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Climate change influences foliar nutrition and metabolism of red maple (*Acer rubrum*) trees in a northern hardwood forest

Megan Blagden,[1] Jamie L. Harrison,[1,4] Rakesh Minocha,[2,]† Rebecca Sanders-DeMott,[1,3] Stephanie Long,[2] and Pamela H. Templer[1]

[1]*Department of Biology, Boston University, 5 Cummington Mall, Boston, Massachusetts 02215 USA*
[2]*USDA Forest Service, Northeastern Research Station, Durham, New Hampshire 03824 USA*
[3]*Woods Hole Coastal and Marine Science Center, Woods Hole, Massachusetts 02543 USA*

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Abstract. Mean annual air temperatures are projected to increase, while the winter snowpack is expected to shrink in depth and duration for many mid- and high-latitude temperate forest ecosystems over the next several decades. Together, these changes will lead to warmer growing season soil temperatures and an increased frequency of soil freeze–thaw cycles (FTCs) in winter. We took advantage of the Climate Change Across Seasons Experiment (CCASE) at the Hubbard Brook Experimental Forest in the White Mountains of New Hampshire, USA, to determine how these changes in soil temperature affect foliar nitrogen (N) and carbon metabolism of red maple (*Acer rubrum*) trees in 2015 and 2017. Earlier work from this study revealed a similar increase in foliar N concentrations with growing season soil warming, with or without the occurrence of soil FTCs in winter. However, these changes in soil warming could differentially affect the availability of cellular nutrients, concentrations of primary and secondary metabolites, and the rates of photosynthesis that are all responsive to climate change. We found that foliar concentrations of phosphorus (P), potassium (K), N, spermine (a polyamine), amino acids (alanine, histidine, and phenylalanine), chlorophyll, carotenoids, sucrose, and rates of photosynthesis increased with growing season soil warming. Despite similar concentrations of foliar N with soil warming with and without soil FTCs in winter, winter soil FTCs affected other foliar metabolic responses. The combination of growing season soil warming and winter soil FTCs led to increased concentrations of two polyamines (putrescine and spermine) and amino acids (alanine, proline, aspartic acid, γ-aminobutyric acid, valine, leucine, and isoleucine). Treatment-specific metabolic changes indicated that while responses to growing season warming were more connected to their role as growth modulators, soil warming + FTC treatment-related effects revealed their dual role in growth and stress tolerance. Together, the results of this study demonstrate that growing season soil warming has multiple positive effects on foliar N and cellular metabolism in trees and that some of these foliar responses are further modified by the addition of stress from winter soil FTCs.

Key words: amino acids; chlorophyll; HPLC; inorganic nutrients; metabolism; photosynthesis; polyamines; soil freeze–thaw cycles; soil warming; stress; sugars.

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[4]Present address: Lamont–Doherty Earth Observatory, Columbia University, Palisades New York 10964 USA.
† **E-mail**: rakesh.minocha@usda.gov

Introduction

Climate models project increases in mean annual air temperatures and decreases in the depth and duration of winter snowpack in seasonally snow-covered mid- and high-latitude temperate forests over the next several decades Hayhoe et al. (2007). In the northeastern United States, average annual air temperatures have increased by 2.4°C since 1900 (Janowiak et al. 2018) and are projected to increase by an additional 2.9–5.3°C by the year 2100 (Hayhoe et al. 2007). The greatest increases in average air temperatures in this region have been recorded during winter months, which are approximately 1.9°C warmer than they were a century ago (Janowiak et al. 2018), and these trends are expected to continue. With an increased frequency of daily winter air temperatures above freezing (Contosta et al. 2019), the depth and duration of winter snowpack are expected to decrease over the next century (Notaro et al. 2014). This reduction in snowpack is projected to increase the frequency of soil freeze–thaw cycles (FTCs) in this region over the next century (Campbell et al. 2010). These changes in climate across seasons are expected to have large effects on forest growth and function, though the entirety of this impact is not well understood (Templer et al. 2017).

