

RESPONSES TO CLIMATE CHANGE IN THE COLD BIOMES

EDITED BY: Hans J. De Boeck, Erika Hiltbrunner, Anke Jentsch and
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PUBLISHED IN: Frontiers in Plant Science





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ISSN 1664-8714

ISBN 978-2-88945-877-6

DOI 10.3389/978-2-88945-877-6

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RESPONSES TO CLIMATE CHANGE IN THE COLD BIOMES

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Gentiana verna (Ftan, Switzerland). Image: Hans De Boeck.

Climate change is thought to be especially relevant to ecosystems in the cold biomes. Observed warming has been higher in cold climates through various positive feedbacks, especially declining snow and ice cover, and climate projections indicate further rapid warming in the decades to come. Temperature change can have profound impacts in cold biome ecosystems, either directly in terms of impacts on physiology or growing season length, or indirectly via changes in nutrient cycling. The regions focused on here are the (sub)arctic and the (sub)alpine areas, both characterized by short growing seasons and low annual temperatures, but with different radiation environments depending on latitude. Climate change can have

impacts in all seasons. Increased spring temperatures can accelerate snowmelt, leading to an earlier onset of the growing season, while warmer summers may stimulate primary productivity through temperatures closer to metabolic optima and/or increased mineralization rates. Winter warming can lead to the vegetation being damaged because of exposure to harsh frost without insulating snow cover. In all of this, concurrent changes in precipitation also play an important role: increased snowfall can buffer warming-induced advances in snowmelt, a higher ratio of rain to snow can greatly accelerate snowmelt in winter and spring, and summer drought may reverse growth-stimulation by warming directly (drought stress) or indirectly (e.g. impaired nutrient uptake). Micro-climate is crucial in these systems and requires particular attention as it can vary widely across the landscape, creating different growing environments in the space of a few meters or even less.

Interest in cold region responses to climate change does not only arise from the fact that they harbor unique ecosystems that may be endangered, but also because they store large amounts of carbon that may be released under climate change. However, research is challenging because of the remoteness of many of these areas and the harsh conditions during much of the year. In spite of this, some studies have been carried out over an extensive period, spanning decades and yielding information on for example plant community reorganization (including invasions), and changes in phenology above- and/or belowground. Other studies focus on shorter term effects, such as impacts of heat waves, late frosts or other anomalous weather, including longer term (after-) effects that may differ drastically from other regions because of the short growing season in cold climates. Ultimately, models are used to predict future changes in vegetation along latitudinal or elevational gradients, although phenology and microclimatic variation may pose particular challenges.

Contributions to this Research Topic focus on climate change, encompassing both changes in the mean (gradual warming) and variability (heat waves, altered precipitation distribution) in cold biomes. The Topic contains reports on observed changes or events, but also research making use of experimentally imposed environmental changes. The focus is varied, including phenology, physiology, soil and vegetation science and biogeochemistry, with the aim of providing a comprehensive overview of observed and expected responses to climate change in cold biome ecosystems.

Citation: De Boeck, H. J., Hiltbrunner, E., Jentsch, A., Vandvik, V., eds. (2019). Responses to Climate Change in the Cold Biomes. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-877-6

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Editorial: Responses to Climate Change in the Cold Biomes

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Keywords: climate change, alpine, arctic, invasive species, biodiversity, climate extreme, range limits, Antarctic

Editorial on the Research Topic

Responses to climate change in the cold biomes

Climate change is a global phenomenon, with particularly rapid changes observed in many mountain ranges, the arctic, and the Antarctic Peninsula, where temperatures have risen at twice the global average or more (Gobiet et al., 2014; Johannessen et al., 2016). The accelerated warming in these cold biomes is mainly due to various positive feedbacks, such as albedo effects related to declining snow and ice cover (Screen and Simmonds, 2010; Pepin et al., 2015). Apart from temperature, precipitation patterns have been changing in most of these regions as well, with varying impacts on snow pack depths, growing season length and in some regions also on the prevalence of summer drought (Gobiet et al., 2014; Bintanja, 2018). While these rapid environmental changes have the potential to significantly alter responses and dynamics of the local ecosystems (e.g., Niittynen et al., 2018), logistic challenges have constrained research in these often remote locations. In this research topic, we collected a number of studies that have overcome these challenges to report on the responses of cold biome ecosystems to various aspects of climate change.

In trying to predict how ecosystems will respond to rapid climate warming, insights may be gained from warm periods in the past. Tree rings provide important information on historic temperatures at the treeline, where the primary limiting factor for tree growth is considered to be growing season temperature (Paulsen and Körner, 2014). In this research topic, Lange et al. investigated possible distortions of temperature-growth relationships along a transect running from Finland into Western Siberia, and found that the temperature signal of *Pinus sylvestris* differed markedly between climate regimes. This could challenge the assumption that the correlation between temperature and radial tree growth limitation is stable over time. The micro-site climate is likely to play an important role here (Körner et al., 2016), which has implications not only for temperature reconstructions, but also for other applications where absolute growth is important, such as dynamic vegetation models.

Experiments are powerful tools for establishing cause-effect relationships and testing specific hypotheses regarding climate change impacts, but researchers should be aware of the limitations of different methods and techniques (e.g., De Boeck and Nijs, 2017). Yang et al. combined three warming approaches in a single study in the Hengduan Mountains (China) to establish their influence on indirect (biotic) climate effects. They concluded that differences exist especially regarding the rate of colonization by new species, which is more rapid in whole-community transplantation studies compared to experiments employing open top chambers (OTCs). Yang et al. suggest that combined approaches therefore provide opportunities in disentangling direct effects of warming from indirect effects mediated through novel plant-plant interactions.

OPEN ACCESS

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 26 February 2019

Accepted: 07 March 2019

Published: 28 March 2019

Citation:

De Boeck HJ, Hiltbrunner E, Jentsch A
and Vandvik V (2019) Editorial:
Responses to Climate Change in the
Cold Biomes. *Front. Plant Sci.* 10:347.
doi: 10.3389/fpls.2019.00347

Sierra-Almeida et al. used OTCs in the first *in situ* study of the impact of climate warming on the freezing tolerance of the only two naturally occurring vascular species in maritime Antarctica (*Colobanthus quitensis* and *Deschampsia antarctica*). Their results show that temperature increases can lead to a reduction in freezing tolerance, highlighting the complexity of warming effects and the importance of phenotypic plasticity in coping with climate change. In a transplantation experiment, Henn et al. demonstrated that leaf trait plasticity enabled alpine plants to persist within communities exposed to warming. This encompassed mostly convergence toward optima found in the local, pre-adapted plants when transplants were moved to warmer conditions. The authors note that the extent and importance of trait plasticity is species-specific, as was also reported in the study of Cui et al. who found low plasticity in leaf area of *Viola biflora* var. *rockiana*. For this species, the key to survival under climate warming seems to hinge on being able to avoid competitive effects from the surrounding community. Both studies demonstrate that climate change is likely to lead to range contraction and extinction especially for those Himalayan alpine species that are unable to adjust important traits, unless competitive exclusion is avoided by management intervention (e.g., mowing or grazing). The declining species richness in response to warming observed by Yang et al. is a further illustration of the risk of biodiversity loss in mountain ecosystems, with alpine-restricted species seemingly more sensitive than wide-ranging species according to Winkler et al.

While uncovering the mechanisms that determine plant distribution ranges along elevational gradients is highly relevant, fewer studies exist on latitudinal range limits. Liu et al. investigated the variation in non-structural carbohydrates and nutrients in *Quercus variabilis* along a 1,500 km north-south transect in China. They conclude that carbon or nutrient availability are likely not the main driving factors of range limits of this species, while recruitment limitation seems more important at the species' northern edge (Gao et al., 2017). Species ranges are affected not only by climate, but also by direct human intervention. Vetter et al. looked into the effects of *Lupinus nootkatensis*, a high-latitude invader introduced in Iceland in the 1940s, on the native flora. The invasive legume tended to decrease plant species richness in the most species-rich habitat (heathland), while promoting especially late-successional and ruderal species in woodland and grassland. As the habitat suitable to lupine could double the next 50 years due to climate change, its role in altering competitive interactions and facilitating succession implies that it may further accelerate climate change-related ecological transition in cold biomes (cf. Hiltbrunner et al., 2014).

The studies discussed so far addressed shifts in baseline (average) temperatures, yet climate warming also leads to a higher incidence of unusual short-term warm spells. Bokhorst et al. focused on warm episodes in winter, which can cause shallow snow packs to melt, subsequently exposing the otherwise sheltered (insulated) vegetation to potentially harmful low temperatures (Treharne et al., 2019). In their experimental study in northern Norway, Bokhorst et al. found that defoliation due

to a larval outbreak likely obscured potential winter warming effects, suggesting that impacts in this case were limited to the event exerting the strongest pressure. On the other hand, De Boeck et al. found that two pressures worked synergistically in an alpine grassland. While the impact of a summertime heat wave or drought was insignificant or moderate, respectively, strong impacts were observed when heat and drought coincided (as is often the case naturally), both in the short (De Boeck et al., 2016) and longer term (De Boeck et al.). The finding that a climate extreme led to legacy effects regarding plant cover and community composition years after the original event, highlights that climate change can cause significant impacts in cold biomes other than through long-term changes in mean conditions.

The interactions between warming and changes in precipitation were further explored by Li et al. Their experimental study on the Qinghai-Tibetan Plateau showed that responses to warming very much depended on concomitant changes in precipitation: drier conditions led to a general deterioration of the grassland (especially due to lower productivity), while wetter conditions increased biomass production while preserving forage quality. It is thus critical for sustainable management of these systems that local precipitation trends and variability are taken into consideration in decision-making (e.g., setting maximum grazing intensities). In cold biomes, snowmelt timing is highly important for the onset of the growing season (Klein et al., 2016). Winkler et al. showed that spatial variation in composition and peak cover of an alpine grassland was driven by the timing of snowmelt and the associated dry-down during summer. Some regions may face lower winter snowfall under climate change (Gobiet et al., 2014), but at high latitudes, the opposite is likely, at least in the near future (Christensen et al., 2013). This could affect arctic communities both through increased mineralization rates (Schimel et al., 2004) and changes in the onset of the growing season (Wipf and Rixen, 2010). D'Imperio et al. looked into effects of both increased winter snow amounts and higher summertime temperatures, focusing on root dynamics, which are critically important in these nutrient poor environments. They found that increased winter snowfall and summer warming elicited opposing effects belowground, with the former suppressing and the latter stimulating root growth in this arctic wetland. Because alpine and arctic soils hold important carbon stocks (Hugelius et al., 2014), improving our knowledge on belowground dynamics is important in determining changes in the carbon balance.

To elucidate these and other climate change related issues, long term studies are essential (Knapp et al., 2012). The final contribution to our research topic has done exactly that. Angulo et al. report on an experiment that went on for 10 years in the Spanish Pyrenees and in which *Pinus uncinata* seedlings (later saplings) were exposed to warming, one-time NPK fertilizer addition and/or interaction with the dominant shrub *Rhododendron ferrugineum*. Interestingly, they find that the nature of interactions with this shrub changed as the experiment went on, switching from facilitation by the shrub to competition and ultimately competitive release. Moreover, the impacts of warming and fertilization (including their interactions) differed depending on presence or absence of *R. ferrugineum*, highlighting

the importance of considering species interactions in studying global change impacts.

This research topic has shown that the speed with which climate change is happening, encompassing long-term warming but also short-term temperature anomalies and concurrent changes in precipitation, may overwhelm some species and alter community composition and functioning also in the resilient ecosystems of the cold biomes. Persistence in a community will likely depend on the plasticity to cope with both changes in the abiotic and the biotic environment, including interactions with novel (invasive) species. Short and longer term responses may differ, which is why more experiments are needed that monitor communities for multiple years. This is challenging in these often remote locations, and made more difficult as the baseline

climate is shifting so rapidly. Understanding, disentangling and predicting the longer-term outcomes of global change impacts in cold biomes will therefore likely remain a frontier in plant ecology.

AUTHOR CONTRIBUTIONS

HD wrote the first draft of the editorial, with all co-authors jointly editing the final version.

ACKNOWLEDGMENTS

We thank the authors, reviewers, and the Frontiers Editorial Office for their support in creating this research topic.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Climate Regimes Override Micro-Site Effects on the Summer Temperature Signal of Scots Pine at Its Northern Distribution Limits

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 01 June 2018

Accepted: 15 October 2018

Published: 08 November 2018

Citation:

Lange J, Buras A, Cruz-García R, Gurskaya M, Jalkanen R, Kukarskih V, Seo J-W and Wilmking M (2018) Climate Regimes Override Micro-Site Effects on the Summer Temperature Signal of Scots Pine at Its Northern Distribution Limits. *Front. Plant Sci.* 9:1597. doi: 10.3389/fpls.2018.01597

Tree growth at northern boreal treelines is generally limited by summer temperature, hence tree rings serve as natural archives of past climatic conditions. However, there is increasing evidence that a changing summer climate as well as certain micro-site conditions can lead to a weakening or loss of the summer temperature signal in trees growing in treeline environments. This phenomenon poses a challenge to all applications relying on stable temperature-growth relationships such as temperature reconstructions and dynamic vegetation models. We tested the effect of differing ecological and climatological conditions on the summer temperature signal of Scots pine at its northern distribution limits by analyzing twelve sites distributed along a 2200 km gradient from Finland to Western Siberia (Russia). Two frequently used proxies in dendroclimatology, ring width and maximum latewood density, were correlated with summer temperature for the period 1901–2013 separately for (i) dry vs. wet micro-sites and (ii) years with dry/warm vs. wet/cold climate regimes prevailing during the growing season. Differing climate regimes significantly affected the temperature signal of Scots pine at about half of our sites: While correlations were stronger in wet/cold than in dry/warm years at most sites located in Russia, differing climate regimes had only little effect at Finnish sites. Both tree-ring proxies were affected in a similar way. Interestingly, micro-site differences significantly affected absolute tree growth, but had only minor effects on the climatic signal at our sites. We conclude that, despite the treeline-proximal location, growth-limiting conditions seem to be exceeded in dry/warm years at most Russian sites, leading to a weakening or loss of the summer temperature signal in Scots pine here. With projected temperature increase, unstable summer temperature signals in Scots pine tree rings might become more frequent, possibly affecting dendroclimatological applications and related fields.

Keywords: *Pinus sylvestris*, tree-ring width, maximum latewood density, micro-site, climate change, climate regime, treeline, boreal forest

INTRODUCTION

Growth of trees at northern boreal treelines is primarily limited by summer temperature due to the short and cool growing season prevailing in the subarctic (Körner, 1998, 2012; Holtmeier, 2009). Consequently, annually resolved tree-ring width (TRW) and maximum latewood density (MXD) from treeline conifers are regularly used as natural archives to reconstruct past summer temperatures over several centuries and sometimes millennia (e.g., Briffa et al., 1990, 2002a,b; Grudd et al., 2002; Hantemirov and Shiyatov, 2002; Linderholm and Gunnarson, 2005; Grudd, 2008; McCarroll et al., 2013; Porter et al., 2013; Matskovsky and Helama, 2014). However, large-scale studies of tree growth-climate sensitivity at treeline ecotone sites point to a non-uniform reaction of treelines to summer temperature (e.g., Harsch et al., 2009; Ohse et al., 2012; Hellmann et al., 2016), up to a regionally occurring complete loss of the summer temperature signal – a phenomenon which has been intensely discussed as the divergence problem, often attributed to changing climatic conditions (e.g., Wilmking et al., 2004; Driscoll et al., 2005; Wilmking et al., 2005; Lloyd and Bunn, 2007; D'Arrigo et al., 2008; Porter and Pisaric, 2011; Seo et al., 2011; Juday et al., 2015).

Scots pine (*Pinus sylvestris* L.) is widely distributed across the Eurasian boreal forest and survives across a broad range of ecological and climate conditions (e.g., it tolerates very dry sites, and acidic and wet peatland conditions; Ellenberg and Leuschner, 2010), making it a popular species for temperature reconstructions (e.g., Esper et al., 2002, 2016; Grudd et al., 2002; Linderholm and Gunnarson, 2005; Grudd, 2008; Helama et al., 2009; McCarroll et al., 2013; Matskovsky and Helama, 2014). Despite this ability to adapt, it was shown repeatedly that ecological micro-site conditions can affect the climatic signal of Scots pine at northern boreal treelines in different ways. Previous investigations indicate that the summer temperature signal of Scots pine in northern Fennoscandia was reduced on wet micro-sites, i.e., at lakeshores, riparian sites or peatlands, when compared to neighboring dry sites, with this response being attributed to stressful anaerobic and nutrient poor soil conditions (Linderholm, 2001; Linderholm et al., 2002, 2014; Matskovsky and Helama, 2014; Duthorn et al., 2015, 2016). Conversely in northern Sweden, Duthorn et al. (2013) have found a weaker summer temperature signal in Scots pine growing on dry sites relative to wet sites and attributed this finding to drought conditions.

In addition to these ecological effects, a climatically induced loss of summer temperature sensitivity in the second half of the 20th century has been reported for Scots pine growing in Finnish Lapland, presumably provoked by drought stress in the early growing season due to high temperatures and below-average precipitation sums (Seo et al., 2011; Franke et al., 2017). One study points to a complex interaction of both ecological and climatic effects: Only the combination of wet lakeshore conditions and cold/wet climatic conditions prevailing during the Little Ice Age led to reduced growth and a presumably weak climatic signal in Scots pine from altitudinal treeline in central Sweden, while all other combinations (wet soil – medieval

warming period; dry soil – any of the two periods) showed no effect (Linderholm et al., 2014).

However, similar studies investigating both ecological and climatological effects simultaneously on temperature sensitivity of Scots pine from northern treelines are entirely missing, as are studies documenting micro-site effects from northern treelines outside Fennoscandia. Further, despite the stronger summer temperature signal of MXD over TRW (e.g., Grudd, 2008; Tuovinen et al., 2009), only one study has investigated micro-site effects on MXD of Scots pine so far (Duthorn et al., 2016). A heterogeneous response to climate due to micro-site effects might generally question the a priori suitability of Scots pine trees for temperature reconstructions and other dendroclimatological applications (e.g., Linderholm et al., 2014; Duthorn et al., 2015; Edvardsson et al., 2015; Hellmann et al., 2016).

Here, we investigated the simultaneous effect of ecological (micro-sites) and climatological differences on the summer temperature signal of Scots pine TRW and MXD on a broad spatial scale. Over the period of 1901–2013 and along a 2200 km gradient from oceanic Finland to continental Western Siberia, we analyzed a well replicated dataset of twelve Scots pine micro-sites, including dry and wet sites at the northern distribution limit of the species. We applied a novel approach to explore the effect of differing climate regimes on temperature-growth relationships by comparing dry/warm and wet/cold years that were equally distributed across the study period. In particular, we tested if there is a difference in strength and direction of temperature-growth relationships between (i) treeline and forest sites, (ii) dry and wet micro-sites, and (iii) dry/warm and wet/cold climate regimes, and their combinations. Assuming that temperature limits growth at our sites, we expect a strong and significant positive summer temperature signal at all sites, being slightly lower at forest than at treeline sites. Due to the growth-limiting role of temperature, we further hypothesize that effects of the climate regime are stronger than micro-site effects, and that the former are overall stronger at Russian sites than in Finland due to higher summer temperatures as a consequence of its continentality.

MATERIALS AND METHODS

Site Selection and Sampling

We analyzed 348 Scots pine trees from three regions at their northern distribution limit in Eurasia: Finnish Lapland (FIN), north of European Russia (RUS), and Western Siberia (SIB) (Figure 1). In each region, a treeline (“T,” located at the northern treeline or northern distribution limit of Scots pine) and a forest site (“F,” located 50–140 km south of the treeline site) were selected for sampling. At each treeline and forest site, trees from neighboring dry (“D”) and wet (“W”) micro-sites were sampled, resulting in twelve different sampling locations (Table 1). Dry micro-sites were characterized by well drained sandy soils with sparse ground cover (mainly *Cladonia* sp.), while micro-sites with peaty soil, visibly upcoming groundwater and a high abundance of indicator species for peatland (e.g., *Vaccinium* sp., *Andromeda polifolia* L.) were defined as wet sites.

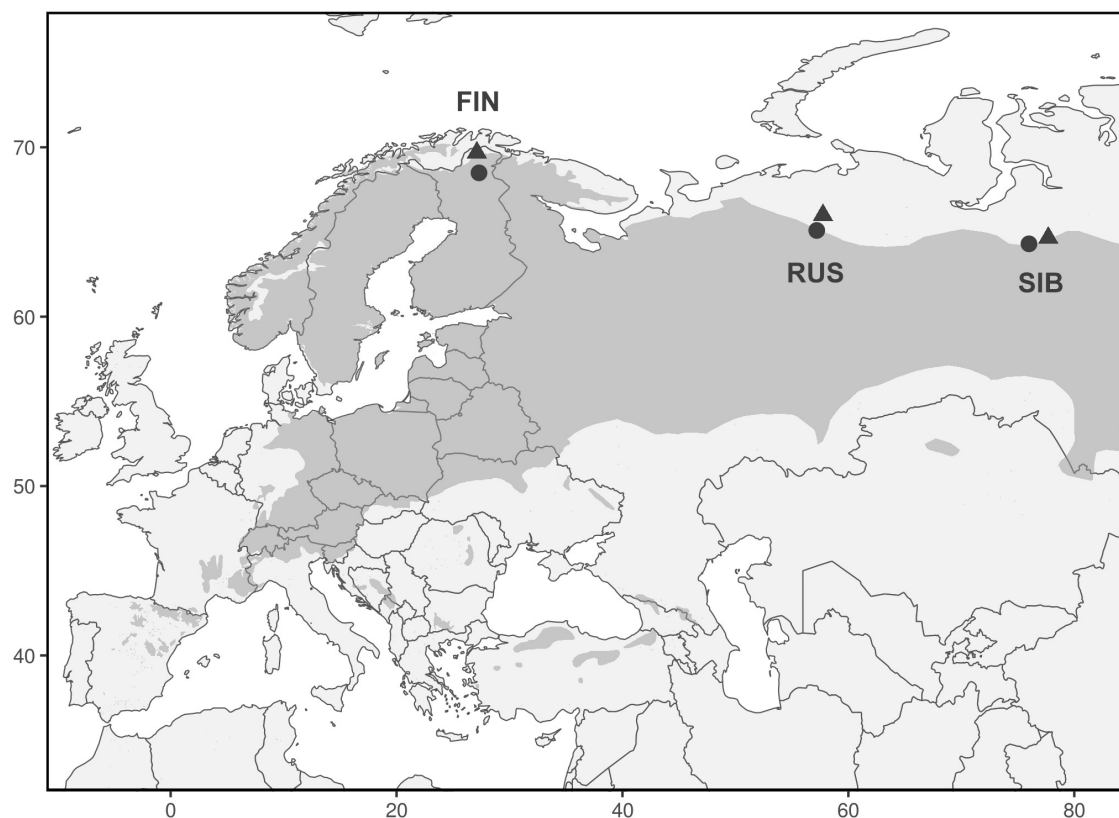


FIGURE 1 | Range of *Pinus sylvestris* (dark gray) and locations of treeline (triangles) and forest (circles) sites. Each triangle and circle represents one pair of dry and wet micro-sites.

TABLE 1 | Description of the study sites and number of analyzed trees.

Region (code)	Site	Latitude	Longitude	Elevation (m.a.s.l.)	N	Mean age
Finland (FIN)	TD	69°41' N	27°06' E	200	26	190
	TW	69°41' N	27°06' E	200	20	190
	FD	68°28' N	27°18' E	270	20	301
	FW	68°30' N	27°18' E	260	16	261
North of European Russia (RUS)	TD	66°00' N	57°51' E	45	35	147
	TW	66°00' N	57°40' E	60	19	182
	FD	65°06' N	57°14' E	50	39	164***
	FW	65°05' N	57°10' E	60	31	132***
Western Siberia (SIB)	TD	64°40' N	77°41' E	45	29	123**
	TW	64°40' N	77°41' E	40	27	183**
	FD	64°18' N	75°59' E	65	29	211***
	FW	64°18' N	75°59' E	65	28	140***

Mean tree age was tested for significant difference between each pair of dry and wet micro-sites using a Wilcoxon rank-sum test or a Student's *t*-test (depending on whether data was normally distributed). Significant differences are marked with an asterisk (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). Site abbreviations: T, treeline; F, forest; D, dry; W, wet.

All Russian sites as well as the Finnish wet sites were mostly flat, ranging between 40–65 and 200–260 m a.s.l., respectively, while Finnish dry sites were located on slightly inclined south or west facing slopes (**Table 1**). All sites were free of permafrost. Only dominant, mature and straight growing trees without visible signs of damage or illness were selected for sampling, the typical

tree form and status used for dendroclimatology. Two increment cores (A and B; 5.15 and 12 mm in diameter, respectively) were taken per tree at breast height perpendicular to each other to lower a possible bias due to irregular/eccentric growth. Diameter at breast height (dbh) and tree height were recorded for each tree.

Dendrochronological Methods

The surface of core A was smoothed using a WSL sledge microtome (Gärtner and Nievergelt, 2010) in order to make annual rings visible, and scanned visually using a conventional scanner. TRW was measured on the scanned image with 0.001 mm precision using the software CooRecorder v. 7.7 (Cybis Elektronik and Data AB). After securing core B to a wooden holder, laths of 1.25 ± 0.1 mm thickness were cut out of the core using a twin-bladed circular saw (Dendrocut 2003, Walesch Electronics¹) and subsequently boiled for 24 h in a Soxhlet extractor filled with 96% Ethanol to remove resins and other soluble substances from the wood (Schweingruber, 1978). After a minimum drying and acclimatization time of 12 h under controlled air temperature (20°C) and relative humidity (50%) conditions, the laths were X-rayed under the same controlled conditions using an ITRAX MultiScanner (Cox Analytical Systems) with an exposure time of 25 ms, an intensity of 30 kV/50 mA, and in steps of 20 μ m. A standard calibration plexi ladder was used to calibrate gray-level light intensity to wood density in g/cm³ for each X-ray scan. MXD and TRW were measured on the obtained gray-scale images using the WinDENDRO software v. 2014b (Regents Instruments Inc.). The mean thickness of each wooden lath was measured using electronic calipers and subsequently used for calibration of the respective sample to account for possible deviations in thickness among the individual laths. All TRW and MXD series were visually cross-dated in CDendro (Cybis Elektronik and Data AB). Series from blurred images and with visible growth disturbance unrelated to climate, such as knotholes, were excluded from further analysis to avoid noise due to non-climatic effects ($n = 29$, corresponding to on average 2.4 excluded sampled trees per micro-site). Consequently, only trees with both TRW and MXD series available were used for the subsequent analysis ($n = 319$). All series were truncated after 2013 to exclude effects from the bark/coring process on MXD (cores were taken in 2014 and 2016) and to ensure that the same period was used for all sites and for both TRW and MXD. Cross-dating was verified and series were standardized using the dplR-package (Bunn, 2008) in the software environment R version 3.5.1 (R Core Team, 2018). We used a 30-year cubic smoothing spline with a 50% frequency cut-off to remove trends unrelated to

climate, such as age and stand effects (Cook et al., 1990). Tree chronologies were prewhitened to remove autocorrelation and subsequently averaged into site chronologies for each micro-site using the biweight robust mean. Well-established descriptive statistics serving as quality criteria for the homogeneity of a tree population in dendrochronology, such as the expressed population signal (EPS), mean interseries correlation (\bar{r}) and Gleichlaufigkeit (glk), were calculated for all micro-sites and both proxies (Eckstein and Bauch, 1969; Wigley et al., 1984).

Climate Data

Monthly resolved temperature means, precipitation sums and the SPEI (standardized precipitation evaporation index, Vicente-Serrano et al., 2010; integrated over 1, 3, and 6 months) were obtained for each site from the CRU TS 3.22 to 3.24 datasets at 0.5° resolution (Harris et al., 2014) for the respective nearest grid and the period 1901–2013. This CRU data was compared with the respective climate data from the nearest meteorological station obtained from the NOAA global database². Due to strong correlations between modeled and station data for the overlapping period (0.97 to 0.99 for temperature and 0.85 to 0.97 for precipitation) and the overall shorter and sometimes discontinuous record of station data (station records start in 1936–1977, depending on the respective site) we decided to use the CRU dataset for climate-growth analysis in this study.

Averaged over the 1901–2013 CRU-data period, July and January were the warmest and coldest months, respectively, at all sites (except at Finnish forest sites where the mean temperature of January and February was identical), with the July temperatures being 0.5–1.3°C higher in the forest than at treelines and 1.2–2.1°C higher in Russia than in Finland. The smaller temperature range between the coldest and warmest months in Finland compared to Russia points to maritime influence in Finland. July precipitation sums are similar across sites (Table 2).

Statistical Analyses

All statistical analyses were performed in the R software environment version 3.5.1 (R Core Team, 2018). First, to statistically validate the selection of micro-sites and to test for possible heterogeneity within micro-sites, a Principal Component Gradient Analysis (PCGA, Buras et al., 2016) was

¹www.walesch.ch

²<https://www.ncdc.noaa.gov/>

TABLE 2 | Mean T_Jul, T_JA, and sum of P_Jul during the two climate regimes.

Region	Site	T_Jul wet/cold (°C)	T_Jul dry/warm (°C)	T_JA wet/cold (°C)	T_JA dry/warm (°C)	P_Jul wet/cold (mm)	P_Jul dry/warm (mm)
FIN	TD, TW	11.1	13.0	10.3	11.7	86	47
	FD, FW	12.5	14.5	11.7	13.1	83	46
RUS	TD, TW	13.0	15.6	12.1	13.9	67	47
	FD, FW	14.1	17.1	13.0	15.0	77	45
SIB	TD, TW	13.4	15.5	12.6	13.6	89	41
	FD, FW	13.6	16.0	12.8	14.2	87	44

The same climate data was used for neighboring dry and wet micro-sites as they were located within one 0.5° CRU grid. Site abbreviations: T, treeline; F, forest; D, dry; W, wet.

performed with the standardized tree-ring series of each pair of dry and wet micro-sites. PCGA makes use of the loadings as derived from the first two principal components of an ordinary PCA to define a gradient of similarity among trees. To test whether the PCGA-loadings indicate micro-site specific growth signals, we applied a Wilcoxon rank-sum test. That is, we tested the similarity of locations (i.e., the non-parametric mean) of the loadings' polar-coordinates between the respective micro-sites. A significant test indicates a true location shift (i.e., difference of non-parametric means) between the PCGA-loadings of the different micro-sites. Based on empirical evidence, PCGA is able to identify micro-sites expressing differing climate–growth relationships correctly (Rehseh et al., 2017; Buras et al., 2018). Due to high accordance between micro-sites defined in the field and those identified by the PCGA for TRW (93% of the IDs were correctly assigned to their micro-site on average, **Figure 2**), we assumed that the differentiation between micro-sites was justified and that micro-sites were homogenous in themselves.

In order to identify the climate proxies and months with the strongest effect on radial tree growth at our micro-sites, we calculated Pearson correlation coefficients between micro-site chronologies and each climate proxy (i.e., temperature, precipitation and the SPEI) and month from June of the previous year until September of the year of growth for the period 1901–2013, using the R-package *treeclim* (Zang and Biondi, 2015). Micro-site chronologies were additionally correlated with seasonal summer temperature values expressed as the mean of June–July (JJ), July–August (JA) and June–August (JJA) temperatures. Stationary bootstrapping (Politis and Romano, 1994) was applied to all climate–growth correlations. For further analysis, we selected July temperature (hereafter T_Jul; for TRW) and the mean of JA temperature (hereafter T_JA; for MXD), both of the year of growth, at all sites as these were the climate proxies with the strongest signal at most of the sites.

To explore whether and to what extent differing climatic conditions prevailing before and during the growing season affect summer temperature–growth relationships, the period of 1901–2013 was divided into dry/warm and wet/cold years (hereafter called climate regimes; for the R code of the following analysis steps please refer to **Supplementary Data Sheet 1**). To this aim, the monthly water balance was calculated and averaged for April–July for each year of the period 1901–2013. Water balance was calculated by subtracting the potential evapotranspiration (PET) from precipitation. PET was calculated using the Thornthwaite equation (Thornthwaite, 1948) in the R-package SPEI (Begueria and Vicente-Serrano, 2017). Subsequently, the period of 1901–2013 was divided into years with a high and a low water balance, respectively, based on the aforementioned April–July mean. Due to significant positive Pearson correlations with precipitation (0.52 to 0.76; $p < 0.001$) and significant negative correlations with temperature (−0.49 to −0.58; $p < 0.001$) of the same April–July mean, years with a water balance above its 1901–2013 median were defined as wet/cold climate regime, while years with a water balance below its 1901–2013 median were defined as dry/warm climate regime accordingly. The classification of these two climate regimes was

carried out for each site separately in order to consider the respective climatic span; a global classification of climate regimes valid for all sites was not viable due to insufficient overlap between sites. For subsequent analysis we selected the 25% most extreme years per climate regime and site (i.e., the 25% driest/warmest and 25% wettest/coldest years per site), resulting in 28 years per climate regime and site. This was done to restrict the analysis to the most extreme years but at the same time keep the sample size reasonably high. Years of both climate regimes were equally distributed across the period 1901–2013 at all sites. Across all sites, summer temperature was 1.9–3.0°C (T_Jul) and 1.0–2.0°C (T_JA) higher in dry/warm years compared to wet/cold years (**Table 2**). July precipitation was overall 20–48 mm lower in dry/warm years, corresponding to 46–70% of the precipitation falling in cold/wet years (**Table 2**).

Finally, Pearson correlation coefficients were calculated for the relationships between TRW and T_Jul, and between MXD and T_JA, by selecting only those years from the TRW and MXD chronologies and the respective climate data that were assigned to the dry/warm and the wet/cold climate regimes. In this way, correlations were performed separately for (i) years assigned to the dry/warm climate regime vs. years assigned to the wet/cold climate regime and (ii) for dry vs. wet micro-sites. Again, stationary bootstrapping (Politis and Romano, 1994) was applied. Additionally, Pearson correlations for the same combinations of micro-sites and climate regimes were performed for individual trees in order to investigate the variability of the summer temperature signal within micro-sites. In order to test if correlations differed significantly between micro-sites and between climate regimes, a pairwise Wilcoxon rank-sum test was carried out using correlation values of the individual tree-ring series. Tests were restricted to pairs where at least one correlation score was significant. The Holm correction for adjusted p -values was applied (Holm, 1979).

RESULTS

Climate Signals

Both proxies, TRW and MXD, showed generally similar results: Correlation scores were similar in sign and of comparable strength across most sites and climate regimes. We observed a tendency of somewhat stronger temperature–growth correlations for TRW in Finland and for MXD at RUS and SIB sites when compared to the respective other proxy. Overall, summer temperature signals were strongest in Finland and Western Siberia and weaker to non-significant in the north of European Russia (**Figures 3, 4**).

In the majority of cases, correlation values of site chronologies were stronger than the median of correlations of the individual trees at that site. The range covered by correlation values of individual trees slightly differed between micro-sites, climate regimes and the two proxies TRW and MXD without following a clear pattern, but with a tendency of higher variability for SIB MXD compared to TRW.

It is worth mentioning that correlations with precipitation over the whole 1901–2013 period, even though by far weaker

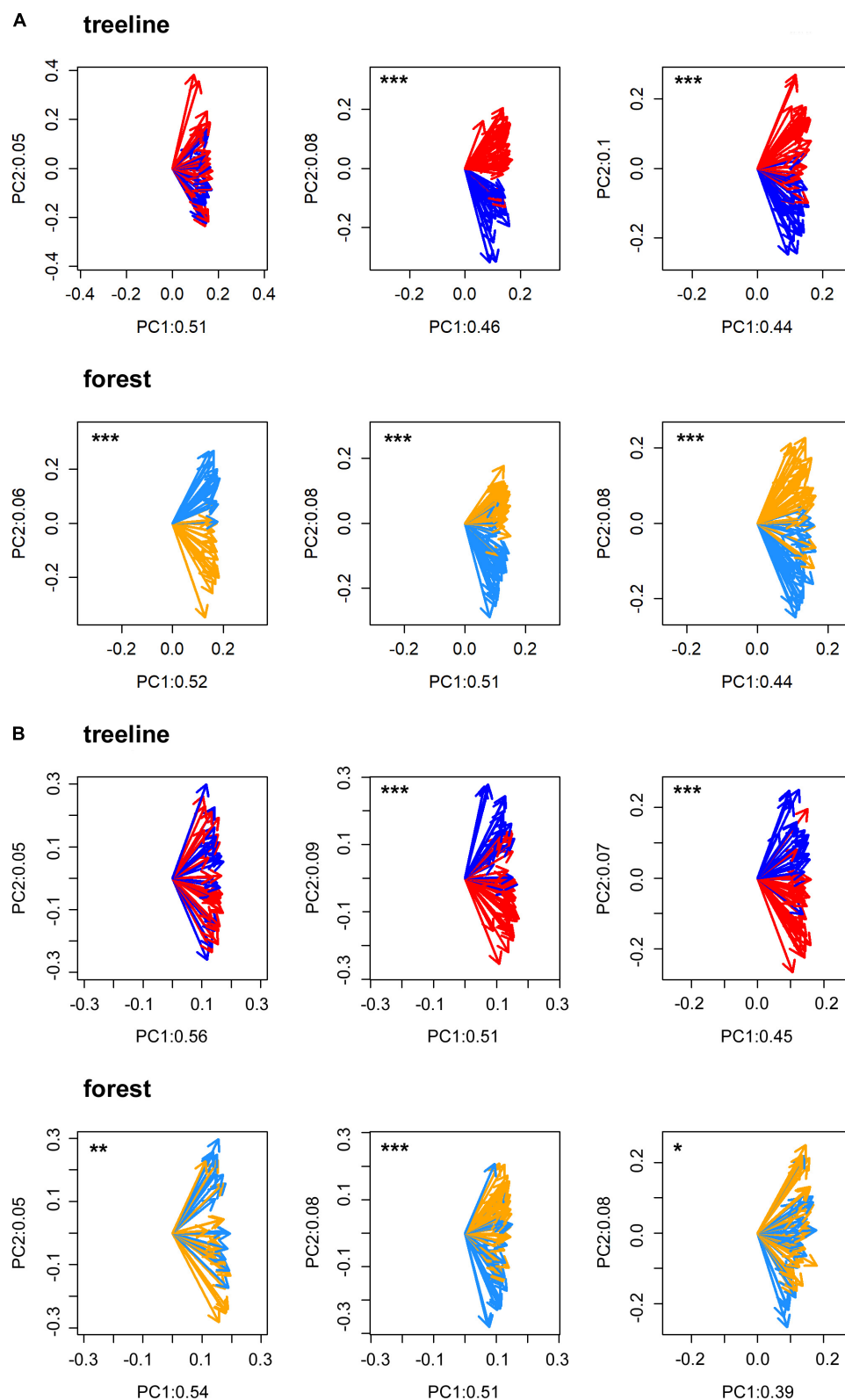


FIGURE 2 | PCGA clearly separated the loadings of dry (treeline: red arrows and forest: orange arrows) and wet (treeline: dark blue arrows and forest: light blue arrows) micro-sites for tree-ring width **(A)** and maximum latewood density **(B)** in FIN (left), RUS (middle) and SIB (right). Labeling values of the principal components (PC) refer to the amount of total variance explained by the respective PC. Significant separations of loadings according to micro-sites are indicated with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

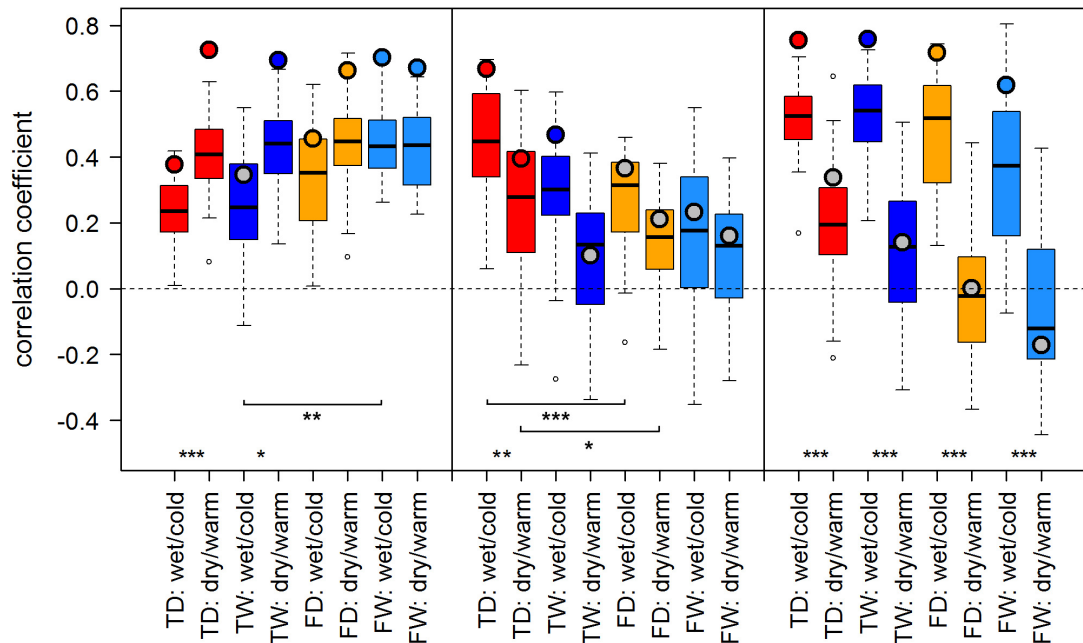


FIGURE 3 | Temperature-growth correlations between MXD and T_{JA} are shown for each micro-site under dry/warm and wet/cold climate regimes for FIN (left), RUS (middle), and SIB sites (right). Boxplots represent the correlation values of individual trees of a micro-site. Colored circles represent the correlation value of the respective site chronology. Color code: red: treeline dry (TD); dark blue: treeline wet (TW); orange: forest dry (FD); light blue: forest wet (FW). Insignificant correlations of site chronologies are represented by a gray circle. Significant differences between dry/warm and wet/cold climate regimes, dry and wet micro-sites and between treeline and forest are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

than correlations with summer temperature, were significantly positive for May of the year of growth for TRW in Finland (up to $r = 0.36$, data not shown). Correlations with precipitation and the SPEI1, 3 and 6 over the whole 1901–2013 period were mostly significantly negative for the summer months (June to August of the year of growth) at FIN and SIB sites (but almost never at RUS sites) for MXD and sometimes TRW (data not shown).

Effect of the Climate Regime

The effect of dry/warm and wet/cold climate regimes was strongest at all Siberian sites (Figures 3, 4): While correlations were strong and significant in wet/cold years, they were non-significant in dry/warm years. This climate signal loss between the two climate regimes in Western Siberia was significant for all micro-sites and results were consistent for both proxies. In Finland, correlation strength between dry/warm and wet/cold years varied marginally for TRW and stronger for MXD: It was stronger in dry/warm years and weaker in wet/cold years. However, variations between the two climate regimes were significant in only two micro-sites for MXD and one micro-site for TRW (Figures 3, 4). At the central site (RUS), only MXD at the dry treeline site showed significant climate signals differing between the two climate regimes with a pattern similar to SIB sites.

In terms of absolute growth, on average, TRW was larger and MXD was higher in dry/warm years than in wet/cold years at all sites.

Effect of Micro-Sites and Treeline-Forest

Even though micro-site chronologies were clearly separated into dry and wet sites by the PCGA (Figure 2), significant differences regarding the summer temperature signal were not found between micro-sites. However, it is worth to mention that even though differences were insignificant, some correlation values were appreciably lower on wet compared to dry micro-sites (particularly for MXD at RUS treeline sites for both climate regimes). The comparison of treeline-forest revealed significant differences in four cases: Correlation strength was significantly higher at the dry treeline than at the dry forest site for MXD in RUS, in both climate regimes, exemplified by dry/warm and wet/cold years (Figure 3). Conversely, correlations were significantly higher at wet forest sites compared to wet treeline sites, for FIN MXD during wet/cold years and for RUS TRW during dry/warm years.

In terms of absolute growth, on average, TRW was larger on dry sites independent of the presence and direction of significant age differences (Tables 1, 3). Further, dry site trees were generally significantly thicker (except at Finnish treeline sites) and taller than wet site trees. Glk for both TRW and MXD was slightly higher on dry than on wet micro-sites at all but one of the Russian sites (Table 3).

Summarized, in terms of temperature-growth correlations five out of twelve or 42% (TRW) to seven out of twelve or 58% (MXD) of the micro-sites were significantly affected by shifts of the growing season climate regime, while only one (TRW) to three (MXD) micro-site pairs were significantly

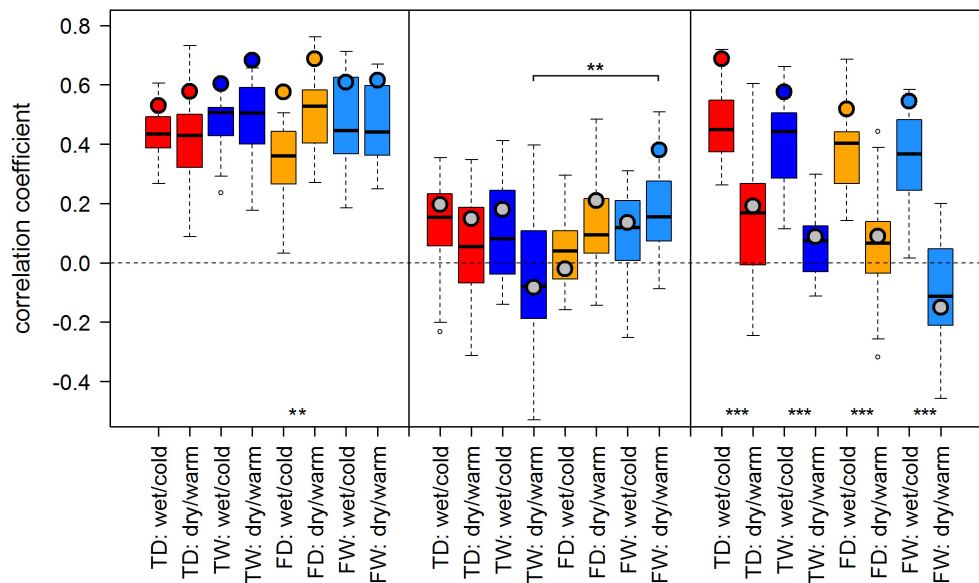


FIGURE 4 | Temperature-growth correlations between TRW and T_{Jul} are shown for each micro-site under dry/warm and wet/cold climate regimes for FIN (left), RUS (middle), and SIB sites (right). For details, please refer to **Figure 3**.

TABLE 3 | Tree metadata and descriptive statistics of TRW and MXD chronologies per micro-site.

Region	Site	dbh (cm)	Tree height (m)	Mean TRW (mm)	glk		EPS		rbar	
					TRW	MXD	TRW	MXD	TRW	MXD
FIN	TD	41.2	13.3***	0.91	0.73	0.71	0.97	0.97	0.61	0.54
	TW	38.1	11.4***	0.84	0.73	0.72	0.96	0.96	0.54	0.53
	FD	49.6***	21.1***	0.70	0.69	0.67	0.96	0.95	0.58	0.49
	FW	35.3***	13.6***	0.58	0.70	0.69	0.96	0.97	0.62	0.64
RUS	TD	37.7***	17.3***	1.22	0.70	0.73	0.97	0.97	0.50	0.49
	TW	26.0***	11.6***	0.68	0.65	0.67	0.97	0.92	0.67	0.42
	FD	34.8***	15.4***	0.92	0.72	0.68	0.98	0.97	0.50	0.47
	FW	26.5***	13.7***	0.82	0.68	0.64	0.98	0.94	0.64	0.36
SIB	TD	34.8*	12.1***	1.08	0.66	0.68	0.99	0.96	0.75	0.46
	TW	31.2*	9.6***	0.70	0.66	0.67	0.97	0.96	0.57	0.48
	FD	38.5***	13.8***	0.68	0.67	0.64	0.95	0.94	0.41	0.36
	FW	24.6***	10.8***	0.65	0.65	0.63	0.96	0.95	0.45	0.40

Glk, EPS, and rbar were calculated for the period that is relevant for climate-growth analysis (1901–2013). Dbh and tree height were tested for significant difference between each pair of dry and wet micro-sites using a Wilcoxon rank-sum test or a Student's *t*-test (depending on whether data was normally distributed). Significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Site abbreviations: T, treeline; F, forest; D, dry; W, wet.

affected by the difference between treeline and forest, and no temperature-growth correlation was significantly affected by micro-site differences.

DISCUSSION

In this study, we clearly demonstrate that the strength of the summer temperature signal in Scots pine is primarily affected by inter-annually differing climate regimes at our sites, while ecological micro-site conditions play a minor role. More particularly, growing season climate variability led to significantly different temperature–growth correlations at about half of our

sites (mostly in Russia), while differing micro-sites significantly affected absolute tree growth, but not the climatic signal. We discuss possible reasons for this differentiated reaction to growing season climate in space and time and evaluate the role of micro-site conditions.

Inter-Annual Climate Variability Drives Spatially Differing Tree-Growth Responses to Climate

Coupled to large-scale circulation systems, regional climate is undergoing natural inter-annual variations in temperature and precipitation within a certain range. Trees as living but immobile

organisms are able to adapt to these variations to a certain degree by reducing or enhancing radial growth under unfavorable or favorable climatic conditions, respectively (Fritts, 1976).

The present study shows explicitly that as a consequence of this growth adaptation to climate, the temperature signal preserved in annual growth rings varies accordingly across years and has a spatially differentiated pattern. At most sites in Russia (RUS and SIB), wet/cold years seem to be growth limiting, leading to narrow annual rings, a low MXD and a strong summer temperature signal. Correspondingly, dry/warm years promoted radial growth at Russian sites, leading to wider rings, a high MXD and a weak or absent summer temperature signal. This temperature-dependent growth pattern fits well with the growth limitation hypothesis for near-treeline locations (Körner, 1998, 2012; Holtmeier, 2009), even though it is surprising that this inter-annual variation of climate regimes seems sufficient to trigger significantly different growth reactions. It is unlikely that this reduction in summer temperature signal in dry/warm years was caused by drought stress, unlike similar studies (e.g., Seo et al., 2011; Dũthorn et al., 2013), as ring growth was enhanced (larger TRW) during dry/warm years and correlations with summer precipitation were either significantly negative or absent when correlated over the whole 1901–2013 period. Since neither temperature nor precipitation seem to be growth limiting here in dry/warm years, other factors might become growth limiting here, such as, e.g., competition for light and nutrients (Fritts, 1976).

Correspondingly, we would have expected the same pattern, i.e., a stronger summer temperature signal in wet/cold than in dry/warm years also in Finland. However, we found that the climatic signal between the two climate regimes differed significantly only at few sites in Finland, being stronger during dry/warm years in contrast to Russia. As summer temperatures and correlation strength were similar between wet/cold years in Russia and dry/warm years in Finland (Table 2) – this was possible because the two climate regimes have been defined relative per site – we conclude that Scots pine growth is limited by similar thermal conditions in Russia and in Finland. Regarding the differences in correlation strength between the two climate regimes in Finland particularly found for MXD at treeline, wet, cold, and cloudy conditions during the growing season might have led to a reduction of the summer temperature signal in wet/cold years as reported in previous treeline studies, e.g., due to a late onset of cambial activity caused by late snowfall and late snowmelt (Kirdyanov et al., 2003), a reduced photosynthetic productivity caused by high precipitation rates/high cloud cover (Dũthorn et al., 2016), or changed hydrological conditions in the soil (Linderholm et al., 2014).

In contrast to MXD, ring-width formation is particularly affected by climatic conditions of the early growing season (Rossi et al., 2011, 2012), generally occurring between mid-May and mid-June in the area of our Finnish forest sites (Rossi et al., 2008; Seo et al., 2008, 2011). Significant positive correlations of TRW with May precipitation over the 1901–2013 period suggest a high importance of water availability during the early growing season at our Finnish sites, which is corroborated

by findings of Seo et al. (2011) in the same area. This positive effect of wet conditions in spring might counteract the negative effect of wet/cold conditions during summer months, possibly explaining the slightly stronger summer temperature signal in wet/cold years in TRW compared to MXD in Finland.

The effect of differing climatic conditions was strongest at the easternmost site (SIB) and weakest in Finland for both tree-ring proxies, as initially hypothesized. This is possibly related to absolutely higher summer temperatures occurring at the more continental Russian sites (Table 2), which are located further east and south than the Finnish sites, following the northern distribution limit of Scots pine (Figure 1 and Table 1). Growth limiting conditions are possibly more easily and more strongly exceeded in Russia, while in Finland both climate regimes seem to be closer to growth limiting climatic conditions (Table 2). Higher summer temperatures in Russia might further explain the low to insignificant correlation values at all central (RUS) sites. Even though not being the southernmost location in this study, RUS forest sites exhibited the highest summer temperatures among all forest sites under both climate regimes (Table 2), which possibly lowers the effect of temperature–growth limitation. The reason for the missing temperature signal in TRW at RUS treeline sites remains unclear so far and needs further investigation.

Growth Adaptation to Ecological Site Conditions Hardly Interferes With the Summer Temperature Signal

Unlike previous assessments of micro-site effects on Scots pine radial growth (e.g., Moir et al., 2011; Dũthorn et al., 2013; Linderholm et al., 2014; Matskovsky and Helama, 2014), ecological site conditions only had minor effects on the summer temperature signal of Scots pine in this study, suggesting that possible ecological effects were largely removed during the standardization process. Different standardization methods might lead to different results when comparing micro-site effects though (Dũthorn et al., 2013, 2015), with the choice of the most appropriate standardization method for the respective dataset being one of the main quality criteria for climate reconstructions (Esper et al., 2016).

However, tree height and dbh were significantly reduced on wet sites. This growth adaptation was likely caused by unfavorable and stressful growing conditions, such as anaerobic soils and low nutrient availability, and is in line with other studies on Scots pine from peatlands (e.g., Moir et al., 2011; Smiljanić et al., 2014) or wet sites (Dũthorn et al., 2013, 2015). Just as in similar studies (e.g., Smiljanić et al., 2014; Blanchet et al., 2017), we experienced difficulties in cross-dating ring parameters from trees growing at wet sites due to frequently occurring narrow and missing rings and found a slightly lower agreement of tree-ring series (i.e., glk) within wet micro-sites in Russia (e.g., Linderholm et al., 2014; Edvardsson et al., 2015; Nõjd et al., 2017) in both TRW and MXD. Even though the PCGA clearly identified differing ecological conditions between pairs of dry and

wet micro-sites, comparability between micro-sites across regions and with other micro-site studies cannot be assured.

Implications and Outlook

We showed that differing climate regimes significantly impacted the temperature signal of Scots pine at about half of our sites, for both TRW and MXD, overriding micro-site effects on the temperature signal. With the significant weakening of the summer temperature signal in Scots pine under certain climatic conditions (particularly in Western Siberia under dry/warm climate regimes) our findings contribute to the divergence phenomenon and loss of temperature sensitivity discussion (D'Arrigo et al., 2008). Although the temperature signal overall was slightly stronger in MXD (T_JA) than in TRW (T_Jul), both tree-ring proxies were similarly affected by the loss of temperature sensitivity in our study. Since reconstructions of summer temperature based on TRW and MXD rely on the assumption that radial tree growth is limited by temperature and that this growth limitation is stable over time, the suitability of the present dataset for dendroclimatological applications such as temperature reconstructions remains to be tested, for both TRW and MXD (but see Tuovinen et al., 2009). Even though we hardly found significant effects of micro-site differences on the summer temperature signal at our sites, we noticed a tendency of weaker correlations at most Russian wet sites.

Concluding, we recommend a thorough examination of possible instabilities stemming from past climate fluctuations and ecological site differences despite an apparently stable signal before attempting temperature reconstructions. An evaluation of micro-site effects might be particularly relevant for the calculation of global vegetation and carbon cycle models, where absolute growth is important. Depending on the respective interplay of temperature and precipitation, the projected future increase of both climate proxies at higher latitudes (IPCC, 2014) might have regionally differing consequences for the future climatic signal of near-treeline trees, but will likely be dominated by temperature increase, thus probably resulting in a reduction of the summer temperature signal in Scots pine in those areas of the Eurasian boreal forest that are most affected by global warming.

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DATA AVAILABILITY STATEMENT

Datasets are available on request: the raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JL, MW, and J-WS designed the study with input from MG, RJ, and VK. JL, RC-G, MG, RJ, VK, and MW collected the data and assisted JL with measurements. JL analyzed the data with input from AB and MW. AB performed the PCGA. JL wrote the first draft of the manuscript with contributions of MW. All authors contributed to manuscript revision, read, and approved the submitted manuscript.

FUNDING

JL, RC-G, and MW were supported by the German Research Council (project DFG WI 2680/8-1). RC-G was supported by a DAAD-Conacyt Mexican Government scholarship. MG and VK were supported by the State Contract of the Institute of Plant and Animal Ecology, UB RAS and Russian foundation of basic research (Grants Nos. 14-04-91356 and 15-04-04933). This study is a contribution to DFG RTG 2010 Response.

ACKNOWLEDGMENTS

The authors are grateful to Marko Smiljanić for assisting with **Figure 1** and to Jill Harvey for language editing and general comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01597/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transplants, Open Top Chambers (OTCs) and Gradient Studies Ask Different Questions in Climate Change Effects Studies

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OPEN ACCESS

Edited by:

Sebastian Leuzinger,
Auckland University of Technology,
New Zealand

Reviewed by:

Christian Koerner,
Universität Basel, Switzerland
Iván Prieto,
Centro de Edafología y Biología
Aplicada del Seguro (CEBAS), Spain

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equally to this work

Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 18 May 2018

Accepted: 09 October 2018

Published: 02 November 2018

Citation:

Yang Y, Halbritter AH,
Klanderud K, Telford RJ, Wang G and
Vandvik V (2018) Transplants, Open
Top Chambers (OTCs) and Gradient
Studies Ask Different Questions
in Climate Change Effects Studies.
Front. Plant Sci. 9:1574.
doi: 10.3389/fpls.2018.01574

Long-term monitoring, space-for-time substitutions along gradients, and *in situ* temperature manipulations are common approaches to understand effects of climate change on alpine and arctic plant communities. Although general patterns emerge from studies using different approaches, there are also some inconsistencies. To provide better estimates of plant community responses to future warming across a range of environments, there have been repeated calls for integrating different approaches within single studies. Thus, to examine how different methods in climate change effect studies may ask different questions, we combined three climate warming approaches in a single study in the Hengduan Mountains of southwestern China. We monitored plant communities along an elevation gradient using the space-for-time approach, and conducted warming experiments using open top chambers (OTCs) and plant community transplantation toward warmer climates along the same gradient. Plant species richness and abundances were monitored over 5 years addressing two questions: (1) how do plant communities respond to the different climate warming approaches? (2) how can the combined approaches improve predictions of plant community responses to climate change? The general trend across all three approaches was decreased species richness with climate warming at low elevations. This suggests increased competition from immigrating lowland species, and/or from the species already growing inside the plots, as indicated by increased biomass, vegetation height or proportion of graminoids. At the coldest sites, species richness decreased in OTCs and along the gradient, but increased in the transplants, suggesting that plant communities in colder climates are more open to invasion from lowland species, with slow species loss. This was only detected in the transplants, showing that different approaches, may yield different results. Whereas OTCs may constrain immigration of new species, transplanted communities are rapidly exposed to new neighbors that can easily colonize the small plots. Thus, different approaches ask slightly different questions, in

particular regarding indirect climate change effects, such as biotic interactions. To better understand both direct and indirect effects of climate change on plant communities, we need to combine approaches in future studies, and if novel interactions are of particular interest, transplants may be a better approach than OTCs.

Keywords: alpine grasslands, experimental warming, integrated approaches, space-for-time, southwestern China

INTRODUCTION

Alpine ecosystems are temperature limited systems and have long been predicted to be sensitive to climate change (Walker et al., 2006; Elmendorf et al., 2015). The projected rate of future warming in the alpine region is also faster than the global average (IPCC, 2013). Thus, understanding how climate warming affects alpine plant communities is especially important for our ability to predict impacts of future climate change.

An increasing number of studies report that climate warming is driving essential changes in alpine and arctic plant communities, such as phenology (Wolkovich et al., 2012; Li et al., 2016), species distribution ranges (Van der Wal et al., 2012; Grytnes et al., 2014), species abundances (Elmendorf et al., 2012a,b) and species richness (Pauli et al., 2012; Wipf et al., 2013; Steinbauer et al., 2018). Although there are general patterns emerging, there are also some inconsistencies. For example, experimental warming by open top chambers (OTCs) in Tibet observed delayed reproductive phenology and decreased number of inflorescences of dominant species (Dorji et al., 2014), whereas warming by transplantation to lower elevations found advanced flowering dates (Wang et al., 2014). Furthermore, whereas warming by OTCs decrease alpine and arctic species diversity (Elmendorf et al., 2012a), studies resampling historical data find increased species richness in alpine regions (Steinbauer et al., 2018). Part of this variation can be due to different methods (Elmendorf et al., 2015), and typically four primary approaches have been used to study impacts of climate warming on alpine plant community properties: studies along natural elevation gradients (space-for-time substitution), resampling or monitoring over time, experimental warming by OTCs, and whole-community transplantation to warmer climates.

Elevation gradient studies allow investigating community responses to a broader range of both abiotic and biotic environmental conditions, including direct and indirect effects of climate change. These communities do, however, reflect responses over longer time scales, and may therefore overestimate plant community responses to current climate changes (Elmendorf et al., 2015). Resampling or long-term monitoring can provide important information on how plant communities respond to both short-term changes in weather and long-term changes in climate (Gottfried et al., 2012; Wipf et al., 2013). However, these approaches require historical data, or several years of monitoring, and, as both resampling and long-term monitoring are observational approaches, they cannot disentangle the different factors driving the changes observed. Experimental warming can better explore cause-and-effect relationships between plant communities and climate

change, as well as provide a mechanistic understanding of short-term responses of ecosystems to climate warming (Rustad, 2006). *In situ* passive warming approaches, such as OTCs, have widely been applied to warm extant vegetation in alpine and arctic regions (Elmendorf et al., 2012a). The walls of the OTCs, may, however, constrain immigration and migration of plant species in the same way as they have been shown to affect pollination by wind and insects (Totland and Eide, 1999; Richardson et al., 2000). Thus, OTCs may not take into account novel interactions from new colonizers in warmer climates (Alexander et al., 2015). OTCs are also conservative warming devices, with an increase of mean daily temperature by c. 1.5°C in alpine and arctic tundra (Marion et al., 1997), which in some areas, is low compared to variations between years (Hollister and Webber, 2000). They therefore need to be installed for a long time to affect the vegetation (Hollister et al., 2005; Hudson and Henry, 2010). Indeed, high stability and resistance observed in low-productivity alpine and arctic plant communities to warming by OTCs can be due to the minor warming (Hudson and Henry, 2010; Keuper et al., 2011). Importantly, the warming effect of OTCs can be confounded with changes in the microenvironment such as soil moisture, wind or snow accumulation (e.g., Marion et al., 1997; Hollister et al., 2005). Another approach that contrasts the traditional *in situ* warming experiments is plant community transplantation (Nooten and Hughes, 2017), following classical transplants of individual plants (Clausen et al., 1948). Intact turfs of whole plant communities are moved to a lower elevation exposing it to a warmer climate in combination with a new neighborhood community. In other words, transplantation may change both the abiotic and the biotic environment, and can therefore examine the net effect of both direct and indirect impacts of climate warming (Alexander et al., 2015).

Thus, different approaches address specific questions in their own way (Elmendorf et al., 2015). To provide better estimates of plant community responses to future warming across a range of environments, there is a call for integrating different approaches within single studies (Dunne et al., 2004; Rustad, 2008; Menke et al., 2014). Here, we used two experimental warming approaches parallel and integrated them with a gradient approach in a single study in the Hengduan Mountains of southwest China to test congruency among them. We conducted warming experiments using *in situ* OTCs, and community transplantation along an elevation gradient, and we monitored control plots (space-for-time approach) over 5 years. Plant species richness and cover were measured each year from 2012 to 2016. Our study addresses two questions: (1) how do plant communities respond to the three different climate warming

approaches? (2) how can the combined approaches improve predictions of plant community responses to climate change?

MATERIALS AND METHODS

Study Area

The study was conducted in the Hengduan Mountains, in southwestern China. The study sites are located in Kang-Ding Valley, northwest of Mt. Gongga (**Supplementary Figure S1**), which is characterized by steep vertical elevational gradients, and vegetation belts changing from mixed coniferous-broadleaves forest and subalpine coniferous forest, to shrubs and alpine meadows with increasing elevation (Liu et al., 1985; Shen et al., 2001). Long term climate data extracted from Worldclim version 2.0 from the period 1970–2000 in the area show a mean annual temperature of 11.6°C and mean annual precipitation of c. 800 mm (Fick and Hijmans, 2017). We selected four perennial grassland sites spanning from the mixed leaf forest to the alpine climatic zone, differing on average by 1.75°C (range: 1.5–2.1°C) mean air temperature (June–August) between the sites (5.3°C between the lowest to the highest site), including a Low (3000 m a.s.l.), Middle (3500 m a.s.l.), Alpine (3850 m a.s.l.), and High alpine (4130 m a.s.l.), site (**Table 1** and **Supplementary Figure S1**). The geographical distance between adjacent sites is on average 10 km. The sites were selected to be as similar as possible in terms of vegetation, soil and grazing regime to enable between-site comparisons. The vegetation in all sites is dominated by grasses (*Festuca* spp., *Poa* spp.), sedges (*Carex* spp., *Kobresia* spp.), forbs (e.g., *Anaphalis nepalensis*, *Clinopodium polycephalum*, *Geranium pylzowianum*, *Polygonum viviparum*, *Potentilla leuconata*, *P. stenophylla*, and *Saussurea* spp.), and some shrubs (*Rhododendron* spp.) at the highest sites (see **Supplementary Table S2** for species list). All the sites are associated with mountain gray-brown soil originating from granite (He et al., 2005). There is moderate livestock grazing by yak, sheep, or horses in all the sites, and fences were used during the study to prevent grazers inside the plots. We simulated grazing by cutting vegetation to ca 5 cm to avoid any fence effects.

Experimental Design

Seven blocks were randomly positioned in each of the sites in 2012, covering an area of ca 400 m². The distance between the replicate blocks ranges from 4 to 6 m. In each block, we randomly positioned four 25 cm × 25 cm plots. One was used for *in situ* warming by OTC, one was transplanted to warmer climates, one was transplanted locally within the same block to control for any transplant effect, and one was used as an untouched control (**Supplementary Figure S1**). Thus, the blocks provided seven replicates for each of the three approaches.

Analysis of the local transplant and the untouched control plots across all years show that there were no differences between them, and thus no unwanted effects of the turf cutting and transplanting, as also shown in a similar transplant experiment using the same approach (Guittar et al., 2016). For the gradient study, we therefore used both the local transplant and the untouched control plot as space-for-time substitutions along the

elevation gradient with ca. 1.5°C temperature difference between each of the sites (**Table 1**).

The OTCs were placed upon one plot in each block, with the plot in the center. The OTCs are 40 cm tall, and the distance between parallel sides is 106 cm at the base and 60 cm at the top. Generally, OTCs increase mean daily air temperature by c. 1.5°C (Marion et al., 1997), which we could not test due to climate logger failures in our experiment and therefore refer to the expected warming in the literature. For the transplant experiment, one plot at each block per site was transplanted to the corresponding block of the site at the lower elevation with c. 1.75°C warmer summer temperature (i.e., temperature difference between each site). We permanently marked each corner of the plots with plastic poles. For the transplanted plots, the upslope center seen from the front of each turf was marked with a plastic flag, to ensure that the turfs were placed in the same position relative to the slope and block orientation at the target site. We used a knife to cut the transplanted plots 2 cm outside the margins, providing a buffer zone for possible edge effects, and at a depth of 20 cm, unless the soil was shallower, as was the case for some of the High alpine plots. After excavation, the plots were packed into boxes and transported to their respective target sites within 1 or 2 days. The turfs were fitted into the gaps created by excavating turfs at the target site and carefully checked that the soil surface was in plane with the surrounding vegetation, and that the edges of the excavated plot was in good contact with the edges of the gap. If necessary, loose soil was carefully removed from the underside of the turf, or local soil was added to the gap or around the edges to achieve this. In total there were 108 plots at the four sites along the elevational gradients, but four plots in the High alpine site were destroyed by yak in 2014.

Data Collection

A climate station (U30-NRC, Onset, United States) at each site recorded air temperature at 2 m and soil moisture at –5 cm in 10 min intervals since September 2012 and during the whole study period. We measured biomass at each of the sites in 2015 to have an estimate of productivity along the gradient (**Table 1**). We did this by harvesting all above ground biomass from 13 0.5 m × 0.5 m plots at the High alpine site and 20 plots from the other sites, and oven-dried it at 60°C for 72 h before weighing.

All vascular plant species in each plot were surveyed in 2012 (before treatment), and annually between 2013 and 2016. Percent cover of each vascular species was visually estimated during the peak of the growing season using a 25 cm × 25 cm frame with a grid of 5 cm × 5 cm subplots. Mean vegetation height for each plot was measured at five points in 2013 using a ruler. Forbs were identified to species level, whereas many of the graminoids were identified to genus level, i.e., *Carex* spp., *Poa* spp., *Kobresia* spp., and *Festuca* spp., because of a lack of a detailed flora from the study region and thus difficulties with identification of sterile graminoids.

Data Curation and Statistical Analyses

Over the 5 years of collecting this extensive data set, different people were involved, which increases the risk of observation errors. In particular, species can be misidentified (i.e., sterile

TABLE 1 | The four study sites with elevation, geographical coordinates, summer mean temperature (June–August) measured at 2 m between 2012 and 2016, long term annual precipitation from Worldclim version 2.0 for 1970–2000 (Fick and Hijmans, 2017), soil moisture (June–August) measured at 5 cm below ground between 2012 and 2016 and productivity measured as biomass per 0.5 m × 0.5 m plots in the Hengduan Mountains, China.

Site	Elevation (m a.s.l.)	Latitude (°N)	Longitude (°E)	Summer mean temperature (°C)	Annual precipitation (mm)	Soil moisture (%)	Productivity g/0.5 m ²
High alpine	4130	29.91	102.01	6.7	797	0.36	34.2 ± 2.2
Alpine	3850	29.89	102.02	8.4	821	0.38	62.2 ± 4.1
Middle	3500	29.86	102.04	9.9	775	0.46	89.2 ± 6.3
Low	3000	29.84	102.03	12.0	784	0.38	67.9 ± 4.4

graminoids) or might be overlooked in one of the observations. These errors will result in pseudo-turnover in the plant community data. To detect such errors, we compared each recorded species in each subplot over the 5 years. We used the subplots to assign unidentified or missing species if there was a record of the species in the previous and following year. Further, we re-estimated species cover in cases where cover was either too low or high to be real when comparing with other years, and replaced these values with the mean cover from the previous and following year. We did such re-estimates in totally 48 occasions (c. 1%) of the whole 5 years dataset.

To test how plant community properties change along the elevational gradient (i.e., represented as change in mean summer temperature among sites) and/or respond to the two different warming treatments (i.e., mean summer temperature contrasts between control and warming treatment), we fit linear models independently for each warming approach with species richness, evenness, or proportion of graminoids as response variable. We checked the fulfillment of the model assumptions visually and evenness had a heavy tail of negative residuals (2–3 observations). For the gradient approach, we fit two models: an intercept-only null model (no effect) and a model with mean summer temperature at each site (effect). For the two experimental warming treatments, we fitted three models: one containing the experimental site (no effect), one with experimental site and temperature contrast (effect) and one with the interactions of experimental site and temperature contrast (interaction). The effects of experimental site and temperature were tested by comparing the difference in Akaike information criterion (AIC) score Δ_i among these models differing in their fixed effects (Burnham and Anderson, 2002). A difference in AIC scores of $\Delta_i > 2$ between models indicates strong support for the model with lower AIC score (Burnham and Anderson, 2002). The model with the lowest AIC score was chosen as the best model, except when competing models differed by $\Delta_i < 2$, then the model with fewest parameters was selected.

To quantify and visualize the temporal changes in species composition between the warming approaches, we used principle response curves (PRC; Van den Brink and Ter Braak, 1999). PRC is the multivariate equivalent of repeated measures ANOVA, and analyses the community response through time to one or more treatments relative to a control. It is a partial RDA where treatments and time are included as factorial variables in a model analyzing the effects of the time × treatment interaction while including time as a covariate to control for any overall temporal trends. Treatment effects (C_{dt}) quantify the

compositional difference between treated plots and controls at each sampling date, and temporal trends can be visualized by plotting C_{dt} against time. We performed two separate PRCs on the forb community only because of the more detailed taxonomic resolution in the forbs. Rare species, that occurred less than three times in the whole data set were removed for this analysis.

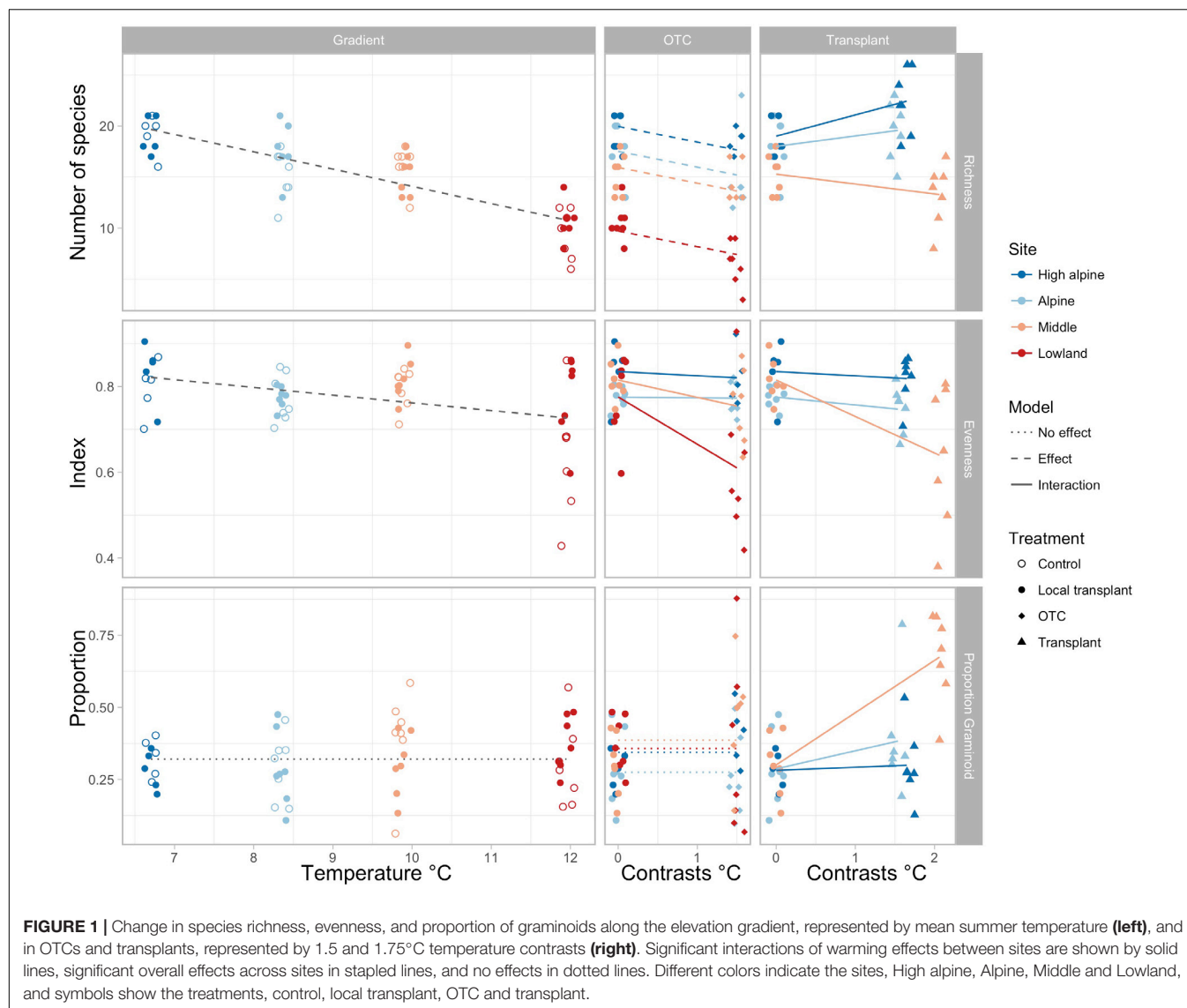
First, we quantified how much the species communities responded to the two warming approaches by moving “away” from the origin control communities (i.e., how much does the species community in the OTC or the transplant at the High alpine site differ from the control communities at the High alpine site). For this, we performed a PRC using species cover from 2012 to 2016 from the controls at the origin site and the two warming treatments. The analysis was done separately for each site and the two warming treatments. To test if the communities in the warming approaches differed from the origin controls communities, we used a permutation test. We used the species scores to assess and compare the species responses to the individual treatments.

Second, we tested if the species communities in the two warming treatments moved “toward” the target communities (i.e., to what extent does species community in the OTC at the High alpine site and the community transplanted from the High alpine to the Alpine site become similar to the target control communities at the Alpine site). We used a similar PRC approach as above, but run one model for each origin site with both warming approaches and the target control communities as treatments. The result was visualized by plotting the treatment effects C_{dt} for each of these treatments against time.

All analyses were performed in R 3.4.4 (R Core Team, 2018) using the vegan packages (Oksanen et al., 2018). All data and R code will be made available at OSF repository (doi: 10.17605/OSF.IO/F3KN, <https://osf.io/f3knq/>).

RESULTS

Temperature measurements show that mean annual and summer temperatures increase along the gradient from the high to the low elevation sites by ca 1.75°C between each site (Table 1). Biomass increased toward lower elevations along the gradient, with a peak in the Middle site ($F_{3,69} = 19.02$, $P < 0.001$; Table 1). Vegetation height after 1 year of treatment increased in the OTCs compared to the control plots by 3.01 (SE 0.19) cm in the High alpine, 4.21 (SE 0.26) cm in the Alpine, 8.58 (SE 0.89) cm in the Middle, and 8.22 (SE 1.13) cm in the Low elevation site ($F_{1,54} = 5.75$, $P = 0.02$).



Species richness gradually decreases toward warmer climates, both in the ambient plots along the elevation gradient and in the OTCs (**Figure 1** and **Supplementary Tables S3, S4**). Transplanting to warmer climates appears to increase species richness in the coldest sites, but decrease species richness in the warmest site. Evenness decreased in OTCs at the warmest site and in plots transplanted to a lower elevation in the warmest climates, i.e., from the Middle to the Low elevation site. The proportion of graminoids increased toward warmer climates only in plots transplanted to the warmest elevation (**Figure 1** and **Supplementary Tables S3, S4**).

