.14**	**1.9**	**70**	**7**	**0.64**	**0.047**
M2	**Vegetative Growing Season**	**−6.3**	**2.50**	**40**	**29.34**	**6.15**	**0.020**

Warming effect estimate is the number of days the warming treatments advanced or delayed (+ or – sign, respectively) the timing of B. tectorum germination, flowering, senescence, or the length of the vegetative growing season. Negative values show earlier timing of phenophases in the warming relative to ambient plots and positive values show later timing. Degrees of freedom, F values, and p values were determined from Type-II ANOVAs. Significant (p < 0.05) treatment effects on phenophases are shown in bold font.

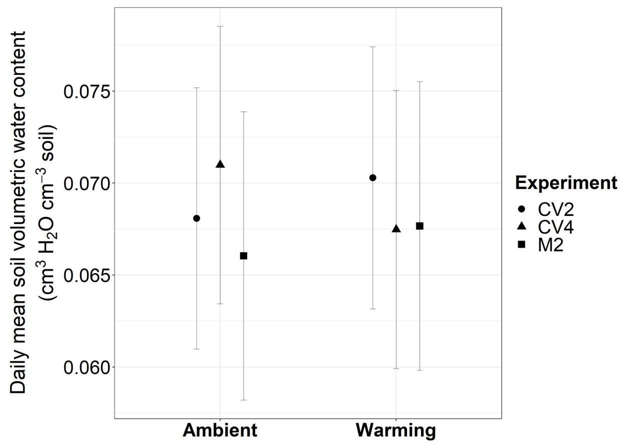

FIGURE 1 | Daily mean soil volumetric water content estimated from marginal means of linear mixed-effects models from 2009–2019 in the CV4, CV2, and M2 experiments. Experiments are represented with different shaped polygons: circles for CV2, triangles for CV4, and squares for M2. Error bars are 95% confidence intervals calculated on marginal means. There were no significant differences in mean soil volumetric water content between warmed and ambient treatments in any of the three experiments.

treatment effects on germination, warming did significantly advance *B. tectorum* flowering and senescence, resulting in a shortening of the warmed plants' vegetative growing season (**Figure 3** and **Table 2**). In contrast, we did not detect any effects of the altered precipitation treatments on *B. tectorum* phenology. This is most likely due to the fact that altered precipitation treatments occurred outside of the *B. tectorum* growing season and that these treatments were only ongoing during two years of this study. Non-etheless, we did not observe changes in *B. tectorum* phenology in response to altered precipitation treatments or the legacy of those treatments.

In tracking *B. tectorum* phenology for seven years across a decade in multiple climate manipulation experiments, we found warming treatments had no significant effect on the timing of germination. This may be partially explained by research showing that the timing of germination is genetically restricted and cannot be explained by interannual precipitation and temperature variation, although the rate of germination is controlled by environmental conditions (Beckstead et al., 1996). However, while overall treatment effects on germination were not significant and varied among years, germination was the only phenophase to be delayed rather than advanced in the warming plots, and this was true for each experiment. The relationship between mean annual soil temperature and germination corroborates these treatment effects as warmer years were associated with later germination events (**Table 3**). *Bromus tectorum* seeds have been shown to shift thermal optimums for germination with changing levels of soil moisture: at low levels of soil moisture emergence rates are decreased by higher temperatures, but at higher levels of soil moisture emergence rates are increased by higher temperatures (Meyer and Allen, 1997). This speaks to the interactive effects of soil moisture and temperature on germination and lends insight into why warming treatments had larger effects in some years than others.

In contrast to germination, both flowering and senescence were significantly advanced by the warming treatments in all three climate manipulation experiments. On average, *B. tectorum* flowering in the +4 °C warmed plots occurred ∼8 days earlier than for plants in the unwarmed plots, whereas senescence occurred ∼3 days earlier. For both flowering and senescence, we observed significant effects of the degree of warming, with larger effects with higher warming levels (e.g., the 4 °C warming in CV4 vs. the 2 °C warming in CV2) (**Table 2**). Thus, these data

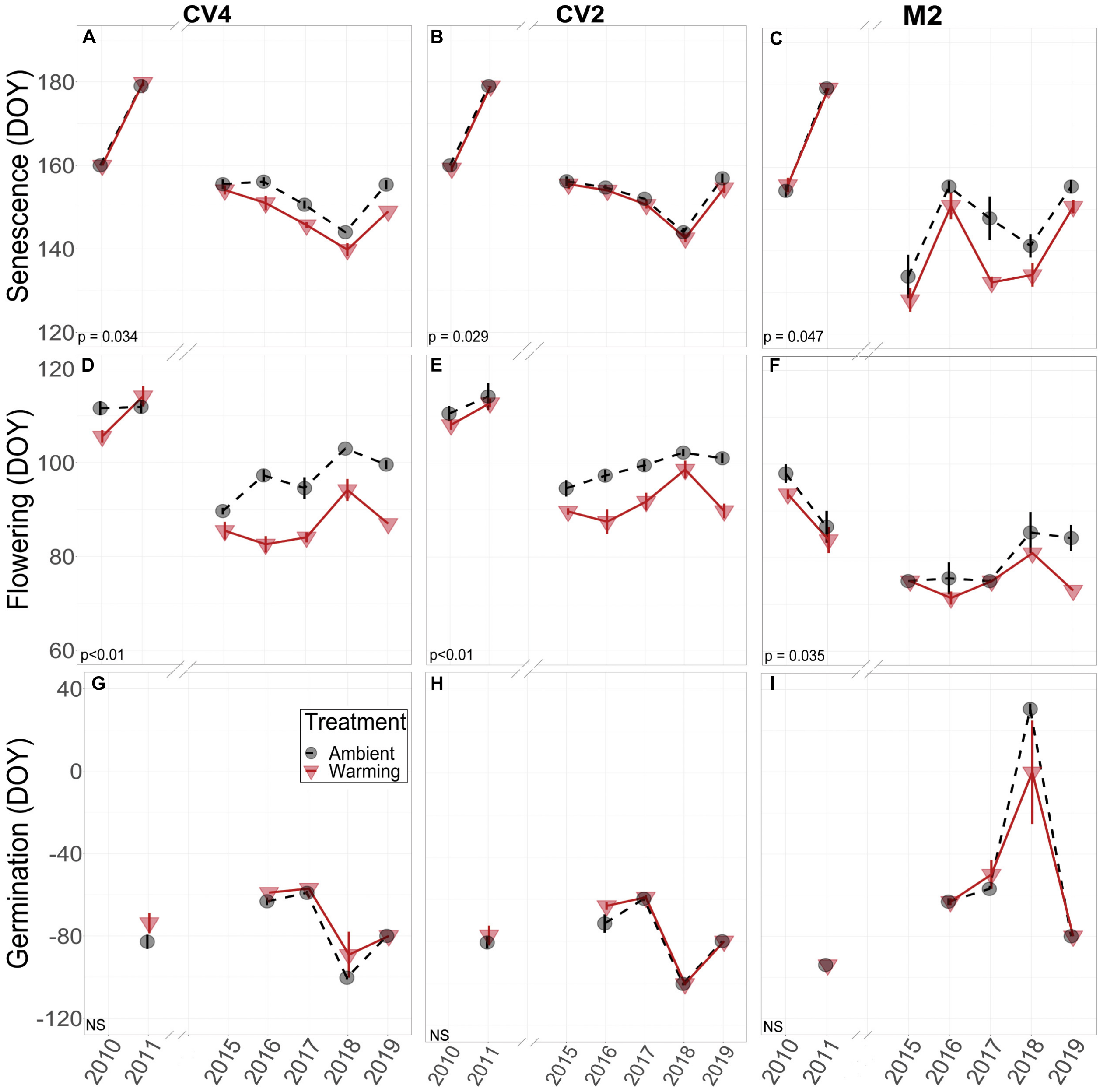

FIGURE 2 | Empirical data for *B. tectorum* senescence **(A–C)**, flowering **(D–F)**, and germination **(G–I)** timing measured weekly in 2010, 2011, and 2015–2019. All phenophases are shown as the average day of year that each phenophase was first observed in each treatment. A value of 0 for day of year corresponds to January 1. A lower value on the y-axis represents earlier timing and a higher value represents later timing. Negative values for day of year indicate that the phenological event took place that number of days prior to January 1. For example, most germination events took place in the Fall and they are shown as negative days for that year (i.e., germination in 2011 took place in the Fall of 2010). Warming treatments are depicted as red triangles and are connected with solid red lines. Ambient treatments are shown with black circles and are connected with dashed black lines. Standard errors of the means are shown with vertical bars associated with the appropriate polygon. Significance of treatment effects is shown in the bottom left of each panel, with NS denoting non-significant effects. Significant differences between the treatment means were determined by Type-II ANOVAs.

suggest that the amount of warming effects the magnitude of the phenological response even across only a 2–4 °C range. The patterns of advancing flowering and senescence under warmer conditions was supported by trends in the soil microclimate analysis across years, which showed that mean annual 10 cm soil temperature was significantly related to flowering and senescence timing at all sites and warmer years had earlier occurrence of these phenophases (**Table 3**). Soil moisture may be playing an important role in the timing of flowering and senescence phenology, but we did not observe this relationship in our analysis. Responses to warming may also be influenced by competitive dynamics with native species in our plots (e.g.,