Experiments simulating decreased winter snowpack and increased soil freezing depth and duration demonstrate fine root damage in red maple (*Acer rubrum*) (Campbell et al. 2014, Sanders-DeMott et al. 2018) and *Acer saccharum* (sugar maple) trees (Cleavitt et al. 2008, Melillo et al. 2011, Comerford et al. 2013), alongside decreased aboveground growth in sugar maple forests (Reinmann et al. 2019). Additionally, increased soil freezing has been shown to decrease soil microbial biomass and rates of soil net nitrogen (N) mineralization in the following growing season (Durán et al. 2014, Sorensen et al. 2018), which may limit microbial decomposition and soil N availability in the future. However, while winter soil FTCs damage roots and reduce plant N uptake (Sanders-DeMott et al. 2018), warmer growing season temperatures increase rates of soil microbial N cycling and tree N uptake (Bai et al. 2013, Harrison et al. 2020*a*, *b*). Changes in forest N cycling have the potential to alter primary production in temperate, northeastern U.S. forests facing a changing climate, as N availability and uptake by trees are closely linked with foliar N allocation and rates of photosynthesis in predominantly N-limited forests. Understanding the mechanisms behind the relationship between N availability and carbon (C) sequestration capacity via primary and secondary metabolism is critical for future carbon budgeting and climate modeling. The reprogramming of primary and secondary metabolism in response to N availability has been linked to C sequestration, as well as cell expansion and growth in Arabidopsis and in trees (Scheible et al. 2004, Minocha et al. 2015).

Common polyamines ([PAs]: putrescine [Put], spermidine [Spd], and spermine [Spm]) are known to be required for an array of diverse functions within cells including cell division, growth, and stress tolerance in all living forms (Galston and Sawhney 1990, Minocha et al. 2014, Handa et al. 2018, Seo et al. 2019, Sobieszczuk-Nowicka et al. 2019). Many studies have also demonstrated an increase in the foliar concentrations of Put and Spd and amino acids (AAs) arginine (Arg), γ-aminobutyric acid (GABA), proline (Pro), and glutamine (Gln) in response to increased soil N availability (Ericsson et al. 1993, Alcázar et al. 2006, Minocha et al. 2015, 2019). These findings suggest that these metabolites could also act as metabolic sinks for N when tree uptake exceeds immediate demand, thereby improving tolerance to a variety of stress factors. Thus, Foliar PA and AA concentrations may play an important role in adapting to changes in N availability and environmental stresses with climate changes that occur across seasons.

Foliar concentrations of N drive the synthesis of chlorophyll (Millard and Grelet 2010). Foliar N, chlorophyll, and carotenoid concentrations are often viewed as predictors of photosynthetic capacity and gross primary production (Ollinger and Smith 2005, Gururani et al. 2015*a*, *b*). Soluble sugars, on the other hand, are mainly synthesized from newly assimilated carbon in the leaves and perform a myriad of functions in plants, including osmoregulation, plant defense, transport, and export as a substrate to support soil microbes (Du et al. 2020 and references therein). More recently, growing season soil warming and winter soil FTCs have been reported to affect soil and/or foliar nutrient

concentrations (Wipf et al. 2015, Marty et al. 2019, Qin et al. 2021, Zhao et al. 2021), which in turn affect the cellular metabolism and photosynthesis in different tree species (Minocha et al. 1997, 2000, 2010, 2019, Bauer et al. 2004), the reason for this being their interdependence on each other within cells to perform critical life-sustaining functions. The potentially additive, antagonistic, or synergistic effects of changes in climate in the growing season and/or winter effects on N cycling (Templer et al. 2017) may also alter N metabolism and C uptake in temperate seasonally snow-covered ecosystems. Identifying the particular mechanisms behind the response of trees to multiple simultaneously acting stress factors in the natural environment remains a key challenge for physiologists and ecologists (Minocha et al. 2014). Regulation of cellular concentrations of metabolites may be positively or negatively impacted by each of the multiple environmental stress factors that co-occur. Thus, the concentrations of cellular metabolites at any given point in time reflect the net effects of all combined stressors on the function of cells. By simultaneously looking at rates of photosynthesis, concentrations of foliar nutrients, and a suite of metabolic parameters (stress level indicators) that are all sensitive and highly responsive to environmental stresses, researchers can determine whether cellular machinery is responding positively or negatively to multiple environmental factors.