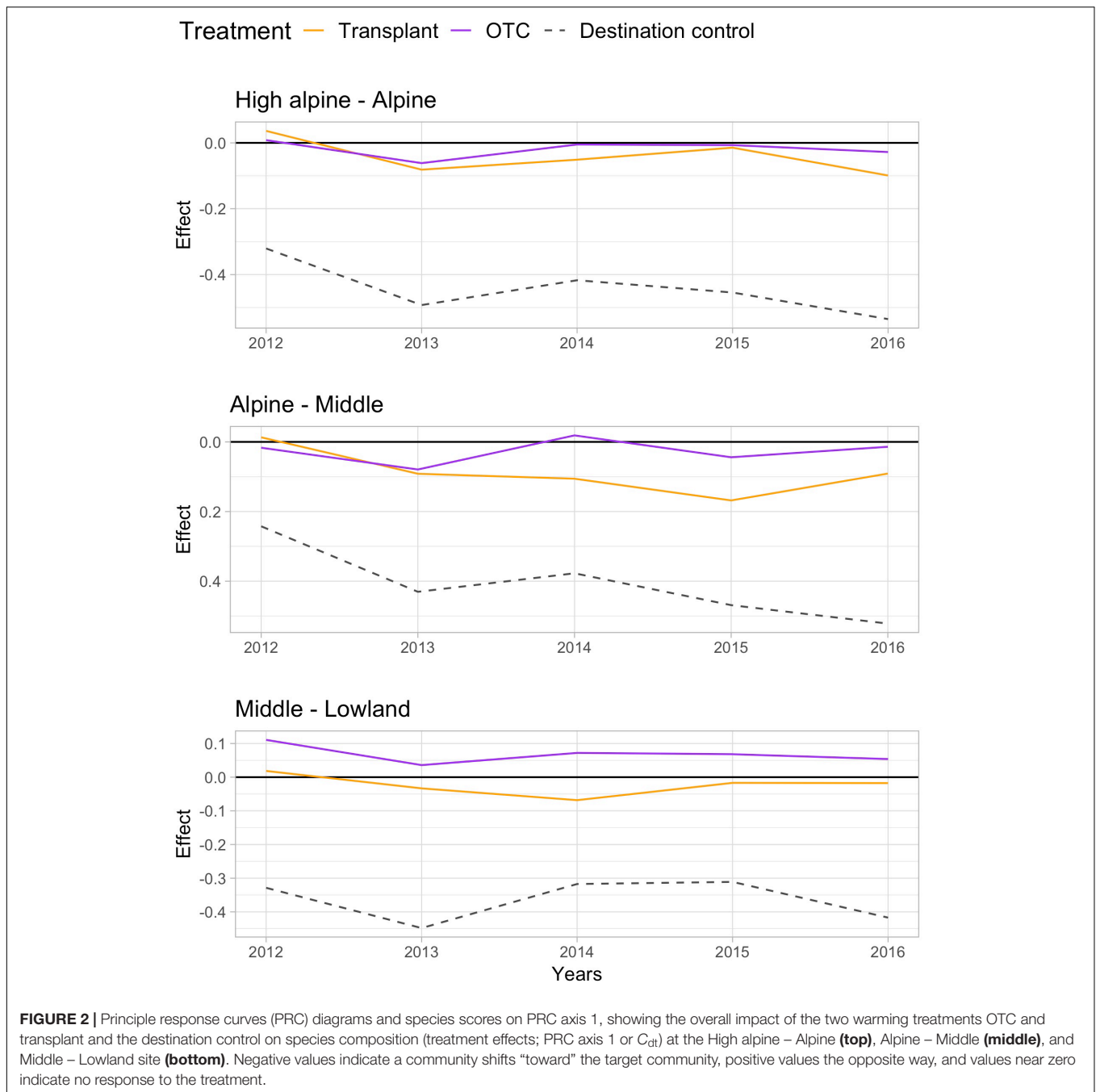
Warming by both OTCs and transplants changed the species communities over the 5 years “away” from the control communities at the origin sites, with the change being stronger at lower elevations (**Table 2**). Species composition in the OTC responded less to warming along the elevation gradient compared to the transplanted communities, i.e., they differed

significantly stronger from the control communities at the Middle site. Interestingly, species composition in the OTCs and in plots that have been transplanted to warmer climates seem to change in slightly different directions. Transplanted

TABLE 2 | Results of the PRC showing the proportion of explained variation by the treatment and treatment × time interaction for each site.

Origin site	OTC	Transplant
High alpine	0.079	0.072
Alpine	0.078	0.077*
Middle	0.107**	0.094**
Lowland	0.072	—

Separate models for each warming treatment were used. Numbers in bold and with asterisk show significant differences in plant communities between the warming treatment and origin control. Bold values and asterisk indicate that the treatment differs significant from the origin control community. * $p < 0.05$, ** $p < 0.01$.



plots appear to move more consistently “toward” the target community, i.e., the species composition in the control plot of the site in which they have been transplanted to (Figure 2, orange line moves more toward the gray dashed line). More species showed a stronger positive or negative response the warming treatment in the transplants, i.e., *Persicaria vivipara*, *Saussurea ceterach* at the High alpine and Alpine site and *Pedicularis davidii* at the Alpine and Middle site (Supplementary Table S5). Across all sites, both the transplants and OTC communities were far away from converging toward the target community even after 5 years. And indeed, at the Middle site

the OTC showed no indication of moving toward the target community.

DISCUSSION

Here we combined three climate warming approaches in a single study and find similar responses in terms of plant community properties, with some exceptions. The general trend, in the elevation gradient and both experimental warming approaches, was decreased species richness and evenness with climate

warming in the warmest sites at low elevation. This is in line with previous studies, suggesting increased competitive effects from the taller vegetation canopy of the species already growing in the plots and/or from lowland immigrating species, under warmer climates (e.g., Elmendorf et al., 2012a,b; Alexander et al., 2015). In our study, increased intensity of competition is indicated by the taller vegetation in the OTCs along the whole gradient, and the increase in proportions of graminoids in plots transplanted to warmer climates in the lowest elevations. The decrease in species richness and evenness observed toward warmer and lower elevation sites along the gradient in our study is also likely due to increased competition, as biomass increase, and the plants are generally bigger toward warmer sites, and thus fewer individuals and hence fewer species can fit into the plots.

In the colder climates at the higher elevations, on the other hand, plant community responses depended on the warming approach. In the transplants, species richness appeared to increase in plots transplanted toward warmer climates from the High alpine to the Alpine, and from the Alpine to the Middle site. This suggests that alpine vegetation is open to invasion by species from lower elevation, confirming the range shift literature that lowland species are moving to higher elevations (e.g., Klanderud and Birks, 2003; Gottfried et al., 2012; Pauli et al., 2012; Steinbauer et al., 2018). Higher invasibility of alpine plant communities has also been shown in a transplant experiment in Norway, where seedling recruitment was higher in alpine vegetation compared to vegetation at lower elevations (Meineri et al., unpublished). Moreover, species appear to have migrated into the transplanted plots in our study from the new neighborhood plant community, but without immediately outcompeting the species inhabiting the plots. Thus, it appears that alpine species are resistant to invasion and increased competitive effects in the short term, and that loss of species is a slow process in these cold environments. This is in line with a similar transplant experiment in alpine Norway (Vandvik et al., unpublished), and with resampling and long-term monitoring studies, showing that migration by lowland species to alpine sites is faster than the extinctions of the alpine specialists, resulting in increased species richness at high elevations, at least in the short term (e.g., Klanderud and Birks, 2003; Gottfried et al., 2012; Pauli et al., 2012; Steinbauer et al., 2018). Contrastingly, warming by OTCs decreased species richness across all sites in our study, which agrees with previous studies using OTCs in alpine and arctic tundra (e.g., Walker et al., 2006; Elmendorf et al., 2012a). This decrease in species richness is previously explained by increased competition inside the plots when the height of the vegetation canopy increase (Klanderud and Totland, 2005; Walker et al., 2006; Elmendorf et al., 2012a). Another possible explanation, in addition to competition, can be that new species have difficulties with entering the plots inside the OTCs because the walls may act as migration barriers. In addition, immigration of new species is likely lower in OTCs than in transplants in our study because of the longer distance to source populations. This can probably also explain the steep increase of graminoids in plots transplanted to the lowest elevation site, but no effects on graminoids in the OTCs. Transplants from colder climates are rapidly exposed to lowland graminoids that can enter the plots.

For the plots in the OTCs on the other hand, it might take more time before graminoids already present inside or outside the OTCs increase in abundance or enter the plots. Thus, transplants are likely better means than OTCs in detecting effects of species migrations and novel interactions due to climate change (Suttle et al., 2007; Blois et al., 2013; Alexander et al., 2015).

Previous explanations of, in some cases, lack of responses of alpine and arctic plant communities to warming by OTCs have been that the OTCs only provide minor warming in comparison to year-to-year variability (Hollister and Webber, 2000; Hudson and Henry, 2010; Keuper et al., 2011). The lack of warming by OTCs during nights, and higher maximum temperatures during the day (Marion et al., 1997; Klein et al., 2005; Sierra-Almeida and Cavieres, 2010; Godfree et al., 2011), also results in larger diurnal ranges in the OTCs than in the transplants and in the communities along the natural gradient in our study. Drought stress induced by high temperatures and, in some cases slightly (not statistical significant) lower soil moisture inside OTCs (Bokhorst et al., 2013), may decrease alpine plant species richness (Klein et al., 2004). We don't think, however, that this explains the decrease in species richness inside OTCs in our study, as humidity is relatively high in this area, and the plots are positioned on slopes where the water moves easily through the soil. Our study shows that plant communities respond slightly differently to approximately the same degree of mean summer temperature increase, although the warming effect of the OTCs was likely lower than the other approaches also in our study. This suggests, however, that other factors than temperature *per se*, such as species ability to migrate in and out of the plots, and species interactions, may explain the different responses of the plant communities between the different approaches in our study. Other factors not measured in our study, such as soil properties, herbivory, microclimate, or pathogens may also have affected our results, but we did not observe any signs of this in the field.

Novel interactions from lowland species that are tracking climate warming and hence immigrating into the alpine communities are likely to be important for the performance of alpine species in the future (Alexander et al., 2015). The approaches studied in this paper differ in their ability to mimic, such indirect impacts of climate warming on plant communities. Whereas OTCs may act as barriers to species migration, transplanting plots to a new environment provide a very rapid exposure to new potential colonizing species. The realistic future scenario is probably somewhere between the recruitment limitations by the OTCs and the extreme exposure to new neighbors in the transplants. The elevation gradient approach reflects long-term responses to species migrations and biotic interactions, and over much longer time scales than can be explained by short-term experiments (Elmendorf et al., 2015). However, slowly changing plant communities along spatial temperature gradients may lag behind the more rapid anthropogenic climate change (Pickett, 1989). Combining and integrating more than one approach is likely the best tool to examine responses of both direct warming effects, and indirect effects of changes in biotic interactions due to species migrations. Our results show, however, that the choice of approach depends on the research question of the particular project. If the aim

is to understand not only direct effects, but also indirect effects of climate warming on plant communities, such as biotic interactions, transplantations will probably examine the role of novel species interactions in a better way than OTCs.

AUTHOR CONTRIBUTIONS

YY secured funding for the project, ran the experiment, and wrote the manuscript. AH analyzed data and wrote the manuscript. KK designed the experiment, conceived the idea of this paper, and wrote the manuscript. RT prepared and analyzed the data and commented on the manuscript. GW secured funding for the project and IMHE research infrastructure and commented on the manuscript. VV designed the experiment, conceived the idea of this paper, and commented on the manuscript.

FUNDING

This study was funded by the Foundation of Key Laboratory of Mountain Surface Processes and Ecological Regulation, CAS;

Key Research Program of Frontier Sciences, CAS (QYZDJ-SSW-DQC006), the 135 Strategic Program of the Institute of Mountain Hazards and Environment (No. SDS-135-1707), and the Norwegian Center for International Cooperation in Education (SIU) projects UTF-2013/10074 and HNP-2015/10037.

ACKNOWLEDGMENTS

We thank Fei Ran, Mari Jokerud, Christine Pötsch, Haijun Cui, and Ruiying Chang for setting up the field sites and Yang Yang, Haijun Cui, Li Zhang, Ahui Peng, Xiaoli Chen, and Zengyuan Hu for collecting data. We are grateful to Prof. Hai He for the identification of species in the field and the Alpine Ecosystem Observation and Experiment Station of Mt. Gongga, CAS for providing accommodation and lab space.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01574/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Warmer Temperatures Affect the *in situ* Freezing Resistance of the Antarctic Vascular Plants

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OPEN ACCESS

Edited by:

Hans J. De Boeck,
University of Antwerp, Belgium

Reviewed by:

Charles L. Guy,
University of Florida, United States
Aud Helen Halbritter,
University of Bergen, Norway

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 01 June 2018

Accepted: 12 September 2018

Published: 08 October 2018

Citation:

Sierra-Almeida A, Cavieres LA
and Bravo LA (2018) Warmer
Temperatures Affect the *in situ*
Freezing Resistance of the Antarctic
Vascular Plants.
Front. Plant Sci. 9:1456.
doi: 10.3389/fpls.2018.01456

Although positive effects on growth and reproduction of Antarctic vascular plants have been reported under warmer temperatures, it could also increase the vulnerability of these plants to freezing. Thus, we assessed *in situ* whether warming decreases the freezing resistance of *Colobanthus quitensis* and *Deschampsia antarctica*, and we compared the level and mechanism of freezing resistance of these species in the field with previous reports conducted in lab conditions. We assessed the freezing resistance of *C. quitensis* and *D. antarctica* by determining their low temperature damage (LT₅₀), ice nucleation temperature (NT) and freezing point (FP) in three sites of the King George Island. Plants were exposed during two growing seasons to a passive increase in the air temperature (+W). +W increased by 1K the mean air temperatures, but had smaller effects on freezing temperatures. Leaf temperature of both species was on average 1.7K warmer inside +W. Overall, warming decreased the freezing resistance of Antarctic species. The LT₅₀ increased on average 2K for *C. quitensis* and 2.8K for *D. antarctica*. In contrast, NT and FP decreased on average c. 1K in leaves of warmed plants of both species. Our results showed an averaged LT₅₀ of −15.3°C for *C. quitensis*, and of −22.8°C for *D. antarctica*, with freezing tolerance being the freezing resistance mechanism for both species. These results were partially consistent with previous reports, and likely explanations for such discrepancies were related with methodological differences among studies. Our work is the first study reporting the level and mechanisms of freezing resistance of Antarctic vascular plants measured *in situ*, and we demonstrated that although both plant species exhibited a great ability to cope with freezing temperatures during the growing season, their vulnerability to suffer freezing damage under a warming scenario increase although the magnitude of this response varied across sites and species. Hence, freezing damage should be considered when predicting changes in plant responses of *C. quitensis* and *D. antarctica* under future climate conditions of the Antarctic Peninsula.

Keywords: Antarctica, climate change, *Colobanthus quitensis*, *Deschampsia antarctica*, freezing events, LT₅₀, photoinactivation, warming

INTRODUCTION

Antarctica is the coldest and windiest landmass on Earth (Robinson et al., 2003). Mean air temperatures in the coastal zone of the Antarctic Peninsula and adjacent islands (also called Maritime Antarctica) seldom exceed 0 or +5°C during the summer (Smith, 2003), with daily temperature ranges from −10 to +15°C for the same period (Convey, 2013). Thus, Antarctic plants are constantly dealing with low temperatures, even during the growing season (Convey, 1996; Convey et al., 2014). For this reason, low temperature stress seems to be part of the explanation for its lower plant species diversity compared to the Arctic (Convey, 2006). Only two vascular plants have been able to establish natural populations in the maritime Antarctica: The pearlwort *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) and the hair grass *Deschampsia antarctica* Desv. (Poaceae) (Smith, 2003).

The constant low temperatures that characterize the growing period in the Maritime Antarctica are likely to be near the minimum thresholds for many physiological processes. This suggests that in the context of climate change, small increments in the temperature experienced by plants in this environment will have a relatively greater biological impact than the same increment experienced in a less extreme environment (Convey, 2001, 2006). During the last part of the past century air temperatures in the Antarctic Peninsula increased at a faster rate than the rest of Antarctica and the globe (Vaughan et al., 2003; Turner et al., 2014). This warmer climate caused longer growing seasons with higher temperatures, ice retreats and higher frequency of rains, which promoted the expansion and increase of population sizes and numbers of *C. quitensis* and *D. antarctica* along the Peninsula (Fowbert and Smith, 1994; Gerighausen et al., 2003; Torres-Mellado et al., 2011; Cannone et al., 2016). Apparently, warmer temperatures favored plant growth and reproduction by providing more favorable thermal conditions for different physiological processes (e.g., photosynthesis), but also by increasing plant nutrient availability via stimulation of soil microbial activity in N-cycling (Wasley et al., 2006; Yergeau et al., 2012). However, these positive effects of warmer temperatures in Antarctica could be negligible because warming can decrease plant freezing survival (Inouye, 2000).

The ability to survive freezing temperatures (i.e., freezing resistance) is highly related to the ambient temperature that plants experience (Beck et al., 2004; Bannister et al., 2005). Thus, warmer daytime temperatures due to climate change may decrease the ability of plant to survive freezing conditions. This is particularly important for high-latitude and -elevation plants to resist freezing temperatures, where it has been shown that warming turned even the most freezing-resistant species more vulnerable to damage by freezing (Loveys et al., 2006; Woldendorp et al., 2008; Rixen et al., 2012). For example, Marchand et al. (2006) reported a reduction of plant performance (i.e., leaf relative chlorophyll content, maximum efficiency of photosystem II, stomatal conductance) of four Arctic plant species after they were exposed to consecutive heat waves. Apparently, warmer conditions reduced cold acclimation of

plants, resulting in damage after the exposure to the natural low temperatures of the Arctic. Likewise, Sierra-Almeida and Cavieres (2010) reported that *in situ* warmer temperatures decreased on average 4K the freezing resistance of seven alpine species of central Chilean Andes. Although Turner et al. (2016) reported that warming in the Antarctic Peninsula (AP) has stopped in the last decade; they warned that new warming episodes are likely to occur in the future. In addition, Lee et al. (2017) pointed out that the recently paused warming observed in the AP is a consequence of short-term natural climate variability and that a new warming phase will be observed across the AP. Thus, to assess whether warmer temperatures reduce the ability of Antarctic plants to resist freezing is crucial to predict their vulnerability to future warming events.

Chronically low temperatures of the Antarctica suggest that *C. quitensis* and *D. antarctica* are morphological and physiologically adapted to cope with these stressful conditions. Overall, plants have two physiological mechanisms of freezing resistance: avoidance and tolerance. Freezing avoidance (FA) prevents the ice formation through freezing point depression or by supercooling, meanwhile freezing tolerance (FT) is defined as the ability of plants to survive the extracellular freezing (Larcher, 2003). In the case of Antarctic plants, they have exhibited freezing avoidance and tolerance mechanisms (Bravo et al., 2001; Reyes-Bahamonde, 2013). Molecular and biochemical aspects of the freezing resistance have been studied in these species, especially in *D. antarctica* (e.g., Bravo and Griffith, 2005; Olave-Concha et al., 2005; Piotrowicz-Cieślak et al., 2005; Bravo et al., 2009; Zúñiga-Feest et al., 2009). However, studies dealing with the freezing temperature causing injury to these species are scarce (Bravo et al., 2001; Gianoli et al., 2004; Chew et al., 2012). Studies with laboratory grown plants have reported that the freezing resistance of *C. quitensis* fluctuates between −14 and −4.8°C, whilst for *D. antarctica* it fluctuates between −26.4 and −12°C. Discrepancies among those studies on the level of freezing resistance of Antarctic plants have been attributed to methodological issues. Particularly, the time and temperature that plants were maintained under greenhouse and/or growth chambers before freezing injury assays varied enormously among them. Therefore, *in situ* determinations are required to unveil the real level of freezing resistance of the Antarctic vascular plants and the mechanisms involved. In addition, manipulative field experiments are needed to assess the likely effect of warming in this important trait.

In this study, we conducted a field experiment in the King George Island, where we increased the air temperatures experienced by *C. quitensis* and *D. antarctica* during two growing seasons to assess their *in situ* vulnerability to freezing damage under different thermal conditions. Specifically, our aims were: (1) to assess the *in situ* level of the freezing resistance of *C. quitensis* and *D. antarctica* and whether warming decrease this ability (i.e., leaf NT, FP and/or LT₅₀ of warmed plants should occur at higher temperatures than of unwarmed plants); and (2) to compare the level and mechanism of freezing resistance of these Antarctic plants

species in the field with previous reports conducted under lab conditions.

MATERIALS AND METHODS

Study Area

This study was carried out in the King George Island, South Shetland Archipelago, nearby the Henryk Arctowski Polish Station (62°09' S, 58°28' W). Plants were obtained from three sites, which differed in soil nutrients, plant cover and relative abundance of the Antarctic vascular species (**Supplementary Table S1** and **Figure 1**). Site 1 (62° 9'43.33"S; 58°27'58.80"W) was located near the beach, about 90 m from the coast line thus receiving sea spray, plant cover is >90% and vegetation is dominated by *D. antarctica*. This site receives great inputs of guano and feces because of the activity of sea birds and mammals (see **Supplementary Table S1** for soil nutrient contents). Site 2 (62° 9'49.15"S; 58°28'9.60"W) was located 200 m distant of the site 1, plant cover is 100% and it is dominated by a compact and continuous moss carpet where *C. quitensis* and *D. antarctica* are growing interspaced (Cavieres et al., 2018). This site seems to be favorable for plant growth because of well drained soils and nutrient availability (**Figure 1** and **Supplementary Table S1**). Site 3 (62° 9'52.90"S; 58°28'21.31"W) is a typical fellfield located 550 m from the beach and at 30 m a.s.l. Plant cover is <10%, and the scarce vegetation is dominated by lichens and only isolated individuals of *C. quitensis* and *D. antarctica* species are present across a stony and rocky soil matrix (**Figure 1**).

The growing season in the study area usually starts with the snowmelt in December and finishes in March with the first snowfall. During two growing periods we collected microclimatic data (83 and 55 days for the first and second growing season, respectively), period over which the daily mean air temperature was 1.8°C, with mean maximum and minimum temperatures of 4.7 and −0.4°C, respectively, with night freezing temperatures occurring frequently during the entire growing seasons (**Table 1**). Precipitation occurs as rain in summer, with estimations that range from 350 to 750 mm (Green et al., 2007).

Plant Species

Studied species were the pearlwort *C. quitensis* (Kunth) Bartl. (Caryophyllaceae) and the hair grass *D. antarctica* Desv. (Poaceae). *C. quitensis* is a long-lived perennial herb. It forms low, compact, discrete cushions with densely packed shoots and a log taproot (Greene and Holtom, 1971). Its geographical distribution comprises from Mexico and from the Andes mountains of Ecuador down to c. 68°S in the Maritime Antarctica (Moore, 1970). Despite its wide latitudinal distribution, this species inhabits sites with similar conditions, characterized by sparsely vegetated, sheltered, moist, and well-drained mineral soils (Smith, 2003). *D. antarctica* is a long-lived perennial herb that forms low, caespitose shallow-rooted tufts (Greene, 1964; Moore, 1979). *D. antarctica* distributes from central Chile and Argentina (33°S) to the Terra Firma Islands

southwestern Antarctic Peninsula (68°S; Smith and Poncet, 1987). In Antarctica this species colonize habitats ranging from mineral to organic soils, from well drained to waterlogged areas, and from nutrient-deficient to highly nutrient-enriched habitats (Smith, 2003).

Experimental Design

In December 2013, on each site we selected seven plant individuals of *C. quitensis* and *D. antarctica*. On each individual, we placed a hexagonal Open Top Chamber (thereafter OTC), similar to those used in the International Tundra Experiment (ITEX). Each OTC was made with transparent Plexiglass® walls of 40 cm height, 115 cm in basal diameter, and reinforced with aluminum profiles. OTCs walls were punched with 25 holes of 1.5 cm diameter each to allow some wind to pass through and hence avoid an excessive increase in air temperature. OTCs were secured to the ground with ropes to avoid being moved and/or destroyed by the strong winds. Another seven individuals per species were randomly selected at 2 m distant from the nearest OTC. These individuals were growing under natural temperatures conditions. Hence, we obtained two experimental conditions with 7 replicates each: warmed (+W) and unwarmed control plants (-W) repeated in three sites. The spatial arrangement of both OTC and control plots was random, taking care that distance between OTC is enough to avoid any possible effects of OTCs on the neighboring control plots by affecting wind or snow deposition. Although the use of passive warming systems such as OTC has been controversial (e.g., Kennedy, 1995; De Boeck et al., 2012), some authors arguably consider that OTCs are a reasonable analog of regional warming for remote areas such as polar habitats (Hollister and Webber, 2000; Bokhorst et al., 2013).

Microclimatic conditions were monitored in warming and control plots during two growing seasons (**Table 1**). For this, a weather station was installed on each site (2 units HOBO®U-30 Station, Onset Computer Co., Bourne, MA, United States; 1 Em50 Data Logger, Decagon Devices Inc., Pullman, WA, United States). Air and soil temperature sensors were placed at 5 cm ($n = 1$) above and 5 cm below ($n = 1$) ground surface, and temperature conditions were recorded every hour. In addition, leaf temperature was measured for warmed and unwarmed plants of *C. quitensis* and *D. antarctica*. For this, each leaf temperature sensor was placed beneath a leaf ($n = 1$) and were connected to the weather station programmed to record temperature every hour. Missing leaf temperatures data in site 1 during 2015 were absent because sensors were destroyed. A similar situation occurred with air and leaf temperature sensors in site 3 during 2014. Due to logistic limitations to access the study area, weather stations were installed and uninstalled in the field for each growing season. Thus, records of microclimatic conditions in the **Table 1** started in December 10th 2013 and finished in March 8th 2014 for the first growing season. For the second growing season records started in January 10th (sites 2 and 3) and 13th (site 1) and finished in March 4th 2015.

Air temperature data were used to estimate growing degree days (GDDs; McMaster and Wilhelm, 1997). GDDs were used as a measure of the accumulated amount of heat (in °C)

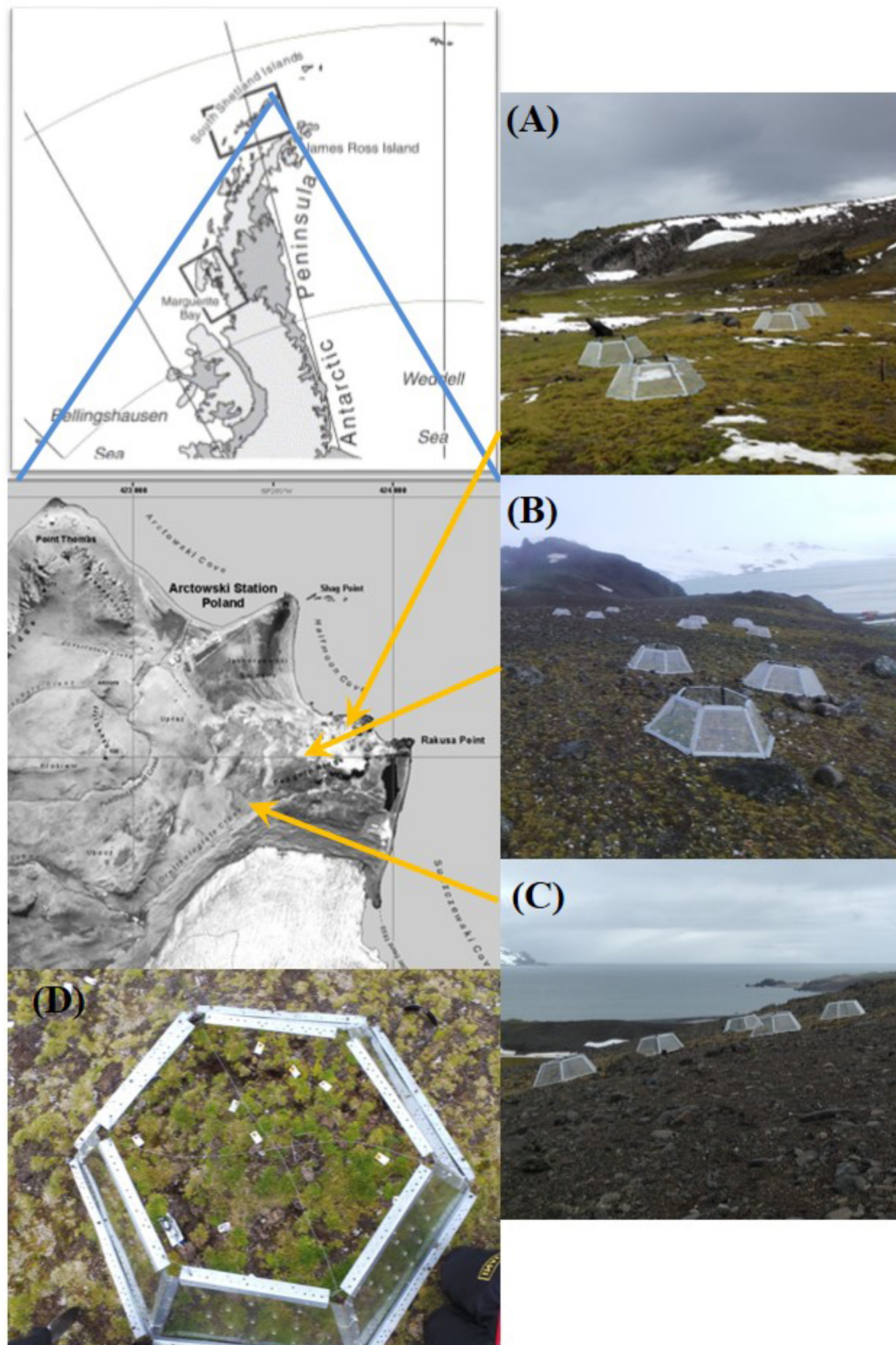


FIGURE 1 | Study area in the King George Island (South Shetland Archipelago) where experimental warming was installed. Photographs correspond to **(A)** Site 1, nearest the beach; **(B)** Site 2, and **(C)** Site 3; **(D)** showed several individuals of *C. quitensis* inside an OTC (see Materials and Methods sections for site descriptions and experimental design).

TABLE 1 | Thermal conditions of Antarctic plants exposed to experimental warming during two growing seasons in the King George Island, Maritime Antarctica (Antarctic Peninsula).

	Variable	Site 1		Site 2		Site 3	
		–W	+W	–W	+W	–W	+W
2014	Length of the growing season (days)	77		89		83	
	Mean air temperature (T_{mean} , °C)	1.3 ± 0.1	2.7 ± 0.1*	0.9 ± 0.1	2 ± 0.1*	–	–
	Maximum air temperature (T_{max} , °C)	4.5 ± 0.4	9.1 ± 0.6*	3.9 ± 0.3	6.6 ± 0.4*	–	–
	Minimum air temperature (T_{min} , °C)	–1 ± 0.2	–0.8 ± 0.2	–1.3 ± 0.2	–0.8 ± 0.2	–	–
	GDD ₀ (°C day ^{–1})	116.6	288.5*	115.1	254.5*	–	–
	Frequency of freezing events (F_{freq} , %)	77	73	80.9	75.3	–	–
	Intensity of freezing events (F_{int} , °C)	–1.7 ± 0.2	–1.6 ± 0.2	–1.9 ± 0.2	–1.5 ± 0.1	–	–
	Absolute min temperature (°C)	–6.5	–4.9	–6.8	–5.8	–	–
	Duration of freezing events (F_{dur} , h)	11.9 ± 0.8	9.9 ± 0.8	17.6 ± 0.8	13.1 ± 0.6*	–	–
	Minimum soil temperature (°C)	3.4 ± 0.2	4.8 ± 0.3*	1.7 ± 0.1	2.4 ± 0.1*	3.1 ± 0.1	4.3 ± 0.1*
	Leaf temperature of <i>C. quitensis</i> (°C)	4 ± 0.1	4.9 ± 0.1*	2.3 ± 0.1	3.8 ± 0.1*	–	–
	Leaf temperature of <i>D. antarctica</i> (°C)	3.7 ± 0.1	5.3 ± 0.1*	2.4 ± 0.1	4.9 ± 0.2*	–	–
2015	Length of the growing season (days)	53		55		55	
	Mean air temperature (T_{mean} , °C)	2.7 ± 0.1	3.7 ± 0.1	2.1 ± 0.1	2.9 ± 0.1	2.1 ± 0.1	3.1 ± 0.1
	Maximum air temperature (T_{max} , °C)	5.3 ± 0.4	9.2 ± 0.6*	4.9 ± 0.4	7.8 ± 0.5*	5.1 ± 0.4	8.4 ± 0.5*
	Minimum air temperature (T_{min} , °C)	0.6 ± 0.3	0.3 ± 0.3	0 ± 0.3	–0.1 ± 0.3	–0.1 ± 0.3	–0.02 ± 0.3
	GDD ₀ (°C day ^{–1})	152.2	242.4*	128.2	206.5*	138.3	220.5*
	Frequency of freezing events (F_{freq} , %)	27.5	37.3	47.3	49.1	60	41.8
	Intensity of freezing events (F_{int} , °C)	–2.2 ± 0.4	–1.9 ± 0.3	–1.8 ± 0.3	–1.8 ± 0.3	–1.4 ± 0.4	–1.8 ± 0.3*
	Absolute min temperature (°C)	–5	–5.6	–6.2	–6.2	–6.4	–5.8
	Duration of freezing events (F_{dur} , h)	10.6 ± 2.5	10.1 ± 2	11.7 ± 2.4	10.7 ± 2.2*	11.2 ± 2.3	11.4 ± 1.8
	Minimum soil temperature (°C)	3.5 ± 0.3	4.4 ± 0.3*	1.6 ± 0.2	2.1 ± 0.2	2.5 ± 0.2	2.4 ± 0.2
	Leaf temperature of <i>C. quitensis</i> (°C)	–	–	3.4 ± 0.1	3.8 ± 0.1	3.2 ± 0.1	5.1 ± 0.1*
	Leaf temperature of <i>D. antarctica</i> (°C)	–	–	3 ± 0.1	4.7 ± 0.2*	3.8 ± 0.1	5.7 ± 0.1*

Values correspond to mean ± SE ($n = 1$), and they are shown for natural temperature conditions (–W) and warming (+W) at three sites in the study area. Asterisks indicate significant differences between natural and warm conditions (χ^2 , $P < 0.05$).

above a base temperature to represent a cumulative index of the energy available to growing plants, according to the formula:

$$GDD = [(maximum\ daily\ temperature + minimum\ daily\ temperature)/2] - base\ temperature$$

The daily GDDs were summed per each entire growing season. We used 0°C as a conservative base growing temperature (the temperature above which plants can perform metabolic functions, e.g., photosynthesis, cell elongation), because plants from cold climate generally vary in their absolute base growing temperature, and this value encompasses this variability (Körner, 2011).

Freezing Resistance Determinations

Plant Material Collection

We collected seven plant samples replicates for each species, experimental condition and site, excepting by the site 3 where we collected six replicates. Plant samples corresponded to complete individuals with at least seven modules (small rosettes or tillers). We collected all plant material between 11:00 AM and 12:00 PM. Plant samples were placed in plastic boxes with

belowground organs wrapped in wet paper to prevent changes in tissue water content and mechanical damage. Samples were then transported to a field laboratory at the Polish Scientific Station, less than 10 min away from the study sites. We kept plant samples outdoor but protected from wind until freezing resistance determinations were performed within 24 h of collection, which were carried out between February 25th and March 5th 2015.

Low Temperature Damage

For each species, experimental condition and site, we estimated the freezing temperature producing 50% damage (LT₅₀). For this, we selected and detached six rosettes/tillers from different plant samples, and they were separated into six subsamples. One subsample was used as control and stored at 2°C and darkness during 24 h. Remaining five subsamples were separately placed in a small plastic bag, which was then placed in a larger plastic bag with a weight to ensure that each subsample was submerged in a cryostat (F25-ME, Julabo Labortechnik GmbH, Germany) with antifreeze solution (Polycool Mix 25, PolyScience, IL, United States). Cryostat was cooled previously at five different target temperatures: –8, –12, –16, –20 and –25°C. All subsamples were transferred from outdoor to the cryostat and incubated during 2 h to reach homogeneous leaf

temperatures. After that freezing treatment, subsamples were removed from the cryostat and placed back into cold room, under darkness and at 2°C during 24 h for thawing. In most studies dealing with plant freezing resistance, samples are cooled gradually to determine LT₅₀ (2–5 K h⁻¹; e.g., Hekneby et al., 2006; Ladinig et al., 2013; Briceño et al., 2014). However, given that cooling rates used by previous studies dealing with freezing resistance of Antarctic vascular plants varied from 1 to 17 K h⁻¹ (Bravo et al., 2001; Gianoli et al., 2004; Chew et al., 2012), it makes impossible to find a consensus cooling rate for comparative purposes. Although sudden cooling can lead to increased tissue damage owing to anomalous water diffusion and ice crystal formation (Guy, 2003; Wisniewski et al., 2014), our procedure induced similar plant damage as a cooling rate of 16 K h⁻¹ (see Pescador et al., 2018 for details of cooling rates assay), enabling to assess cooling directly as proxy for natural and immediate freezing exposure (Larcher et al., 2010).

Leaf damage was assessed as percentage of photoinactivation (*Phi*) of the photosystem II as described by Larcher (2000). For this, we measured the ratio of variable to maximum fluorescence (*Fv/Fm*) of dark-adapted leaf by using a chlorophyll fluorometer (MINI-PAM, Walz, Germany). LT₅₀ corresponds to the temperature at which *Phi* reaches a 50% value in subsamples, and was determined by linear interpolation using the temperature of the highest *Phi* of <50% and the temperature of the lowest *Phi* of >50% (Bannister et al., 2005). *Phi* was chosen because it measures changes in photosynthetic performance that correlates very well with direct measurements of tissue damage (i.e., visual freezing injuries and vital stain; Boorse et al., 1998; Neuner and Buchner, 1999) and because is an easy, rapid and cheap method to work in areas with difficult logistic as it is the Antarctica.

Thermal Analyses

A small module (rosette or tiller) was removed from each of six-seven plant samples taken from each species, experimental condition and sites. Each module was attached to a thermocouple (Gauge 30 copper-constantan thermocouples; Cole Palmer Instruments, Vernon Hills, IL, United States), and immediately enclosed in a small, tightly closed cryotube. The cryotubes were placed in a cryostat (F25-ME, Julabo Labortechnik GmbH, Germany), and the temperature was decreased from 0 to -20°C, at a cooling rate of 2 K h⁻¹. The temperature of individual module was monitored every second with a Personal Daq/56 multi-channel thermocouple USB data acquisition module (IOtech, Cleveland, OH, United States). The sudden rise in leaf temperature (exotherm) produced by the heat released during the extracellular freezing process was used to determine two variables: the ice nucleation temperature (NT), which corresponds to the lowest temperature before the exotherm, indicating the onset of ice crystal formation, and the freezing point (FP), the highest point of the exotherm, indicating the freezing of water in the apoplast, including symplastic water driven outward by the water potential difference caused by the apoplastic ice formation (Larcher, 2003). We chose this cooling rate because it is

the same used by previous studies where thermal analyses were carried out (Bravo et al., 2001; Reyes-Bahamonde, 2013).

Statistical Analyses

Differences in air (i.e., mean, maximum, minimum, intensity, and duration of freezing events) and leaf temperatures between warming and control conditions were assessed by Chi square (χ^2) tests. Differences in the effect of warming and site on LT₅₀, NT and FP were assessed by using Factorial ANOVAs as well (See details in **Supplementary Table S2**). Differences between NT and LT₅₀ in determining freezing resistance mechanisms for each species and experimental conditions were assessed with *t*-tests. Data were checked for normality before analyses.

RESULTS

Microclimatic Conditions

Air, soil and leaf temperatures during both growing seasons were affected by warming (**Table 1**). Although minimum air temperatures (*T*_{min}) and the intensity of freezing events (*F*_{int}) were similar between +W and -W conditions (average *T*_{min} ranged from -13 to 0.6°C and average *F*_{int} was -1.8°C in both growing periods), the frequency (*F*_{freq}) and duration of freezing events (*F*_{dur}) tended to be lower and of shorter duration inside +W plots (**Table 1**). The maximum air temperature (*T*_{max}) was consistently higher inside +W plots. For example, in 2014 +W increased 4.6 and 2.7°C the air *T*_{max} in the sites 1 and 2, respectively. This *T*_{max} increase was of 3.9, 2.9, and 3.3°C in the sites 1, 2, and 3 in 2015. Regarding the growing degree days above 0°C (GDD₀), in 2014 GDD₀ were 59.6 and 54.8% greater in +W than in -W conditions in the sites 1 and 2, respectively (**Table 1**). In 2015, +W increased GDD₀ on average 37% in the three sites (**Table 1**).

Freezing Resistance in the Field Under Warming Scenario

Antarctic plant species exhibited different ranges of freezing resistance in the field (**Supplementary Table S3**). Considering all sites together, average of ice Nucleation Temperature (NT) and Freezing Point (FP) of *C. quitensis* were -3.7 ± 0.1°C and -2.2 ± 0.1°C, respectively (**Figure 2**). Freezing temperature producing 50% photoinactivation (LT₅₀) ranged from -12.4 ± 0.1°C in site 2 to -17.4 ± 0.5°C in site 1 (**Figure 2**; *F*_{2,32} = 172.5, *P* < 0.0001). For *D. antarctica*, NT ranged from -3.6 ± 0.3°C in site 1 to -5.4 ± 0.3°C in sites 2 and 3 (**Figure 3**; *F*_{2,34} = 24.1, *P* < 0.0001). Similarly, FP ranged from -2.5 ± 0.3°C in site 1 to -4 ± 0.6°C in site 3 (**Figure 3**; *F*_{2,32} = 12.2, *P* < 0.001). In contrast, LT₅₀ decreased (more negative) from -20.4 ± 0.8°C in site 3 to -24 ± 0.5°C in sites 1 and 2 (**Figure 3**; *F*_{2,34} = 22.7, *P* < 0.0001). It seems noteworthy that NT were sharply higher than LT₅₀ on *C. quitensis* and *D. antarctica* at any site, suggesting that both species are able to tolerate ice formation within their leaf tissues. Thus, the

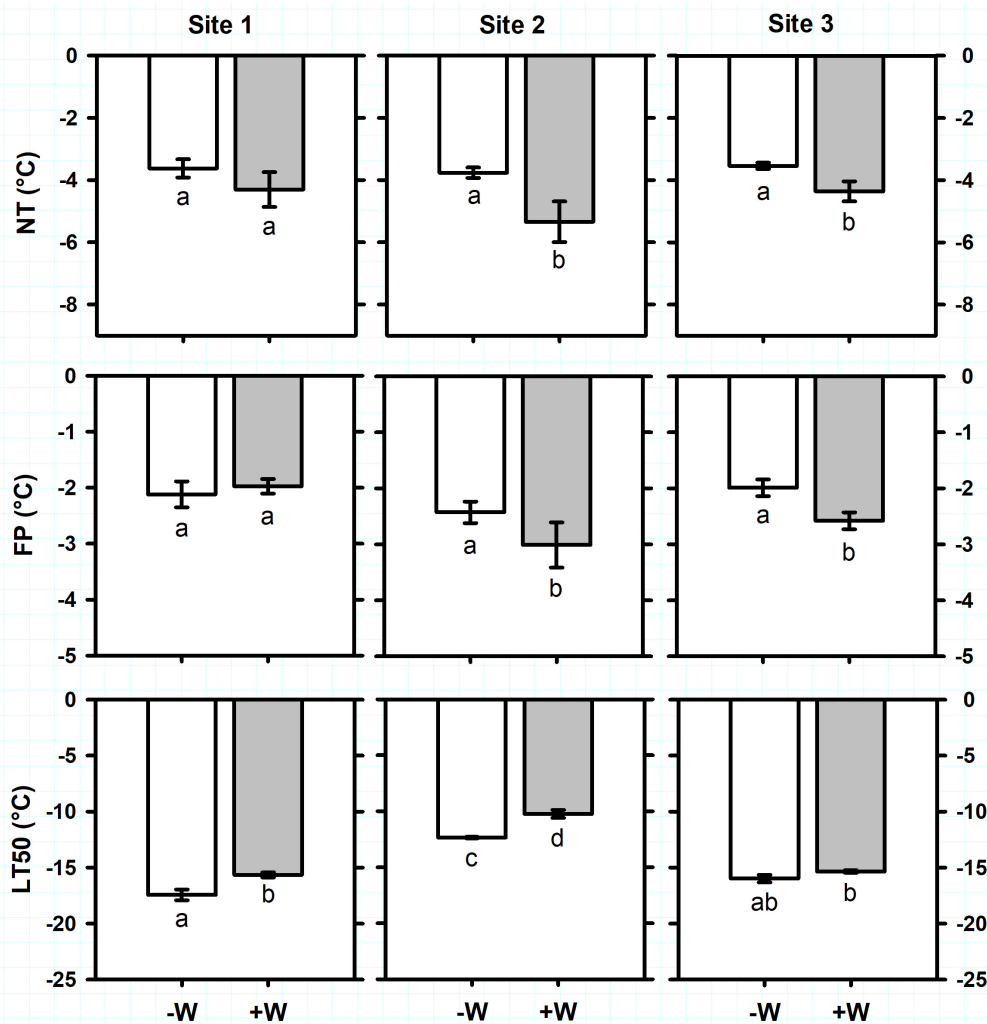


FIGURE 2 | Freezing resistance of *Colobanthus quitensis* measured in plants growing in three sites in the King George Island. Freezing parameters measured were: NT, ice nucleation temperature (°C); FP, freezing point (°C); and LT₅₀, freezing temperature producing 50% photoinactivation (°C). Values correspond to mean \pm SE ($n = 6-7$). Treatments were: -W, plants under natural temperature conditions; +W, plants under warm temperature conditions. Less negative values indicate higher freezing resistance. Significant differences between natural and warm conditions are shown as different lowercases ($P < 0.05$).

mechanism of freezing resistance did not vary with site and was freezing tolerance for both *C. quitensis* and *D. antarctica* (Supplementary Table S3).

Antarctic plants varied their vulnerability to suffer freezing damage with warming (Supplementary Table S3). For *C. quitensis*, warming (+W) increased (less negative temperature) on average 2K the LT₅₀ of plants growing in sites 1 and 2 (Figure 2; $F_{1,32} = 34$, $P < 0.0001$). In contrast, +W decreased on average 1.2K the NT ($F_{1,32} = 6.8$, $P = 0.014$) and 0.6K the FP ($F_{1,32} = 2.6$, $P = 0.012$) of plants growing at sites 2 and 3 (Figure 2). For *D. antarctica*, +W effects on freezing resistance varied with site (Figure 3). For example, LT₅₀ increased 2.8K with +W but only on plants from site 2 ($F_{2,34} = 6.7$, $P = 0.003$). In contrast, +W decreased 0.8 and 1.9 K the NT of plants growing in sites 1 and 3 ($F_{1,34} = 5.4$, $P = 0.026$), respectively, but no effects on NT were observed in plants at site

2 (Figure 3). Similarly, FP of *D. antarctica* inside +W occurred at temperatures 2K more negative than control plants but only at site 3 ($F_{1,34} = 8.4$, $P = 0.007$). Despite the opposite effects of warming on NT and LT₅₀ of Antarctic plants, both *C. quitensis* and *D. antarctica* were always classified as freezing tolerant species (Supplementary Table S3).

DISCUSSION

Sufficient levels of resistance to freezing temperatures during the summer is key for the survival, growth and reproduction of *C. quitensis* and *D. antarctica* in the Maritime Antarctica (Cavieres et al., 2016). Paradoxically, the regional warming that promote the growth and reproduction of these species (Cannone et al., 2016) could reduce their survival ability making even the

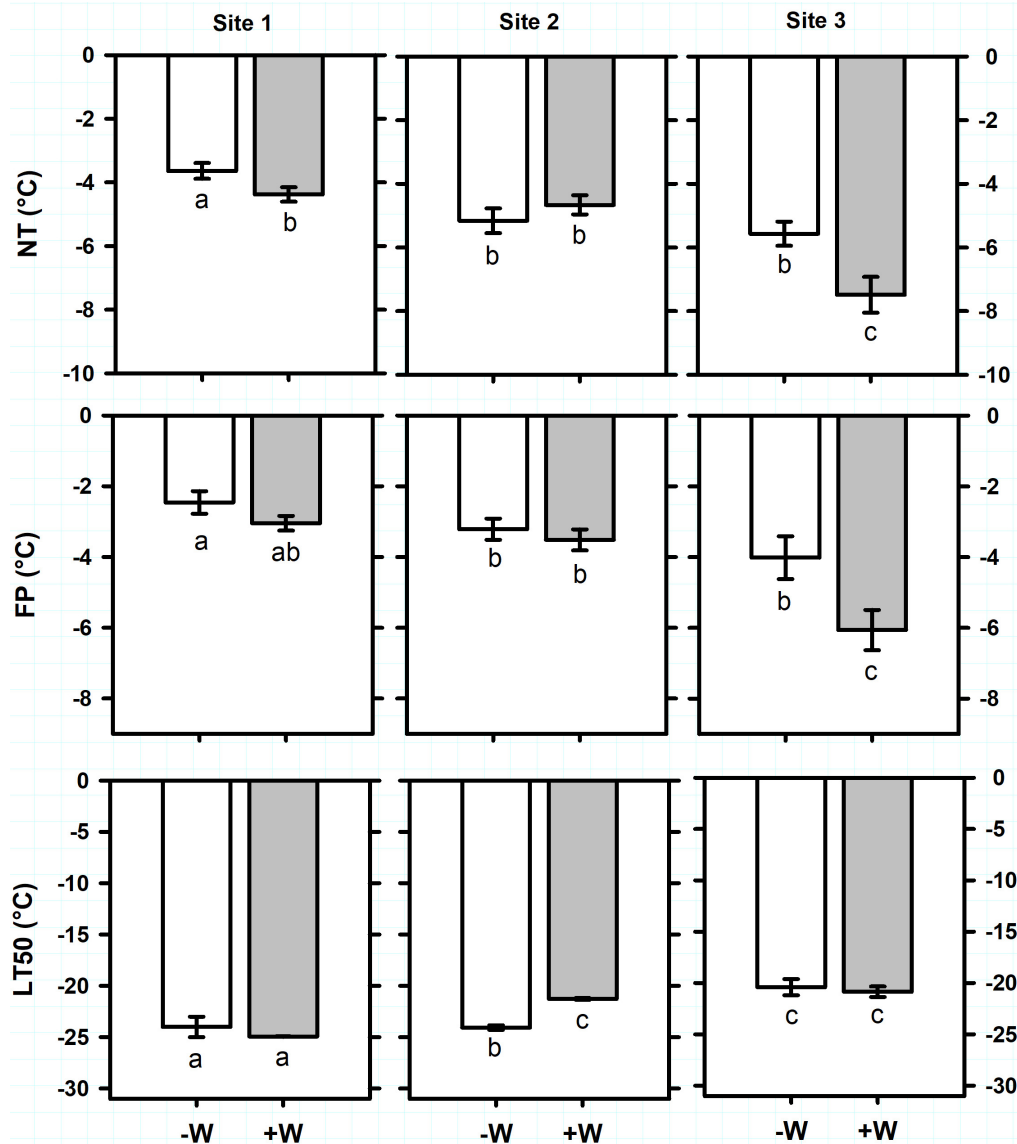


FIGURE 3 | Freezing resistance of *Deschampsia antarctica* measured in plants growing in three sites in the King George Island. Freezing parameters measured were: NT, ice nucleation temperature (°C); FP, freezing point (°C); and LT₅₀, freezing temperature producing 50% photoinactivation (°C). Values correspond to mean \pm SE ($n = 6-7$). Treatments were: -W, plants under natural temperature conditions; +W, plants under warm temperature conditions. Less negative values indicate higher freezing resistance. Significant differences between natural and warm conditions are shown as different lowercases ($P < 0.05$).

best cold adapted plants more susceptible to damage by freezing temperatures (Woldendorp et al., 2008; Ball et al., 2011). As far as we are aware, our work is the first study reporting the level and mechanisms of freezing resistance of Antarctic vascular plants measured *in situ*, and we demonstrated that both plant species exhibited a great ability to cope with freezing temperatures during the growing season. Nonetheless, increases in the temperatures experienced by plants during the growing season changed this functional trait suggesting increases in their vulnerability to suffer freezing damage under warmer temperature scenarios.

Overall, warmer conditions decreased the freezing resistance of both Antarctic species. That is, LT₅₀ occurred at higher

(less negative) temperatures in warmed plants of *C. quitensis* and *D. antarctica* (Supplementary Table S3 and Figures 2, 3). The LT₅₀ increased on average 2K for *C. quitensis* and 2.8K for *D. antarctica*, despite mean air temperatures increased only by 1K with OTCs. This suggests that further increases in ambient temperature, as those projected in future climate scenarios could lead to greater changes in this plant functional trait. Whilst LT₅₀ increases in warmer conditions were observed at all sites for *C. quitensis*, for *D. antarctica* it showed site specific responses (Supplementary Tables S2, S3). These results highlight two aspects that have to be considered. First, *C. quitensis* would be more vulnerable to freezing damage than *D. antarctica*

with warmer conditions. Several studies have reported that the Antarctic vascular plants respond differently to warmer conditions (Xiong et al., 2000; Cannone et al., 2016). For example, whereas in *D. antarctica* no effects of warming on leaf carbon gain and plant growth has been observed, in *C. quitensis* warmer temperatures promoted both plant traits (Sáez et al., 2018). Our microclimatic data showed that although freezing temperatures events were frequent and of long duration during the Antarctic summer, the intensity of those events were relatively mild (average of -2°C , with absolute records *c.* -7°C). Previous studies on the climate of the study area have reported summer freezing events of -5 and -7.8°C (Cygan, 1981; Araźny et al., 2013). However, air temperatures suddenly decrease in autumn, with some records of -13°C in April (Araźny et al., 2013). If we considered that snow duration and cover are highly unpredictable in the area because of topography and especially of wind speed (Angiel et al., 2010), Antarctic plants could be frequently exposed to such freezing temperatures during the summer–autumn transition. In that scenario, *D. antarctica* has a temperature safety margin of seldom 10K because warmed plants exhibited an averaged LT_{50} of -22.4°C . However, this safety margin doesn't exist for *C. quitensis*, which exhibited an averaged LT_{50} of -13.8°C . Although, both species have the ability to cope with summer freezing events in the area, if ambient temperatures continue to rise, as some authors propose (Lee et al., 2017), they might have negative consequences for plant survival of *C. quitensis* but not for *D. antarctica*.

Secondly, a site-dependent response of *D. antarctica* to warming was observed, where warming increased LT_{50} only in plants from site 2. This site is dominated by moss carpets and presents a permanent water-saturated but well drained substrate, abundant in organic matter and N content of 18–40 ppm (Kozeretska et al., 2010; **Supplementary Table S1**). According to substrate preferences of this species, moss carpets is where this species is more abundant and frequent (Casanova-Katny and Cavieres, 2012; Park et al., 2013), and where individual plants grow bigger (Casanova-Katny and Cavieres, 2012; Cavieres et al., 2018). Given that this site presents a greater availability of resources (i.e., water and nutrients) compared to the other sites, and where the presence of neighbors (moss carpets) can ameliorates the harsh climatic conditions (see Casanova-Katny and Cavieres, 2012; Cavieres et al., 2018), the site-dependent LT_{50} response to warming of *D. antarctica* could be attributed to the tradeoff between plant growth and stress resistance, where warmer temperatures are favoring plant allocation to growth. The absence of better soils (site 3), the presence of sea spray and animal disturbance (site 1) and the absence of moss carpets (sites 1 and 3) generate that plants on these sites are constantly dealing with stress, even under warmer conditions.

Contrary to our expectations, NT and FP occurred at lower (more negative) temperatures in leaves of warmed plants of both species. FP and NT depend on specific properties of the plant tissues and may vary according to the cell sap concentration and/or the accumulation of water-binding substances inside the cell (Sakai and Larcher, 1987). NT decreases in plant tissues with small cell sizes, relative low water content, and/or little or no intercellular space for nucleation (Sakai and Larcher, 1987).

Sáez et al. (2018) reported that *in situ* warmer temperatures induced changes in morpho-anatomical leaf traits of *C. quitensis* and *D. antarctica* that might relate with the changes in freezing resistance reported here, but further studies are needed to unveil their relation and consequences for the plant freezing resistance. In addition, several studies have reported that water-soluble carbohydrates depress FP, and their accumulation is positively related to abiotic stress survival, which is also the case of these two Antarctic plant species (Bravo et al., 2001; Pastorczyk et al., 2014). We expected that lower FP values contributed to increase the freezing resistance of Antarctic plants, by decreasing their LT_{50} . However, this was not the case. This could be related with the fact that +W plants were exposed to warmed temperatures during days but similar cold temperatures as –W plants during the nights. This has two implications. First, plants under +W may be exposed to more frequent freeze/thaw events than plants under –W. This may cause recurrent xylem embolism and cell dehydration (Pearce, 2001), which may induce the warmed plants to keep some freezing avoidance capability such as lower NT and FP than unwarmed plants. It has been observed that the degree of frost hardening may be a function of the number of freezing events (Beck et al., 2004). Second, warmer days imply better conditions for CO_2 assimilation in the +W treatment (e.g., Sáez et al., 2018) but similar respiration rates during the night on both +W or –W. Then, there is a higher probability that carbohydrates synthesized exceeded carbohydrates respired in +W than in –W treatment with the consequent higher accumulation of non-structural carbohydrate in +W, which can act as compatible solutes reducing the FP, but these putative explanations remain to be elucidated.

Previous studies have measured the freezing resistance of *C. quitensis* and/or *D. antarctica* of plants grown under controlled conditions in the lab. However, there were discrepancies in the level (i.e., LT_{50} values) and mechanism of freezing resistance (i.e., freezing avoidance or tolerance), as well as in their capacity for cold acclimation. For instance, Bravo et al. (2001) reported that *C. quitensis* avoided freezing by supercooling, that non-acclimated plants of *C. quitensis* experienced freezing injury at -4.8°C , when ice nucleation was induced by silver iodine, and its LT_{50} decreased only 1K after cold acclimation at 4°C for 21 days under the same measurement condition. In contrast, LT_{50} of *C. quitensis* decreased from -7 to -15°C with a similar cold-acclimation period without using ice nucleator according to Gianoli et al. (2004) and Reyes-Bahamonde (2013), and they classified *C. quitensis* as a freezing tolerant species (**Table 2**). In the case of *D. antarctica*, all previous studies classified it as a meanly freezing tolerant plant (**Table 3**). According to Bravo et al. (2001), cold-acclimation decreased LT_{50} from -12 to -26.6°C . However, Chew et al. (2012) found that LT_{50} of *D. antarctica* decreased from -12°C in non-acclimated to -17°C in cold-acclimated plants, whilst Reyes-Bahamonde (2013) found that this species exhibited a LT_{50} of -16.5 and -18.4°C in non- and cold-acclimated plants, respectively (**Table 3**).

Considering all sites together our results showed an average LT_{50} of -15.3 and -22.8°C for *C. quitensis* and *D. antarctica*, respectively, and that both species exhibited freezing tolerance as the mechanism of freezing resistance. In the case of *C. quitensis*

TABLE 2 | Previous studies where freezing resistance of Antarctic plants has been reported.

Reference	<i>Colobanthus quitensis</i>		<i>Deschampsia antarctica</i>		Freezing injury method
	A	NA	A	NA	
Casanova-Katny, 1997 lab	—	—	−26.4	−14.8	Photoinactivation
Field	—	—	−27	—	Photoinactivation
Bravo et al., 2001	−5.8 (FA)	−4.8 (FA)	−26.6 (FA/FT)	−12 (FA/FT)	Ion leakage
Gianoli et al., 2004	−15	−7	—	—	Plant survival
Chew et al., 2012	—	—	−17	−12	Survival and regrowth
Reyes-Bahamonde, 2013	−14.9 (FT)	−7 (FT)	−18.4 (FT)	−16.5 (FT)	Photoinactivation
This study	−15.3	—	−22.8	—	Photoinactivation

Values correspond to mean $LT_{50} \pm SE$, obtained for non-acclimated (NA) and cold acclimated plants (A) after 21 days to 2–5°C, excepting for Chew et al. (2012), which corresponded to cold acclimation period of 14 days. Freezing mechanisms are shown between brackets: Freezing Avoidance (FA) and Tolerance (FT).

our LT_{50} are similar to those previously reported for cold-acclimated plants, which is reasonable considering temperature conditions recorded in the field when the determinations were carried out. For *D. antarctica*, however, LT_{50} were relatively different from previous reports. Likely explanations for the discrepancies in the level and mechanism of freezing resistance of Antarctic vascular plants found here with those previously reported arise from methodological differences among studies. For example the method used to assess freezing injury can lead to important differences. Bravo et al. (2001) estimated LT_{50} of *C. quitensis* and *D. antarctica* from electrolyte leakage by freezing-induced cell lysis, whereas Reyes-Bahamonde (2013) LT_{50} estimations were based on photoinactivation. On the contrary, Gianoli et al. (2004) calculated LT_{50} of *C. quitensis* with plant survival percentage, and Chew et al. (2012) LT_{50} estimations of *D. antarctica* were based on tiller survival from re-growth. It has been reported that photoinactivation method agrees very well with the results obtained by methods that directly measure plant tissue damage (i.e., survival, visual assessment of freezing injuries and vital staining; Boorse et al., 1998; Neuner and Buchner, 1999). Our estimations coincided with similar LT_{50} reported for *C. quitensis* with those methods (see Table 2 for references). The electrolyte leakage method can lead to confusing results, because on one hand, it can overestimate leaf damage given that cellular solutes other than electrolytes may be induced by freezing, and on the other hand, coriaceous leaves do not release electrolytes readily, which can lead to spurious estimates of LT_{50} using this method (Boorse et al., 1998; Bannister, 2007). However, Antarctic vascular plants exhibited similar LT_{50} regardless they were calculated by ion leakage or photoinactivation methods as it is shown in the Table 3. In the case of *D. antarctica*, our LT_{50} values were intermediate compared to previous studies. Nevertheless, this result have to be taken with caution as some replicates did not reach the 50 percent damage (i.e., six replicates in the site 1). Thus, average LT_{50} in the field could be even more negative than we reported. This point is consistent with Casanova-Katny (1997) who found that the LT_{50} of *D. antarctica* was below −27°C in the field.

As mentioned, the ambient temperatures experienced by plants affect their ability to resist freezing temperatures (Beck et al., 2004). Thus, a second methodological aspect

that differed among studies and that could underlie the discrepancies in the freezing resistance of Antarctic species is the residence time and temperature used on growth chambers. For example, Bravo et al. (2001) and Gianoli et al. (2004) collected adult plants from Antarctica that were vegetative propagated at 15°C for a couple of years before the freezing determinations. Chew et al. (2012) obtained adult plants from seeds collected in the field, while Reyes-Bahamonde (2013) used plants grown at constant 11°C during 2 months after their collection in the field. However, although Bravo et al. (2001) and Gianoli et al. (2004) used similar plants residence time and growth temperature for determinations, they found different LT_{50} for *C. quitensis*. A similar situation occurred for *D. antarctica* (Table 2). Probably, multiple factors could influence the level of freezing resistance. Such discrepancies reinforce the importance of *in situ* determinations as we did here.

Finally, our results clearly showed that warmer temperatures affect the freezing resistance of Antarctic vascular plants. These results are in line with previous studies conducted in alpine and arctic plant where similar plant responses to warming (decreases in freezing resistance with warming) were reported (e.g., Loveys et al., 2006; Marchand et al., 2006; Woldendorp et al., 2008; Sierra-Almeida and Cavieres, 2010; Rixen et al., 2012). The ability to withstand freezing

TABLE 3 | A comparison of two criteria for LT_{50} determinations in *Colobanthus quitensis* and *Deschampsia antarctica*.

Species	Origin	LT_{50} Phl	LT_{50} IonL	Z	p
<i>Colobanthus quitensis</i>	Arctowski	−7.1 ± 0.5	−6.6 ± 0.4	0.94	0.345
	Punta Arenas	−6.3 ± 0.4	−6.9 ± 0.5	1.48	0.138
	La Parva	−6.7 ± 0.3	−7.3 ± 0.6	2.02	0.053
<i>Deschampsia antarctica</i>	Arctowski	−19.9 ± 1.5	−20.1 ± 1.9	0.41	0.686

Plants were collected from different localities, cultivated in growth chambers, and measured in laboratory conditions at the University of Concepción. LT_{50} values (mean ± SE, n = 5) were obtained by using two methods: Photoinactivation (Phl) and Ion Leakage (IonL). Statistical differences were assessed by using Wilcoxon matched paired tests. Followed criteria are described by Flint et al. (1967) and Bannister (2005) respectively.

temperatures is a key feature of species inhabiting cold climates, hence if new warming phases occur in the Antarctic Peninsula due to climate change, the survival of *C. quitensis* could be threatened. Whilst *D. antarctica* seems to be unaffected by warming on this trait, other aspects of its biology could be altered by warming (e.g., increases in respiration). Nevertheless, more research is needed to unveil the likely consequences of global warming on plants from cold biomes where *in situ* determinations of plant freezing resistance are crucial to understand the physiological mechanisms underlying plant adaptations to current and future climatic scenario for the Antarctic in particular.

AUTHOR CONTRIBUTIONS

AS-A conceived and designed the study, conducted the data collection, analyzed the data, and edited the manuscript. LC contributed to the study conception, designed and installed experimental setup and climatic sensors, and edited the manuscript. LB designed and installed experimental setup and climatic sensors, and contributed to the data analysis and manuscript approval.

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FUNDING

This work was supported by PIA-Conicyt under grant ART-1102; MINECOM F ICM under grant P02-005; and CONICYT under PFB-23 supporting the IEB, and Fondecyt 11150710 (AS-A) 1151173 and NEXER-UFRO (LB).

ACKNOWLEDGMENTS

We thank Oscar Viel icebreaker crew, the Chilean Army, and Arctowski Station crew for their help and logistic support during 51th Antarctic Scientific Expedition (ECA) of the Chilean Antarctic Institute (INACH). We thank Ana Sanhueza for her help with climatic data analyses and Claudia Reyes for methodological comparisons for LT₅₀ determinations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01456/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Intraspecific Trait Variation and Phenotypic Plasticity Mediate Alpine Plant Species Response to Climate Change

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OPEN ACCESS

Edited by:

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Science and Technology, China
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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 15 June 2018

Accepted: 03 October 2018

Published: 13 November 2018

Citation:

Henn JJ, Buzzard V, Enquist BJ, Halbritter AH, Klanderud K, Maitner BS, Michaletz ST, Pötsch C, Seltzer L, Telford RJ, Yang Y, Zhang L and Vandvik V (2018) Intraspecific Trait Variation and Phenotypic Plasticity Mediate Alpine Plant Species Response to Climate Change. *Front. Plant Sci.* 9:1548. doi: 10.3389/fpls.2018.01548

In a rapidly changing climate, alpine plants may persist by adapting to new conditions. However, the rate at which the climate is changing might exceed the rate of adaptation through evolutionary processes in long-lived plants. Persistence may depend on phenotypic plasticity in morphology and physiology. Here we investigated patterns of leaf trait variation including leaf area, leaf thickness, specific leaf area, leaf dry matter content, leaf nutrients (C, N, P) and isotopes ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) across an elevation gradient on Gongga Mountain, Sichuan Province, China. We quantified inter- and intra-specific trait variation and the plasticity in leaf traits of selected species to experimental warming and cooling by using a reciprocal transplantation approach. We found substantial phenotypic plasticity in most functional traits where $\delta^{15}\text{N}$, leaf area, and leaf P showed greatest plasticity. These traits did not correspond with traits with the largest amount of intraspecific variation. Plasticity in leaf functional traits tended to enable plant populations to shift their trait values toward the mean values of a transplanted plants' destination community, but only if that population started with very different trait values. These results suggest that leaf trait plasticity is an important mechanism for enabling plants to persist within communities and to better tolerate changing environmental conditions under climate change.

Keywords: functional traits, phenotypic plasticity, alpine plants, climate change, intraspecific variation

INTRODUCTION

Understanding and predicting how plants will respond to accelerating climate change is paramount for conservation and the maintenance of ecosystem function (Chapin et al., 2000; Davis et al., 2005). Ecological theory suggests that plant functional traits (characteristics related to life history strategies) should be related to the ability of a species to survive and reproduce in a given set of environmental conditions (Funk et al., 2017). For example, the leaf economic spectrum is a set of leaf traits that characterize

a continuum from fast to slow photosynthetic and tissue turnover rates (Wright et al., 2004; Reich, 2014; Díaz et al., 2016). Traits like those included in the leaf economics spectrum should be related to a species ability to persist under changing conditions. However, it has been difficult to test these predictions because variation in functional traits occurs at both inter- and intra-specific levels and can be affected by evolutionary history, environmental context, genetic constraints, and plasticity (Messier et al., 2010; Violle et al., 2012). Specifically, intraspecific trait variation can contribute approximately a quarter of the community trait variation in leaf and wood traits (Albert et al., 2010, 2012; Hulshof and Swenson, 2010; Jung et al., 2010; Messier et al., 2010; De Bello et al., 2011). This has also attracted attention as a potentially key component of community assembly (Hart et al., 2016; Des Roches et al., 2017; Hausch et al., 2018).

Intraspecific variation arises from both heritable differences and plasticity (Matesanz et al., 2012) and might be very important for adaptation in response to changing environmental conditions (Norberg et al., 2001; Björklund et al., 2009). For example, those traits that exhibit low variation in variable environments may respond slowly and lag behind shifting optimal trait values. This suggests that the relative extent of variation in a trait that is due to intraspecific variation as opposed to interspecific variation might be informative in predicting how species will respond to climate change (Dunne et al., 2003; Anderson and Gezon, 2015; Moran et al., 2015; Malyshev et al., 2016).

The distribution of traits within a community is expected to reflect variation around a mean optimal phenotype for fitness and/or growth rate (Norberg et al., 2001; Enquist et al., 2015). This idea follows from a central paradigm in ecology (Whittaker, 1972) and evolutionary biology (Levins, 1968) where observed shifts in phenotypes, species' abundances, and composition across environmental gradients reflect the arrangement of phenotypes or species that maximizes fitness in different environments. Abiotic filters such as temperature or moisture that limit successful survival strategies promote convergence of traits around this optimal local phenotype (Keddy, 1992; Weiher and Keddy, 1999; Violle et al., 2012). On the other hand, when competitive interactions are a dominant factor in species survival or environmental conditions are highly variable at a scale smaller than the study unit, there could be advantages to expressing more variable phenotypes that are not clumped at the optimal phenotype (Pacala and Tilman, 1994; Grime, 2006). However, tests of this theory have shown mixed results with some evidence for higher fitness (Lajoie and Vellend, 2018) or occurrence (Muscarella and Uriarte, 2016) when species are nearer the community trait mean while other cases showed no tendency for species in their preferred habitats to have trait values closer to the community mean (Mitchell et al., 2018). The importance of intraspecific variation in this dynamic is also unclear.

Phenotypic plasticity can play a role in the persistence of individuals under changing climate conditions (Nicotra et al., 2010) but the extent of phenotypic plasticity can be limited by ecological and evolutionary constraints (Valladares et al., 2007). If phenotypic plasticity promotes a tendency to converge on community mean traits (Ghalambor et al., 2007), it could help

predict the likely effects of climate change on plant communities. These patterns can be examined by transplanting communities and comparing phenotypes under different environmental conditions (Dunne et al., 2003; Anderson and Gezon, 2015; Guittar et al., 2016; Cui et al., 2018).

Here we use the results from a transplant experiment along an alpine elevation gradient on Gongga Mountain, Sichuan Province, China to provide a novel basis to assess how functional trait variation is structured and mediates responses to climate change. Specifically, we address three questions:

- (1) For various plant functional traits, how does the amount of intraspecific variation compare to interspecific variation across an alpine elevation gradient?
- (2) How do community functional trait distributions change across an alpine elevation gradient?
- (3) When plants are transplanted to new environments, how plastic are their traits and does trait plasticity promote divergence or convergence toward community mean trait values?

To answer these questions, we measured leaf functional traits in a reciprocal transplant experiment across an elevation gradient where a transplantation down the gradient simulates a warmer climate (+1.5°C) and transplantation up the gradient simulates a cooler climate (−1.5°C). This amount of warming is consistent with the smallest projected changes for the next 20–40 years in nearby Himalayan regions (Wu et al., 2017). We conducted both warming and cooling treatments to examine the range of possible plant responses under contrasting environmental changes. As environmental conditions are changing rapidly in alpine environments, understanding the plasticity of leaf functional traits will help improve predictions of future plant community change.

MATERIALS AND METHODS

Study Area and Experimental Setup

This study was situated in the Kang-Ding Valley of Gongga Mountain in the Hengduan Mountains in western Sichuan Province, China. The study region has a mean annual temperature of 11.6°C and mean annual precipitation of 800 mm (Fick and Hijmans, 2017). We studied plant communities at four sites along an elevation gradient: 3000 m (29.843469°, 102.034283°), 3500 m (29.86192°, 102.036°), 3800 m (29.88911°, 102.0173°), and 4130 m (29.85742°, 102.0118°) above sea level (**Supplementary Figure S1**). There is an approximately 1.5°C mean annual temperature difference between each of the sites. The vegetation at all sites is grassland characterized by a mix of subalpine and alpine species including grasses like *Festuca* spp. and *Poa* spp., sedges like *Carex* spp. and *Kobresia* spp., and forbs like *Anaphalis nepalensis*, *Clinopodium polycephalum*, and *Saussurea* spp. (Yang et al., in press). All study sites are on mountain gray-brown soil (He et al., 2005) and are grazed by yak, sheep, and horses. There is low variation in the timing of snowmelt along this gradient, as little snow falls during winter.

A whole-community turf transplant experiment was established at these sites in 2012 when exclosures were erected to prevent grazing (**Supplementary Figure S1**). Each transplanted turf measured 25 cm × 25 cm and were excavated to 20 cm depth or to where the soil ended, if that was shallower than 20 cm as was the case in the highest site. To control for the effect of transplantation, turfs were transplanted in the same site of origin. No differences were found between transplanted and untransplanted control plots, so both plot types were used as control plots in this study. Transplanted turfs were blocked such that each turf came from close to the control plots in the same block. In total, there were seven replicate turfs for each type of treatment and control at each site (except the highest site, where there are five replicates of each plot type, totaling of 92 plots used in this project). The local plant community composition and biomass was recorded in a separate set of 20, 50 cm × 50 cm plots (except for the highest site, where 13 plots were measured) at each site (for a total of 73 plots). These plots were used for characterizing the unmanipulated community composition at each site and are separate from the experimental transplant plots because biomass of each species was measured destructively in 2015. These community surveys were also used to characterize the regional species pool for partitioning of interspecific and intraspecific trait variation.

Community Description

We collected leaves from the most common species in the plant community at each of the four sites in August 2015 and 2016. Leaves ($n = 2,873$) were collected from 164 species outside of the experimental plots and we aimed to collect one healthy, fully expanded leaf from up to five individuals for each species at each site where they occurred. However, this was not possible for all species at all sites. To avoid sampling clones, we selected individuals that were visibly separated from other stems of that species. Most species were sampled in only 1 year and species sampled over both years were unlikely to be from the same individuals, as the area from which we sampled was large. Numbers of leaves per site varied from 533 to 850 ($L = 809$, $M = 867$, $A = 664$, $H = 533$). All these leaves were used to assess leaf structural trait variance partitioning (LA, LT, SLA, and LDMC) while a subset of 209 observations from all sites combined were used to assess leaf nutrient and isotope variance partitioning. Between 57 and 85% of the biomass in the 50 cm × 50 cm plots used for community composition consisted of species with trait data, with between 97 and 100% of the biomass at each site comprised by genera with trait data (**Supplementary Table S1**). All taxa names were standardized using the Taxonomic Name Resolution Service (Boyle et al., 2013).

Intraspecific Variation

Ten of the most common species along the gradient were selected for sampling in experimental plots during August 2016. These include *Artemisia flaccida*, *Epilobium fangii*, *Geranium pylzowianum*, *Hypericum wightianum*, *Pedicularis davidii*, *Persicaria vivipara*, *Plantago asiatica*, *Potentilla leuconota*, *Veronica szechuanica*, and *Viola biflora* var. *rockiana*. Of these

species, only *P. leuconota* and *V. szechuanica* were present across the whole elevation gradient, so all other species were found in transplanted turfs outside of the elevation range where they are common. These species were selected to avoid species that readily spread clonally to prevent measuring individuals that did not originally occur on the experimental turf. Because many species in this system display at least some clonal reproduction and distinguishing genetic individuals is impossible without destructive sampling, we worked at the ramet level in each plot, as in Cui et al. (2018). We only selected species that were present in turfs prior to transplantation and that had remained present in transplanted turfs. For these species, up to five healthy, fully expanded leaves were collected from individuals in each experimental plot (i.e., control, locally transplanted control, warmed, and cooled) where that species occurred. A total of 2,246 leaves were collected and measured with 112–350 individuals per species to assess intraspecific variation for these 10 species.

Functional Trait Measurements

We measured 11 functional traits related to potential physiological rates and environmental tolerance of plants. These include leaf area (LA, cm²), leaf thickness (LT, mm), leaf dry matter content (LDMC, g/g), specific leaf area (SLA, cm²/g), carbon (C, %), nitrogen (N, %), phosphorus (P, %), carbon:nitrogen (C:N), nitrogen:phosphorus (N:P), carbon13 isotope ratio ($\delta^{13}\text{C}$, ‰), and nitrogen15 isotope ratio ($\delta^{15}\text{N}$, ‰). Measurements were made based on standardized protocols from Pérez-Harguindeguy et al. (2013).

All leaves for trait measurements were collected and stored in plastic bags and coolers in the field before transport to the lab. At the lab, leaves were measured for leaf area, leaf thickness, and fresh mass. Leaf area was measured on Canon LiDE 220 scanners at 300 dpi. Following scanning, ImageJ (Schneider et al., 2012) and LeafArea package were used to calculate leaf area (Katabuchi, 2017). Leaf thickness was measured using calipers at three random locations on each leaf and the average taken for further analysis. Fresh mass was measured on a balance within 24 h of collecting leaves. Leaves were then dried for at least 72 h at 65°C before dry mass was measured. A subset of leaves was then ground into a fine powder and analyzed for nutrients and isotopes including P, N, C, $\delta^{15}\text{N}$, and $\delta^{13}\text{C}$ at The University of Arizona. Total phosphorus concentration was determined using persulfate oxidation followed by the acid molybdate method (APHA, 1992). Phosphorus concentration was then measured colorimetrically with a spectrophotometer (ThermoScientific Genesys20, United States). Carbon, nitrogen, and their stable isotope ratios were measured by the Department of Geosciences Environmental Isotope Laboratory at The University of Arizona on a continuous-flow gas-ratio mass spectrometer (Finnigan Delta PlusXL) along with an elemental analyzer (Costech). Samples of 1.0 ± 0.2 mg were combusted and standardization was based on acetanilide for N and C concentration, NBS-22 and USGS-24 for $\delta^{13}\text{C}$, and IAEA-N-1 and IAEA-N-2 for $\delta^{15}\text{N}$. Ratios between C:N and N:P were also calculated and analyzed. Prior to analysis, samples with apparent measurement errors that resulted in unrealistic trait values were removed. This included leaves with

leaf dry matter values higher than 1 g/g, leaves with specific leaf area values less than 5 cm²/g or greater than 500 cm²/g and leaf nitrogen values higher than 6.4%. The nitrogen cutoff values was chosen based on the highest published leaf nitrogen values found in the Botanical Information and Ecology Network (Enquist et al., 2009) for the genera in our study.