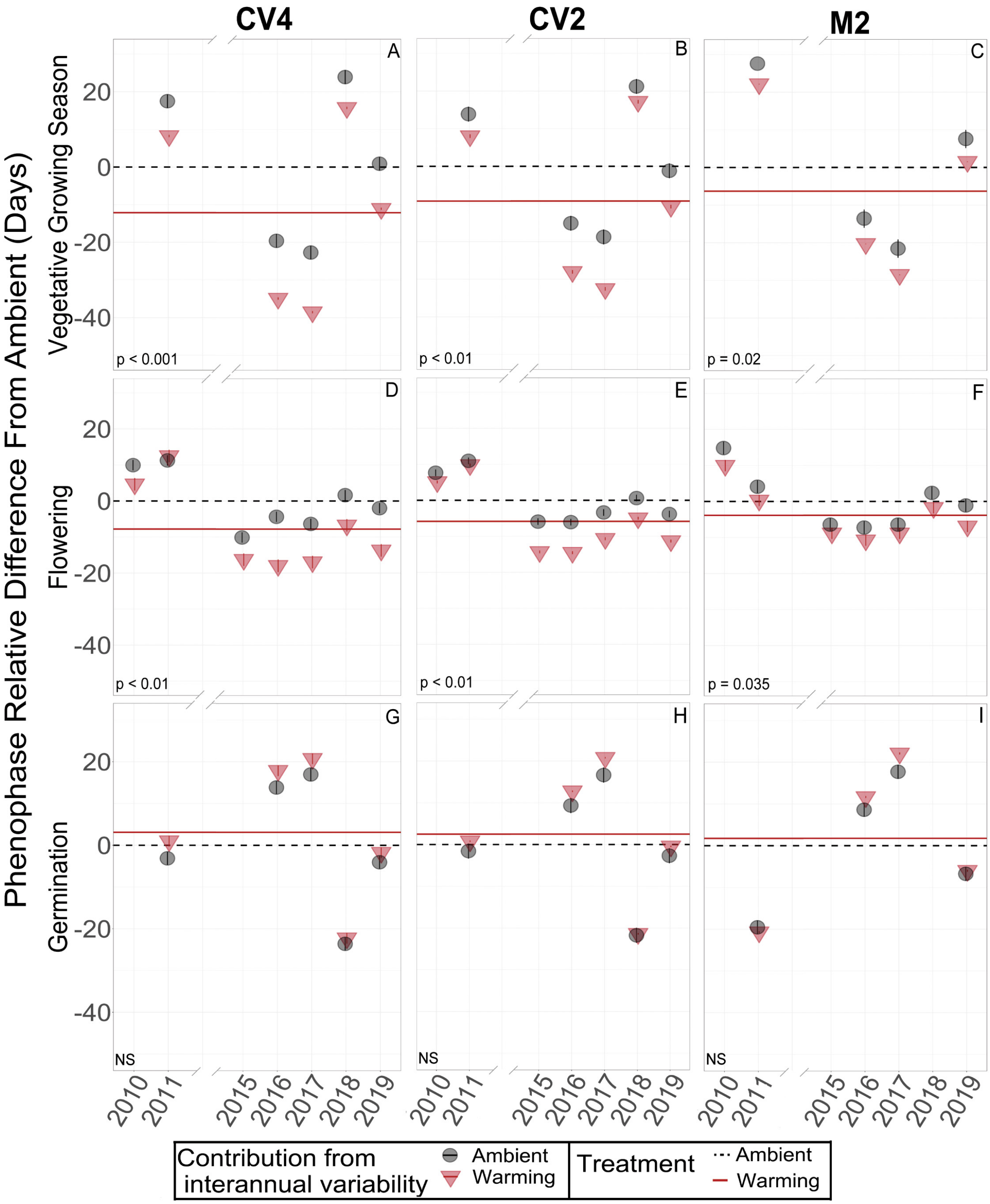

FIGURE 3 | Model decompositions for vegetative growing season **(A–C)**, flowering **(D–F)**, and germination **(G–I)** of *B. tectorum* in each of the three climate manipulation experiments. The estimated mean number of days each phenological stage was delayed or advanced are represented by the horizontal lines. A lower value on the y-axis represents earlier timing and a higher value represents later timing. The estimated means for the ambient plots are represented by the dashed black horizontal lines and are set to zero as all data are shown relative to the ambient plots for each site and each phenological stage. Solid red horizontal lines represent the mean difference in timing (in Julian days) of the warmed plots relative to the ambient plots (black dashed line). When the solid red line is below the dashed black, line there is an estimated warming-induced advance of that phenophase. When the solid red line is above the dashed black line, there is an estimated warming-induced delay of that phenophase. Significance of treatment effects is shown in the bottom left of each panel, with NS denoting non-significant effects. Significant differences between the treatment means were determined by Type-II ANOVAs. The conditional modes of the random effects for the ambient and warming treatments in each year are shown with black circles or red triangles, respectively. Estimated confidence intervals for these conditional modes are shown with vertical bars within the circles or triangles. Interannual variability of these conditional modes are estimated by their difference each year from the horizontal lines of the means.

TABLE 3 | Results from mixed effects models analyzing the effects of 10 cm soil moisture and temperature on the timing of *B. tectorum* germination, flowering, senescence, and length of vegetative growing season.

Site	Phenophase	Mean annual soil moisture parameter estimate (DOY/%VWC)	Mean annual soil moisture standard error	Mean annual soil temperature parameter estimate (DOY/°C)	Mean annual soil temperature standard error	Marginal r^2	Conditional r^2
CV4	Germination	0.71	1.24	**1.27**	0.52	0.01	0.87
CV2	Germination	−0.21	0.81	1.79	0.94	0.01	0.84
M2	Germination	−0.11	0.99	1.99	1.4	0.01	0.89
CV4	Flowering	−1.11	0.8	**−3.13**	0.36	0.21	0.75
CV2	Flowering	−0.89	0.55	**−2.98**	0.6	0.16	0.66
M2	Flowering	−0.22	0.4	**−3.32**	0.8	0.17	0.73
CV4	Senescence	**−1.06**	0.48	**−1.28**	0.21	0.04	0.92
CV2	Senescence	−0.29	0.25	**−0.75**	0.29	0.01	0.95
M2	Senescence	−0.65	0.55	**−3.51**	1.1	0.06	0.84
CV4	Vegetative growing season	−2.14	1.62	**−4.76**	0.68	0.09	0.88
CV2	Vegetative growing season	−0.64	1.01	**−4.87**	1.17	0.06	0.85
M2	Vegetative growing season	0.54	1.32	**−5.82**	1.87	0.08	0.86

Model parameters show the relationship between 10 cm soil moisture and soil temperature in units of DOY/%VWC and DOY/°C, respectively. DOY is the Julian day of year and %VWC is the percent volumetric water content of soils at 10 cm depths in units of cm^3 H_2O / cm^3 *soil. When model parameters are negative increases in moisture or temperature correspond to earlier phenology, when parameters are positive increases in moisture or temperature correspond to later phenology. Significance of the relationship between either soil moisture or temperature and B. tectorum phenology timing are determined with Type-II ANOVA and model parameters are italicized when p values < 0.1 and > 0.05 or bolded when p values < 0.05. Marginal and conditional* r^2 *values were determined with the r.squaredGLMM function in the 'car' package (Nakagawa and Schielzeth, 2013; Fox and Weisberg, 2019). Marginal* r^2 *values show how much variation in phenology timing can be explained by the additive effects of 10 cm soil moisture and soil temperature. Conditional* r^2 *values show how much variation in phenology timing is explained by other aspects of interannual variation and by spatial variation across plots.*

Walther et al., 2009) and future work would benefit from exploring these interactions further.

The strongest effects of the warming treatment were seen for the total number of vegetative growing days, with 12.1 fewer days of vegetative growth with warming in the CV4 experiment, 9.2 fewer days in the CV2 experiment, and 6.3 fewer days in the M2 experiment. By advancing the timing of the later phenological stages (flowering and senescence) without a corresponding shift in the timing of germination, the vegetative growing season of *B. tectorum* was effectively shortened under warmer conditions. Here too the effects of warming scaled with the magnitude of the temperature treatment, with larger effects in the +4°C treatments compared with plant responses in the +2 °C treatment (**Table 2**). The trends of shorter vegetative growing seasons under warmer conditions were also seen in our soil microclimate analysis across years, with significant negative relationships between mean annual soil temperature and growing season length in all experiments (**Table 3**). Although *B. tectorum* can cope and persist with increased temperatures, a shorter growing season in warmer environments may be evidence of increased stress in a warmer world, which could reduce *B. tectorum* success relative to ambient conditions. For example, in a Great Basin Desert study, shorter vegetative growing seasons led to decreased seed production (Mack and Pyke, 1983). Additionally, in a global meta-analysis, herbaceous species that flowered earlier were shown to exhibit shorter heights, decreased seed sizes, shallower roots, and smaller less dense leaves, showing that earlier phenology is tied with metrics that determine a plant's success (Wolkovich and Cleland, 2014). Future research elucidating the interactive controls of phenology, growing season length, plant fitness, and seed production, as well as explorations of competitive interactions between *B. tectorum* and native plants under altered climate, would improve predictions of climate change's effect on this common invasive grass. In light of *B. tectorum's* large negative effects on invaded ecosystems (Young et al., 1987; Melgoza and Nowak, 1991; Anderson and Inouye, 2001; Humphrey and Schupp, 2001; Svejcar and Sheley, 2001) and of the substantial resources used attempting to control the invasive plant, an improved understanding of how and where *B. tectorum* will be affected by climate change would be of great value.

Contrary to the strong effects of warming, the altered precipitation treatments in our experiments did not significantly affect any of *B. tectorum's* phenological stages in the years the experimental watering occurred (2009–2012) or in the years following. Additionally, in the microclimate analysis, mean annual 10 cm soil moisture was only significantly related to one phenophase in one experiment (senescence in CV4; **Table 2**). Determining how precipitation affects phenology is difficult, in part due to the strong interactions between temperature and soil moisture (Cleland et al., 2007). However, greenhouse experiments have shown no effect of watering amount on phenological differences for multiple source populations of *B. tectorum*, except for a delay in senescence (Rice et al., 1992). Conversely, the timing of water application has been shown to have strong effects on *B. tectorum* phenology, with winter moisture being particularly important (Prevéy and Seastedt, 2014). With this in mind, the lack of altered precipitation treatment effects in our study may have been due to the fact that the treatment was designed to ask questions about the monsoon and thus water was applied in summer months, which are effectively outside the *B. tectorum* growing season. Additionally, altered precipitation treatments occurred only in two of the years used for this analysis and therefore the analysis is better suited to examine legacy altered precipitation effects, and no legacy effects were seen. It is also possible that, although water is clearly a dominant driver of numerous ecological patterns in water-limited drylands, temperature may exert larger controls over *B. tectorum's* phenology. Invasive plant phenology may be more strongly tied to temperature in systems where precipitation is variable, sporadic, and difficult to predict (Marushia et al., 2012; Winkler et al., 2018).

In addition to asking questions about how *in situ* climate manipulation affected the phenology of this invasive grass, the longer-term nature of the experiment also allowed us to ask questions about how phenology varied with interannual variations in background climate. We observed substantial interannual variation in the timing of all four measured phenological stages. Interestingly, while germination phenology showed little response to experimental warming, there were large differences in germination timing across years. For example, differences in the timing of germination initiation among years varied by up to 40 and 94 days in the CV experiments and the M2 experiment, respectively. In comparison, the maximum differences in germination timing between warmed and ambient plots within a year were 11 days at CV4 and 30.6 days at M2. In other words, the largest effects of experimental warming were small relative to the range in timing seen across years for *B. tectorum* germination. We observed similar patterns in the effects of experimental warming vs. interannual variation for senescence and flowering. Annual plants are particularly plastic in their response to interannual variation in temperature and precipitation compared to perennials (Wainwright et al., 2012). The patterns we observed across years and with treatments align with other studies suggesting that phenotypic plasticity in *B. tectorum* may make the plant particularly adept at dealing with extreme interannual climate variation, except in the cases where insufficient winter moisture is available (Funk, 2008; Wainwright et al., 2012; Prevéy and Seastedt, 2014). Indeed, studies suggest *B. tectorum* senesces prior to the driest summer conditions as a drought avoidance strategy that allows the species to persist in arid environments (Thill et al., 1984; Rice and Mack, 1991). *Bromus tectorum's* ability to alter senescence, and the timing of flowering, shows a flexibility that could help the species closely track changing climatic conditions. Further, global data suggest plant species that flower earliest in the growing season have the highest phenological temperature sensitivities, thus, part of the strong temperature responses we observed in flowering and senescence may be due to *B. tectorum* life history traits (Sherry et al., 2007; Wolkovich et al., 2012; Wolkovich and Cleland, 2014). Accordingly, although the shortened growing season with warming treatments and in warmer years may point to higher plant stress as temperatures rise, the large phenological plasticity observed for *B. tectorum* could also suggest success for the invasive plant in a world where climate is changing.