We measured rates of photosynthesis and foliar metabolites and nutrients in red maple foliage in a seasonally snow-covered forest ecosystem located in New Hampshire, USA. We took advantage of the Climate Change Across Seasons Experiment (CCASE) at the Hubbard Brook Experimental Forest (Templer et al. 2017) to evaluate how projected changes in soil temperatures across seasons affect foliar N and C metabolism since these two elements are the basic building blocks for most metabolites (Minocha et al. 2019). In this experiment, two plots are warmed 5°C in the growing season and two others are warmed 5°C in the growing season and have multiple soil FTCs induced in winter. Our past work from this study shows that growing season warming has a net positive effect on soil N availability (Sanders-DeMott et al. 2018) and foliar N (Harrison et al. 2020*b*) even when trees experience an increased frequency of winter soil FTCs. However, foliar nutrients and partitioning of N and related C compounds into specific foliar metabolites may differ between red maple trees experiencing growing season soil warming (a potential positive event) alone vs. those that experience an increased frequency of winter soil FTCs (a potential stressful event) in addition to soil warming, as the effects of winter climate may further impact tree C and N metabolism. For this reason, we hypothesized that warmer soils in the growing season have a positive effect on foliar nutrients, rates of photosynthesis, and concentrations of foliar PAs, AAs, chlorophyll, carotenoids, and soluble proteins, but that stress from winter soil FTCs offsets these positive effects.

Methods

Site description

We sampled foliage from red maple trees at the CCASE experiment (Templer et al. 2017), which is located at Hubbard Brook Experimental Forest in the White Mountain National Forest in Woodstock, NH, USA (43°56 N′, 71°45′ W). The rate of mean annual precipitation is 1400 mm. Snowpack typically lasts from mid-December until mid-April (Campbell et al. 2010). Mean winter air temperature from December to March was −4°C, with annual temperatures ranging from an average minimum of −12.9°C during January (1956–2000) to an average maximum of 23.8°C during July (1956–2000) (Bailey et al. 2003). Other environmental conditions for this site (temperature, relative humidity, and soil moisture, etc.) are archived at: https://hubbardbrook.org/d/hubbard-brook-data-catalog. Soils consist of base-poor spodosols, specifically coarse-loamy Typic Haplorthods (Dahlgren and Driscoll 1994) that developed in glaciofluvial sand and gravel, bedrock is approximately 14 m below the soil surface (Winter et al. 2008).

Experimental design and temperature manipulation

We established CCASE in summer 2012 (Templer et al. 2017), which is at approximately 250 m elevation in a site dominated by red maple trees, which makes up 63.1 ± 6.9% of the basal area with an understory composed mostly of American beech (*Fagus grandifolia*) saplings. The

experiment consists of six 11 × 13.5 m plots, each centered upon three mature red maple trees. Two plots experience growing season soil warming of 5°C above ambient air temperatures (hereafter referred to as *warmed* treatment), two experience growing season soil warming of 5°C coupled with snow removal and FTCs in winter (*warmed + FTC* treatment), and two plots experience ambient temperatures year-round (*reference* treatment). Ambient air temperature is measured from two air temperature sensors (model CSI 215 Campbell Scientific, Logan, Utah, USA), placed adjacent to the *reference* and *warmed* plots, respectively. Due to the cost and infrastructure required to implement the experimental treatments, it was not possible to have more than two plots for each treatment. However, since the plots are large (11 × 13.5 m) enough to encompass large portions of the root systems of the sampled trees, and the two plots within a given treatment are separated by only several meters, we treated the measured canopy responses of each tree as an independent unit for analysis by treatment (n = 4 trees per plot, n = 8 trees per treatment).

To heat the *warmed* and *warmed + FTC* plots, buried heating cables were installed in July 2012 by cutting the soil with a flat shovel to a depth of 10 cm and burying cables. *Reference* plots were similarly cut to mimic cable installation disturbance, but no cable was installed. Belowground mixing of roots across treatments was prevented by installing roofing membrane material to 30 cm depth between plots that had different treatments (Templer et al. 2017). The first soil FTCs were initiated in December 2013, and the experiment is ongoing.

While aboveground warming most realistically mimics projected increases in air temperature, obtaining consistent aboveground warming in tall statured forests is challenging both logistically and financially, thus buried heating cables are commonly used for warming experiments in forest ecosystems (Sanders-DeMott and Templer 2017). Our experimental design precludes an evaluation of how expected changes in air temperature and vapor pressure deficit will influence foliar N and metabolites, but our treatments mimic projected climate change at the plant–soil interface where many unanswered questions about ecosystem responses to climate change are focused. Therefore, we believe that assessing the foliar response to changes in soil temperature and their demonstrated effects on roots, microbial activity, and soil water and nutrients has value for better understanding future tree function.