Analyses

To quantify the extent of intraspecific vs. interspecific variation of leaf traits along the elevation gradient, we performed a variance partitioning analysis. This analysis assesses the variation in traits at different taxonomic levels (i.e., within-species, species, genus, family, and order) and between sites (populations) across the elevation gradient. We log transformed the data for the multiplicative growth traits (i.e., LA, LDMC, and LT) of all non-experimental leaves and performed a nested ANOVA using the *lme* (Bates et al., 2015) and *varcomp* (Qu, 2017) functions in R (Messier et al., 2010). For each level, the function first calculates the group mean. It then compares the variance around the group mean to the mean of the next level (e.g., variance of genus level is compared to the mean of family level). We used taxonomy as a substitute for phylogeny in the analysis. As a result, trait variance may be influenced by the loss of information about the ages of species, genera, families, and orders in relation to each other.

To describe community trait distributions at our sites, biomass-weighted community trait distributions were calculated for each site using non-parametric bootstrapping (Enquist et al., 2015, 2017). At each site we calculated 1000 replicate distributions. For each replicate, trait data for each species within the site was randomly sampled with replacement from the set of available trait data for that species. Trait data was not available for each species at each site, and so we prioritized trait data to use as follows: (1) focal species data from unmanipulated conditions at the focal site, (2) focal genus data from unmanipulated conditions at the focal site, (3) focal species data from any unmanipulated conditions, and (4) focal genera data from any unmanipulated conditions. When congener trait data were used as a proxy for focal species trait data, we randomly rarefied this data to ensure that each congener was equally likely to be sampled.

To examine phenotypic plasticity, we took the average trait value of each species in each plot to make comparisons between the trait values of plants in plots that had been transplanted with plants in plots that hadn't been moved. We only compared plots from the same block to reduce variation due to microtopographic and microclimatic variation at each site. First, we assessed if the experimental treatment influenced the relative plasticity of each trait, P_R by calculating the extent to which the traits of the plants in transplanted plots (T) changed with respect to plants in plots in their original home (H). This is a simplified Relative Distance Plasticity Index (Valladares et al., 2006), which is useful for comparing plasticity of traits or species under different environmental conditions and was done by calculating:

$$P_R = |(H - T)| / H$$

where H is the mean trait value of all individuals within the home control plot and T is the mean trait value of all individuals within the transplanted turf. The absolute value of the numerator is taken to standardize all potential trait shifts while the absolute

value of the whole quotient is to standardize trait values that are negative ($\delta^{13}\text{C}$). Low values of P_R indicate that the observed intraspecific variation in each trait in an experimental plot shows no change in trait mean relative to the home populations. In contrast, high values of P_R indicate a change in intraspecific mean trait value induced by the experiment. We calculated P_R for each species where we had paired observations between their home (H) and transplanted (T) site. We tested whether traits differ in their response to transplantation by using linear mixed effect models where P_R was modeled as a function of trait, transplant type (warming vs. cooling), and their interaction with species and site as random effects to account for multiple samples from each species and site. We used the *lmer* function with Satterthwaite estimations for degrees of freedom for hypothesis testing from the *lmerTest* R package (Kuznetsova et al., 2017).

Next, to assess whether transplanted plants shifted their functional traits toward the community mean of their transplant community, we assigned observations to several binary groups including:

- (1) Whether the trait value of plants converged on, or diverged from, the destination site community trait means after transplantation. “**Converging**” corresponds to plants whose trait values moved closer to their transplantation destination community trait mean after transplantation. In contrast, “**Diverging**” corresponds to plants whose trait values moved further from their destination community trait mean after transplantation.
- (2) Whether the transplant was to higher or lower temperatures. “**Warming**” corresponds to transplants to higher temperatures (+ ~1.5°C) and “**Cooling**” corresponds to transplants to lower temperatures (– ~1.5°C).
- (3) Whether the trait values of plants were closer to their home community trait mean or the transplant destination community trait mean prior to transplantation. “**Home**” corresponds to plants who started with trait values closer to their home community mean value (further from their destination mean trait value) and “**Destination**” corresponds to plants who started with trait values closer to the transplant destination community mean value (further from their home mean trait value).

We used log-likelihood ratio tests (G-tests, Signorell and Al et mult, 2018) to determine whether the proportion of comparisons converging or diverging (category 1 above) is dependent on transplant type (category 2 above) and whether the trait value started closer to the home or destination community values (category 3 above) for all traits together. We also determined whether transplantation induced a significant response in trait values by calculating the 99% confidence interval assuming a t-distribution around the mean value of each trait for each species under unmanipulated conditions at each site. If the mean value of a species trait value in a transplanted site fell outside of the 99% confidence interval from its home site, then it was considered a significant plastic response at the $p < 0.01$ level. All analyses were conducted in R 3.4.2 (R Core Team, 2017).

RESULTS

Intraspecific Variation

The variance partitioning analysis shows that nitrogen-related leaf traits (except for $\delta^{15}\text{N}$) tended to have very high intraspecific variation with >75% of variation being found within species or sites (**Figure 1**). A set of other traits including specific leaf area, leaf dry matter content, leaf thickness, %P, and isotope ratios had intermediate intraspecific variation, ranging from 25 to 40% of variation. Finally, %C and leaf area had less than 25% of their variation at the intraspecific level (**Figure 1**).

Traits Along Gradient

All traits varied between sites across the elevation gradient, but few had directional shifts along the elevation gradient (**Figure 2**). While there is substantial overlap in trait variation in many of the traits across the elevation gradient (overlap in the confidence intervals for all traits between sites, indicating few significant differences in mean trait values between elevations) there are some consistent shifts in the community biomass-weighted mean trait values with elevation.

Phenotypic Plasticity

Phenotypic plasticity varied substantially by trait where $\delta^{15}\text{N}$, leaf area, and %P were most plastic while C and $\delta^{13}\text{C}$ were least plastic in response to transplantation (**Figure 3**). For three traits, the amount of phenotypic plasticity varied by transplant treatment, where C:N ($\text{df} = 704.6$, $t = 2.41$, $p = 0.016$), leaf area ($\text{df} = 708.4$, $t = 1.90$, $p = 0.058$), and leaf dry matter content ($\text{df} = 708.4$, $t = 2.56$, $p = 0.011$) all tended to change more when transplanted to cooler locations compared to warmer locations (**Figure 3**). Species and origin site random effects both had significant variance (species: $\chi^2 = 13.9$, $\text{df} = 1$, $p < 0.001$; origin: $\chi^2 = 10.3$, $\text{df} = 1$, $p = 0.001$) indicating

that phenotypic plasticity varied by species and by origin site.

Slightly more transplants showed divergence in their trait value from the destination community mean after transplantation ($n = 279$) than transplants that showed convergence toward their destination community mean ($n = 277$) while 180 transplants did not significantly change (**Figure 4**). Additionally, slightly more transplants had functional trait values closer to their home community prior to transplantation ($n = 400$) compared to transplants where functional trait values were closer to their destination community prior to transplantation ($n = 336$). When combined, individuals were more likely to converge with their destination community trait mean if they started further away from their destination community mean (**Figure 4**, $G = 36.54$, $\text{df} = 2$, $p < 0.001$). Warming transplants were slightly more likely to result in convergence while cooling transplants were slightly more likely to result in divergence but this was not a significant difference ($G = 2.49$, $\text{df} = 2$, $p = 0.29$). Between traits, the proportions of individuals converging and diverging varied substantially and this depended on both the type of transplant (warming or cooling) and where the individual trait value started relative to their destination trait community mean (**Supplementary Figure S2**). Foliar %C showed consistently high rates of convergence under all conditions except when transplanted plants were warmed and they started closer to their home trait values. Those traits that showed greatest convergence did not tend to be those traits that varied more in community biomass-weighted mean values along the elevation gradient.

DISCUSSION

Phenotypic plasticity will likely be a very important way for organisms to tolerate changing future climate conditions

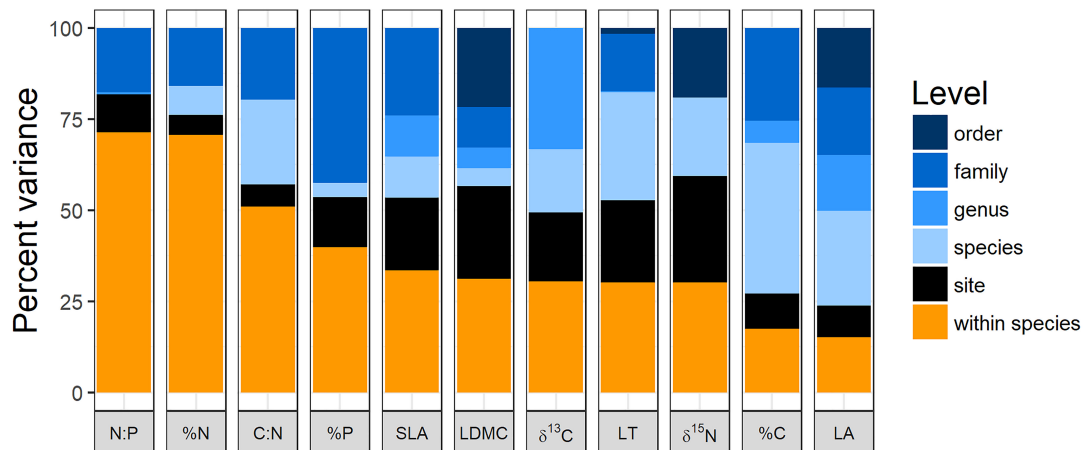
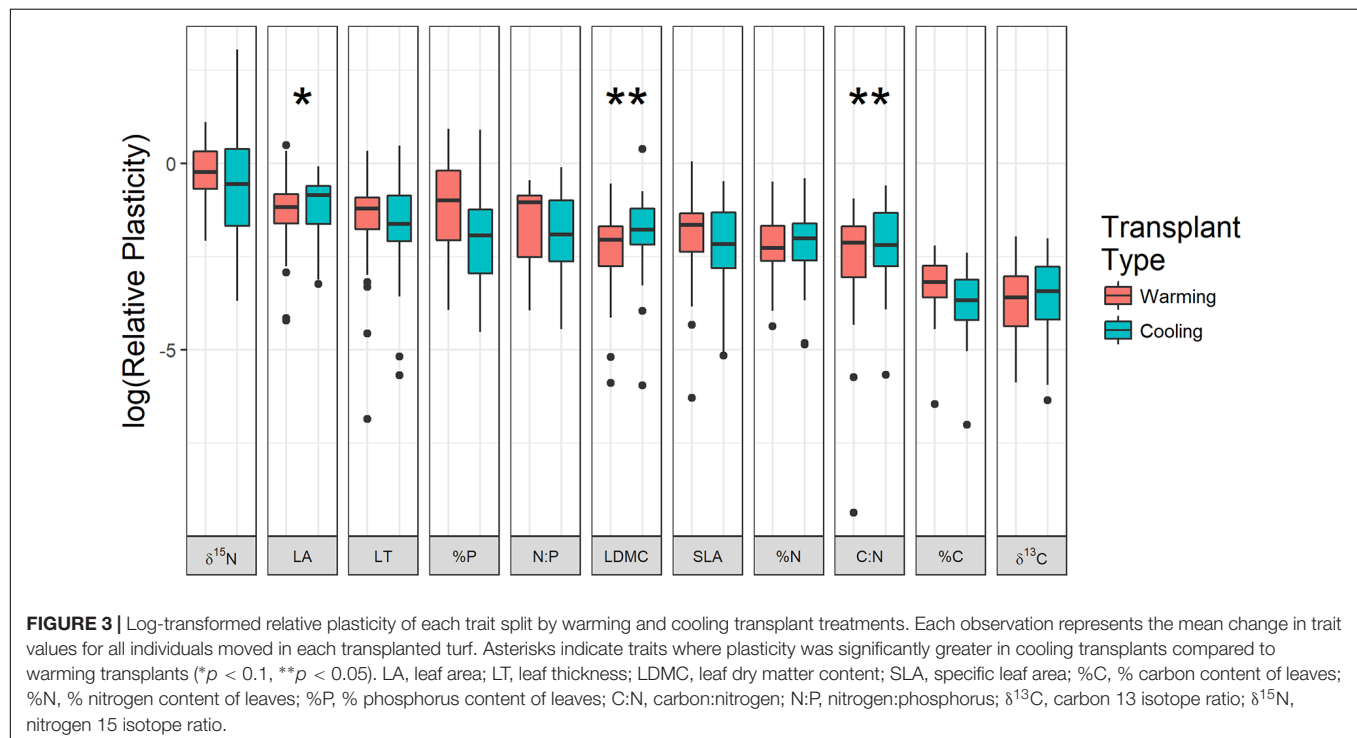
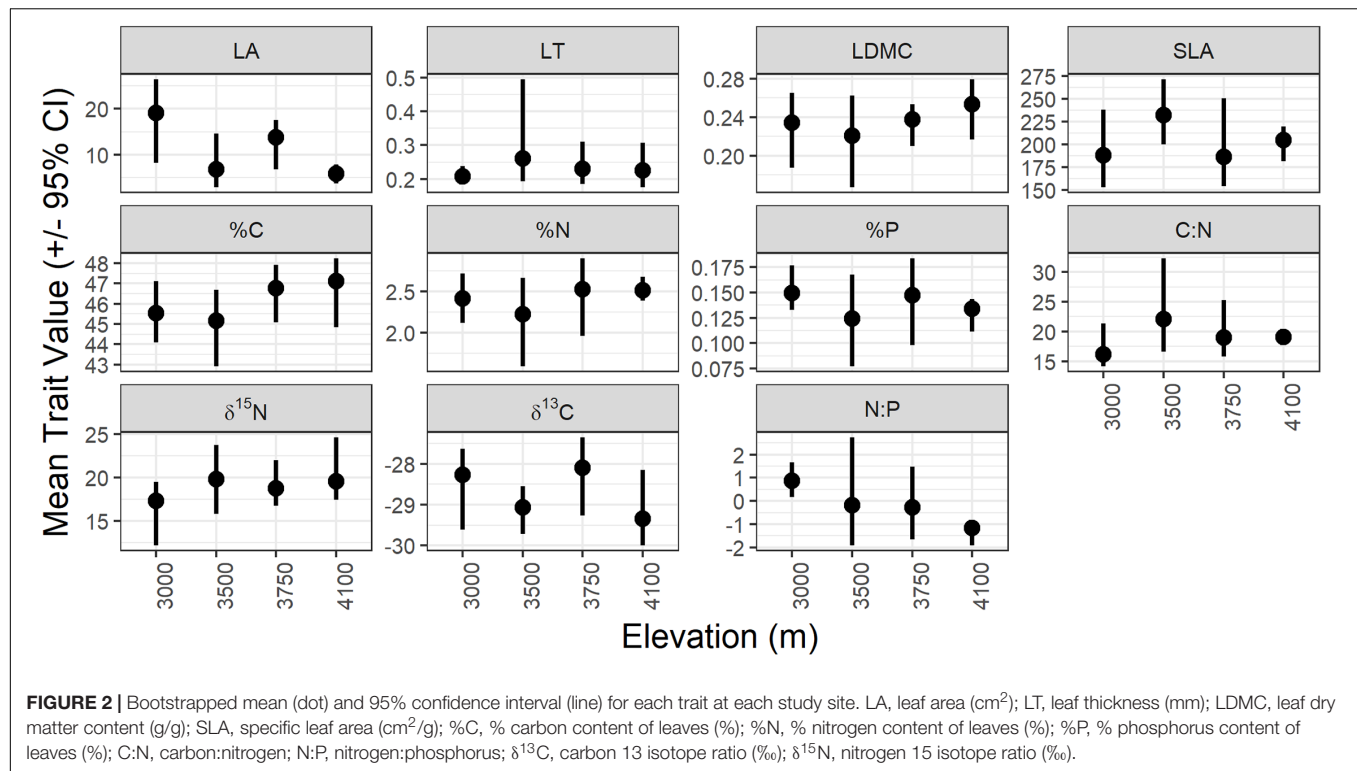


FIGURE 1 | Variance partitioning of leaf traits at different taxonomic levels and between populations across sites along the elevation gradient. LA, leaf area; LT, leaf thickness; LDMC, leaf dry matter content; SLA, specific leaf area; %C, % carbon content of leaves; %N, % nitrogen content of leaves; %P, % phosphorus content of leaves; C:N, carbon:nitrogen; N:P, nitrogen:phosphorus; $\delta^{13}\text{C}$, carbon 13 isotope ratio; $\delta^{15}\text{N}$, nitrogen 15 isotope ratio. Data for LDMC, LT, and LA were log transformed prior to analysis.



(Nicotra et al., 2010; Valladares et al., 2014). The combination of a transplant experiment and an elevation gradient have allowed us to examine the extent and directionality of phenotypic plasticity and intraspecific trait variation under simulated climate change.

This experiment reveals that all traits studied showed some level of plasticity in response to changing climate and a general trend of convergence toward the trait mean of their new community when that mean was far from their home trait value.

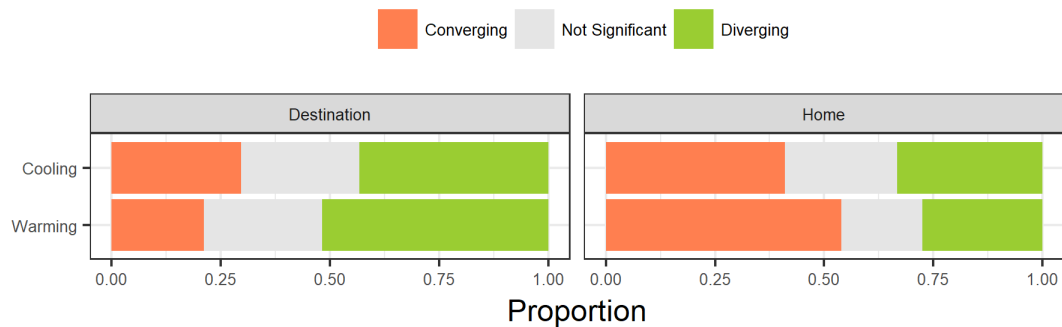


FIGURE 4 | Proportions of observations where transplanted plant functional trait values converged or diverged relative to community mean trait values in transplant destination communities. Rows correspond to warming and cooling treatments. Columns correspond to whether the trait value of a species was closer to its **destination** community trait mean or the **home** community trait mean prior to transplantation. Gray colors indicate comparisons where trait values of transplanted plants fell within the 99% confidence interval of the trait values for untransplanted plants in their home (no significant difference, i.e., no plastic response).

Intraspecific Variation and Phenotypic Plasticity

There was little evidence that traits that have more intraspecific variation at the regional scale also have greater plasticity in response to climate manipulations. The structural leaf trait with most intraspecific variation (SLA) showed moderate plasticity while the trait with lowest intraspecific variation (leaf area) showed high plasticity (Figures 1, 3). Nitrogen-related traits with high intraspecific variation also tended to have moderate plasticity. Trait variation in the species pool involves long-term community assembly processes that are generating or constraining variation over long time-scales and is likely subject to different constraints compared to phenotypic plasticity (Zobel, 1992). On these long time-scales there are costs and benefits to having high intraspecific variation (Valladares et al., 2007). Our measure of phenotypic plasticity, on the other hand, is a response to rapid change on the scale of years where only the individuals who can respond quickly can survive. Second, there are only certain traits that are likely to respond to the types of environmental change imposed on the transplanted plants in our study. For example, with decreasing mean temperature, there was an overall shift toward dominance by more conservative strategies (lower leaf area, higher thickness and leaf dry matter content, increased %C content, and lower %N). Further, colder sites are more dominated by plants with lower foliar N:P and $\delta^{15}\text{N}$. Specific leaf area, on the other hand, showed no directional change along the gradient, indicating that other factors than temperature are the most important determinants of optimal SLA values. These factors could include other abiotic conditions like differences in growing season length or biotic conditions like differences in soil microbe communities between sites (Dunne et al., 2003; Tomiolo et al., 2015). Finally, our calculations of inter- versus intra-specific trait variation did not include measurements from transplanted individuals because only a subset of species were transplanted. If these transplanted individuals were included, we would expect much higher measures of intraspecific variation, as phenotypic plasticity can substantially weaken

phylogenetic signal in functional traits (Burns and Strauss, 2012).

Extent of Phenotypic Plasticity

The functional traits that showed the most plasticity, including leaf area and leaf thickness, are both related to leaf lifespan, where thicker and smaller leaves have higher leaf mass per area and are thus more likely to have longer leaf lifespans and more conservative strategies (Wright et al., 2004; Reich, 2014). The observed plasticity in these traits could be driven by the imposition of additional stress when transplanted to colder climates, or the release of that stress when transplanted to warmer climates, as there is some tendency toward more acquisitive leaf strategies at lower elevations (Cornwell and Ackerly, 2009; Read et al., 2014). However, we found that the extent and importance of plasticity in traits varies by species. This is similar to Cui et al. (2018), who found that *Viola biflora* var. *rockiana* had low plasticity in leaf area in response to transplantation in the same study system. Plasticity in leaf P is likely to be more related to P availability in the soil along the elevation gradient. Leaf P may be less genetically controlled compared to environmentally controlled by access to that nutrient so movement to new places results in different P availability. $\delta^{15}\text{N}$ also had high plasticity, potentially for similar reasons to %P. Variation in foliar $\delta^{15}\text{N}$ has been linked to variation in soil N supply and nitrogen sources are primarily determined by local differences in N fixation, uptake, and outside sources (Craine et al., 2015) which could vary substantially between sites. Carbon-related traits (%C and $\delta^{13}\text{C}$) showed the lowest degree of plasticity in response to transplantation. This indicates that these traits might be more genetically controlled with little response to the environmental conditions in this experiment. Little variation in $\delta^{13}\text{C}$ suggests that moisture conditions do not vary substantially between transplant sites, resulting in little change in isotope ratios (or water use efficiency) between sites.

It is important to note that while we refer to trait shifts with transplantation as plastic, our comparisons rely on the assumption that transplanted plants started with similar phenotypes to the other plants at the site where they originated.

This means that we cannot rule out the importance of maternal effects and epigenetic inheritance. Additionally, the observed phenotypic plasticity in this study may or may not be adaptive and the extent to which phenotypic plasticity is adaptive is difficult to quantify (van Kleunen and Fischer, 2005; Ghalambor et al., 2007).

Trait Convergence and Divergence

According to theory (Enquist et al., 2015), phenotypic plasticity may be adaptive if it results in movement toward a trait optimum that is adaptive for a specific environment. We did find evidence that transplanting to a new community results in a plastic shift in trait values toward the new community dominant trait value. This was especially true when the transplanted species differed more from the members of its new community before transplantation. This central tendency is consistent with the results found by Muscarella and Uriarte (2016) and Lajoie and Vellend (2018). However, when the difference between the transplanted species and the community mean trait value was small, phenotypic plasticity tended to result in divergence from the community mean. This could be a signature of biotic interactions where occupying space away from the mean trait value could be advantageous. On average, convergence was more common when transplants were moved to warmer conditions, especially when those plants started far from their destination community. This could be because competition is limiting the extent of higher elevation species at lower elevations and that this filter is stronger than the lower temperatures at higher elevations. Species moving to lower elevations can only survive if they can express the most competitive phenotype under these warming conditions.

Observations of convergence and divergence as plastic response to climate change could be due to other factors that we could not measure in this study. For example, phenotypic plasticity can be expressed as a result in interaction with new neighbors (Lipowsky et al., 2015; Abakumova et al., 2016) and transplanted plants were exposed to changing communities through time (Yang et al., in press). Our study also did not fully address the potential for fine-scale niche partitioning within communities. Such small-scale processes may be an important mechanism for the maintenance of local functional diversity (Stark et al., 2017). For example, microclimatic environmental conditions can vary within sites including soil depth, chemistry, water availability, light variation, and exposure to sun. Further, we did not assess multivariate shifts in traits between populations which may also better reveal the multivariate nature of community assembly (see Kraft et al., 2015). As climate continues to change, the limits of this plasticity will be important to consider, but this study demonstrates the importance of considering both a species' traits and the plasticity in those traits when considering their ability to tolerate climate change.

If environmental controls on community assembly were strong along the gradient we would predict more consistent patterns of trait convergence toward community means. In our study site, like many other gradient studies (Wright et al., 2004; Muscarella and Uriarte, 2016), there is substantial overlap between mean trait values from site to site in most trait values

(Figure 2). If there were substantial or directional trait differences between each site, those difference are likely to be driven mostly by species turnover. Under that scenario, transplantation would likely result in death, and only the most extreme plastic responses would promote survival. Since we are only working with species who have survived in their new conditions after transplantation for 5 years, all the intraspecific variation and plastic responses represent relatively successful strategies. We are not able to assess whether the individuals that did not survive had lower phenotypic plasticity or different tendencies than the winners reported here, but Guittar et al. (2016) found that community trait values responded to transplantation by converging toward local trait values over time as species composition changed.

CONCLUSION

A trait-based approach to community ecology is providing valuable insight into both the physiological mechanisms underpinning species' broad-scale geographical distributions and patterns of local diversity (McGill et al., 2006). Indeed, resolving patterns of trait–environment relationships and intra- and interspecific trait variation is critical for developing predictive models in community ecology (Laughlin et al., 2012; Violle et al., 2012). The ability of a species to adjust its phenotype as the climate changes rapidly will be very important in plant persistence under new conditions. This is especially important in cold biomes, where climate is changing most rapidly.

We assessed if the traits that are more variable will respond more quickly to environmental change. Our results show that a variety of alpine species had substantial phenotypic plasticity, although this plasticity was not necessarily related to intraspecific variation of these traits. Traits with high intraspecific variation did not correspond to traits that showed the highest plasticity in response to transplantation. We also assessed if patterns of intraspecific variation supported either community ecology models of intraspecific trait convergence or divergence. On the one hand, our results provide support of the community-weighted mean optimality hypothesis and support the assumption often made in community ecology that trends in intraspecific trait variation tend to mirror trends in interspecific variation (Muscarella and Uriarte, 2016). Specifically, when species are moved into new climates, traits tend to shift toward local optima. This suggests that there is some advantage to adopting a similar phenotype to other species, but only if a plants' phenotype started different to the members of its new community. On the other hand, our results also support trait divergence hypotheses (Pacala and Tilman, 1994; Valladares et al., 2007), but only if traits from the transplanted population are already close to the new community mean trait value. These seemingly contradictory results indicate that hypotheses of trait convergence and divergence may not be mutually exclusive and instead are dependent on the context of the underlying processes (Grime, 2006). Together, our results indicate that trait plasticity is an important mechanism for enabling plant populations to persist within communities and to better tolerate changing environmental conditions under climate change.

AUTHOR CONTRIBUTIONS

VV and KK designed the transplant experiment. YY secured funding and implemented the experiment. VB, JH, BE, KK, CP, SM, VV, and YY designed and implemented the field sampling design. BM, KK, JH, LS, CP, and LZ collected plant trait data. LZ collected community composition and biomass data. AH, CP, and RT cleaned and processed the data. BM, JH, and LS conducted analyses. JH wrote the manuscript with input from BE, BM, and VV. All authors have commented on and approved the final manuscript. Author order is first-last-emphasis with alphabetical order in the middle.

FUNDING

This research was supported by the Alpine Ecosystem Observation and Experiment Station of Mt. Gongga, CAS for providing accommodation and lab space. This study was funded by the Foundation of Key Laboratory of Mountain Surface Processes and Ecological Regulation, CAS; Key Research Program of Frontier Sciences, CAS (Grant No. QYZDJ-SSW-DQC006), the 135 Strategic Program of the Institute of Mountain Hazards and Environment (Grant No. SDS-135-1707) and the

Norwegian Center for International Cooperation in Education (SIU) projects UTF-2013/10074 and HNP-2015/10037. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program (Grant No. DGE-1747503) with additional support from the Graduate School and the Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin-Madison, Wisconsin Alumni Research Foundation, and the Integrative Biology Department John Jefferson Davis Travel Awards.

ACKNOWLEDGMENTS

We are grateful for the assistance of the Plant Functional Traits Course 1 and 2 members, specifically F. Knoop, and L. Nordås, for field work help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01548/full#supplementary-material>

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- The reviewer CD declared a shared affiliation, though no other collaboration, with one of the authors SM to the handling Editor.
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Plastic Population Effects and Conservative Leaf Traits in a Reciprocal Transplant Experiment Simulating Climate Warming in the Himalayas

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OPEN ACCESS

Edited by:

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Zhejiang Agriculture and Forestry
University, China

Reviewed by:

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 02 February 2018

Accepted: 02 July 2018

Published: 30 July 2018

Citation:

Cui H, Töpper JP, Yang Y, Vandvik V
and Wang G (2018) Plastic Population
Effects and Conservative Leaf Traits
in a Reciprocal Transplant Experiment
Simulating Climate Warming
in the Himalayas.
Front. Plant Sci. 9:1069.
doi: 10.3389/fpls.2018.01069

Climate warming poses considerable challenges for alpine plant species, especially for competitively inferior ones with resource-conservative adaptations to cold climates. The Himalayas are warming at rates considerably faster than the global average, so it is particularly important to assess how and through which mechanisms alpine plant species are affected there. We employed a demographic approach in a climate change experiment, where vegetation turfs were transplanted reciprocally between the central parts of the study species' (*Viola biflora* L. var. *rockiana*) range and the warmer range margin, with a temperature difference of ca. 1°C. In addition, turfs were also transplanted outside the range to warmer habitats, simulating two different scenarios of climate warming, +1 and +4°C. Transplanting to warmer sites negatively impacted population growth rates (λ), survival and clonality, but did not affect growth and fecundity, while the productivity of the plant community increased. The reciprocal transplants to the colder habitat showed the opposite effects, for both *V. biflora* and the plant community, indicating plastic responses of the study species, driven by changes in plant-plant competition. However, the leaf traits underlying the modeled population growth rates were origin-site specific and not affected by the climate-change treatments over the study period, suggesting local adaptation of growth form to competition in the warmer range margin, and to climate adversity in the colder range center. The transplants outside the present species' range showed consistently stronger reductions in population growth rate and survival, with mortality of 90–100% in the +4°C treatment. This illustrates that climatic changes beyond species' present climatic ranges pose a serious risk for range contraction and extinction for Himalayan alpine species in the near future. As *V. biflora* seems mostly limited by competition under warming, its persistence in a future climate may become increasingly dependent on keeping competitive effects from the surrounding community low, for instance by management interventions like grazing and mowing.

Keywords: climate change, alpine, reciprocal transplant experiment, altitudinal gradient, population growth rate, vital rate, *Viola biflora* var. *rockiana*, leaf traits

INTRODUCTION

Under global climate warming, especially alpine plant species with narrow ranges and locally adapted populations face a considerable risk of extinction (Holt, 1990; Theurillat and Guisan, 2001; Thomas et al., 2004). In contrast, in species where responses to climatic variability are plastic there may be time for evolutionary changes (Jump and Peñuelas, 2005). Thus, it is important to consider local adaptation and phenotypic plasticity of plant populations when estimating their vulnerability to climate warming (Matesanz et al., 2010; Nicotra et al., 2010). A powerful tool for studying local adaptation and phenotypic plasticity is the reciprocal transplant approach, where individuals of a focal species are transplanted reciprocally between two contrasting habitats within the species' range. In such set-ups, opposed outcomes of the two transplant directions (i.e., negative effects in one transplant direction, positive effects in the other) indicate plasticity, whereas negative effects in both transplant directions indicate some degree of local adaptation (Claussen et al., 1940; Reznick and Travis, 1996; Kawecki and Ebert, 2004).

Environmental variation, and hence also experimental alteration, may affect various vital rates in a species' life cycle, from germination (Levine et al., 2008) and survival probability (Simons et al., 2010), to flowering dynamics (Inouye et al., 2002; Pfeifer et al., 2006). Moreover, climatic effects on different vital rates often differ and may even be in opposed directions (Hutchings, 2010; Nicolé et al., 2011), so that even in cases where the overall population growth rate is not affected by climatic change, there may thus be strong effects on the underlying responses in vital rates. Detailed demographic analyses may therefore yield valuable information on environmental change impacts on plants beyond the directly observable effects on population size and abundance.

In this paper, we combine a reciprocal transplant experiment along an altitudinal gradient with a demographic study on a small alpine forb to investigate potential future responses of a typical alpine species under climate warming in the Gongga mountains in China. Vegetation turfs were transplanted from the species' range center to its lower altitudinal range margin and vice versa. We collected demographic data on all individuals of *Viola biflora* L. var. *rockiana*, referred to as *V. biflora* from here on, in the experimental turfs to examine whether and how the species responds in both population abundance and underlying vital rates to a climatic change of ca. 1°C, the moderate regional 30-year prediction (Ding et al., 2007; Chen et al., 2013), within the species' present altitudinal range. In order to explore impacts of warming on range edge populations and impacts of extreme warming, we complemented the reciprocal transplant setup with transplantations to beyond the species current climatic range, simulating warming of +1.4 and 3.9°C, respectively. This is especially relevant in the Himalaya region, which has experienced about twice the temperature rise as compared to the global average (Chen et al., 2013). *V. biflora* is a relatively weak competitor (Olsen et al., 2016), and we expect it to respond negatively to the warming treatments as the release from cold-temperature stress leads to higher productivity of competitive species in the turfs, more biomass

and thus increased competition for light (cf. Hautier et al., 2009). We disentangle potential plastic responses from local adaptations by testing for opposite responses vs. all-negative responses of transplants in both directions (Kawecki and Ebert, 2004). Moreover, under plastic responses, we expect increasingly stronger detrimental effects in the +1.4 and +3.9°C transplants outside the species' range than in the 1°C warming within the range.

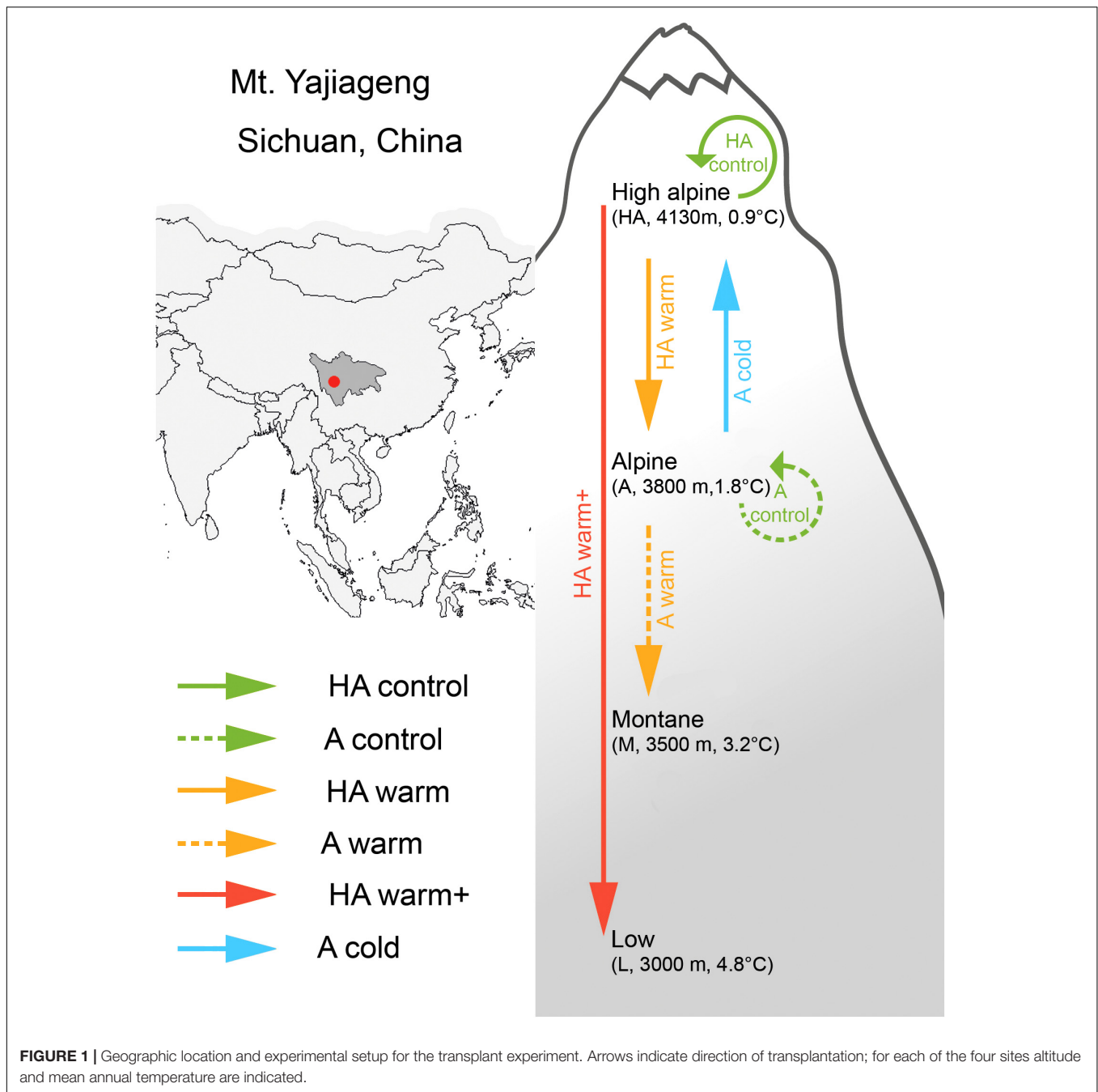
MATERIALS AND METHODS

Study Species

Viola biflora is a perennial, clonal forb species, which is common in snowbeds and leesides, grazed upland pastures, stream banks, and birch forests in the alpine and high alpine zone. It grows in open, relatively nutrient rich habitats with good access to moisture, and is characterized as a weak competitor (Evju et al., 2010).

Study Sites

The study was conducted at Mount Yajiageng on the eastern fringe of the Tibetan Plateau from 2012 to 2015. We selected four sites in the natural open grasslands along the western slope of the mountain at altitudes from 3000 to 4130 masl, a low site (L, 3000 m), a montane site (M, 3500 m), an alpine site (A, 3800 m), and a high alpine site (HA, 4130 m). Sites A and HA are located in the natural shrub and grass ecotone, where the study species, *V. biflora*, is common and relatively widespread. The vegetation at these sites is dominated by *Kobresia uncinoides*, *Kobresia royleana*, *Potentilla stenophylla*, *Saussurea ceterach*, *Saussurea stella*, and *Primula* spp. (site HA), and *Polygonum viviparum*, *Potentilla leuconota*, *Carex laeta*, and *Carex atrata* (site A). Sites L and M lie below the natural tree line, where the study species does not occur naturally. At these sites, the vegetation is dominated by *Deyeuxia scabrescens*, *Halenia elliptica*, *Pedicularis davidii*, *Pedicularis sima*, *Geranium pylzowianum*, and *Anaphalis nepalensis* (site M), and *Carex finitima*, *Carex nubigena*, *Plantago asiatica*, *P. leuconota*, *Trifolium repens*, and *Clinopodium polyccephalum* (site L). The study area is located in the transition zone between subtropical humid monsoon climate of eastern China and cold climate of Tibetan Plateau, with a mean annual precipitation of ca. 1000–2000 mm/a concentrated in May to October (Gongga Mountain Alpine Ecosystem Observation Station; cf. Zhou et al., 2013). The four sites are characterized by a pronounced temperature gradient (Figure 1). While precipitation decreases along this altitude gradient as well, largely due to lower July precipitation in A and HA, air relative humidity and soil moisture do not vary systematically with altitude (Supplementary Table S1). All sites are characterized by mountain dark brown soil and the vegetation of the four sites is all upland grasslands under low-intensity extensive grazing from horses, cattle, sheep, and yak. The sites were fenced in July 2012 to avoid animal damage of the experimental plots. After fences were erected, the vegetation was cut annually to avoid biomass buildup.



Experimental Design

In sites HA and A, we established five experimental blocks, and in each block three semi-randomly placed 25 cm × 25 cm plots (they had to contain the study species). In September 2012, two plots from each block of site HA were transplanted to site A and site L, representing a temperature change of +0.9°C within the species range and +3.9°C outside the species range, respectively. In addition, two plots from each block of site A were transplanted to site M and site HA, representing a temperature change of +1.4°C outside the species range and of −0.9°C within the range, respectively. For simplicity, we hereafter refer to these

transplants as the “HA warm” (i.e., +0.9°C warming within the range), “HA warm+” (i.e., +3.9°C warming outside the range), “A warm” (i.e., +1.4°C warming outside the range), and “A cold” (i.e., −0.9°C cooling, from range margin to range center) treatment, respectively. The fifth plot was transplanted within the original site and block, as a control (we hereafter refer to the controls at HA and A as “HA control” and “A control,” respectively). The transplanted turfs measured 25 cm × 25 cm and were 20 cm deep. After excavation, the turfs were placed in wooden boxes and replanted into their respective target sites within 1 day.

Data Collection

As a clonal plant, *V. biflora* produces long lateral rhizomes, each with multiple flowering ramets, on the same genetic individual. As the below-ground connections cannot be assessed under non-destructive sampling, we used ramets as our working unit (Hegland et al., 2010). In July/August 2012, prior to transplanting, we non-destructively marked all *V. biflora* ramets within each plot with toothpicks, measured a selected set of vegetative traits and counted the number of flowers and capsules. The vegetative traits “number of leaves” and “length of the longest leaf” were used to estimate plant biomass (hereafter referred to as “size”), based on a regression model tested on 236 destructively sampled individuals (outside the experimental plots) from sites HA and A in 2012 and 2013 (Supplementary Table S2). In the summers of 2013, 2014, and 2015, we recorded the survival of the previous year ramets, tagged new ramets and seedlings, and repeated the measures of vegetative and of reproductive traits for all life ramets. For *V. biflora* it is impossible to non-destructively assess how ramets are connected and new clones got therefore the closest ramet (but not seedlings) assigned as “parent.” *V. biflora* exhibits vegetative dormancy with ramets being able to resprout after having been dormant for up to 4 years (Evju et al., 2010). Therefore, the limited period of the study does not allow disentangling mortality from “going dormant” and clonality from “resprouting.” We hence regarded all newly appearing non-seedling ramets as clonal offspring and all disappearing ramets as “dead.”

To get an estimate of productivity and competition for light in the experimental sites and treatment plots we measured overall community height (see Hautier et al., 2009) and cover in 2012, prior to transplanting, as well as in 2013, 2014, and 2015. Community height was determined as the average of five measurements of the foliage height per plot, and cover was determined as the percentage of the plot covered by all vascular plant species.

Statistical Analyses

Population Models for *V. biflora*

To assess the population dynamics of the study species and estimate population growth rates (λ) we used integral projection models (IPMs) (Easterling et al., 2000; Ellner and Rees, 2006, 2007). IPMs are continuous analogs of matrix models (Caswell, 2001) based on regressions of vital rates (survival, growth, clonality, and fecundity) against a continuous state variable (size, weight, age, etc.) describing each individuals' state (Easterling et al., 2000; Ellner and Rees, 2006, 2007; Merow et al., 2014; Rees et al., 2014). We used R version 2.15.3 (R Core Team, 2014) to build IPMs separately for each control and treatment population. For the vital rate regressions the IPMs are built from, we examined the effects of plant size in a given year on survival, size in the following year (i.e., growth), probability of producing clonal offspring, number of clonal offspring produced, size of clonal offspring, flowering probability, and number of flowers produced. This was done separately for each transplant treatment and the controls across all sites and transitions (i.e., the time between the annual censuses: 2012–2013, 2013–2014,

and 2014–2015) using generalized linear mixed effects models (GLMMs, R-package lme4; Bates et al., 2014). In addition to assessing “size” as the deterministic “fixed effect,” these models allow specification of stochastic “random effects” enabling us to model the stochastic variation caused by the spatial structure of the experimental setup and the repeated measures on the same plants. All vital rate models were first fitted with linear terms for size in the fixed effects, as well as random intercepts and slopes for every combination of site and annual transition (SiteTrans in Supplementary Material S3). Additionally incorporating block and plot as random effects over-parameterized the models and lead to non-convergence, and we therefore dropped these random effects. The appropriate minimum model structure for both fixed and random effects was found with a backward selection procedure using likelihood ratio tests (significance level 0.05). In this procedure, we considered dropping the linear terms for size in the fixed effects, and the random slopes, whereas the random intercepts were always kept as the minimum random structure. The variables “number of clones” and “number of flowers” showed too little variability to warrant estimation of random effects and for these we thus used simpler generalized linear models instead. For the models on probability of survival, clonal reproduction, and flowering, we used a binomial error distribution with logit link, for the models on number of clonal offspring and flowers we used a Poisson error distribution with log link, and for the models on growth and size of clonal offspring we used a Gaussian error distribution with identity link. Where necessary, over-dispersion in the binomial and Poisson models was accounted for by extending the error structure with an observation-level random effect (Maundonald and Braun, 2006). More detailed documentation of the vital rate models can be found in Supplementary Material S3.

Some reproductive traits such as the number of seeds per flower, seedling establishment, the probabilities for entering and staying in the seed bank, and seedling size cannot be related to plant size under non-invasive data collection. These values were obtained from another study on the focal species in a climatically similar environment (Olsen et al., 2016) and used as constants in all models.

Using the R-package IPMPack (Metcalfe et al., 2013) we built IPMs from the above-described regression models for the vital rates growth, survival, clonality (based on probability of producing clonal offspring, number of clonal offspring produced, and size of clonal offspring) and fecundity (based on flowering probability, number of flowers produced, number of seeds per flower, the probability of seed germination and seedling establishment and entering the seed bank, as well as the mean size of seedlings). The seed bank is a discrete stage in an otherwise continuous population model, and was represented by a model describing transitions between the continuous distribution of plant sizes and the discrete seed bank (probability of staying in the seed bank, leaving the seed bank with subsequent seedling establishment and leaving the seed bank with subsequent seedling establishment failure) (Metcalfe et al., 2013). These five vital rate models were then used to construct growth-survival (P), clonality (C), and fecundity (F) matrices (the discrete transition seed bank model goes into the P-matrix) with size ranges from the observed

minimum and maximum sizes minus/plus an increment of 1% of the minimum/maximum size as described in, e.g., Metcalf et al. (2013). The matrices were of the bin dimensions 101×101 with the first bin representing the seed bank transitions and the bins 2–101 representing the continuous part of the size range. Finally, these matrices were combined into a full IPM from which the dominant eigenvalue λ , representing population growth rate in population projection matrices, could be calculated. Separate IPMs were constructed for each transplant treatment and the controls in every site and every year.

We estimated the uncertainty around the λ 's by bootstrapping. Individual ramets were sampled with replacement to construct a resampled dataset containing the same number of observations as the original dataset. Regression modeling, construction of IPMs and calculation of λ were then repeated as described above using the resampled dataset. Performing this procedure 2000 times generated a set of bootstrap λ estimates, which were used to assess the significance of differences in λ between transplants and controls. Pairwise independent transplant and control bootstrap λ samples were subtracted from each other (control-treatment). A qualitative difference in λ was accepted as significant at the 0.05 level when it occurred in more than 95% of the bootstrap sample pairs.

Finally, we used life table response experiments (LTRE) to determine how much changes in the vital rates contributed to the differences in λ between the transplants and their respective controls. The contribution of a given vital rate was calculated as the sum of the differences between the vital rate matrices of the transplant and control treatments multiplied by the sensitivity of a matrix midway between the full IPM matrices of the two treatments (i.e., transplant and control) (Caswell, 2001). We separated growth and survival, which together make up the P -matrix, by setting the probability of survival to one for all sizes. The contribution of growth alone could then be calculated using the method outlined above. By subtracting the contribution of growth from the total growth-survival contribution we found the contribution from survival alone.

Leaf Traits of *V. biflora*

To address origin-related differences in plant architecture we assessed differences in number of leaves, leaf size, and length of the leaf stalk from all plants in all years in our study. For this, we modeled all three variables as a function of origin (fixed effect) and with random intercepts for all treatment–origin combinations and random intercepts for year. We used GLMMs with Poisson errors and log-link for number of leaves, and with Gaussian errors and identity-link for leaf size and leaf stalk length.

Community Responses

The difference in community height or cover between transplant plots and controls was analyzed for each transplant treatment by linear mixed effects models with Gaussian error structure. We used year as fixed effect to assess whether or not the difference observed in 2013, 2014, and 2015 were significantly different from prior to transplanting in 2012. The model intercept represents 2012 and indicates whether or not the observed difference in that year is significantly different from zero.

RESULTS

Population Growth Rate (λ)

Both the high alpine and the alpine native populations of *V. biflora* were stable ($\lambda \sim 0.9$ – 1.1 and $\lambda \sim 0.96$ – 0.99 , respectively) throughout the study period and all transplant treatments showed clear and significant differences from this stable state (Figures 2A–D and Table 1). Transplanting to warmer sites reduced λ in populations originating from both the high alpine and the alpine site, but the magnitude of the effect varied. λ was around 0.7–0.8 in the “HA warm” treatment, 0.7 in the “A warm” treatment, and as low as 0.3–0.5 in the “HA warm+” treatment. As the population size at site A was much smaller than at site HA (see Table 1) uncertainty was higher in the transplants originating from site A, potentially masking statistical significance of the effects of the “A warm” treatment. The transplants from the alpine site to the colder high alpine site led to a λ increase of $\sim 30\%$ which was significant for all transitions. In the “HA warm+” transplants to the low alpine site, λ was unchanged in the first transition but then dramatically dropped in the following years.

Vital Rates

Changes in survival and clonality were the main contributors to differences in λ in all treatments (Figures 2E–H). Similarly to λ , the negative survival contributions in the warming treatments increased progressively from the “HA warm” treatment, via the “A warm” treatment and to the “HA warm+” treatment. The “A cold” and the “HA warm” treatments were complementary with respect to survival, the respective positive and negative contributions to changes in λ being comparable in effect size.

Growth did not significantly contribute to changes in λ between treatments, while fecundity slightly increased in the “HA warm+” transplants and slightly decreased in the “A cold” transplants.

Height and Cover of the Plant Community

Community height was higher under warming than in the controls in the course of the experiment, by ~ 3 cm under both the “HA warm” and the “A warm” treatments (Figures 3B,C), and by ~ 16 cm in the “HA warm+” transplants from the second transition on (Figure 3A). In the “A cold” transplants, community height was lower than in the controls by ~ 5 cm already in the first transition, the effect size increasing in the following transitions to ~ 10 cm (Figure 3D). Cover was mainly unaffected by the climate treatments (Figures 3E–H), but was significantly lower in the “A cold” transplants in the last transition (Figure 3H). In site HA, cover was highly variable (ranging from 50 to 90%), whereas it was constant and high in the transplants (both to site A and site L) originating from this site (Figures 3E,F).

Leaf Traits

Number of leaves, leaf size, and leaf stalk length had different values in the high alpine and alpine habitats. In the high alpine,

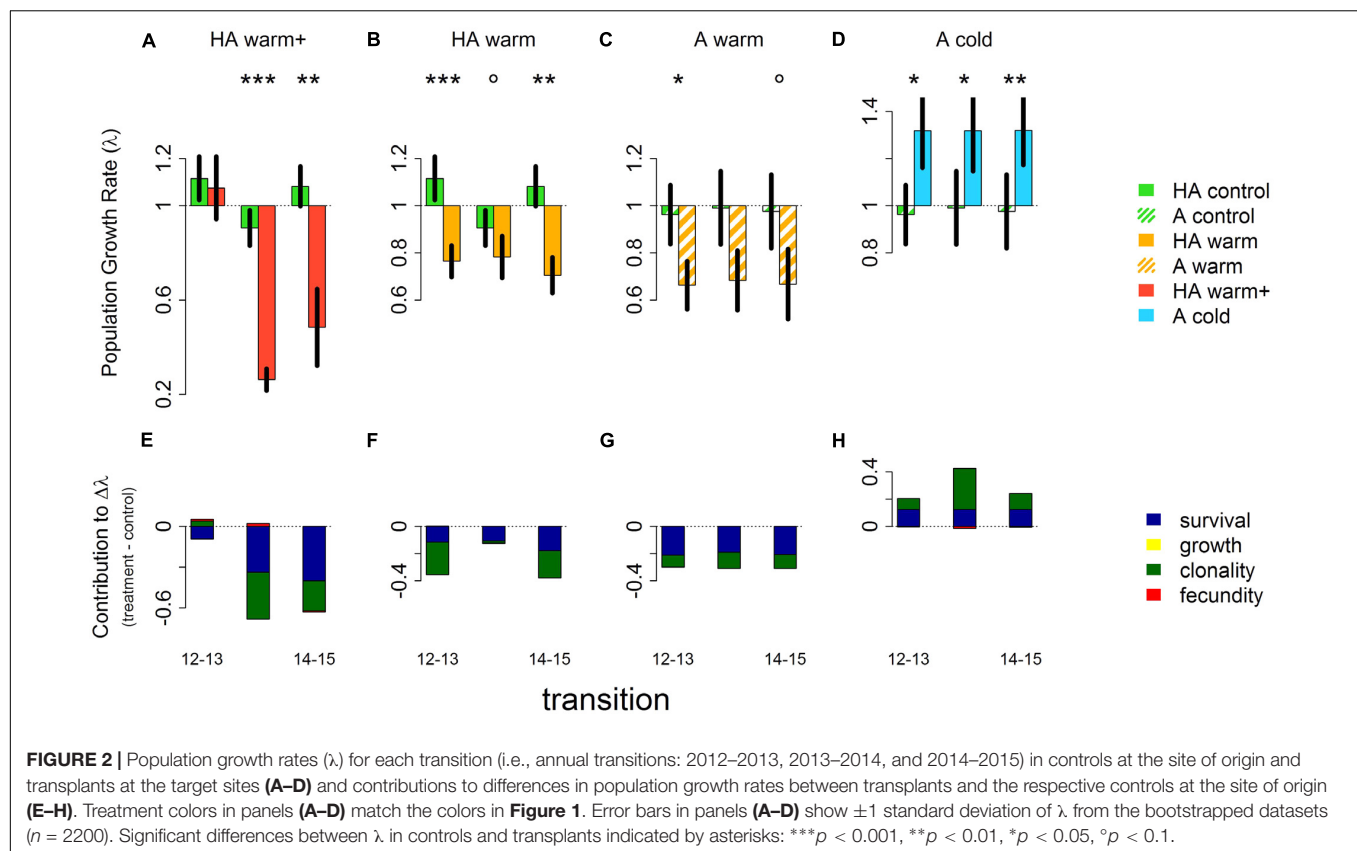


TABLE 1 | Population sizes in all transplant treatments and sites in the four study years.

Year	HA control	A control	HA warm	A warm	HA warm+	A cold
2012	98	16	121	21	75	23
2013	127	13	92	14	87	21
2014	106	12	69	9	18	38
2015	131	11	59	8	10	54

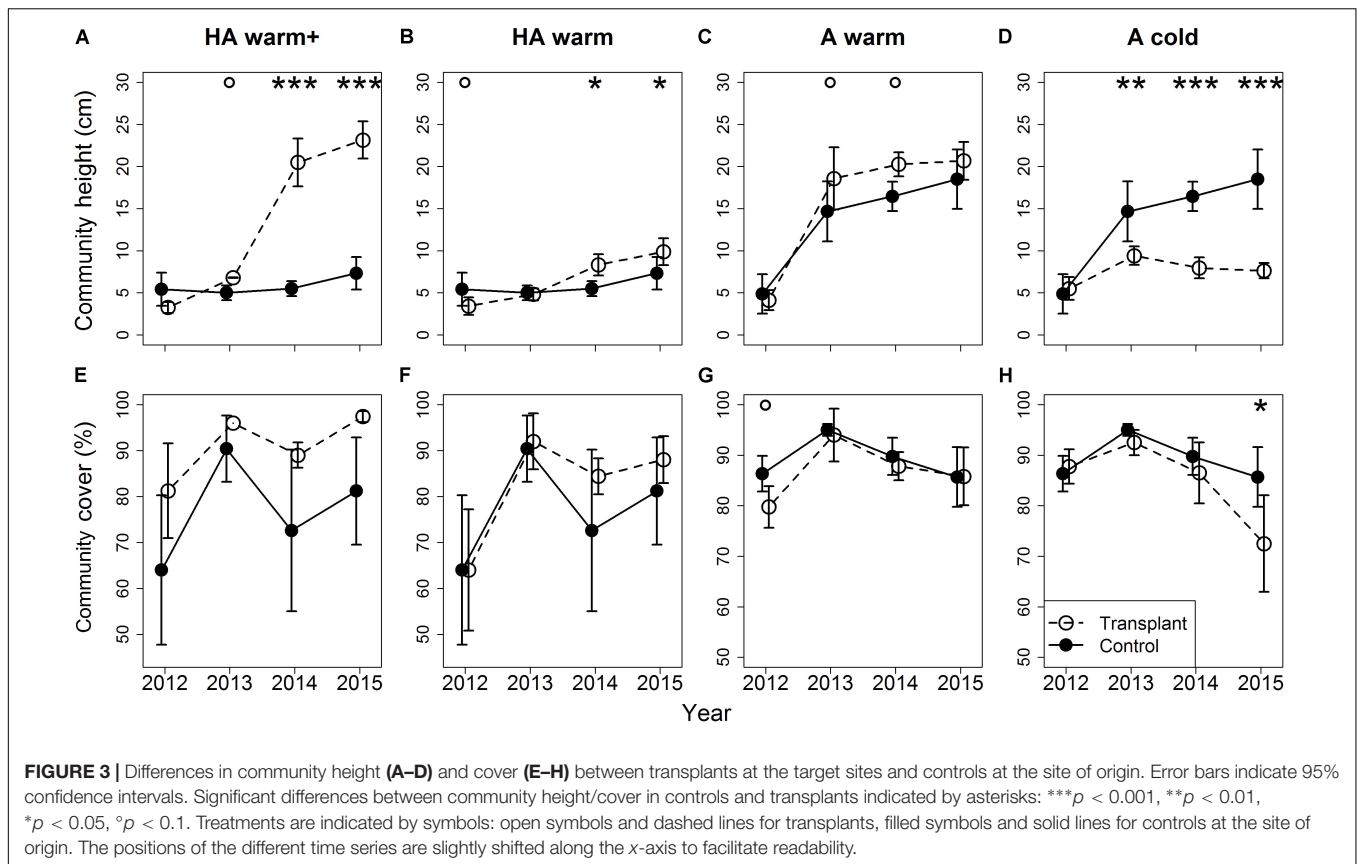
HA and A depict the origin of the transplants, i.e., high alpine site and the alpine site, respectively.

the plants produced more, but smaller and lower growing leaves, and in the alpine the plants produced fewer, larger and higher growing leaves (Figure 4 and Table 2). For number of leaves and leaf size, the transplanted populations retained their original trait values even after transplantation to climates differing with $\sim 1^\circ\text{C}$ (Figures 4A,C). In the “HA warm+” treatment, however, these two traits changed from the “high alpine values” in direction of the “alpine values.”

DISCUSSION

The results of this transplant study clearly show negative effects of warming on the population dynamics of the small herb *V. biflora*. This contrasts the general positive impacts on plant growth and productivity commonly found as a response to

warming (Myneni et al., 1997; Rustad et al., 2001; Walker et al., 2006; Wu et al., 2011; Elmendorf et al., 2012). These positive impacts are associated with direct physiological effects of higher temperatures on photosynthesis (Sage and Kubien, 2007), although warming-caused drying may reverse these impacts (Barber et al., 2000; Ciais et al., 2005). However, a general increase in productivity often leads to a shift in dominance structure in the vegetation (Harte and Shaw, 1995), penalizing species with a resource-conservative, cold climate strategy that cannot utilize the higher temperatures effectively (Klanderud and Totland, 2005; Klanderud et al., 2015). *V. biflora* is a relatively weak competitor for light (Evju et al., 2010), and therefore we suggest that the observed impacts are indirect effects via increased competition from the more productive plant community under warming (Figure 3), although we acknowledge that our study design does not explicitly test for indirect competition effects vs. direct climate effects. Higher vegetation canopies reduce light availability in the vegetation sward and thus penalize smaller, less competitive species (Hautier et al., 2009). In line with this, we found changes in growth to play an inferior role in *V. biflora*, while changes in survival and clonal growth constituted the main drivers of changes in population growth rates. Decreased survival and clonality, without any delay via prior changes in plant sizes, translate into swift reductions on population size, which in turn increases the chance for local extinctions of *V. biflora* populations (cf. Morris and Doak, 2002), especially in the species’ warmer range margin (cf. Hampe and Petit, 2005).



Plasticity vs. Local Adaptation

The results from our reciprocal transplant treatments, ca. +1°C warmer and −1°C colder for high alpine and alpine populations, respectively, clearly show that both populations perform better in the high alpine habitat than in the alpine. The opposite effects of warming and cooling on lambda with similar effect sizes and similar suites of vital rate contributions to changes in lambda support neither the “local vs. foreign” or “home vs. away” criterion for detection of local adaptation and hence suggest a plastic response to altered temperature (Kawecki and Ebert, 2004). Nevertheless, the control populations were similarly stable ($\lambda \sim 1$) in both habitats. This seemingly contradicts the plastic effects found in the reciprocal transplants, from which we could have expected lower fitness in the alpine population than in the high alpine population. However, this discrepancy can make sense in the light of the stress-gradient-hypothesis (Choler et al., 2001; Pugnaire and Luque, 2001; He et al., 2013; Klanderud et al., 2017) which suggests that plant species are rather limited by cold temperatures at the adverse end of their temperature range but by competition at the favorable end of their temperature range. At both ends any established populations would persist at locally suitable habitats that are not too harsh in the high alpine and not too competitive in the alpine. When transplanted, however, a release from competition seems to be positive in spite of a harsher climate in the high alpine, whereas an increase in competition seems to be negative in spite of a more favorable climate in the alpine.

These two limitations are reflected by different growth forms of *V. biflora* individuals in the alpine and high alpine habitats in our study (Figure 4). In the high alpine sites, the plants produce several, small and low standing leaves, a classical growth form strategy in climatically harsh environments (Larcher, 2003); in the alpine site, the plants grow fewer, but larger leaves on higher leaf stalks, a good strategy under higher competition for light (Weiner and Thomas, 1992; Weiner, 2004; Poorter et al., 2012). Both leaf size and number of leaves were very conservative in our data, with individuals in both the controls and the transplants originating from the same site sharing similar values despite the temperature difference after transplantation. This indicates that these leaf traits in *V. biflora* do not change plastically with climate but are at least to some degree adapted to the relevant local stressors, climatic adversity and competition. We have no information about the actual biomass associated with size of leaves and leaf stalks and we thus do not know whether the observed differences reflect patterns in allocation or organ morphology, but plants have been shown to be less able to adjust allocation than organ morphology (Poorter et al., 2012). Based on these results, one could expect that transplanted populations failing to acclimate their individuals' growth form to the locally prevalent stressors should be penalized. However, in our study this was only the case for the small growing individuals from the high alpine transplanted into the alpine habitat, whereas the taller growing individuals from the alpine habitat thrived better when transplanted to the high alpine, even when compared to the local

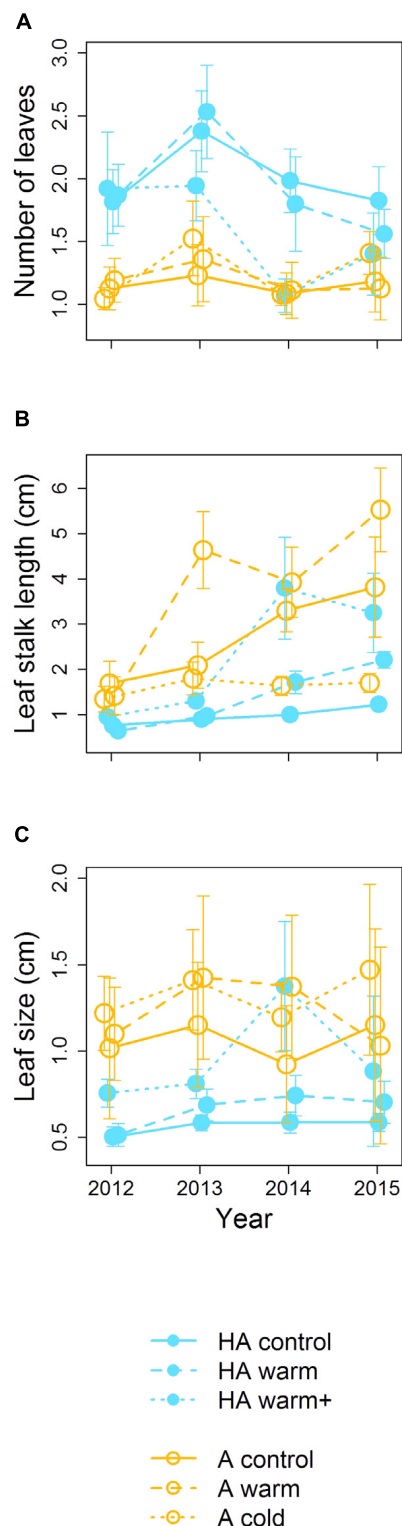


FIGURE 4 | Leaf traits (A–C: Number of leaves, Leaf stalk length and Leaf size) in transplants (dashed and dotted lines) and controls (solid lines) from high alpine (blue, filled symbols) and alpine origin (orange, open symbols) over the study period. Error bars indicate 95% confidence intervals. The positions of the different time series are slightly shifted along the x-axis to facilitate readability.

TABLE 2 | Differences in leaf traits between the high alpine (HA) and the alpine (A) sites.

Leaf trait	Effect (HA–A)	SE	N	p-Value	SD treat	SD trans
# Leaves	0.40	0.10	876	<0.001	0.08	0.13
Leaf-stalk length	–1.53	0.96	876	0.19	1.17	0.40
Leaf size	–0.54	0.13	868	0.01	0.13	<0.001

Shown are the regression statistics on the differences (effect) in number of leaves (# leaves), leaf-stalk length, and leaf size between the high alpine (HA) and the alpine (A) sites, as well as the associated standard errors (SE), sample sizes (N), and p-values. In this analysis, the leaf trait data for all treatments and years were lumped together and analyzed with specifying random intercepts for the different treatments (HA control, A control, HA warm, A warm, HA warm+, and A cold). SD treat and SD trans indicate the standard deviation among treatments and transitions, respectively.

high alpine controls. This could suggest that climatic adversity is a less limiting factor in our study sites than competition, but without any further experimental evidence to support such a conclusion, this remains speculative.

In a climate change perspective, conservative leaf traits may be rather detrimental, as this prevents plastic adjustments to a higher vegetation under a warmer climate and hence causes range contractions (Anderson et al., 2009; Jones and Gilbert, 2016). However, under the extreme “HA warm+” treatment, number of leaves and leaf size seemingly displayed less conservatism than indicated by the other treatments, as their values changed in the 3 year (Figures 4A,C). From the second transition (i.e., 2nd to 3rd year) on, mortality in this treatment was very large, reaching 94 and 100% in the last two transitions. Hence, the remaining population in the last 2 years consisted mostly of new recruits, which adopted the above indicated altered growth form as an ontogenetic acclimatization to their environment (Mason et al., 2013). Although mitigation of negative effects at the species level is still deemed unlikely for alpine plants especially under stronger warming scenarios (Parmesan, 2006), this may facilitate local persistence and acclimatization to the environmental conditions in the short-term or under moderate climate change. In species with clear patterns of local adaptation, as, e.g., *Erysimum capitatum* in North America (Kim and Donohue, 2013), regional persistence under climate warming would need to rely on niche-tracking and gene-flow, or ultimately evolutionary adaptation.

Warming Within the Temperature Range vs. Outside the Range

Warming that takes the plants outside their present temperature range caused a stronger decrease in lambda based on stronger reductions in survival when compared to the same temperature rise within the range. This mirrors the results from a study in Norway, where populations of *V. biflora* from the lower altitudinal range margin performed worse than populations from the range center when transplanted to sites with warmer and wetter climate (Töpfer et al., 2018). The concurrent increase in community height, however, was similar under ca. 1°C warming both within and outside the species range in our study. This suggests that populations growing in the leading range margin of

an environmental stressor (here, competition in alpine habitats) experience an increase in that stressor more adversely than more central-range populations (Anderson et al., 2009; Hargreaves et al., 2014).

In line with Gavazov et al. (2014), who found that warming of +4°C had more negative impacts on sub-alpine pastures in Switzerland than more moderate warming, the extreme “HA warm+” transplants in our study showed a considerably lower λ in *V. biflora* than in the controls based on even more reduced survival. However, these reductions showed a lag of 1 year, which was not present in the other, more moderate, warming treatments. Interestingly, change in plant-plant competition, expressed as change in community height, also showed a lagged increase, probably due to a general (i.e., for all species) need to adjust the photosynthetic temperature optimum under warming of 4°C (Yamori et al., 2014). Thus, the *V. biflora* population did not experience an increase in competitive stress during the first transition, which may explain how the population could remain relatively stable in the 1st year after transplantation. Nevertheless, once the community height increased, this increase was drastic and likewise was the reduction in survival and λ . With only 10 individuals left in 2015 (all clonal recruits), the persistence of this *V. biflora* population at the low site is highly unlikely (Morris and Doak, 2002). While no alpine species has yet got extinct from the transplanted turfs in our study, colonization from the surrounding local vegetation has occurred frequently (Yang et al., unpublished). These “novel competitors” may constitute a significant part of the community height increase and thus contribute to shaping the responses of alpine species like *V. biflora* (Alexander et al., 2015).

During this lagged response phase, changes in clonality were, surprisingly, contributing positively to the difference in λ . In our study, such increase in clonality may also be due to increased re-sprouting after vegetative dormancy. Such re-appearance of dormant plants has been linked to re-mobilization of stored resources (Gremer et al., 2010), which may be interpreted as a stress response contributing to the apparent stabilization of this *V. biflora* population during the first transition. As both dormancy and clonality may be susceptible to effects of climate change (Carlsson and Callaghan, 1994; Symstad and Tilman, 2001), a separation of dormancy and clonal reproduction based on a longer term study would be enlightening.

Limitations

While yielding many important insights into climate-change driven responses on alpine plants, our study also has two limitations worth discussing. First, as mentioned above, vegetative dormancy is a factor complicating non-destructive population studies. If the demographic time series is long enough and the temporal extent of the dormant phase short enough, then dormancy can be identified and modeled with satisfactory levels of uncertainty (Kery et al., 2005; Evju et al., 2010). In short-term studies, as the one at hand, identification of dormant stages is impossible for most cases of “missing” observations: is a new individual in year 2 a clone or a previously dormant

plant? Is an individual missing the last year dead or dormant? These questions consider 1-year dormancy, which hence would complicate demographic studies over up to four transitions, but 2- and 3-year dormancy is just as common (Spindelböck and Olsen, 2013), ultimately increasing the required number of transitions for reasonably sound inference on dormancy. The approach adopted in this study, ignoring dormancy by treating all missing individuals as dead and all re-appearing individuals as clones (Olsen et al., 2016; Töpfer et al., 2018), constitutes a simplification of the species’ life cycle and ecology. However, while this needs to be considered when dealing with vital rate contributions, λ still reflects the population-size dynamics above ground, which still harbors the crucial life stages for assessing ecological performance as green plants failing to re-sprout also increase their risk to die (Shefferson, 2006).

Second, our experiment is replicated locally but not regionally, which in principle limits the degree to which our results can be generalized beyond the mountain our study was performed on to alpine systems in general. However, our overall finding of competition-limitation in *V. biflora* is well supported by other population studies on this species from Europe (Evju et al., 2010; Olsen et al., 2016; Töpfer et al., 2018), and the responses found in our study thus do not seem to be “locally special” for Mountain Gongga.

CONCLUSION

The average regional predictions for climate warming in China lie around 1–2°C until about 2050, while extreme models predict an increase by more than 4°C in the second half of the 21st century (Ding et al., 2007), which is double the climatic change predicted for the planet on average (Chen et al., 2013). This study illustrates how these scenarios may affect alpine herbs in the region and elaborates on the underlying mechanisms of population change. Our results show that the study species, the alpine herb *V. biflora*, clearly is sensitive to climate warming, which is threatening especially populations at the warmer species range margin, where competition already acts limiting for survival and establishment of new recruits in the species. There, the species’ persistence will likely become increasingly dependent on reducing competition from the plant community, e.g., through management interventions like grazing and mowing (Evju et al., 2010). Should the global warming trajectory follow the worse scenarios, also more central populations are at high extinction risk during this century, as these climatic changes would take the populations well out of their present environmental range.

AUTHOR CONTRIBUTIONS

VV, YY, and GW conceived and designed the experiment. JT designed the demographic study. HC and YY performed the experiment with input from VV. GW and YY provided climate data. JT and HC analyzed the data. JT wrote the paper with

contributions from HC and VV. All the authors read and approved the final manuscript.

FUNDING

This research was supported by the Key Research Program of Frontier Sciences, CAS (QYZDJ-SSW-DQC006) and by the Foundation of Key Laboratory of Mountain Surface Processes and Ecological Regulation, CAS and by the Chinese CAS/SAFEA International Partnership Program for Creative Research Teams (KZZD-EW-TZ-06), the Norwegian Center for International Cooperation in Education (SIU) projects 2013/10074 and HNP-2015/10037, and the Southwest Forestry University Research Start-up Fund (111813).

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ACKNOWLEDGMENTS

We thank Christine Pötsch and Mari Jokerud for their fieldwork contributions and invaluable inputs to the demographic study. We are grateful to the Alpine Ecosystem Observation and Experiment Station of the Gongga Mountain of the Chinese Academy of Sciences for field assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01069/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Snowmelt Timing Regulates Community Composition, Phenology, and Physiological Performance of Alpine Plants

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OPEN ACCESS

Edited by:

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University of Antwerp, Belgium

Reviewed by:

Thomas Abeli,
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Roman Zweifel,
Swiss Federal Institute for Forest,
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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 15 May 2018

Accepted: 13 July 2018

Published: 31 July 2018

Citation:

Winkler DE, Butz RJ, Germino MJ,
Reinhardt K and Kueppers LM (2018)
Snowmelt Timing Regulates
Community Composition, Phenology,
and Physiological Performance
of Alpine Plants.
Front. Plant Sci. 9:1140.
doi: 10.3389/fpls.2018.01140

The spatial patterning of alpine plant communities is strongly influenced by the variation in physical factors such as temperature and moisture, which are strongly affected by snow depth and snowmelt patterns. Earlier snowmelt timing and greater soil-moisture limitations may favor wide-ranging species adapted to a broader set of ecohydrological conditions than alpine-restricted species. We asked how plant community composition, phenology, plant water relations, and photosynthetic gas exchange of alpine-restricted and wide-ranging species differ in their responses to a ca. 40-day snowmelt gradient in the Colorado Rocky Mountains (*Lewisia pygmaea*, *Sibbaldia procumbens*, and *Hymenoxys grandiflora* were alpine-restricted and *Artemisia scopulorum*, *Carex rupestris*, and *Geum rossii* were wide-ranging species). As hypothesized, species richness and foliar cover increased with earlier snowmelt, due to a greater abundance of wide-ranging species present in earlier melting plots. Flowering initiation occurred earlier with earlier snowmelt for 12 out of 19 species analyzed, while flowering duration was shortened with later snowmelt for six species (all but one were wide-ranging species). We observed >50% declines in net photosynthesis from July to September as soil moisture and plant water potentials declined. Early-season stomatal conductance was higher in wide-ranging species, indicating a more competitive strategy for water acquisition when soil moisture is high. Even so, there were no associated differences in photosynthesis or transpiration, suggesting no strong differences between these groups in physiology. Our findings reveal that plant species with different ranges (alpine-restricted vs. wide-ranging) could have differential phenological and physiological responses to snowmelt timing and associated soil moisture dry-down, and that alpine-restricted species' performance is more sensitive to snowmelt. As a result, alpine-restricted species may serve as better indicator species than their wide-ranging heterospecifics. Overall, alpine community composition and peak % cover are

strongly structured by spatio-temporal patterns in snowmelt timing. Thus, near-term, community-wide changes (or variation) in phenology and physiology in response to shifts in snowmelt timing or rates of soil dry down are likely to be contingent on the legacy of past climate on community structure.

Keywords: distribution, elevation, flowering, Niwot Ridge, photosynthesis, snowmelt gradient, spatio-temporal dynamics, water relations

INTRODUCTION

Recent climate warming coupled with regional declines in winter precipitation have led to an advance in the timing of snowmelt, one of the fastest changing environmental factors in alpine systems worldwide (Dyer and Mote, 2006; Barnett et al., 2008; Clow, 2010; Stocker et al., 2013; but see Mote et al., 2018). This has potentially serious implications for alpine plant communities (Wipf et al., 2006; Wipf and Rixen, 2010) and the ecosystem services they provide (e.g., carbon sequestration, mountain resilience; Bowman and Fisk, 2001; Barni et al., 2007). Spatial variation in the timing of snowmelt generates hydroclimate gradients over very short distances, structuring alpine plant communities (e.g., moist meadows, dry meadows, snowbeds; Walker et al., 1993; Taylor and Seastedt, 1994; Choler et al., 2001; Bruun et al., 2006; Jonas et al., 2008; Litaor et al., 2008). These gradients within the alpine zone include differences in timing and duration of soil water- and nutrient-availability, pH levels, and soil organic matter content (Walker et al., 1993; Stanton et al., 1994). Hydroclimate gradients represent natural experiments that can be used to better understand ecological processes in alpine systems (e.g., Cabrera et al., 1998; Michalet et al., 2014) and can also potentially act as space-for-time substitutions (Dunne et al., 2004).

Combined with relatively short growing season lengths (<3 months), these hydroclimate and associated environmental factors result in a landscape mosaic of vegetation communities, which vary in species composition, productivity, and physiological performance (Billings and Bliss, 1959; Stanton et al., 1994; Galen and Stanton, 1995; Germino and Smith, 2001; Winkler et al., 2016a). For example, sites with earlier snowmelt are typically more productive (Kudo et al., 1999) and can have greater species richness corresponding with better soil fertility (Stanton et al., 1994). This higher richness also reflects the larger number of wide-ranging species in relatively lower elevation and/or early melting sites (Kammer and Möhl, 2002; Bruun et al., 2006; Lenoir et al., 2008; Erschbamer et al., 2009). Typically, later melting sites are where alpine-restricted or specialist species occur (Odland and Munkejord, 2008; Pickering et al., 2014). While as many as 25% of species in alpine zones can also be found below treeline (Rundel, 2011), this leaves 75% of alpine species susceptible to competition with wide-ranging species as conditions change (Bruun et al., 2006; Steinbauer et al., 2018). However, to date, no studies have compared the potentially distinct phenological and physiological sensitivities of alpine-restricted and wide-ranging species.