In exploring drivers of *B. tectorum* phenological plasticity across years, we found soil temperature to be significantly associated with the timing of germination, flowering, and senescence, and with the length of the vegetative growing season. Mean annual soil temperature was significantly related to the timing of all phenophases except germination in M2. In contrast, mean annual soil moisture was only significantly linked to senescence timing in CV4 (**Table 3**). Additionally, the marginal r^2 values showed that mean annual soil moisture and temperature do not explain a large portion of the variation in timing of all phenophases, suggesting additional controls. Here it is important to note that by estimating soil temperature and moisture at the annual scale, we are almost certainly missing cues and events that play an important role in promoting phenological advance. This is particularly important in the context of seasonal rain events that would not be captured well by an annual average. For instance, multiple studies have shown that the timing of germination is tied with large precipitation events, and that late season precipitation can delay senescence (Sakai et al., 2001; Winkler et al., 2018). Soil moisture is likely a component driving phenological responses in our experiments, but this relationship was difficult to detect, in part because for most of the year the soils in these dryland experiments were dry. However, while the experimental warming effects on soil moisture did not lead to significant deviations from ambient soil (**Figure 1**), there is the likelihood that warming-induced soil drying could contribute to the warming effects on *B. tectorum* phenology and previous research at the CV site showed that warming dried soils at 5 cm depths in both the CV4 and CV2 experiments during 2010 and 2011 (Wertin et al., 2015). Nevertheless, more work elucidating climate controls on and predictions for finer-scale patterns of *B. tectorum* phenology would improve forecasts of how future temperature and precipitation patterns will alter *B. tectorum* phenology.

We also observed differences across the two sites in the responses of *B. tectorum* phenology to experimental warming and annual microclimate variation. When comparing the +2 °C warming effects in the CV2 experiment with the +2 °C warming effects in the M2 experiment, we found that the flowering advances with warming were nearly 1.5 times higher in the CV2 experiment, while advances in senescence were five times greater in the M2 experiment (5 day warming treatment-induced advance in M2 vs. a 1 day advance in CV2). Similarly, senescence in the M2 experiment was more sensitive to natural inter-annual temperature fluctuations than senescence in the CV2 experiment: a 1°C increase was related to a 3.5 day advance in M2 and only a 1.28 and 0.75 day advance in CV4 and CV2, respectively (**Figure 4**). This suggests different sites will vary in not only the magnitude of their *B. tectorum* phenology response to warming, but also in the degree to which specific aspects of phenology are affected (i.e., warming had a larger effect on flowering phenology in CV2 than M2, but a smaller effect on senescence). Soil texture may help explain the between-site variation in the controls of soil moisture and temperature. The limited water-holding capacity of coarse, sandy soils, such as those found at the M2 site, often drive higher variability in soil moisture and may lead to greater variability in soil temperature between rain events (Santos et al., 2019). It has been suggested that *B. tectorum* abundance and cover are higher in finer-textured soils compared to more coarse, sandy soils (Belnap et al., 2016) and the increased variation of soil moisture in sandy soils may help explain this

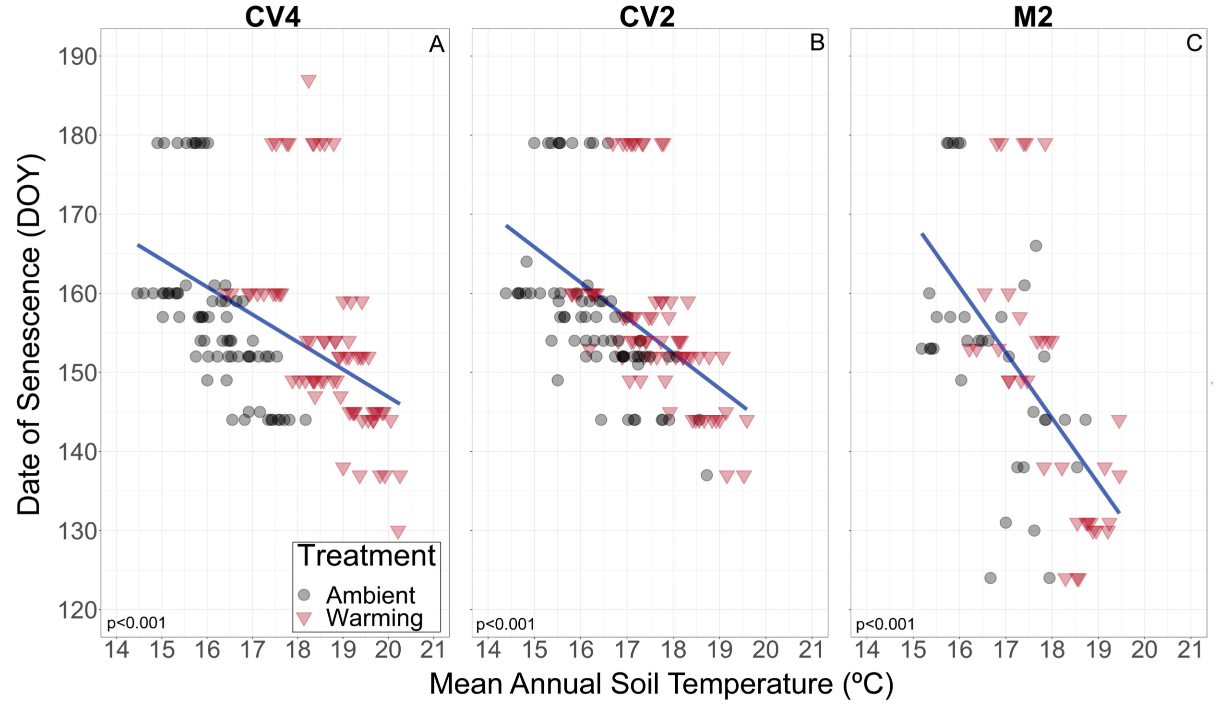

FIGURE 4 | Plot level relationship of date of *B. tectorum* senescence and mean annual soil temperature at 10 cm depth in the CV4, CV2, and M2 experiments (**A–C**, respectively). Values from warmed and ambient plots are shown with red triangles and black circles respectively. The linear fits are represented by blue lines and were determined by the geom_smooth function in ggplot2. The linear fits incorporate data from all plots (Ambient and Warming) in each site. R^2 values for each linear fit is shown in the bottom left of each panel.

preference. A better understanding of how *B. tectorum* phenology responds to changing temperatures across multiple sites and texture gradients is necessary for predicting the future success of the invasive plant.

Although our data point to a shortened *B. tectorum* growing season with both 2 and 4°C increases in temperature, the large phenological flexibility we observed suggest warmer conditions could also afford *B. tectorum* a competitive advantage over the perennial plants it lives amongst if those perennial plants are not able to respond to changing climate as quickly. We did not analyze phenological responses to warming of co-occurring species here, however, previous research in these experiments has shown a dominant native perennial species has experienced dramatic declines in cover in response to warming, potentially enabling *B. tectorum* to take advantage of this newly opened space and further invade the site (Winkler et al., 2019). Due to the large effect of *B. tectorum* on native perennials, any climate change effects on the invasive grass could, in turn, affect the success of native plants (Willis et al., 2010). For example, *B. tectorum* winter growth has been shown to inhibit germination success of co-located native perennials (Harris, 1977; Aguirre and Johnson, 1991). *Bromus tectorum's* roots also develop rapidly after fall germination, and as temperatures cool in the winter the plant becomes semi-dormant (Klemmedson and Smith, 1964; Aguirre and Johnson, 1991). If increased warming is sufficient to cancel dormancy effects of colder winter temperatures, warming could allow *B. tectorum* to stay metabolically active, and continue root growth, permitting increased exploitation of resources before natives are active (Ashbacher and Cleland, 2015). Although we did not find advances in the timing of fall germination with experimental warming, the substantial advance of flowering suggests that *B. tectorum* shifted its life stages earlier in response to increased temperatures. Such a shift would be expected to include the uptake of limited soil resources, and thus these changes could affect plant competition. In all, the large flexibility we observed in *B. tectorum* with warming and across years suggests a strong potential to track climate in way that may support the plant's success even in the face of climate change.

Large changes in the timing of *B. tectorum* life stages represent management challenges that additional data could help address. In particular, understanding the fate of this invasive grass with future climate would be of significant use to land managers, as would specific predictions of how phenology will change across time. Management of *B. tectorum* often focuses on spraying or grazing the plant prior to it setting seed (Whitson and Koch, 1998; Lehnhoff et al., 2019). If increasing temperatures create both earlier seed set and increased variability of the timing of seed set, this would create additional logistical challenges for determining when and where to treat *B. tectorum* across the landscape. The unique climate manipulation experiments presented here provide insight into the mechanisms through which this highly successful invasive plant may respond to changes in climate, and the effects of experimental warming on *B. tectorum* phenology offer a glimpse into how the common invasive grass will respond to predicted future climate conditions. The reduced vegetative growing season, the high plasticity observed, and the differences seen across sites improve our understanding of *B. tectorum's* ability to closely track climatic conditions, which provides forecasting power for the timing of key life stages. Overall, the findings from these experiments can inform predictions of when germination, flowering, and senescence may occur in order to more effectively manage invaded areas and to help identify which ecosystems may be prone to invasion with the continued effects of climate change.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AH synthesized and collected the data and wrote the first draft of the manuscript. AH, BM, and MP led the statistical analyses of the data. DW contributed a broader global change perspective to data interpretation. SR acquired funding and provided project leadership. All authors contributed to the idea development, data interpretation, and the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.570001/full#supplementary-material

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METHODS
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Assessing the Effectiveness of *in-situ* Active Warming Combined With Open Top Chambers to Study Plant Responses to Climate Change

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Temperature manipulation experiments are an effective way for testing plant responses to future climate conditions, especially for predicting shifts in plant phenological events. While passive warming techniques are widely used to elevate temperature in low stature plant communities, active warming has been applied less frequently due to the associated resource requirements. In forest ecosystems, however, active warming is crucial to simulate projected air temperature rises of 3–5 K, especially at the warm (i.e., southern and low elevation) range edges of tree species. Moreover, the warming treatment should be applied to the complete height of the experimental plants, e.g., regenerating trees in the understory. Here, we combined open top chambers (OTCs) with active heat sources, an electric heater (OTC-EH) and warming cables (OTC-WC), and tested the effectiveness of these set-ups to maintain constant temperature differences compared to ambient temperature across 18 m^2 plots. This chamber size is needed to grow tree saplings in mixture in forest gaps for 3 to 10 years. With passive warming only, an average temperature increase of approx. 0.4 K as compared to ambient conditions was achieved depending on time of the day and weather conditions. In the actively warmed chambers, average warming exceeded ambient temperatures by 2.5 to 2.8 K and was less variable over time. However, active warming also reduced air humidity by about 15%. These results underline the need to complement passive warming with active warming in order to achieve constant temperature differences appropriate for climate change simulations under all weather conditions in large OTCs. Since we observed considerable horizontal and vertical temperature variation within OTCs with temperature differences of up to 16.9 K, it is essential to measure and report within-plot temperature distribution as well as temporal temperature variation. If temperature distributions within large OTCs are well characterized, they may be incorporated in the experimental design helping to identify non-linear or threshold responses to warming.