To monitor and control soil temperature, each plot is equipped with six thermistors (Betatherm type 10K3A1; Betatherm, Shrewsbury, Massachusetts, USA) at 10 cm depth that continuously measures soil temperature and indicate if heating cables need to be turned on or off to maintain +5°C soil temperatures in the *warmed* and *warmed + FTC* plots throughout the snow-free season. Additionally, four volumetric soil moisture sensors (Campbell Scientific CS616; Campbell Scientific) were installed in each plot to measure soil moisture across a 0–30 cm depth profile. For additional technical details on experimental treatments and methodology, refer to (Templer et al. 2017).

We operationally define the onset of the growing season as the time in spring during which daily soil temperature begins to naturally ramp up alongside air temperatures and decreased snow cover (Groffman et al. 2012). Based on this cue, we initiate warming in the *warmed* and *warmed + FTC* treatments and maintained soil temperatures 5°C above ambient soil temperatures throughout the growing season until mean daily air temperatures are below 0°C or December 1 in a given year, whichever occurs first, and then, the heating cables are turned off. In 2015, heating cables were turned on April 21 and off December 1, 2015, making a total duration of growing season treatment equal to 224 d. In 2017, heating cables were turned on April 14 and off on November 15, 2017, making a total growing season treatment duration of 215 d.

Winter soil FTCs are achieved by manually removing snow within 48 h of snowfall to expose the soils to below-freezing air temperatures and induce soil freezing. A 3–5 cm layer of snow is left on the plots each winter to reduce disturbance and maintain the albedo of the forest floor. Once soil temperatures are below −0.5°C (to account for depressed freezing point of soil water due to solute concentration) for 72 h, the heating cables are turned on to thaw soils to 1°C for an additional 72 h, this full cycle comprises one FTC. Heating cables are turned on to initiate FTCs multiple times during the winter to achieve

four successful FTCs each winter, based on those projected for the region by the end of the next century (Campbell et al. 2014).

During each winter, we made weekly measurements of soil frost and snow depth at four locations in each plot from December through April. Frost depth was measured throughout winter using PVC frost tubes (Ricard et al. 1976), which were inserted vertically into soil to a depth of ~50 cm in December 2012. Snow depth was measured within 0.5 m of each frost tube using a meter stick placed vertically into the snowpack. Soil thaw date (defined as the day in which no soil frost remains past 0 cm in the frost tubes of each plot) at the end of the winter was calculated by assuming 3.5 d (mid-point between weekly measurements) past the last date with recorded values of soil frost depth exceeding 0 cm for each frost tube.

Foliage sampling and photosynthesis

Sunlit canopy leaves were collected from four red maple trees in each of the six plots using a shotgun on August 3, 2015, and August 14, 2017, during the second and fourth years of treatment in this study, respectively. From each of the canopy branches shot down, we measured rates of photosynthesis. In our previous measurements that compared intact to excised red maple leaves, we found that rates of photosynthesis did not change for at least ten minutes following the excision of branches from trees. Therefore, to minimize potential artifacts associated with measuring photosynthesis on excised branches in this study, we submerged the ends of excised branch shoots in water, cut 2–3 cm above the break to reduce the impact of embolisms on gas exchange rates, and measured rates of photosynthesis within 5–7 min of excision from the trees. We also allowed leaves to stabilize for approximately 1–2 min and then made five measurements of photosynthesis and conductance at 15-s intervals for 1 min and utilized the mean of these five measurements (Harrison et al. 2020*a*).

Photosynthetic measurements were made on three leaves per tree with a portable photosynthesis system (LI-6400; LICOR, Lincoln, Nebraska, USA). Photosynthetic rates were measured at saturating light levels (1000 μmol photons·m^{-2}·s^{-1}) to reflect the high light conditions at the top of the canopy using a built-in LED light source, the carbon dioxide concentration in the reference analyzer was set to 400 μmol/mol, and relative humidity and temperature in the sample chamber were equilibrated to ambient levels.