Changes in growing season length may negatively influence plant production and sexual reproduction indirectly via shifts

in phenological cues including snowmelt timing (Kudo et al., 1999; Hülber et al., 2006; Venn and Morgan, 2007; Baptist and Choler, 2008). Individual alpine species vary in their ability to initiate growth immediately following snow retreat as a result of variation in traits like bud preformation, tolerance to chilling and photoinhibition, ability to develop under or around snow, photoperiodism, seed dormancy, and speed of development (Amen, 1965; Hamerlynck and Smith, 1994; Meloche and Diggle, 2001; Germino and Smith, 2000; Keller and Körner, 2003). Thus, snowmelt timing is expected to have varied effects on alpine plant phenology, including flowering duration; yet, we can expect that the strongest effects can be observed at early melting sites where diversity is typically higher and composed of a relatively larger number of wide-ranging species (Holway and Ward, 1963; Kudo, 1991, 1992; Kudo and Hirao, 2006; Sherwood et al., 2017). For example, Venn and Morgan (2007) found that individual species varied in their response to snowmelt timing, with some species synchronously flowering regardless of snowmelt timing due to bud preformation and others producing flowers shortly after snowmelt, thus closely tracking snowmelt. However, it remains unknown how species respond to differences in snowmelt timing as a function of their distributions.

While plant physiological performance also likely responds to snowmelt timing, this relationship has rarely been explicitly quantified as it has in phenological research (but see Oberbauer and Billings, 1981; Germino and Smith, 2001). In some temperate alpine systems, where soils rapidly dry down following snowmelt, and as environmental conditions change throughout the growing season, plants are exposed to intense radiative forcing and drier atmospheric conditions that can lead to desiccation as the season progresses (Geller and Smith, 1982; Smith and Johnson, 2009). Species-specific ecophysiological responses to snowmelt timing and soil dry-down rates may further explain patterns of alpine community composition and productivity. Adaptation to water stress and photosynthetic capacity of individual species likely contribute to individual presence in a community, with species exhibiting the highest water potentials in the wettest sites with earliest snowmelt (Oberbauer and Billings, 1981), and could be linked to whether they are alpine-restricted or wide-ranging species. Species that are wide-ranging across elevation gradients (i.e., broad environmental tolerance) may also be relatively well suited to adjust performance across small-scale gradients generated by snowmelt timing. For example, the wide-ranging *Bistorta vivipara* successfully adjusted (i.e., exhibited increased photosynthetic rates) when water was experimentally added at the end of the growing season when soils were driest (Enquist and Ebersole, 1994) but was unresponsive to watering earlier in the season when soils were likely still saturated with snowmelt

runoff from higher slopes or when summer precipitation events were frequent. This may explain a similar lack of photosynthetic response observed during early- and mid-season sampling events in a similar experiment that added water above ambient precipitation levels (Bowman et al., 1995). We might further expect that alpine-restricted species are at risk of local extinction if they are not able to physiologically adjust in response to earlier snowmelt and associated exposure outside of their typical optima (Lenoir et al., 2008). Yet, whether these potential plant relationships with snowmelt gradients can be attributed to plant-soil water relationships remains an unanswered question in alpine ecology.

In this study, we utilized a ca. 40-day snowmelt gradient in the Colorado Rocky Mountains to assess the associations between snowmelt timing and alpine community composition and function. We asked how community diversity and richness varied due to snowmelt timing. We predicted, as others have shown at similar sites (Litaor et al., 2008), that community composition at this site would be tightly correlated with snowmelt patterns. We also asked how flowering phenology and plant peak % cover varied due to snowmelt date. Given the short growing season length in this and similar systems, we expected that species would initiate growth shortly after snowmelt, and develop earlier with earlier snowmelt. We expected earlier melting plots, therefore, to have longer growing seasons resulting in higher species richness and peak % cover. Finally, we asked if alpine-restricted species (*Lewisia pygmaea*, *Sibbaldia procumbens*, *Hymenoxys grandiflora*) and wide-ranging species (*Artemisia scopulorum*, *Carex rupestris*, *Geum rossii*) differed in the sensitivity of plant water potential and leaf-level gas exchange to snowmelt timing. We expected that plant water potential and gas exchange would remain high across the gradient in wide-ranging species due to these species' presumed broad environmental tolerances. Conversely, we expected that alpine-restricted species would experience lowered water potentials and gas exchange by the end of the growing season in early melting plots reflecting greater moisture limitation compared to wide-ranging species.

MATERIALS AND METHODS

Study Site

Our alpine research site is located at Niwot Ridge, at 3545 m in elevation in the Front Range of the Colorado Rocky Mountains (40° 03' 14.84'' N, 105° 35' 37.71'' W). The site is on a 15° south-southeast facing slope, 400 m above local treeline. This site is characterized by a short growing season that typically lasts from

June through September (Greenland, 1989), high inter-annual variability in monthly mean temperatures and precipitation, and low growing season precipitation (Walker et al., 1995). Climate data recorded at the nearby Niwot Ridge LTER Saddle weather station show mean annual air temperature was -2.15°C and mean annual precipitation was 966 mm from 1981 to 2009. Mean microclimate data were calculated across the summer growing season (1 June–30 September; Table 1). Approximately 80% of the precipitation falls as snow at Niwot Ridge (Blanken et al., 2009). Snow depth at the site is spatially variable and controlled by topography and westerly wind (Walker et al., 1995; Litaor et al., 2008). The soils, developed on glaciofluvial deposits or residuum derived from igneous and metamorphic rock, are mapped as Moran family and classified as lithic Cryorthents. Community composition at the site is similar to moist and dry meadow community types (May and Webber, 1982; see Winkler et al., 2016a for a detailed description of the community).

We established 20 3 m diameter plots stratified by local elevation and aspect, as well as total plant cover. We divided plots into 4 1 m² quadrants to account for any fine-scale microtopography within plots (Supplementary Figure S1). The average slope of individual plots was 16% with a range of 8.5–21.5% across plots. The first plot was snow free on May 29 and the last plot on June 22. Variation in snow depth and snowmelt timing across plots results from prevailing winds from the west, the south-easterly aspect of the site, as well as microtopography within the site.

Field Measurements

All data were recorded during the 2009 growing season, before the plots became part of the Alpine Treeline Warming Experiment. We defined the growing season as the time from the date of snowmelt until all plants had senesced in late September. Date of snowmelt was determined by manual snow surveys carried out three times per week starting at the onset of spring snowmelt. Digital SLR planimetric photographs were taken 1.5 m above each quadrant to determine the date of snowmelt, defined as the first snow free day when all subsequent snow cover events lasted less than four continuous days.

We conducted vegetation surveys at peak community productivity (determined by weekly inspection of plant flowering phenology in each plot; Negi et al., 1992). Surveys began in the middle of July and concluded by early August following the ca. 40-day snowmelt gradient from the lowest to highest elevation plots at the site. We visually estimated peak % cover as an indirect estimate of productivity (Winkler et al., 2016a), first for the entire community and then for individual species using a 1 m² survey

TABLE 1 | Summary of microclimate data for summer 2009 (Jun 1 – Sep 30).

Snowmelt dates	R_{solar} (W m^{-2})	T_{air} ($^{\circ}\text{C}$)	T_{min} ($^{\circ}\text{C}$)	T_{max} ($^{\circ}\text{C}$)	T_{soil} ($^{\circ}\text{C}$)	# $T_{\text{night}} < 0^{\circ}\text{C}$	VPD (kPa)
149–173	441 (8)	7.9 (0.3)	3.5 (0.3)	13.4 (0.4)	6.7 (0.2)	16	0.43 (0.01)

Microclimate data except for snowmelt dates are from an LTER climate station located nearby (<500 m away and approximately at the same elevation). Snowmelt dates indicate the plot-level range in snowmelt in Julian days in the study site. Solar radiation (R_{solar} ; 300–3000 m), air (T_{air}), minimum air (T_{min}), maximum air (T_{max}), and soil (T_{soil}) temperatures, and air vapor pressure deficit (VPD) are summer daily means. # $T_{\text{night}} < 0^{\circ}\text{C}$ is the number of nights during the summer when air temperatures dropped below 0°C . Numbers in parentheses are SE.

grid divided into 10 cm² units. We also estimated % cover of all other surface types (i.e., solid rock, lichen cover, bryophyte cover, bare ground, fine litter, and woody debris). We measured 45 species across our site in 2009 (Supplementary Table S1). We conducted flowering phenology surveys for all species in each quadrant weekly beginning at snowmelt and continuing until flowering ceased toward the end of the growing season. These measures were used to determine the date of first flower and flowering duration for each species in each quadrant.

We selected six species for physiological measurements during the growing season, including three alpine-restricted species that only occur in the alpine zone (*L. pygmaea*, *S. procumbens*, and *H. grandiflora*) and three wide-ranging species that occur in the alpine zone but also occur at or below treeline (*A. scopulorum*,

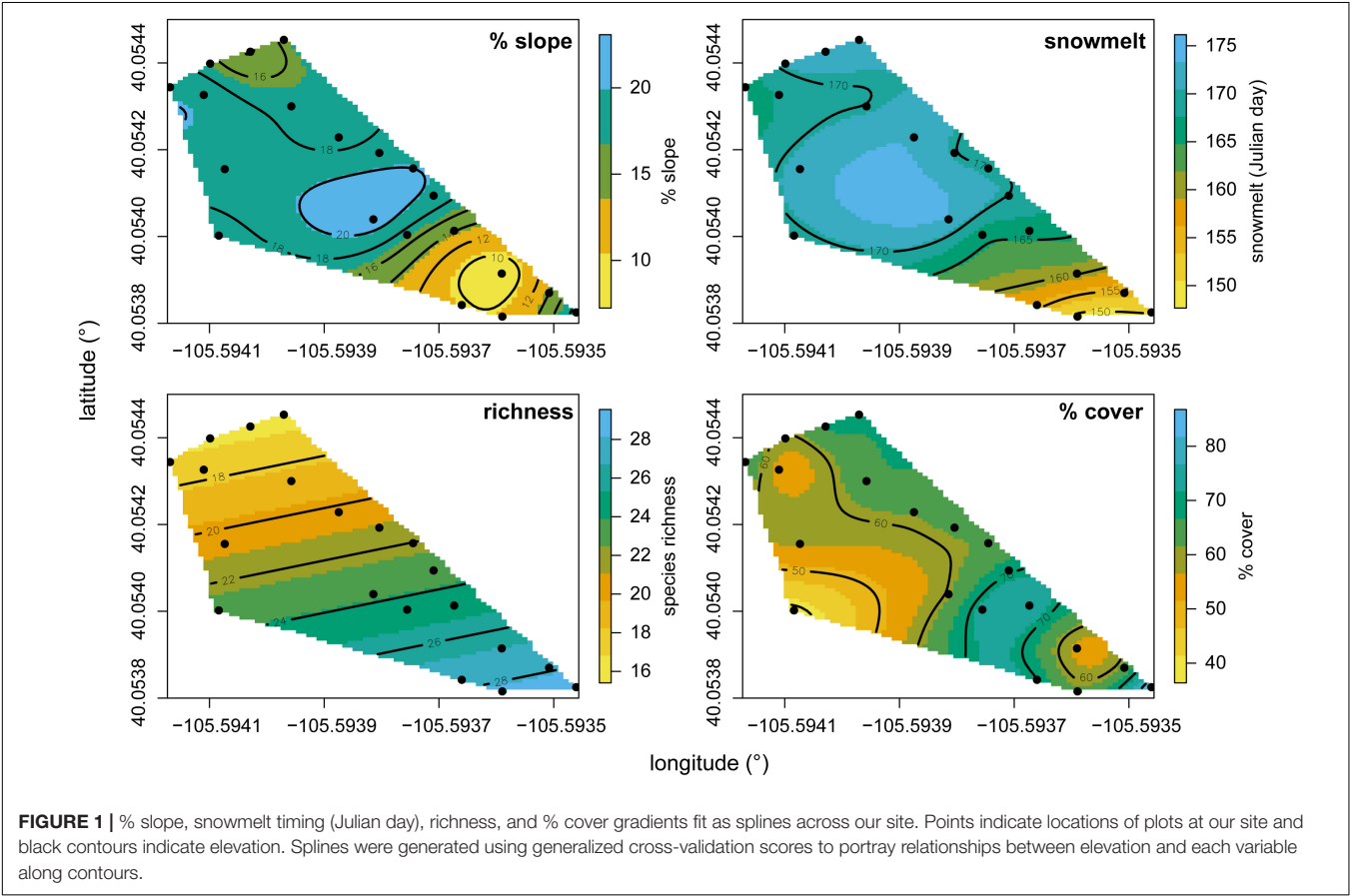
C. rupestris, and *G. rossii*). We measured gas exchange in the field on intact leaves during three sampling events each lasting 2 days (early July, *n* = 19; early August, *n* = 63; and early September, *n* = 55). We randomly shuffled species and plots sampled to avoid effects of sampling time (none were detected). All measurements were conducted during the hours of peak sunlight (>90% of maximum light) using an infrared gas analyzer photosynthesis system (LI-6400XT; Li-Cor Biosciences, Lincoln, NE, United States) equipped with a 2 cm × 3 cm leaf chamber with an internal LED light source. During all measurements, chamber conditions were set to a photon flux density of 1500 μmol m⁻² s⁻¹ and CO₂ concentration of 400 μl mol⁻¹. Vapor pressure was kept at ambient levels during all measurements and temperature inside the chamber was set to match ambient air temperature using the flow of air inside the chamber. Gas exchange was calculated on a projected leaf area basis, with leaf area in the chamber determined using digital photographs of the portion of leaf area inside chamber gaskets and using image processing software (ImageJ; Scion Co., Frederick, MD, United States). During the July and September measurement events, conditions were partially cloudy; during the August measurements, conditions were sunny.

Net CO₂ assimilation (*A*_{net}), stomatal conductance (*g*), internal concentration of CO₂ (*C*_i), and transpiration (*E*) were calculated according to Ball (1987) and Farquhar and von Caemmerer (1982), and water-use efficiency (WUE) was

TABLE 2 | Species diversity of the entire alpine plant community at our site.

Diversity measure	Value
Alpha	17
Beta	2.65
Gamma	45
Shannon's <i>H</i>	2.10
Pielou's <i>J</i>	0.75

Diversity indices are defined in the "Materials and Methods" section of the text.



calculated as A_{net}/E . Following an initial measurement of A_{net} under saturating light, the chamber was darkened (light intensity of $0 \mu\text{mol m}^{-2} \text{s}^{-1}$), and dark respiration (R_d) was recorded after CO_2 ceased to increase in the chamber and used as an indication of growth and maintenance respiration (Atkin et al., 2000). R_d under full sun conditions can be less than in what would be measured at night (Krömer, 1995), and consequently our calculated values may be overestimated. During the third sampling event (early September), we also increased CO_2 to $800 \mu\text{mol mol}^{-1}$ ($n = 55$) to generate information on stomatal limitation to photosynthesis (greater increases in photosynthesis with increased CO_2 at $800 \mu\text{mol mol}^{-1}$ indicate greater stomatal limitation to photosynthesis at $400 \mu\text{mol mol}^{-1}$; Sage, 1994). One individual per species was sampled for gas exchange measurements in each plot when possible (not all species occurred in all plots). Last, we measured plant water potential on detached leaves at pre-dawn (0400–0600 h local time) using a Scholander type pressure chamber (PMS-1000; PMS Instruments, Corvallis, OR, United States). Due to the long-term nature of our research plots, pre-dawn water potential measurements were made outside of the plots on 2–3 individuals of each species at each sampling date near early-, mid- and late-melting plots.

Statistical Analyses

We calculated alpha (average species richness per plot), beta (a measure of species turnover among plots calculated as the ratio of gamma over alpha), and gamma (total species richness across all plots) diversity, and Shannon's H and Pielou's J (Pielou, 1969) as measures of evenness. Diversity metrics are reported in Table 2. All analyses were carried out in R 3.3.2. (R Core Team, 2014). We displayed the relationships between %

slope of plots, snowmelt timing, richness, and % cover across the elevation gradient of our site using the Tps command from the fields package (Nychka et al., 2015). This command uses generalized cross-validation scores to portray relationships between elevation and each variable along contours. We also used non-metric multidimensional scaling (NMS) ordination to examine associations among snowmelt date and cover variables across plots. We did the same for presence/absence of individual species, log-transforming data to account for zero values and high skew (McCune and Grace, 2002). NMS ordinations were conducted using PC-ORD (MjM Software Design; Gleneden Beach, OR, United States).

Species that occurred in ten or more plots were selected for phenological analyses ($n = 19$ spp.). We used a linear mixed effects model to determine whether snowmelt timing explains date of first flower across all 19 species, with snowmelt timing as a fixed effect and plot, quadrant, and species as nested random effects to account for pseudoreplication across quadrants and species. We compared this model to a null (intercept-only model) and used the change in Akaike Information Criterion corrected for small sample sizes (ΔAICc ; Johnson and Omland, 2004; Aho et al., 2014). We subsequently used linear regression to test for correlations between date of first flower and snowmelt timing, and flowering duration and snowmelt timing for individual species. We used an alpha value with Bonferroni correction ($\alpha = 0.025$) to account for non-independence, but note marginally significant values ($\alpha = 0.05$) when appropriate to address our increased concern over Type II error.

We used linear mixed effects models to test for differences in A_{net} with snowmelt timing (i.e., early, mid, and late) and group (i.e., alpine-restricted vs. wide-ranging species), their interaction, and sampling event (i.e., July, August, and September) as fixed

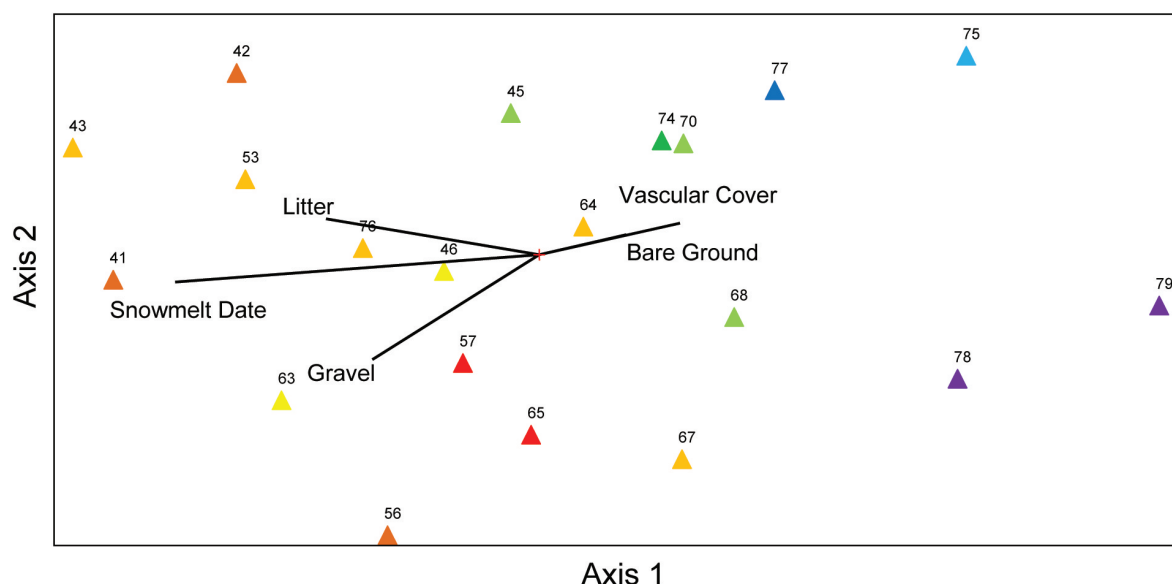


FIGURE 2 | Non-metric multidimensional scaling (NMS) ordination of % cover measurements (i.e., vascular cover, bare ground, litter, gravel, and snowmelt) across the field site illustrating within community gradients. Numbers correspond to plot identifiers and colored triangles indicate snowmelt timing of each plot (warmer colors indicate later snowmelt).

effects. Similar to our phenology models, plot, quadrant, and species were included as nested random effects to account for pseudoreplication across quadrants and species. We compared the full model to simpler versions and used the same ΔAICc approach described above to determine the best-fit model. We carried out the same analysis for R_d , g , C_i , E , and WUE. When full models indicated a fixed effect was important for predicting performance, we compared least-squares means of groupings adjusted for repeated measures using the Tukey method (e.g., July vs. August sampling events, alpine-restricted vs. wide-ranging species, etc.). Last, we used the same mixed model strategy for our pre-dawn water potential measurements. Models were evaluated using the nlme and lsmeans packages (Lenth, 2016; Pinheiro et al., 2018).

RESULTS

Community Responses

Species richness and total % cover by vascular plants differed in their sensitivity to elevation and snowmelt timing (Figure 1), with richness more closely tracking elevation and % cover more closely tracking snowmelt timing. NMS ordination of plot level % cover achieved the greatest reduction in stress with just two axes (Figure 2). The proportion of variance explained by the first two axes was 0.812 and 0.093 (total 0.906 explained). Vascular plant cover, litter, snowmelt timing, and gravel were significantly associated with the ordination axes. Plots with earlier snowmelt exhibited a higher percentage of vascular cover, and lower percentages of litter and gravel cover. Species compositions of plots were determined by snowmelt timing, with ca. 20% of the 45 species occurring only in the earliest or latest melting plots (Figure 3). A subset of ca. 25 species were found across the entire snowmelt gradient and are clustered at the center of the NMS ordination (Figure 3).

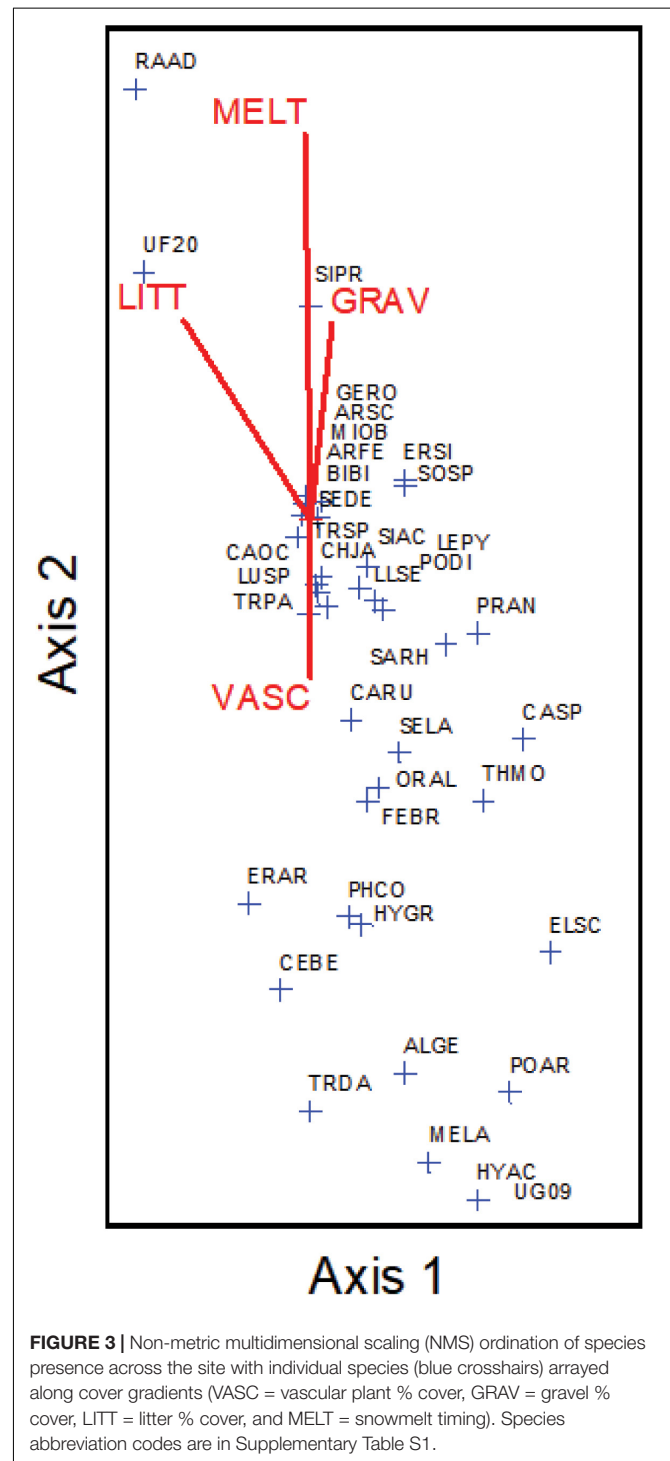
Phenological Responses

Linear mixed models predicting date of first flower with snowmelt timing ($\text{AICc} = 2164.77$) outperformed a null model ($\text{AICc} = 2196.75$, $\Delta\text{AICc} = 31.99$), suggesting that snowmelt timing has predictive power in explaining the date of first flower across our community. The same was true for the duration of flowering across our site (full model $\text{AICc} = 2201.78$, null model $\text{AICc} = 2226.49$, $\Delta\text{AICc} = 24.71$). Of the nineteen species analyzed, twelve showed a significant positive correlation between date of first flower and snowmelt timing ($P \leq 0.025$; with *C. rupestris* and *Trifolium parryi* $P \leq 0.05$; Table 3), while only six species showed a significant negative correlation between flowering duration and snowmelt ($P \leq 0.025$). Of the twelve that showed significant correlations between date of first flower and snowmelt timing, a majority of these species are wide-ranging species (*A. scopulorum*, *B. bistortoides*, *C. rupestris*, *Castilleja occidentalis*, *Erigeron simplex*, *G. rossii*, *Lloydia serotina*, and *Potentilla diversifolia*, *T. parryi*; Table 3) whereas only three are alpine-restricted species (*Chionophila jamesii*, *L. pygmaea*, and *Silene acaulis*). Of the six species that showed a significant correlation between flowering duration and snowmelt, five are

wide-ranging species (*A. scopulorum*, *B. bistortoides*, *Castilleja occidentalis*, *E. simplex*, and *Lloydia serotina*) and only one is alpine-restricted (*L. pygmaea*; Table 3).

Physiological Responses

Plant gas exchange varied substantially through time but was invariant across species and the spatial snowmelt gradient



(Figure 4). The interaction of group and snowmelt timing with sampling event as an additional covariate best predicted photosynthesis ($w_i = 0.99$; Supplementary Table S2). However, photosynthesis did not differ between alpine-restricted and wide-ranging species and photosynthesis declined in all species > 50% throughout the growing season (Figure 4A). Compared to in July, A_{net} was significantly lower in August ($t = 5.41$, $P < 0.001$) and September ($t = 5.20$, $P < 0.001$). Photosynthesis increased when measured at $800 \mu\text{mol mol}^{-1} \text{CO}_2$ compared to $400 \mu\text{mol mol}^{-1} \text{CO}_2$ in September (Figure 5), especially in wide-ranging species (though not statistically different). Increases in photosynthesis were greater in plots that had melted out late in the summer (1.05-fold increase at 800 compared to $400 \mu\text{mol mol}^{-1}$) compared to plots that had melted mid-summer (0.89-fold increase) or in early summer (0.65 increase; Figure 5), though these differences were not statistically significant.

Dark respiration was also best predicted by the full model with the interaction of group and snowmelt timing, with sampling event as an additional covariate ($w_i = 0.70$; Supplementary Table S3). Although respiration decreased by ~50% in all species throughout the growing season, these decreases were not significantly explained by either species groupings or snowmelt timing (Figures 4B,H). Internal-concentration of leaf CO_2 was also predicted best by the full model ($w_i = 0.99$; Supplementary Table S4), but values for the two species groups tracked each other, slightly declining throughout the season. Transpiration was also best predicted by the full model ($w_i = 0.90$;

Supplementary Table S5). Transpiration similarly showed no difference between groups but significantly declined throughout the season; July and August values were 30–50% higher than September values (July: $t = 4.269$, $P < 0.01$; August: $t = 6.70$, $P < 0.001$; Figures 4E,K).

Stomatal conductance and WUE were best explained by group and sampling event (g: $w_i = 0.98$; WUE $w_i = 0.40$; Supplementary Tables S6, S7). Stomatal conductance was higher for wide-ranging species than alpine-restricted species, mostly due to differences early in the summer ($t = -2.05$, $P = 0.04$; Figures 4C,I), though all species exhibited declines in stomatal conductance from early to late in the growing season (all *post hoc* pairwise tests had $P < 0.001$). WUE models with only snowmelt timing and sampling event performed just as strongly ($w_i = 0.36$, $\Delta\text{AICc} = 0.21$; Supplementary Table S7), suggesting that sampling event is largely driving the model's predictive power. WUE values significantly declined from July to August ($t = -5.04$, $P < 0.001$; Figure 4F) but there were no differences between groups, nor were there differences associated with snowmelt timing (Figure 4L). Pre-dawn water potential was best explained by snowmelt timing and sampling event ($w_i = 0.60$; Supplementary Table S8). Last, pre-dawn leaf water potential for all species increased (became less negative) by ca. 50% from July to August ($t = -4.68$, $P < 0.001$; Figure 6) and then significantly decreased by ca. 150% from August to September ($t = 9.00$, $P < 0.001$). However, there were no statistically significant differences due to snowmelt date (early vs. late melt: $t = -2.69$,

TABLE 3 | Linear regressions of date of first flower (Julian day) and flowering duration (number of days) as functions of date of snowmelt (Julian day).

Species	<i>n</i>	First flower					Flowering duration			
		$\bar{x} \pm \text{SEM}$	r^2	Slope	<i>P</i>		$\bar{x} \pm \text{SEM}$	r^2	slope	<i>P</i>
<i>Arenaria fendleri</i>	W	20	198 ± 3	0.129	0.136	0.121	41 ± 5	0.099	0.201	0.176
<i>Artemisia scopulorum</i>	W	20	193 ± 8	0.510	0.657	<0.001***	24 ± 8	0.235	−0.449	0.030*
<i>Bistorta bistortoides</i>	W	19	194 ± 9	0.478	0.782	0.001***	35 ± 9	0.296	−0.579	0.016*
<i>Carex rupestris</i>	W	16	194 ± 12	0.315	0.760	0.037*	28 ± 17	0.100	−0.597	0.271
<i>Castilleja occidentalis</i>	W	14	183 ± 6	0.539	0.537	0.001***	37 ± 15	0.439	−1.144	0.005**
<i>Chionophila jamesii</i>	A	20	197 ± 8	0.326	0.574	0.009**	20 ± 9	0.183	−0.454	0.060
<i>Erigeron simplex</i>	W	20	197 ± 6	0.766	0.670	<0.001***	11 ± 6	0.394	−0.478	0.003**
<i>Festuca brachyphylla</i>	W	15	219 ± 4	0.169	0.169	0.128	12 ± 3	0.172	−0.147	0.124
<i>Geum rossii</i>	W	20	186 ± 7	0.838	0.781	<0.001***	39 ± 6	0.032	−0.142	0.449
<i>Lewisia pygmaea</i>	A	20	184 ± 7	0.604	0.678	<0.001***	21 ± 8	0.269	−0.524	0.019*
<i>Lloydia serotina</i>	W	16	188 ± 8	0.724	0.833	<0.001***	15 ± 8	0.549	−0.668	0.001***
<i>Luzula spicata</i>	W	18	217 ± 1	0.022	0.023	0.554	7 ± 3	0.001	0.015	0.890
<i>Minuartia obtusiloba</i>	A	20	189 ± 3	0.010	0.039	0.680	47 ± 8	0.072	0.264	0.252
<i>Potentilla diversifolia</i>	W	16	194 ± 7	0.411	0.473	0.007**	26 ± 14	0.114	−0.533	0.200
<i>Sedum lanceolatum</i>	W	14	221 ± 8	0.023	0.133	0.604	14 ± 5	0.235	−0.284	0.079
<i>Silene acaulis</i>	A	10	199 ± 5	0.544	0.484	0.015*	10 ± 6	0.003	0.044	0.879
<i>Sibbaldia procumbens</i>	A	12	187 ± 5	0.010	0.216	0.758	26 ± 11	0.124	1.567	0.261
<i>Trifolium parryi</i>	W	10	194 ± 7	0.414	0.539	0.045*	11 ± 7	0.241	−0.404	0.150
<i>Trisetum spicatum</i>	W	20	202 ± 7	0.089	0.269	0.201	32 ± 7	0.005	−0.055	0.773

Mean Julian day of first flower and standard error ($\bar{x} \pm \text{SEM}$) are calculated for each species across all plots it occurred in (*n*). (For \bar{x} , day 183 = 2 July; day 221 = 9 August). First flower: linear regression of date of first flower of each species in each plot as a function of date of snowmelt of each plot. Mean flowering duration (mean number of Julian days: \bar{x}) and standard deviations ($\pm s$) are calculated for each species across all plots it occurred in. Flowering duration: linear regression of flowering duration of each species in each plot as a function of date of snowmelt of each plot. *** ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 . W near a species name indicates that it is a wide-ranging species. A near a species name indicates that it is an alpine-restricted species.

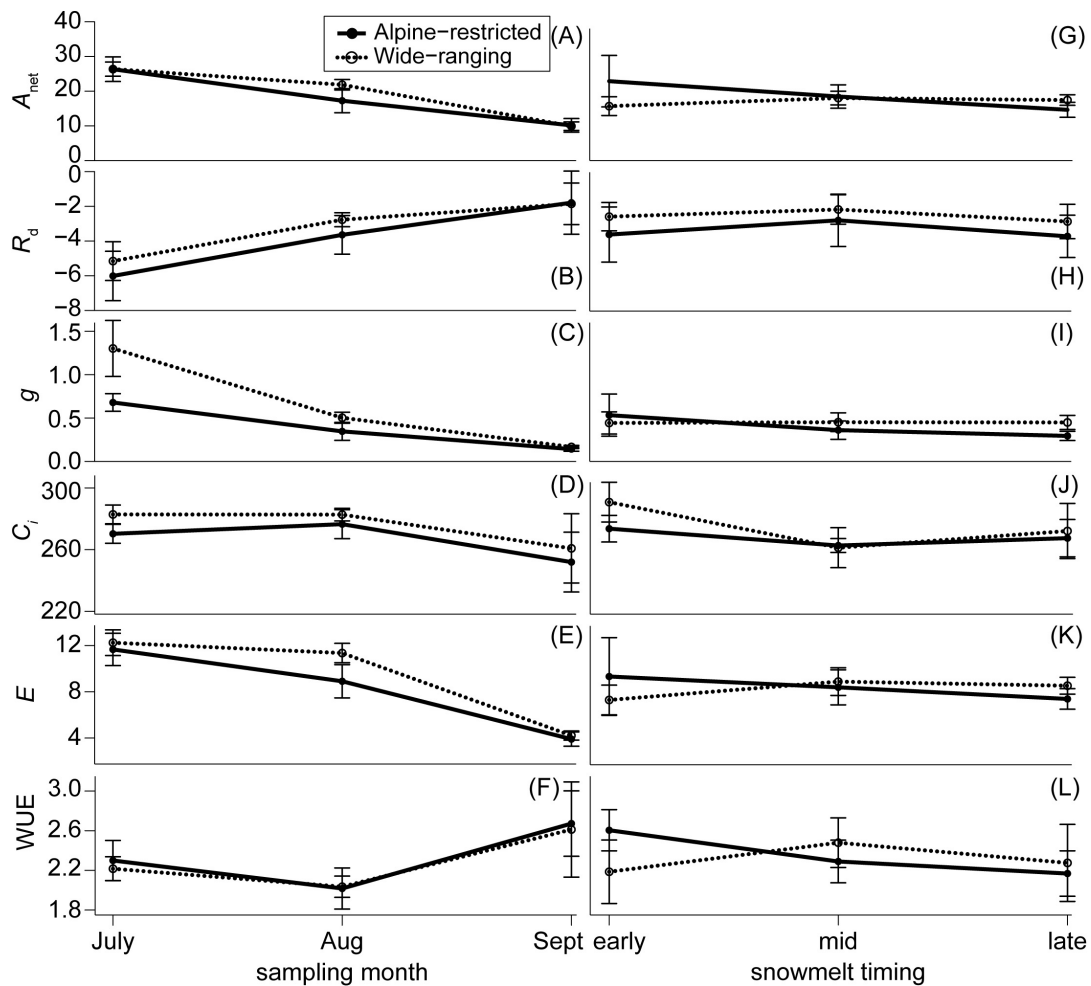


FIGURE 4 | Physiological measurements separated by alpine-restricted (closed circles and solid lines) and wide-ranging species (open circles and dotted lines) by sampling month (A–F) and snowmelt timing (G–L). Means and standard errors are presented for photosynthetic rates (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), dark respiration (R_d ; $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$), stomatal conductance (g ; $\text{mol m}^{-2} \text{s}^{-1}$), CO₂ assimilation (C_i ; PPM), transpiration (E ; $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$), and water-use efficiency (WUE; $\mu\text{mol mmol}^{-1}$).

$P = 0.14$; early vs. mid melt: $t = -2.38$, $P = 0.19$; late vs. mid melt: $t = 0.20$, $P = 0.98$; **Figure 6**).

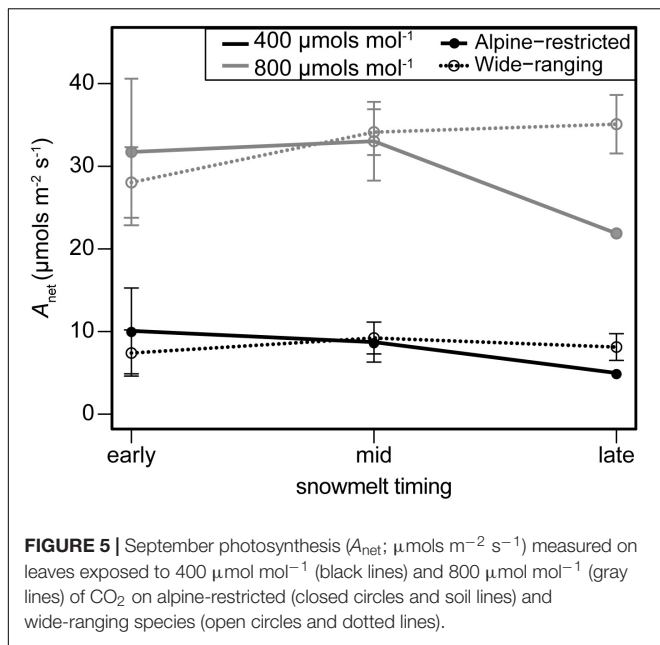
DISCUSSION

Our findings reveal that spatial variation in the composition and peak % cover of this moist alpine meadow community is driven by snowmelt timing. Flowering phenology was also strongly related to snowmelt timing for a majority of species. Leaf physiology tracked seasonal soil dry-down similarly among species, regardless of whether they were alpine-restricted or wide-ranging species. While early season stomatal conductance was higher in wide-ranging species, there were no associated differences in photosynthesis or transpiration, suggesting no strong differences between these groups in physiology.

Previous research in the Mosquito Range in Colorado shows that species richness and total community cover were

significantly greater in early melting microsites (Stanton et al., 1994). Our results corroborate these findings but also suggest that richness can more closely follow elevation gradients than snowmelt. Species richness and peak % cover increased with earlier snowmelt, due to a larger number of wide-ranging species present in earlier melting plots. Interestingly, peak % cover typically declined in areas where topographic depressions led to later snowmelt.

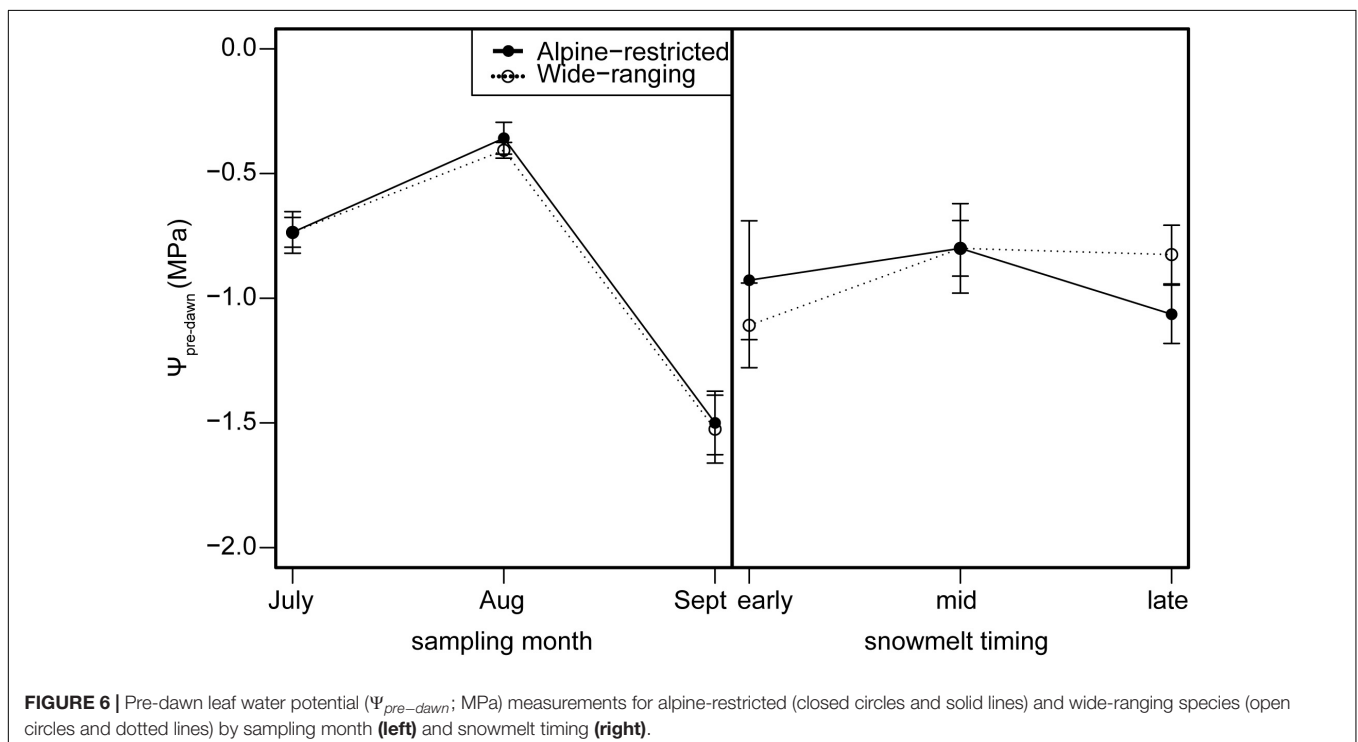
Future climate scenarios project a decrease in the snow-to-rain ratio during winter as a result of warmer temperatures (Stocker et al., 2013). Decreases in snowpack combined with warmer temperatures will ultimately lead to an advance of snowmelt and this has already been observed in montane systems (Barnett et al., 2008; Clow, 2010). Changes in snowmelt timing across a gradient or within a patch, and the associated changes in soil dry-down rates have implications for community structure and function (Pickering et al., 2014). Not only can we expect shifts in productivity, perhaps leading to a more productive alpine



(Kullman, 2010; but see Baptist et al., 2010), but an overall compositional shift that will result in novel alpine communities is also possible (Alexander et al., 2015). Productivity in this system has previously been shown to be sensitive to changes in soil moisture (Winkler et al., 2016a). This was shown using a warming experiment at our site that advanced snowmelt and subsequently led to more rapid soil dry-down rates, thus influencing productivity. Our current study illustrates a similar

effect of snowmelt timing but also reveals spatial structuring of the community across the gradient that contributes to productivity differences. If species are able to locally shift upward in elevation (Lenoir et al., 2008; Kiełtyk, 2017) or seek refuge in nearby microclimates (e.g., local depressions) where snowmelt changes are less pronounced (Opedal et al., 2015), productivity changes will reflect both the immediate effects of earlier snowmelt and the longer term effects of community change.

The flowering phenology of many alpine species worldwide closely tracks environmental cues including snowmelt timing, presumably as a result of pressure from relatively short growing seasons (Kudo, 1991; Walker et al., 1995; Studer et al., 2005; Kudo and Hirao, 2006; Björk and Molau, 2007). Our study reveals that flowering phenology can vary depending on whether a species' range is restricted to the alpine or extends below treeline. We show that wide-ranging species have more flexible flowering phenologies and, in most cases, will flower longer with earlier snowmelt (nine out of twelve species with significant correlations between snowmelt timing and timing of flowering initiation were wide-ranging species) whereas alpine-restricted species appear more conservative with initiation (only 3 out of 12 species showed significant correlations with snowmelt). The same was true for duration of flowering with only one alpine-restricted species and five wide-ranging species showing a significant correlation with snowmelt. Although our results are within one community type, other studies have shown that entire communities can exhibit this relationship where more diverse communities flower earlier and more consistently (i.e., those similar to microsites where snowmelt occurs earlier in our community), while less diverse communities composed primarily of specialists (i.e., those similar to the microsites with



late snowmelt in our community), are largely at the whim of snowmelt timing (Kudo and Hirao, 2006; Venn and Morgan, 2007; Baptist et al., 2010).

Many of the trends we observed in flowering phenology across the snowmelt gradient were species-level responses to snowmelt timing and related abiotic drivers. That said, these plant-environment relationships can sometimes be carried up to functional groups (Iversen et al., 2009; but see Henry and Molau, 1997). For example, nearly all of the graminoid species in our site flowered irrespective of snowmelt timing, flowering later in the season than other species on average and highlighting their late-season, drought-avoidance strategies (Rosbakh et al., 2017). However, these strategies might not always be dependent on environmental conditions experienced during the current growing season. It is estimated that ca. 50% or more of alpine plant species pre-form leaf and flower buds before the growing season begins and oftentimes 1+ years prior (Theodose et al., 1996; Körner, 2003). Since much is theorized and little is known about the prevalence of bud preformation and whether or not it occurs in many of our study species, it is difficult to say to what extent previous-years climate plays a role in the initiation of growth and flowering. However, in order to take advantage of early snowmelt, early flowering species need to maintain a high metabolic readiness while under the snow, which is dependent upon bud preformation (Körner, 1999). *G. rossii* date of first flower had the strongest correlation with date of snowmelt. *G. rossii* is not only a dominant species in our plots, but also has a long period of growth for each leaf and inflorescence (3 years) from initiation through senescence as a result of bud preformation (Meloche and Diggle, 2001). Along with temperature, the role of photoperiodism is certainly a main driver in the initiation of seasonal growth in many alpine plants (Keller and Körner, 2003). However, our data suggest that wide-ranging species may be taking advantage of growth initiation immediately after snowmelt in order to establish themselves as dominants in the community (both *G. rossii* and *C. rupestris* alternate dominance or co-dominate in our plots in terms of cover). Further studies are needed to elucidate the role of bud preformation in species at Niwot Ridge in order to concretely say what the extent of the impact of earlier snowmelt will be on these species, as well as those species whose phenologies are not correlated with snowmelt date.

Advances in snowmelt timing may provide a longer period for growth, but may also expose plants to an increased number of spring frost events that hinder growth and performance (Wipf et al., 2009; Sierra-Almeida and Cavieres, 2010). Few studies have examined alpine plant physiological connections to snowmelt timing but several studies have looked at related environmental variables that track snowmelt gradients like the one in our study (e.g., early season temperatures, species temperature optima, soil moisture status; Germino and Smith, 2000, 2001; Shen et al., 2009; Shi et al., 2010). However, our results suggest that snowmelt timing does not have a large impact on plant performance and, instead, physiological adjustment occurs in both alpine-restricted and wide-ranging species as the season progresses and environmental conditions change.

It is likely that the species in our community are displaying some level of physiological compensation by altering assimilation rates and water use along the snowmelt gradient (Ren et al., 2010). In addition to influencing composition and phenology, microclimate can also regulate plant physiological performance (Germino and Smith, 2001). We did detect higher stomatal conductance rates in wide-ranging species compared to alpine-restricted species early in the season. Even so, species overwhelmingly adjusted photosynthetic, conductance, transpiration rates, and leaf water potentials as water availability declined during the season. Water-use efficiency similarly declined from early to mid-season but increased late in the growing season when soils were driest and plants switched to investing in reproductive structures. Together, our physiological data confirm that alpine plant species are highly adapted to large amounts of environmental variation (Körner, 1999) and that responses to short-term change may not be as apparent if physiological thresholds are not crossed (Walther et al., 2002).

In this sense, the potential for species replacement or invasion increases as temperatures warm or other driving factors (e.g., soil moisture status) change with shifts in snowmelt timing. For example, snowmelt changes and subsequent increases in soil dry-down rates have already led to the encroachment of an invasive subalpine dwarf bamboo in Hokkaido, Japan (Winkler et al., 2016b). Changes in microclimates in alpine settings have the potential to negatively impact current community members by creating unfavorable conditions and creating stepping stones for non-native species to invade (Lembrechts et al., 2017). Similarly, warmer temperatures may lead to shifts in the position of local treeline (Kueppers et al., 2017), though these shifts may largely depend on available soil moisture (Moyes et al., 2013, 2015). Nonetheless, our results suggest that alpine community composition and peak % cover are strongly structured by spatio-temporal patterns in snowmelt timing, and may not change as rapidly as climate due to long generation times.

AUTHOR CONTRIBUTIONS

LK, MG, KR, and RB conceived and designed the experiments. RB collected the data. DW and RB analyzed the data. DW, LK, and RB wrote the manuscript. MG and KR provided editorial advice.

FUNDING

This research was supported by the Office of Science, (BER), United States Department of Energy. Additional funding was provided by the Environmental Systems Graduate Group and Graduate Division at the University of California, Merced.

ACKNOWLEDGMENTS

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the United States Government. We thank the Mountain Research

Station and Niwot Ridge LTER at the University of Colorado, Boulder for logistical support. Additionally, we thank all who assisted in data collection and maintenance of the experiment, especially M. Barlerin, S. Barlerin, S. Ferrenberg, A. Dixon, A. Faist, and A. Farnham.

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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.2018.01140/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Concentration of Non-structural Carbohydrates, N, and P in *Quercus variabilis* Does Not Decline Toward Its Northernmost Distribution Range Along a 1500 km Transect in China

OPEN ACCESS

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 26 January 2018

Accepted: 12 September 2018

Published: 17 October 2018

Citation:

Liu J-F, Deng Y-P, Wang X-F,
Ni Y-Y, Wang Q, Xiao W-F, Lei J-P,
Jiang Z-P and Li M-H (2018) The
Concentration of Non-structural
Carbohydrates, N, and P in *Quercus
variabilis* Does Not Decline Toward Its
Northernmost Distribution Range
Along a 1500 km Transect in China.
Front. Plant Sci. 9:1444.
doi: 10.3389/fpls.2018.01444

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Understanding the mechanisms that determine plant distribution range is crucial for predicting climate-driven range shifts. Compared to altitudinal gradients, less attention has been paid to the mechanisms that determine latitudinal range limit. To test whether intrinsic resource limitation contributes to latitudinal range limits of woody species, we investigated the latitudinal variation in non-structural carbohydrates (NSC; i.e., total soluble sugar plus starch) and nutrients (nitrogen and phosphorus) in mature and juvenile Chinese cork oak (*Quercus variabilis* Blume) along a 1500 km north-south transect in China. During the growing season and dormant season, leaves, branches, and fine roots were collected from both mature and juvenile oaks in seven sites along the transect. Tissue concentration of NSCs, N, and P did not decrease with increasing latitude irrespective of sampling season and ontogenetic stage. Furthermore, higher levels of NSCs and N in tissues of juveniles relative to mature trees were found during the dormant season. Partial correlation analysis also revealed that during the dormant season, soluble sugar, NSC, the ratio of soluble sugar to starch, and tissue nitrogen concentration were correlated positively with latitude but negatively with precipitation and mean temperature of dormant season. Our results suggest that carbon or nutrient availability may not be the driving factors of the latitudinal range limit of the studied species. Further studies should be carried out at the community or ecosystem level with multiple species to additionally test the roles of factors such as regeneration, competition, and disturbance in determining a species' northern distribution limit.

Keywords: *Quercus variabilis*, latitudinal distribution range, ontogeny, non-structural carbohydrate, nutrient

INTRODUCTION

In the context of climate change, altitudinal and latitudinal gradients can be used as natural laboratories to deduce species' responses to global warming (Körner, 2007; De Frenne et al., 2013). Nowadays, northward and upward shift of species distribution has been observed in a wide range of studies (Kelly and Goulden, 2008; Chen et al., 2011; Boisvert-Marsh et al., 2014; Desprez et al., 2014; Rinas et al., 2017; Sittaro et al., 2017), but the underlying physiological mechanisms are under debate. Therefore, we may use geographical gradients to explore the mechanisms for species' distribution under current conditions, and thus to understand and forecast the responses of species' distribution to predicted climate change.

Compared to a few eco-physiological evidences for the formation of a species' northernmost distribution, altitudinal tree-line formation has attracted lots of studies during the last several decades, and several important hypotheses have been proposed (Li and Krauchi, 2005). Notably, two mutually exclusive hypotheses: the Carbon Limitation Hypothesis (CLH) (Stevens and Fox, 1991) and the Growth Limitation Hypothesis (GLH) (Körner, 1998; Hoch and Körner, 2003), have been extensively tested, as they both have the potential to be applied worldwide (Körner, 1998) due to intrinsic response or adaptation strategies of plants to a variety of environmental factors at the alpine tree-line. Non-structural carbohydrates (NSC, sum of starch and total soluble sugars) and nutrients (nitrogen or phosphorus) of plant tissues were generally used to evaluate the carbon or nutrient status along altitudinal gradients to test the hypotheses mentioned above (Hoch and Körner, 2003, 2012; Li et al., 2008a; Sullivan et al., 2014; Fajardo and Piper, 2017). NSC, formed during photosynthesis, could act as substrate for respiration to provide the energy needed for growth and maintenance processes (Körner, 2003); and the level of its two main components, soluble sugars and starch, could reflect the balance between carbon gain and carbon utilization and loss within a plant, representing a tree's capital for growth and acting as a buffer during insufficient source activities due to environmental stress (Li et al., 2002; O'Brien et al., 2014). Meanwhile, nitrogen and phosphorus are the two most limiting elements to terrestrial vegetation (Reich and Oleksyn, 2004), which are not only directly related to carbon assimilation and allocation but also indirectly to stress tolerance or resistance (DeHayes et al., 1989; Villagra et al., 2013; Yan et al., 2016). However, at a single species level, whether the factors in determining the latitudinal distribution range are also associated with carbon and nutrient status still remain unclear.

Apart from the vital roles of carbon and nutrients to support plant survival and growth, ontogenetic variations in responses to environment variability or stress have gained more attention (Niinemets, 2010; le Roux et al., 2013; Klockmann et al., 2017). To date, numerous studies have already reported ontogenetic variations in carbon assimilation and allocation (Portsmouth et al., 2005; Steppe et al., 2011), resource use strategies (Gedroc et al., 1996; Rivas-Ubach et al., 2012), and stress tolerances (Cavender-Bares and Bazzaz, 2000; Niinemets, 2010). Compared to adult trees, however, less attention has been paid to early life stages (e.g., seedlings or saplings), which are more sensitive to

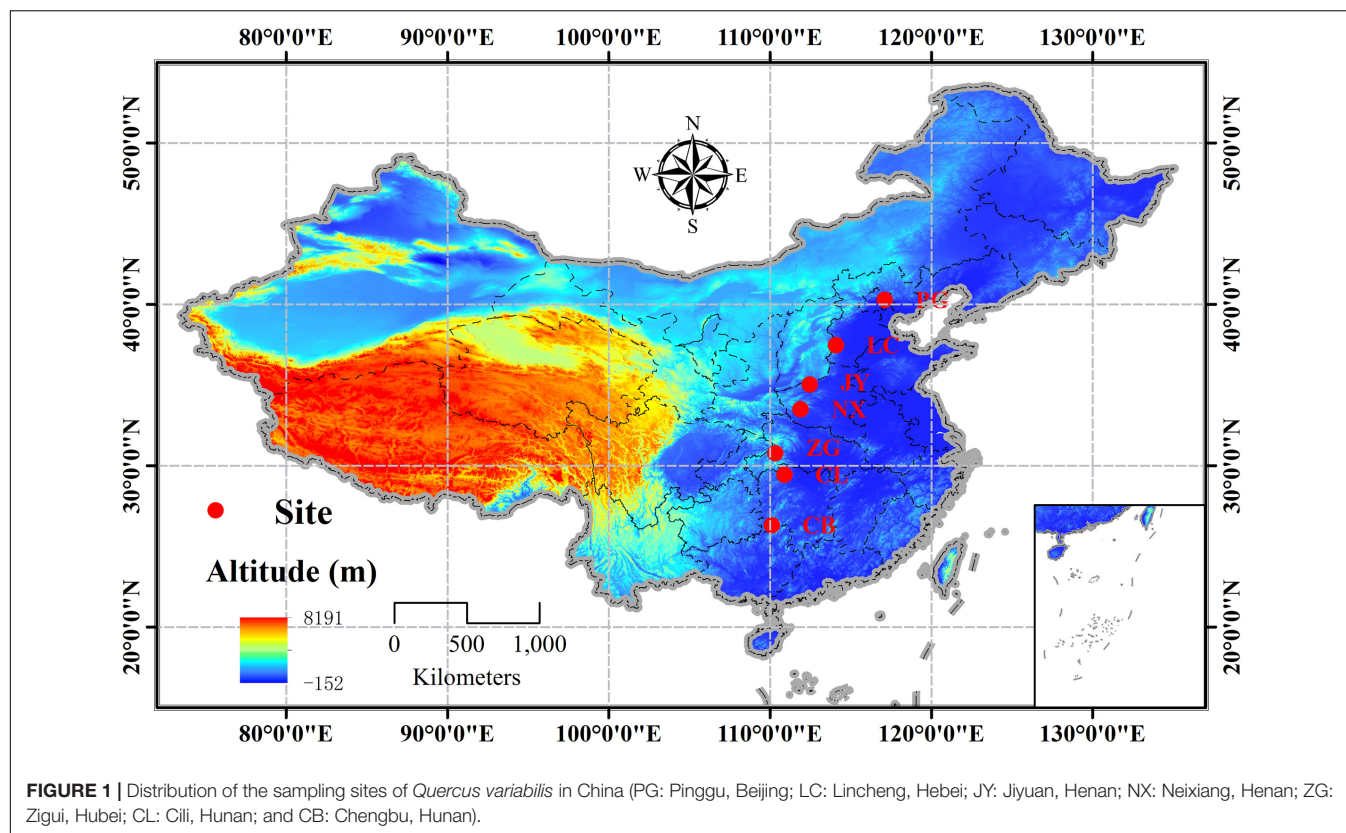
environmental changes or stresses (Niinemets, 2010). Although adult trees may persist over hundreds of years, if seedlings or saplings fail to keep pace with the rate of rising temperature or climate change (Zhu et al., 2012; Bell et al., 2014; Máliš et al., 2016), in the long-term the distribution range of a species will shrink. Hence, to predict the responses of tree species to climate change, disentangling ontogenetic variations along environmental gradients is indispensable for a more profound understanding of adaptation strategies among different life stages, as well as for a better understanding of the mechanisms in determining the latitudinal range.

The Chinese cork oak (*Quercus variabilis* Blume) is one of the most widely distributed tree species in eastern Asia, stretching from approximately 24° to 40°N and 97° to 140°E (Chen et al., 2012). The different regions where the species is present differ greatly in terms of climatic and edaphic conditions, providing an ideal situation for studying the spatial patterns of carbon or nutrient allocation within a single widespread species. In the present study, leaves, branches, and fine roots in healthy juvenile and mature trees in seven sites along a designed 1500 km north-south transect in China, were collected and analyzed. Our aims were to answer the questions of (1) how the carbon or nutrient status vary along the south-north transect; and (2) whether there is a consistent difference in NSCs and nutrients between juvenile and mature trees across latitudes. Our results will contribute toward understanding and predicting the impacts of climate change on the range dynamics of woody plants along latitudinal gradients.

MATERIALS AND METHODS

Sampling Protocol

A south-north transect covering 14 latitudinal degrees (~26°–40°N) (c.1500 km) was setup in the natural distribution range of *Q. variabilis* in China, and seven sites were designed along the transect with a latitudinal interval of ~2° (**Figure 1** and **Supplementary Table S1**). Using the geographical location-based method by Gong and Jian (1983), we calculated the phenological date for each site (**Supplementary Table S1**), so that we collected samples at the same phenological stage across the seven sites to avoid effects of plant phenological variation or bias on carbon or nutrient status (Bansal and Germino, 2009; Bazot et al., 2013). The sampling tasks were conducted on two age classes (juvenile and mature) of natural stands during the mid-growing season (August, 2014) and the dormant season (Dec, 2014). At each site, three 50 m × 50 m temporal plots ($n = 3$), with a minimum distance of 10 km from each other, were set up. Within each plot, three to four canopy trees of the same age class, without browsing and other damages, were selected. The sampling processes were identical for the two sampling seasons. From each sample tree, three to four upper and outer-most sun-exposed branches were cut to collect the leaves (only for growing season) and branches (1-2a). Fine roots (<5 mm in diameter) attached to coarse roots of each sample tree were manually excavated using a mini-spade and carefully collected. All samples were stored in a cool box until they were taken to the laboratory. The same tissue from



individuals in the same age class collected from the same site was pooled as one mixed sample. All samples were heated in a microwave oven at 600 W for 60 s, and then dried at 65°C for 72 h and ground to pass a 0.20 mm sieve for further analyses.

Biochemical Analysis

Total Soluble Sugars and Starch

The powdered material (~0.10 g) was put into a 10 ml centrifuge tube, and 5 ml of 80% ethanol was added. The mixture was incubated at 80°C in a water bath shaker for 30 min, and then centrifuged at 4000 rpm for 5 min. The pellets were extracted two more times with 80% ethanol. Supernatants were retained, combined, and stored at -20°C for soluble sugar determinations. The ethanol-insoluble pellet was used for starch extraction. Glucose was used as a standard. Soluble sugars were determined using the anthrone method (Seifter et al., 1950). The starch concentration was measured spectrophotometrically at 620 nm using anthrone reagent, and was calculated by multiplying the glucose concentrations by the conversion factor of 0.9 (Osaki et al., 1991). The concentration of soluble sugars and starch was described on a dry matter basis (mg g^{-1} DW).

Total Nitrogen and Phosphorus

For the determination of tissues' nitrogen (N) and phosphorus (P) concentrations (mg g^{-1} DW), finely ground material (~50 mg) was first digested with H_2SO_4 and then H_2O_2 for further analysis. The nitrogen concentration was then measured using the Kjeldahl method (Kjeltec 2200, FOSS, Sweden),

while the phosphorus concentration was determined with the molybdenum blue spectrophotometric procedure (6505 UV spectrophotometer, United Kingdom) (Page, 1982).

Environmental Data

The monthly climatic data (2014) were interpolated with the kriging method (Matheron, 1963) from 675 national weather stations around China (download from¹) using GIS software (ArcGIS v10.0, Esri, United States). The soil data was obtained from the gridded Global Soil dataset (30 arc-second resolution) which was developed by Shangguan et al. (2014)². The special values of each sampled plot were then extracted according to the geolocation information (latitude and longitude) of the plot. The climatic variables, monthly mean temperature (°C) and monthly mean precipitation (mm), were divided into two parts as one for the growing season (April to September; represented as gT mean and gpPT) and another for the dormant season (October to December; denoted as dT mean and dpPT). The soil variables, on the other hand, included total nitrogen (SN, %) and total phosphorus (SP, %) for each plot.

Statistical Analyses

All statistical analyses were conducted using R statistical software (RStudio version 1.0.143³). Shapiro-Wilk and Bartlett's tests

¹<http://data.cma.cn>

²<http://globalchange.bnu.edu.cn>

³<http://www.rstudio.com/>

were used to test for normality and homogeneity of variances, respectively, and it was found that all data met the assumption for further variance analysis. We utilized a linear mixed effects model with tree age (juvenile and mature trees), sampling site (latitudinal gradient), and season (growing season: Aug; and dormant season: December) as fixed effects and individuals as a random effect (R package 'lme4'). The response variables included soluble sugar, starch, NSC, nitrogen, and phosphorus in the current-year leaves, branches, and fine roots. The mean and standard error were given if necessary. For both the sampling seasons, a partial pairwise correlation analysis was performed to explore the correlations between the response variables with the environmental factors (e.g., climate or soil), where bio-factors (tree age and tree tissue) were treated as covariant (R package 'psych'). The contributions of all the environmental and biological factors to variations of responsible variables were performed by the redundancy analysis (RDA). The variation partitioning was achieved by means of partial redundancy analysis (pRDA) to extract the pure effect of environment factors and the pure effect of bio-factors (R package 'vegan').

RESULTS

Non-structural Carbohydrate and Its Components

The tree age significantly affected leaf soluble sugars and leaf NSC concentration, as well as branch starch, but did not impact root NSCs (Table 1). The sampling season and the interaction between sampling season and tree age significantly influenced the NSCs in the branches and fine roots (Table 1). For instance, root NSC in both juveniles and mature trees was significantly higher in December than in August ($p < 0.001$) (Supplementary Figure S1). However, during the growing season, root NSC in juveniles ($30.34 \pm 1.46 \text{ mg g}^{-1}$) was significantly lower than that in mature trees ($42.79 \pm 3.06 \text{ mg g}^{-1}$, $p < 0.05$), whereas it showed an opposite result ($80.61 \pm 4.79 \text{ mg g}^{-1}$ for juveniles and $60.52 \pm 2.94 \text{ mg g}^{-1}$ for mature trees, $p < 0.001$) during the dormant season. On the other hand, the NSCs including the ratio of soluble sugars to starch varied strongly with latitude and tissue type (Figure 2 and Supplementary Figures S2–S4). The results from simple linear models indicated that the NSC of various tissues for both life stages did not show any decreasing trend with increasing latitude (Figure 2). Inversely, the NSC in the branches and roots of juveniles significantly or marginally significantly increased with the increasing latitude for both sampling seasons (Figure 2).

Nitrogen, Phosphorus Concentrations and Their Ratios

The tree age had significant impacts on N, P, and N:P ratio of the studied tissues with exception of leaf N and branch N (Table 1 and Supplementary Figure S5). Mature trees generally had higher P concentration across tissues than juveniles, especially during the dormant season (Supplementary Figure S5). During the growing season, the N, P, and N:P ratio in the leaves of

mature trees were $18.93 \pm 0.32 \text{ mg g}^{-1}$, $1.63 \pm 0.18 \text{ mg g}^{-1}$, and 14.27 ± 1.48 , respectively, whereas, the corresponding values for juveniles were $18.54 \pm 0.36 \text{ mg g}^{-1}$, $1.13 \pm 0.07 \text{ mg g}^{-1}$, and 17.71 ± 1.08 , respectively (Supplementary Figure S5). The P and N:P ratio in various tissues were interactively affected by tree age and latitude (Table 1), and N:P ratio in the leaves and roots of mature trees during the growing season increased significantly with latitude (Supplementary Figure S6). The tissues' N varied significantly with latitude (Table 1 and Figure 3), while root N of both life stages increased with increasing latitude for both sampling seasons with the exception of mature trees' roots in the dormant season (Figure 3).

Correlation Between Non-carbohydrates, Nutrients, and Environmental Factors

The season transition strongly affected the intensity of correlations between dependent variables with environmental variables (Figures 4, 5). During the growing season, tissues' NSC and N have no significant correlations with the environmental factors, e.g., geographical, soil, or climatic variables (Figure 4A). The ratios of soluble sugar to starch were negatively correlated with precipitation of growing season. The ratios of N to P were positively correlated with latitude and longitude but negatively correlated with elevation, soil N, soil P, precipitation, and mean temperature of growing season (Figure 4A). The partial RDA revealed that only a total of 26.2% variations of dependent variables were jointly explained by environmental factors (17.5%) and bio-factors (8.3%) (Supplementary Figure S7A). Nevertheless, during the dormant season, soluble sugar, NSC, the ratio of soluble sugar to starch and tissue nitrogen were correlated positively with latitude and longitude but negatively with elevation, soil N, soil P, precipitation, and mean temperature of dormant season (Figure 4B). A total of 48.2% variations of dependent variables were jointly explained by environmental (27.5%) and bio-factors (20.6%) (Supplementary Figure S7B).

DISCUSSION

The present study showed that NSCs (total soluble sugars, starch, and NSC) in woody tissues (branches and fine roots) of both life stages had higher concentrations in the dormant season than in the growing season along the latitudinal gradient (Supplementary Figure S1). Our results were consistent with Martínez-Vilalta et al. (2016) who compiled data from 121 studies including 177 species under natural conditions and found that NSCs varied seasonally, with a general increase during winter months. Compared to evergreen species, deciduous trees generally stored more NSC in the tissues (e.g., stem or root) to withstand low temperature in the coming winter and to support bud-break and shoot growth in the early spring (Klein et al., 2016). On the other hand, higher level of NSC in reserved tissues during the dormant season may attribute to lower level of growth and maintenance respiration due to low temperature in the winter, with more starch broken down into soluble sugars to promote cold tolerance by adjusting the intracellular osmotic concentration (Morin et al., 2007), as shown by an increase in the

TABLE 1 | Effects of age (*juvenile* and *mature*), latitude, sampled season (growing season: Aug, 2014; and dormant season: Dec, 2014) and their interactions on NSCs, nutrients over tissues revealed by linear mixed effects models with individuals as random factors.

Source of variation		Soluble sugar			Starch			NSC			SS			Nitrogen			Phosphorus			N:P		
		df	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P				
Leaf																						
Age	1	9.705	0.004	1.526	0.227	10.683	0.003	7.415	0.011	1.449	0.232	10.571	0.003	7.447	0.011	0.000						
	6	4.747	0.002	5.247	0.001	5.973	0.000	3.241	0.015	5.524	0.001	3.228	0.015	6.666	0.000	0.000						
	6	0.714	0.642	1.873	0.121	0.281	0.941	1.136	0.367	4.135	0.005	2.174	0.076	2.820	0.028	0.000						
Branch																						
Age	1	3.042	0.087	4.820	0.032	0.094	0.761	2.161	0.147	0.094	0.761	118.33	0.000	35.730	0.000	0.000						
	6	20.027	0.000	18.205	0.000	7.386	0.000	24.459	0.000	6.468	0.000	14.958	0.000	16.909	0.000	0.000						
	1	455.16	0.000	175.37	0.000	607.45	0.000	2.022	0.161	4.920	0.031	98.364	0.000	15.422	0.000	0.000						
Age × latitude	6	1.111	0.368	2.507	0.032	1.464	0.207	1.325	0.261	1.255	0.293	12.790	0.000	5.282	0.000	0.000						
	1	3.996	0.050	15.175	0.000	0.136	0.714	9.841	0.003	3.528	0.066	90.777	0.010	16.029	0.000	0.000						
	6	6.261	0.000	10.860	0.000	5.602	0.000	10.797	0.000	1.528	0.186	10.381	0.000	2.563	0.029	0.002						
Age × latitude × season	6	0.549	0.769	0.768	0.598	0.567	0.755	0.166	0.985	0.882	0.514	11.385	0.000	3.907	0.002	0.000						
	Root																					
	Age	1	0.015	0.903	1.378	0.246	1.093	0.301	3.741	0.059	4.051	0.049	47.490	0.000	93.730	0.000	0.000					
6		10.473	0.000	2.674	0.025	8.357	0.000	3.331	0.008	6.754	0.000	2.342	0.044	13.648	0.000	0.000						
1		347.83	0.000	34.088	0.000	263.20	0.000	6.886	0.011	16.685	0.000	28.173	0.000	0.183	0.670	0.001						
Age × latitude	6	1.374	0.243	4.389	0.001	2.646	0.026	4.572	0.001	0.534	0.780	2.990	0.013	4.253	0.001	0.000						
	1	69.450	0.000	5.537	0.022	49.532	0.000	7.979	0.007	2.950	0.091	23.975	0.000	34.849	0.000	0.000						
	6	5.011	0.000	4.091	0.002	5.023	0.000	3.310	0.008	1.297	0.274	2.722	0.022	3.571	0.005	0.000						
Age × latitude × season	6	1.489	0.219	2.309	0.070	1.271	0.293	1.556	0.200	1.026	0.418	2.057	0.073	1.500	0.196	0.000						

Significance levels of less than 0.05 are identified in bold.