Keywords: air temperature, electric heater (EH), phenology, relative humidity, spatial temperature distribution, warming cables

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INTRODUCTION

Climate change affects the structure and function of ecosystems (Walther et al., 2002; Thomas et al., 2004; Parmesan, 2006) by altering growth rates, physiology, survival, and distributions of individuals, populations, species, and communities (Dukes and Mooney, 1999; Hobbie et al., 1999; Morin et al., 2008; Reich et al., 2015; Gruner et al., 2017). Understanding and predicting biological effects of climate change are among the key challenges of current ecological research. Temperature manipulation experiments are an effective way of testing and quantifying plant responses to climate change (Ettinger et al., 2019). They are urgently needed to improve and validate models that predict climate driven shifts in phenological events.

Ecosystem warming experiments can employ passive or active warming methods and the efficiency of the different methods has been reviewed several times (e.g., Shaver et al., 2000; Rustad et al., 2001; Aronson and McNulty, 2009; Wolkovich et al., 2012; De Frenne, 2015; Ettinger et al., 2019). Passive warming methods include greenhouses or open top chambers (OTCs) that elevate daytime temperatures via a greenhouse-effect by transmitting solar radiation and trapping the heat within the chamber (Kennedy, 1995; Marion et al., 1997) or IR reflective curtains that increase night-time soil temperatures by reducing the infrared radiation heat loss from vegetation and soil surface at night (Beier et al., 2004; Emmett et al., 2004). Warming chambers for ecosystem manipulation studies consist of variously sized and shaped greenhouses, tents and OTCs. The latter were initially developed for gas exchange experiments. The passive warming of these chambers was an unintended side effect making them interesting for climate warming research (e.g., Drake et al., 1989). Due to their low infrastructure, maintenance and budget requirements OTCs have been widely used to elevate temperature in open low stature plant communities, such as remote arctic and alpine tundra ecosystems (e.g., Elmendorf et al., 2012), grassland steppe (Klein et al., 2005), temperate grasslands (Carlyle et al., 2011) and saltmarshes (Gedan and Bertness, 2009), but they were rarely used in taller-stature plant communities (Welshofer et al., 2018). OTCs are ineffective without solar irradiance, and thus have only limited potential for applications in forest ecosystems (De Frenne et al., 2010).

Active warming methods employ an external heat source (e.g., Cleland et al., 2006; Kimball et al., 2008; Dawes et al., 2011). They are independent of solar radiation and warming is implemented by either constant energy output (wattage), or a heating system with a feedback control system, which maintains a constant temperature difference between warmed and control treatments (Ettinger et al., 2019). The extensive associated energy demands (e.g., Pelini et al., 2011) as well as infrastructure and maintenance requirements limit the circumstances under which active warming techniques can be applied (Aronson and McNulty, 2009), such as their implementation in natural forest ecosystems.

IR heaters are the most frequently used external heat source for active warming of short-stature plant communities (e.g., Harte and Shaw, 1995; Luo et al., 2001; Shaw et al., 2002; Kimball et al., 2008), but they have recently also been used for the heating of a tropical forest understory (Kimball et al., 2018; Wood et al., 2019). Another valuable, often used active warming field method are soil warming cables (e.g., Peterjohn et al., 1993; Rustad et al., 2001; Melillo et al., 2017). However, both methods have difficulties in achieving target temperatures under unfavorable weather conditions, such as strong winds or rainstorms (Ettinger et al., 2019).

Only few climate manipulation experiments achieve warming with electric free air heaters that are combined with OTCs. Yet, such actively warmed chambers can provide a more accurate quantitative temperature control and have the potential to simulate non-linear and threshold responses to warming (Jentsch et al., 2007; Amthor et al., 2010). Norby et al. (1997) established an air-warming method by using a fully regulated active open-top chamber, which was heated by passing warmed air through the chamber. Bronson et al. (2008) built larger actively warmed chambers to study the effects of elevated temperatures on boreal black spruce (*Picea mariana*) and combined the air warming approach with soil-warming cables. In the framework of the whole ecosystem experiment SPRUCE (Spruce and Peatland Response Under Climatic and Environmental Change) a promising system of new active air warming chambers has been developed that can achieve multiple levels of experimental warming in combination with deep soil warming in a black spruce-*Sphagnum* peat bog in northern Minnesota (Barbier et al., 2013; Hanson et al., 2017; Richardson et al., 2018). Another whole ecosystem experiment, the Climate Change Experiment (CLIMEX), used fully enclosed air warming chambers consisting of large-scale greenhouses that enclosed an existing intact boreal forest ecosystem with mature trees and shrubs (Beerling, 1999). Medhurst et al. (2006) implemented a series of experiments using fully enclosed whole-tree chambers with air warming of single adult boreal spruce trees (*Picea abies*) in Norway. The same system has thereafter been deployed for *Eucalyptus* studies in Australia (Barton et al., 2010) and has subsequently been adapted to improve temperature control system (Crous et al., 2013; Drake et al., 2019).

Ettinger et al. (2019) reviewed 17 studies that applied active warming methods and included either multiple levels of warming or precipitation treatments. Their meta-analysis showed that warming treatments were confounded with a suite of indirect and feedback effects that are likely to affect biological responses studied in climate change experiments such as growth or phenology. They also underline the importance of considering spatial and temporal variation in plot temperatures. However, the study of Ettinger et al. (2019) only addressed among-plot variation while a better understanding of within-plot temperature variation, which may have similar ecological implications, would be equally important.

In this study, we aimed at developing an infrastructure suitable to warm the canopy of young temperate forest communities, i.e., 3- to 10-year-old tree seedlings growing in forest gaps which can be easily and relatively autonomously be deployed in the field. We also wanted to assess the feasibility of a warming experiment with a large number of tree saplings in the field. We combined OTCs with two different active heat sources, an electric heater (hereafter referred to as “OTC-EH”) and warming cables (hereafter “OTC-WC”), and tested the effectiveness of these set-ups to maintain a constant temperature difference of 3 K above ambient temperature across an 18 m^2 plot (5.2 m maximum basal diameter, 2.1 m OTC panel height). We measured air temperature and humidity distribution in the passively warmed OTC plot (hereafter “OTC-CTRL”), as well as in the two actively warmed OTC plots (OTC-EH and OTC-WC) and determined the achieved warming and humidity changes relative to ambient conditions (hereafter “Full-CTRL”) in order to test the efficiency of the different set-ups. Testing empty OTCs allowed us to focus on physical effects while minimizing biotic disturbances. Spatial and temporal temperature variation within plots and among treatments was analyzed based on more than 180,000 temperature records. Specifically, we asked the following questions: (1) How much warming can be achieved with (a) an OTC (passive warming only) and (b) an OTC combined with two different heat sources (active warming)? (2) Are the achieved warming effects constant over time? (3) If the achieved warming is not constant, what environmental factors cause variation in the effects? (4) Is the temperature evenly distributed across plots?

MATERIALS AND METHODS

Experimental Design

Four experimental plots were installed on a grassland in the outdoor area of the research facility WSL in Birmensdorf, Switzerland (47.3616°N, 8.4556°E, 560 m a.s.l.), in August 2019. The four hexagonal plots had a maximum basal diameter of 5.2 m and a height of 2.1 m. The six corners were marked with roof battens stacked into the soil. Three of the plots were framed with a 2.1 m × 16 m plastic foil (180 µm thick PP foil and 200 µm thick Lumisol clear AF) forming a tall OTC, whereas the fourth plot served as full control. The grass-covered soil surface of the plots was covered with a water-permeable lining and a 0.05 m thick layer of wood chips to prevent interference from transpiring plants. The first plot was experimentally warmed with an electric heater (plot 1, OTC-EH; **Figure 1A**), the second plot contained electrical resistance-heating cables laid out on the ground surface (plot 2, OTC-WC; **Figure 1B**), the third plot was passively warmed through the greenhouse effect of the OTC (plot 3, OTC-CTRL; **Figure 1C**) and the fourth plot was a full control

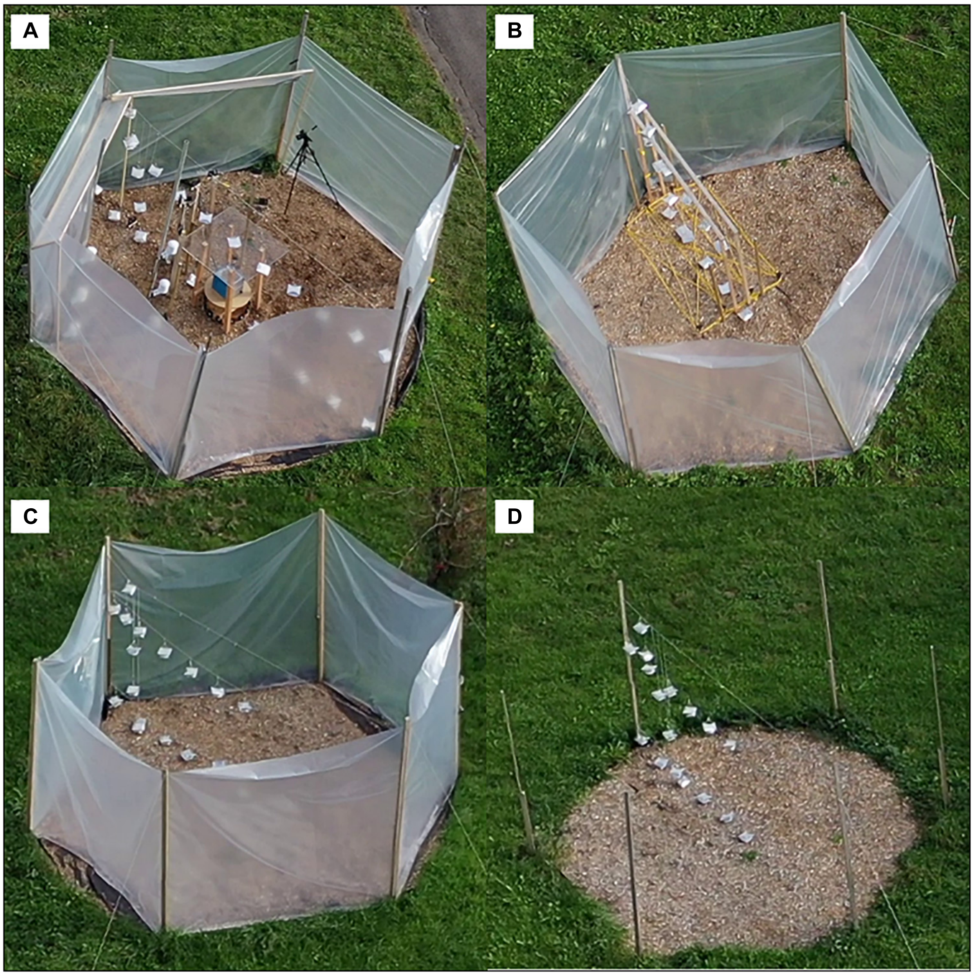

FIGURE 1 | Aerial view of experimental OTC-EH plot 1 **(A)**, OTC-WC plot 2 **(B)**, OTC-CTRL plot 3 **(C)**, and Full-CTRL plot 4 **(D)**.

plot without OTC, in which only the ground was covered with the water-permeable lining and wood chips (plot 4, Full-CTRL; **Figure 1D**).