Leaves used for photosynthesis measurements were transported on ice and analyzed for fresh mass and leaf area within 24 h of excision. Leaves were then dried to constant mass at 60°C and dry mass was recorded. Three dried leaves from each tree (n = 8 trees per treatment) with petioles intact were homogenized with a mortar and pestle for analysis of N and C concentration via flash combustion (Thermoquest NC 2500 autoanalyzer; Thermo Scientific, Waltham, Massachusetts, USA).

We processed leaves from the same branch that was used for photosynthesis to measure dilute acid-soluble (5% perchloric acid, PCA, also referred to as free or exchangeable) PAs, AAs, and inorganic elements. Briefly, immediately after sampling, a pool of ~0.3 g of 6 mm leaf discs were collected from 2 to 3 leaves per tree using a paper puncher. Approximately 0.2 g of foliage was placed in pre-weighed 2 mL microfuge tubes with 1 mL of cold 5% PCA for extraction of AAs, PAs, and inorganic elements. The remainder of the pooled leaf discs was placed into separate microfuge tubes to be used for chlorophyll, soluble protein, and sugar analyses. Samples were kept on ice during transport to the laboratory where they were frozen at −20°C until further analyses. Samples in 5% PCA were frozen and thawed three times before various analyses to disrupt cell membranes and release cell contents following Minocha et al. (1994).

Foliar metabolite analyses

Free PAs and AAs were dansylated and quantified via reverse-phase HPLC per Minocha and Long (2004) with minor modifications described in (Majumdar et al. 2018). Polyamines and AAs were analyzed, and the data were processed using Perkin Elmer (Waltham, Massachusetts, USA) TotalChrom software (version 6.2.1).

Using 2 leaf discs, chlorophyll a, chlorophyll b, and total carotenoids were extracted in 95% ethanol and analyzed according to Minocha et al. (2009) with a Hitachi U2010 spectrophotometer (Hitachi Ltd., Tokyo, Japan; spectral bandwidth 2 nm, wavelength accuracy of +0.3 nm, wavelength setting reproducibility of ±0.1 nm; with Hitachi UV Solutions software version 2.0) by

scanning absorbance in the range of 350–710 nm. Equations from Lichtenthaler (1987) were used for quantification of total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids.

Soluble protein was extracted from 50 mg of leaf tissue in 500 μL of Tris extraction buffer by 3 freeze–thaw cycles (Minocha et al. 2019). The supernatant was quantified for soluble protein concentration per Bradford (1976) method using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, California, USA). Absorbance was recorded at 595 nm with a Hitachi U2010 spectrophotometer (Hitachi Ltd., Tokyo, Japan; spectral bandwidth 2 nm, wavelength accuracy of +0.3 nm, wavelength setting reproducibility of ±0.1 nm), and data were analyzed with Hitachi UV Solutions software version 2.0.

Soluble sugars were extracted from 50 mg of leaf tissue and analyzed, by the method described here. Briefly, soluble sugars were extracted in 80% ethanol at 65°C for 30 min. The extract was then filtered using a 0.45 μm nylon syringe filter. The sugar profiles were determined using reverse-phase HPLC coupled with a refractive index detector (HPLC-RID; Shimadzu Scientific Instruments Inc, Columbia, Maryland, USA). For sugar separation, an isocratic mobile phase of 80% acetonitrile at a 2 mL/min flow rate and a Luna NH_2 column (250 × 4.6 mm, 5 μm, Phenomenex Inc, Torrance, California, USA) was used. Each sugar was quantified using a 5-point external standard curve (0.125–2 mg/mL). The chromatographs were analyzed, and the data were processed using Perkin Elmer TotalChrom software (version 6.2.1). To quantify glucose+galactose (the two peaks did not separate), the areas and concentrations of each were added together to create a combined standard curve.

Analysis of soluble/exchangeable inorganic ions (defined as the fraction of total ions within cells that is extractable in 5% PCA), including calcium (Ca), potassium (K), phosphorous (P), magnesium (Mg), manganese (Mn), aluminum (Al), iron (Fe), and zinc (Zn), was conducted using a simultaneous axial Inductively Coupled Plasma Optical Emission Spectrophotometer (Vista CCD, Varian, Palo Alto, CA, USA) and Vista Pro software (version 4.0). Supernatants of 3x frozen and thawed samples in PCA were diluted (100×) with distilled deionized water for estimation of soluble ions. Analyses and quantitation of the elements were conducted using a simultaneous axial Inductively Coupled Plasma Optical Emission Spectrophotometer (Vista CCD, Varian, Palo Alto, California, USA) and Vista Pro software (version 4.0) per EPA SW-846 compendium, method 6010.