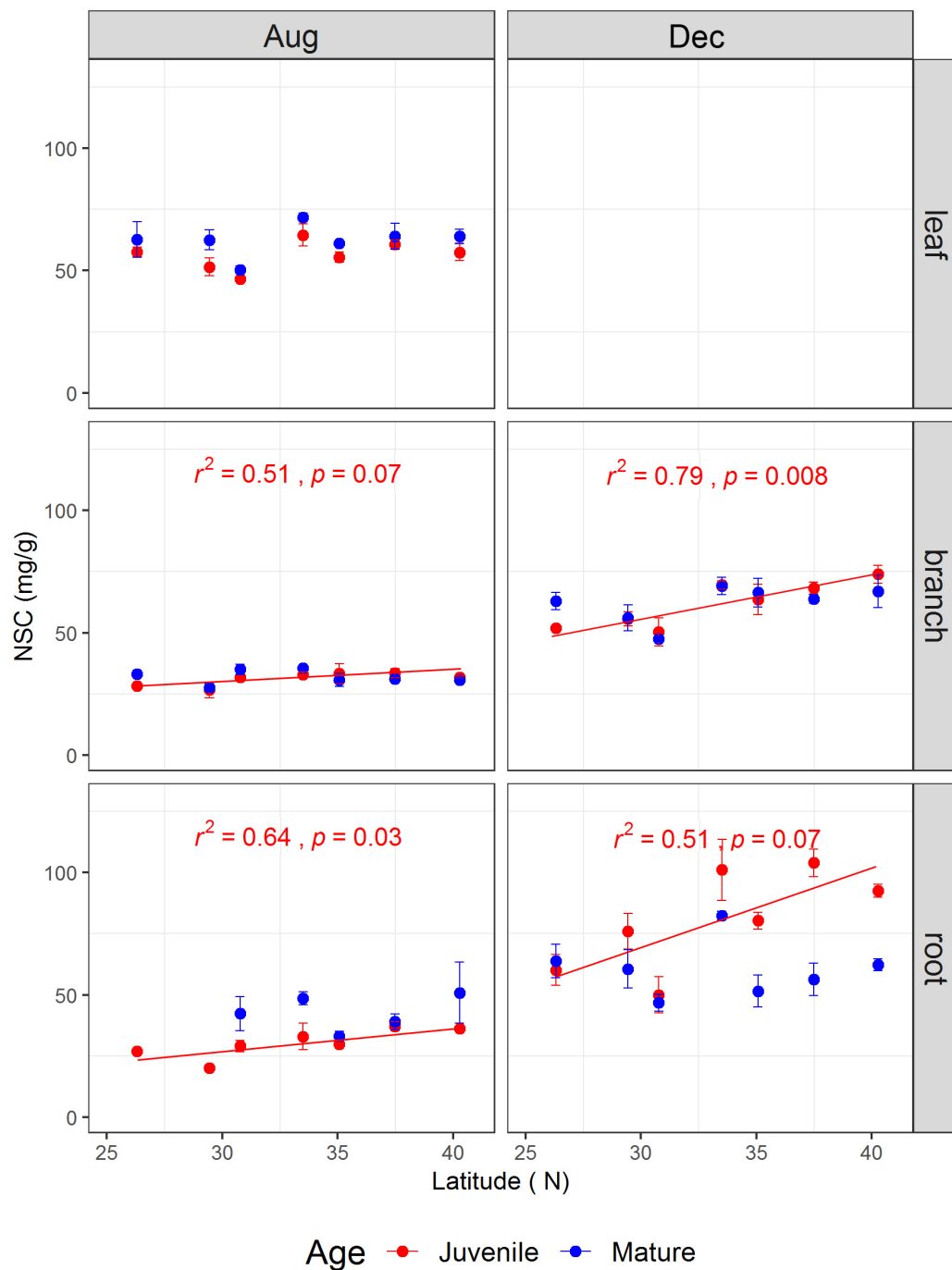
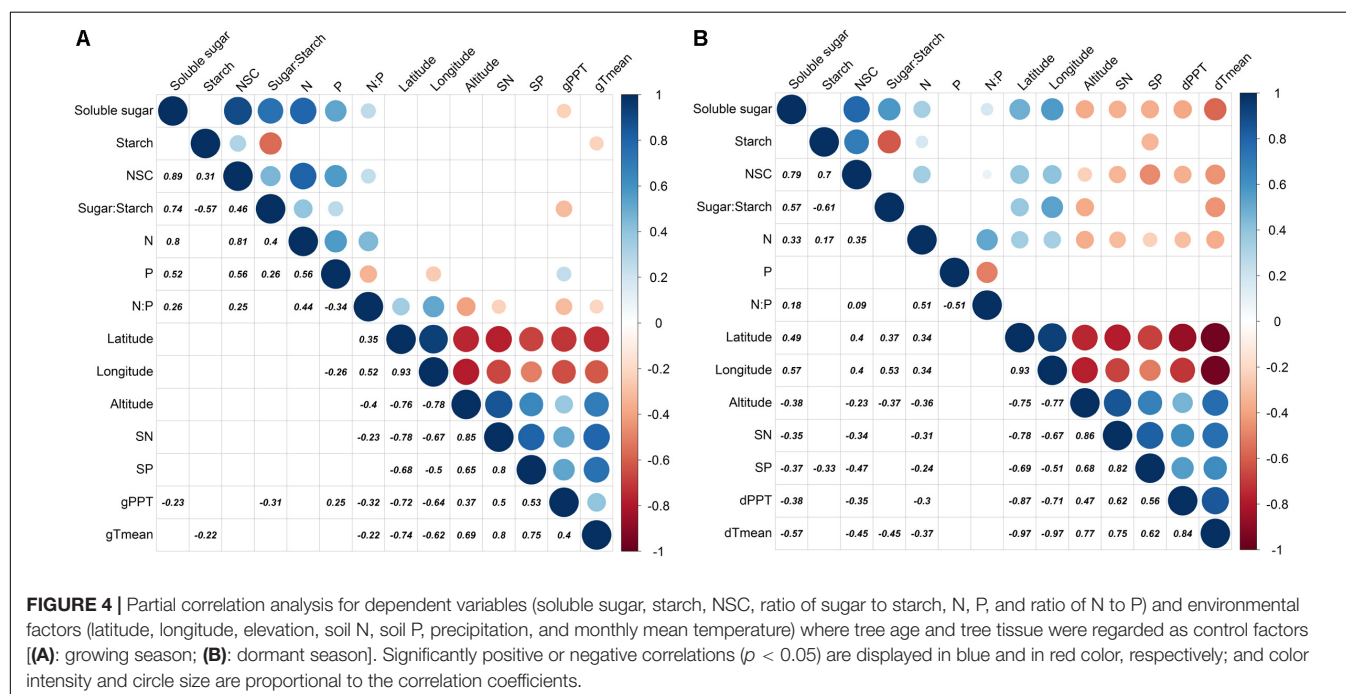
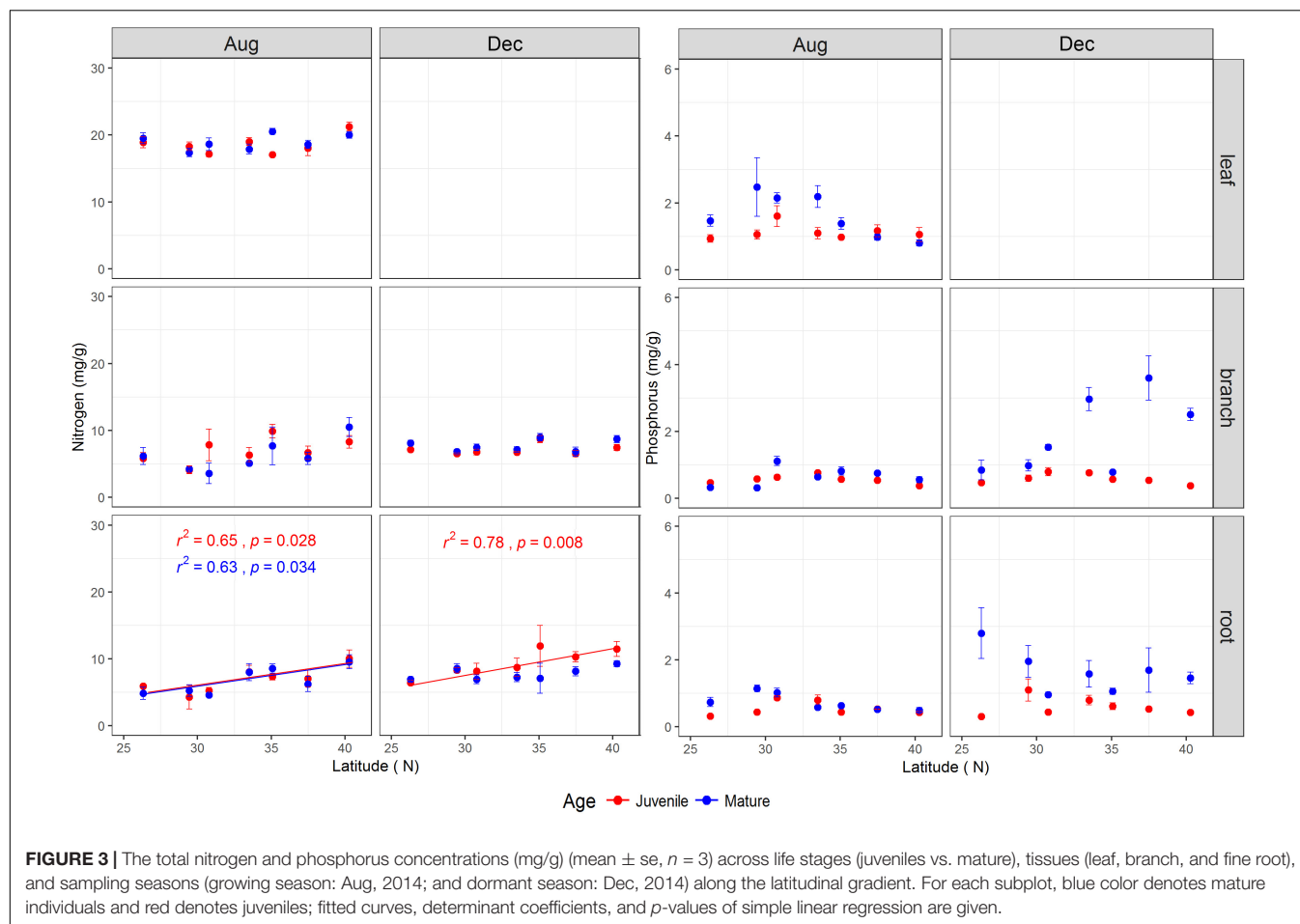
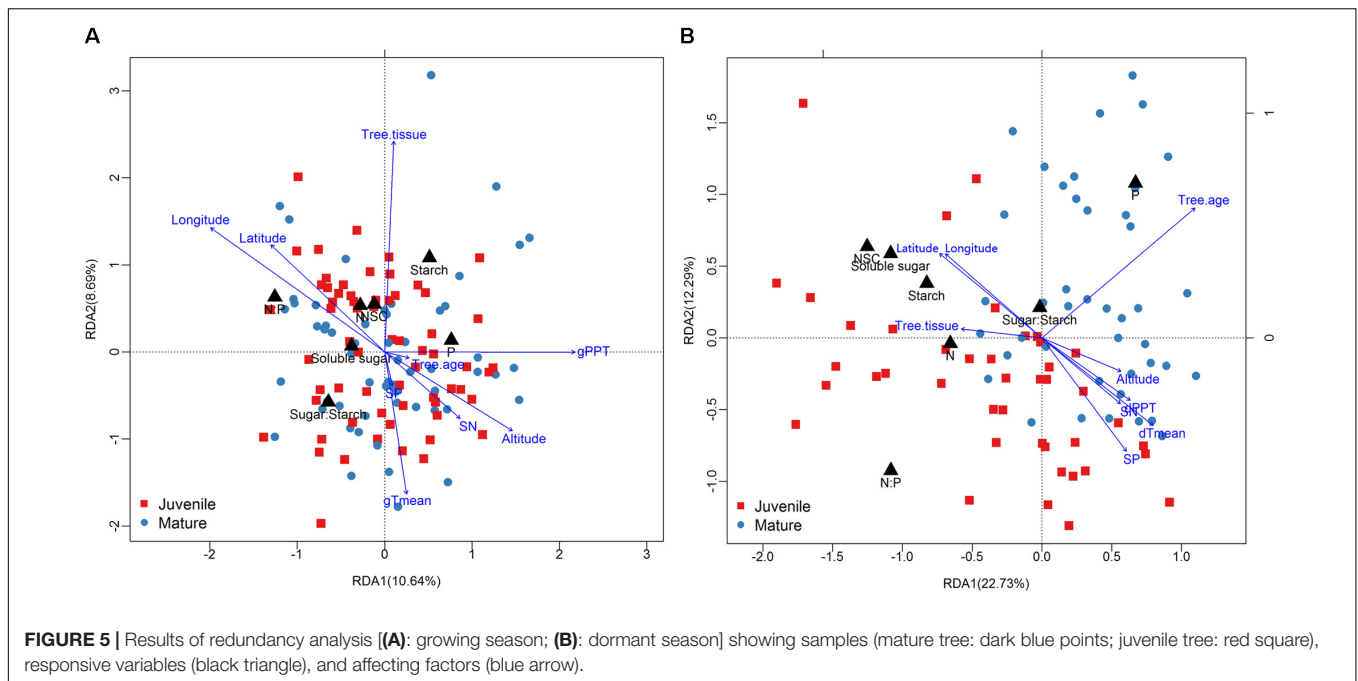


FIGURE 2 | The non-structural carbohydrate concentrations (mg/g) (mean \pm se, $n = 3$) across life stages (juveniles vs. mature), tissues (leaf, branch, and fine root), and sampling seasons (growing season: Aug, 2014; and dormant season: Dec, 2014) along the latitudinal gradient. For each subplot, blue color denotes mature individuals and red denotes juveniles; fitted curves, determinant coefficients, and p -values of simple linear regression are given.

sugar-starch ratios in juvenile roots in our study (**Supplementary Figure S4**). Juveniles' roots had lower NSC concentration in the growing season but higher NSC concentration in the dormant season than that of mature individuals, especially for north populations (**Figure 2**), indicating that juveniles are more sensitive to seasonal transition than mature trees. This further

suggests that juveniles may use a strategy to invest more NSC into fast growth (thus leading to lower level of NSC in juveniles than in mature trees during the growing season) to get a competitive advantage with relatively larger body size in a community. During the dormant season, juveniles generally had a higher level of NSC to cope with low temperature because of their





relatively younger tissue and smaller body size (i.e., smaller carbon pool size) (Bansal and Germino, 2009, 2010). On the other hand, Li et al. (2008b) proposed that trees growing at the elevational or the latitudinal climate limit rely not only on the total NSC concentration but also require a sufficiently high sugar-starch ratio to overwinter successfully. Our results support this view, as both life stages maintained comparable high ratio of soluble sugars to starch in the dormant season (**Supplementary Figure S1**).

Although there exists considerable disparities between altitudinal and latitudinal gradients (Jump et al., 2009), both gradients are mainly dominated by temperature. In this regard, the altitudinal tree-line hypotheses (e.g., GLH and CLH) present important reference values to deduce the driven mechanism for latitudinal range. In the present study, we found that tissue NSC did not exhibit decreasing trends for both life stages along the latitudinal gradient. Nevertheless, NSC in the woody tissues of juveniles increased significantly with increasing latitude during both the sampling seasons, suggesting that carbon limitation cannot act as a determinant driver to north latitudinal range of the species studied. This result fails to support the CLH along latitudinal gradients. Li et al. (2016) also observed weekly increasing latitudinal trends of leaf NSCs from tropical to cold temperate forests at the levels of species and plant functional groups. Alternatively, other factors, such as recruitment limitation, rather than NSC at the north edges may contribute to the oak's north-latitudinal limit formation. Our previous study found that the relative densities of seedlings of the species studied were significantly lower in the northern edge than in the core populations (Gao et al., 2017).

Apart from the role of carbon status in determining the range limit, nutrient shortage is another important factor to elucidate the issue which not only limited carbon assimilation

but also limited tissue development (McNown and Sullivan, 2013; Sullivan et al., 2014). Ontogenetic variations in tissue P but not in tissue N were found in this study (**Supplementary Figure S5**), where mature trees had higher tissue P concentration than juveniles, especially during the dormant season, consequently leading to relatively lower N:P ratio occurring in mature trees. This result is inconsistent with Noh et al. (2007) who found that N and P concentrations in tissues of *Q. acutissima* significantly decreased with tree age or size. Indeed, plant nutrient demand and morphological structure (e.g., root morphology), which vary with ontogeny (Álvarez-Yépez et al., 2014), may contribute to the discrepancies mentioned above. For example, mature trees featured with larger and deeper root system could help to uptake more available soil P which mainly originates from rock weathering, while available soil N mostly comes from atmospheric deposition in nature ecosystems (Verhoeven and Schmitz, 1991; Güsewell, 2004).

Leaf nutrient concentrations were generally closely correlated with soil available nutrients (Koerselman and Meuleman, 1996; Tessier and Raynal, 2003; Ordoñez et al., 2009; Wang et al., 2017). The mean value of the leaf N:P ratio in the present study was 14.3 ± 1.48 for mature trees and 17.7 ± 1.08 for juveniles for the growing season (**Supplementary Figure S5**), which may indicate that the juveniles are slightly limited by P according to the threshold for P-limitation established by Koerselman and Meuleman (1996), but this case is still not limited by P availability according to the relaxed threshold suggested by Güsewell (2004). Sun et al. (2015) found that the mean N, P concentration and the N:P ratio of Chinese cork oak leaves were $19.00 \pm 0.26 \text{ mg g}^{-1}$, $1.03 \pm 0.03 \text{ mg g}^{-1}$, and 20.48 ± 0.63 , respectively, across the distribution range of that species in China. Hence, leaf N:P ratio in the present study was lower than that of Sun et al. (2015) but similar to that of Wu et al. (2012) (16.56 for *Q. variabilis*).

In line with our results of P concentration ($1.13 \pm 0.07 \text{ mg g}^{-1}$ for juvenile, $1.63 \pm 0.18 \text{ mg g}^{-1}$ for mature), Han et al. (2005) pointed out that low leaf P across China's flora, compared to the global average revealed by Reich and Oleksyn (2004) ($P: 1.77 \text{ mg g}^{-1}$), is a result of low soil P content in China. On the other hand, we found that the N:P ratio in leaves and roots of mature trees increased with latitude (**Supplementary Figure S6**), which is a result of increased tissue N accompanied by relatively stable P concentration across latitudes. A higher leaf N concentration in plants in colder habitats is generally considered as an adaptation mechanism that enhances the metabolic activity and growth rates under low temperatures or short growing season (Reich and Oleksyn, 2004; Soolanayakanahally et al., 2009; El Zein et al., 2011). Fajardo and Piper (2017) also found that nutrient limitation is not likely to be involved in the carbon limitations and could not be an explanation for altitudinal tree-line formation. Our recent field investigation found that the Chinese cork oak showed increasing annual basal increment with increasing latitude from south to north (Gao et al., 2018). We therefore speculate that higher availability of resources (e.g., NSC, N, and P) found in northern populations supports higher growth rate, which in turn decreases the resource storage and remains the resource availability at a stable level.

The fact that more pronounced negative correlations between resource storage and climatic variables (e.g., mean temperature) were found in the dormant season (**Figure 4B**) implies that the projected climate warming could alter trajectories in plant resource allocation into growth and regeneration (De Frenne et al., 2012; Lapenis et al., 2013; Carón et al., 2015), especially as the climate warming in China is predicted to be strongest during the winter months (Ge et al., 2013). This in turn may result in distribution range shift. The results presented here are based on one species with two sampling dates, and should be supplemented by further studies carried out at the community or ecosystem level with multiple species and spanning multiple years, to take into account species-specific responses to environmental variations (Drobyshev et al., 2013; Boisvert-Marsh et al., 2014) as well as their inter-annual fluctuations (Scartazza et al., 2013). Meanwhile, the other factors such as recruitment potential (Graginc et al., 2014), competitive interaction (Ettinger and HilleRisLambers, 2013; Liang et al., 2016), and disturbance (Slaton, 2015; Renwick et al., 2016) had been found to affect distribution range, which should be considered in further exploring the mechanism determining tree species' northern distribution range other than resource availability.

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CONCLUSION

In the present study, from the perspective of carbon and nutrient allocation strategy, a whole-tree approach integrating with two distinct life stages was used to explore the mechanisms that determine tree latitudinal range limit, with a case study of Chinese cork oak. Our results indicate that tissue NSCs (soluble sugar, starch and sum of soluble sugar and starch, NSC) and nutrient concentrations (nitrogen and phosphorus) in both mature and juvenile trees did not decrease with increasing latitude across seasons, and even root nitrogen and root NSC in the juvenile trees increased with latitude. Our results suggest that available carbon, N, and P may not be the determinant factors driving the latitudinal range limit of the species studied. These findings will greatly improve our understanding of the mechanism involved in determining the latitudinal range limit, and help to understand and predict the dynamics of the northern range under global warming.

AUTHOR CONTRIBUTIONS

J-FL, M-HL, and J-PL conceived the experiment. Y-PD, X-FW, and Y-YN conducted the experiment and analyzed the samples. J-FL, M-HL, QW, W-FX, and Z-PJ wrote the paper. All authors contributed critically to the drafts and gave final approval for publication.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 41371075 and 41371076), the Fundamental Research Funds for the Central Non-profit Research Institution of CAF (CAFYBB2018ZB001 and CAFYBB2014ZD001), the Natural Science Foundation of Beijing (No. 8152032), and the China Scholarship Council (No. 201303270003).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01444/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Invasion of a Legume Ecosystem Engineer in a Cold Biome Alters Plant Biodiversity

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OPEN ACCESS

Edited by:

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Hebrew University of Jerusalem, Israel

Reviewed by:

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 23 December 2017

Accepted: 11 May 2018

Published: 05 June 2018

Citation:

Vetter VMS, Tjaden NB, Jaeschke A,
Buhk C, Wahl V, Wasowicz P and
Jentsch A (2018) Invasion of a
Legume Ecosystem Engineer in a
Cold Biome Alters Plant Biodiversity.
Front. Plant Sci. 9:715.
doi: 10.3389/fpls.2018.00715

Plant ecosystem engineers are widely used to combat land degradation. However, the ability of those plants to modulate limiting abiotic and biotic resources of other species can cause damage to ecosystems in which they become invasive. Here, we use *Lupinus nootkatensis* as example to estimate and project the hazardous potential of nitrogen fixing herbaceous plants in a sub-polar oceanic climate. *L. nootkatensis* was introduced to Iceland in the 1940s to address erosion problems and foster reforestation, but subsequently became a high-latitude invader. In a local field survey, we quantified the impact of *L. nootkatensis* invasion at three different cover levels (0, 10–50, and 51–100%) upon native plant diversity, richness, and community composition of heath-, wood-, and grasslands using a pairwise comparison design and comparisons of means. Afterward, we scaled impacts up to the ecosystem and landscape level by relating occurrences of *L. nootkatensis* to environmental and human-mediated variables across Iceland using a species distribution model. Plant diversity was significantly deteriorated under high lupine cover levels of the heath- and woodland, but not in the grassland. Plant species richness of the most diverse habitat, the heathland, linearly decreased with lupine cover level. The abundance of small rosettes, cushion plants, orchids, and small woody long-lived plants of the heath declined with invader presence, while the abundance of late successional species and widespread nitrophilous ruderals in wood- and grasslands increased. Distribution modeling revealed 13.3% of Iceland's land surface area to be suitable lupine habitat. Until 2061–2080, this area will more than double and expand significantly into the Central Highlands due to human mediation and increasingly favorable climatic conditions. Species-rich habitats showed a loss of plant species diversity and richness as well as a change in community composition even in low lupine cover classes. The future increase of suitable lupine habitat might lead to the displacement of cold-adapted native plant species and will certainly challenge conservation as well as restoration of ecosystems in the cold climate of Iceland, but also elsewhere. Lupine invasion speeds up succession, which may be additive with climate change effects, and accelerates ecological change in cold biomes.

Keywords: disturbance, field experiment, high latitude invader, Maxent, plant community reorganization, sub-arctic climate, transformer species, vegetation dynamics

INTRODUCTION

Invasive plants are globally threatening ecosystems and island floras leading to species endangerment and extinction (Pejchar and Mooney, 2009; Harter et al., 2015). Especially invasive ecosystem engineers can strongly influence native ecosystems by altering energy, water and/or nutrient fluxes, which consequently leads to altered ecosystem-level properties (e.g., *Myrica faya* a nitrogen fixing tree invasive in Hawaii; Vitousek et al., 1987; Vitousek and Walker, 1989). Ecosystem engineers (Jones et al., 1994) are often intentionally introduced to new environments by humans, e.g., for soil and water conservation, to restore degraded ecosystems or to solve the problems of deforestation (Lazzaro et al., 2014; Ayanu et al., 2015). They generally possess traits that can positively influence soil stability, nutrient and hydrological cycling, and light infiltration (Ayanu et al., 2015) and show protective characteristics, e.g., reduced erosion (Fei et al., 2014). But if they become invasive, those positive traits of the respective alien ecosystem engineer can have negative and long-lasting effects on native communities and ecosystem properties (Richardson et al., 2000; Catford et al., 2012; Fei et al., 2014) that often extend far beyond its life span and/or presence (Richardson et al., 2000; Ehrenfeld, 2003, 2010). Ecosystem engineers that have become invasive, are called “transformer species” (Richardson et al., 2000). Invaders that are introduced for management purposes, such as the ecosystem engineers, are usually widely and deliberately applied by humans and are thus able to spread into large areas right at the beginning of the invasion process with many starting points for the invasion.

Lupinus nootkatensis DONN ex SIMS acts as an ecosystem engineer in the sub-polar ecosystems of its invasive range Iceland. Originally from Alaska and Canada, this high-latitude invader was introduced to Iceland in 1945 for soil amelioration and reforestation. Due to repeated human introductions, *L. nootkatensis* has a high propagule pressure and is rapidly spreading across the Icelandic lowlands (Magnusson, 2010). *L. nootkatensis* stabilizes slopes and modulates limiting abiotic resources of other species by fixing atmospheric nitrogen, thus changing the nutrient cycling of invaded habitats. Cold biomes show a rapid saturation in the ecosystem's capacity to retain N, making them prone to N₂ fixers (Hiltbrunner et al., 2014). Such changes caused by the accumulation of atmospheric nitrogen in the soil and subsequently in the plant community composition are persistent and continue even after the removal of the legume from the ecosystem or its replacement by other species (Hiltbrunner et al., 2014). The increased soil nitrogen content in old lupine stands facilitates the settlement of further invasive species, such as demonstrated for, e.g., *Anthriscus sylvestris* and *Ribes rubrum* in Iceland (Magnusson et al., 2008; Magnusson, 2010). *L. nootkatensis* modulates biotic factors such as plant-plant interactions by forming dense patches, affecting plant establishment and succession of arctic plant species via direct competition effects (Magnusson et al., 2008; Magnusson, 2010). *L. nootkatensis* is also a habitat generalist, and widely occurs across Icelandic lowland habitats (Magnusson, 2010). It transforms the native vegetation, e.g.,

heathlands, into *Poa pratensis* dominated grasslands (Magnusson et al., 2008), thus directly affecting plant establishment and succession. However, *L. nootkatensis*' ability to facilitate soil enrichment and succession, by building up nutrients, organic matter, and water storage capacity of soils is perceived as one solution to combat the manmade and massive problem of severe land degradation and desertification in Iceland (Arnalds and Runolfsson, 2008), which also may be exacerbated by future climate change.

The combination of species invasion and climate change might lead to negative synergistic effects, which are more powerful than the additive effects of the two single stressors. Despite the buffering effects of the surrounding oceans, climate change will lead to profound alterations of the environmental conditions on islands, which might positively affect the establishment and spread of alien species in various ways (Harter et al., 2015).

We investigate lupine invasions in different plant communities on a local scale field study and scale up to the ecosystem and landscape level using a correlation model. It is currently under debate which factors are mainly responsible for the ecosystem engineer's ongoing spread in Iceland and how climate change will affect these factors in the future. Although, there are existing studies concerning the community impact, the invasion success and the future distribution of *L. nootkatensis* in Iceland, most of these studies only concern one or few factors of the same kind, e.g., different climate variables or biotic interactions. Here, we set out to address and quantify the relative influence of a variety of abiotic, biotic and human-mediated factors, which are probably determining the actual distribution pattern of *L. nootkatensis* across Iceland and project the likelihood of lupine-free areas to become invaded in the near future. The rapid spread, ability to alter its local environment, and its habitat generality make *L. nootkatensis* an interesting case study for invasion processes in cold biomes, e.g., the consequences of exotic invasion in niche construction (Fei et al., 2014). Combining experimental studies of local communities with predictive modeling at a landscape level, provides a more accurate overview of the potential range of the species in Iceland (Stricker et al., 2015). The spatially enclosed system of Iceland is well-suited for our approach because of its insularity, the excessive spread of *L. nootkatensis* into a great variety of plant communities of the Icelandic lowlands and the relatively precise documentation of its introduction into the sub-polar system (Magnusson et al., 2008; Magnusson, 2010).

We aim to (a) quantify the current invasion status of *L. nootkatensis* on Iceland using a distribution map of the year 2016, (b) quantify the invasion impacts of the ecosystem engineer on the native vegetation (hereafter: biotic characteristic) in Iceland, (c) understand the abiotic and biotic filters decisive for the recent invasion success, and (d) robustly project the invasion range of *L. nootkatensis* in Iceland under current (reference period: 1960–1990) and future (2061–2080) climate conditions based on the findings of a and b. We use two distinct data sets: (1) a field study to test the biotic characteristics and (2) a distribution map to model the abiotic characteristics as well as the invasion process.

MATERIALS AND METHODS

Study Species

Lupinus nootkatensis (Fabaceae) is a long-lived (up to 20 years) herbaceous plant originating from coastal regions of the Aleutian Islands and from Queen Charlotte Island, Alaska to Vancouver Island, British Columbia, Canada (Magnusson, 2010). *L. nootkatensis* prefers open habitats of frequent natural disturbance (Fremstad and Elven, 2008), e.g., early successional stages with vegetation destruction and soil erosion. In Iceland, the lupine is primarily recorded from gravel bars along the coast and rivers, roadsides, dry slopes and sandy beaches. But it is also found in disturbed landscapes, as well as in dwarf shrub-heathlands (Magnusson, 2010).

Biotic Filter Experiment and Propagule Pressure

Study Area

The study area of the local field survey, Morsádalur, is located in the Vatnajökull National Park in South-East Iceland (Figure 1). The Vatnajökull area is greatly influenced by glacial and volcanic processes (Steinthorsson et al., 2000; Björnsson, 2003; Björnsson and Pálsson, 2008). Within the sub-polar oceanic climate of Iceland, the valley Morsádalur is characterized by a mild climate with warm temperatures (Björnsson et al., 2007) and high annual precipitation (Crochet et al., 2007; Björnsson and Pálsson, 2008). We chose three different habitat types, which are characteristic for the native vegetation of Iceland and most dominant, and are currently invaded by *L. nootkatensis*: a heathland on the mountain slope Réttargil, a grassland with occasional trees in the valley Morsádalur and the old birch forest (*Betula pubescens*) Bæjarstadaraskógur on the adjacent western slope of Morsádalur.

Sampling Design and Methods

To test the effect of lupine invasion on plant community composition among three different habitats a pairwise comparison design, between the cover levels within and among each habitat type, was employed.

First, we defined three different levels of lupine cover density: “none,” which had no lupines in the vegetation cover, “low” which had 10–50% lupine cover, and “high” which had 51–100% lupine cover (Magnusson et al., 2008). Areas with 1–9% of lupine cover were excluded from the analysis because these areas are mainly occupied by immature lupine plants. This gradient in lupine invasion succession was observed along transects from the center to the edge of a lupine patch. While the center represents late invasion stages with high lupine cover, the edges of a lupine patch represent early invasion stages with relatively low lupine cover (Magnusson et al., 2008).

Three plots of 2 m × 2 m size for each of the three lupine cover density levels were randomly assigned to the lupine patches of each habitat (in total = 27 which consist of 3 × 3 = 9 plots per habitat). The plot size of 2 m × 2 m was determined by a minimum area analysis to cope with the heterogeneity of the habitats and represents the largest minimum area found in the

heathland. Plots of the same density level were not placed within the same lupine patch, although where possible, different density levels did occur within the same patch.

Soil seed bank of *L. nootkatensis* was estimated by taking one soil core of 5 cm diameter and depth per plot. Thus, soil samples were replicated three times per cover level of each habitat ($n = 27$). All soil samples were taken at the end of the field season in August within one single day. For levels “low” (10–50%) and “high density” (51–100%) the core was randomly taken at 40 cm distance to the lupine chosen as reference for the nearest neighbor analysis. Samples were air-dried and sieved through three sieves with mesh sizes of 16, 8, and 4 mm. We sorted and counted the lupine seeds by hand.

We additionally analyzed plant community composition and nearest neighbor growth patterns of the three habitats to pinpoint potential differences between lupine cover levels (see Appendix Figures A1–A3).

Statistical Analyses

As a measure of alpha diversity within habitats and plots the Simpson (diversity) index, also called Simpson concentration, was calculated separately for each of the three plots per lupine cover level and habitat (Simpson, 1949; Lande, 1996):

$$\lambda = \sum_{i=1}^R p_i^2$$

R is the richness of each habitat type, p_i is the squared relative abundance of the respective species and λ is the probability of two randomly chosen specimen to belong to the same species. Thus, a Simpson index of 0 represents highest diversity, while a value of 1 represents no diversity.

Analyses were conducted using the statistical software R 3.4.2 (R Core Team, 2017). The effects of habitat and lupine cover level within habitats on the alpha diversity, plant species richness, seed abundance and soil depth were tested via ANOVA and *post hoc* Tukey-test in case of normally distributed data with variance homogeneity (Hothorn et al., 2008). The Kruskal–Wallis test for multiple comparisons (Giraudeau, 2017) was applied to data with an inhomogeneous variance or residuals that did not follow the normal distribution. We used the Bartlett-test and the Shapiro–Wilk test to check for variance homogeneity among the groups and normal distribution of the residuals respectively.

Modeling the Spatial Distribution of *L. nootkatensis* in Iceland

We used the model algorithm Maxent (Phillips et al., 2017) version 3.4.1 to relate occurrences of *L. nootkatensis* to environmental variables across whole Iceland.

Species Occurrence Data and Environmental Variables

Abiotic, biotic and human-mediated environmental variables, which are associated with the range limits of *L. nootkatensis* in Iceland according to literature, were pre-selected by expert

TABLE 1 | Environmental predictor variables pre-selected by expert knowledge.

Category	Variables	Source	Reference
Climate data	Annual mean temperature, temperature seasonality, maximum temperature of warmest month , minimum temperature of coldest month, minimum temperature of May, mean temperature of wettest quarter , mean temperature of warmest quarter, annual precipitation, precipitation of driest month, precipitation seasonality , precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter	Bioclimatic variables WorldClim1.4 – Global Climate Data of the current (reference period 1960–1990) climate conditions. Bioclimatic variables for future climate scenarios (CMIP5): NorESM1-M (RCP 4.5, RCP 8.5) MPI_ESM-LR (RCP 4.5, RCP 8.5) (Hijmans et al., 2005)	Magnusson et al., 2008; Magnusson, 2010; Wasowicz et al., 2013
Topography	Altitude Aspect and slope	Bioclimatic variables WorldClim1.4 – Global Climate Data (Hijmans et al., 2005). Manually calculated from altitude in R	Own consideration in accordance with Magnusson, 2010
Soil	Age of substrate Soil type	Icelandic Institute of Natural History (http://en.ni.is/). Accessed October 17, 2016. Agricultural University of Iceland (provided February 27, 2018)	Own consideration in accordance with Sigurdardottir, 2008; Magnusson, 2010 Personal communication Dr. Olafur Arnalds; Arnalds, 2015
Land cover	Vegetation types: grassland and cultivated land, birch woodland, moss heathland Surface water: rivers, waterbodies, glaciers Substrate: sand, lava, gravel plains	Icelandic Institute of Natural History (http://en.ni.is/). Accessed October 17, 2016.	Hultén, 1968; Fremstad and Elven, 2008; Magnusson, 2010
Human vector	Distance to nearest road Human influence index (human population pressure; human land use and infrastructure; human access)	Calculated based on the road map obtained from the NLSI: National Land Survey of Iceland (http://www.lmi.is/en/). Accessed January 04, 2017. Wildlife Conservation Society – WCS, and Center for International Earth Science Information Network – CIESIN – Columbia University, 2005. Last of the Wild Project, Version 2, 2005 (LWP-2). Palisades, NY: NASA SEDAC. doi: 10.7927/H4BP.00QC. Accessed January 04, 2017.	Magnusson, 2010

Variables in bold were further selected by Pearson Correlation Coefficient, Jackknife and AIC and used to calibrate the species distribution model 1 (Maxent) of *Lupinus nootkatensis*. In model 2, we omitted the variable “distance to nearest road” but kept all other settings constant.

knowledge (Table 1) to determine the most influential variables.

We used climate data together with characteristics of the terrain (e.g., aspect and slope), soil type, geology, native vegetation cover, and aspects of human interference (Table 1) as a proxy to test how much of Iceland’s land surface area is threatened by lupine invasion. Aspect and slope in combination with the climate variables control for the self-propagation of the invader species (Magnusson, 2010), while all other variables are potential factors determining the distributional range of *L. nootkatensis* (see e.g., Magnusson et al., 2008; Magnusson, 2010; Wasowicz et al., 2013).

Climate data for current and future conditions was obtained from Worldclim 1.4 (Hijmans et al., 2005) at a spatial resolution of 30 arc seconds (≈ 1 km). To predict the potential future distribution of the legume invader in Iceland, downscaled and calibrated climate data from the global climate models (GCM) NorESM1-M and MPI_ESM-LR for the years 2061–2080 was used. Both, the medium stabilization (RCP 4.5) (Thomson et al., 2011) and very high baseline emission (RCP 8.5) (Riahi et al.,

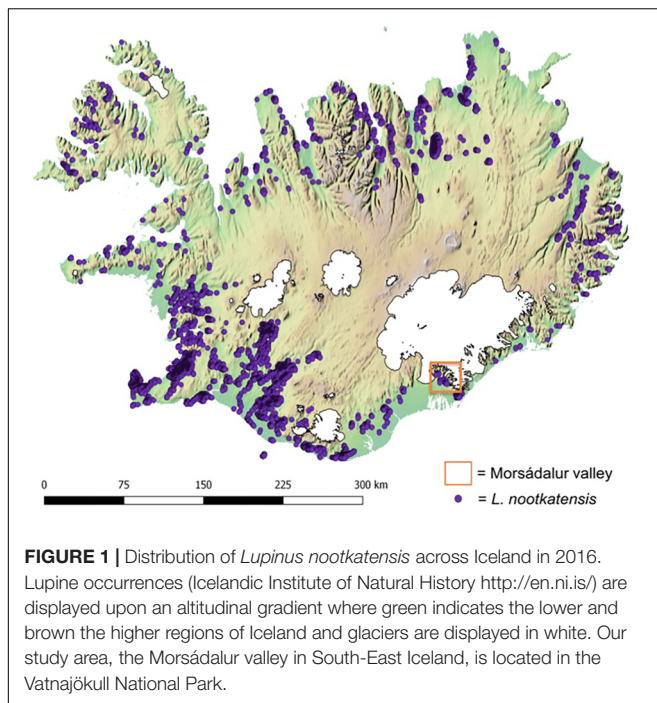
2011) representative concentration pathways of the IPCC’s fifth assessment report were used.

If necessary, other variables were projected to WGS84, rasterized and re-sampled (Hijmans, 2016) to the 1 km spatial resolution of the climate variables.

The species occurrence data was obtained from the Icelandic Institute of Natural History¹ in the form of spatial polygons representing *L. nootkatensis* patches derived from high-resolution satellite imagery. We converted the spatial polygons to a raster of the same spatial resolution and dimensions as the environmental data using the “rasterize” function in R (Hijmans, 2016). The center point of each grid cell containing *L. nootkatensis* patches were then used to derive the needed occurrence records for Maxent. A total of 5709 species occurrences were compiled across Iceland (Figure 1).

We used the open source software R version 3.4.2 (R Core Team, 2017) and QGIS 2.16.3 in order to prepare the species occurrence records as well as all environmental variables (background data) as spatial data layers.

¹<http://en.ni.is/>



Species Distribution Model

We calculated Pearson correlation coefficients (r) in R to derive a set of fairly uncorrelated environmental variables. Because Maxent copes well with collinearity (Elith et al., 2011), cross-correlation was used as a selection criterion only to exclude the highest correlative variables ($r > 0.8$).

The remaining variables were used to calculate principal component analyses (PCAs) based on which we measured spatial heterogeneity of the environment. The derived grids of environmental heterogeneity were then used to spatially rarefy our species occurrence points ("Spatially Rarefy Occurrence Data for SDMs" tool, SDMtoolbox; Brown, 2014). Overall, 98 unbiased species occurrences were used in Maxent.

For invasive species, the absence of occurrences means not necessarily a "true absence" due to, e.g., the unsuitability of the respective habitat, but rather a reflectance of the fact that the species has not yet reached its equilibrium distribution in the new habitat. Therefore, we treated our species data as presence-only data. Maxent is a common and very effective methodology to model the ecological niche of species with presence-only data (Elith et al., 2006; VanDerWal et al., 2009; Phillips et al., 2017) but it needs to be provided with a set of background data (VanDerWal et al., 2009; Barve et al., 2011). As the dispersal potential of the invasive species might be large, e.g., due to human traffic or targeted propagation by humans, we opted for a buffer-based approach for background sampling. Following the example of VanDerWal et al. (2009), we produced a series of test models using buffer zones with radii of 1 km (size of one raster grid cell), 5, 10, 25, and 50 km. In our case, a buffer zone with a radius of 25 km gave the best result.

Jackknife testing within Maxent along with the Akaike Information Criterion (AIC) implemented in R, were used to select the final environmental variables for the species distribution model (model 1, **Table 1**). We gradually removed all variables whose regularized training gain was less than 0.1, unless the AIC and AUC of model 1 significantly deteriorated. To evaluate model performance, we ran a 10-fold cross-validation (cv) after each simplification.

The ENMeval package in R (Muscarella et al., 2014) was used to tune Maxent settings, as well as for model validation. We tested regularization multiplier (RM) values of 1, 2, 5, 10, 15, 20 (Warren and Seifert, 2011; Merow et al., 2013; Shcheglovitova and Anderson, 2013) together with different combinations of the Maxent feature classes linear (L), quadratic (Q), and hinge (H) (Merow et al., 2013; Phillips et al., 2017) with block-wise data partitioning (Roberts et al., 2017).

We fitted two final models using all of the spatially rarefied species occurrences, RM = 5, LH features, and a maximum of 1000 iterations. The cloglog output format was chosen for both models (Phillips et al., 2017). Model 1 was used to evaluate the environmental variables decisive for the actual spread pattern as well as to predict the potential distribution of *L. nootkatensis* across Iceland under current and future climate conditions. To evaluate the potential maximum area of suitable habitat available for *L. nootkatensis* under current and future climate conditions, without the restriction to roads as the vectors of propagation, we fitted a second model and calculated difference maps based on the predictions of both models (see Appendix). Model 2 was fitted with the same settings as model 1, but without the variable distance to nearest road.

The cloglog output format gives probabilities of occurrences for the respective species varying between 0 and 1. We used the maximum training sensitivity plus specificity threshold, a threshold selection method which is not affected by pseudo-absences (Liu et al., 2013), to reclassify the cloglog output in suitable ($>$ threshold) and unsuitable habitat ($<$ threshold).

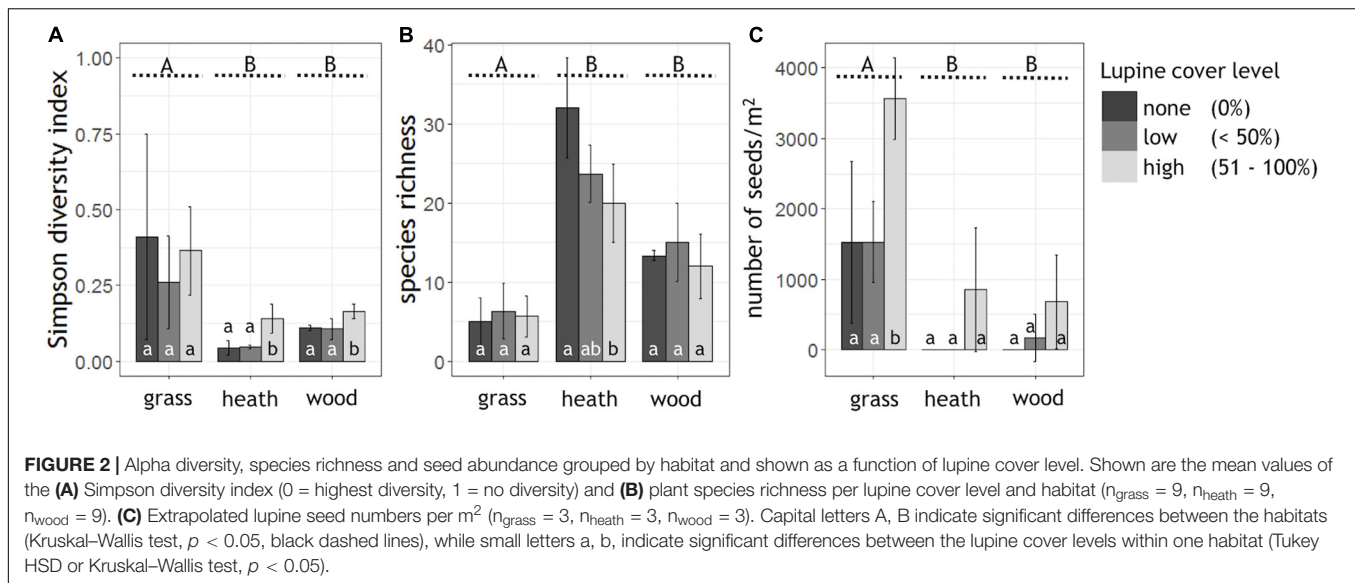
To assess the accuracy of our species distribution model we calculated partial receiver operating characteristics (Peterson et al., 2008; Tjaden et al., 2017) with 1000 bootstrapping iterations on 50% of the test data and an expected error rate of 5%.

RESULTS

Biotic Filter Experiment and Propagule Pressure

High lupine cover levels significantly reduced the alpha diversity of the heath- and woodland (Simpson diversity index; **Figure 2A**). In the grassland, lupine cover did not have a significant effect on alpha diversity. Plant species richness of the heathland, the most diverse habitat, decreased linearly with lupine cover level (**Figure 2B**). In the woodland as well as in the grassland, species richness showed a slightly hump-shaped pattern from none to high lupine cover.

Typical heath species such as *Calluna vulgaris*, *Empetrum nigrum*, and *Arctostaphylos uva-ursi* decreased in their abundance with proceeding lupine invasion. The percentage



cover of *Calluna vulgaris* was halved in both, low and high cover classes of *L. nootkatensis* (Appendix Figure A1). Small rosettes (*Silene acaulis*), cushion plants (*Armeria maritima*), orchids (*Listera cordata*, *Dactylorhiza maculata*, *Platanthera hyperborea*) and small woody long-lived plants (e.g., *Salix herbacea*) of the heathland were absent in the presence of the invader, even in low lupine cover classes (Appendix Table A1). In the heathland as well as in the woodland, the abundance of late successional species, e.g., *Betula pubescens*, increased with lupine cover (Appendix Figure A1). In high lupine cover classes widespread nitrophilous plants – *Taraxacum* sp. in the woodland and *Geranium sylvaticum* in the grassland – appeared, while they were not present in low cover classes or without the invader (Appendix Table A1). *Poa pratensis*, the most abundant grass in the grassland decreased remarkably, while a contrasting trend was observed for *Angelica archangelica*, another late successional species (Appendix Figure A1).

Abundance of *L. nootkatensis* seeds in the soil differed significantly among habitats. The most diverse habitat, the heathland, had the lowest abundance of lupine seeds while the least diverse habitat, the grassland, showed highest seed numbers (Figure 2C). Propagule pressure of lupine seeds tended to be highest in patches with 51–100% lupine cover while it was indifferent in the cover classes “none” and “low,” although this effect was only statistically significant in the grassland but not in the other two habitat types. Only in the woodland, the expected trend toward no seeds without lupine cover, few seeds with low lupine cover and increased seed abundance in high lupine cover stands was observed (Figure 2C).

Modeling the Spatial Distribution of *L. nootkatensis* in Iceland

Both Maxent models had a good predictive ability as measured by the area under the curve ($\text{AUC}_{\text{model1}} = 0.84$, $\text{AUC}_{\text{model2}} = 0.79$) and the AUC ratios of the partial receiver operating characteristics (mean $\text{AUCratio}_{\text{model1}} = 1.76$, mean

TABLE 2 | Percent contribution and permutation importance of the environmental variables used in the final models.

Predictor variable	Contribution [%]	Permutation importance
Distance to nearest road	72.3 (–)	53.4 (–)
Maximum temperature of warmest month	12.1 (52.6)	24.3 (54.7)
Land cover	6.3 (22.8)	5.0 (7.5)
Mean temperature of wettest quarter	5.6 (13.1)	14.6 (22.0)
Precipitation seasonality	3.7 (10.0)	2.7 (15.6)
Human influence index	0 (1.3)	0 (0.2)

Results of model 2 are given in brackets. The higher the relative information of a single variable, the more decisive it is for the current pattern of propagation.

$\text{AUCratio}_{\text{model2}} = 1.70$). All values ≥ 0.531 and 0.553 respectively (maximum training sensitivity plus specificity threshold) were interpreted as suitable lupine habitat. The five most important variables influencing the distribution of *L. nootkatensis* across Iceland were distance to nearest road, maximum temperature of warmest month, land cover, mean temperature of wettest quarter, and precipitation seasonality (Table 2).

Under current climate conditions, a total of 13.3% of Iceland's land surface area was projected to be suitable lupine habitat (Figure 3 and Table 3). *L. nootkatensis* was mainly found in habitats close to roads (≤ 0.5 km). The predicted probability of presence shows an optimum at 14.4°C for the maximum temperature of the warmest month, at 8.2°C for the mean temperature of the wettest quarter, and at 2.7 for the precipitation seasonality (Appendix Figure A4). *L. nootkatensis* was found in all land cover classes across Iceland, but the invasion risk was projected to be highest for grassland/cultivated land and lowest for moss heath and wetlands (Appendix Figure A4).

Independent from the tested emission scenarios both GCMs projected a more than double increase in the amount of suitable lupine habitat for the years 2061–2080 (Table 3). With proceeding climate change, the environmental suitability of Iceland was

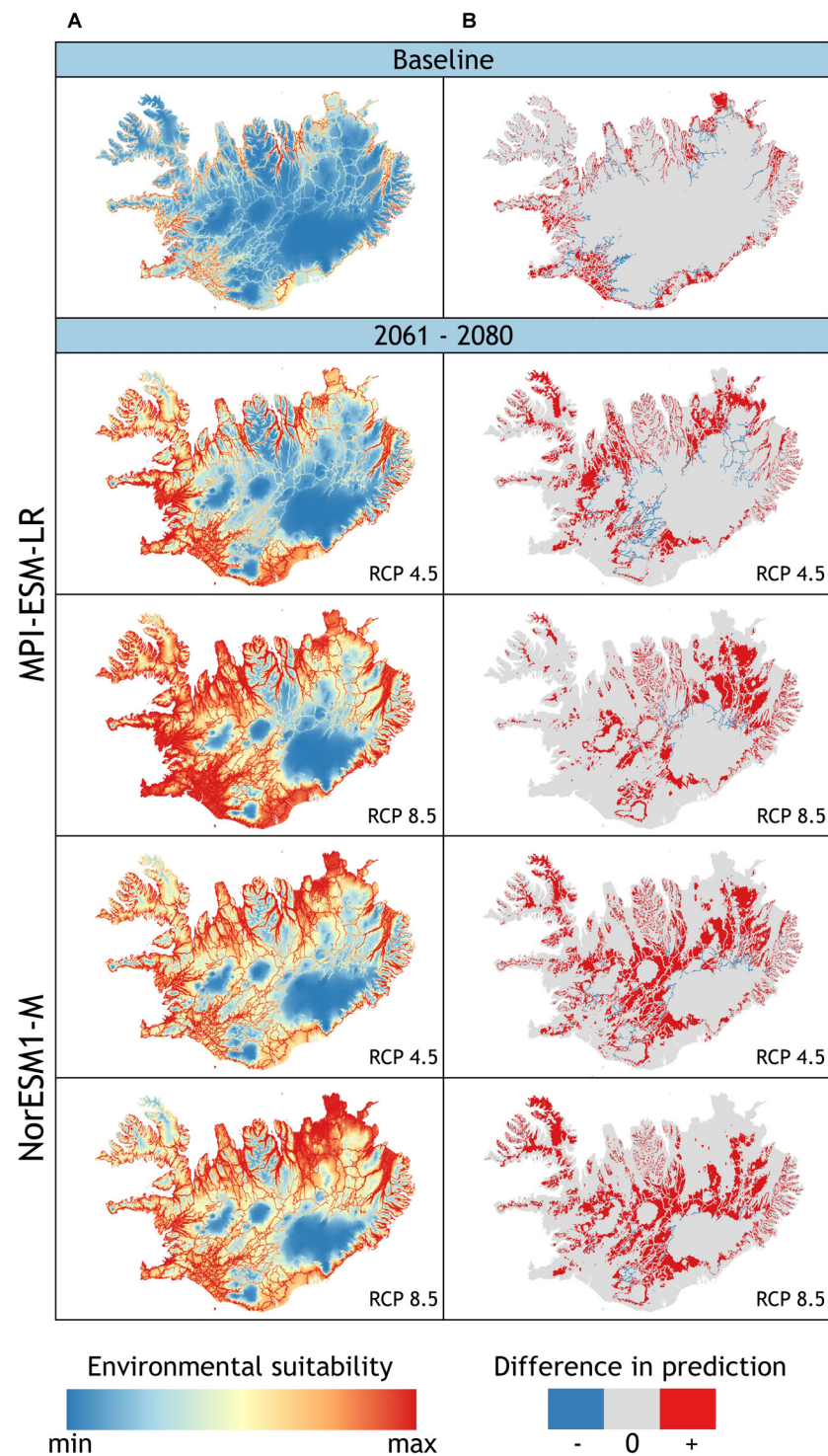


FIGURE 3 | (A) Projected potential distribution (model 1) of *L. nootkatensis* across Iceland under current climate conditions (baseline) and future climate conditions modeled with the global climate models NorESM1-M and MPI-ESM-LR each in the medium stabilization (RCP 4.5) and very high baseline emission scenario (RCP 8.5). Environmental suitability ranges from: minimum = 0 to maximum = 1 with maximum training sensitivity plus specificity threshold (MTSS) = 0.531. **(B)** Difference in prediction between the two models used to project *L. nootkatensis*' potential future distribution [difference = binary output (model 2 – model 1)] (see Appendix). The models only differ in the presence of the propagation vector “distance to nearest road”: model 1 vector present, model 2 absent. Areas projected to be suitable habitat only by model 2, shown in red, are an addition to the projections of model 1 (+); areas in gray (0) are equally projected from both models, while areas in blue (–) are solely projected by model 1. Together with the projections of model 1, red areas show the maximum of suitable lupine habitat without restriction to “roads” as the vectors of propagation.

TABLE 3 | Percentage amount of land surface area of Iceland projected to be suitable habitat for *L. nootkatensis* under current and future climate conditions.

Time scale	Concentration pathway	Projected amount of suitable habitat [%] (% increase compared to current)	
		Model 1	Model 2
Current	–	13.3	20.1
MPI_ESM-LR	RCP 4.5	39.1 (+ 25.8)	53.2 (+ 33.1)
	RCP 8.5	61.7 (+ 48.4)	76.7 (+ 56.6)
NorESM1-M	RCP 4.5	50.1 (+ 36.8)	72.6 (+ 52.5)
	RCP 8.5	58.0 (+ 44.7)	81.2 (+ 61.1)

The percentage point increase in suitable habitat compared to current climate conditions is given in brackets.

projected to expand into the Central Highlands, thus the potential distribution range of *L. nootkatensis* will enlarge. For example, in 2016 *L. nootkatensis* occurred on altitudes up to 572 m but was projected to reach heights of 1087–1119 m (MPI_ESM-LR RCP 8.5, NorESM1-M RCP 8.5) in the future. *L. nootkatensis* is likely to spread from its current main distribution area – along the coasts and near the human settlements – following the main valleys and roads into the Central Highlands. While roads serve as vectors of propagation (model 1, **Figure 3A**), *L. nootkatensis*' occurrence is not dependent upon the presence of roads under future climate conditions (**Figure 3B**). The future spatial focus was projected to lie in the northern to northeastern and southwestern parts of the island.

DISCUSSION

The restrictive factor(s) controlling lupine colonization is depending on the respective area. Low propagule pressure, is impeding lupine spread in areas without major human interference, e.g., the highlands. *L. nootkatensis* is a very effective disperser, in terms of durability and amount of produced seeds, and in addition its spread is accelerated by human interference (Magnusson, 2010; Wasowicz, 2016). We detected a large quantity of seeds even in areas where currently no or only a few lupines are growing. The large amount of seeds in the rather low lupine cover classes of the grasslands are either deposited by the river Morsá which floods the valley of Morsádalur at irregular intervals – an important avenue for propagule dispersal facilitated by frequent disturbance dynamics (Magnusson, 2010) – or are part of an old seed bank (Svavarsdóttir et al., 2008). Additionally, our results imply, that a high plant species diversity seems to go along with a lower overall seed abundance of *L. nootkatensis*, potentially reducing risk of invasion. However, in high lupine cover levels the natural diversity decreases and the invader is able to build up persistent seed banks. The missing significance of these results might be due to the extreme patchiness of seed banks. Increasing the number of soil samples per plot could overcome this obstacle. Consequently, propagule pressure, one of the key drivers and a prerequisite for successful invasion (Lockwood et al., 2005; Colautti et al., 2006; Catford et al., 2009) is not limiting but

delaying *L. nootkatensis*' distribution across the highlands and mountainous areas of Iceland. Biotic competition in areas void of disturbance such as the grassland, seems to impede colonization of *L. nootkatensis*, however, as the lupine seeds are durable, it is only a matter of time until disturbance occurs and colonization is facilitated (Sigurdsson and Magnusson, 2008). Abandonment of traditional management practices, e.g., free-ranging sheep grazing, might further facilitate lupine establishment as sheep graze on small seedlings and thus prevent the lupine from establishing (Magnusson et al., 2008). Based on the SDM projections, sheep grazing could now systematically be used to restrict the predicted potential distribution of *L. nootkatensis* across Iceland, while maintaining a traditional management system.

Our results suggest that *L. nootkatensis* may benefit from anthropogenic influences, though is not necessarily dependent on human presence. Initially, areas close to human infrastructure (e.g., roads) are exposed to a higher invasion risk, but as the invasion progresses, the lupine increasingly decouples from the roads as primary vectors of propagation and begins to penetrate large areas of the Central Highlands. Since propagule pressure increases with time and due to *L. nootkatensis*' long residence time in Iceland, starting with its first introduction in 1945 (Magnusson, 2010), seed swamping around human settlements can be assumed (Colautti et al., 2006; Catford et al., 2009). Human-mediated disturbance along with sufficient propagule pressure creates invasion windows as disturbances reduce competition, increase space and subsequently resource availability (Catford et al., 2009). Based on our results we are able to verify the recently postulated relation between human disturbance and occurrence of invasive species (Wasowicz, 2016) for *L. nootkatensis*.

All current hot-spots of invasive plant species occurrences in the Central Highlands are linked to human disturbance, e.g., tourism and the related infrastructure (Wasowicz, 2016). Tourism in general but also the number of visitors of the Icelandic highlands is sharply increasing in recent years (Icelandic Tourist Board, 2017). Thus, one of the last wilderness areas of Europe (Sæþórsdóttir and Saarinen, 2015) becomes gradually more accessible for propagules and at the same time more disturbed by human visitors (Wasowicz, 2016).

Arctic and sub-arctic regions will be affected by climate change in a twofold way: (1) the cold-adapted native plants will be expelled and forced to migrate with their shifting climatic niche, e.g., upwards or northwards (Phoenix and Lee, 2004; Parmesan, 2006), (2) due to the temperature increase the (sub-) arctic regions will become more and more accessible to alien plants (Crumpacker et al., 2001; Kreyling, 2010). As projected by our model – and in accordance to recent publications (Wasowicz et al., 2013) – with proceeding climate change the potential suitable habitat of *L. nootkatensis* will expand significantly into the high elevation ecosystems of Iceland during the years 2061–2080, potentially due to warming and a prolonged growing seasons. In accordance to Wasowicz et al. (2013) we found human-mediation and temperature-related variables to be the most important factors shaping the distribution of *L. nootkatensis* across Iceland under current climate conditions. Wasowicz et al.

(2013) interpreted this pattern as a limitation of the alien plant due to the harsh climatic conditions of Iceland. Although this explanation is probably true for most alien plant species of Iceland, it might not be applicable to the “high-latitude invader” lupine as the climate envelope of the native versus invasive range is very similar: both range from a cold temperate (boreal) to sub-polar climate (Wasowicz, 2016). Single plants and small lupine stands are not detected by the remote sensing technique used to derive our species occurrence data set, but are already recorded as present and invasive in the Icelandic highlands and mountainous areas (Wasowicz, 2016). Although the majority of lupine patches occur in the lowlands, the invader might not be limited to these climatically favorable regions close to manmade infrastructure. Our model neither confirmed a dependency of *L. nootkatensis* toward areas with high precipitation as indicated by Magnusson (2010), nor did the precipitation parameters show a high relative contribution to the Maxent model. We therefore assume that *L. nootkatensis* is already adapted to the climate of Iceland, but the predicted invasive range under the current climate conditions is biased, i.e., underestimates the potential distribution, due to the manmade distribution together with a dispersal lag of the invader. To partially exclude this bias as well as to estimate the maximum area at risk to be changed by the invader, we calculated two separate models one with and one without human infrastructure as propagation vectors.

The question arises whether the Central Highlands subsequently lose their function as a refuge for cold-adapted native species due to the projected habitat expansion and induced succession of *L. nootkatensis*. The Central Highlands and mountainous regions, especially of northern Iceland, are biodiversity hot-spots (Wasowicz et al., 2014). They are habitat to many native, cold-adapted plant species (Wasowicz et al., 2014), which are adjusted to the harsh climate (Wasowicz et al., 2013; Wasowicz, 2016) and nutrient-poor soils of arctic environments (Arnalds, 2004; Liška and Soldán, 2004; Dowdall et al., 2005). Via the accumulation of litter and atmospheric nitrogen *L. nootkatensis* eventually increases soil quality and depth (Sigurdardottir, 2008; Magnusson, 2010) and finally induces succession (Magnusson et al., 2008). Thus, the invasive ecosystem engineer pursues niche construction (Fei et al., 2014) and might act as a transformer species. In our experiments, species-rich habitats like the heathland showed a decrease in plant species diversity and richness as well as a change in community composition as soon as lupine invasion occurs, while species-poor habitats, e.g., grassland and woodland, showed an increase. There are reasons to believe that Arctic plant species probably do not tolerate elevated N as caused by lupine invasion and might be poorer competitors compared to non-native nitrophilous plants (Chapin et al., 1986; Lilleskov et al., 2002; Hofland-Zijlstra and Berendse, 2009). For example, elevated nitrogen levels lead to a decrease in the mycorrhiza community and, combined with shading, to a reduced production of phenols and tannins, resulting in a diminished competitive ability of heathland plants (Lilleskov et al., 2002; Hofland-Zijlstra and Berendse, 2009). Thus, as shown for the heath communities, a loss of plant species diversity and richness must

be assumed. Additionally, elevated soil nutrients may lead to a facilitated settlement of further invasive species (Simberloff and Von Holle, 1999), which has already been demonstrated for old lupine stands (Magnusson et al., 2008; Magnusson, 2010). By altering plant community organization and by inducing succession (Appendix Figures A1, A2 and Table A1) *L. nootkatensis* changes the functional integrity of the respective habitats.

Most species will not be able to keep pace with the rapidly changing climate as their migration rates are considerably lower than the expected range shifts (Cunze et al., 2013). This is especially relevant for ecosystems in cold biomes such as Iceland, where suitable climate space is limited. On the other hand, invasive species may benefit from climate warming allowing accelerated spread. Both lead to significant changes in the native vegetation and therewith to the loss of unique ecosystems. The changes in soil properties and succession induced by lupine invasion will further speed up vegetation changes induced by climate change. It is unlikely that the native vegetation is able to adapt fast enough to those ecosystem changes.

In current as well as in future climate conditions, the amount of projected suitable habitat for *L. nootkatensis* will mainly cover areas without native vegetation (Appendix Table A2). Thus, the ecosystem engineer *L. nootkatensis* could induce the urgently needed succession to higher plant communities, which are able to stabilize the barren and sometimes degraded soils and subsequently reduce desertification and dust storms on Iceland (Arnalds and Runolfsson, 2008; Magnusson et al., 2008; Riege, 2008). However, up to 86.9% of the area currently domicile to the native vegetation of Iceland is projected to become suitable lupine habitat in future climate conditions and thus will be at risk of being permanently changed to a secondary vegetation. It is very probable that the emerging plant community differs in structure and composition from native plant communities of Iceland (Magnusson et al., 2008). The maps of the potential distribution of *L. nootkatensis* across Iceland only show the amount of projected suitable habitat, thus they give an estimate of which areas are generally endangered by lupine invasion. Those projected areas are not necessarily simultaneously covered by *L. nootkatensis* as succession might eventually lead to the displacement of the invader (Magnusson et al., 2008). However, as the emerging vegetation does not necessarily correspond to the original native vegetation of Iceland, the SDM projections predict the maximum potential area at risk to be permanently changed by *L. nootkatensis*. In addition, not only the plants, but also invertebrates and birds are affected by lupine induced homogenization (Davidsdottir et al., 2016).

CONCLUSION

Invasion of an ecosystem engineer into a sub-polar environment can induce very different effects. In heavily degraded habitats it can cause a fast increase in plant species richness and diversity, while in native, cold-adapted habitats it might lead to a reduction in plant

species richness by outcompeting more sensitive species. In areas where positive aspects prevail, ecosystem engineers might carefully be used for restoration purposes, e.g., to induce succession toward a stable vegetation cover on severely degraded soils. However, the spread beyond such areas is very likely leading to altered energy and nutrient fluxes. The resulting changes in ecosystem-level properties are, due to the low conversion rates of those ecosystems, long-lasting, or permanent. A change in the limiting factors, e.g., due to climate change, might lead to a massive expansion of the potential habitat, which additionally hampers the targeted application of the ecosystem engineer and facilitates invasion.

AUTHOR CONTRIBUTIONS

VV and AJe conceived the ideas. VV and VW designed methodology. VV, NT, AJa, VW, and PW collected and analyzed the data. VV led the writing of the manuscript. NT, AJa, VW, CB, PW, and AJe helped with the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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ACKNOWLEDGMENTS

We thank the Icelandic Institute of Natural History (<http://en.ni.is/>) and the National Land Survey of Iceland (NLSI, <http://www.lmi.is/en/>) for providing us with the species occurrence data of *Lupinus nootkatensis* and additional environmental predictor variables. We thank Prof. Póra Ellen Þórhallsdóttir and Oliver Bechberger from the University of Iceland, Kristín Svavarsdóttir from the Soil Conservation Service of Iceland, and the team and rangers of the Vatnajökull National Park for their support and advice during field work. Many thanks to Prof. Dr. Olafur Arnalds for the kind permission to use the soil data provided by the Agricultural University of Iceland as well as for his valuable suggestions. Additionally, we would also like to thank the two reviewers for their thoughtful recommendations, which helped to further improve our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.00715/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Impact of Multiple Ecological Stressors on a Sub-Arctic Ecosystem: No Interaction Between Extreme Winter Warming Events, Nitrogen Addition and Grazing

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OPEN ACCESS

Edited by:

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Reviewed by:

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 12 April 2018

Accepted: 16 November 2018

Published: 30 November 2018

Citation:

Bokhorst S, Berg MP, Edvinsen GK, Ellers J, Heitman A, Jaakola L, Mæhre HK, Phoenix GK, Tømmervik H and Bjerke JW (2018) Impact of Multiple Ecological Stressors on a Sub-Arctic Ecosystem: No Interaction Between Extreme Winter Warming Events, Nitrogen Addition and Grazing. *Front. Plant Sci.* 9:1787. doi: 10.3389/fpls.2018.01787

Climate change is one of many ongoing human-induced environmental changes, but few studies consider interactive effects between multiple anthropogenic disturbances. In coastal sub-arctic heathland, we quantified the impact of a factorial design simulating extreme winter warming (WW) events (7 days at 6–7°C) combined with episodic summer nitrogen (+N) depositions (5 kg N ha⁻¹) on plant winter physiology, plant community composition and ecosystem CO₂ fluxes of an *Empetrum nigrum* dominated heathland during 3 consecutive years in northern Norway. We expected that the +N would exacerbate any stress effects caused by the WW treatment. During WW events, ecosystem respiration doubled, leaf respiration declined (–58%), efficiency of Photosystem II (Fv/Fm) increased (between 26 and 88%), while cell membrane fatty acids showed strong compositional changes as a result of the warming and freezing. In particular, longer fatty acid chains increased as a result of WW events, and eicosadienoic acid (C20:2) was lower when plants were exposed to the combination of WW and +N. A larval outbreak of geometrid moths (*Epirrita autumnata* and *Operophtera brumata*) following the first WW led to a near-complete leaf defoliation of the dominant dwarf shrubs *E. nigrum* (–87%) and *Vaccinium myrtillus* (–81%) across all experimental plots. Leaf emergence timing, plant biomass or composition, NDVI and growing season ecosystem CO₂ fluxes were unresponsive to the WW and +N treatments. The limited plant community response reflected the relative mild winter freezing temperatures (–6.6°C to –11.8°C) recorded after the WW events, and that the grazing pressure probably overshadowed any potential treatment effects. The grazing pressure and WW both induce damage to the evergreen shrubs and their combination should therefore

be even stronger. In addition, +N could have exacerbated the impact of both extreme events, but the ecosystem responses did not support this. Therefore, our results indicate that these sub-arctic *Empetrum*-dominated ecosystems are highly resilient and that their responses may be limited to the event with the strongest impact.

Keywords: cryptogam, CO₂ fluxes, fatty acids, frost, geometrid moth, herbivory, multiple stress, snow

INTRODUCTION

The Arctic is experiencing extreme weather events more frequently due to climate change, causing high mortality rates among species when events surpass survival thresholds (Liston and Hiemstra, 2011; Kivinen et al., 2017). Especially during the winter period more winter extreme events are expected, such as rain on snow, unseasonal warm periods, ground ice formation and loss of snow cover (Vikhamar-Schuler et al., 2016). Abrupt changes in winter snow cover and depth following mid-winter thaw events (e.g., due to temperature rise from -20°C to $+5^{\circ}\text{C}$ in just 24 h) negatively affect plant survival, detectable across landscapes through remote sensing, because of reduced snow insulation against low temperature extremes (Bokhorst et al., 2009, 2018). In addition, loss of snow cover during a midwinter melt can induce plant physiological activity (i.e., respiration and fluorescence), disrupting winter dormancy of plants, and often resulting in high mortality due to drought and frost stress (Ögren, 1996; Schaberg et al., 1996; Bokhorst et al., 2009, 2010). These extreme winter warming events (WW) occur against a background of gradually increasing temperature and extreme events such as intense herbivory by seasonal population explosions of defoliating insects (Jepsen et al., 2008) and nitrogen (N) deposition events originating from the industrialized regions further south (Karlsson et al., 2013). A combination of stressors may enhance the individual effects (Crain et al., 2008; Phoenix et al., 2012; Liess et al., 2016) and therefore have large impacts on plant development, such as phenology (Bokhorst et al., 2008), but also the plant community composition and its role in the sub-arctic terrestrial carbon budget. As such, a combination of these stressors may, through changing the dominance of plant functional types, affect the carbon balance of these sub-arctic ecosystems with potential feedbacks to greenhouse gas induced climate warming (De Deyn et al., 2008). Moreover, these community responses may differ greatly from summer warming responses and we therefore, need to understand the species and community responses for future sub-arctic vegetation predictions.

Recent experimental field studies of WW events in the sub-arctic have shown great vulnerability of evergreen dwarf shrubs to such events while deciduous plants and cryptogams are less affected (Bjerke et al., 2011, 2017a; Bokhorst et al., 2015), which may govern future sub-arctic vegetation changes (Bokhorst et al., 2015). Differences in vulnerability between plant types are partly determined by differences in exposure of overwintering tissue to freezing, and in part by physiological adaptations, such as winter dormancy and changes in the membrane fatty acid composition (Bokhorst et al., 2018). Higher N availability

can also affect the plants' cell and physiological characteristics associated with drought and frost susceptibility (Carroll et al., 1999; Schaberg et al., 2002). Nitrogen input from agricultural practices, fossil fuel burning, and biomass burning can reach high latitudes (Forsius et al., 2010; Karlsson et al., 2013) and make sub-arctic plants more vulnerable to the temperature variability of WW events (Power et al., 1998; Phoenix et al., 2012). However, it is unclear which plant types will be most vulnerable to the combination of high N availability and WW events and whether this will result in a vegetation regime shift.

To address the impact of the combined stressors of N input and WW events on the community composition of sub-arctic vegetation we initiated an experiment that combined these factors in sub-arctic Norway. The simulation of WW events was done during three consecutive winters in February (2014–2016), while N additions were applied during the summer months. However, during the growing season directly following the first WW simulation (2014), the field sites were subject to intense grazing by caterpillars of the geometrid moths *Epirrita autumnata* and *Operophtera brumata* (Bjerke et al., 2017b, 2018; Pepi et al., 2017). Such grazing pressure greatly affect the growth response of dwarf shrubs, as these are targeted by the caterpillars that drop onto the ground cover once the birch trees have been defoliated (Lehtonen and Heikkinen, 1995; Malmström and Raffa, 2000; Jepsen et al., 2013; Karlsen et al., 2013). Grazing pressure can result in sub-arctic vegetation regime shifts where dominant dwarf shrubs are suppressed and herbs and grasses emerge (Tømmervik et al., 2004; Olofsson et al., 2013). This therefore resulted in a factorial field experiment where the impacts on sub-arctic heath vegetation of +N and WW simulations were compared but also included intense grazing by caterpillars across all treatments. We hypothesized that (1) summer N additions will negatively impact the winter plant physiological adaptations to frost and drought. Because N additions are known to exacerbate other stressors (Power et al., 1998; Phoenix et al., 2012) we expect N additions to result in an increase of the damage caused by WW events and grazing impact. The grazing and WW effects will be strongest on evergreen dwarf shrubs because this plant type appears most vulnerable to WW events (Bokhorst et al., 2015, 2018) while also being heavily grazed when caterpillars fall to the ground (Jepsen et al., 2013; Karlsen et al., 2013). Therefore, we hypothesize that in response to these extreme events (2) the plant community may start to shift from an evergreen dwarf shrub dominated community to one with higher dominance of cryptogams, grasses, herbs and deciduous plants with potential greater turnover of carbon flux rates and limiting carbon sequestration of these sub-arctic ecosystems.

MATERIALS AND METHODS

The study site was located on the small island Håkøya, situated in the fiord Balsfjorden between the larger island Kvaløya and the mainland (Tromsø, Norway, 69.66° N 18.78° E, 30 m a.s.l.). The western part of Håkøya, where this study was located, has a mosaic of open deciduous woodland dominated by *Betula pubescens* Ehrh. and treeless heathland dominated by the dwarf shrub *E. nigrum* L. The climate is relatively mild for these latitudes due to the warm Norwegian current (which is a branch of the North Atlantic current), resulting in mean summer and winter temperatures of 12°C and −4°C, respectively (Førland et al., 2009). Annual precipitation is ca. 1000 mm and the winter snowpack typically reach 60–80 cm depth. The experimental site was situated in an area with sparse distribution of birch trees (*B. pubescens*) and dominated by *E. nigrum* with a dense cover of the moss *Pleurozium schreberi* (Willd. ex Brid.) Mitt. Sub-dominant plant species included *Vaccinium vitis-idaea* L., *V. uliginosum* L., *V. myrtillus* L., *Cornus suecica* L., *Avenella flexuosa* (L.) Drejer, the moss *Polytrichum commune* Hedw. and *Cladonia* lichens.

The experiment consisted of 24 plots (1 m × 2 m) with four treatments replicated six times: control (C), N addition (+N), extreme winter warming events (WW), and WW with N addition (WW+N). Ammonium nitrate solutions (5 kg N ha^{−1}) were applied by watering can (2 L volume) across each +N treatment plot three times during the growing season at monthly intervals. The N additions were at the lower limit of effect doses for most plants (Phoenix et al., 2012) and chosen to reflect realistic scenarios of N input resulting from airborne transport for these sub-arctic regions (Karlsson et al., 2013). The WW simulations were implemented by using four infrared heaters (800 W emitting at 3 μm; HS 2408, Kalglo Electronics Co., Bethlehem, PA, United States) that were suspended in parallel (65 cm apart) from wooden frames. This produced a thermal radiation flux of 270 W m^{−2} to the plots (at zero wind speed). Lamps were on between 9 and 16 February 2014, 13–20 February 2015, and 11–18 February 2016. The snow pack (60–80 cm deep) gradually melted out during 3 days after which the lamps remained on for another 4 days. Lamps were adjusted in height above the surface to achieve leaf temperatures of ca. 5°C. Leaf temperatures were monitored at least twice per day by obtaining a reading of a thermocouple attached to the underside of a plant leaf in each plot. Lamps were turned off after 7 days and removed from the frames. The experimental plots were left exposed to ambient conditions and build-up of a new snowpack for the remainder of the winter. We marked out an additional 12 quadrats (1 m × 1 m) which were treated as control (*n* = 6) and nitrogen additions (*n* = 6) from which we could sample winter plant tissues and measure ecosystem gas fluxes without disturbing the main C and +N experimental plots. Temperature at canopy height was monitored throughout the year by temperature loggers (Hobo UA-001-08, Onset Computer Corporation, MA, United States) recording at hourly intervals in four control plots and four plots exposed to extreme WW events. Loggers were shielded from direct sunlight by a white dome cover.

Leaf Phenology and Vegetation Composition

Vegetative bud development was monitored during the subsequent growing season (early June onward). For this, 10 randomly selected shoots of the dwarf shrubs *E. nigrum*, *V. vitis-idaea*, and *V. myrtillus* were tagged in each plot and surveyed every week or more frequently depending on the speed of development. Due to the presence of large caterpillar numbers of geometrid moths, a large proportion (see results) of tagged shoots were grazed and new unaffected shoots were selected in spring 2014. Vegetative bud development was recorded by noting when the bud had burst and the first leaf had fully expanded (Bokhorst et al., 2008). During the 2015 WW event we quantified bud elongation of *V. myrtillus*, as it represents a first step in bud development (Bokhorst et al., 2010). The abundance and cover of plant species was quantified using the point intercept method in a quadrant (30 cm × 30 cm) in the middle of each plot during peak plant biomass (August). A total of 121 point counts at 2.5 cm intervals were made of the vegetation in each square by counting the number of times a vertical pin touched plant parts (Jonasson, 1988). Cryptogams were counted as present or absent, while vascular plants could be hit more than once by each vertical pin. For *E. nigrum* only shoots were counted rather than every leaf hit to avoid overrepresentation due to the high number of tightly packed, small needle-like leaves. Point intercept counts of vascular plants were converted to biomass using regression formulas (Supplementary Table S1) (Jonasson, 1988; Bokhorst et al., 2011). Species cover was quantified from point count survey based on presence or absence at each point. Changes in plant biomass and cover were compared from 1 year to the next starting at August 2013.

Normalized Difference Vegetation Index (NDVI)

As a measure of vegetation activity (“health”) across the experimental plots we quantified NDVI by using two different handheld proximate sensor. We used a Maxmax-modified Canon camera (LDP LLC, Carlstadt, NJ, United States) where an infrared sensor replaced the normal sensor (the blue channel records the visible light and the red channel the near infrared). In addition, we used a GreenSeeker (Trimble Navigation Ltd., Sunnyvale, CA, United States) which generates its own radiation for NDVI measurements, while the MaxMax camera is a passive instrument, using reflected and incident radiation (Sakamoto et al., 2012). Plot pictures were taken during peak vegetation biomass, the second week of August, each year (2013–2016). During 2015 and 2016, the two types of NDVI measurements were also done at weekly intervals to monitor changes in plot-level greenness during the growing season (May–August).

CO₂ Fluxes

Respiration rates of *V. vitis-idaea* (individual leaves) and shoots (2 cm) of *E. nigrum* (2014 and 2015) and the moss *P. schreberi* (2015 only) were quantified on an Infrared Gas Analyser (IRGA) (GFS-3000, Walz GmbH, Effeltrich, Germany). Measurements were done in closed cuvettes in complete darkness, at 7000 ppm

H₂O, with 380-ppm base level of CO₂ and temperature of the measuring head kept at 5°C. We used the mean respiration rates of nine measurements taken at 15 s intervals for each sample. Samples were collected during the last day of warming, after the maximum exposure period, and 3 days after the lamps had been turned off.

Ecosystem CO₂ fluxes were measured during the WW events and in the following growing seasons (2014–2016) from May till August. Measurements were made by placing a clear chamber (20 cm × 20 cm × 20 cm) made from poly-methyl methacrylate over the vegetation and monitoring the rate of change in headspace CO₂ concentration, across nine measurements at 10 s intervals, using an IRGA (EGM-4 PP Systems, Amesbury, MA, United States). To minimize internal chamber air exchange with the external environment, plastic skirts (20 cm wide) were attached to a square frame – onto which the chamber could be attached – and weighed down with chains (and snow in winter). The square frame was slotted onto four metal pins that were fixed in the plots to ensure that CO₂ fluxes were measured at the same spot in each plot. Snow was removed 2 h before CO₂ measurements from the additional C and +N plots, to allow any build-up of CO₂ underneath the snow layer to diffuse out (Grogan and Jonasson, 2006).

Plant Physiological Measurements

As a measure of winter plant physiological activity we quantified potential activity of PSII using a mini-PAM (Walz, Effeltrich, Germany) for *E. nigrum* and *V. vitis-idaea* using leaf clips, in the experimental warmed plots and from underneath the snow as controls during early morning (06:00–06:30) when the sun was below the horizon and PAR levels were zero. Measurements were done during the last 2 days of warming when plants had the longest exposure to the warming, to quantify potential release from winter dormancy, and in addition 4 days after the lamps had been turned off to quantify the response of PSII following freezing. One leaf (*V. vitis-idaea*) or shoot (*E. nigrum*) was measured in each experimental plot during each measuring day.

To quantify changes in the fatty acid composition of the cell membranes as a result of the warming and freezing, we collected leaf samples ($n = 5$), while plants had been exposed to WW for 4 days and 4 days after the WW treatment had been turned off. Samples from control plots were retrieved during either of these sampling events. All samples were brought back to the laboratory (within 1–2 h) and frozen at –20°C, freeze-dried, ground, and analyzed for fatty acids following the direct methylation procedure (Browse et al., 1986). Samples of 5–20 mg were dissolved in 1 mL methanolic hydrochloric acid (HCl) (1M) and an internal standard (heptadecanoic acid, C17:0) was added to a glass tube. The solution was heated to 80–100°C for 1 h, and after cooling, 0.4 mL hexane and 1 mL of 0.9% sodium chloride (NaCl) were added to each sample. The fatty acid methyl esters (FAMES) were extracted into the hexane phase by vigorous shaking. The tubes were centrifuged for 10 min to separate the phases completely, and a sample was then taken directly from the hexane phase. Samples were stored at –20°C until gas chromatography (GC) analyses, according to Mæhre et al. (2013). The instrument used was an Agilent 6890N equipped

with a flame ionization detector (FID) (Agilent Technologies Inc., Santa Clara, CA, United States) and a CP7419 capillary column (50 m × 250 μm × 0.25 μm nominal, Varian Inc., Middelburg, Netherlands). The fatty acids were identified by comparing against the commercial fatty acid standards PUFA 1, 2, and 3 (Sigma-Aldrich Chemicals Co., St. Louis, MO, United States) and the GLC standards 80, 411, and 412 (NuChec Prep. Inc., Elysian, MN, United States). The amount of each fatty acid was calculated by comparing peak area with the known amount of an internal standard (C17:0). The proportions of the single fatty acids were used in further analysis. We calculated the unsaturation to saturation ratio (U/S ratio) as the ratio between the total proportion of all unsaturated fatty acids and the total proportion for all saturated fatty acids (van Dooremalen et al., 2011). The detected fatty acid composition differed greatly between plant species, which limited direct comparisons of specific fatty acids between study species. However, changes in fatty acid composition could be quantified in response to the treatments.

Calculations and Statistical Analyses

We used repeated measures ANOVA to detect differences in leaf phenology, NDVI, and CO₂ fluxes between treatments (WW and +N) during the growing seasons of 2014–2016. To identify changes in plant biomass and cryptogam cover across the measurement years (2013–2016) we used a linear mixed effects model with treatments (WW vs. C and –N vs. +N) and years as fixed factors and block as a random factor. *P*-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. A Principal Component Analyses (PCA) was used to summarize changes in vegetation composition of vascular plant and cryptogam cover between treatments, and the first two PCs were analyzed in the same way as the plant biomass. Treatment effects on fatty acid concentrations and U/S ratio were tested using a factorial ANOVA with Treatment and +N as fixed factors. We used Tukey HSD tests at $P = 0.05$ to identify differences in means between WW and C (with and without +N) whenever the interaction effect was significant. A visual inspection did not show any patterns in the residuals. All statistical analyses were carried out using R 3.3.0 (R Core Team, 2015).