The OTC-EH plot 1 was equipped with a 3-kW electric heater (TROTEC TDE25) located in the center of the plot and protected by a 100 cm × 100 cm transparent acrylic glass plate at 115 cm height. Ten large ventilators (DC 119 × 119 × 25 mm 24 V 195 m^3/h, NMB) and 17 small ventilators (DC 80 × 80 × 25 mm 12 V 70 m^3/h) were arranged in the Eastern half of the plot around the electric heater in order to distribute the warmed air within the OTC (**Figures 2A,B**). For the last experimental run (see section "Experimental Runs"), the whole plot 1 was equipped by 24 large and 24 small ventilators and wall-shields creating a 30° frustum to direct the warm air toward the ground (**Figures 2A,C**). In OTC-WC plot 2, 100 m soil heating cable (CAMPLEX, 11 W/m) was laid out as double-string and in spirals on the ground surface with 20 or 40 cm distance between neighboring cables, covering an area of a total of 6 to 9 m^2 in the Eastern half of the plot (**Figure 2D**). OTC-CTRL plot 3 was not equipped with any heating device to test passive warming effects of the OTC. Full-CTRL plot 4 was only equipped with the six corner roof battens and the permeable lining with the wood chips layer on the ground that allowed for identical plot temperature measurements as in plots 1–3 (for material details in **Supplementary Table S1**).

Temperature and Humidity Measurements

We measured air temperatures and humidity in each plot with 68 iButton loggers (36 DS 1922L Thermochron loggers, which measured only temperature and 32 DS 1923 Hygrochron loggers, which measured temperature and air humidity, Maxim Integrated, San Jose, CA, United States). To determine spatial temperature and humidity variation within each plot, two strings were spanned diagonally across each plot at 0.1 m and 1.5 m height. Six iButton loggers were attached to each diagonal string in distances of 0.5 m from the plot center to the Eastern plot edge. Additionally, five vertically spanned strings of different lengths (0, 0.5, 1.0, 1.5, and 2.0 m) were attached to a third diagonal string at 2.0 m height in a distance of 1.8 m from the plot center to the Eastern plot edge. Five iButton loggers

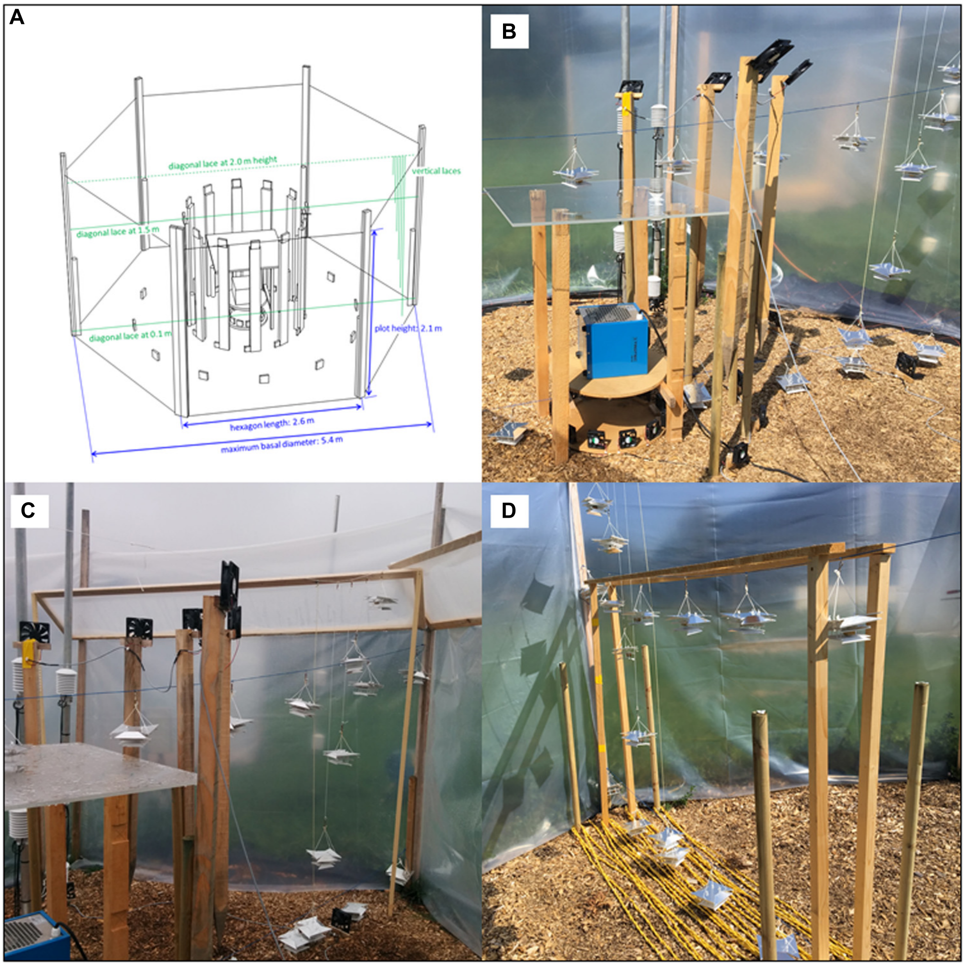

FIGURE 2 | Schematic view of OTC-EH plot 1 with full-plot set-up **(A)** and side-views of OTC-EH plot 1 with different set-ups **(B,C)** and OTC-WC plot 2 **(D)**.

were attached to these vertical strings. Each iButton logger was protected from direct solar radiation with a custom-fabricated radiation shield (referred to as "Small-Radshield" in Terando et al., 2017). We showed in a preliminary test under open field conditions that this type of radiation shield was more efficient in reducing heating up of the temperature sensors by solar radiation than the radiation shield described by Holden et al. (2013) or a manufacturer-recommended protective cover for outdoor temperature transmitters (TFA Dostmann GmbH & Co. KG). The iButtons protected with the small radiation shields most closely followed the temperatures measured at the climate station of the Model Ecosystem Facility/MODOEK nearby, an ATMOS 41 All-in-One Weather station from METER Group (**Supplementary Figure S1**). Even though the iButtons recorded temperatures approximately 0.5°C above the climate station during nighttime and 2°C during daytime, this does not affect absolute differences between individual iButtons (temperature resolution 0.0625°C) since they were all covered by the same type of radiation shield. The iButton loggers were set to record temperature and air humidity every 10 min.

Experimental Runs

Six experimental runs were conducted between August 23rd and December 6th 2019 featuring four slightly different set-ups. During the runs the effects of the arrangement of ventilators in the OTC-EH plot and the spacing of warming cables in the OTC-WC plot was tested (for details **Table 1**).

Data Analysis

All statistical analyses were conducted using R version 3.6.1 (R Core Team, 2019). We first calculated means, standard deviations, and ranges (=differences between highest and lowest values) of all logger temperatures in each plot. Based on these, we calculated the temperature differences $\mathrm{TD_{Treatment}} = \mathrm{T_{Treatment}} - \mathrm{T_{Full\text{-}CTRL}}$ between each treatment and the Full-CTRL. As an aggregated measure of the temperature increase achieved in the treatments with respect to ambient temperature, we determined the mean temperature difference (hereafter referred to as meanTD) between each treatment and Full-CTRL for each experimental run as $\mathrm{meanTD} = \frac{1}{n}\sum_{i=1}^{n} \mathrm{TD_{Treatment,\ i}}$. The meanTD between OTC-CTRL and Full-CTRL describes the passive warming effect of the OTC (without additional heating), whereas the meanTDs between the actively heated plots and Full-CTRL reveal the total achieved warming, which comprises the combination of passive and active warming effects. Similarly, we calculated the mean difference in relative humidity between treatments and Full-CTRL (meanRHD).

Student's *t*-tests (Welch Two Sample *t*-tests) were used to check if temperature differences between treatment and full control differed between day and night. Resulting *t*-values were Bonferroni corrected for multiple comparisons. To test whether the effectiveness of the warming treatments depended on weather conditions, we used linear mixed effects models and multi-model inference (Burnham and Anderson, 2002). The hourly average temperature differences between treatments (OTC-EH, OTC-WC, OTC-CTRL) and Full-CTRL were related to the following predictors: time of the day (day or night), hourly mean air temperature, rain (yes or no), and hourly mean wind speed (log transformed). Relative humidity and global radiation were not included in the models because they were highly correlated with air temperature. Because time of the day significantly interacted with temperature, rain and wind speed in an overall model, we decided to fit separate models for day (from sunrise to sunset) and night (from sunset to sunrise). Predictor and response variables were aggregated to hourly values. Linear mixed effect models were calculated with the lme-function in package nlme (Pinheiro et al., 2020). The identifier of the experimental run was included as random effect. Continuous predictor variables were standardized to a mean of zero and unit standard deviation using the function decostand from the package vegan (Oksanen et al., 2019). Automatic backward model selection using the function dredge (package MuMIn; Bartoń, 2019) was applied to find the simplest model explaining the highest proportion of variation. We report all models with a ΔAICc < 2 from the best fitting model. The times of sunrise and sunset for each day at the experiment location

TABLE 1 | Details of the six experimental runs conducted from August to December 2019, including their duration as well as the arrangement of the five ventilators at 170 cm height in OTC-EC and the warming cables in OTC-WC.