Statistical analysis

All statistical analyses were conducted in R version 3.1.2. The effects of experimental treatment on growing season soil temperature and soil moisture, winter snow depth, soil frost depth, and minimum soil temperature, and rates of photosynthesis within each year (2015 and 2017) were assessed with linear mixed-effects models with the plot as the random effect and treatment as the fixed effect using the package "nlme" in R (Pinheiro et al. 2016). Chlorophyll, foliar nutrients, PAs, and AAs were similarly analyzed using linear mixed-effects models. Some data were not normally distributed, and we, therefore, specified the data to have a gamma distribution in the models. Comparisons of all analytes in foliage were also compared between *reference* trees in 2015 and 2017 using linear mixed-effects models with the plot as the random effect and year as the fixed effect. Post hoc pairwise comparisons among treatments within each year (2015 and 2017) were calculated using a general linear model using the package "lsmeans" in R (Lenth 2016).

Most field studies conducted using mature trees that include measurements of foliar metabolites utilize a sample size of 10–20 trees per treatment (Minocha et al. 2010, 2015, 2019). Tree to tree variation due to each tree's unique genetic composition, microsites variability within each plot, and plot to plot soil chemistry variability add dimensions of variability to the highly sensitive and responsive metabolite data, thus demanding a much larger sample size relative to other variables being studied. A larger number of plots and samples would have strengthened our experimental design, but these were not feasible due to logistical and financial constraints of our experimental design and infrastructure. For this reason, while *P*-values equal to and <0.05 are typically considered statistically significant, in this study, we considered differences where the *P*-values were between 0.05 and 0.1 to still hold meaning for this dataset (Minocha et al. 2021).

Results

Snow depth, soil frost, and soil temperature

Since December 2013, temperature of the soils in the *warmed* and *warmed + FTC* plots have been elevated by 5°C throughout the growing season and multiple soil freeze/thaw cycles have been induced in winter in the *warmed + FTC* plots. The winter of 2017 was milder than 2015. In the winter of 2015, four soil FTCs were applied in the *warmed + FTC* plots, while in 2017, only one soil FTC was applied because soils did not freeze below −0.5°C for 72 h for additional FTCs (Table 1). Soil temperatures in the *warmed + FTC* plots were below freezing for a shorter period in 2017 than 2015 winter; soils experienced approximately 79 ± 28 h of soil freezing to 10 cm depth in 2017 compared to 289 ± 82.5 h in 2015 (Table 1). The *warmed + FTC* plots had significantly less maximum snow depth, greater maximum soil frost depth, and lower minimum soil temperatures than the *reference* and *warmed* plots in both years (Table 1). Air temperatures were warmer in the winter and growing season of 2017 (−4.1 ± 0.08°C and 13.4 ± 0.07°C, respectively) than 2015 (−6.8 ± 0.09 and 12.9 ± 0.07°C, respectively).

Photosynthesis and foliar N and C

In 2015, photosynthetic rates were elevated by approximately 50% in the *warmed* relative to the *warmed + FTC* treatment and were significantly different from the other two treatments. In 2017, although photosynthetic rates were elevated by approximately 30% in the *warmed + FTC* treatment relative to the *warmed* and *reference* treatments, they were not significantly different (Table 2). Across all treatments, rates of photosynthesis were higher in 2017 than in 2015.

Foliar N concentrations in *warmed + FTC* treatment were elevated compared to the *reference* treatment in both 2015 and 2017, but only elevated in the *warmed* treatment relative to *reference* in 2017 (Fig. 1A). With higher N concentrations, foliage in the *warmed* and *warmed + FTC* treatments had lower C:N ratios than the *reference* plots, although significant differences among treatments were observed in 2017 only (Fig. 1B).

Table 1. Values of snow depth, frost depth, and minimum soil temperature are mean ± standard error (n = 4 per plot).