RESULTS

Temperature Effects of Winter Warming Treatment

Canopy temperature increased to 6.3–7.2°C in the WW plots while canopy temperatures underneath the snow were around freezing (–0.1–0.1°C) in the control plots (**Figure 1**). Minimum temperatures were somewhat lower in WW compared to C during 2014 (–11.8°C vs. –9.7°C) and 2016 (–6.6°C vs. –1.0°C) but did not differ during 2015 (–9.1°C). The number of freeze thaw cycles increased following WW by 67% and 57% during 2014 and 2015, respectively, and from 1 in C to 29 in WW

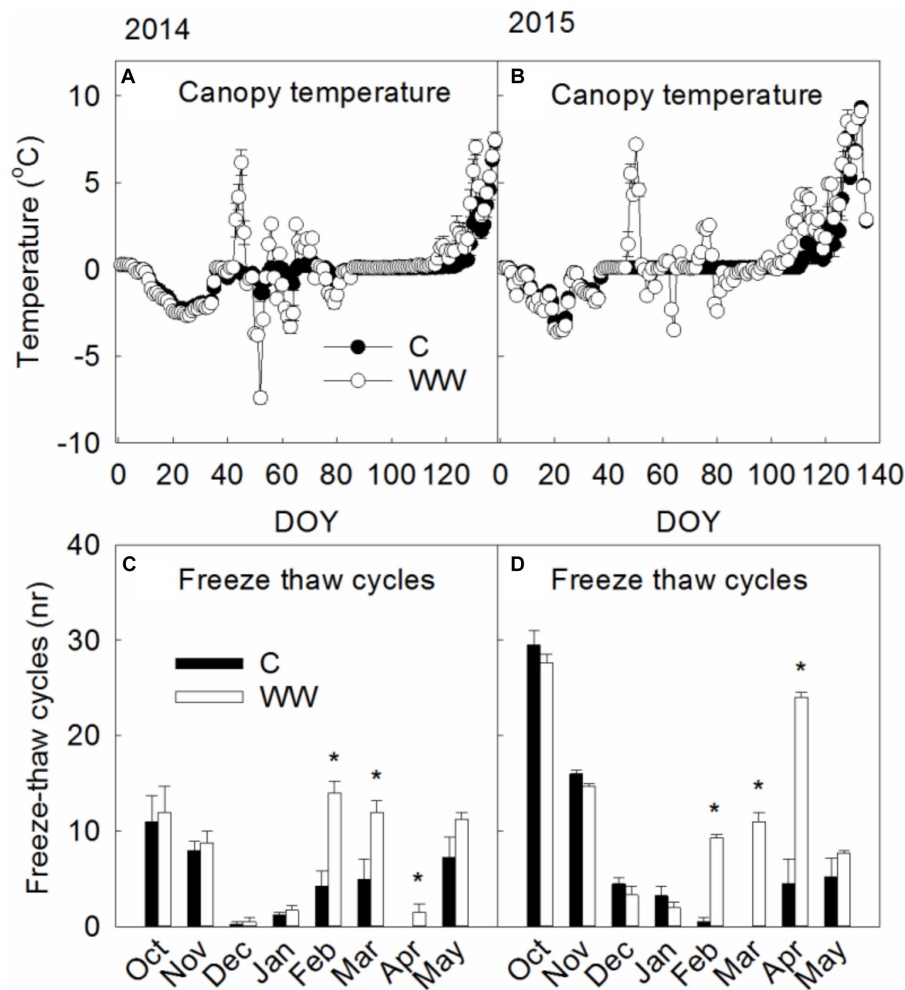


FIGURE 1 | (A,B) Show canopy temperatures measured during 2014 and 2015 respectively in control plots (C) and those exposed to extreme winter warming (WW). **(C,D)** Show the monthly number of freeze-thaw cycles during 2014 and 2015 respectively. Mean daily canopy temperatures and monthly number of freeze thaw cycles during the winters of 2014 and 2015. Data points and bars are means of $n = 4$ with SE as error bars. * indicate significant ($P < 0.05$) differences between treatments. Canopy temperatures were measured following the extreme winter warming event of 2016 but not during the winter period before therefore, these are not shown.

during 2016 (Figures 1C,D). Leaf and moss temperatures were on average 5 and 10°C, respectively, during the WW treatments.

Winter Physiology and Ecosystem Responses

Efficiency of Photosystem II (Fv/Fm) of *E. nigrum* increased ($P < 0.001$) by 77 and 80% in the WW (0.59) and WW+N (0.59) treatments, respectively, as compared to C (0.33) during 2014, indicating a release from winter dormancy. Fv/Fm of *V. vitis-idaea* increased ($P < 0.01$) by 26 and 33% in the WW (0.61) and WW+N (0.64) treatment, respectively, compared to C (0.48) during 2014. Fv/Fm no longer differed between the treatments after the warming lamps was turned off. Fv/Fm was unaffected by WW during 2015, and values across treatments were 0.68, 0.65, and 0.69 for *E. nigrum*, *V. vitis-idaea*, and *P. schreberi*, respectively. Winter leaf

respiration decreased by 58% for *E. nigrum* ($F_{3,19} = 3.8$, $P = 0.027$) and with 43% for *P. schreberi* ($F_{3,19} = 6.4$, $P = 0.005$) in WW compared to C during 2015 but did not differ after lamps were turned off, and there were no differences between WW and WW+N. *Vaccinium vitis-idaea* leaf respiration was unaffected by the treatments ($F_{3,19} = 2.0$, $P = 0.155$).

Bud length of *V. myrtillus* increased by 12% during the 2015 warming event, representing an increase of 0.14 mm and 0.17 mm in WW and WW+N, respectively.

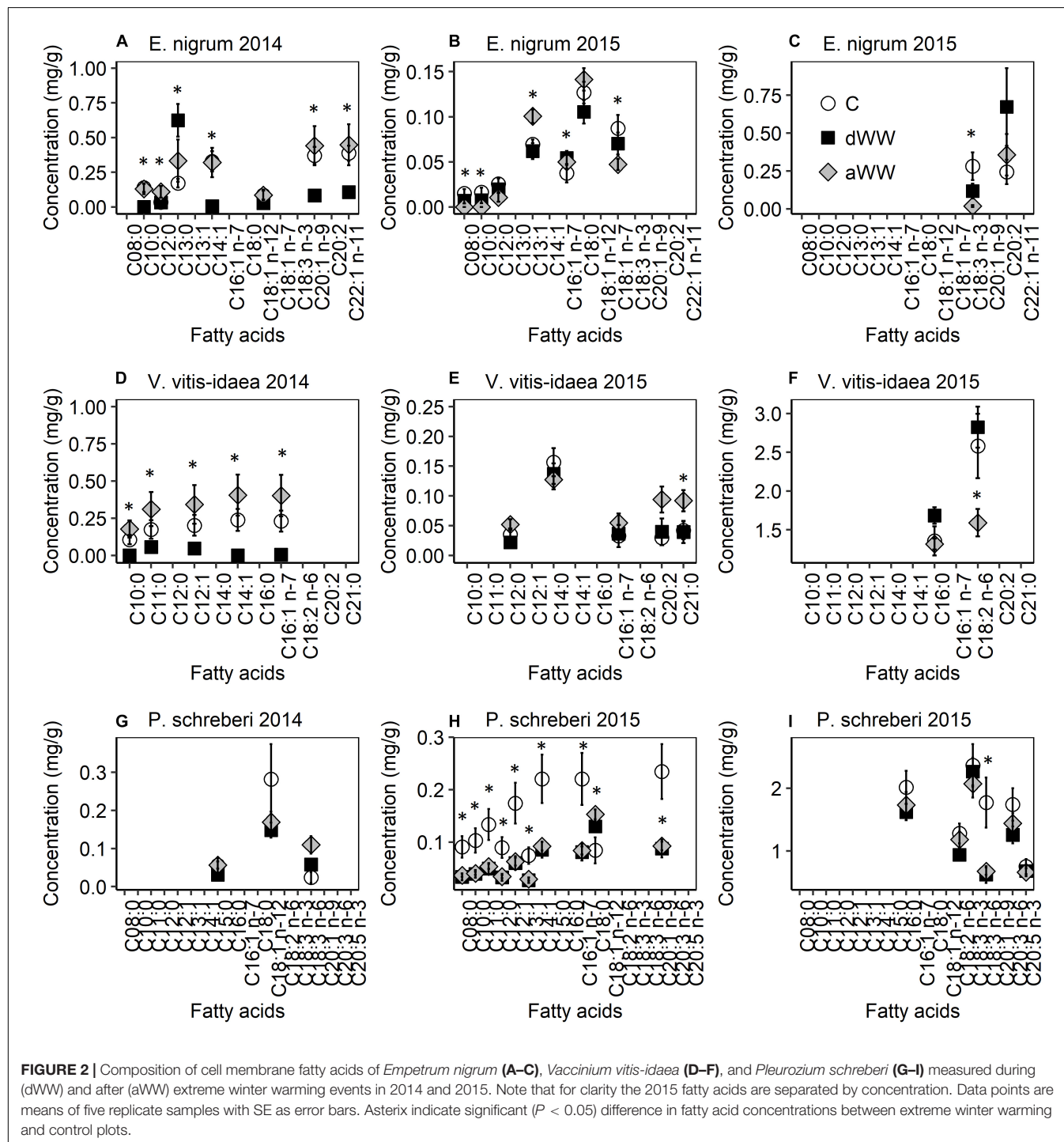
Winter ecosystem respiration was twice as high in WW (WW: $14.0 \pm 1.9 \text{ mg C m}^{-2} \text{ h}^{-1}$ and WW+N: $16.9 \pm 4.1 \text{ mg C m}^{-2} \text{ h}^{-1}$) compared to C ($6.4 \pm 2.6 \text{ mg C m}^{-2} \text{ h}^{-1}$) during the last day of warming in 2014 ($F_{2,9} = 9.8$, $P = 0.005$), but no respiration differences were found during the 2015 WW event ($F_{3,2} = 7.0$, $P = 0.074$). There was no effect of +N on winter ecosystem CO_2 fluxes. Snow fungi were observed in the plots during 2014 (but

not quantified), while during 2015, snow fungi covered 26% (SE: 4.3) and 47% (9.1) of the surface of WW and WW+N ($F_{1,9} = 3.7$, $P = 0.088$).

Membrane Fatty Acids

The ratio of unsaturated to saturated fatty acids was not affected by the treatments for any of the study species during 2014 or 2015 (data not shown). The concentration of various membrane

fatty acids declined for *E. nigrum*, *V. vitis-idaea*, and *P. schreberi* in response to WW during 2014 (Supplementary Table S2 and Figure 2). However, during the second WW event (2015), the response varied between treatments and species and not all fatty acids were found during both years. Five affected fatty acids were consistently lower (from 3 to 100%) during the WW of 2014 in *E. nigrum* compared to control plots and following the WW (Figure 2A). However, C13:0 was increased (263%)



during WW compared to control plots. The fatty acids C16:1n-7, C18:1n-7, and C18:3n-3 were all lower following the 2015 WW compared to control plots, while C8:0, C10:0, and C12:0 were not found in detectable concentrations. C13:1 had highest concentrations (45% higher) following WW compared to control samples (**Figures 2B,C**). Nitrogen increased C18:1 n-12 by more

than three times during 2014 while C12:0 was reduced by 69% during 2015. C18:0 and C20:2 (eicosadienoic acid) responded to WW in combination with +N; for C18:0 there were no Tukey *post hoc* differences, while C20:2 concentrations were consistently higher during WW without N compared to the other treatments.

TABLE 1 | Repeated measures ANOVA results (*F*-values) of leaf phenology, plot NDVI values and CO₂ gas fluxes in response to extreme winter warming events (WW), nitrogen additions (N) and their combination during three growing seasons. * <0.05, ** <0.01, *** <0.001.

		N	WW	Date	N:WW	N:Date	WW:Date	N:WW:Date
<i>E. nigrum</i>	2014	0.0	4.2	77.6***	0.5	0.0	3.6*	0.7
	2015	0.4	0.2	14.7***	0.7	0.6	0.4	0.4
	2016	0.1	0.9	50.8***	0.1	1.8	2.3	0.1
<i>V. myrtillus</i>	2014	0.5	0.3	30.7***	0.1	0.5	1.2	0.4
	2015	1.3	1.2	18.4***	0.4	1.1	1.4	0.4
	2016	1.3	0.2	44.1***	0.5	2.1	0.4	0.4
<i>V. vitis-idaea</i>	2014	5.7*	0.1	1.1	1.0	0.5	1.1	0.1
	2015	0.0	0.0	150.3***	0.3	2.4	0.5	1.2
	2016	1.6	1.0	53.0***	3.1	2.8*	1.5	0.6
NDVI	2013	0.8	3.4		0.1			
	2014	0.8	0.2		0.7			
	2015	1.2	0.9	4.5**	0.0	1.4	0.9	1.1
	2016	0.3	0.0	935.1***	0.8	0.7	3.0***	0.5
Greenseeker	2015	1.9	0.3	58.8***	0.0	0.4	0.0	0.5
	2016	3.7	0.6	255.6***	1.8	0.5	1.2	0.8
ER	2014	1.3	3.1	96.7***	0.0	1.6	0.9	0.5
	2015	0.0	0.4	10.3***	0.0	0.2	0.6	0.3
	2016	0.0	0.4	10.3***	0.0	0.2	0.6	0.3
NPP	2014	1.9	2.0	12.8***	0.9	2.7**	1.1	1.4
	2015	1.0	1.2	0.9	0.3	0.7	0.2	1.5
	2016	0.7	3.6	31.0***	0.3	0.5	1.2	0.2
GPP	2014	0.0	3.9	48.1***	0.3	2.6**	1.0	1.0
	2015	0.4	1.1	7.2***	0.1	0.1	0.4	0.1
	2016	0.0	3.2	23.6***	0.2	0.3	0.4	0.3

TABLE 2 | Mixed-effect model output of changes in plant biomass (number of point intercept hits) and cryptogam cover between years in response to extreme winter warming events (WW) and nitrogen additions.

	WW		Nitrogen		WW × N		Year	
	χ ²	P	χ ²	P	χ ²	P	χ ²	P
Vascular plants (Biomass change %)								
<i>Cornus suecica</i>	3.8	0.148	2.9	0.233	2.8	0.093	30.7	<0.001
<i>Avenella flexuosa</i>	4.1	0.130	3.3	0.194	1.2	0.268	3.9	0.144
<i>Empetrum nigrum</i>	2.5	0.285	2.4	0.305	2.4	0.124	50.1	<0.001
<i>Vaccinium myrtillus</i>	5.1	0.080	0.4	0.803	0.2	0.632	24.7	<0.001
<i>Vaccinium uliginosum</i>	0.1	0.933	0.8	0.656	0.1	0.886	7.5	0.024
<i>Vaccinium vitis-idaea</i>	1.0	0.611	2.3	0.320	1.0	0.322	3.4	0.182
Cryptogams (cover change)								
<i>Cladonia uncialis</i>	0.1	0.999	0.2	0.927	0.0	0.969	4.3	0.119
<i>Pleurozium schreberi</i>	0.2	0.855	2.2	0.339	0.2	0.676	30.1	<0.001
<i>Polytrichum commune</i>	0.0	0.996	0.1	0.968	0.0	1.000	7.7	0.021
<i>Ptilidium ciliare</i>	0.1	0.979	0.1	0.945	0.1	0.898	1.3	0.526
PC1	2.9	0.238	3.8	0.152	2.7	0.103	0.2	0.641
PC2	1.0	0.603	1.0	0.596	1.0	0.318	0.2	0.659

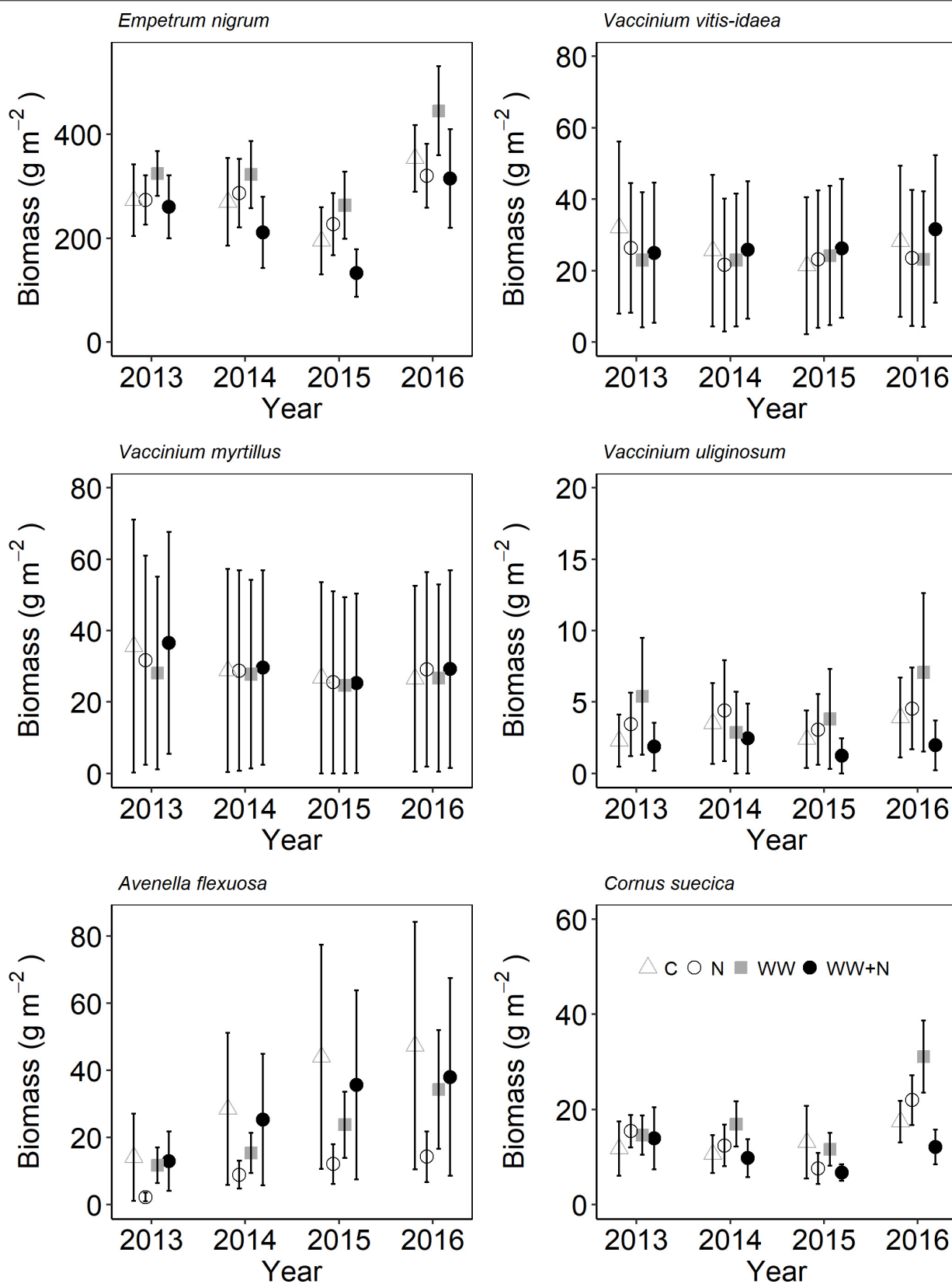
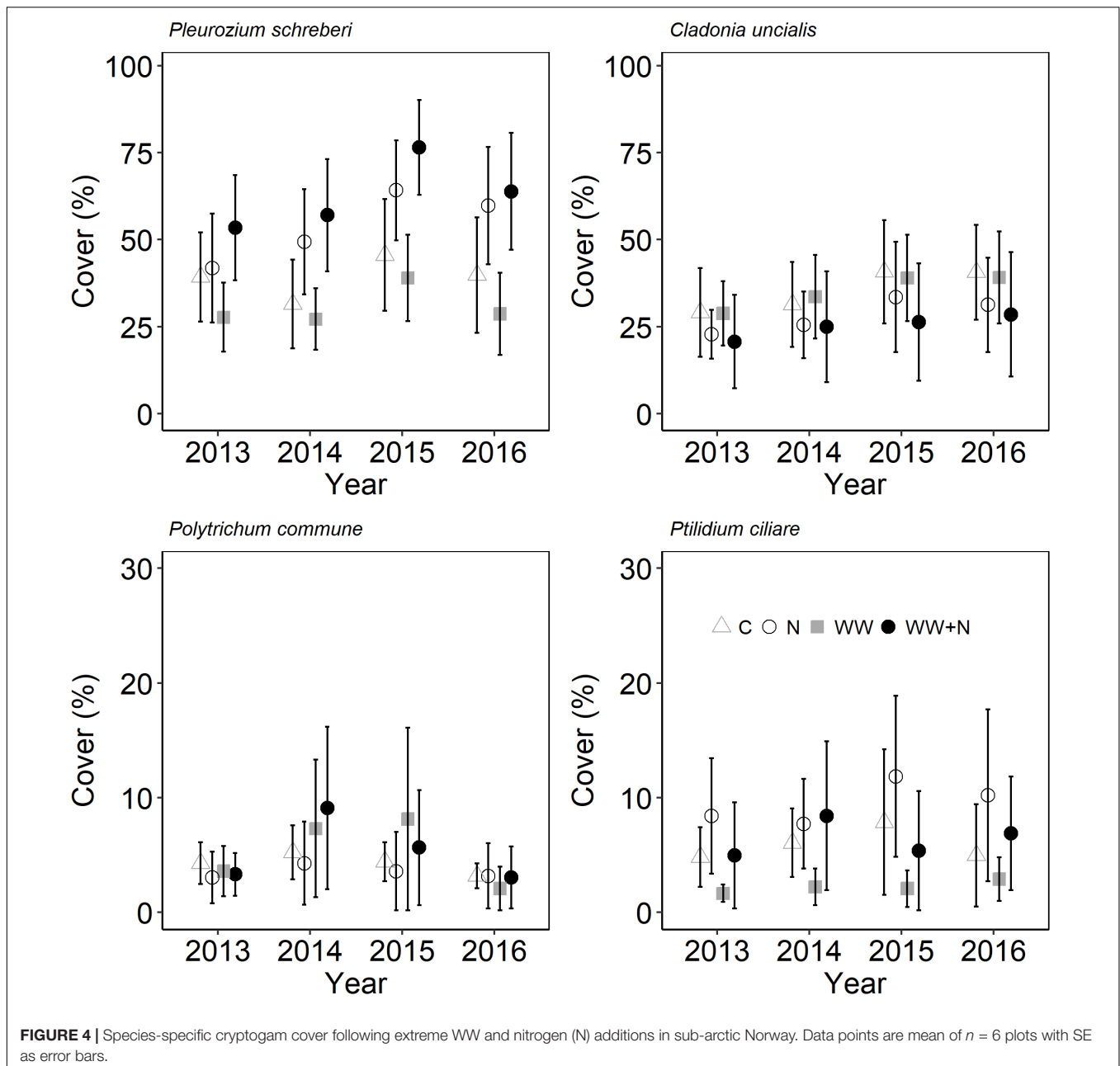


FIGURE 3 | Species-specific plant biomass following extreme winter warming (WW) and nitrogen (N) additions in sub-arctic Norway. Data points are mean of $n = 6$ plots with SE as error bars.



Affected fatty acids ($n = 5$) were consistently lower (from 67 to 100%) during the WW of 2014 in *V. vitis-idaea* compared to frozen samples from control plots and following the WW (Figure 2D). During 2015, C18:2 n-6 declined by 38% following the WW event compared to control samples, while C21:0 and C20:2 increased by 219 and 118%, respectively, following the WW event (Figures 2E,F). The remaining near-significant differences ($P > 0.05$) in fatty acid concentrations were found during and after the WW event. Nitrogen did not affect fatty acid concentrations during 2014. During 2015, C21:0 and C20:2 were both reduced (58 and 54%, respectively) when +N was applied. C14:0 had a significant WW \times N interaction, but Tukey *post hoc* testing did

not indicate significant treatment differences (Supplementary Table S2).

There were three affected fatty acids (C15:0, C18:1 n-12, and C18:3 n-6) responding to the interaction between WW and +N during 2014 in *P. schreberi* but all in a different way (Figure 2G). Concentrations of C15:0 were approximately 10 times higher in C +N compared to C without N but not compared to any of the other treatments. Concentrations of C18:1 n-12 were highest in C (without +N) compared to all other treatments, while concentrations of C18:3 n-6 were highest for WW+N compared to all other treatments. During the winter of 2015, the majority of the affected fatty acids ($n = 16$) were consistently higher (9–65%) in control plots, while only C18:1 n-12 was higher during and

after the WW event (54 and 81%, respectively) (**Figures 2H,I**). Nitrogen increased the concentration of C20:3 n-6 and C20:5 n-3 by 31% during 2015, while the fatty acids C16:0, C18:2 n-6, C18:3 n-3, and C20:3 n-6 had the highest concentrations in the control plots that also received +N.

Ecosystem Responses in the Growing Season

Leaf emergence timing was not affected by the treatments during any of the years, although there were differences in the percentage of emerged leaves between treatments across the measuring periods (**Supplementary Figure S1** and **Table 1**). *Empetrum nigrum* had higher total percentage of emerged leaves in WW (65%) compared to control (33%) at the final measuring date in 2014. There were no treatment effects on *V. myrtillus* leaf emergence during any of the years. While *V. vitis-idaea* had highest percentage (40%) of total fully developed leaves in the +N treatment at the final measuring dates in 2016.

Plant biomass and cryptogam cover were not affected by WW, +N or their interaction (**Table 2**). Instead, biomass of most vascular plant species declined following the second WW events in all plots (including C) due to the caterpillar outbreak. Especially the dwarf shrubs were targeted by the caterpillars resulting in a large proportion of grazed shoots of *E. nigrum* (81%), *V. myrtillus* (87%), and *V. vitis-idaea* (17%) during 2014. There were no significant differences in the number of grazed shoots between treatments. *Avenella flexuosa* and *C. suecica* biomass were unaffected by the treatments and did not appear to be affected by caterpillar grazing. Plant biomass increased again during the last (2016) growing season (**Figure 3**). Moss cover (*P. schreberi* and *P. commune*) reached its peak following the second WW event (summer 2015) but declined again in 2016 (**Figure 4**). The lichen *Cladonia uncialis* and the liverwort *Ptilidium ciliare* had a stable cover between years and were unaffected by the treatments (**Table 2** and **Figure 4**). The PCA analysis indicated that the vegetation community did not change between treatments or years (**Table 2**).

Plot NDVI was not consistently affected by WW, +N or their interaction. During 2016 WW plots were 5% lower NDVI values compared to C in spring while later on in the season there was no difference (**Table 1** and **Supplementary Figure S2**). NDVI plot values were on average 38% lower during August 2015 compared to starting values (**Table 1**). CO₂ fluxes increased with time during the growing seasons (**Table 1**), but there were no differences in ecosystem ER, NPP, and GPP between the treatments during the growing seasons (**Supplementary Figure S3**), with the exception of some lower NPP values under +N treatment in 2016.

DISCUSSION

We anticipated a vegetation regime shift from evergreen dwarf shrubs (heath) to one of cryptogams, grasses, and deciduous plants (meadow) as a result of WW with this being exacerbated by the N treatment. Although evergreen dwarf shrubs were reduced in biomass following the grazing and WW events, the

communities did not significantly differ in species composition between treatments and years, indicating a strong resistance to change in these *E. nigrum* dominated ecosystems. Furthermore, there was no strong evidence for any interaction between the multiple extreme events, indicating that multiple stresses do not necessarily lead to accumulation of biological stress responses (Thompson et al., 2008; Jackson et al., 2016).

We expected that +N would exacerbate the effects of WW and grazing pressure but no such responses were observed. We expected that N additions would promote plant growth, but simultaneously increase vulnerability to freezing stress due to changes in cell and physiological characteristics associated with drought and frost susceptibility (MacGillivray et al., 1995; Carroll et al., 1999; Schaberg et al., 2002). However, the added N-levels (5 kg ha⁻¹) did not lead to measurable responses in the control plots suggesting that these coastal *E. nigrum* vegetation types should be able to withstand N depositions events (Forsius et al., 2010; Karlsson et al., 2013). In addition, mid-winter bud swelling, Fv/Fm and leaf respiration, indicators of a potential release from winter dormancy responded similarly to WW with or without +N. The moss *P. schreberi* did show changes in membrane fatty acids to +N, but not consistently in combination with WW. The fatty acid C20:2 increased in WW–N compared to WW+N for both *E. nigrum* and *V. vitis-idaea*, indicating that soil N availability may affect cell membrane adaptations to freezing stress. However, as we did not find consistent phenology and plant biomass responses to WW+N in the following growing seasons, it appears that the role of N on the plants' winter physiology appears limited in this study. Nitrogen additions have been shown to increase insect damage to *Calluna vulgaris* communities (Power et al., 1998) but this was not observed in our study. Overall, the combination of stress events and low dose N deposition does not appear to seriously affect these coastal sub-arctic *E. nigrum* communities.

In support of our second hypothesis, the evergreen dwarf shrub *E. nigrum* declined in response to the grazing by geometrid caterpillars while the herb *C. suecica*, not often grazed upon, increased over the study period. *Empetrum nigrum* is frequently targeted by caterpillars when their main food plant birch trees have been defoliated (Jepsen et al., 2013), which explains the decline of this dwarf shrub over time. The increase of *C. suecica* was also found elsewhere in northern Fennoscandia as a combined effect of grazing pressure, increased precipitation and deposition of nitrogen through precipitation and caterpillar fecal matter (Tømmervik et al., 2004; Karlsen et al., 2013). However, we did not find any such response from N additions in our study, which indicates that the applied levels were insufficient to elicit a measurable plant growth response or that caterpillar fecal N input was much higher. In addition, moss cover was highest during 2015, reflecting the opening of the vascular plant cover canopy, as also observed following similar extreme events in physiognomically comparable open birch woodland in a more continental upland region of northern Scandinavia (Bokhorst et al., 2015). However, the evergreen species, such as *E. nigrum* and *V. vitis-idaea*, quickly recovered their biomass to starting levels in this study, indicating that this coastal *E. nigrum* dominated vegetation were fairly resistant to the combination

of WW events, N additions and invertebrate grazing. The ecosystem CO₂ fluxes measured during the growing season also did not show differences between treatment plots indicating that the plant community shifts were too limited to impact this ecosystem's carbon balance (Luyssaert et al., 2007; De Deyn et al., 2008), although the 2016 values were notably higher than during the 2 previous years (Supplementary Figure S3).

The muted response from the plant community, and NDVI values to the extreme WW events, may result from the intensive grazing impact overall, including the control plots, which could have diluted the effect of the imposed winter stress. The relative importance of such events, and their interaction, in shaping sub-arctic plant communities remains a complex interplay depending in large part on intensity, timing and duration (Olofsson et al., 2013). Furthermore, sub-dominant species cover was not affected enough to affect plot communities dominated by *E. nigrum* and *P. schreberi*, while high shoot defoliation of dwarf shrubs recorded during 2014 could have been partly compensated later on in the season (Bokhorst et al., 2011). In addition, studies on *C. vulgaris*-dominated ecosystems show that plant community responses to multiple environmental drivers can be affected by successional stage and disturbance regimes across Europe (Kröel-Dulay et al., 2015). As such, the high dominance of *E. nigrum* in our field site may reflect a successional stage that is more resistant than stages where other species comprise a larger proportion of the community or these coastal habitats have plants with greater physiological adaptability to cope with multiple stressors than habitats with more stable climates. With respect to the WW events, more pronounced plant responses have been reported when temperatures were lower (Bokhorst et al., 2009; Bjerke et al., 2017b), which is an important factor for frost drought-induced plant mortality (Kreyling et al., 2008; Bokhorst et al., 2010, 2018). The relative mild freezing temperatures may have allowed for winter physiological adaptations by *E. nigrum* and *V. vitis-idaea*, such as the greater proportion of longer chain fatty acids (Dalmannsdóttir et al., 2001; Schaberg et al., 2002). The N addition levels were chosen at the lowest concentration effect-response by most plant communities (Phoenix et al., 2012) as a realistic scenario, while higher N levels for a longer duration (>3 years) may result in additive biological responses (Power et al., 1998; Phoenix et al., 2012). Similarly, grazing intensity on plants is not consistent during each herbivore outbreak (Olofsson et al., 2013) indicating that sub-arctic plant community responses to multiple extreme events may depend predominantly on the event with the strongest intensity.

CONCLUSION

The findings indicate that coastal sub-arctic dwarf shrub plant communities and growing season CO₂ fluxes appear largely

unaffected to a combination of extreme WW events, grazing and nitrogen additions in summer. This response may in part be the result of inherent physiological adaptations, such as changing the membrane fatty acids (Dalmannsdóttir et al., 2001), during winter and the mild winter freezing temperatures (Bokhorst et al., 2018). The grazing impact appears to have overshadowed all plant responses to the WW and +N treatments indicating that multiple extreme events, that in theory can enhance each other's effects (Power et al., 1998; Phoenix et al., 2012), do not necessarily increase biological stress responses.

AUTHOR CONTRIBUTIONS

SB and JB designed and conducted the experimental work. All authors contributed to the experimental design, field and laboratory work, wrote or commented on the main text and supplementary notes.

FUNDING

This work was financed by a grant from the Research Council of Norway (Grant No. 225006) to JB and SB, and by financial support from the Flagship Programme "Effects of climate change on terrestrial ecosystems, landscapes, society and indigenous peoples" of FRAM – High North Research Centre for Climate and the Environment (Grant No. 362206), and the Norwegian Institute for Nature Research.

ACKNOWLEDGMENTS

We would like to thank all people who provided invaluable assistance during the field and lab work conducted for this research: Manuel Ballesteros, Dick Bokhorst, Ellen Elverland, Vigdis Frivoll, Trond Vidar Johnsen, Marit Jørgensen, Kristian Laustsen, Leidulf Lund, Geraldine Mabile, Kjell Nilsen, Ilona Peltoniemi, Martina Schwartzmüller, Laura Stendardi, Leif Einar Støvern, Rachael Treharne, Grzegorz Wierzbinski, and Matthias Zielke. We thank the two reviewers for their constructive comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01787/full#supplementary-material>

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Legacy Effects of Climate Extremes in Alpine Grassland

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OPEN ACCESS

Edited by:

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Auckland University of Technology,
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Reviewed by:

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 14 May 2018

Accepted: 11 October 2018

Published: 30 October 2018

Citation:

De Boeck HJ, Hiltbrunner E,
Verlinden M, Bassin S and Zeiter M
(2018) Legacy Effects of Climate
Extremes in Alpine Grassland.
Front. Plant Sci. 9:1586.
doi: 10.3389/fpls.2018.01586

Climate change is particularly apparent in many mountainous regions, with warming rates of more than twice the global average being reported for the European Alps. As a result, the probability of climate extremes has increased and is expected to rise further. In an earlier study, we looked into immediate impacts of experimentally imposed heat waves in alpine grassland, and found that these systems were able to cope with heat as long as enough water was available. However, concomitant drought led to increased stress, and reduced aboveground biomass production and green plant cover. Here, we studied the legacy effects (lag-effects) of the imposed climate extreme to see whether delayed responses occurred and how fast the alpine grassland could rebound from the initial changes. Green cover continued to be suppressed the two following years in communities that had been exposed to the most intense hot drought, while aboveground biomass production had returned to control levels by year 2. The initial lower resistance of the forb fraction in the communities was not compensated by faster recovery later on. This resulted in alpine communities that became (and remained) relatively enriched with graminoids, which resisted the original extreme better. The responses of alpine grassland to heat extremes with or without drought observed in this study resemble those typically found in lowland grassland in the short term. However, alpine grassland exhibited longer legacy effects from an annual perspective, with delayed recovery of aboveground production and persistent changes in community composition. This suggests that once initial resistance thresholds are exceeded, impacts may be longer-lasting in alpine grassland, where recovery is constrained by both the short growing season and difficult seedling establishment.

Keywords: biomass, drought, heat wave, mountain, recovery, resistance, stress, warming

INTRODUCTION

Ongoing climate change is characterized by a relatively modest rise in global surface temperatures to date, yet this has led to a much more pronounced increase in the number of heat waves. The current 0.85 K warming has made preindustrial 0.1-percentile heat extremes ~5 times more likely (Fischer and Knutti, 2015). With continued anthropogenic warming (IPCC, 2013), the number of

heat waves will increase further, making current extreme events common occurrences by the second half of the twenty-first century (Christidis et al., 2015). Moreover, concurrent heat and drought extremes are thought to occur more frequently than their single likelihoods would suggest. Indeed, as the water and energy balance are entwined, temperatures tend to rise during drought as energy dissipation shifts from latent to sensible heat (De Boeck and Verbeeck, 2011), while the anticyclonic conditions associated with most heat waves result in gradual drying through low precipitation and high atmospheric demand for water vapor (Trenberth and Shea, 2005; De Boeck et al., 2010).

Climate extremes can profoundly impact plant communities and ecosystems. The aforementioned heat and drought can result in reduced primary productivity (Zscheischler et al., 2014), altered community composition (Hoover et al., 2014), and even system state shifts (Fensham et al., 2009), with effects of the combination of heat and drought generally exceeding the sum of their individual impacts (De Boeck et al., 2011). Some of these effects manifest in the short term, but delayed responses that affect ecosystems after the end of the initial extreme (legacy effects) are also often observed (e.g., Sala et al., 2012; Anderegg et al., 2015). The most pronounced legacy effects are usually found where the initial impacts were highest (Yahdjian and Sala, 2006), but it is also possible that effects of the extreme are small or insignificant in the short term and become apparent only later (Vanoni et al., 2016). Determining how long antecedent conditions can affect plant and ecosystem responses remains challenging. Using a modeling approach, Ogle and co-workers found that antecedent effects explained between 18 and 28% of the response variables in semi-arid and arid ecosystems (Ogle et al., 2015).

The rapid increase in frequency and severity of climate extremes together with the high-impact potential has led to increased scientific interest. Nevertheless, many regions are underrepresented in extreme event studies (Beier et al., 2012), among which the cold biomes with their short growing season. This contrasts with observations of rapid climate change in these biomes, with warming rates significantly exceeding those recorded globally (Gobiet et al., 2014; Overland et al., 2017). At the same time, droughts are expected to become more frequent, longer and more intense the coming decades, also in mountain areas (Calanca, 2007; Gobiet et al., 2014), although regional differences are likely (Beniston et al., 2018). In addition, most

extreme event studies focus on responses in the short term (initial resistance and within-season recovery), while reports on longer term impacts, including legacy effects, are less common (Knapp et al., 2012).

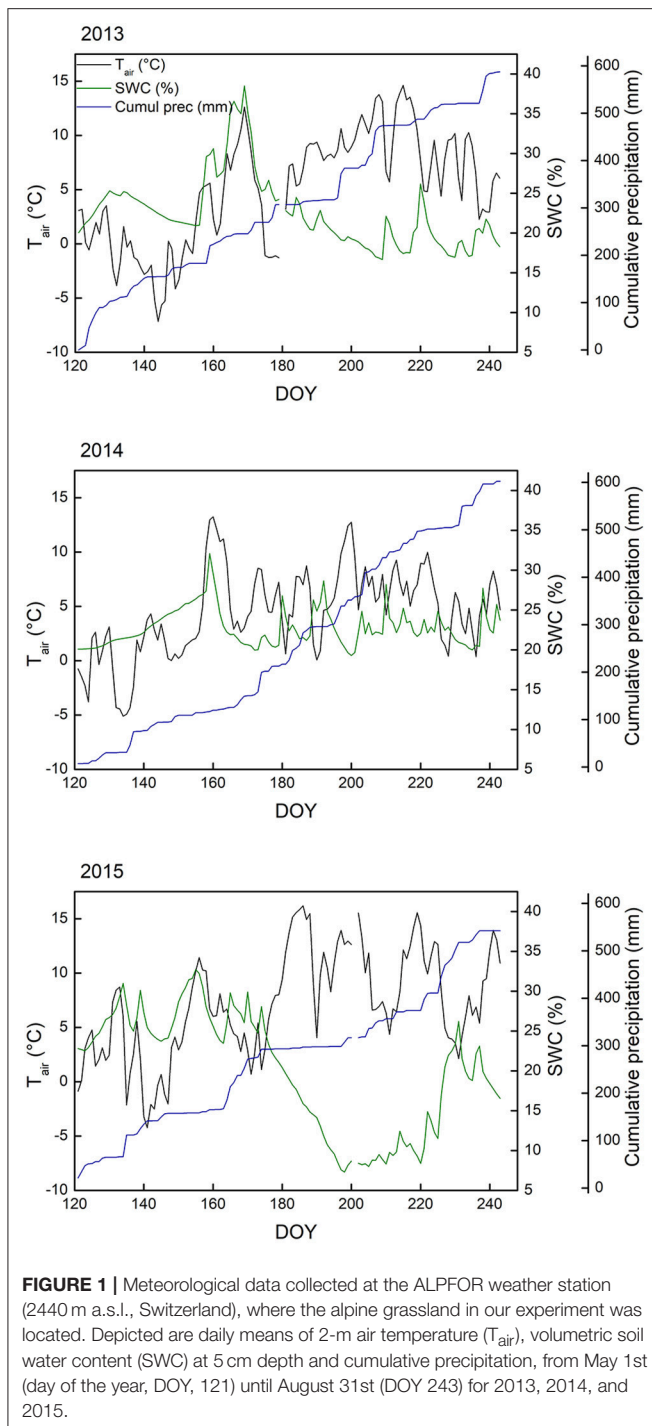
In an earlier extensive experimental study on heat and drought extremes in alpine grassland, we found that exposure to heat waves of variable intensity provoked little direct response unless the heat coincided with drought (De Boeck et al., 2016). Both F_v/F_m (a non-invasive measurement of photosystem II efficiency used as a general stress indicator) and canopy greenness decreased linearly with increasing average vapor pressure deficit (a combined measure of temperature and dryness of the air) under drought, resulting in lower aboveground biomass (−40% to −75%). Graminoids initially seemed more resistant than forbs, a trend also observed by Cremonese et al. (2017) following a natural hot drought. Although community-scale green cover was reduced dramatically (−80%) at the hottest site under drought conditions, it was unclear if mortality was widespread or if it was mainly a phenological response that prevented further water loss. In the latter case, this would allow plants to rebound the following growing season, in particular as the majority of alpine plant species are perennials.

In the current study, we focus on the legacy effects of the imposed heat and/or drought. While recovery after climate extremes is often fast in temperate grassland (Dreesen et al., 2014; Griffin-Nolan et al., 2018), the short growing season in the (sub)alpine zone is likely to constrain immediate recovery (De Boeck et al., 2016; Cremonese et al., 2017). This means that, although short-term responses to heat and drought, both as single factors and in combination, were found to be similar in alpine and temperate grassland, the different recovery dynamics may lead to divergent responses in the longer term. However, research on the legacy effects of climate extremes in cold biomes has been scant thus far. Here, we discuss these longer-term responses by reporting on changes in plant cover, aboveground phytomass and community composition during the 2 years (2014 and 2015) following the imposed extremes (2013) in alpine grassland. We hypothesize that (i) decreases in green cover during the extremes were caused by mortality rather than phenological responses, i.e., lower cover should also be apparent the following year; (ii) recovery is slow and legacy effects are predominantly negative; and (iii) the initially more resistant graminoids are able to maintain their increased relative success (De Boeck et al., 2016) also

TABLE 1 | Overview of measured air temperature and precipitation during the three growing seasons in this study.

	2013			2014			2015		
	June	July	August	June	July	August	June	July	August
T_{air} average (°C)	4.2	9.1	8.0	5.9	6.6	5.5	6.3	11.0	9.2
T_{air} min (°C)	−4.0	2.3	0.8	−1.9	−1.6	−1.6	−1.9	0.3	0.0
T_{air} max (°C)	15.8	16.4	19.4	18.0	16.0	12.4	15.4	19.8	18.2
Precipitation (mm)	105	139	138	107	215	171	136	64	185

Average, minimum (min), maximum (max) 2-m air temperature (T_{air}) and cumulative precipitation per month at the ALPFOR weather station (2440 m a.s.l., Switzerland) are given.



the following years, whereas forb biomass and species richness remains suppressed.

MATERIALS AND METHODS

Location and Set-Up

This experiment is a follow-up from an earlier study (De Boeck et al., 2016), in which swards of alpine grassland including

their main rooting horizons (monoliths) were transplanted along an extended (c. 1,800 m) elevation gradient. Monoliths of comparable species composition were collected at the ALPFOR research station, situated at 2,440 m near the Furka pass in the Swiss central Alps (46°34'N 8°25'E). These 590 cm² monoliths were selected on the basis of the presence of five species (the graminoids *Nardus stricta* and *Carex curvula*, and the forbs *Homogyne alpina*, *Potentilla aurea* and *Geum montanum*) and similarity in cover, to avoid bias between treatments due to differences in community composition. After excavation (early July 2013) the monoliths were transported to three lower-elevation sites: Oberwald (1,390 m a.s.l., 46°32'N 8°21'E), Bister (1,040 m a.s.l., 46°21'N 8°04'E), and Visp (660 m a.s.l., 46°17'N 7°53'E). One batch of monoliths was installed at the site of origin (Furka) as a reference (control). Half of the 48 monoliths was regularly irrigated, the other half was covered by a rainout shelter and was not irrigated. The climate extreme lasted 17 days (15 July–1 August 2013), after which all monoliths were transported back to the reference site, were immediately irrigated and reinstalled into the soil on their original locations on August 19 after aboveground phytomass harvest. A more elaborate description can be found in De Boeck et al. (2016).

Measurements

Meteorology

Meteorological measurements were made by an automatic weather station at the Furka site (operated by ALPFOR) that contributes to the network of the Swiss Federal Office of Meteorology and Climatology (MeteoSwiss). These include air and relative humidity (RH; Rotronic MP102H and Hygroclip HC2-S3, Switzerland) incoming and reflected global radiation (CM11 pyranometer, Kipp & Zonen, The Netherlands), precipitation (Lambrecht 1518H3, Germany), soil temperature (−10 cm, −25 cm; T107 probe Campbell Scientific, USA) and volumetric soil water content (−5, −15 cm; CS616 water content reflectometer, Campbell Scientific, USA).

Plant and Community Responses

Measurements were made following the same protocols used in the first study (De Boeck et al., 2016). The percentage of total green cover (between 0 and 100%), used as an indicator of leaf growth, expansion and senescence, was visually estimated for every monolith by the same observer and without looking at previously recorded data to avoid bias. Standing plant matter (phytomass) was clipped at ~2 cm height, separated per functional group (graminoids, herbs and (one) nitrogen fixer) and into living (green; biomass) and dead (brown; necromass), dried at 70°C for 2 days and then weighed. Cover estimates and phytomass harvests were carried out around the peak of the growing season, and were completed in the first week of August 2014 and 2015, which is ~1 week earlier than in 2013. This was done to reflect the earlier vegetation development due to earlier snow melt in 2014 and 2015 compared to 2013, i.e., to ensure that measures were comparable between years. To complement data on changes in the contribution of functional groups to phytomass, we assessed which species were present in each monolith in 2015.

TABLE 2 | Overview of the outcome of the statistical analyses (ANCOVA) for the response variables 1 and 2 years after the 2013 extreme.

		2014						2015					
		VPD		Irrig		VPD x Irrig		VPD		Irrig		VPD x Irrig	
group		<i>F</i> _{1,2}	<i>P</i>	<i>F</i> _{1,42}	<i>P</i>	<i>F</i> _{1,42}	<i>P</i>	<i>F</i> _{1,2}	<i>P</i>	<i>F</i> _{1,42}	<i>P</i>	<i>F</i> _{1,42}	<i>P</i>
Biomass	all	43.92	0.022	1.79	0.188	2.67	0.110	0.00	0.975	2.22	0.144	0.22	0.642
	graminoids	12.11	0.074	1.17	0.286	0.28	0.603	4.36	0.172	0.13	0.721	0.32	0.573
	forbs	8.81	0.097	2.87	0.098	8.40	0.006	0.60	0.519	10.98	0.002	4.67	0.036
Necromass	all	0.17	0.723	1.60	0.213	0.17	0.680	0.00	0.985	0.80	0.376	0.02	0.883
Phytomass	all	51.31	0.019	1.79	0.199	2.59	0.115	0.00	0.988	2.31	0.136	0.20	0.66
Green cover		10.30	0.085	23.60	<0.001	3.4	0.072	6.39	0.127	9.82	0.003	4.42	0.041
Species richness	total							2.25	0.272	4.22	0.046	4.06	0.05
	graminoids							0.81	0.462	0.00	0.947	0.27	0.609
	forbs							3.41	0.206	5.54	0.023	6.20	0.017

The site-specific average daytime vapor pressure deficit (VPD) during the 2013 extreme was used as a continuous fixed factor, the irrigation treatment in 2013 (Irrig) as a categorical fixed factor, and monolith (nested within site) as a random factor. *F*-values (including degrees of freedom) and *P*-values are given with significant ($P < 0.05$) or marginally significant ($P < 0.10$) indicated in bold.

Statistics

All statistical analyses were performed with the R statistical package (version 3.4.3; R Development Core Team, 2017). The design was a two-factor split-plot arrangement with monoliths nested within sites ($n = 6$ replicates). The categorical factor irrigation included two levels (irrigation vs. no irrigation). The continuous factor heat included four levels (sites). Two different measures of heat were used as explanatory variables: vapor pressure deficit (VPD) and air temperature (T_{air}). In both cases we used the average daytime values during the period of the extremes. These were 0.55 kPa and 14.1°C (Furka), 1.08 kPa and 19.8°C (Oberwald), 1.56 kPa and 24.5°C (Bister) and 1.19 kPa and 23.8°C (Visp), respectively (for further information, see De Boeck et al., 2016). Separate models were fitted for each of these variables because the two are correlated ($r = 0.95$). We consider VPD as the most relevant explanatory variable, as it combines temperature and humidity, two important parameters in assessing heat and drought effects (De Boeck et al., 2011). All response variables were transformed to achieve normal distribution of the residuals and then tested using ANCOVA with VPD or T_{air} as a continuous fixed factor, irrigation treatment as a categorical fixed factor, and monolith (nested within site) as a random factor. Separate analyses were performed for data collected in 2014 and 2015. Nitrogen fixers were not analyzed separately as this group contained only one species (*Trifolium alpinum*), and was therefore lumped together with the other dicots ('forbs').

RESULTS

Meteorology

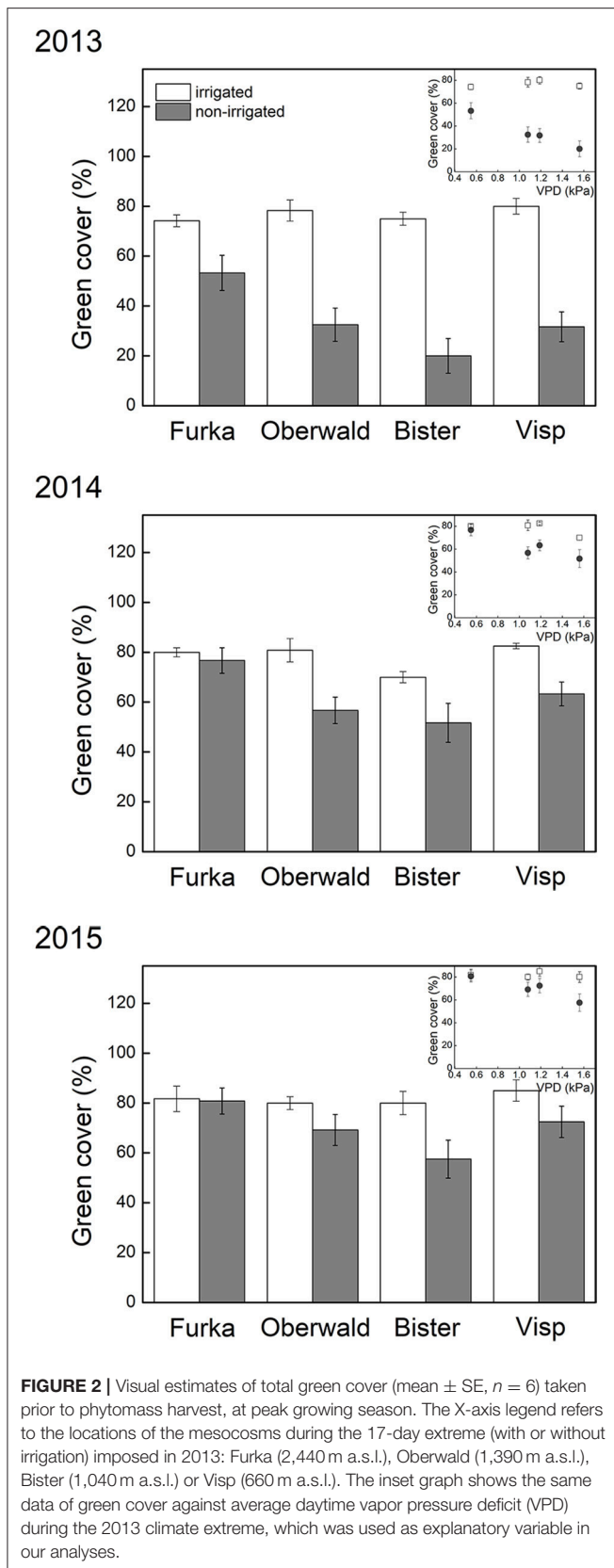
General environmental conditions varied in several ways during the 3 years this study spans (Table 1, Figure 1). One of the most relevant differences was the date the ground was free of snow, which is highly important regarding the start of the growing season (Körner, 2003). The spring of 2013 was characterized by generally cool conditions, with several late snowfall events. The

snowpack was only gone June 19 (DOY 170), whereas snowmelt came markedly earlier in 2014 (DOY 159) and 2015 (DOY 157). While 2013 (after the cool spring) and especially 2015 had warm summers (mean June–August air temperature of 7.1°C and 8.9°C, respectively), 2014 was cooler for the same period (6.0°C) and also less sunny (−18 and −12% direct radiation compared to 2013 and 2015, respectively). The 2015 (early) summer saw little precipitation, resulting in soil surface drying (Figure 1). Relative air humidity was 74% in 2015, compared to 78% in 2013 and 83% in 2014. It is important to keep in mind that these variables describe the general meteorological conditions, while the microclimate the plants experience deviates from the weather station measurements (see Körner and Hiltbrunner, 2017).

Plant and Community Responses

The short-term effects of climate extremes on total green cover detected in 2013 (De Boeck et al., 2016) were still reflected in the observations one and two growing seasons later (Table 2). Communities that had been exposed to heat waves showed very similar green cover (non-significant VPD effect), unless the heat wave had coincided with drought. Indeed, green cover was still significantly reduced 1 and 2 years after drought, and this effect was especially pronounced in monoliths of high-VPD sites, as demonstrated by the marginally significant (2014) and significant (2015) interaction with VPD (Table 2, Figure 2). In other words, 2 years after drought, cover remained suppressed, especially in communities that had been exposed to high atmospheric demand for water (VPD).

Contrary to green cover, biomass and phytomass at the community scale had rebounded to control levels by 2015 (no VPD or irrigation effect anymore in 2015, cf. Figure 3). However, there was a trend for drought-suppressed forb biomass in 2014, an effect that had become significant in 2015. Furthermore, we detected a significant interaction between drought and VPD for this group, indicating that the negative drought impact on forb biomass the years after the extreme increased with VPD.



Although forbs made up less than half of the community biomass in 2015 (controls), more forb species were present (11 in total) than graminoid species (4 in total) 2 years after the 2013 extreme. Both total species richness and species richness of forbs were significantly reduced by drought, especially in monoliths of high-VPD sites (significant interaction), whereas species richness of graminoids was unaffected by the different treatments (**Figure 4**). These effects mirror those found for the biomass of both groups (**Figure 3**).

DISCUSSION

Temperatures in mountain regions are generally rising faster than the global mean, which also increases the probability for exceptionally warm periods (Gobiet et al., 2014). At the same time, changes in precipitation patterns are expected, with more frequent droughts a distinct possibility in the Alps (Heinrich et al., 2014). We here studied if heat waves and/or drought in alpine grassland would lead to discernible legacy effects regarding productivity (here: above-ground biomass formation), green plant cover and species composition the years after the event, and whether these were closely linked to the immediate impacts. The short-term responses to heat waves of varying intensity were subdued and insignificant (De Boeck et al., 2016). We speculated that thresholds for heat stress had not been exceeded because tissue temperatures were moderated via latent heat transfer (transpiration; Larcher, 2003; De Boeck et al., 2011), with stomatal conductance not being affected by warming as such in our experiment (De Boeck et al., 2016). Moreover, alpine plants do experience relatively high tissue temperatures also in their native environment, as a result of high radiation and atmospheric decoupling (Körner, 2003; Neuner and Buchner, 2012; Dietrich and Körner, 2014). The initial lack of responses to heat in the absence of drought was also observed the following growing seasons. Both green cover and biomass did not significantly differ from control in the years after the imposed heat wave. Likewise, no significant changes in species richness or the balance between the functional groups present were observed. This also implies that potential indirect effects from the heat wave, for example through warming-induced changes in nutrient availability (Schmidt et al., 2002; Cross et al., 2015) or shifts in allocation to storage organs (Xu et al., 2016), were minor or absent.

Whereas these alpine grasslands were stable in both the short and longer term when faced with heat waves under non-limiting soil water, this changed when drought co-occurred. Drying soils and high VPD led to closure of stomata and loss of transpirational cooling, likely resulting not only in drought stress, but also heat stress (cf. Buchner et al., 2017) which in turn caused significant decreases in green cover and biomass, and increases in necromass (De Boeck et al., 2016). Our measurements demonstrate that trends observed on the short-term persisted the following 2 years, especially regarding green cover (**Figures 2, 3**). This implies that the initially observed senescence was not, or not merely, a

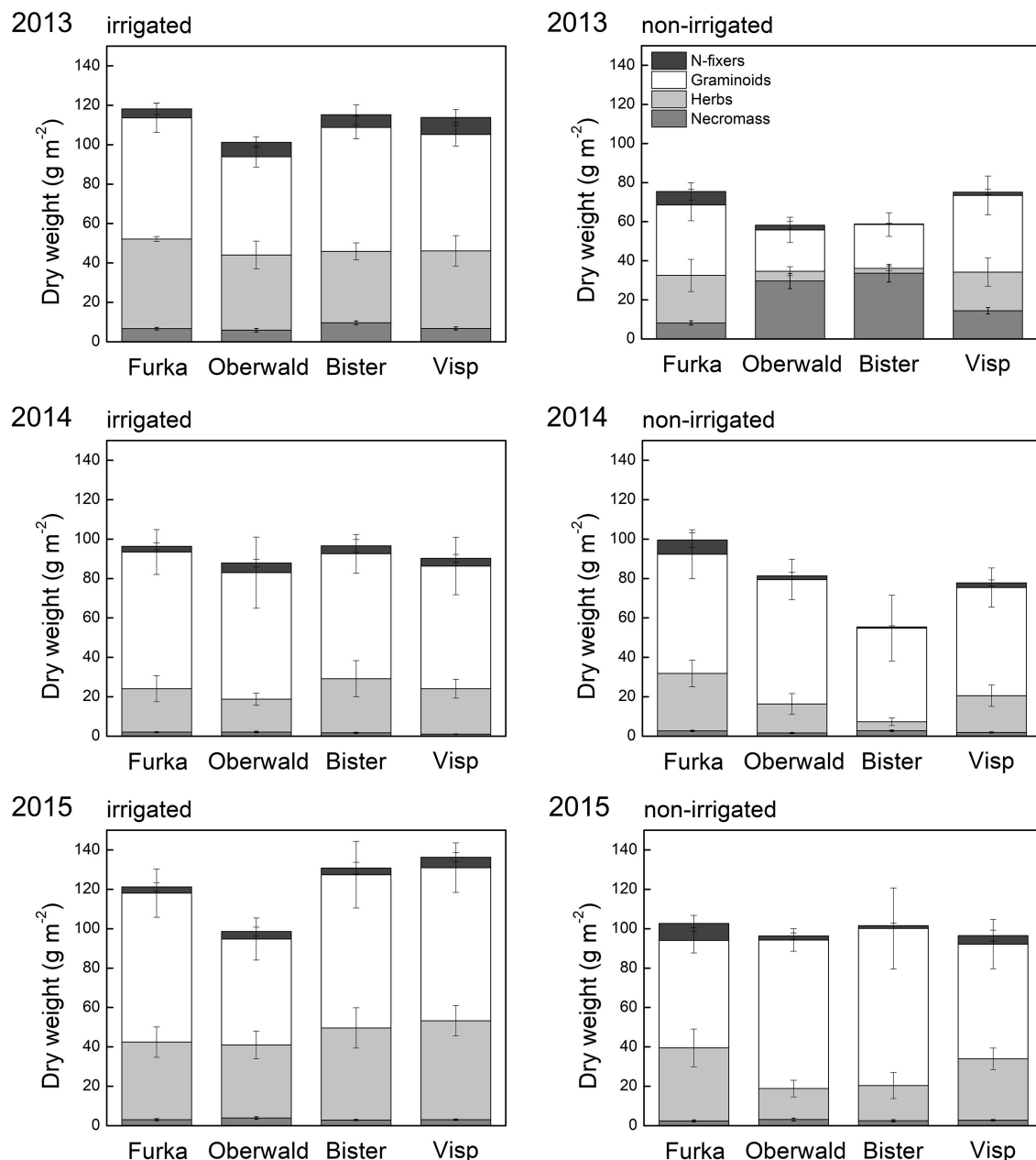
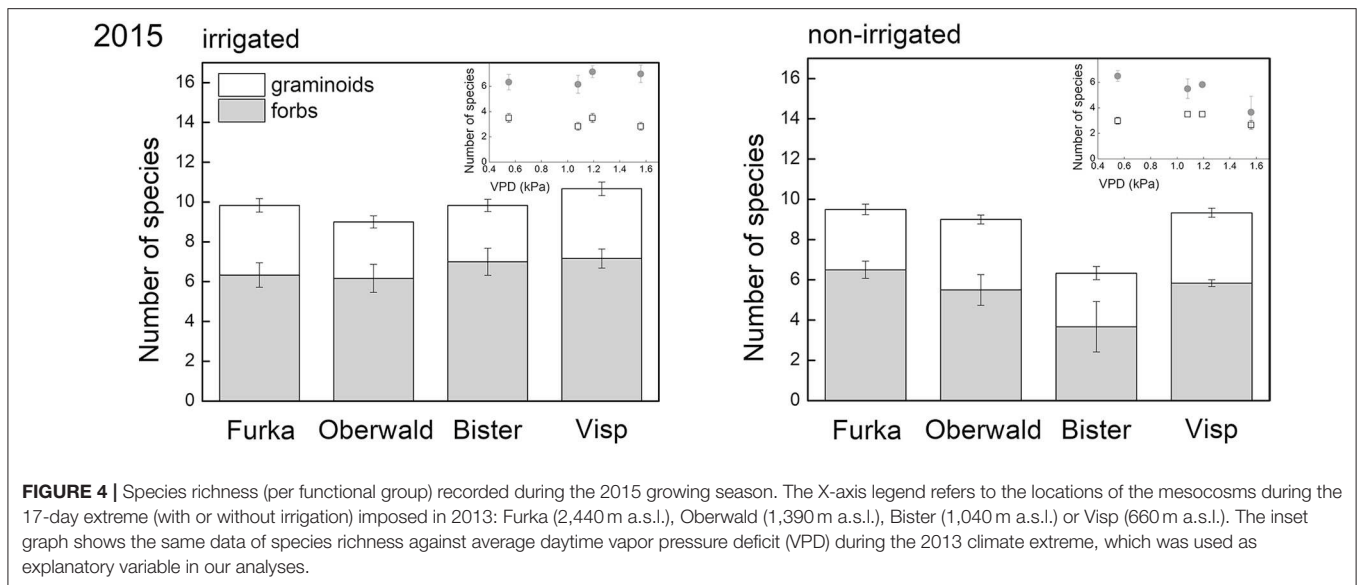


FIGURE 3 | Aboveground phytomass of all monoliths collected around the peak of the growing season, separated per functional group and into living (biomass) and dead (necromass). Error bars depict the standard error (6 monoliths per site and per irrigation treatment). The X-axis legend refers to the locations of the mesocosms during the 17-day extreme (with or without irrigation) imposed in 2013: Furka (2,440 m a.s.l.), Oberwald (1,390 m a.s.l.), Bister (1,040 m a.s.l.) or Visp (660 m a.s.l.).

response that avoided further dehydration so that cell integrity in meristems could be maintained, but an indicator of vulnerability to the imposed stress (Zwicke et al., 2015; Stampfli et al., 2018), confirming hypothesis 1. The significant interaction of green cover and VPD (i.e., site) reflects the complete recovery for the communities that experienced only drought ("Furka"), while cover continued to be below reference (control) levels elsewhere, especially in the communities that had been exposed to the hottest and driest conditions ("Bister"). Reduction in

cover may increase the exposure of soils to heavy precipitation events (Zuazo and Pleguezuelo, 2009), in turn affecting nutrient availability and soil stability. Here, the relatively large root biomass in alpine grassland (Körner, 2003) would decrease the vulnerability to such impacts, however. Productive temperate grasslands tend to bounce back to prior growth levels rapidly, often within weeks of the extreme (Dreesen et al., 2014; Zwicke et al., 2015). Fast recovery is stimulated in such grasslands by generally high available soil nutrients, especially immediately



following a drought (the Birch effect, cf. Birch, 1958; Ingrisch et al., 2018). However, rapid recovery was not observed in our alpine grassland. It is possible that low nutrient availability played a role (Gessler et al., 2017), and it is likely that phenological constraints severely limited potential (re)growth later in the (2013) growing season (Körner and Pelaez Menendez-Riedl, 1989).

The slow (literal) recovery and lack of new species observed in communities with the highest mortality suggests that gaps tended to close through encroachment rather than recruitment from seed. The alpine grassland in our study consists mostly of long-lived individuals that often reproduce clonally (De Witte et al., 2012) and lacks annual species that can quickly colonize open niches. This implies that compensation for negative impacts of extremes through recruitment, as observed in communities where dominant species have a short life cycle or produce abundant seedlings (Aragón et al., 2010; Lloret et al., 2012), is limited here. Such fast compensation is more important in temperate grasslands, where the longer growing season presents a longer window of opportunity for recovery (De Boeck et al., 2011). Moreover, the sometimes harsh microclimatic environment in gaps in high alpine grassland (Lembrechts et al., 2015) makes seedling establishment more difficult, and thus a limiting factor in these systems. It should be noted that whereas our imposed extremes affected only the monoliths and not the landscape (an inherent problem of manipulation experiments, see De Boeck et al., 2015), a natural, landscape-wide hot drought could affect propagule rain (Zeiter et al., 2006; Ertl, 2013), with implications for recruitment from seed.

In spite of slow restoration of green cover after extremes, the productivity of the alpine grassland in our study did bounce back faster. Cover and biomass are not necessarily correlated, as the latter also depends on plant height, canopy structure and community composition (Axmanová et al., 2012; Jiang et al., 2017). In our case, the graminoid fraction in the vegetation,

with its more erect growth form, likely compensated for the reduced share of forbs compared to communities not exposed to drought, which led to restored community productivity in spite of lower green cover. Both the extremes and the changes in community composition could have altered root biomass and vertical distribution of roots (Liu et al., 2018), but were not studied here.

The relative success of graminoids was more pronounced in treatments where the remaining (living) biomass was lowest directly after the extreme event and necromass highest (Oberwald and Bister, Figure 3). The damage to forbs had been significantly higher than to graminoids under those hot and dry conditions, possibly resulting from leaf size (larger) and orientation (more perpendicular to midday sun) and ensuing higher tissue temperatures (Dietrich and Körner, 2014; De Boeck et al., 2016). In addition, the forb species present in our study had a significantly higher specific leaf area than the graminoids (abundance-weighted means, data not shown). Specific leaf area has been linked to water and nutrient economy, suggesting that higher values are more common for fast-growing (resource acquisitive) species, while lower values are found more often in slow-growing (resource conservative) species (Reich, 2014). Indeed, the dominant graminoids in this alpine grassland, *Carex curvula* and *Nardus stricta*, are known to have high leaf longevity, which is inversely related with specific leaf area (Körner, 2003). This conservative strategy has been associated with increased resistance to drought compared with the resource acquisitive strategy (Lepš et al., 1982; Grime et al., 2000) and could thus help explain the higher survival of the graminoid fraction in our communities in the face of hot droughts. An increased proportion of graminoids vs. forbs after drought has been observed in other grassland studies in the Alps (Ingrisch et al., 2018; Stampfli et al., 2018), but also elsewhere (Mulhouse et al., 2017). Of special note is that our experimental findings were corroborated by the observational study of Cremonese et al.

(2017), who found that a pronounced natural hot drought in the Italian Alps impacted forbs (of which several species were identical to our study) significantly more than grasses. Forbs showed some signs of recovery in absolute terms the years after our imposed extreme, but remained suppressed compared to controls with their share around 20% (compared to c. 35% in controls). Other than forbs making up a reduced proportion of community biomass, forb species richness had also significantly declined. The ability of graminoids to rapidly resprout from basal meristems and “grab” the available nitrogen (Volaire et al., 2014) after the mortality of especially forbs may have helped to consolidate their increased share and increase community production back to control levels two growing seasons after the extremes.

Changes in the balance between functional groups can have profound implications for ecosystem functioning. In our case, above-ground biomass production was restored, but other functions may have been altered, such as seed and pollinator value. Carnicer et al. (2011) reported that herbivory, as well as pest or pathogen density can be reduced after mortality of a dominant plant species, although we have no indications for that here. Nutrient cycling may be affected by compositional changes cascading to soil communities via altered inputs (Wardle et al., 2004; De Deyn and Van der Putten, 2005). Nevertheless, the recovery of biomass production seems to indicate that at least initially, any changes in nutrient cycling did not play a major role in this alpine grassland. Another potential consequence of changes in community composition is an altered response to new extremes. As graminoids resisted the original extreme events best, new episodes of heat and drought combined may have less effect in such “pre-adapted” communities and could thus prolong graminoid dominance. In the future, climate extremes are projected to not only become more intense, but also more frequent. Such changes in the weather regime may thus perpetuate shifts arising from single extreme events (cf. Kreyling et al., 2011). This is an important aspect of the impacts climate change may have in these alpine grasslands that should be considered next to the effects of changes in the mean climate, such as species migration and altered phenology, including the ensuing changes in competitive interactions (Alexander et al., 2016; Rumpf et al., 2018; but see Scherrer and Koerner, 2010 on the importance of microtopography).

In conclusion, even though the immediate responses to heat extremes with or without drought seemed similar to

the ones repeatedly observed in lowland grassland (De Boeck et al., 2011; Dreesen et al., 2014), alpine grassland showed longer legacy effects (cf. Wu et al., 2018). The short growing season and difficult seedling establishment constrained recovery, at least from the annual perspective (see Körner, 2003 and Körner, 2013 regarding annual versus growing season points of view). Return to normal functioning regarding aboveground biomass production took 2 years, though green cover was still suppressed in some treatments, while the prior (“normal”) state was not reached from the perspective of diversity and community structure. Our data suggest that these alpine systems may become relatively enriched with graminoid species following hot droughts while forbs would decline, both in terms of biomass and species richness. While the ecosystem should return back to its prior state slowly, a new climate regime with more frequent extreme events could perpetuate the shift in community composition we observed. The relative enrichment with resource conservative graminoids may improve the system’s resistance in the face of new climate extremes, while some of its functioning is bound to be different. Slow recovery after mortality hints at the importance of initial resistance to a climate extreme in shaping these alpine grasslands the years after the event.

AUTHOR CONTRIBUTIONS

HD designed the study. HD and MV performed the measurements, with help from EH, SB and MZ. MZ analyzed the data. HD and MZ wrote the first draft. All authors contributed substantially to revisions and gave final approval for publication.

FUNDING

HD was supported by the Fund for Scientific Research—Flanders. Additional support was provided by the Swiss National Science Foundation (project number 149862).

ACKNOWLEDGMENTS

We thank the scientists and staff at Alpine Research and Education Station Furka (ALPFOR) for practical help, the community of Obergoms, the Zeiter family, and Landwirtschaftszentrum Visp for providing locations for our study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Productivity and Quality of Alpine Grassland Vary With Soil Water Availability Under Experimental Warming

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OPEN ACCESS

Edited by:

Hans J. De Boeck,
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Reviewed by:

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Stefano Chelli,
University of Camerino, Italy

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 21 May 2018

Accepted: 19 November 2018

Published: 14 December 2018

Citation:

Li C, Peng F, Xue X, You Q, Lai C, Zhang W and Cheng Y (2018) Productivity and Quality of Alpine Grassland Vary With Soil Water Availability Under Experimental Warming. *Front. Plant Sci.* 9:1790. doi: 10.3389/fpls.2018.01790

The plant productivity of alpine meadow is predicted to generally increase under a warming climate, but it remains unclear whether the positive response rates will vary with soil water availability. Without consideration of the response of community composition and plant quality, livestock grazing under the current stocking rate might still lead to grassland degradation, even in meadows with high plant biomass. We have conducted a warming experiment from 2010 to 2017 to examine the interactive effects of warming and soil water availability on plant growth and forage quality at individual and functional group levels in an alpine meadow located in the permafrost region of the Qinghai–Tibetan Plateau. Warming-induced changes in community composition, biomass, and forage quality varied with soil water availability. Under dry conditions, experimental warming reduced the relative importance of grasses and the aboveground biomass by 32.37 g m⁻² but increased the importance value of forbs. It also increased the crude fat by 0.68% and the crude protein by 3.19% at the end of summer but decreased the acid detergent fiber by 5.59% at the end of spring. The increase in crude fat and protein and the decrease in acid detergent fiber, but the decrease in aboveground biomass and increase the importance value of forbs, which may imply a deterioration of the grassland. Under wet conditions, warming increased aboveground biomass by 29.49 g m⁻² at the end of spring and reduced acid detergent fiber by 8.09% at the end of summer. The importance value of grasses and forbs positively correlated with the acid detergent fiber and crude protein, respectively. Our results suggest that precipitation changes will determine whether climate warming will benefit rangelands on the Qinghai–Tibetan Plateau, with drier conditions suppressing grassland productivity, but wetter conditions increasing production while preserving forage quality.

Keywords: climate warming, community composition, importance value, biomass, forage quality, alpine meadow, desertification

INTRODUCTION

The Qinghai–Tibetan Plateau (QTP) is the highest and largest plateau worldwide with a mean elevation of more than 4000 m above sea level (Wang et al., 2016). Alpine meadows cover approximately 7×10^5 km², accounting for about 50% of the total usable grassland on the QTP (You et al., 2017), and serve as the principal base of livestock husbandry for the local ethnic minority. However, alpine meadows have been severely degrading due to climate change and over-grazing activities (Chen et al., 2014; Wang et al., 2016). The warming on the QTP is predicted to be earlier and higher than other areas at the same latitude (Piao et al., 2004; Xu et al., 2014), and the livestock grazing intensity of the QTP has largely increased in the past 30 years (Song et al., 2009; Shang et al., 2014). For instance, the grassland area available per head of cattle declined from 13.73 ha in 1959 to 2.93 ha in 1999 in the Naqu region (Min and Cheng, 2001).

Climate warming will profoundly influence not only the above-ground biomass (AGB, Sullivan and Welker, 2005; Polley et al., 2013) but also the plant community composition and other ecosystem functions (Cardinale et al., 2012; Zhang et al., 2014). This can lead to changes in the nutritional status of individual species, and overall rangeland quality (Xu et al., 2018). For instance, an increase in legume abundance generally improves rangeland quality due to a high crude protein (g g^{-1} , CP) in the legumes (Cantarel et al., 2013). Thus, the investigation of forage production, forage quality, and plant community composition in a changing climate, is fundamental for the sustainable use of the alpine meadow ecosystem.

Previous studies have reported increases (Wan et al., 2005; Sullivan et al., 2008; Lin et al., 2010; Li et al., 2011), decreases (De Boeck et al., 2007, 2008; Klein et al., 2007), and no change (Saleska et al., 2002; Liu et al., 2018) in AGB in response to a warmer climate. These different responses may be partly related to water availability, which has been shown to affect the warming impact on plant productivity and ecosystem carbon fluxes (Yang et al., 2009; Peng et al., 2015). For example, in cold and humid ecosystems with abundant soil water, warming generally has positive effects on plant growth directly by stimulating temperature-driven physiological processes and indirectly by extending the growing season length (Wan et al., 2005; Liu et al., 2018). In contrast, in high-elevation ecosystems that face water shortage, warming will cause further water limitation for the carbon uptake process, and negatively influence plant growth (Xue et al., 2009; Chelli et al., 2017).

Plant community composition can mediate the response of AGB and forage quality to warming and the warming-induced change in soil moisture because of the different traits of different plant types. For example, grasses are more affected by drought than forbs in alpine systems because they have a low relative reduction in stomatal conductance (Bolling and Feller, 2014; Wellstein et al., 2017; Ganjurjav et al., 2018). Forbs have high root biomass, and superior interspecific competition ability, which could make them better able to cope with a warmer and drier climate (Dunnett and Grime, 1999; Klein et al., 2007; Zhang et al., 2014). Any increases in forage production may be compromised

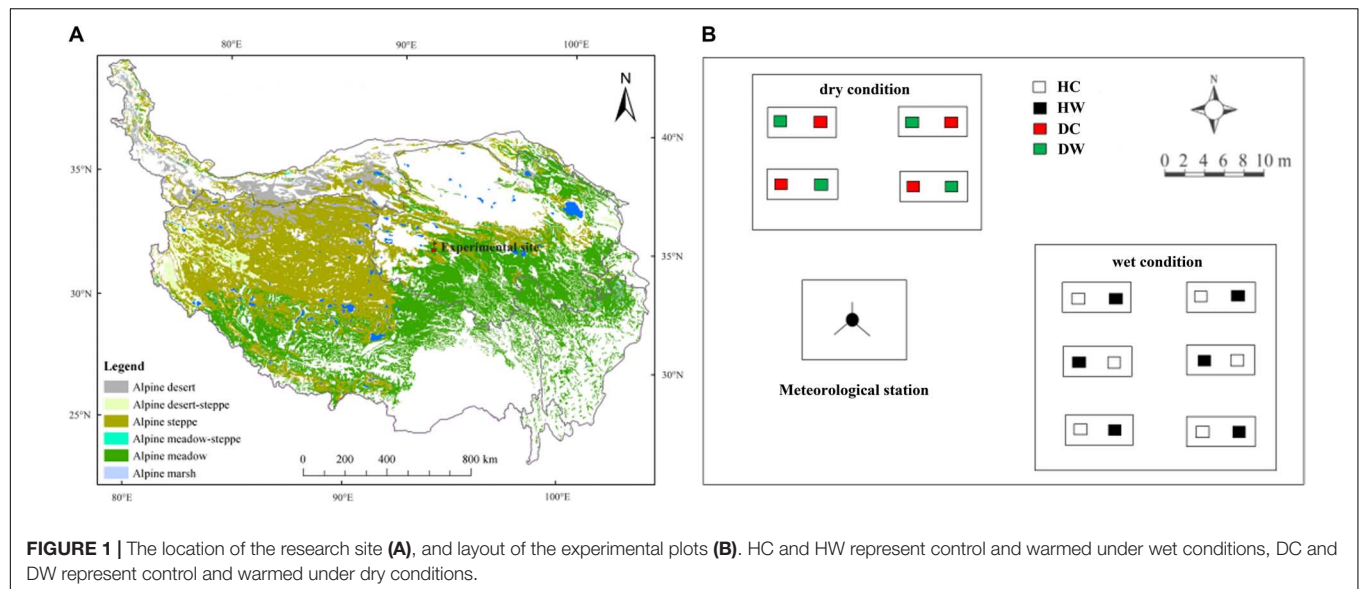
by a decrease in forage quality via nutrient-dilution effects (Shi et al., 2013). For example, a meta-analysis for grassland showed an increase in structural carbohydrates and lignification even though the forage production was enhanced in a warming climate (Dumont et al., 2015). CP, crude fat (EE), and acid detergent fiber (ADF) are indicators that can be used to reflect the forage quality. Pastures with higher CP and EE have higher nutritional value, while the higher the ADF content in pastures, the poorer the nutritional value of the pasture (Deak et al., 2007; Shi et al., 2013; Xu et al., 2018).