Run	OTC-EH Ventilator arrangement	OTC-WC Distance between warming cables	Start date	End date	Duration [hours]
1	5 ventilators at 170 cm above ground blowing toward the ground at a 30° angle, no frustum (**Figure 1A**)	20 cm	August 23	August 26	60
2	Same as run 1	40 cm	September 5	September 18	330
3	Same as run 1	40 cm	October 10	October 15	105
4	5 ventilators at 170 cm height blowing horizontally toward a 30° frustum designed to direct the warm air toward the ground (**Figure 1B**)	40 cm	October 18	October 21	60
5	Same as run 4	40 cm	October 26	November 1	138
6	12 ventilators at 170 cm height, blowing horizontally toward a 30° frustum designed to direct the warm air toward the ground, whole plot ventilated	40 cm	December 2	December 6	72

were determined using the R package suncalc (Thieurmel and Elmarhraoui, 2019). Weather parameters from two climate stations located within a distance of 100 m from our experimental plots were used: the LWF Uitikon Freiland station belonging to the Swiss Long-term Forest Ecosystem Research program LWF[1], which is part of the UNECE Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests ICP Forests[2] (Schaub et al., 2011): LWF Uitikon Freiland station (for precipitation and global radiation), and the Model Ecosystem Facility/MODOEK, WSL climate station, an ATMOS 41 All-in-One Weather station from METER Group (for air temperature and wind speed).

As measures for spatial variability of temperature within each experimental plot, the mean within-plot temperature standard deviation for each experimental run (hereafter referred to as MTSD) were calculated as MTSD $=\frac{1}{n}\sum_{i=1}^{n}\sigma\left(T_{\text{Treatment},\ i}\right)$. We also determined mean and maximum range of plot temperatures for each experimental run (hereafter referred to as meanTR and maxTR). Similarly, we calculated the mean within-plot standard deviation of relative humidity (MRHSD). We calculated radial temperature gradients at 10 cm height from the center toward the outer edge of the experimental plot as the difference between the temperatures measured at 250 and 50 cm from the center divided by the horizontal distance between the two logger positions and reported the mean gradients for each experimental run (hereafter referred to as mean gradient).

RESULTS

Passive and Active Warming Effects

Average plot temperatures, i.e., the mean temperatures of all individual temperature logger measurements within a plot at a given point in time, were mostly higher in the two actively warmed plots (OTC-EC and OTC-WC) than in the passively warmed plot (OTC-CTRL) and the full control plot (Full-CTRL; **Figure 3**). Determining passive and active warming effects turned out to be challenging during sunrise and sunset: OTC-EC and OTC-WC were located approximately 10 m to the west of OTC-CTRL and Full-CTRL, which means that the actively heated plots were hit by direct sunlight earlier than the control plots in the morning. The opposite effect occurred during sunset in the evening. Consequently, pairwise temperature difference between the heated plots and Full-CTRL peaked for a short period of time in the early morning (**Figure 3B**), while temperatures in OTC-CTRL and Full-CTRL were higher than in the actively warmed plots around sunset (**Figure 3A**) seemingly leading to lower heating effect with respect to Full-CTRL in actively heated plots than in OTC-CTRL (**Figure 3B**).

Nighttime temperatures in the actively warmed OTCs were elevated in comparison to OTC-CTRL and Full-CTRL. Average plot temperature of the passively warmed OTC-CTRL showed a different pattern than the actively warmed plots: during the night, the temperatures in OTC-CTRL were similar to those in Full-CTRL, whereas during daytime the former warmed more rapidly than the latter, as shown by

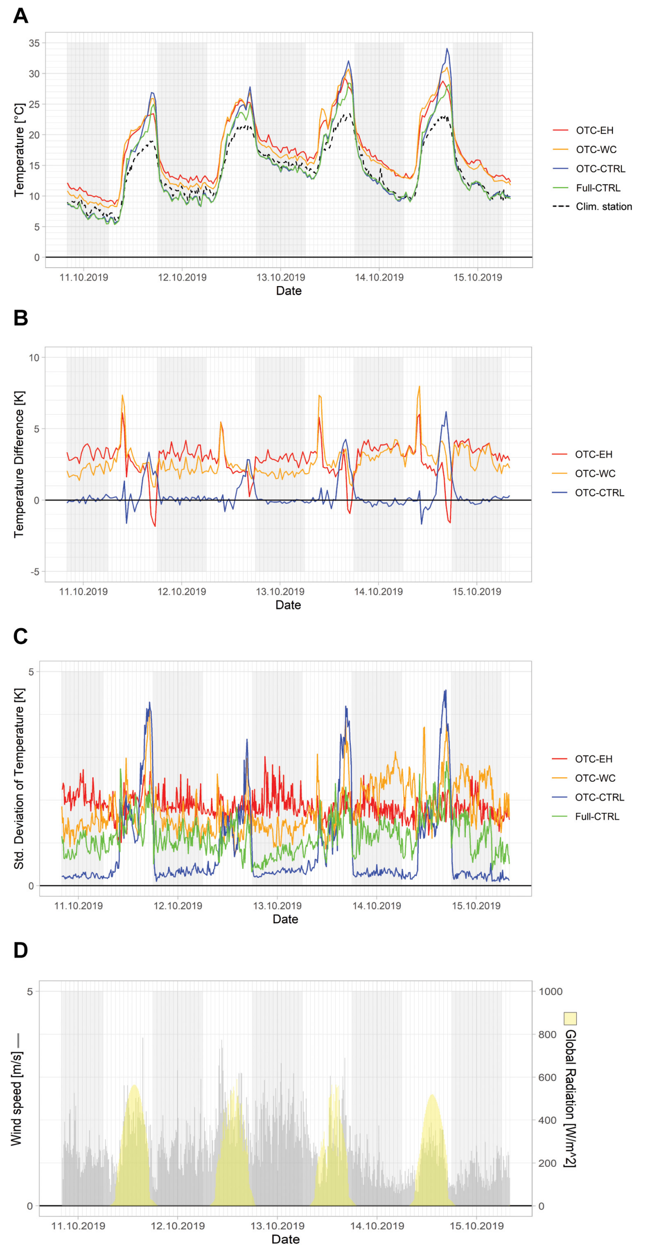

FIGURE 3 | Temperature measurements during experimental run 3 (October 10–15, 2019): **(A)** Mean absolute plot temperatures (Clim. Station: air temperature measured at the MODOEK climate station), **(B)** temperature differences with respect to Full-CTRL, **(C)** standard deviations of within plot temperatures, and **(D)** average wind speed and global radiation measured at the climate stations. The gray bars indicate wind speed and the yellow bars global radiation from climate stations. Gray shading indicates the time intervals between 6 pm and 6 am (not actual dark hours).

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[1] www.lwf.ch

[2] www.icp-forests.net

the increasing temperature difference between OTC-CTRL and Full-CTRL (**Figure 3**). We also found seasonal variation in the achieved passive warming: While temperatures in OTC-CTRL reached the temperatures of the actively warmed plots for part of the day in August and September, the achieved temperature difference became smaller toward winter because of the shorter day length and the lower elevation angle of the sun (**Table 2**).

The average warming measured as mean temperature difference (meanTD) relative to Full-CTRL (mean difference between warmed plot and full control) in the six experimental runs between August and December 2019 ranged from 2.58 to 3.02 K with the electric heater (OTC-EH), from 1.99 to 3.57 K with the warming cables (OTC-WC), and from 0.09 to 0.94 K with passive warming only (OTC-CTRL; **Table 2**).

Weather Effects on Achieved Warming During Day and Night

The meanTD of OTC-EH and Full-CTRL was 0.88 K larger during the night than during the day (3.25 K vs. 2.37 K, $t = 31.46$, $P < 0.001$). During the day, the meanTD was primarily affected by rain events and air temperature: the warming effect was generally greater during dry than rainy weather (**Table 3A**) and when ambient temperatures were lower. Higher wind speed also resulted in larger temperature differences, i.e., more warming, although the effect was smaller than that of rain and air temperature. At night, weather acted again as the main driver of temperature differences, with smaller differences during rain events. The effects of temperature and wind speed, by contrast, were inverse to their effects during the day: While higher ambient temperatures increased temperature differences, high wind speed led to smaller warming effects.

The meanTD of OTC-WC and Full-CTRL was 0.21 K larger during the night than during the day (2.48 K vs. 2.28 K, $t = 6.71$, $P < 0.001$). During the day, two models rendered almost equal goodness of fit (ΔAICc < 2). In both models, temperature differences, i.e., the amount of warming, was negatively affected by rainy weather, while air temperatures showed a much smaller but positive effect (estimate of −0.6 for rain and 0.1 for temperature, respectively; **Table 3B**). Although wind speed was included in the top model, it did not add much explanatory power (ΔAICc = 1.6). At night, rainy conditions considerably decreased warming effects and higher air temperature again resulted in larger temperature differences. In contrast to daytime, wind speed resulted as the most important factor during the night, significantly reducing warming effects.

For OTC-CTRL, the meanTD was 0.58 K smaller during the night than during the day (0.09 K vs. 0.67 K, $t = -23.70$, $P < 0.001$). During daytime, temperature differences increased first and foremost with air temperature (**Table 3C**) and to a lower degree with higher wind speeds. Interestingly, rain events were negligible (included in model 2 but not significant). At night, higher air temperatures increased temperature differences, whereas higher wind speeds reduced them.

Spatial Temperature Variation

Temperature substantially varied within the plots. We found greatest spatial temperature variation in OTC-EH and OTC-WC with average standard deviations of within-plot temperatures (MTSD) of 1.64 to 3.50 K and 1.64 to 2.84 K, respectively (**Table 4**). The corresponding mean range between the highest and the lowest temperature (meanTR) in these plots amounted from 4.64 to 9.65 K and 4.59 to 7.42 K, respectively, with greatest differences (maxTR) reaching up to 10.98 and 16.93 K. In Full-CTRL the spatial temperature varied about half as much as in the actively warmed plots with MTSDs of 0.35 to 1.38 K and meanTR of 0.95 to 3.64 K. The maxTR observed in Full-CTRL was 11.21 K. Overall, the spatial temperature variation was smallest in OTC-CTRL with MTSDs of 0.23 to 0.88 K, meanTR of 0.71 to 2.70 K, and maxTR of 5.62 to 15.51 K. The mean radial temperature gradients at ground level (i.e., the temperature gradient from the plot center to the plot wall) in OTC-EH ranged between +4.43 and +1.56 K/m. In the other plots, much smaller gradients were observed with +0.81 to −0.12 K/m in OTC-WC, +0.17 to −0.60 K/m in OTC-CTRL, and +0.28 to −0.05 K/m in Full-CTRL.

In the actively warmed plots, temperatures were 2.28 and 2.46 K higher (OTC-EH and OTC-WC, respectively) at 10 cm as compared to 150 cm above ground. In the two plots without active warming (i.e., OTC-CTRL and Full-CTRL), however, vertical temperature differences were small and temperatures at 10 cm were slightly lower than at 150 cm above ground (OTC-CTRL: $\Delta T = -0.18$ K; Full-CTRL: $\Delta T = -0.52$ K; **Table 5**).

Air Humidity Variation Between and Within Treatments

Relative air humidity in the actively warmed plots (OTC-EH and OTC-WC) was on average 13.4% lower than ambient humidity (**Table 6**), whereas in OTC-CTRL, air humidity was only reduced by 2.1% on average (**Table 6**). Relative humidity varied considerably in actively warmed

TABLE 2 | Mean temperature differences (meanTD) between treatments and Full-CTRL for the six experimental runs.

Parameter	Plot	Experimental run						All runs		
		1	2	3	4	5	6	Average	Min	Max
meanTD [K]	OTC-EH vs. Full-CTRL	2.79	2.58	2.91	2.96	2.88	3.02	2.86	2.58	3.02
	OTC-WC vs. Full-CTRL	3.57	2.31	2.72	2.14	1.99	2.35	2.51	1.99	3.57
	OTC-CTRL vs. Full-CTRL	0.94	0.55	0.39	0.18	0.24	0.09	0.40	0.09	0.94

TABLE 3 | Model selection results for differences between treatments and Full-CTRL separately for day (defined as time from sunrise to sunset) and night (defined as time from sunset to sunrise).

Top LMM	Explanatory variable	Linear mixed model			Multi-model inference	
		Estimate	**SE**	***P***	**df**	**AICc**
(A) Temperature difference between OTC-EH and Full-CTRL						
Day						
1	rain	−0.47	0.20	0.020	343	994.24
	air temperature	−0.29	0.07	<0.0001		
	wind speed	0.11	0.05	0.035		
Night						
1	rain	−0.60	0.08	<0.0001	414	559.97
	air temperature	0.42	0.05	<0.0001		
	wind speed	−0.29	0.02	<0.0001		
(B) Temperature difference between OTC-WC and Full-CTRL						
Day						
1	rain	−0.66	0.18	0.0003	343	925.12
	air temperature	0.15	0.07	0.027		
	wind speed	−0.09	0.05	0.054		
2	rain	−0.69	0.18	<0.0001	344	926.74
	air temperature	0.15	0.07	<0.0001		
Night						
1	wind speed	−0.58	0.03	<0.0001	414	609.94
	rain	−0.49	0.08	<0.0001		
	air temperature	0.19	0.05	<0.0001		
(C) Temperature difference between OTC-CTRL and Full-CTRL						
Day						
1	air temperature	0.84	0.07	<0.0001	344	1009.26
	wind speed	0.11	0.05	0.044		
2	air temperature	0.87	0.08	<0.0001	343	1010.91
	rain	0.15	0.21	0.469		
	wind speed	0.11	0.06	0.051		
Night						
1	wind speed	−0.04	0.01	<0.0001	415	−302.44
	air temperature	0.03	0.01	0.030		

The full model included the following explanatory variables: air temperature (continuous), presence of rainfall (binomial), and average wind speed (continuous, log-transformed). Experimental run was included in the models as random effect. Starting with the full model, multi-model inference was used to select the simplest models explaining the highest proportion of variation. The top linear mixed models with $\Delta AICc < 2$ *are shown* ($N_{Day} = 352$, $N_{Night} = 423$).

plots with average within-plot standard deviations of 7.7 and 10.0% in contrast to the two control plots with average within-plot standard deviations of only 2.5 and 2.2 % (**Table 7**).

DISCUSSION

Our findings show that complementing large OTCs with active warming equipment resulted in an average achieved warming of 2.5 to 2.9 K and that the warming effect varied over time. It was strongly reduced by rain and also partly influenced by air temperature and wind. Substantial horizontal and vertical within-chamber temperature variation was observed. Relative air humidity in general showed opposite patterns compared to temperature changes.

Passive and Active Warming Effects

The average warming achieved in the six experimental runs between August and December 2019 was around six to seven times higher in the actively warmed plots with an electric heater (meanTD = 2.86 K) or warming cables (meanTD = 2.51 K) as external heat source than in the passively warmed OTC-CTRL (meanTD = 0.40 K; **Table 2**). One of the main reasons

TABLE 4 | Within plot spatial temperature variation as characterized by the mean standard deviation (MTSD), mean (meanTR) and maximum (maxTR) temperature range of all loggers in a plot.

Parameter	**Plot**	**Experimental run**						**All runs**		
		1	**2**	**3**	**4**	**5**	**6**	**Average**	**Min**	**Max**
MTSD [K]	OTC-EH	1.88	1.83	1.85	1.64	1.74	3.50	2.07	1.64	3.50
	OTC-WC	2.84	1.64	1.80	1.72	1.67	2.34	2.00	1.64	2.84
	OTC-CTRL	0.88	0.69	0.82	0.26	0.40	0.23	0.55	0.23	0.88
	Full-CTRL	1.38	1.09	1.15	0.40	0.50	0.35	0.81	0.35	1.38
meanTR [K]	OTC-EH	5.93	5.90	5.68	4.64	5.17	9.65	6.16	4.64	9.65
	OTC-WC	7.42	4.59	5.04	4.84	4.86	6.69	5.57	4.59	7.42
	OTC-CTRL	2.70	2.00	2.44	0.84	1.15	0.71	1.64	0.71	2.70
	Full-CTRL	3.64	2.88	2.94	1.12	1.40	0.95	2.15	0.95	3.64
maxTR [K]	OTC-EH	9.23	10.98	9.16	8.68	10.97	10.65	9.95	8.68	10.98
	OTC-WC	15.54	16.93	12.37	7.63	8.59	13.79	12.48	7.63	16.93
	OTC-CTRL	14.62	15.51	14.55	6.62	13.54	5.62	11.74	5.62	15.51
	Full-CTRL	11.21	10.71	9.86	7.90	8.79	5.95	9.07	5.95	11.21
mean temperature gradient [K/m]	OTC-EH	2.23	2.25	2.18	1.56	1.99	4.43	2.44	1.56	4.43
	OTC-WC	0.81	−0.07	0.01	−0.12	0.03	0.77	0.24	−0.12	0.81
	OTC-CTRL	0.04	0.17	−0.60	−0.14	−0.10	0.11	−0.09	−0.60	0.17
	Full-CTRL	0.12	0.23	0.28	−0.05	0.01	0.06	0.11	−0.05	0.28

Values are indicated for the individual experimental runs and summarized over all runs. For the mean radial temperature gradient from the center to the edge of the plot only measurements at 10 cm height were considered.

explaining this difference is that passive warming was only effective during daylight hours when the plots were warmed by solar irradiance (meanTD$_{Day}$ = 0.67 K). Therefore, passive warming in OTC-CTRL was obviously negligible at night (meanTD$_{Night}$ = 0.09 K; **Figure 3**). The greatest passive warming effect (maxTD between OTC-CTRL and Full control) was reached around 4 pm. It therefore lagged 2.5–3 h behind the peak solar irradiance, which occurred around 1 pm CEST (**Figure 3D**). This is the same time of the day when also ambient air temperature reached its maximum (dashed line in **Figure 3A**). The passive warming effect is due to "trapping" of infrared radiation emitted by the ground surface inside the OTC (greenhouse effect). In particular, since the degree of passive warming is correlated with the ground surface temperature and it takes some time to heat the ground, air temperature lags behind solar irradiance. Other studies with OTCs at a High Arctic site as well as at a site in Montreal confirmed that warming occurred mainly during day-time hours (Marion et al., 1997; Dabros et al., 2010) and that warming correlated positively with solar irradiance at the site (Bokhorst et al., 2013). In contrast, active warming can be effective 24 h a day and we found an even greater warming effect at night (OTC-EH: meanTD$_{Day}$ = 2.37 K vs. meanTD$_{Night}$ = 3.25; OTC-WC: meanTD$_{Day}$ = 2.28 K vs. meanTD$_{Night}$ = 2.48 K). We suggest that the active warming is more effective because of increased convection effects at night that support efficient air mixing inside the OTCs.

TABLE 5 | Mean temperatures, standard deviation of temperatures and differences between mean temperatures at 10 and 150 cm over all six experiment runs.

Plot	**Height above ground [cm]**	**Mean temperature [°C]**	**Standard deviation [K]**	**ΔT [K]**
OTC-EH	10	17.45	6.59	2.28
	150	15.17	7.36	
OTC-WC	10	16.75	7.45	2.46
	150	14.29	7.99	
OTC-CTRL	10	13.08	7.94	−0.18
	150	13.26	7.12	
Full-CTRL	10	13.21	7.78	−0.52
	150	13.73	8.22	

Our results demonstrate that topography and the relative positioning of the reference temperature measurement are important shortly after sunrise and before sunset, particularly in fall when solar elevation above the horizon is low. This resulted in short periods of time when seemingly erratic temperature differences between the two actively warmed OTCs (OTC-EH, OTC-WC) and Full-CTRL were measured (**Figure 3B**), which were due to earlier sunrise and earlier sunset in actively warmed OTCs as compared to OTC-CTRL and Full-CTRL. Topography and the location of the reference temperature measurement should be evaluated carefully when designing a warming experiment with large OTCs that are potentially scattered over an area of several hundred square meters, particularly if the degree of warming is regulated relative to the control. Other sources of confounding variation might include shading by the OTC construction or other technical equipment.

Environmental Influences on Warming

The mixed model analysis revealed that air temperature had the greatest influence on passive warming (OTC-CTRL)

TABLE 6 | Mean differences in relative humidity (meanRHD) between treatments and Full-CTRL for the six experimental runs.

Parameter	Plot	Experimental run						All runs		
		1	2	3	4	5	6	Average	Min	Max
meanRHD [%]	OTC-EH vs. Full-CTRL	−10.98	−10.13	−12.69	−15.26	−18.47	−12.69	−13.37	−18.47	−10.13
	OTC-WC vs. Full-CTRL	−15.79	−10.63	−13.81	−12.98	−13.49	−13.75	−13.41	−15.79	−10.63
	OTC-CTRL vs. Full-CTRL	−3.44	−3.36	−1.17	−0.78	−2.36	−1.44	−2.09	−3.44	−0.78

TABLE 7 | Spatial variability of relative humidity within plots measured as mean standard deviation of relative humidity (MRHSD) for the six experimental runs.