Although rangeland quality has been investigated in terms of decline of medicinal and non-palatable forbs (Klein et al., 2007), to our knowledge, no studies have examined rangeland quality at the community level. From previous studies in the same ecosystem, we know that the duration of soil-thaw period has extended (Xue et al., 2014), which suggests a long growing season, and the high availability of soil nitrogen in the warming treatment (Peng et al., 2016). However, the warming effect on ecosystem carbon fluxes and AGB varies with the annual rainfall amount (Peng et al., 2014). The AGB increased only when the annual rainfall was much higher than the long-term average. The gross ecosystem production and ecosystem respiration positively relate to the soil moisture when it is less than $\sim 15\%$ but negatively relate to soil moisture when it is higher than the threshold (Peng et al., 2014). Warming will decrease surface layer soil moisture (Xue et al., 2014) because of elevated evapotranspiration (Peng et al., 2015). We expect warming to lead to a decrease of grass species but increase in forb species in localities where soil moisture is low. Thus, we hypothesize that a warmer climate would stimulate growth, leading to improved forage quality where local soil water availability is high. Conversely, growth would be inhibited in drier areas, leading to poorer forage quality.

MATERIALS AND METHODS

Site Description

The experimental site is located near the QTP Research Base of the State Key Laboratory of Permafrost Engineering, Chinese Academy of Sciences ($34^{\circ}49'34''$ – $34^{\circ}49'37''$ N, $92^{\circ}55'57''$ – $92^{\circ}56'06''$ E), with a mean elevation of 4635 m above sea level (Figure 1A). Based on meteorological station data (daily observations) collected from January 2010 to December 2013, the mean annual temperature is -3.8°C , with monthly air temperature ranging from -27.9°C in January to 19.2°C in July. Mean annual precipitation is 290.9 mm, over 95% of which falls from May to October. Mean potential annual evaporation is 1316.9 mm, mean annual relative humidity is 57% and mean annual wind velocity is 4.1 m s^{-1} (Xu et al., 2015). The frozen period lasts from September to April and the depth of the seasonally frozen soil generally ranges from 2 to 3 m (Wang et al., 2007). The study site is a summer grazed range, dominated by alpine meadow vegetation, such as *Kobresia pygmaea* (sedge), *Kobresia capillifolia* (sedge), and *Carex moorcroftii* (sedge). *Polygonum viviparum* (forb) and *Stipa purpurea* (grass) are also common. The mean plant height is about 5 cm at a community level. Plant roots are mainly within the 0–20 cm soil layer. Soil



development is weak, and soils are classified as Mattic Cryic Cambisols (Alpine meadow soil, as Cambisols in FAO/UNESCO taxonomy) with a mattic epipedon at a depth of approximately 0–10 cm and an organic-rich layer at 20–30 cm (Wang et al., 2007). The experimental field was on a mountain slope with a mean inclination of 5°.

Experimental Design

Experimental plots were established in a typical alpine meadow within about 300 m of the Research Base. The experimental setup was finished in June 2010 and the plots were protected by a fence with hard iron wires to keep large herbivores away. A completely randomized split-plot experiment design was used (Figure 1B). Detailed information about soil properties in the 0–20 cm layer, soil temperature and soil moisture in the 0–10 cm layer, and plant features under dry and wet conditions are presented in **Supplementary Table S1**. Species composition was similar throughout the experimental plots but with lower coverage and plant height under dry than wet conditions. The average elevation difference between plots under dry and wet conditions was about 1 m.

In the experiment, the study site was split into large blocks with dry (6.55% annual mean in the 0–10 cm layer, v/v%) and wet (11.08%, v/v%) soils. Each block was split into small sub-plots in which warming treatments were applied. In the dry block, there were four pairs of control (DC) and warmed plots (DW); in the wet block, there were six pairs of control (HC) and warmed (HW), which represents four and six replicates under dry and wet conditions, respectively. There were 20 plots in total. Each plot occupied a 2 m × 2 m area. In each warmed plot, one 165 cm × 15 cm infrared heater (MR-2420, Kalglo Electronics Inc., Utah, United States) was suspended in the middle of the plot at 1.5 m above the ground with a radiation output of 150 W m⁻². Compared with the control, warming increased soil temperature by 1.8°C at 10 cm soil depth (Xue et al., 2014). The heating worked yearly-round since July 1st, 2010 and continued until

October 1st, 2017. Each control plot had a “dummy” radiator (no heating element) with the same dimensions as the infrared radiators suspended at a similar height to rule out any effects of shading by heaters (Xue et al., 2011). Moreover, in an open and flat area, a permanent meteorological observation station of 4 m height was installed to automatically record the climate variables outside of the experimental plots.

Temperature and Moisture Measurements

A thermo-probe (Model 109, Campbell Scientific, Inc, Utah, United States) was installed at the 15 and 30 cm soil depth in the center of each plot to monitor the soil temperature. Volumetric soil water content (v/v%) was measured by frequency domain reflectometry (FDR, EnviroSmart sensor, Sentek Pty Ltd., Stepney, Australia) at depths of 0–10, 10–20, and 20–40 cm. The daily average soil temperature and moisture were recorded with a CR 1000 data logger (Campbell Scientific, Inc., Utah, United States) at 10-min intervals. The 10-min recorded data were then averaged into daily data. The warming effects on soil temperature and soil moisture were analyzed according to the daily data. Although the warming experiment started in 2010, the species composition was investigated in June and September 2017. Thus, the warming impact on soil moisture was reported based on data for 2017. Soil temperature at 15 and 30 cm soil depth in the experimental sites was also reported based on data in 2017.

Vegetation Characteristic Measurements

In the growing season of 2017 (early June and late September), the vegetation characteristics of each plot were determined by the following methods. The experimental field (2 m × 2 m) was divided equally into four parts diagonally. Ten individuals were measured for height randomly in each part. The coverage of each part was measured with a frame with the interior dimensions

of 27 cm × 27 cm. The heights and coverage from the four parts were averaged for the height and coverage of each plot. The AGB was measured by cutting all the visible individuals above the ground (30 cm × 30 cm) in the center of each plot. Each species was identified then put in different envelopes. The collected biomass was then air dried, and then sent back to the laboratory and put into an oven to dry for 48 h at 75°C.

The height and frequency of each species were measured in a 20 cm × 20 cm small subplot within each plot. A frame with 100 small quadrants (1 cm × 1 cm) was used to measure the frequency. The height of each species was assessed based on the number of individuals for the species. If the number of individuals of a given species was less than 20, the height of the species was averaged by the number of heights measured. Otherwise, the height of that species was derived by 20 measurements with a ruler. The measured heights were then averaged for each species. Changes in importance values (IV) of species can reflect the variation of plant community composition (Zhang et al., 2014; Xu et al., 2015; Peng et al., 2017). The IV was initially developed to investigate the community composition and structure for forestry studies. The parameters used to calculate this index are density, basal area of each tree, and the frequency of a specific species. In the case of herbaceous vegetation, IV should be calculated based on the aboveground biomass. The height, coverage, and frequency are the most important factors to determine the biomass of each species of the herbaceous plants. Thus, we used the modified version of IV to characterize the community. The IV of each species was derived from relative coverage, relative height, and relative frequency. The relative height of a species is the ratio of the average height of that species to the summed height of all the species in the plot. The IV of each plant functional group was the sum of the IVs of any species belonging to that group.

$$IV_i = (rc + rh + rf)/3$$

where IV_i is the importance value of a specific species, rc is the relative coverage of species, rh is the relative height of species, and rf is the relative frequency of species.

The root biomass was sampled with a soil corer (7 cm internal diameter). In the 2016 growing season (late August), the soil with roots was extracted every 10 cm to a depth of 50 cm in each plot. The soil cores were placed in the cooler immediately and then transported to the laboratory by train. In the laboratory, soil samples were air-dried and crumbled by hand to pass through a 2-mm diameter sieve to remove large particles from the finer soil in distilled water. Then, the fine living roots were hand-picked based on their color and consistency in a distilled water bath, and the separated roots were dried at 75°C for 48 h.

Forage Quality Measurement

Plant species were classified into the three functional groups: grasses, sedges, and forbs. Only one legume species (*Astragalus polycladus*) was found in the study site and it was rare, therefore, we grouped it with forbs. Because of the lower stature of alpine plants and the need to avoid the interactive effect of warming and clipping, we only collected aboveground biomass for quality

measurement in a 20 cm × 20 cm subplot, therefore, the biomass was not enough to measure the CP, ADF, and EE of each functional group. As a result, the CP, ADF, and EE of the AGB mixture were measured for each plot. The CP was determined with an Automatic Kjeldahl Nitrogen Determination Apparatus (Kjeltec 8100, FOSS, Höganäs, Sweden). The ADF was determined by a sequential detergent fiber analysis (Goering and Van Soest, 1970), and the EE was determined by the Soxhlet extraction method (ANKOM XT15i, United States).

Data Analysis

A split-plot analysis of variance (ANOVA) was used to examine the main and interactive effects of warming and soil moisture conditions on the community AGB; belowground biomass; AGB of individual species; plant coverage; the IV of grasses, sedges, and forbs; and CP, ADF, and EE. One-way ANOVA was used to examine the effect of warming under dry and wet conditions on soil moisture; soil temperature; AGB; belowground biomass; AGB of individual species; plant coverage; individual species coverage; the IV of grasses, sedges, and forbs; and CP, ADF, and EE. Simple linear regression analyses were conducted to examine the correlation between the IV of grasses and ADF, the IV of forbs, and CP. All the above-mentioned statistical analyses were conducted using SPSS.17.0 for Windows (SPSS, Inc., Chicago, IL, United States).

RESULTS

Microclimate

The mean annual soil temperature was lower under wet conditions (15 cm, 0.96°C; 30 cm, 2.32°C) than dry conditions (15 cm, 1.60°C; 30 cm, 3.35°C; **Figure 2A**). Experimental warming significantly increased the soil temperature by 1.43 and 1.52°C at 15 and 30 cm soil depth, respectively, in the HW compared with the HC plots. It increased the soil temperature by 1.87 and 1.85°C at 15 and 30 cm soil depth, respectively, in the DW compared with DC plots ($P < 0.001$; **Figure 2A**). Soil water content was lower under dry conditions (0–10 cm, 6%, v/v%; 20–40 cm, 7%, v/v%) than wet conditions (0–10 cm, 11%, v/v%; 20–40 cm, 13%, v/v%) (**Figure 2B**). These values represent a significant decrease of 1% and 2% (v/v%; $P < 0.001$) in HW and DW plots in 0–10 cm layer, respectively, and increase by 4% and 1% (v/v%; $P < 0.001$) in HW and DW plots in the 20–40 cm layer, respectively (**Figure 2B**).

Biomass and Plant Coverage

Warming had no overall effect on the total AGB and plant coverage of a plot (**Table 1**). However, the interaction between warming and measuring month had a significant effect on the total AGB ($P < 0.05$). A significant interaction between warming and soil moisture condition on the plant coverage was observed ($P < 0.001$; **Table 1**). The plant coverage and AGB of a plot decreased by 15% ($P < 0.05$) and 32.37 g m⁻² ($P < 0.1$) in September in the DW plots but they increased by 11% ($P < 0.05$) and 29.49 g m⁻² ($P < 0.05$) in June in the HW plots (**Figures 3A,B**). Neither warming nor the interaction of

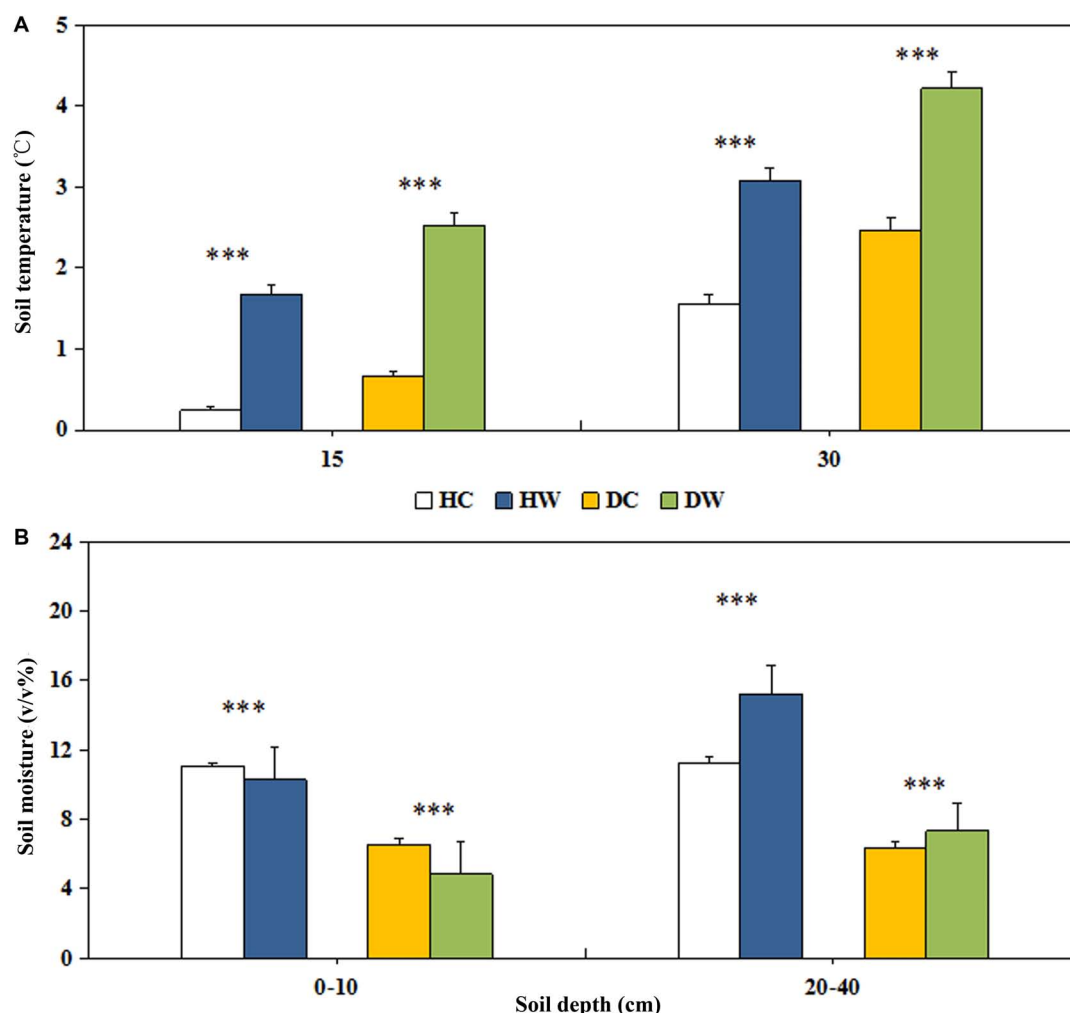


FIGURE 2 | Effects of warming on soil temperature at the depth of 15cm and 30cm **(A)**, soil moisture at the depth of 0–10 cm and 20–40 cm **(B)** under wet and dry conditions. See **Figure 1** for meanings of abbreviations. The error bars indicate standard error. Significance: *** $P < 0.001$.

TABLE 1 | Results (F -values) of split-plot ANOVA analysis of the effect of soil moisture condition (P), warming treatment (W), and their interactions on aboveground biomass (AGB), plot average plant coverage (PC), below-ground biomass (BGB), individual aboveground biomass (IAGB) of *Carex moorcroftii* (C.m), *Kobresia humilis* (K.h), *Kobresia pygmaea* (K.p), *Aster asteroides* (A.a), and *Astragalus polycladus* (A.P).

Variance source	AGB	BGB	PC	IAGB	C.m	K.h	K.p	A.a	A.p
P	5.47*	0.79	29.42***	8.94**	0.80	0.21	0.16	0.00	0.19
W	0.03	1.54	0.82	0.40	7.13*	0.64	0.82	9.43**	1.25
P*W	1.70	0.62	22.63***	0.03	0.29	4.32†	7.91*	0.67	6.18*

Significance: † $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

warming with soil moisture condition affected the AGB of each individual species (**Table 1** and **Figure 4A**). Warming significantly increased the coverage of *C. moorcroftii* by 0.12% in the HW plots (sedge; $P < 0.05$, **Table 1**) and *Aster asteroides* by 0.86% in the DW plots (forb; $P < 0.01$, **Table 1**). However, it decreased the coverage of *Poa pratensis* by 0.24% ($P < 0.01$) in the DW plots (**Figure 4B**). The interaction between warming and soil moisture condition had a significant effect on the coverage of *K. humilis* (sedge; $P < 0.1$), *K. pygmaea* (sedge; $P < 0.01$) and

A. polycladus (forb; $P < 0.05$, **Table 1**). The coverage of *K. humilis* decreased by 2.74% ($P < 0.1$) but that of *A. polycladus* increased by 0.18% ($P < 0.05$) in the DW plots and the coverage of *K. pygmaea* increased by 5.06% ($P < 0.01$) in the HW plots (**Figure 4B**).

Neither warming nor the interaction of warming with soil moisture condition had any significant effect on the total belowground biomass (**Table 1**). Belowground biomass decreased by 970 g m⁻² ($P < 0.1$) at 10–20 cm soil depth in the HW plots

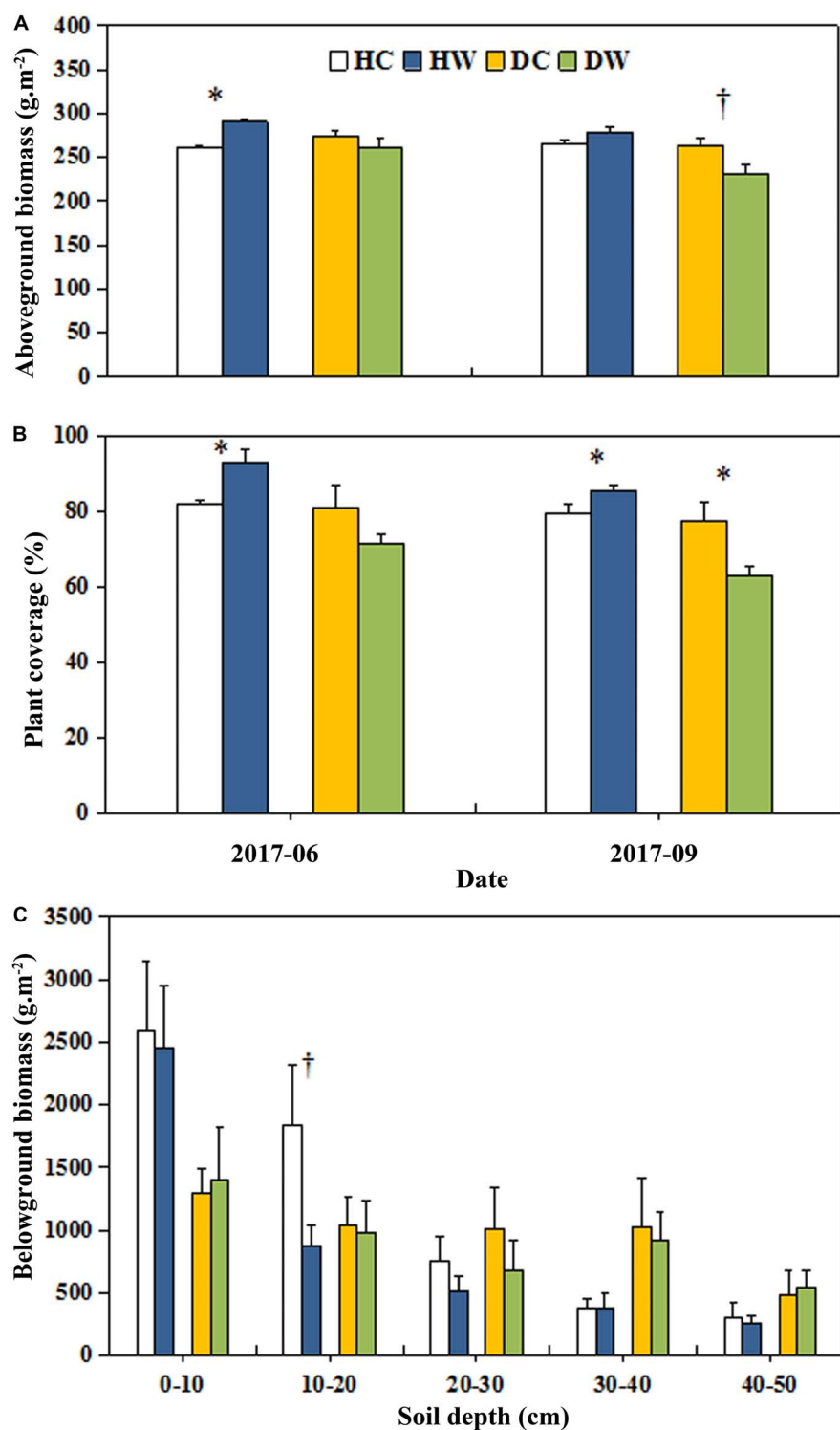
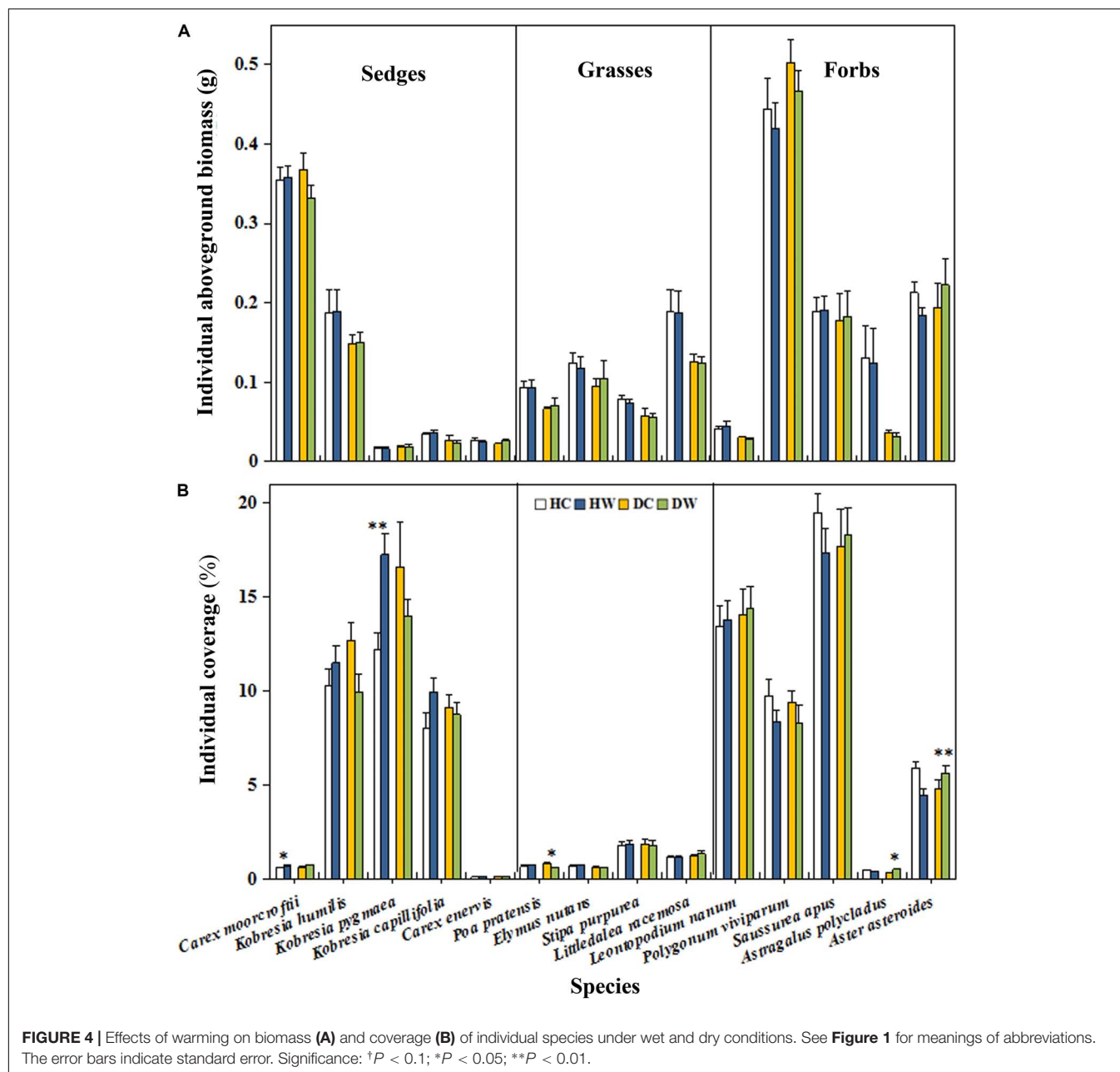


FIGURE 3 | Effects of warming on AGB (A), plant coverage (B), and belowground biomass (C) under wet and dry conditions. See Figure 1 for meanings of abbreviations. The error bars indicate standard error. Significance: $^{\dagger}P < 0.1$; $^*P < 0.05$.

but no change in the DW plots (Figure 3C). The proportion of the belowground biomass at 0–20 cm soil depth was lower but it was higher at the depth of 30–50 cm under dry conditions (the

average belowground biomass of DC and DW plots) than wet conditions (the average belowground biomass of HC and HW plots, Figure 3C).



Plant Community Composition

Warming significantly decreased the total IV of grasses ($P < 0.05$; **Table 2**). The interaction between warming, month, and soil moisture condition had a significant effect on the IV of grasses ($P < 0.05$) but no significant effect on the IV of sedge. The average IV of grasses was lower in DW plots (0.20) compared with DC plots (0.24, $P < 0.1$) in September 2017 (**Figure 5A**). The average IV of sedges under dry conditions was lower than wet conditions (**Figure 5B**), but the average IV of forbs reversed (**Figure 5C**). Warming increased the total IV of forbs ($P < 0.1$; **Table 2**). The interaction of warming with soil moisture condition had no significant effect on the IV of forbs (**Table 2**). The IV of forbs increased by 0.05 in DW plots (0.40) compared with the DC plots

(0.35, $P < 0.05$) in September but there was no change in the HW plots (**Figure 5C**).

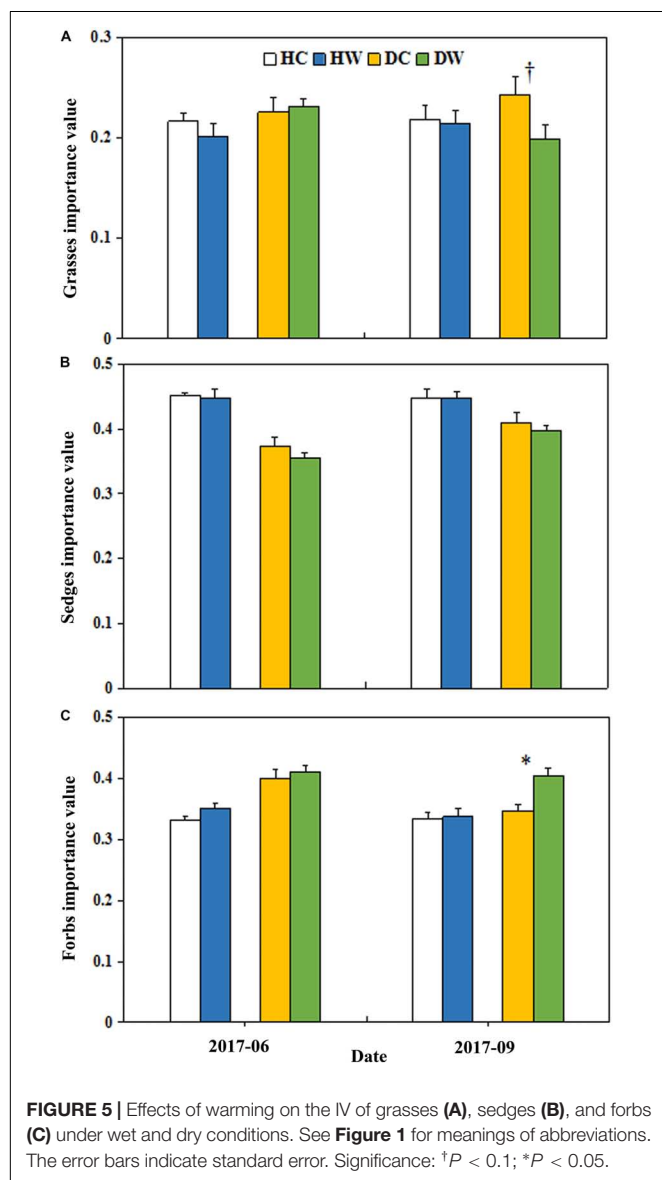
Forage Quality

Warming significantly reduced the ADF ($P < 0.01$; **Table 2**). No interactive effects of warming with soil moisture condition were observed on ADF (**Table 2**). The ADF decreased in June by 5% in the DW plots (48%) compared with the DC plots (43%, $P < 0.05$) and it reduced only in September by 8% in the HW plots (42%) relative to the HC plots (34%, $P < 0.05$, **Figure 6A**). Warming had no significant effect on the total CP (**Table 2**). The interaction between warming and soil moisture condition significantly affected the CP ($P < 0.01$, **Table 2**), which increased

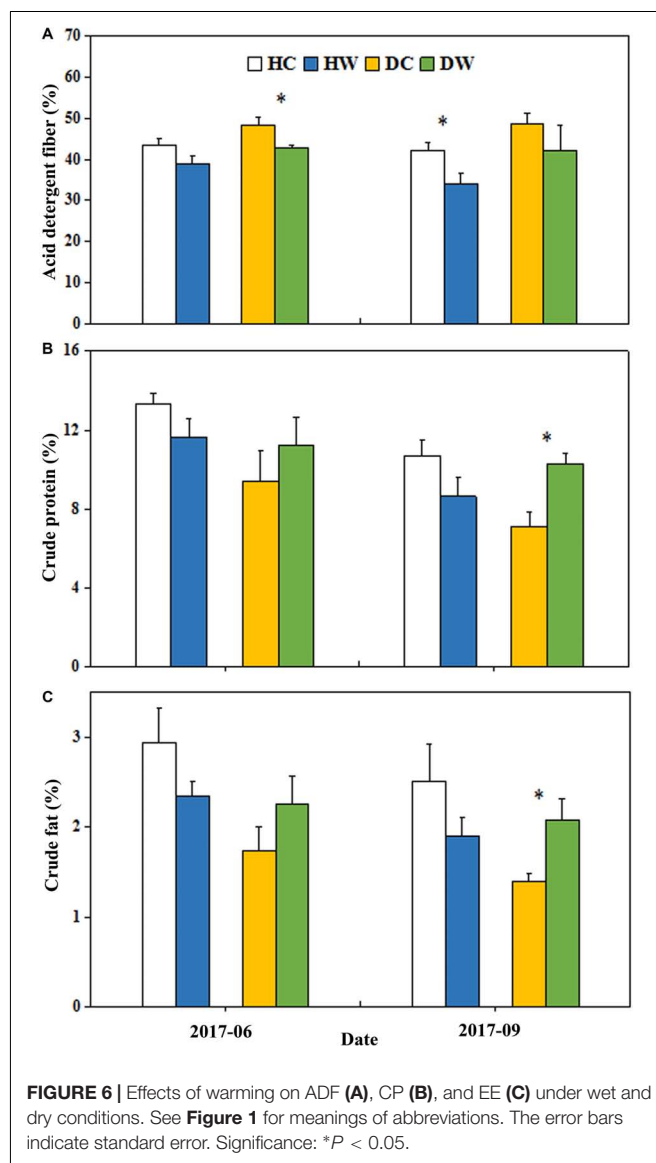
TABLE 2 | Results (*F*-values) of split-plot ANOVA analysis of the effect of soil moisture condition (P), warming treatment (W), and their interactions on ADF, CP, EE, and importance value of different functional groups (IV).

Variance source	ADF	CP	EE	Grasses IV	Sedges IV	Forbs IV
P	10.08**	5.30*	6.56*	5.63*	39.17***	26.36***
W	10.85**	0.20	0.00	5.51*	0.44	3.91 [†]
P*W	0.01	10.18**	7.74**	1.49	0.00	0.47

Significance: [†] $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



by 3% in the DW plots (10%) compared with the DC plots (7%, $P < 0.05$) in September 2017 (Figure 6B). Warming had no significant effect on the total EE (Table 2). The interaction between warming and soil moisture condition had a significant effect on EE ($P < 0.01$, Table 2), which was 1% higher in the DW plots (2%) relative to the DC plots (1%, $P < 0.05$) in September (Figure 6C).



Relationship Between Forage Quality and Importance Value of Different Groups

The pooled data showed a positive correlation between the ADF and the IV of grasses in the control and warming plots ($P < 0.001$, Figure 7A), and a positive correlation between the CP and the IV of forbs ($P = 0.02$, Figure 7B).

DISCUSSION

Response of Soil Moisture to Warming

The hydrological cycle in the ecosystem is driven by energy transfer. Soil surface evaporation and plant transpiration always accompany heat transfer from the soil to the atmosphere. In our study system, experimental warming increased soil surface temperatures, and temperature gradients between the soil surface and the atmosphere (Xue et al., 2014). This led to a higher evaporation and a reduction in soil water in the upper layers (**Figure 2B**), where most of the plant roots were located (0–20 cm, **Figure 3C**). Warming may foster a less favorable belowground environment for plant species with respect to soil temperature and water content. The study area is located in an area with continuous permafrost and the thickest active layer in China (Xue et al., 2014; Peng et al., 2017). Experimental warming enhanced the thawing process in the active layer during the warming season (Xue et al., 2014). Longer thaw duration and the higher active layer thickness might be responsible for increased soil water content in the deeper soil layers (**Figure 2B**).

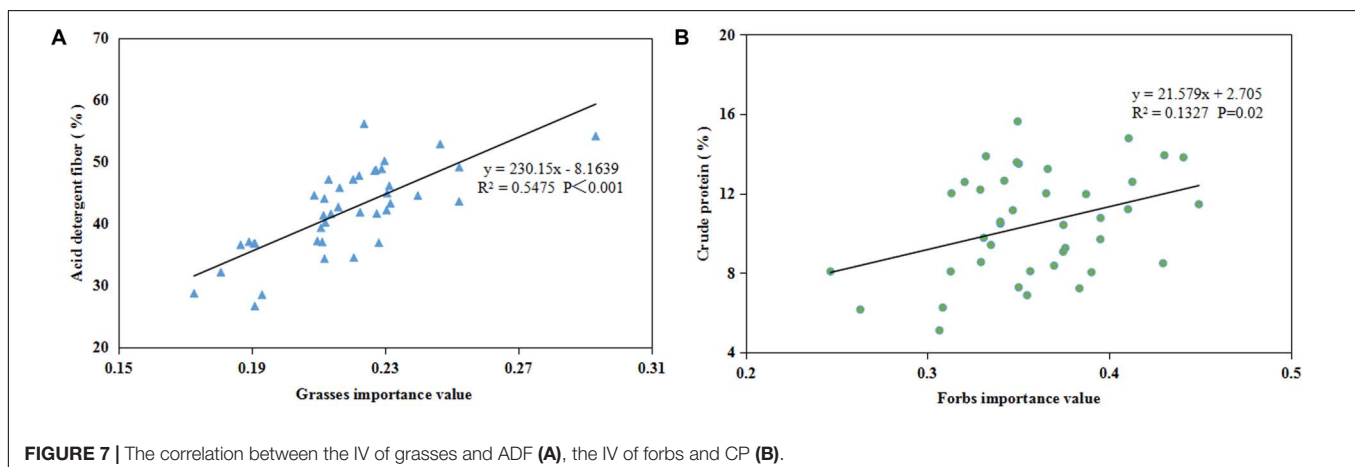
Response of Biomass to Warming

An increase in temperature could stimulate plant growth, but the associated soil moisture decline might offset the positive effect of increased temperature on plant biomass (De Boeck et al., 2008; Chelli et al., 2016; Liu et al., 2018). This might explain the non-detectable overall effect on AGB as soil moisture declined in our study (**Figure 2B**). Warming decreased the AGB in end of summer under dry conditions and increased the AGB at the end of spring under wet conditions (**Figure 3A**). This confirmed the hypothesis that a warmer climate would stimulate growth where local soil water availability is high, while growth would be inhibited in drier areas. Although soil moisture declined at 0–10 cm soil depth under both dry and wet conditions, the soil moisture in HW plots was higher than that in DC plots (**Figure 2B**). The reduced soil moisture might not limit the plant growth under wet conditions but constrains it under dry conditions. We did not directly measure the growing season duration; however, the advanced thawing of the active layer

in spring (Xue et al., 2014) indicates an early start of the growing season in the permafrost region, thus resulting in the enhanced AGB in end of spring under wet conditions. Even the freezing of active layer delays (Xue et al., 2014) plants cannot make use of the extended period of thermally favorable conditions, probably because of an established life cycle (Yu et al., 2010) due to the low temperature (Dong et al., 2009). The long-term evolution and adaptation of alpine plants cause them to have low heat requirements even in the summer developmental stages (Yu et al., 2010). Warming in summer thus rapidly accelerates plant development, leading to earlier completion of the reproductive cycle of species (Yu et al., 2010). The possible early end of growing period under the high-magnitude soil temperature increase, under dry conditions (**Figure 2A**) might explain the decline in AGB in the DW plots at the end of summer.

Given no change in the individual aboveground biomass of the species in either the DW or HW plots (**Table 1** and **Figure 4C**), the enhancement in AGB under wet conditions and reduction under dry conditions can be partly ascribed to the changes in community composition (Zhang et al., 2014; Xu et al., 2018). In general, the height of grass species is greater than that of forb species. The decrease in the IV of grasses due to the reduction in plant height and coverage of grasses (**Figures 5A,C**) in our study might partly bring about a decrease in AGB under dry conditions. Under wet conditions, the relatively higher soil moisture (**Figure 2B**) might be adequate to offset the negative effects of surface drying after warming, and the elongation of the growing season could support the increased AGB in wet locations.

The warming-induced decrease in belowground biomass is mainly due to drought stress in the surface layer (De Boeck et al., 2007; Klein et al., 2007). Plant belowground biomass was mostly distributed in upper soil layers in our experimental sites (**Figure 3C**), which might explain the decrease in belowground biomass at 0–20 cm soil depth in the HW plots. Forb species have deep roots (Peng et al., 2017). The higher IV of forbs under dry than wet conditions (**Figure 5C**), thus may be responsible for the higher proportion of the belowground biomass at 30–50 cm soil depth under dry than wet locations (**Figure 3C**).



Response of the Community Composition to Warming

The abundance of different plant functional groups responds distinctly to climate warming depending on the traits that can enhance survival. For example, the abundance of grasses decreased with the presence of forbs under dry conditions (Dunnett and Grime, 1999) because forbs are more adaptable to climate warming than grasses species because of their high interspecific competitive ability for resources (Dunnett and Grime, 1999; Klein et al., 2007; Klanderud, 2008). The competitive advantage of forbs (Klein et al., 2007) could therefore result in the decrease in IV of grasses but the increase in IV of forbs in the DW plots (**Figure 5C**). This confirms the hypothesis that warming could lead to a decrease in grass species but increase in forb species in areas of low soil moisture.

The responses of different plant functional types could vary with precipitation or nutrient availability due to the niche difference or complementary use of moisture and nutrients (Peng et al., 2017; Xu et al., 2018). For example, the frequency and abundance of grasses decreased in the relatively dry sites in a sub-alpine meadow warming study (Rudgers et al., 2014) but that of sedge and grasses increased in a wet tundra (Elmendorf et al., 2012). The soil water conditions in the sub-alpine meadow and the wet tundra may explain the opposite responses of grasses and sedge abundance to warming. Warming can aggravate soil drought stress (Xue et al., 2017) on plant growth under dry sites, which likely causes plants to distribute more biomass belowground to obtain more nutrients (Zhang et al., 2014). The competitive disadvantage of grasses leads to the reduction in IV of grass species (Wang et al., 2010; Xu et al., 2014). Enhancement of water uptake capacity of forbs under drought stress was reported in an alpine ecosystem on the QTP (Ganjurjav et al., 2018), whereas forbs are less resistant to drought than grasses in alpine grassland with an elevation less than 3000 m (De Boeck et al., 2016, 2018).

In our study system, we found a higher average IV of forbs under dry than wet conditions (**Figure 5C**), which suggests the dependence of variation in response of plant groups to warming on the site conditions. In alpine ecosystems, under wet conditions, adequate moisture can satisfy the growth of grasses and forbs, therefore weakening the inter-specific competition (Wang et al., 2010; Zhang et al., 2014). This could partly support the non-significant change in the IV of grasses and forbs under wet conditions (**Figures 5A,C**). One study carried out in the alpine grassland ecosystems on the eastern QTP found that both experimental warming and drought treatments increased grass abundance and decreased sedge and forb abundance (Liu et al., 2018). The difference between our results and those of Liu et al. might be attributed as follows first, different community composition in two study sites. In Liu et al.'s study, grasses are the dominant functional groups, the grass abundance (about 0.6) was significantly higher than that of forbs (about 0.3) and sedges (about 0.1). However, there were fewer grasses in our study site, the IV of forbs (about 0.4) and sedges (about 0.4) was significantly higher than that of grasses (about 0.2). Warming is expected to increase biotic interactions

and competition among species under dry conditions (Olsen and Klanderud, 2014). Dominant species generally have a more competitive position than other functional groups (Dunnett and Grime, 1999; Klanderud, 2008). Second, in Liu's study, mean annual precipitation is 489 mm. Grass abundance is highly significantly correlated with water condition across the altitude gradient on the QTP (Dorji et al., 2014) and decreases with the presence of forbs under dry conditions (Dunnett and Grime, 1999). This suggests a threshold after which graminoids are no longer able to compete with forbs if precipitation decreases further in alpine systems where few grasses exist compared with forbs. The low mean annual precipitation (290.9 mm) in our study area might also be the cause of the reduction in IV of grasses and increased in IV of forbs under dry conditions.

Response of Forage Quality to Warming

Rangeland quality was determined by community nutrient production, which included two aspects: forage production (AGB) and forage quality (Xu et al., 2018). Forage production provides edible fodder to livestock and directly affects grassland carrying capacity, while forage quality determines the efficiency of pasture utilization and affects livestock growth (Shi et al., 2013). Shifts in plant community structure caused by warming could have further effects on forage quality (Dumont et al., 2015). Grass species contains more ADF than other plant functional groups (Xu et al., 2018). In general, the higher the EE and nitrogen-free extracts, especially the CP in the pasture, the better the nutritional value, and the higher the ADF content, the poorer the pasture's nutritional value (Shi et al., 2013). In general, CP and ADF are positively and negatively correlated with livestock productivity, respectively (Lee et al., 2016). In our experiment, warming reduced the ADF content both under dry and wet conditions but increased the CP content under dry conditions. The positive correlation between ADF and IV of grasses (**Figure 7A**) and the decrease in IV of grasses indicate the change in grass abundance may have a positive effect on the forage quality. Alpine plants have a series of adaptive mechanisms in response to warming and drought (Shi et al., 2013), such as decreasing structural-carbohydrate content and increasing CP content to reduce damage caused to the plant by warming and drought (Shi et al., 2013; Xu et al., 2018). Results on the northeastern QTP show that legumes have the highest nutritive value, followed by non-legume forbs, sedges, and grasses according to the nutrient content ranking (Xu et al., 2018), which supports our observation of the positive correlation between ADF and IV of grasses. The positive correlation between CP and the IV of forbs (**Figure 7B**) could lead to the CP increase as IV of forbs increased in the DW plots at the end of summer (**Figure 6B**). The negative correlation between EE and precipitation (Shi et al., 2013) could support the EE increase in the DW plots.

In our study, warming improved forage quality due to reduced ADF content and increased EE and CP content under dry conditions. However, warming had no significant effect on the CP and EE under wet conditions, which was not as we expected. The forb increase may contribute to improved forage quality due

to the higher CP content of forbs than grasses. No change in community composition may result in no significant response of CP content to warming under wet conditions. Warming may improve forage quality owing to stimulation of legume growth on the northeastern QTP (Wang et al., 2012; Xu et al., 2018). In our experimental site, there was only one legume species, and it was rare. Klein et al. (2007) showed that warming reduced rangeland quality due to increased production of the non-palatable forb *Stellera chamaejasme* and the decreased production of the medicinal forb *Gentiana straminea*. However, neither of these species have been found at our experimental site.

Rangeland Management Implications

Our study provides experimental evidence that the effect of warming on forage production and forage quality change seasonally under different moisture conditions. Warming improved the rangeland quality by increasing forage production and there was no change of CP under wet conditions in alpine grassland on the QTP. However, warming might decrease the rangeland quality by decreasing relative importance of grasses but increasing that of forbs and decreasing AGB under dry conditions. Thus, local governments need to take some efficient adaptation strategies, such as setting the grazing intensity according to the grassland forage production and forage quality, and reducing livestock numbers to avoid rangeland degradation, especially in dry regions.

CONCLUSION

Experimental warming reduced the average IV of grasses and increased the average IV of forbs under dry conditions, which may indicate a shift in community composition toward fewer grass species and more forb species in alpine systems with few grasses present relative to forbs. A future warmer climate might bring about the increased AGB under wet conditions and cause decreased AGB due to an earlier end of the growing season under

dry conditions. Precipitation changes on the QTP will determine whether climate warming is going to benefit rangelands, with drier conditions suppressing grassland productivity, but wetter conditions increasing production while preserving forage quality.

AUTHOR CONTRIBUTIONS

FP, QY, and XX proposed the idea and designed the experiment. CLi, FP, CLa, and YC conducted the study. CL and FP wrote the manuscript. QY, XX, CL, and YC revised the manuscript. All authors contributed substantially to revisions and gave final approval for publication.

FUNDING

This study was financially supported by the State Key Laboratory of Grassland Agro-ecosystem (SKLGAE201709) and National Natural Science Foundation of China (41771229 and 31560151).

ACKNOWLEDGMENTS

We thank Alex Boon, Ph.D., from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript. We also thank Guilong Wu and Ji Chen in the Beiluhe Research Station for their support during the field sampling and measurement, and Qingqing Ma and Linrong Chai in the Lanzhou University for assisting the analysis of forage quality.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01790/full#supplementary-material>

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fast Responses of Root Dynamics to Increased Snow Deposition and Summer Air Temperature in an Arctic Wetland

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OPEN ACCESS

Edited by:

Hans J. De Boeck,
University of Antwerp, Belgium

Reviewed by:

Ivika Ostonen,
University of Tartu, Estonia
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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 14 May 2018

Accepted: 09 August 2018

Published: 30 August 2018

Citation:

D'Imperio L, Arndal MF, Nielsen CS,
Elberling B and Schmidt IK (2018)
Fast Responses of Root Dynamics
to Increased Snow Deposition
and Summer Air Temperature in an
Arctic Wetland.
Front. Plant Sci. 9:1258.
doi: 10.3389/fpls.2018.01258

In wet tundra ecosystems, covering vast areas of the Arctic, the belowground plant biomass exceeds the aboveground, making root dynamics a crucial component of the nutrient cycling and the carbon (C) budget of the Arctic. In response to the projected climatic scenarios for the Arctic, namely increased temperature and changes in precipitation patterns, root dynamics may be altered leading to significant changes in the net ecosystem C budget. Here, we quantify the single and combined effects of 1 year of increased winter snow deposition by snow fences and summer warming by open-top chambers (OTCs) on root dynamics in a wetland at Disko Island (West Greenland). Based on ingrowth bags, snow accumulation decreased root productivity by 42% in the 0–15 cm soil depth compared to ambient conditions. Over the growing season 2014, minirhizotron observations showed that root growth continued until mid-September in all treatments, and it peaked between the end of July and mid-August. During the season, plots exposed to experimental warming showed a significant increase in root number during September (between 39 and 53%) and a 39% increase in root length by the beginning of September. In addition, a significant reduction of root diameter (14%) was observed in plots with increased snow accumulation. Along the soil profile (0–40 cm) summer warming by OTCs significantly increased the total root length (54%), root number (41%) and the root growth in the 20–30 cm soil depth (71%). These results indicate a fast response of this ecosystem to changes in air temperature and precipitation. Hence, on a short-term, summer warming may lead to increased root depth and belowground C allocation, whereas increased winter snow precipitation may reduce root production or favor specific plant species by means of reduced growing season length or increased nutrient cycling. Knowledge on belowground root dynamics is therefore critical to improve the estimation of the C balance of the Arctic.

Keywords: arctic tundra, minirhizotrons, open top chambers (OTC), root dynamics, snow fence, warming, wetland

INTRODUCTION

Wetlands are widespread in the Arctic and cover about 7% of its vegetated area (Walker et al., 2005). In these ecosystems, the accumulation of soil organic carbon (SOC) exceeds the rates of decomposition due to the low soil temperature and lack of oxygen during periods of high water-table (Sullivan et al., 2008). Consequently, wetlands represent a vast reservoir of C (Hugelius et al., 2014). In a warmer and in particular drier future climate, this C stock may become available for biological decomposition and arctic wetlands consequently play a central role in balancing the uptake and release of carbon dioxide (CO₂) and methane (CH₄) on a global level (Swindles et al., 2015). Links between plant communities, soil parameters and processes represent an important control over the C cycle (Wookey et al., 2009). In the Arctic, the belowground plant biomass exceeds its aboveground counterpart (Mokany et al., 2006; Iversen et al., 2015; Wang et al., 2016a) and therefore represents an important component of soil nutrient cycling and net ecosystem C budget (Iversen et al., 2015). As concluded by Blume-Werry et al. (2016) neither the knowledge from non-Arctic ecosystems, nor projections of aboveground to belowground production, reflect seasonal dynamics of root growth in arctic plant communities and limited data is available on root phenology in this region (Radville et al., 2016).

According to the projected climatic scenarios, air temperatures across the Arctic are expected to increase more than the global average, in particular during winter (McGuire et al., 2012). At high latitudes, increased air winter temperature is expected to trigger an increase in precipitation as snow fall, though with large regional to local variations (Christensen et al., 2013). On the one hand, a thicker snow cover during winter will thermally insulate the soil preventing large fluctuations in soil surface temperature (Morgner et al., 2010) and will enhance nitrogen (N) mineralization rates taking place during the shoulder and cold seasons (Giblin et al., 1991; Schmidt et al., 1999; Schimel et al., 2004). This has been shown to increase the N-availability in the growing season, the N-content of plant leaves and summer season photosynthesis rates (Cooper, 2014; Semenchuk et al., 2015). On the other hand, increased amount of winter snow precipitation will lead to late snowmelt in spring, hence to a delay in the onset of the growing season (Wipf and Rixen, 2010). A late snow melt may affect the growth and reproductive success of early-growing plant species (Cooper et al., 2011; Khorsand Rosa et al., 2015), preventing the plants from taking advantage of the 24-h photoperiod in late spring and the increased soil nutrient availability linked to a thicker snow cover.

In addition, increased summer air temperature can directly influence photosynthesis, nutrient cycling, decomposition processes and increased growing season length (Shaver et al., 1992; Schmidt et al., 2002; Oberbauer et al., 2007; Ernakovich et al., 2014). Hence, the interaction and magnitude of increased winter snow precipitation with increased summer air temperature will determine the length of the growing season, which may be critical for plant phenology and productivity (Fitter et al., 1999; Wipf and Rixen, 2010).

The aboveground production of arctic tundra plants and especially woody shrub species have increased in response to the positive trends in mean annual surface air temperature observed across the Northern-hemisphere (Tape et al., 2006; Elmendorf et al., 2012; Hollesen et al., 2015; Myers-Smith et al., 2015). Potentially, the so called “greening of the Arctic” could lead to climatic feedbacks as variations in vegetation cover may alter several abiotic factors such as surface albedo (Sturm et al., 2005), active soil layer depth, altering nutrient cycling and C storage (Mack et al., 2004). There is also evidence to support a positive relation between root growth and soil temperature, if other growth-related resources are not limiting (Pregitzer et al., 2000). The limited studies available for high latitudes suggest that root growth dynamics might also not directly respond to (small) changes in air temperature regimes. Positive trends of root growth in wet sedge tundra were linked to nutrient availability (Hill and Henry, 2011) and allocation of photosynthate from aboveground (Sullivan and Welker, 2005), which increased in response to increased surface air temperature. However, for some arctic sedge species, well adapted to cold soil temperatures, the length of the photoperiod, rather than air temperature, was identified as main driver of root elongation (Shaver and Billings, 1977).

The objective of this study was to quantify the short-term root dynamics, meaning the sensitivity of root growth (here meant as root elongation), in an arctic wetland to a moderate increase in winter snow precipitation and summer air temperature regimes. *In situ* measurements of root growth were carried out in a wetland in Disko Island (West Greenland) during the growing season 2014. A full factorial experimental set-up with snow fences and open top chambers (OTC) was used to simulate increased winter precipitation as snowfall, summer warming and their combination. We hypothesized that: (i) snow accumulation would have a negative effect on root growth as a consequence of late snowmelt and thus shorter growing season; (ii) summer warming would have at least a short-term positive effect on root growth to support plant uptake of nutrients; (iii) with the combined effect of experimentally increased summer warming and snow accumulation, summer warming would offset the delay in the onset of the growing season due to snow accumulation.

MATERIALS AND METHODS

Site Description

The study site is a wetland in Blåsedalen Valley, Disko Island, on the coast of West Greenland (69°16'N, 53°27'W). Disko Island is located in the transitional zone between the low and high Arctic. According to meteorological data (1991–2011) of nearby Arctic Station (Hansen et al., 2006; Hollesen et al., 2015) mean annual air temperature is $-3.0 \pm 1.8^\circ\text{C}$ (SD), the monthly means of the warmest (July) and the coldest (February–March) months are 7.9 and -14.0°C . The mean annual soil temperature at 5 cm depth is -1.9°C and frozen soil conditions prevail from October to late May. At Arctic Station, 60% of the total annual precipitation is in the form of rain and the overall mean annual precipitation (rain

and snow) has been estimated to be ~ 400 mm (Hansen et al., 2006; Hollesen et al., 2015).

The study site is classified as a graminoid-dominated wetland located in the transition zone between the bioclimatic subzones C and D (Walker et al., 2002, 2005). The wetland has a peat layer of approximately 20–70 cm sitting on glacially rebedded sediments of volcanic basalt. The water table fluctuates from 20 cm below soil surface to 15 cm above (Nielsen et al., 2017).

The vegetation cover is dominated by the sedges *Carex rariflora*, *Eriophorum angustifolium*, *Carex aquatilis* ssp. *stans*, *Carex gynocrates*, and by the shrub *Salix arctophila*. Furthermore, in some areas *Equisetum arvense* is abundant. Pin-point vegetation cover analysis (Jonasson, 1988) was carried out on August 25th and 26th 2014.

Experimental Set-Up

A full-factorial experiment was established in July 2013 and included passive snow accumulation using snow fences, warming by OTCs and their combination. Six replicate blocks, each with a 14.7 m-long and 1.5 m-tall snow fence, were established to create snowdrifts on the leeward (South) side of the fences during winter (snow accumulation). The maximum ambient snow depth in 2014 was 80 cm and the snowdrift at the snow accumulation plots was *ca.* 30 cm deeper than ambient conditions. The site became snow-free on June 18th 2014, approximately a week later than at the ambient deposition side. On each side of the fences two plots (2×2 m) were established ensuring an ambient snow cover depth at the windward side of the fences (6 m from the fence), as well as a maximum snow depth within the drift at the leeward side (3 m from the fence). Half of the plots were covered year-round by 3 mm thick polycarbonate hexagon OTCs (35 cm tall, 150 cm in diameter at the base and 85 cm in diameter at the top) to increase air temperature during summer (Marion et al., 1997). The other half of the plots had ambient summer air regimes. The treatments were identified as: control (C), warming with OTCs (W), snow accumulation (S) and the combination snow + warming with OTCs (SW), all in $n = 6$ replicates (Supplementary Figure S1).

Weather and Soil Parameters

Air temperature at 2 m height was measured every 30 s and logged every 30 min by a meteorological station established at Blåsedalen Valley in July 2013 ($69^{\circ}15' 930''$ N, $53^{\circ} 28' 015''$ W, 97 m asl). Temperature probes (Tinytag, Gemini Data Loggers, Chichester, United Kingdom) and soil moisture probes (HOBO, Onset Computer Corporation, Bourne, MA, United States) were installed in 5 cm depth in all the plots of three blocks. Temperature loggers, protected by waterproof plastic covers, were also placed 2 cm above the ground for measurements of air temperatures within the canopy. The soil moisture was recorded every 10 min and the temperature was recorded hourly.

Soil pH values at the wet sites were based on *in situ* measurements made during August 2015 by inserting the pH probe (WTWTM, SenTixTM 41 pH electrode) directly into the ground at 2.5 and 7.5 cm (Table 1). Volume specific soil samples at 0–5 and 5–10 cm depth were collected in August 2014 at the site and stored at 5°C until further analyses. Prior elemental analyses, the samples were oven dried at 60°C for 48 h. The

total C and N concentrations in the samples (Table 1) were measured in solid samples by Dumas combustion (1020°C) on an elemental analyser (EA Flash 2000, Thermo Scientific, Bremen, Germany). Briefly, 10 mg of grinded and dried material was weighed into tin combustion capsules for elemental analysis. Acetanilide (Merck, Darmstadt, Germany) and soil standards (Elemental Microanalysis, Okehampton, United Kingdom) were used for elemental analyser mass calibration.

Minirhizotron Installation and Image Collection

During July 2013, while setting up the snow fences, we installed 24 minirhizotron tubes made of high-grade transparent acrylic in order to take images of roots in each plot and follow the changes of root parameter over time in a non-destructive way. The tubes were inserted in the soil with a 45 degrees angle to the soil surface (Bragg et al., 1983); they had an inner diameter of 6.40 cm and a maximum length of 1 m. Due to the presence of permafrost the maximum vertical depth reached by the bottom of the tubes ranged between a minimum of *c.* 25 cm and a maximum of *c.* 55 cm, randomly. Foam pipes insulation were placed inside the tubes to protect from changes in temperature and moisture, and were only temporary removed while scanning. Tubes were closed on top with a rubber lid to protect the inner part from water leaks and debris. The part of the tubes protruding aboveground was painted in white to exclude sunlight and to avoid variations in the albedo during the periods of snow cover. In order to avoid an upwards movement of the tubes and a change in the angle due to the freezing – thawing cycles of the active layer, each tube was anchored to a metal bar placed in the soil.

One year after installation of the snow fences (*i.e.*, summer 2014), images of roots were taken with a CI-600 root scanner (CID, Camas, WA, United States) at 600 dpi. The imaging campaigns took place five times during the growing season 2014 on July 2nd and 22nd, August 13th, and September 8th and 17th.

Image and Data Analysis

During each campaign, three to five images were collected in each tube depending on its total length. The images were then analyzed with the software *WinRhizoTron* MF 2014a and *XLRhizoTron* (©Regent Instruments Canada Inc.). In order to be able to load and analyze all the images of a tube at once, the resolution was decreased to 400 dpi (0.06 mm pixel size) so each image was

TABLE 1 | Overview of the main soil characteristics at the site ($n = 6 \pm \text{SE}$).

	Soil depth (cm)			
	0–5	$\pm \text{SE}$	5–10	$\pm \text{SE}$
C (%)	21.08	1.50	18.65	0.63
N (%)	1.30	0.15	1.48	0.09
C:N	17.20	2.90	12.70	1.10
pH	7.20	0.07	6.90	0.09
Bulk density (g cm^{-3})	0.04 ^a	0.01	0.12	0.04

^aThe low bulk density indicates that this depth interval was mainly dominated by undecomposed litter and mosses.

19.6 cm in width and 21.6 cm in length. A total of 308 images were analyzed. The output of the image software provided information on root length, number, and average diameter for each single root in each campaign as well as the surface area calculated assuming perfectly round roots. It was not possible to separate the roots into different plant species. The disappearance of roots between campaigns was indicated as “gone roots” rather than “dead roots” since it was not possible to confirm the latter condition. Moreover, root mortality was not likely to be assessed during a single growing season. For this reason, the final calculations included both the “alive roots” and the “gone roots,” where “gone roots” represented 1.6% of the total number. All roots were clustered in vertical soil depth increments of 10 cm from the soil surface to the bottom of the tubes, and the calculations took into account the 45 degrees angle used to insert the tubes in the soil. Across all the treatment plots, 20 and 66% of the tubes randomly reached the 50–60 and 40–50 cm vertical soil depth, accordingly these soil depth intervals were excluded from the analyses by depth. Consequently, the soil depth of 0–40 cm was chosen to assess root properties and dynamics over the soil profile represented by 86% of the tubes.

The results are reported for each measurement campaign as: total number of roots per tube (0–40 cm depth), total root length and surface area (as sum of each single root length or surface area) were estimated per tube area (cm²) based on the specific length of each tube belowground, and average root diameter is reported by tube.

Total root length growth was estimated as daily rates as follows:

$$L_g = \frac{(L_2 - L_1)}{T_d}$$

where L_g is the root length growth rate (mm cm⁻² d⁻¹), L_2 and L_1 are the root lengths measured at two consecutive sampling dates and T_d is the number of days between sampling dates (Sullivan and Welker, 2005).

The root growth at each vertical soil depth is reported as maximum root growth calculated as the rate per tube surface area between the 2nd of July and the 13th of August (mm cm⁻² d⁻¹).

Fine Root Biomass

At the time of the minirhizotron installation the soil cores excavated ($n = 24$) were brought back to the laboratory, split into specific vertical soil depths (O-horizon, 0–10, 10–20, 20–30, 30–40, 40–50, and 50–60 cm) and stored at 4°C until manual root sorting. The fine roots (<2 mm) were separated from the soil by forceps, gently washed, oven-dried at 55°C for 48 h and weighed to estimate the dry weight (DW).

Root Ingrowth Bags for Fine Root Production

In July 2013, 12 soil cores (4.5 cm diameter) were collected with a 45 degrees angle adjacent to the experimental blocks in an area with comparable vegetation cover. Back in the laboratory, the samples were split into 0–5, 5–10, and 10–15 cm vertical soil depths and stored at 4°C. Within 2 days after collecting the samples, all the roots present in the soil were manually removed

with forceps. Due to insufficient soil conditions (too wet and full of organic material), coarse sand was collected from the same area to mix into the ingrowth bags. The sand was first washed with distilled water and sieved through 0.5 mm mesh. Then, in order to avoid introducing microbial communities not belonging to the “wetland area,” it was set for 30 min into a glass vessel with a solution of distilled water (2 l) and hydrochloric acid (3.7%, 200 ml) and finally rinsed several times with distilled water. The ingrowth bags were made of synthetic textile designed with a length of 21 cm, a width of 4.5 cm and a mesh size of 1 mm. The bags were filled with root-free soil and sand homogeneously mixed together (1:1) to reach the original fresh weight of each portion of the soil core. The three depth-specific subsamples of sorted soil and sand were placed into the ingrowth bags following the exact depth order and they were kept separated by inserting a small piece of mesh cloth in between each soil layer matrix. Prior to installation, the bags were stored in the fridge at 4°C. During the same month the ingrowth bags were placed in the soil at a 45 degrees angle within each control (C) and snow accumulation (S) plot ($n = 6$) 25 cm from the minirhizotron tubes. In September 2014, the bags were retrieved manually by using a knife. In the laboratory, the samples were separated into each depth-specific section and stored in the freezer at –18°C until the time of root sorting. Once the soil samples were thawed at 5°C, the fine roots (≤ 2 mm diameter) were manually sorted, washed and oven-dried at 55°C for 48 h. Live roots were identified by color and elasticity whenever possible (Oliveira et al., 2000), although the preservation of roots in the cold Arctic made this visual inspection difficult. The fine root biomass, which colonized the volume of the ingrowth bags during a year, was used to estimate the belowground net root productivity per soil area. As for the results from the minirhizotron tubes, the calculation of root productivity took into account the 45 degrees angle, and the root depths reported refer to vertical depths.

Fine Root Turnover

Root system turnover was calculated based on the root ingrowth bags (0–15 cm soil depth) as belowground net primary production (g m⁻²) divided by the initial standing belowground biomass of fine roots (g m⁻²) (Gill and Jackson, 2000). The belowground biomass used for the calculations included the O-horizon (ca. 5 cm) and the top 0–10 cm soil depth.

The turnover was estimated only for the roots that grew at ambient condition (C), despite the fact that ingrowth bags were placed also in plots with snow accumulation as main treatment (S). This was done in order to avoid biases derived from different initial conditions between the time of estimation of fine root biomass, prior to the beginning of the S treatment, and the fine root production, which was estimated a year after the snow accumulation experiment was initiated. A timeline of the installations and the measurements carried out at the site can be found in **Supplementary Table S1**.

Statistical Analyses

Possible differences in initial root biomass between the two sides of the snow fences, i.e., the control and snow accumulation plots were tested with a two samples *t*-test.

Significant total and depth-specific differences of fine root productivity, derived from the ingrowth bags, between single treatment snow accumulation (S) and ambient snow (C) were tested with a general linear model (GLM).

The effects of the climate manipulations on the root parameters monitored with minirhizotrons were quantified by taking into account both temporal (during the season) and spatial (over the soil profile) changes. Unless stated otherwise, these analyses were done using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2013) and the SAS Enterprise Guide version 7.1. Least squares *post hoc* tests on significant treatment effects were performed to investigate all pairwise differences of least squares means among factors and Bonferroni adjusted *P*-values were used for multi-comparison correction. Model reduction was done by stepwise exclusion of non-significant terms starting from the highest degrees of interaction ($P > 0.2$).

When necessary, the data were log or square root transformed in order to meet homogeneity of variance and normality. The significant treatment effects are based on $P \leq 0.05$, but tendencies toward significance ($P \leq 0.10$) are also presented. Further, the results of the *F* tests are reported together with the numerator and denominator degrees of freedom, indicated, respectively, by the first and second subscript values.

The error bars shown are one standard error of the mean (SE). In the figures, tendencies and significant treatment effects are indicated by: $^{\dagger}P \leq 0.1$, $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$. All the figures were created using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA, United States).

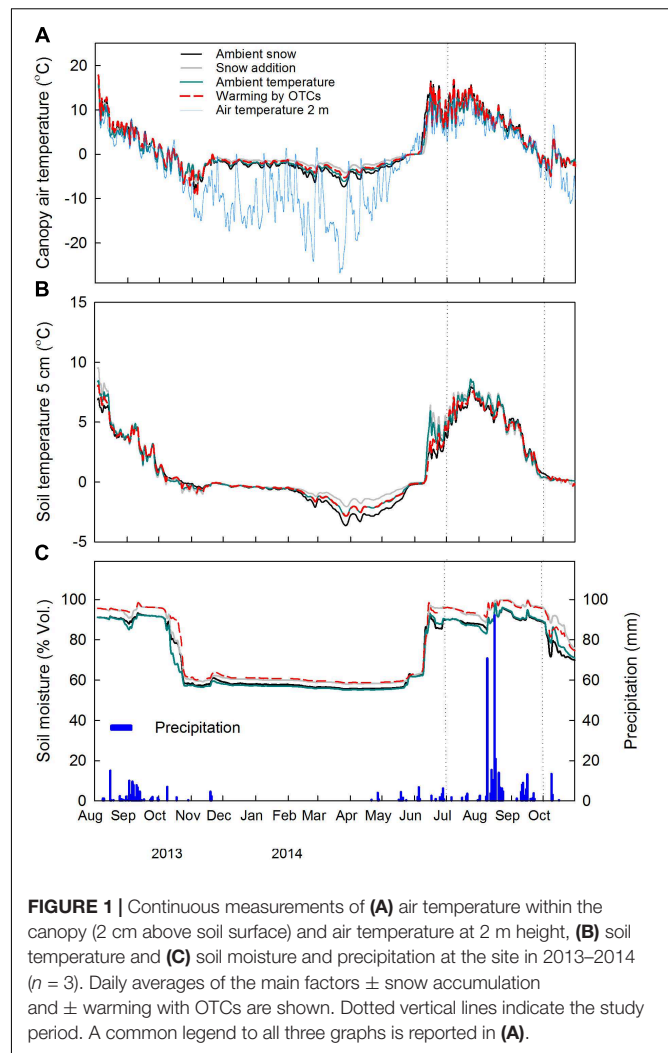
Statistical Tests on Temporal Changes

Overall changes in response to the climate manipulation in root number, length, surface area and growth per tube area as well as averaged root diameter over the whole tube were quantified for the growing season. Accordingly, we used an ANOVA mixed model with random effects “block” and “block \times snow” (where “snow” referred to the side of the block with experimentally increased snow depth) and with date as day of the year (DOY) of each measurement campaign as repeated effect and “plot” as subject identifier. The model included DOY, S and warming (W) as fixed effects in a factorial design. This means that, if for example the outcome of the model indicates warming (W) as significant main effect, all the plots with experimentally increased air temperature (W and SW) are different from plots with ambient air conditions (C and S).

The same ANOVA mixed model, without DOY as fixed factor, was used to test treatment effects on air temperature within the canopy, soil temperature and soil moisture at 5 cm depth.

Statistical Tests on Spatial Changes

To test the treatment effects on the probability of finding roots at specific vertical depth intervals (0–10, 10–20, 20–30, and 30–40 cm), we ran a logistic mixed model on the root number data obtained from the image analysis and included “block” as random effect. This analysis was done using the GLIMMIX procedure of SAS 9.3 (SAS Institute Inc., 2013).



In order to analyze the climate effects on the roots distribution (total number of roots, their lengths and maximum root growth) over the soil profile, we ran an ANOVA mixed model for each measurement campaign. The model included “block” and “block \times snow” as random effects, “soil depth” as repeated effect and “plot” as subject identifier. The fixed effects were soil depth, S and W in a factorial design.

The overall treatment effects on root number and length distribution were quantified by including in the above-mentioned ANOVA mixed model all the measurement campaigns and “DOY \times plot” as random factor to account for time repetition.

Maximum root growth in each depth interval ($\text{mm cm}^{-2} \text{d}^{-1}$) and aboveground plant cover data (%) were assessed with an ANOVA mixed model with only the random effects.

RESULTS

Soil Temperature and Moisture

During the growing season 2014 (21st of May–31st of September), the daily average air temperature within the canopy

TABLE 2 | Vegetation cover identified in August 2014 at each treatment plot here reported as average with standard errors (\pm SE; $n = 6$).

Functional group/species	Cover (%)							
	C	\pm SE	W	\pm SE	S	\pm SE	SW	\pm SE
Cyperaceae	65	14	92	21	48	8	73	14
<i>Carex aquatilis</i> ssp <i>stans</i>	0	—	3	3	1	1	9	7
<i>Carex</i> sp.	1	1	0	—	3	3	0	—
<i>Carex rariflora</i>	38	17	51	23	26	7	44	18
<i>Carex gynocrates</i>	4	3	5	2	11	8	7	4
<i>Eriophorum angustifolium</i>	21	12	33	9	7	4	12	7
Shrub species	9	4	35	9	37	5	29	8
<i>Betula nana</i>	0	—	0	—	2	2	0	0
<i>Salix arctophila</i>	9	4	30	9	35	6	23	9
<i>Salix glauca</i>	0	—	0	—	0	—	3	3
<i>Vaccinium uliginosum</i>	0	—	5	5	0	—	2	2
Equisetaceae ^a	30	13	22	9	26	11	37	18
Polygonaceae ^b	2	1	1	0	3	2	6	3
Mosses	32	16	40	9	30	13	32	9
Peat	14	14	1	1	0	0	1	0
Litter	74	10	71	11	82	8	73	8
Standing dead plant	56	13	57	9	46	8	72	18

The treatment plots are control (C), warming by OTCs (W), snow accumulation (S), and the combination of snow + warming by OTCs (SW). ^a*Equisetum arvense* and *Equisetum scirpoides* ^b*Bistorta vivipara*

was significantly reduced by snow accumulation (-0.9°C) ($F_{1,9} = 10.34$, $P = 0.01$) and increased by warming with OTCs ($+0.9^{\circ}\text{C}$) ($F_{1,9} = 8.91$, $P = 0.02$) (**Figure 1A** and **Supplementary Figure S2A**). During the same period, no significant treatment effect was noticed for the soil temperature at 5 cm depth (**Figure 1B** and **Supplementary Figure S2B**). The effect of increased snow depth on air temperature within the canopy and at 5 cm soil depth was detected in the measurements during winter time (21st November 2013–20th May 2014), as expected from the experimental set-up. Snow accumulation significantly increased air temperature within the canopy by 1.4°C ($F_{1,9} = 5.39$, $P = 0.05$), and tended to increase soil temperature at 5 cm depth by 0.6°C ($F_{1,9} = 4.01$, $P = 0.08$). No significant treatment effect was instead detected on the continuous measurements of soil moisture at 5 cm soil depth (**Figure 1C** and **Supplementary Figure S2C**).

Aboveground Plant Cover

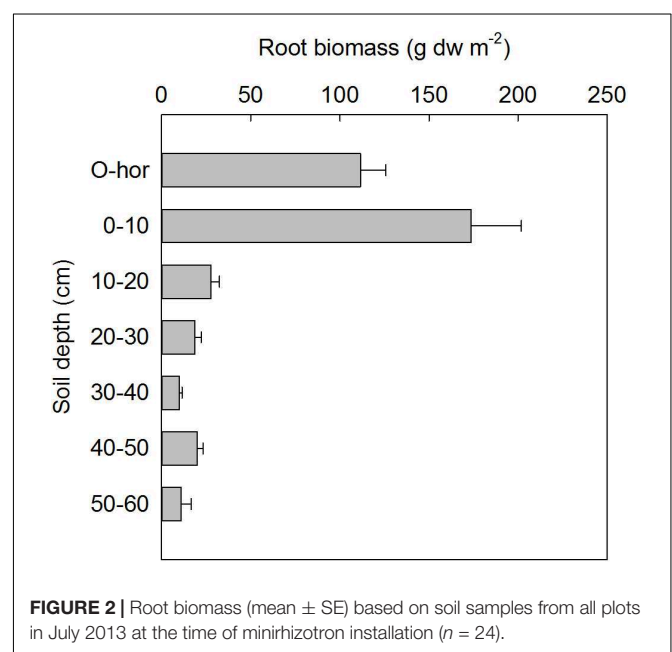
A year after the beginning of the experiment, the total aboveground cover of sedge species was 39% higher in plots with warming by OTCs ($F_{1,11} = 4.74$, $P = 0.05$) (**Table 2**).

Furthermore, the total cover of shrubs was significantly higher with the interaction of warming by OTCs and snow accumulation ($F_{1,10} = 8.61$, $P = 0.015$). No other differences between treatments were detected.

Soil Cores and Ingrowth Bags

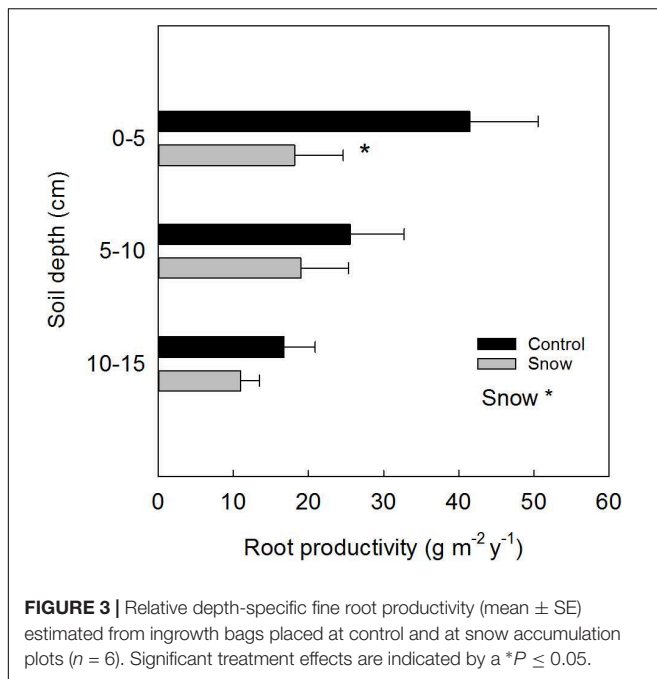
Root Biomass, Productivity and Turnover

Depth-specific root biomass (≤ 1 mm diameter) was estimated based on the soil cores excavated during minirhizotrons



installation at each plot in 2013 (**Figure 2**), therefore these results are representative of the initial standing root biomass at the site in ambient conditions. The largest root biomass was found in the 0–10 cm soil depth with a maximum value of 174 ± 28 g dw m⁻², and roots were found down to 60 cm soil depth. There was no significant difference in the initial root biomass between the two sides of the snow fences.

By adding sand to the ingrowth bags, the soil texture and nutrient concentration changed as compared to the initial



characteristics of the soil. For this reason, the results reported are presented as “relative” fine root production. A year after the installation of ingrowth bags, in 2014, in plots with snow accumulation as main treatment (S), the relative production of fine root biomass (**Figure 3**) was reduced in the 0–5 cm soil depth by 56% ($F_1 = 4.96$, $P = 0.05$). Overall, snow accumulation reduced the total fine root productivity (0–15 cm) by 42% ($F_1 = 4.11$, $P = 0.05$). Within the control plots a tendency toward less fine root production was found in the 10–15 cm soil depth, as compared to the upper 0–5 cm (Tukey’s adjustment for multiple comparisons: $P = 0.09$).

Based on the relative root production derived from the ingrowth bags (year 2014) at the control plots (C) and the initial standing root biomass at the site estimated by the soil cores (year 2013), the root turnover at ambient conditions was $0.29 \pm 0.048 \text{ y}^{-1}$ over the 0–15 cm soil depth.