Parameter	Plot	Experimental run						All runs		
		1	2	3	4	5	6	Average	Min	Max
meanRHSD [%]	OTC-EH	6.59	6.07	7.23	9.51	9.69	7.23	7.72	6.07	9.69
	OTC-WC	12.61	6.89	9.39	10.72	9.47	11.03	10.02	6.89	12.61
	OTC-CTRL	3.04	2.55	3.55	2.52	2.05	1.34	2.51	1.34	3.55
	Full-CTRL	2.90	2.94	3.06	1.44	1.52	1.50	2.23	1.44	3.06

during the day with higher air temperatures resulting in greater warming. A likely explanation for this finding is that air temperature is related to weather conditions: higher temperatures are commonly observed during sunny days when high solar irradiance leads to efficient passive warming of the OTC. Interestingly, rain events did not significantly influence passive warming. However, the effect of rain should be interpreted with caution because there were only very short periods of rainfall during the experiments and during some of the experimental runs rainfall was completely absent. At night, when the warming in OTC-CTRL was nearly zero, the influence of the considered weather factors was only weak (**Table 3**).

The active warming techniques, however, were most importantly influenced by rain, which reduced the effectiveness of active warming by roughly 0.6 K both during day and night. Reduced warming during rainstorms was also observed in an experiment using soil warming cables buried at 10 cm depth (Peterjohn et al., 1993). This suggests that additional heating power is required to maintain constant temperature differences during rain events. Higher wind speed consistently reduced temperature differences in all treatments at night and in OTC-WC also during the day, which is likely due to more efficient mixing of air inside the OTCs with ambient air reducing the warming effect. Numerical simulations showed that the wind-induced mixing effects inside an OTC become stronger with increasing wall heights (Cunningham et al., 2013). Warming effects increased with higher wind speeds during the day in OTC-EH and OTC-CTRL because strong wind cools ambient air in contrast to less circulated air within OTCs, which is warmed by solar irradiance during the day.

Environmental influences and temporal temperature variation might be reduced by installing additional heating combined with a temperature feedback system that controls the power output of the heaters. Temperature variation in such feedback controlled systems has been found to be smaller than in set-ups with constant wattage heating (Ettinger et al., 2019).

Warming Effects on Air Humidity

Warming the air by 2.5 K would lead to approximately 12.5% higher potential evapotranspiration (Dabros et al., 2010) and consequently to reduced soil moisture and air humidity, which may cause drought stress for plants (Amthor et al., 2010). In our experiment, we observed that relative humidity in OTC-EH and OTC-WC was 13.4% lower than in Full-CTRL on average, whereas in OTC-CTRL relative humidity was reduced by only 2.1% (**Table 6**). Similar humidity reductions as in our active warming treatments were observed in the SPRUCE whole ecosystem warming experiment with large OTCs in northern Minnesota (Hanson et al., 2017). According to climate change scenarios, there may be regional and temporal variation in the extent and direction of predicted air humidity changes (Stocker et al., 2013). Thus, several experiments compensated for reduced air humidity by moisturizing the heated air with humidification systems (e.g., Thompson et al., 1992). However, such installations are resource intensive and technically challenging. As an increase of air temperature is naturally associated with a reduction in relative humidity, we believe that both effects should be included in climate change experiments. Since not only temperature but also humidity changes may influence plant physiology, growth and phenology as well as soil microbiology, climate change experiments should not only measure and report air temperature but also soil temperature and moisture as well as air humidity (Amthor et al., 2010; Ettinger et al., 2019).

Spatial Temperature Variation

Our temperature measurements in the full control plot showed that the horizontal and vertical temperature distribution under ambient conditions was relatively homogeneous within the volume of our OTCs (5.2 m diameter, 2.1 m height). The MTSD in this plot was 0.81 K and the meanTR was 2.15 K (**Table 4**). Temperature varied even less in OTC-CTRL with MTSD = 0.55 K and a meanTR = 1.64 K (**Table 4**), which suggests that the OTC had an equalizing effect on within-chamber temperatures. We propose that the OTC walls acted as temperature-equalizing elements, which warmed or cooled all air layers within the OTC similarly whereas the ground affected air warming or cooling only within a few centimeters.

In the actively warmed plots (OTC-EH and OTC-WC), spatial temperature variation was substantially greater than in OTC-CTRL and Full-CTRL with MTSDs of 2.07 and 2.00 K and meanTRs of 6.16 and 5.57 K, respectively (**Table 4**). The overall larger temperature variation in the actively warmed plots as compared to OTC-CTRL can, at least partly, be explained by vertical temperature differences: in the actively warmed plots average temperatures at 150 cm above ground were 2.28 K (OTC-EH) to 2.46 K (OTC-WC) lower than at 10 cm height while they were only marginally lower (0.2 to 0.5 K) in the two control plots (**Table 5**). This demonstrates that both the electric heater and the warming cables caused pronounced warming near the ground but only moderate warming at 150 cm above ground. For the electric heater in OTC-EH this was due to the way it was installed, blowing the heated air toward the ground. Since the warming cables in OTC-WC were laid out on the ground surface, they mostly warmed the near-ground air and the top-soil, which has been demonstrated in other studies (e.g., Hagedorn et al., 2010).

Vertical heat exchange in both set-ups occurred mainly through natural convection, i.e., the buoyancy of warmer, lower density air. This effect may have been enhanced passively by the OTC walls during the day. However, the relatively large vertical within-chamber temperature differences suggest that internal air mixing in the actively warmed OTCs was limited. It is also unclear to what extent air escaped through the open tops of the OTCs. The escape of warm air may be prevented by covering the OTCs at least partly. This may, on the other hand, intercept precipitation depending on the specific design.

Horizontal temperature distribution in OTC-WC was similar to the two control plots, with small mean temperature gradients in horizontal direction between the OTC center and the OTC wall in the range of −0.09 to +0.24 K/m (**Table 4**), which may be explained by the layout of the warming cables that covered the complete radius of the OTC. In contrast, in OTC-EH, the heat source was concentrated in the center of the plot. To compensate for this technical constraint, we distributed the warm air more evenly over the chamber radius by installing small ventilators, which blew the warmed air in radial direction toward the OTC walls. A homogeneous temperature distribution was not completely achieved, though. The horizontal mean temperature gradient at 10 cm height in OTC-EH of 2.44 K/m was clearly higher than in the other plots (**Table 4**), underlining the difficulty to achieve a homogeneous temperature distribution within the OTC using this method. Temperature gradients in OTC-EH were smaller in experimental runs 4 and 5 than in runs 1–3 (1.78 K/m vs. 2.2 K/m), which shows that the 30° frustum added to the setup in runs 4 and 5 helped to achieve a more homogeneous temperature distribution in OTC-EH, which is also reflected in the MTSC values (**Table 4**). The temperature distribution in OTC-EH was clearly less homogenous in experimental run 6 compared to all other runs. This shows that the heating power of the electric heater only insufficiently produced homogenous warming when ventilators were blowing heated air in all directions as compared to only one half of the OTC in the other runs. It might be considered to force air mixing in large OTCs by installing larger or more effective fans. However, the increased air movement caused by these devices may exacerbate the potential side effects of ventilators, i.e., increased evapotranspiration and forced movements of the plants. Alternatively, we suggest to integrate the temperature gradient in OTC-EH in the experimental design. Such a design would allow to test different degrees of warming within a single OTC with strongest warming in the center of the OTC and decreasing degrees of warming toward the walls. A similar concept has been suggested for warming studies on rice paddies by combing an OTC with a solar-heated air-introduction tunnel (Chiba and Terao, 2014).

Ecological Implications of Warming Methods

Climate manipulation experiments are valuable tools in global change ecology. While small passive OTCs can adequately warm low-stature plant communities or early life stages, larger-sized, actively warmed OTCs are required to study long-term warming effects on taller vegetation, such as tree saplings. Our large OTCs in combination with an electric air heater or warming cables as external heat source achieved a mean daytime warming of 2.5 to 2.9 K. This amount of warming approximately corresponds to recent IPCC climate change scenario predictions for the middle of the century (Stocker et al., 2013) and a similar amount of warming has frequently been shown to alter phenology, growth and survival of tundra plant communities in passive warming experiments (e.g., Arft et al., 1999; Elmendorf et al., 2012). Active warming experiments revealed similar responses in forest ecosystems, e.g., physiological acclimation (Drake et al., 2016) and phenological shifts at both ends of the growing season (Bronson et al., 2009; Gunderson et al., 2012; Richardson et al., 2018).

Since ecosystem responses to environmental changes are not always linear (Amthor et al., 2010), experimental designs incorporating environmental gradients are required to identify non-linear or threshold responses. Such a design has been implemented in the SPRUCE experiment with several large chambers that are designed to produce different levels of warming up to +12 K relative to ambient temperature (Richardson et al., 2018). As an alternative, we suggest to plan experiments such that the within-chamber temperature gradients that we observed in the actively warmed OTCs are incorporated in the experimental design for testing the non-linearity in plant and ecosystem responses to warming. Furthermore, the observed influence of the surrounding landscape on temperatures underlines the importance of a robust experimental design with replicate blocks to take such confounding effects into account.

The reported results were measured with empty OTCs in order to minimize biotic inferences with the heating setup and facilitate the understanding of physical effects occurring in the OTCs. We expect that plants in the OTCs will influence the warming depending on plant density, height and species identity.

However, it is important to test the effect of the plants on warming with the actual plants before the start of an experiment. Since our experiments were run during fall and early winter, the reported results are conservative and higher degrees of warming are expected during spring and summer due to more sunshine hours and higher intensity of solar irradiance.

CONCLUSION

Based on our findings for warming experiments with large OTCs we conclude that the temperature distribution in a passive OTC is reasonably homogeneous across space. The maximum amount of warming that can be achieved remains limited, though, and is not constant over time because the passive warming effect is small or inexistent during the night and under cloudy weather conditions. In contrast, the warming that can be achieved in actively warmed OTCs is substantially greater than in passively warmed chambers–provided that sufficient heating power is installed. Temporal variation in the achieved warming is much smaller than in a passive OTC. Nevertheless, the active warming methods have the disadvantage of producing uneven horizontal and vertical temperature distributions, although horizontal temperature variation is considerably smaller when using warming cables. The observed horizontal and vertical within-plot temperature variation can be several times larger than the target temperature difference in the study, which is a critical issue when these methods are used to study plant responses to warming such as changes in phenological or growth traits. Considering these findings, we emphasize the importance of a statistically robust experimental design and suggest to incorporate within-chamber temperature gradients in the experimental design instead of aiming to achieve a homogeneous temperature distribution within the OTCs. This approach requires to measure and report the spatial and temporal temperature distribution within the experimental chambers during the experiment (see also recommendations in Ettinger et al., 2019), and to consider the temperature variation in the interpretation of observed changes in plant growth and phenology.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

ERF, LS, YV, TW, and BM conceived the ideas and designed the methodology. LS and ERF collected the data. ERF, BM, and LS analyzed the data. ERF and BM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.539584/full#supplementary-material

Supplementary Figure 1 | Comparison of temperature readings of unprotected iButtons (red), iButtons protected with a commercial protective cover (yellow), and custom-fabricated radiation shields (orange) to the temperature recorded at the MODOEK climate station in approx. 1 m distance (black).

Supplementary Table 1 | Materials and Equipment: List of required materials.

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