Minirhizotrons

Treatment Effects on Seasonal Root Parameters and Growth

The results of image analysis show a strong seasonality (DOY: $F_{4,83,9} = 41.63$, $P < 0.001$) and clear trends in the total number of roots, total root length, total surface root area and average diameter estimated by minirhizotrons (**Figure 4**). In contrast, the averaged single root length per tube area was not altered by any of the treatments and did not show any seasonality (**Supplementary Table S2**). All the parameters investigated, except for the average diameter, increased in all the treatments during the season reaching a maximum at the beginning of September (**Figures 4A–C**). The decreasing trends observed during the last campaign are due to missing measurements in block 1 (because of adverse weather conditions) rather than an ecological process. However, the abundant presence of roots in

block 1, in comparison to the other replicate blocks, and the missing measurement during the last campaign did not alter the outcome of the statistical analyses, as these aspects are taken into account by the model used (Zuur et al., 2009).

The average root diameter was highest during the first measurement campaign in July across all treatments ($0.67 \pm 0.02 \text{ mm}$) and followed by a decreasing trend until the last campaign in September (**Figure 4D**). Plots with experimental summer warming by OTCs showed a tendency of increased root number (49%) and length (38%) over the season (**Table 3**). The increased snow accumulation instead, significantly reduced the diameter of the roots by 14% (**Table 3**) as also indicated by the significant interaction snow \times warming (S: $t_{18,9} = 3.48$, $P = 0.015$).

The number of roots significantly increased during the last two campaigns with warming with OTCs (**Table 4**). The increase ranged between 39% on September 8th and 53% on September 17th. During the same campaigns, positive tendencies were also detected in total root lengths, which increased significantly (39%) on September 8th (**Table 4**). Root diameter significantly decreased in plots with increased snow deposition (**Table 4**) from July 22nd (17%) to September 17th (24%).

The rate of root growth at ambient conditions peaked between July 24th and August 13th, the same pattern was observed among all treatments (**Figure 5**). Warming with OTCs tended to increase root growth ($F_{1,10} = 4.27$, $P = 0.066$) between August 13th and September 8th, in agreement with the significant increase in total root length observed at the beginning of September.

Root Distribution Along the Soil Profile

The root image analyses showed that the distribution of roots within the soil profiles was not always the same among treatment replicates. It was therefore of interest to understand whether the presence or absence of roots at specific soil depths was related to the effects of the treatments or other factors. The results of the logistic model confirmed that warming by OTCs significantly increased the presence of roots in the deeper layers of the soil profile (20–30 cm: $F_{1,98} = 2.92$, $P = 0.09$; 30–40 cm: $F_{1,98} = 9.30$, $P = 0.003$).

Across the entire soil profile (0–40 cm), the maximum number of roots and length were found in the 0–20 cm depth (data not shown) and in each sampling date, they consistently decreased with soil depth (**Table 5**). During the last two measurement campaigns root length significantly increased with warming with OTCs by 62 and 74%, while the number of roots significantly increased by 59% during the last campaign (**Table 5**). Further tendencies toward a positive experimental warming effect on these parameters are reported in **Table 5**.

No significant effect of the climate manipulation was detected either on the number of roots nor on their total length within the single soil depth intervals.

The overall effects of the climate manipulations on root number and length were also estimated taking into account the possible differences in spatial distribution across the soil profile without focusing on the seasonal patterns. The total number of roots and length significantly increased with

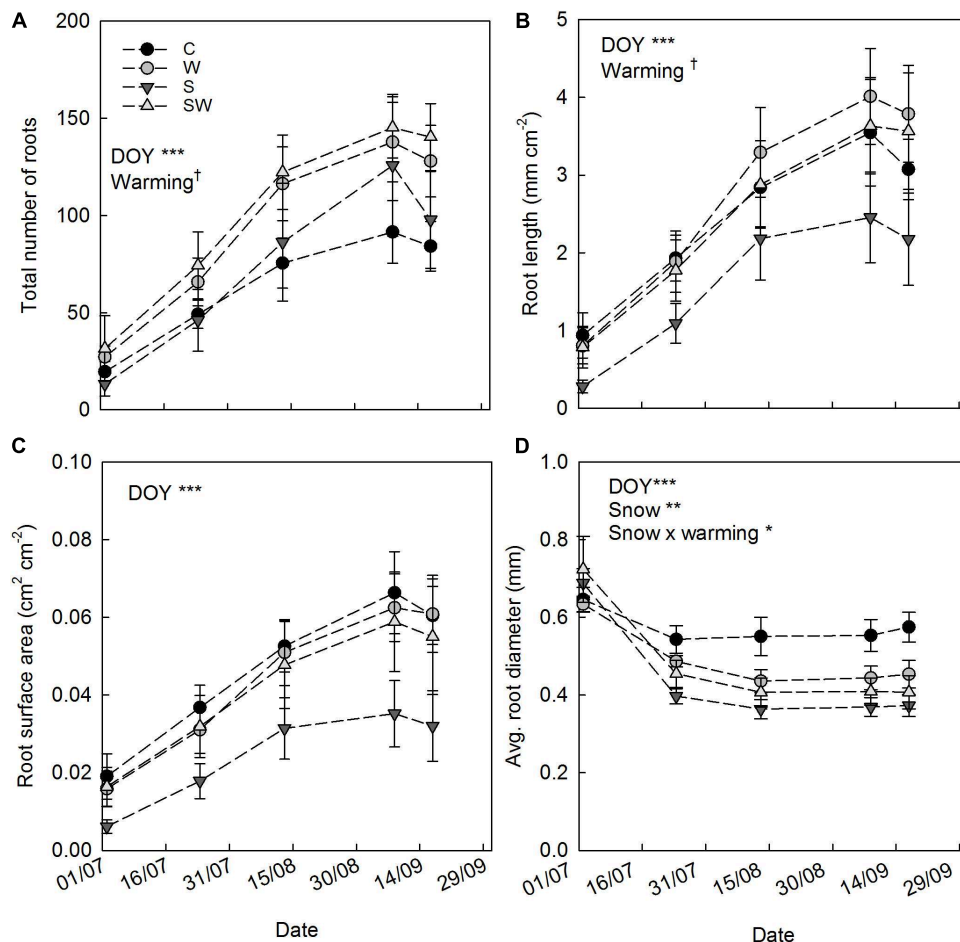


FIGURE 4 | Root parameters measured over the growing season 2014 (mean ± SE). **(A)** Total root number by tube. **(B)** Total root length per tube area. **(C)** Total root surface area per tube area. **(D)** Average root diameter by tube. Tendencies and significant repeated treatment effects are reported for each parameter and indicated by † $P \leq 0.1$, * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. In legend: control (C), warming by OTCs (W), snow accumulation (S), and snow + warming by OTCs (SW).

experimental warming with OTCs over the whole soil profile by 41% ($F_{1,22.2} = 4.45$, $P = 0.046$) and 54% ($F_{1,22.4} = 5.14$, $P = 0.03$). The values of averaged single root length in each soil depth interval are reported in **Supplementary Table S3**.

The depth-specific root length growth estimated at the same soil depth intervals showed a maximum growth within the 0–20 cm soil depth in all the treatment plots ($0.08 \pm 0.01 \text{ mm cm}^{-2} \text{ d}^{-1}$ average across treatments) which decreased significantly (Depth: $F_{3,63} = 16.87$, $P < 0.001$) down to 40 cm soil depth (**Figure 6**). Root growth at 20–30 cm depth significantly increased by 71% with warming by OTCs ($F_{1,9.63} = 6.58$, $P = 0.03$).

DISCUSSION

The results presented in this study quantify the short-term effects (1 year) of snow accumulation, summer warming and their combination on root production and phenology in an arctic

wetland. A key observation was the fast response of root growth to increased snow deposition and summer air warming, despite the fact that an actual temperature increase was detected only at the canopy. This highlights the importance of investigating the initial responses of the belowground root biomass to changes in precipitation (as snow) and temperature regimes, bearing in mind that changes in root growth and depth reveal an important seasonal dynamic that may affect the overall ecosystem C budget and nutrient cycling.

Ambient Root Biomass, Production and Turnover

The fine root biomass estimates at ambient conditions as well as relative root production from ingrowth cores were consistent with values previously reported per unit ground area in arctic wetlands (Sullivan et al., 2007, 2008; Iversen et al., 2015). Fine root production was 84 g m^{-2} in the 0–15 cm vertical soil depth with a dry fine root biomass of 285 g m^{-2} . If steady state is assumed, as suggested in Sullivan et al. (2008), the mean residence time of fine roots in this study was about 3.4 years. In line with

TABLE 3 | *P*-values of treatment effects on root parameters over the measurement season 2014 (ANOVA repeated mixed model).

Variables	Fixed effects	DF	F-value	P-value
Total number	DOY	4, 84	152.17	<0.001 ↑
	W	1, 21.8	3.04	0.095
Total length (mm cm ⁻²)	DOY	4, 80	123.04	<0.001 ↑
	W	1, 20.9	2.96	0.100
	S	1, 20.9	0	0.970
	DOY × W	4, 80	3.43	0.012 ↑
Total surface area (cm ² cm ⁻²)	DOY	4, 76.1	117.32	<0.001 ↑
Average diameter (mm)	DOY	4, 75.4	54.89	<0.001 ↓
	W	1, 18.9	0.82	0.376
	S	1, 18.9	6.98	0.016 ↓
	DOY × S	4, 75.4	6.45	<0.001 ↓
	W × S	1, 18.8	5.22	0.034 ↓

The fixed effects are day of the year (DOY), warming by OTCs (W), snow accumulation (S), and their interactions. Significant *P*-values are reported in bold together with arrows indicating either a significant negative (↓) or positive (↑) treatment effect.

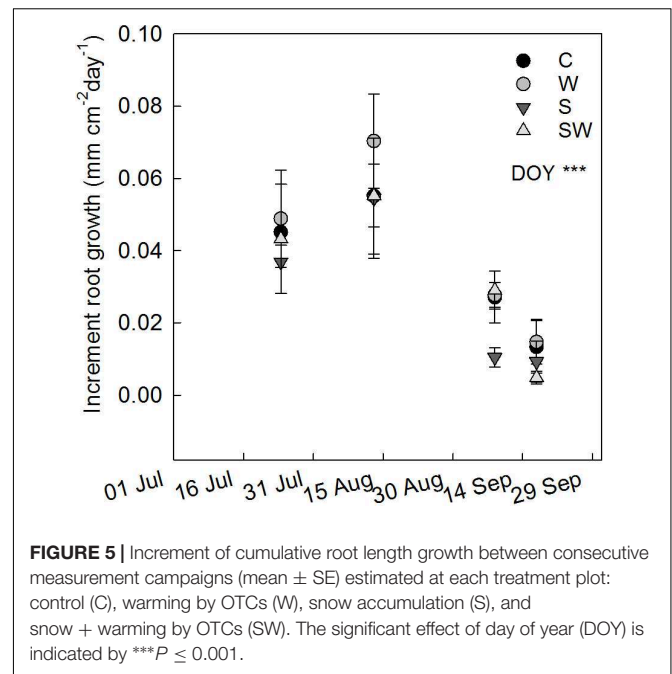
TABLE 4 | *P*-values of main treatment effects and interactions on root parameters during each measurement campaign (ANOVA mixed model).

Date (2014)	Fixed effect	Parameters			
		Number	Length	Surface area	Diameter
02 July	W	0.096	0.119	0.126	0.814
	S	—	—	—	0.198
	W × S	—	—	—	0.637
22 July	W	0.165	0.097	0.100	0.965
	S	—	—	—	0.005 ↓
	W × S	—	—	—	0.050 ↓
13 August	W	0.111	0.090	0.130	0.337
	S	—	—	—	0.008 ↓
	W × S	—	—	—	0.042 ↓
08 September	W	0.043 ↑	0.039 ↑	0.076	0.310
	S	—	—	—	0.004 ↓
	W × S	—	—	—	0.036 ↓
17 September	W	0.020 ↑	0.080	0.237	0.254
	S	—	—	—	0.004 ↓
	W × S	—	—	—	0.049 ↓

The fixed effects are warming by OTCs (W), snow accumulation (S), and their interactions. Significant *P*-values are reported in bold together with arrows indicating either a significant negative (↓) or positive (↑) treatment effect. For the root parameters: number, length and surface area the *P*-values are reported only for W due to exclusion of non-significant ($P > 0.2$) fixed effects from the model.

this estimate, a mean life-span > 3 years has been observed in temperate grasslands (Mommer et al., 2015) characterized by low nutrient availability and low root mortality per year (Van Der Krift and Berendse, 2002; Arndal et al., 2017).

However, it is important to acknowledge that the absolute numbers may be only a rough estimation of the root production at the site. This was because the soil characteristics inside the ingrowth bags were changed by adding sand, and therefore



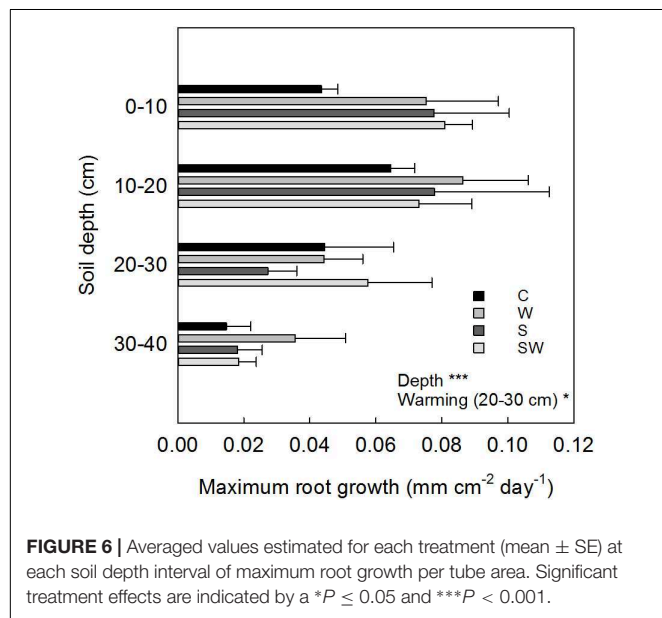
changes in soil bulk densities, nutrient concentration and the reduced root competition in the soil volume inside the bags may have altered the degree of colonization of the finest roots.

A fine root turnover at ambient conditions of $0.29 \pm 0.048 \text{ y}^{-1}$ was in the range of previously estimated turnovers of high latitudes grasslands, which are considered the lowest reported turnover rates across different ecosystems (Lauenroth and Gill, 2003). Gill and Jackson (2000) reported a mean root turnover for graminoids at high latitudes of 0.29 y^{-1} across 16 studies, and accordingly Iversen et al. (2015) estimated an averaged root lifespan of > 5 years across tundra ecosystems mainly dominated by graminoids. In these systems, slow root turnover rates represent an effective advantage to increase the mean residence time of plant nutrients (Berendse and Aerts, 1987). We did not estimate root turnover based on the minirhizotron data as earlier studies suggest a waiting time between 6 months (Hendrick and Pregitzer, 1996) and 4 years (Iversen et al., 2008), to reach quasi-equilibrium in root turnover (Iversen et al., 2012). Clearly, the values of root turnover rates estimated at ambient conditions provide the background knowledge to assess the current status of an ecosystem, which over time, may change in order to adapt to the projected climatic scenarios. Our observations of aboveground plant cover at the experimental plots highlighted a significantly higher amount of sedges (39%) in plots exposed to experimental warming with OTCs. Although, sedges have higher root turnover rates compared to shrub species (Gill and Jackson, 2000), we could expect an accumulation of litter in the deeper layers of the soil as the deeper-rooted sedges will produce more root litter deeper in the soil profile. Due to the low temperature, decomposition is also expected to be low. Hence reduced root decomposition rates may lead, in the long-term to increased C storage, with implications for the overall ecosystem C and N dynamics.

TABLE 5 | *P*-values for differences of Least Squares Means (LSmeans) for main effects in each measurement campaign after exclusion of non-significant fixed effects from the model.

Date (2014)	2 July		22 July		13 August		08 September		17 September	
Fixed effects	Depth	W	Depth	W	Depth	W	Depth	W	Depth	W
Parameters										
Length	0.002↓	0.071	<0.001↓	>0.1	<0.001↓	0.096	<0.001↓	0.041↑	<0.001↓	0.026↑
Number	0.001↓	>0.1	<0.001↓	>0.1	<0.001↓	>0.1	<0.001↓	0.075	<0.001↓	0.018↑

The fixed effects are depth and warming by OTC (W). The arrows indicate either a significant negative (↓) or positive (↑) treatment effect. Here are reported all the effects with $P \leq 0.1$ and significant treatment effects ($P \leq 0.05$) are noticeable in bold.

**FIGURE 6 |** Averaged values estimated for each treatment (mean \pm SE) at each soil depth interval of maximum root growth per tube area. Significant treatment effects are indicated by a * $P \leq 0.05$ and *** $P < 0.001$.

Responses to Increased Snow Deposition

Overall, root dynamics at the snow accumulation plots are complex due to the combined effects of snow accumulation on soil temperature during wintertime (Figure 1), timing of thaw and low air temperature within the canopy during the growing season (Supplementary Figure S2).

In accordance to our hypothesis (i), snow accumulation as main treatment (S) consistently reduced the relative fine root production in the 0–15 cm depth. At these plots, we estimated an annual fine root production of 48 g m^{-2} . The snowdrift behind the fences delayed the onset of the growing season of about a week by shading the aboveground vegetation from sunlight and significantly reducing the air temperature within the canopy. Hence, this might have affected the development of belowground biomass by means of reduced photosynthetically fixed C allocation from aboveground (Lefler and Welker, 2013). Consequently, reduced fine root biomass production could derive from the production of lighter and thinner roots (Figure 4D) than those under ambient snow precipitation, to preserve the capacity of nutrient uptake under C-limited conditions (Van Der Krift and Berendse, 2002).

On the other hand, arctic plant species are well-adapted to cold temperatures (Iversen et al., 2015) and a positive correlation between root production and soil temperature is species-specific and becomes less relevant at high latitudes (Abramoff and Finzi, 2015; Blume-Werry et al., 2016; Sloan et al., 2016; Arndal et al., 2017). An alternative explanation for the observed results could be that due to better insolation under the thicker snow layer, increased soil temperatures (Figure 1) might have stimulated N mineralization (Semenchuk et al., 2015), thereby increasing plant-available N at the onset of the growing season (Jonasson et al., 1999; Schmidt et al., 2002; Schimel et al., 2004; Buckeridge and Grogan, 2010). In accordance, the plants might have changed their rooting strategy by reducing the allocation to roots in accordance to the “functional equilibrium model” (Poorter and Nagel, 2000). In the present study, it was not possible to estimate nutrient availability during spring thaw and further analyses are recommended to link depth-specific root growth and nutrient availability. The consistently small roots diameters we observed (Figure 4D and Table 4) may also suggest that increased snow depth (as single factor and in combination with warming) have favored the belowground growth of plant species, e.g., shallow-rooted shrubs, with thin absorptive roots for a fast acquisition of nutrients (Kong et al., 2016), rather than triggering a change in rooting strategy. This is well in accordance to previous studies in high latitudes, which observed early-season growth of shrub root biomass simultaneously with increased nutrient availability in the surface soil (Wang et al., 2017).

Responses to Increased Summer Air Temperature

The results of the measurement campaign during the growing season 2014 showed, in accordance with our hypothesis (ii), a tendency toward an increased number of roots and root length in the plots with experimental warming (Figure 4). These results may be interpreted in the light of the continuous measurements of soil parameters (Figure 1 and Supplementary Figure S2) and their indirect effects on concentrations of soil nutrients.

Warming with OTCs has previously been observed to be an efficient method to increase the soil surface temperature. However, it may not yield the same effect in the deeper soil layers (Marion et al., 1997; Hobbie and Chapin, 1998; Sullivan and Welker, 2005; Natali et al., 2011). During summer time, the wetland in Blåsedalen is characterized by a horizontal water flow at the soil surface, which comes from a semi-permanent snowdrift to the East of the plots. This horizontal movement

of water is considered to have partly transferred the heat away from the plots, preventing a significant effect of the OTCs on the soil temperature in depth. Hence, the observed root responses in plots with OTCs may be explained by indirect effects of increased temperature within the canopy. In plots exposed to experimental warming, the vegetation had a higher C assimilation, and it likely increased the allocation to the belowground biomass to tackle possible nutrient constraints. Consequently, the number of roots and root length increased to allow the exploitation of a larger volume of soil for nutrient uptake (Pregitzer et al., 2000; Arndal et al., 2014) and this was consistently observed over time, during each campaign (**Table 4**), and over the soil profile (**Table 5**). Similar observations were reported for a fen in Alaska, where a tendency in increased root biomass was linked to increased air temperature within the canopy and increased allocation belowground (Sullivan and Welker, 2005).

The maximum root number, total length and elongation were consistently observed across all treatments in the 0–20 cm depth interval, which is consistent with higher concentrations of plant-available nutrients, as well as higher soil temperature in the upper soil layers as compared to freezing temperatures at the bottom of the active layer (Shaver and Billings, 1977).

The indirect positive effect of warming by OTCs on the number of roots and total root length over the soil profile further suggests an increased C allocation to roots in the deeper soil layers, as confirmed by the significant increase of maximum root growth in the 20–30 cm soil depth (**Figure 6**). A deeper rooting system allows the plant to take up nutrients released at the interface between active layer and permafrost. In previous studies, the root system of the sedge *E. angustifolium* has been observed to reach the top of the permafrost showing tolerance to the cold temperature (Shaver and Billings, 1977; Wang et al., 2016b, 2017). In the long-term, increased summer air temperature could further trigger a switch in the plant community composition of this wet tundra in favor of deep-rooted sedges able to exploit the increased thickness of the active layer (Björk et al., 2007; Wookey et al., 2009).

Concerning the combination of winter snow accumulation and summer air warming, the cumulative observations along the soil profile and during the growing season suggest that the belowground biomass benefitted from the snow insulation during the cold period, which confirms our hypothesis iii. Furthermore, increased summer temperature may counterbalance the increase in snow depth, preventing a delay in the onset of the growing season. Based on our minirhizotrons observations, the combined effects on roots of snow addition and air warming were mainly driven by warming by OTCs for root number and root length and by snow addition for root diameter.

Root Growth and Phenology

The peak of root growth rates was observed between July 24th and August 13th (**Figure 5**). At the same site, Nielsen et al. (2017) measured at ambient conditions a peak in ambient gross ecosystem productivity (GEP) and ecosystem respiration (ER) on August 5th 2014. This indicates an overall match between the period of maximum aboveground activity and belowground production and a late season asynchrony as root

growth continued until mid-September for all treatments after the occurrence of aboveground senescence, as assumed by the reduced rates of GEP (Nielsen et al., 2017). Similar seasonal root growth was reported in a recent study of Sloan et al. (2016) in which the authors observed synchronized maximum leaf and root production in a graminoid-dominated landscape in the sub-Arctic region and a late season root growth after aboveground senescence. A prolonged belowground growth may be paired to C losses in the atmosphere through ER during the shoulder season (Blume-Werry et al., 2016), while instead the GEP is reduced or absent due to the senescence of the aboveground vegetation (Keenan et al., 2014; Toomey et al., 2015). This could cause an imbalance in the net C-flux between soil and atmosphere with the risk of increased C emissions; especially in ecosystems dominated by plant communities more sensitive to soil temperature rather than the length of the photoperiod. At the same time, a prolonged belowground growth may allow re-translocation and uptake of nutrients taking advantage of the maximum active layer depth and the still warm soil temperatures (Schimel et al., 2004; Blume-Werry et al., 2016).

The seasonal pattern of root growth corresponded to a significant increase of air temperature within the canopy with warming with OTCs. This is consistent with other studies carried out during the growing season 2014 at the same site, which measured a significant increase of ER (Nielsen et al., 2017) and GEP (Lindwall et al., 2016) under warming with OTCs. Yet, during the time of this study, it was not possible to witness significant seasonal changes in root growth rates across experimental treatments, but only a tendency between August and September (**Figure 5**). In spite of this, the temporal increase in the number of roots and their lengths corresponded with the patterns in root length growth under experimental warming. This can be explained as an indirect effect of the increased air temperature within the canopy on photosynthetic activity (Oechel and Billings, 1992; Shaver et al., 1992), which may have led to increased allocation belowground of photosynthate to sustain root growth, as also observed by other studies (Fitter et al., 1999; Tierney et al., 2003; Edwards et al., 2004). In contrast, Arndal et al. (2017), observed in a Danish mixed heathland – grassland the opposite effect of experimental warming on root length in the upper 8–15 cm soil. However, in that study, a reduction in root length was simultaneously observed with a reduction of water availability, net photosynthesis and hence C allocation belowground.

IMPLICATIONS AND CONCLUSION

Our results suggest that in an Arctic ecosystem such as the one in Blåsedalen, short-term changes in air temperature and snow precipitation may lead to an increased allocation of C to the roots. However, most ecosystem C-models, which are used to predict net ecosystem responses to climate change in the Arctic, have not incorporated the sensitivity of tundra ecosystems to short-term changes in temperature regimes. Recent data from the Arctic showed dramatic changes in temperature regimes from year to year (Westergaard-Nielsen et al., 2018), hence it is critical

to assess and take into account the short-term effects of plant responses to climate variability in order to look at the stability of ecosystem C-models. In Blåsedalen, the observed effects of snow accumulation on the relative production of roots as well as the patterns of root growth seemed to be triggered by changes in nutrient cycling during winter rather than by the shorter growing season, which contrasts with what we had originally hypothesized. We also conclude that multi-year measurements of single and combined effects of warming, snow and the associated changes in soil nutrient availability are needed to understand the full effects of increased winter and summer air temperature on root dynamics. Thus, by combining the knowledge of root biomass C responses to climate change with the recent improved understanding and mapping of greening of the Arctic (Myers-Smith et al., 2015), we are close to see the hidden part of the iceberg, and thereby provide a more robust and improved measure of the total organic C balance of the Arctic. To assess this for longer terms requires that this type of measurements are continued in the years to come.

AUTHOR CONTRIBUTIONS

LD, MA, BE, and IS planned and designed the study. LD, MA, and CN collected the field data. LD analyzed the data and wrote the paper with contributions from all the authors.

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FUNDING

This study was funded by The Danish National Research Foundation (CENPERM DNRF100) with additional funding provided by the EU-funded FP7 infrastructure INCREASE (Grant #227628) and participating institutions.

ACKNOWLEDGMENTS

We wish to thank Jens Gammeltoft, Per Freiberg, and Paul Christiansen for helping with the preparation and installation of the minirhizotrons; Frida Lindwall, Nynne Marie Rand Ravn, and Kristine Dyrnum for their help in collecting root images in the field. We also wish to thank Jean-Baptiste Borres for helping with the image analysis software and Romain Baudet for assistance in the laboratory.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01258/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tree Sapling Responses to 10 Years of Experimental Manipulation of Temperature, Nutrient Availability, and Shrub Cover at the Pyrenean Treeline

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OPEN ACCESS

Edited by:

Hans J. De Boeck,
University of Antwerp, Belgium

Reviewed by:

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United States
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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 12 May 2018

Accepted: 04 December 2018

Published: 08 January 2019

Citation:

Angulo MA, Ninot JM, Peñuelas J, Cornelissen JHC and Grau O (2019) Tree Sapling Responses to 10 Years of Experimental Manipulation of Temperature, Nutrient Availability, and Shrub Cover at the Pyrenean Treeline. *Front. Plant Sci.* 9:1871. doi: 10.3389/fpls.2018.01871

Treelines are sensitive to environmental changes, but few studies provide a mechanistic approach to understand treeline dynamics based on field experiments. The aim of this study was to determine how changes in the abiotic and/or biotic conditions associated with global change affect the performance of tree seedlings (later saplings) at the treeline in a 10-year experiment. A fully factorial experiment in the Central Pyrenees was initiated in autumn 2006; 192 *Pinus uncinata* seedlings were transplanted into microplots with contrasting environmental conditions of (1) increased vs. ambient temperature, (2) increased nutrient availability vs. no increase, and (3) presence vs. absence of the dominant shrub *Rhododendron ferrugineum*. We assessed the performance of young pines on several occasions over 10 years. The pines were removed at the end of the experiment in autumn 2016 to characterize their morphology and to conduct chemical and isotopic analyses on their needles. Both the warming and the fertilization treatments increased seedling growth soon after the start of the experiment. *R. ferrugineum* facilitated the survival and development of pine seedlings during the early years and affected the chemical composition of the needles. Toward the end of the experiment, the transplanted *P. uncinata* individuals, by then saplings, competed with *R. ferrugineum* for light and nutrients; the presence of the shrub probably altered the strategy of *P. uncinata* for acquiring nutrients and buffered the effects of warming and fertilization. The pines were highly sensitive to all factors and their interactions throughout the entire experimental period. These findings indicated that the interactive effects of several key abiotic and biotic drivers associated with global change should be investigated simultaneously for understanding the contribution of young trees to treeline dynamics.

Keywords: chemical composition, competition, facilitation, fertilization, open-top chamber, *Pinus uncinata* seedlings, Pyrenees, *Rhododendron ferrugineum*

INTRODUCTION

Treeline ecotones are highly sensitive to climatic warming, because air and soil temperatures limit growth at high elevations and latitudes, where the growing season is generally short (Holtmeier, 2009; Körner, 2012). Many studies during the last decade have focused on the potential shifts in the position of treelines in response to climate change that leads to warmer temperatures (Grace et al., 2002; Körner and Paulsen, 2004; Moen et al., 2004; Peñuelas et al., 2007; Harsch et al., 2009; Barbeito et al., 2012; Yadava et al., 2017). Climatic warming, however, is only one aspect amongst several that control the altitudinal or latitudinal movements of treelines (Holtmeier and Broll, 2005; Lyu et al., 2016). Other factors may also shape treeline dynamics: abiotic factors such as wind velocity, solar radiation, and duration of snow cover (Wipf et al., 2009); and increases in nitrogen (N) deposition (Holtmeier and Broll, 2005), atmospheric CO₂ concentration (Hättenschwiler and Zumbunn, 2002; Handa et al., 2006), and ozone concentration (Díaz-de-Quijano et al., 2012; Huttunen and Manninen, 2013); but also biotic factors such as plant–plant interactions (facilitation or competition for abiotic resources) (Germino et al., 2002; Grau et al., 2012; Liang et al., 2016; Lyu et al., 2016), dispersal patterns (Vetaas and Grytnes, 2002), damage caused by herbivory (Munier et al., 2010), and changes in land use (Hofgaard, 1997). Many of these factors generally operate simultaneously and may interact, so providing clear mechanistic explanations for shifts in treelines based on reproducible experiments is difficult. The underlying factors that cause treeline shifts are thus not yet fully understood.

Marked regional displacements of treelines to higher altitudes or latitudes have occurred in the past. For example, treelines migrated upwards during a warm period in the early Holocene in many northern regions, such as the Scandinavian Mountains (Kullman, 1999), northern Eurasia (MacDonald et al., 2000), and central and western Canada (Spear, 1993). No general patterns, however, have been observed at the continental scale during the last century. In fact, a global meta-analysis (Harsch et al., 2009) reported that 87 of 166 treelines had advanced, 77 were stable, and two had receded since 1900, suggesting that the upward displacement of treelines is not a general world-wide phenomenon. Liang et al. (2011) reported that a treeline on the Tibetan Plateau had become denser because of an increase in the number of seedlings, but it had not moved significantly upslope. Gehrig-Fasel et al. (2007) reported that 10% of the treelines studied in the Swiss Alps had shifted upwards between 1985 and 1997 and that the woody vegetation in the other 90% had become denser. A regional densification of treeline vegetation was detected in the Catalan Pyrenees and Andorra during the second half of the 20th century (Batllori and Gutiérrez, 2008). Some Pyrenean treelines, though, shifted upwards by almost 40 m between 1956 and 2006, especially those where the cessation of human activity (livestock grazing, fire, logging) was more evident, whereas some other treelines in this region have responded little or not at all in recent decades (Ameztegui et al., 2015; Camarero et al., 2015). Identifying general patterns over large regions is thus difficult. Here we postulate that this lack of common response is due to other abiotic and biotic drivers

influencing the response of trees to temperature at or near the treeline.

The cover of shrubs and the ‘shrubline’ may also vary along with the changes in tree density or altitudinal/latitudinal shifts observed in some treelines (Hallinger et al., 2010). These changes are relevant because shrubs are potential modifiers of abiotic conditions at the microhabitat scale (Myers-Smith et al., 2011), so the expansion of shrubs across the treeline may play a role in treeline dynamics (Liang et al., 2011; Grau et al., 2013). For example, an increase in shrub cover favors the accumulation of snow leeward of the shrubs (Sturm and Holmgren, 2001; Wipf et al., 2009), thereby protecting tree seedlings from damage caused by low temperatures and snow abrasion (Germino and Smith, 1999; Holtmeier and Broll, 2007). Shrubs can also protect tree seedlings from strong winds or high irradiance during the growing season, which can affect their performance and photosynthetic rates (Akhalkatsi et al., 2006; Batllori et al., 2009; Grau et al., 2013). These tree seedlings, however, do not necessarily form a treeline over time, because most individuals will die or become ‘Krumholz’ trees, despite the facilitative effects of shrubs (Ninot et al., 2008). Furthermore, trees and shrubs may compete for resources such as light, nutrients, or water during later stages of development, so initial facilitation may not necessarily lead to the development of mature trees at the treeline (Wang et al., 2016). This idea was reinforced by Barbeito et al. (2012), who observed that shrubs could inhibit the development of trees in the Swiss Alps. Recent changes in shrub cover may nevertheless have a greater impact on treeline dynamics than recent changes in temperature (Dullinger et al., 2004; Liang et al., 2016); more research is needed to understand the impact of increases in shrub cover on treeline shifts and the interaction between changes in shrub cover and abiotic regimes.

Many of the studies conducted in treeline ecotones have described the observed patterns (e.g., treeline shifts, densification, and stability), but only few studies have analyzed these patterns experimentally. A few studies have investigated the effects of climatic warming on the performance of tree seedlings or samplings at the treeline. Munier et al. (2010) concluded that climatic warming would displace treelines upwards only if viable seeds and suitable substrates were available. Nutrient (especially N) availability at treelines is generally low, because low soil temperatures limit the rates of microbial decomposition and mineralization and nutrient uptake (Chapin, 1983; Birmann and Körner, 2009; Mcnown and Sullivan, 2013). An increase in soil temperatures, however, is expected to increase nutrient availability (Hobbie, 1996) and tree productivity at the treeline (Mack et al., 2004). Sveinbjörnsson et al. (1992) found that the establishment of *Betula pubescens* at a Swedish treeline was favored under increased N availability, and Hoch (2013) found that fertilization doubled the productivity of *Larix decidua* and *Pinus uncinata* when temperature was experimentally increased. It remains unknown, however, how important nutrient limitation is for tree performance at the treeline, and whether the increase of nutrient availability through potential increases in mineralization will compensate the nutritional demands of trees that grow under warmer conditions.

Few studies, have investigated the interactions amongst several of the factors that control the performance of trees at the treeline, such as temperature, nutrient availability, and shrub cover. Experimental studies where all these factors are combined are essential to find out whether nutrient limitation or shrub cover could alter or buffer the effects of temperature. Grau et al. (2012) conducted a multifactorial experiment in Swedish Lapland where *Betula pubescens* seedlings grew under contrasting environmental scenarios involving a full factorial combination of presence vs. absence of the shrub *Vaccinium myrtillus*, increased vs. ambient warming, and increased nutrient availability vs. no increase. This study found that treeline dynamics were driven by complex environmental interactions amongst these factors and that facilitation, competition, herbivory, and environmental changes at the tree seedling stage acted as important filters in structuring the treeline ecotone. Another experiment with the same factorial design was conducted in a more southern treeline, in the Pyrenees (Grau et al., 2013). In this region, mean annual temperatures increased (Cuadrat et al., 2013; Martín-Vide et al., 2017), snow cover decreased (López-Moreno, 2005; Cuadrat et al., 2013; Martín-Vide et al., 2017), and shrub cover expanded over the last decades (Molinillo et al., 1997; Roura-Pascual et al., 2005; Montané et al., 2007; Alados et al., 2011; Ninot et al., 2011; Garcia-Pausas et al., 2017). The tree and shrub used in this experiment, though, were *P. uncinata* and *Rhododendron ferrugineum*, which are dominant across the Pyrenean treeline. The seedlings of *P. uncinata*, which commonly forms the treeline in the Central Pyrenees, were highly sensitive to the simulated environmental changes within 3 years after transplantation (see Grau et al., 2013 for further details). The seedling stage is crucial but is only a short phase in the life of a treeline tree. Saplings, for example, being taller, are likely exposed to different abiotic and biotic environments than seedlings (Körner, 1998; Chrimes, 2004). Here we argue that understanding tree performance and associated treeline dynamics requires determining the complex interactions of abiotic and biotic drivers over time as the trees grow taller.

We re-visited the upper treeline site established in 2006 by Grau et al. (2013) in the Pyrenees 10 years after the onset of the experiment. We assessed the contributions of multiple interactive drivers of tree performance through time and whether the responses of the tree seedlings during the early years after transplantation persisted or varied over time. To our knowledge, this treeline experiment is the first and longest to test the responses of trees to contrasting environmental scenarios involving abiotic and biotic drivers simultaneously. In the initial study (Grau et al., 2013), the *P. uncinata* seedlings responded positively to the presence of *R. ferrugineum* shrubs, which provided protection to seedlings against winter damage. Both higher temperatures and increased nutrient availability had positive effects on seedling development. The positive effects of warming, however, were more marked in the absence of the shrub. In agreement with the observations in the initial study, we hypothesized that tree saplings in the Pyrenean treeline 10 years after transplantation would grow better (better development and higher foliar nutrient content) (1) under the protection of the shrubs, (2) in plots with increased temperatures, and (3) in

plots with increased nutrient availability. We also hypothesized that some factors would interact (4) negatively (e.g., presence of the shrub and warming) or (5) positively (e.g., warming and nutrient addition). We tested these hypotheses to improve our knowledge of the factors that control longer term treeline dynamics in the Pyrenees and to provide a robust mechanistically based framework for extrapolating the impacts of environmental changes on treeline dynamics to other regions.

MATERIALS AND METHODS

Study Area

The experiment was conducted on the north-western slope of Serrat de Capifonts (Pallars Sobirà, 42°33'N, 01°23'E) in Alt Pirineu Natural Park (Central Pyrenees, Catalonia, Spain) (**Supplementary Figure S1**). The experimental area was located in the upper part of the treeline at 2400 m a.s.l. (Carreras et al., 1996), dominated by scattered *P. uncinata* individuals (generally <2 m high) and *R. ferrugineum* shrubs, with patches of grassland that become progressively dominant above the treeline. The macroclimate of this part of the Pyrenees is montane continental due to its intermediate position between the Mediterranean Sea and the Atlantic Ocean (del Barrio et al., 1990). The 0°C isotherm from November to April is at about 1600–1700 m a.s.l., which indicates the lower limit where snow accumulates for a long period (López-Moreno et al., 2009). The growing season is normally about 5 months at the treeline; it generally starts when the air temperature remains above 5°C for more than five consecutive days and ends when temperatures are lower than 5°C for several days (Grau et al., 2013). The nearby meteorological station in Salòria (42°31'N, 01°21'E; 2451 m a.s.l.) recorded a mean annual temperature of $2.45 \pm 6.77^\circ\text{C}$ and a mean annual precipitation of 946 ± 315 mm between 2006 and 2015. The mean maximum daily air temperature in summer (June–August) was $13.43 \pm 4.08^\circ\text{C}$, and the mean minimum daily air temperature in winter (December–February) was $-3.6 \pm 4.77^\circ\text{C}$. The snow cover lasted 160 ± 50.4 days, although the winters in 2005–2006 and 2006–2007 were exceptionally dry and the snow lasted for only about 100 days. The maximum snow depth indicated great interannual variability, with mean depths generally exceeding 1 m in winter (**Supplementary Figure S2**).

Study Species

We transplanted *P. uncinata* seedlings in this experiment because this tree species forms most of the altitudinal treelines on the southern slopes of the Central Pyrenees. This pine species is a long-lived, slow-growing, and shade-intolerant conifer with a wide ecological tolerance for topography (slope, exposure, and elevation) and soil type, because it is highly tolerant to stress (Batllori et al., 2010). *R. ferrugineum* is one of the most abundant shrubs in subalpine environments at elevations around 1600–2200 m a.s.l. (Pornon et al., 2000). It usually covers the understory of subalpine forests up to the treeline and may form scrub upslope, toward the alpine belt. It appears in places where the snow accumulates for an extended period, so it remains protected from low temperatures. It is an ericaceous evergreen

shrub mostly on siliceous soils, and its stems may reach a height of approximately 50 cm at the treeline.

Experimental Design

The *P. uncinata* seedlings had been grown in a nursery from seeds collected in the Central Pyrenees. The seedlings were transplanted in the experimental site in autumn 2006 when they were 8–10 cm tall. We focused on the performance of young trees in this experiment because the early developmental stages are highly sensitive and responsive to environmental changes, thereby strongly determining future treeline dynamics (Barbeito et al., 2012).

The fully factorial experimental design included three factors that simulated contrasting environmental scenarios, with a total of eight treatments and four replicates per treatment. These three binary factors, assumed to be the most critical to the performance of pine seedlings, were: (1) increased temperature (+T) vs. ambient temperature (−T), (2) increased nutrient availability (+F) vs. no increase (−F), and (3) presence (+S) vs. absence (−S) of the dominant shrub *R. ferrugineum* (Supplementary Figure S3). These treatments were distributed randomly over 16 experimental units of the same area (1.32 m²) (Supplementary Figure S4). We had a total of four experimental units (used as replicates) for the treatments, +T, +F, and +T+F, and four −T−F experimental units that were used as controls. Each of these treatments was combined with the presence (+S) or absence (−S) of the shrub, and each experimental unit was placed at the edge of a *R. ferrugineum* patch (approximately 50 cm tall), so that half of the experimental unit was covered by the shrub and the other half was not (two microplots per experimental unit). Six seedlings were transplanted into each microplot, for a total of 192 seedlings (16 experimental units × 2 microplots × 6 seedlings). The vegetation growing inside the microplots without the shrub was regularly cut aboveground to avoid any re-growth. The effect of temperature was simulated with passive warming with OTCs of 1.32 m² in area and 50 cm high, designed based on the hexagonal ITEX model (Marion et al., 1997). Toward the end of the experiment, the upper part of the stems of the saplings in the −S+T+F and the −S+T−F treatments were already taller than the OTC. Although most of the stem was still inside the chamber, we decided to finalize the experiment at this point to avoid that the upper parts of the saplings in these treatments would become exposed to different temperatures than the lower parts. The temperature inside the OTCs was monitored by temperature loggers during the first 4 years of the experiment (iButtons 1-wire Thermochron temperature logger, Dallas Semiconductor Corporation, Dallas, United States). The temperature at ground level was approximately 2°C higher in the warmed experimental units than the control units during the growing season and this increase was consistently detected through the monitoring period. The aboveground plant tissues inside the OTC, though, may have been exposed to higher temperatures than at ground level due to wind speed reduction inside the chamber, and the sensitivity of the saplings to warming was therefore possibly overestimated (De Boeck et al., 2012). The units that did not have an OTC were also hexagonally shaped to maintain the uniformity of all experimental units. The OTCs were removed every winter

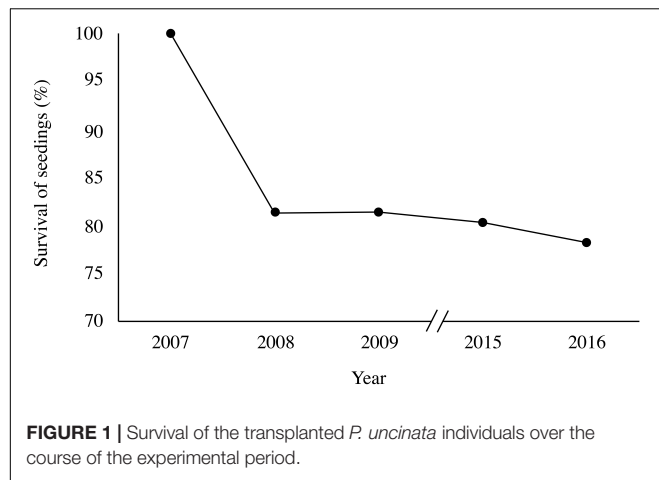
to avoid different patterns of snow accumulation amongst the experimental units, so we did not account for any advance of the snowmelt in spring. We replaced the OTCs soon after the snow had melted to simulate temperature changes only over the growing season. Finally, fertilizer was applied to the ground surface to simulate an increase in the mineralization rate and thus in the availability of nutrients (Rustad et al., 2001). The nutrients were added only once, in June 2007, by adding 200 g of slow-release NPK (10% N, 5% P₂O₅, 20% K) granules in each fertilized microplot.

Data Collection and Laboratory Analyses

Stem height was measured when the seedlings were transplanted in autumn 2006 and yearly from 2007 to 2009 at the end of the growing season (end of September or early October). Stem height was measured again 10 years later, in June and October 2016. We assumed that the heights measured in June 2016, before the onset of the growing season, corresponded to the growth until 2015. We also assessed survival in each survey, and on some occasions we also measured the stem diameter and the number of branches.

The pine saplings were removed from the ground in October 2016 and transported to the laboratory in plastic bags. The roots were cut off, and we measured several morphometric variables: stem height (including only the woody stem without the upper needles), number of primary branches, basal diameter, and number of branches that grew during the last growing season. All saplings were then oven-dried at 65°C to a constant weight (usually 72 h), and total aboveground biomass, biomass of needles grown in 2016 (new needles), and biomass of new stems grown in 2016 were measured.

A few needles from each sapling were collected from each microplot and pooled into a composite sample (a minimum of 2 g per sample, with an equal weight of needles from each sapling in a given microplot). The needles were ground with a MM400 of Retsch (Haan, Germany) and stored in Eppendorf tubes. These samples were used for the analysis of the elemental and isotopic composition of the pine needles. The macro- and microelements were analyzed to determine whether the experimental treatments affected the nutritional status of the trees, their sources of N uptake (δN^{15}), or the water-use efficiency (δC^{13}). We also tested the 'biogeochemical niche hypothesis', which predicts that changes in abiotic and biotic conditions will alter the stoichiometric composition of plant tissues (Peñuelas et al., 2008; Urbina et al., 2017), by analyzing a wide spectrum of chemical elements. The foliar concentrations of Na, Mg, P, S, K, Ca, V, Cr, Mn, Fe, Ni, Cu, As, Sr, Mo, Cd, and Pb were estimated from digested dilutions using inductively coupled plasma mass spectrometry in the laboratories of the Universitat Autònoma de Barcelona. The concentrations of C, N, δN^{15} , and δC^{13} were analyzed by depositing 3.5 mg of dried and ground sample in aluminum capsules, which were sent to a laboratory at the University of California, Davis (UC Davis Stable Isotope Facility). The samples were run on an Elementar Vario EL Cube elemental analyzer (Elementar Analysen systeme GmbH, Hanau, Germany) connected to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, United Kingdom).



Statistical Analyses

The effect of each treatment on each variable for each year was analyzed with a linear mixed model as implemented in R v. 3.3.2 (R Core Team, 2017), using the ‘nlme’ (Pinheiro et al., 2017) and ‘lme4’ (Bates et al., 2015) packages. ‘Microplots’ and ‘shrubs’ (nested within ‘microplots’) were considered as random factors to account for the grouping structure of the data. The interaction terms and factors for a given variable that had no statistical support ($p > 0.05$) were removed from the model. The significance of the remaining factors and interactions was recalculated every time a term was excluded from the analyses, provided that the new model was an improvement ($p < 0.05$) over the more complex model in a likelihood

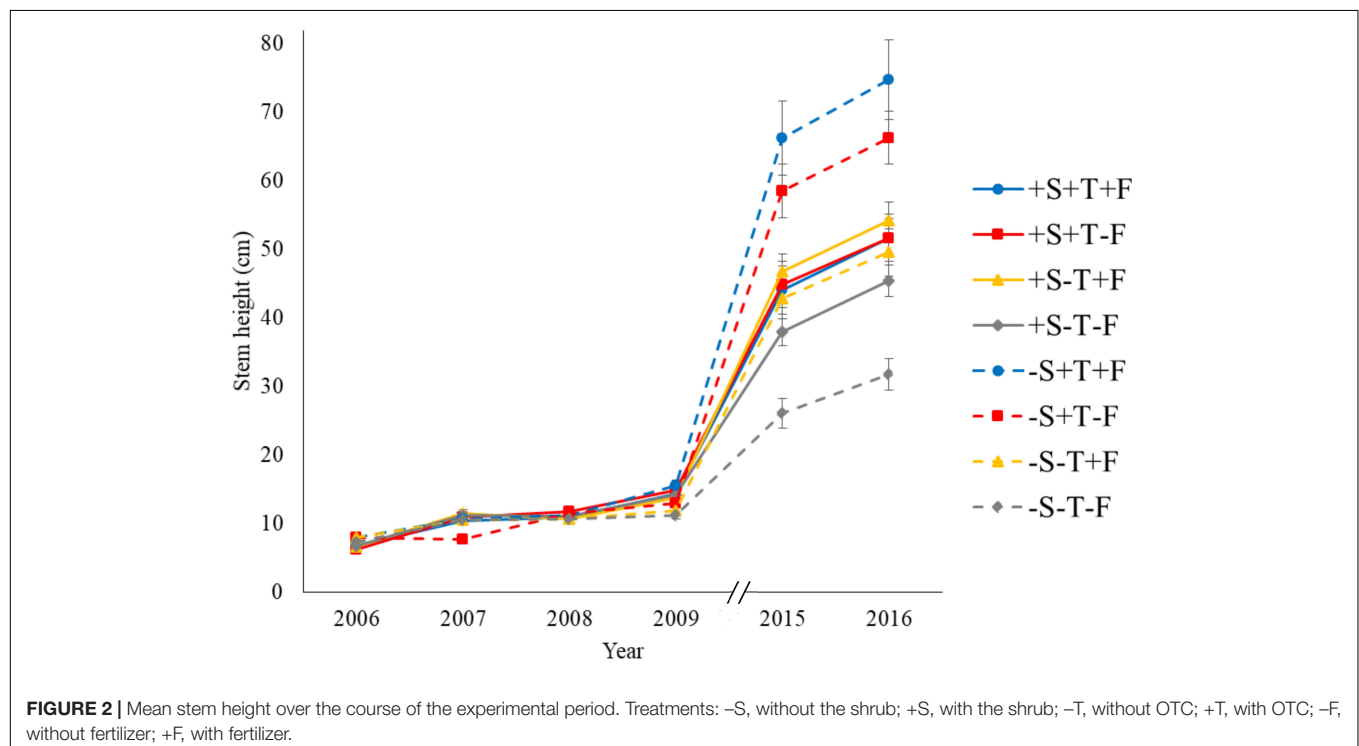
ratio test. The significance of each factor was based on the minimally adequate model. We also conducted pairwise tests with Bonferroni correction (Bland and Altman, 1995) for comparing the effects of the presence vs. absence of the shrub on *P. uncinata* growth for the +T, +F, and +T+F treatments.

In addition, a ‘repeated-measures analysis’ was conducted to determine the influence of time on stem height. The model used in this analysis also included an autocorrelated error term that took into account the repeated measures on the same individuals throughout the experimental period. We compared the models that included the temporal term with the models that did not and chose the minimally adequate model with the lowest Akaike’s information criterion (Akaike, 2011).

We conducted principal component analyses (PCAs) with the R ‘FactoMineR’ package to determine the differences between the trees growing in control plots vs. those in the other treatments based on the measured variables (tree morphometry and foliar nutrient concentration).

RESULTS

Seventy-eight percent of the 192 transplanted seedlings survived until the end of the experiment (Figure 1), but survival did not differ between treatments. Warming and the presence of *R. ferrugineum* influenced most of the other variables measured in the transplanted trees, and most of their effects increased over the course of the experiment. Fertilization had a mostly positive effect (Figures 2, 3 and Supplementary Table S1). The means of the morphometric and chemical variables are presented in Supplementary Tables S2–S6.



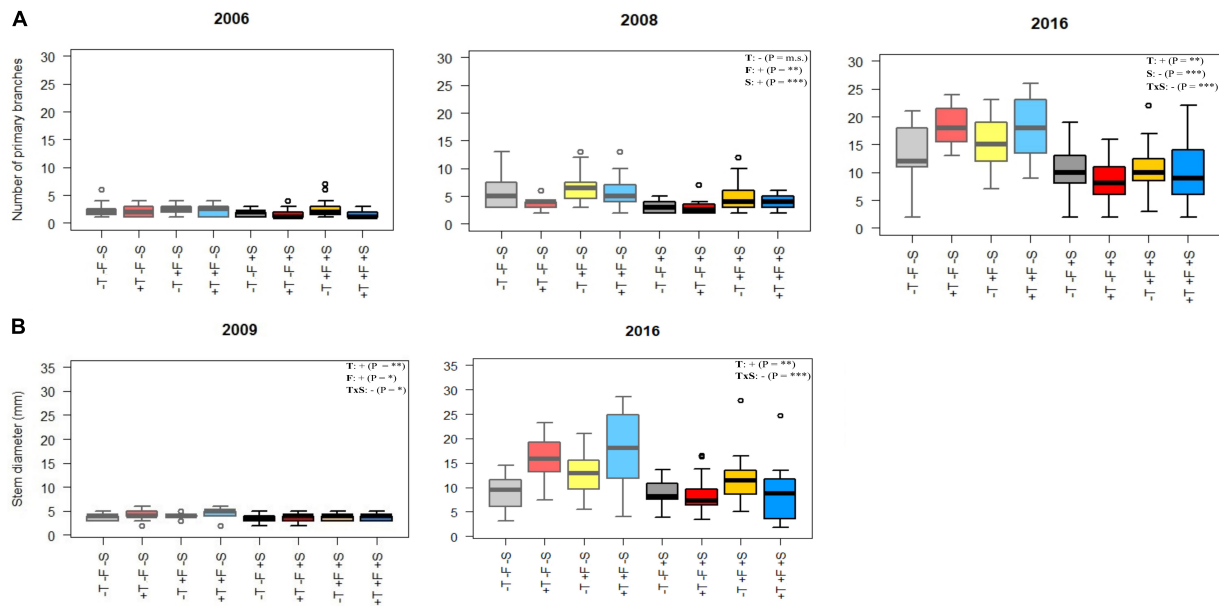


FIGURE 3 | (A) Number of primary branches and **(B)** stem diameter in various sampling periods. Light colors (left half of each plot) represent -S treatments, and darker colors (right half of each plot) represent +S treatments. Gray, control treatment (-T-F); red, warming (+T); yellow, fertilization (+F); blue, warming and fertilization (+T+F). See **Supplementary Tables S3, S4** for the statistical significance of each factor. * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; m.s., marginally significant.

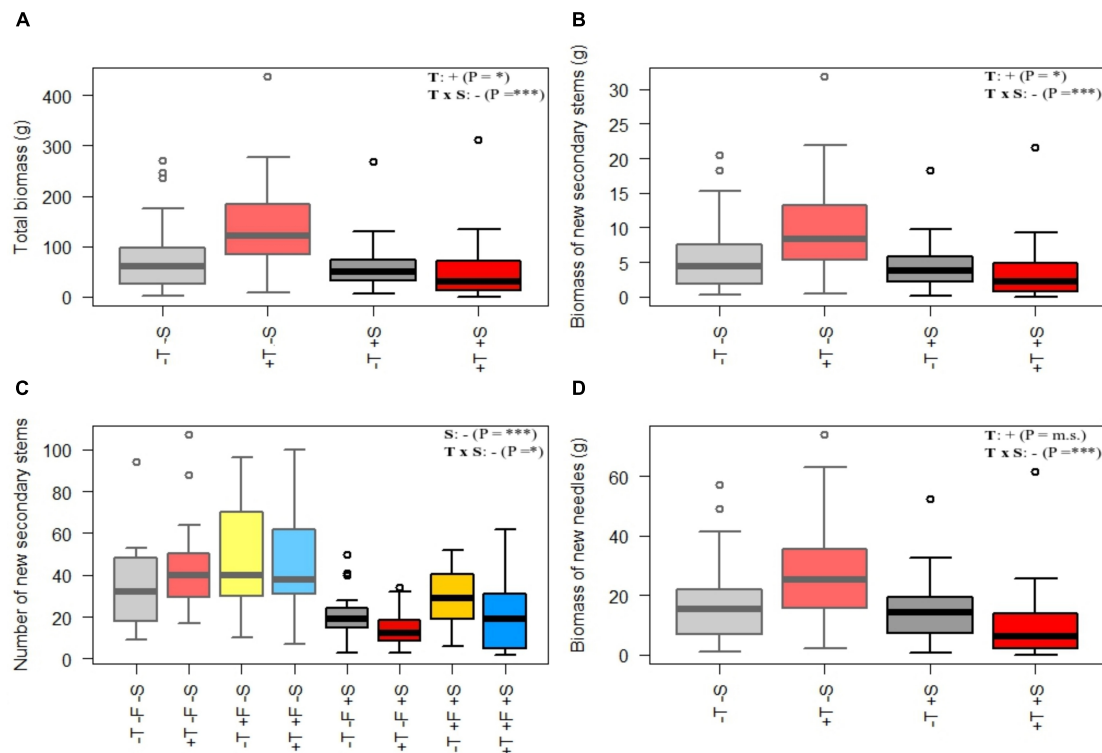


FIGURE 4 | Morphological variables measured in autumn 2016 for each treatment: **(A)** total biomass, **(B)** biomass of new secondary stems, **(C)** number of new secondary stems, and **(D)** biomass of new needles. The statistical differences between treatments are shown in **Supplementary Table S7**. Light colors (left half of each plot) represent -S treatments, and darker colors (right half of each plot) represent +S treatments. Gray, control treatment (-T-F); red, warming (+T); yellow, fertilization (+F); blue, warming and fertilization (+T+F). See **Supplementary Table S5** for the statistical significance of each factor. ** $p \leq 0.01$; *** $p \leq 0.001$; m.s., marginally significant.

Effects of the Experimental Conditions on Sapling Growth and Biomass

Experimental warming had a strong positive effect on stem height (Figure 2) and diameter over time (Supplementary Tables S1A,B). The number of primary branches was also positively affected by warming at the end of the experiment (Figure 3A and Supplementary Table S1C). Warming did not significantly increase the number of new secondary stems, but their biomass and the biomass of new needles increased (Figure 4 and Supplementary Table S7). The warming treatment, however, interacted with the shrub treatment; stem height, basal diameter, the number of primary branches and new secondary stems, the biomass of new needles, and the total biomass increased to a much lesser extent or did not increase in response to warming when *R. ferrugineum* was present.

The addition of fertilizer also increased stem growth over the course of the experimental period (Figure 2 and Supplementary Table S1A) although it interacted with the presence of *R. ferrugineum*, as observed in the warming treatment. The effect of the fertilizer tended to be more evident in 2009, 2015, and 2016 if the shrub was not present. Branching increased significantly only in autumn 2008 (Figure 3A), and stem diameter increased significantly only in 2009 (Figure 3B).

Stem height did not increase significantly in the presence of *R. ferrugineum* in the repeated-measures analyses when the effect of the shrub alone was analyzed. The stems in 2009, 2015, and 2016, however, were significantly longer in the presence of *R. ferrugineum* when each year was analyzed separately (Figure 2 and Supplementary Table S1A). *P. uncinata* saplings growing with the shrub tended to have thinner stems, although this trend was not significant, and their biomass did not vary (Figures 3B, 4A and Supplementary Tables S1B, S7). Trees growing without *R. ferrugineum* developed thicker stems than those growing in the presence of the shrub while stems were short. When stem height was lower than 40 cm, the stem was much thinner in trees growing with the shrub. This difference disappeared, however, as trees became saplings (Figure 5).

The PCA ordination in Figure 6 illustrates the impact of the contrasting experimental conditions on the morphometric characteristics of the saplings 10 years after the onset of the experiment. Trees growing in altered conditions (+T, +F, and +T+F) without *R. ferrugineum* differed much more from the initial conditions (control plots) than the trees growing with *R. ferrugineum*.

Effects of the Experimental Conditions on the Chemical Composition of Sapling Needles

None of the treatments significantly affected foliar C or N concentrations 10 years after the fertilizer was applied. The concentrations of P, K, and Cu increased, and the concentration of Mn marginally decreased. The N:P, C:K, and N:K ratios decreased with fertilization (Supplementary Table S8). Warming significantly increased the concentrations of Mn and Zn and marginally significantly increased the concentrations of K and Cu. The presence of *R. ferrugineum* had a positive effect on K and

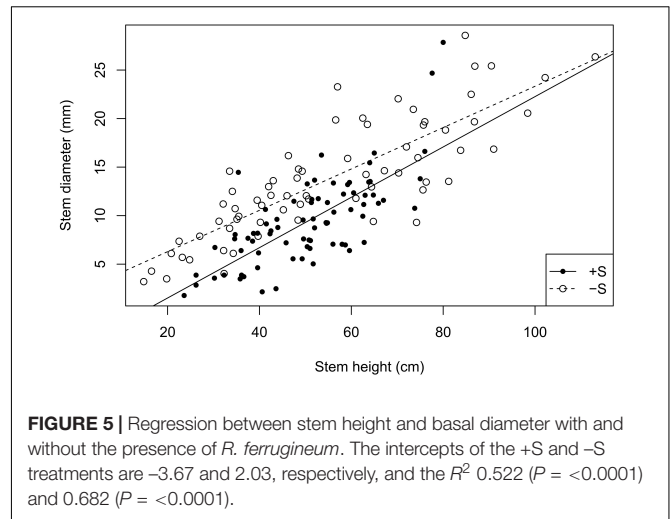


FIGURE 5 | Regression between stem height and basal diameter with and without the presence of *R. ferrugineum*. The intercepts of the +S and -S treatments are -3.67 and 2.03, respectively, and the R^2 0.522 ($P = <0.0001$) and 0.682 ($P = <0.0001$).

Zn concentrations but had a negative effect on Mn concentration. The Sr concentration increased marginally when warming was combined with the presence of *R. ferrugineum*.

The presence of *R. ferrugineum* did not significantly affect needle δN^{15} , but δN^{15} tended to be lower when the presence of the shrub was combined with warming and/or fertilization (Figure 7 and Supplementary Table S9A). We investigated this trend in more detail by comparing the foliar δN^{15} values for the +T, +F, and +T+F treatments with *R. ferrugineum* with the values for the same treatments but without the presence of the shrub using pairwise tests. δN^{15} was lower when *R. ferrugineum* was present for each of the +T, +F, and +T+F treatments.

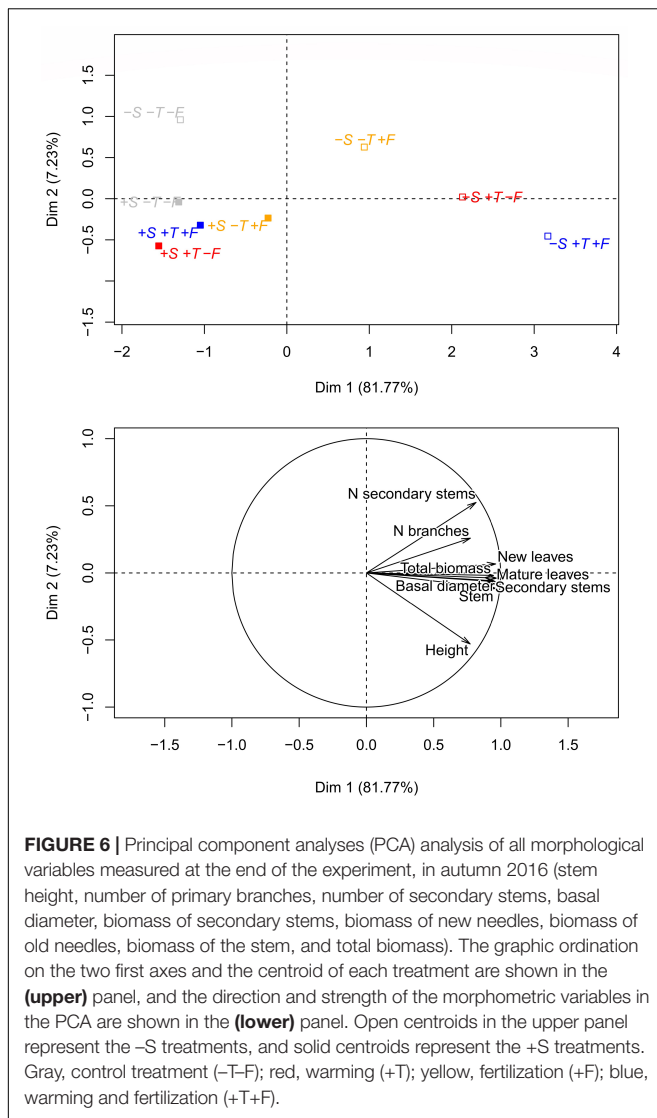
The PCA of the foliar chemical composition (Figure 8) indicated that the saplings growing in altered conditions (+T, +F, and +T+F) generally differed greatly from the control saplings, similar to the morphometric variables. The saplings growing in altered conditions (+T, +F, and +T+F) with *R. ferrugineum*, however, tended to differ more from the control saplings than those growing in altered conditions without *R. ferrugineum*, especially for the +T and +T+F treatments.

Warming had a positive effect on the total contents of P, Mn, Cu, and Zn (the product of the concentration of each element and the total needle biomass of the sapling) when *R. ferrugineum* was not present (Supplementary Table S9B). Fertilization only had a marginally positive effect on total Cu content but had no effect on the total contents of the other elements. The contents of some of the elements analyzed (Na, S, V, As, Cd, and Pb) were below the detection limit and were thus excluded from the analyses.

DISCUSSION

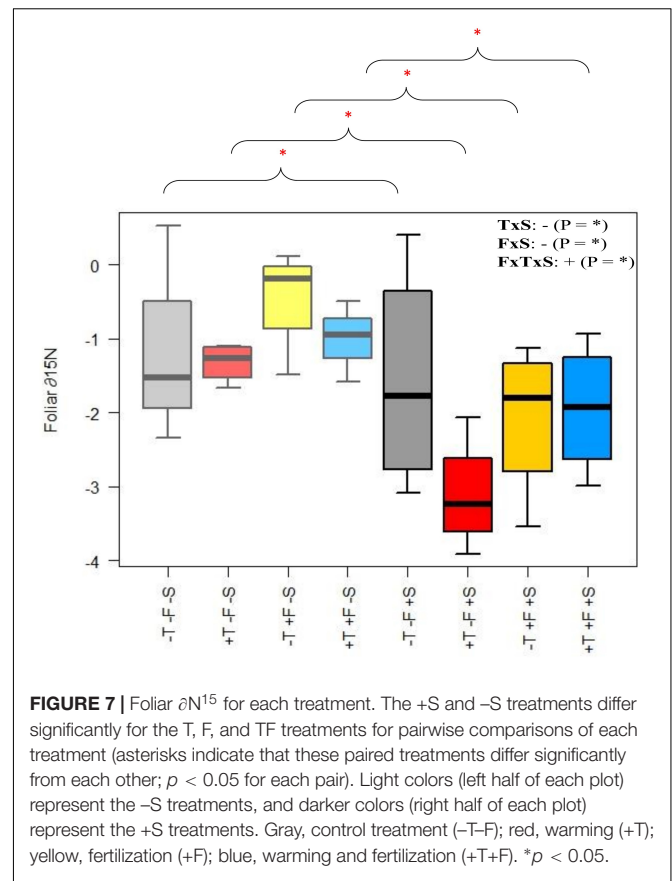
Biotic and Abiotic Manipulations

In the initial study, Grau et al. (2013) reported that the *P. uncinata* seedlings were facilitated by *R. ferrugineum*. This shrub protected the seedlings from damage in the winter of 2007–2008, when snow cover was exceptionally low during the coldest months (Supplementary Figure S2). The accumulation of snow

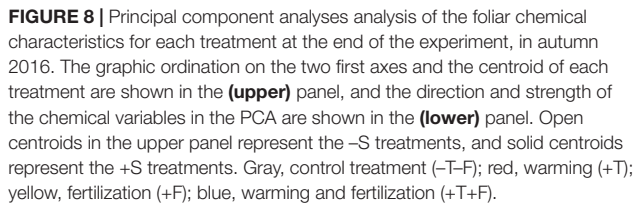


leeward of the shrubs and the insulation capacity of the snow protected the seedlings from snow abrasion and low temperatures (Smith et al., 2003; Neuner, 2007; Ninot et al., 2008). The presence of *R. ferrugineum* was therefore a crucial factor for seedling development during this early stage of development. We found no further evidence of winter facilitation by shrubs after the winter of 2007–2008, possibly because snow cover was not critically low again until the end of the experiment (Supplementary Figure S3). The potential facilitation by the shrub was nevertheless expected to occur, especially during the initial stages of development when the trees were much shorter than the protective shrubs.

We found evidence, however, that the transplanted trees competed with the shrubs for resources over the course of the experiment. *P. uncinata* individuals tended to develop longer but thinner (i.e., etiolated) stems when grown with *R. ferrugineum*, although their total aboveground biomass did not differ significantly from *P. uncinata* grown without the



shrub (Figure 4A and Supplementary Table S7). *P. uncinata* is intolerant of shade (Ninot et al., 2007; Battlori et al., 2010), so this response most likely helped it to adjust to the lack of light when grown in the presence of *R. ferrugineum* (Cranston and Hermanutz, 2013). The trees became progressively less shaded or were no longer shaded when they became taller, and the etiolation tended to disappear (Figure 5). Hypothesis (1), that *P. uncinata* individuals would grow better in the presence of *R. ferrugineum*, was thus only supported for the early stage of life. We observed a sequence over the course of the experiment of a facilitative and then a competitive impact of the shrub, with a final release from competition by the shrub. The competition between *P. uncinata* and *R. ferrugineum* did not significantly affect the concentration of most of the chemical elements of the pine needles. This lack of effect was unexpected, at least for some elements. For example, N is expected to be limiting in cold ecosystems such as treelines (Körner, 2003), where the short growing season and recalcitrancy of plant material limit soil microbial activity and the decomposition of organic matter (Loomis et al., 2006; Macek et al., 2012). Pines, however, nearly always have ectomycorrhizal fungi (Harley and Harley, 1987) and can decompose relatively recalcitrant organic matter, which makes them relatively independent of the availability of inorganic nutrients provided by mineralization or experimental fertilization (Read, 2003). Possibly also indirectly related to mycorrhiza, we observed that the foliar δN^{15} of the saplings in



the +T, +F, and +T+F treatments was lower with than without *R. ferrugineum* in pairwise comparisons (**Figure 7**). Foliar δN^{15} was also significantly lower in the +S control treatment than in the -S control treatment. Some studies have argued that variations in δN^{15} may indicate changes in the strategy of N uptake in plants (Michelsen et al., 1996; Russo et al., 2013). Lower δN^{15} generally indicates more N uptake by ectomycorrhizal or ericoid mycorrhizal fungi (Michelsen et al., 1998), with more recycled N leading to lower N losses from the ecosystem (Garten, 1993; Robinson, 2001; Craine et al., 2009; Anadon-Rosell et al., 2016). *R. ferrugineum* has ericoid mycorrhizal fungi (Straker, 1996), so the lower δN^{15} values when the shrub was present could indicate that the pines had taken up more N that had been recycled by ericoid mycorrhizal fungi in a relatively closed N cycle. This recycled N with low δN^{15} values could still have been mineralized before uptake, but the pines had likely taken up part of their N in organic form derived from *R. ferrugineum* litter, thereby overcoming inorganic-N limitation (Akhmetzhanova et al., 2012). This adaptation may eventually lead to similar foliar N concentrations with or without the presence of *R. ferrugineum*,

even though the strategy of N uptake may differ. None of the treatments had any effect on foliar δC^{13} (**Supplementary Table S6**), suggesting that water-use efficiency did not differ significantly between treatments (Sullivan and Sveinbjörnsson, 2011).

Our hypothesis (2), that trees would grow more with warming, was clearly supported. Stem height increased in the warmed plots [approximately twofold more than the control trees when the shrub was not present (**Supplementary Table S2**)], and basal diameter, number of primary branches, and biomass of the new secondary stems also increased (**Figures 2–4** and **Supplementary Tables S1, S7**). In fact, the *P. uncinata* seedlings were highly sensitive to warming soon after transplantation (Grau et al., 2013), and this sensitivity persisted. Temperature strongly controls photosynthetic rates (Danby and Hik, 2007), root activity (Du and Fang, 2014), meristematic activity and tissue development (Körner, 1998) during the growing season, particularly at the treeline (Körner and Paulsen, 2004). Warming only affected the concentrations of K, Mn, and Zn, but the total contents of P, Mn, Cu, and Zn increased (**Supplementary Tables S9A,B**). The increase in total content but no change in concentration indicated that *P. uncinata* individuals growing in warmed conditions did not suffer from a dilution of their nutrients and that nutrient acquisition kept pace with the increase in biomass. This finding suggests that warming was generally positive and improved the overall performance of *P. uncinata* individuals.

The addition of fertilizer supported our hypothesis (3), that *P. uncinata* would grow better if nutrient availability increased. The addition of fertilizer enhanced the performance of *P. uncinata* over the course of the experiment (**Figures 2, 3B and Supplementary Tables S1A,C**), even though the fertilizer was applied only once in 2007.

The effect of warming on *P. uncinata*, however, was significantly lower when *R. ferrugineum* was present (**Figures 2–4** and **Supplementary Tables S1, S7**), supporting hypothesis (4) that warming and the presence of the shrub could interact negatively. When warming was combined with the presence of *R. ferrugineum*, *P. uncinata* grew significantly less, and foliar nutrient concentration and content did not increase, suggesting that the occurrence of shrubs could strongly buffer or diminish the effects of warming.

The foliar concentrations of P and K were higher and the N:P and N:K ratios were lower in the fertilized than the non-fertilized saplings at the end of the experiment, 10 years after the NPK fertilizer had been applied (**Supplementary Table S6**). This finding suggests that N was more limiting than P and K over the course of the experiment and that the added N was depleted more quickly and could not be accumulated in the needles until the end of the experiment, as for P and K. These results thus support the idea that N is more limiting than other nutrients for trees that grow at the treeline (Körner, 2003) and is available mostly in a recalcitrant organic form. The positive effect of fertilization on stem growth, however, decreased to some extent when fertilization was combined with the presence of the shrub (**Figure 2** and **Supplementary Table S1A**). *R. ferrugineum* thus profited more than *P. uncinata*

from the higher availability of soil nutrients, probably because of its greater biomass (Epstein et al., 2000). We also found some support for hypothesis (5), that warming and fertilization would have an additive effect on tree performance; the trees were largest in the microplots where both treatments were combined. This finding suggests that the growth of the trees that were warmed but not fertilized was limited by nutrient availability, because the warmed trees grew more if they were also fertilized. This additive effect, however, only occurred when the shrub was not present, again suggesting that the presence of shrubs could buffer the effects of other factors.

The chemical composition of the *P. uncinata* needles differed greatly between the control and the warmed and/or fertilized trees (Figure 8). This shift could be explained by the 'biogeochemical niche hypothesis,' which states that the biogeochemical niche should determine the species-specific strategy of growth and uptake of resources when plants are exposed to changes in environmental conditions or suffer from competition with other species (Peñuelas et al., 2008). We would thus observe an expansion of the biogeochemical niche (an increase in stoichiometric differences between treatments), due to changes in the abiotic and biotic conditions (Urbina et al., 2017), suggesting a clear shift in the chemical properties of the needles, which possibly respond to changes in their physiology.

The ordinations in Figures 6, 8 clearly indicate that all experimental manipulations influenced the performance of *P. uncinata*, with important interactions amongst the treatments. The trees responded quickly at the start of the experiment (Grau et al., 2013), and these physiological responses and adaptations persisted, confirming that the trees growing at the Pyrenean treeline are persistently influenced by shrub-tree interactions and changes in temperature and nutrient availability. The mechanisms of these interactions, however, change over time, with facilitation playing an important role at the seedling stage and competition (and release from it) becoming more prominent at the sapling stage, as discussed above. This finding is in agreement with previous studies (e.g., Körner and Paulsen, 2004; Ameztegui and Coll, 2011; Liang et al., 2016) reporting that tree development at the treeline is highly sensitive to changes in both biotic and abiotic conditions.

CONCLUSION

Future Implications for Tree Development at the Pyrenean Treeline

The high sensitivity of *P. uncinata* seedlings (later saplings) to the experimental manipulations suggests that pines at the treeline will most likely respond to current and future changes in abiotic and biotic conditions. The ongoing expansion of shrub cover in this region (Molinillo et al., 1997; Roura-Pascual et al., 2005; Montané et al., 2007; Alados et al., 2011; Ninot et al., 2011; Garcia-Pausas et al., 2017) could favor an intensification of the interactions between shrubs and trees

growing at the treeline. Our results indicate that both facilitation and competition may co-occur under such a scenario at the initial stage of tree development. The expansion of shrubs such as *R. ferrugineum* would favor the availability of safe sites and protect small trees from abiotic damage, especially in years with low snow cover, and enhance survival and tree establishment at the treeline, which could be especially relevant because of the statistically significant reduction of winter precipitation in recent decades (1959–2010) in the Pyrenean region and the increase in interannual variability of winter precipitation (López-Moreno, 2005; Cuadrat et al., 2013; Martín-Vide et al., 2017) that are likely to persist (IPCC, 2013). The shrubs, however, may compete with the trees for resources, especially light and nutrients, and thereby hamper their development. The balance between facilitation and competition between the shrubs and trees will thus strongly determine the establishment and development of new trees at the treeline.

Temperatures have also increased by +0.2°C per decade in recent decades in the Pyrenees, especially during spring and summer (Cuadrat et al., 2013; Martín-Vide et al., 2017). This trend is also predicted to continue (IPCC, 2013). Based on the results of our experiment, where we simulated an increase of ca. 2°C during the growing season, *P. uncinata* seedlings are expected to benefit from this ongoing thermal increase. Warmer conditions, together with the predictable increase in the availability of limiting nutrients such as N (Hobbie, 1996; Rustad et al., 2001), are also expected to favor the development of young *P. uncinata* individuals, based on the findings of our experiment. We predict, though, that nutrient availability will remain an important limiting factor in this system also in a future, warmer climate, as trees that were warmed but not fertilized grew less than those that were warmed and fertilized. The cover of *R. ferrugineum*, however, is expected to buffer these positive warming and fertilization effects on young individuals, suggesting that the interaction between abiotic and biotic factors may play a key role in future treeline dynamics, especially if shrub cover increases. We believe that our findings for dynamic shrub-tree interactions throughout the lifetime of young trees in treeline environments subjected to global change have important implications not only for treelines in the Pyrenees, but also for many other treelines around the world where shrubs and trees co-occur.

AUTHOR CONTRIBUTIONS

OG and JN conceived and designed the study. MA and OG wrote the first version of the manuscript. All authors actively contributed to revisions.

FUNDING

This research was partly funded by the Comissionat per a Universitats i Recerca of the Generalitat de Catalunya, the European Social Fund, and the Synergy grant ERC-SyG-610028 IMBALANCE-P.

ACKNOWLEDGMENTS

We thank the Parc Natural de l'Alt Pirineu for their support and all the people who have helped with the fieldwork over the course of the experiments.

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