Environmental variable	Reference	Warmed	Warmed + FTC
2015			
Snow depth (cm)***	55.3 ± 0.8[a]	51.3 ± 1.1[a]	18.6 ± 0.7[b]
Frost depth (cm)**	14.0 ± 1.5[a]	15.2 ± 1.0[a]	20.1 ± 2.9[b]
Minimum soil temperature (°C)**	−0.14 ± 0.01[a]	−0.23 ± 0.03[a]	−2.12 ± 0.1[b]
Number of soil FTCs	0	0	4
Below freezing soil temperature (hours)	0	0	289 ± 82.5
Date of spring soil thaw	April 4, 2015	April 4, 2015	March 31, 2015
Winter air temperature (°C)	−6.8 ± 0.09		
Growing season air temperature (°C)	12.9 ± 0.07		
Precipitation (mm/yr)	1299.4		
2017			
Snow depth (cm)***	29.68 ± 0.32[a]	24.80 ± 1.24[a]	2.99 ± 0.06[b]
Frost depth (cm)***	3.02 ± 0.72[a]	2.61 ± 0.28[a]	10.74 ± 0.99[b]
Minimum soil temperature (°C)***	0.09 ± 0.006[a]	0.36 ± 0.21[a]	−2.36 ± 0.12[b]
Number of soil FTCs	0	0	1
Below freezing soil temperature (hours)	0	0	79 ± 28
Date of spring soil thaw	April 12, 2017	April 12, 2017	April 6, 2015
Winter air temperature (°C)	−4.1 ± 0.08		
Growing season air temperature (°C)	13.4 ± 0.07		
Precipitation (mm/yr)	1528.2		

Notes: Distinct letters within a row indicate statistically significant differences among treatments ($P \leq 0.10$). Asterisks represent statistical differences in reference plots between years (**$P \leq 0.05$, ***$P \leq 0.001$). Growing season, winter air temperature and annual precipitation rates for each year for Hubbard Brook Experimental Forest are also presented.

Table 2. Photosynthetic rate (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$) by treatment for 2015 ($P = 0.004$) and 2017 ($P = 0.08$).

Year	Reference	Warmed	Warmed + FTC
2015	$3.88 \pm 0.42^{a**}$	4.68 ± 0.69^{b}	2.24 ± 0.08^{a}
2017	6.63 ± 1.12^{a}	7.19 ± 0.66^{ab}	9.33 ± 0.88^{b}

Notes: These measurements were taken at the time of foliar sampling in August of each year. Distinct letters within a row represent statistically significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical differences in reference plots between years ($^{**}P \leq 0.05$). Data are means ± SEs ($n = 8$ trees per treatment).

Foliar metabolites

Soil warming treatments generally had a positive effect on foliar PA concentrations. Specifically, foliar Put was elevated in 2015 in the *warmed + FTC* treatment relative to the *reference* and *warmed* treatments (Fig. 2A). Foliar concentrations of Spd did not differ with treatment, though there was a trend of elevated Spd with *warmed* and *warmed + FTC* treatments (Fig. 2B). Spermine was significantly higher in both the *warmed* and *warmed + FTC* treatments relative to the *reference* treatment in 2015, and though the trend was similar in 2017, the variability was greater, and the differences were not significant (Fig. 2C).

The concentrations of several foliar free AAs increased in the *warmed* or *warmed + FTC* treatments. Glutamic acid (Glu) did not show significant changes with warming (Fig. 3A). In 2015, foliar alanine (Ala) was elevated in both the *warmed* and *warmed + FTC* treatments relative to the *reference* plots, while in 2017, Ala was elevated only in the *warmed + FTC* treatment (Fig. 3B). In both years, foliar GABA was elevated in the *warmed + FTC* treatment relative to the *reference*, while GABA in the warmed treatment foliage was not significantly different from either the *reference* or *warmed + FTC* treatments (Fig. 3C). Foliar Pro, Leu, Val, and Ile were elevated in only the *warmed + FTC* treatment compared to the *reference* treatment in 2015 with no differences among treatments in 2017 (Fig. 3D, E, I, J). In 2015, foliar histidine (His) was elevated in the *warmed* treatment compared to the *reference*, but not compared to the *warmed + FTC* treatment. There were no significant differences in His concentrations in 2017 (Fig. 3F). In 2017, aspartic acid (Asp) was significantly elevated and methionine (Met) decreased in the *warmed + FTC* treatments relative to the *reference*, but not compared to the *warmed* treatment; neither could be quantified in 2015 (Fig. 3G, H). No differences in Val or Ile were observed among

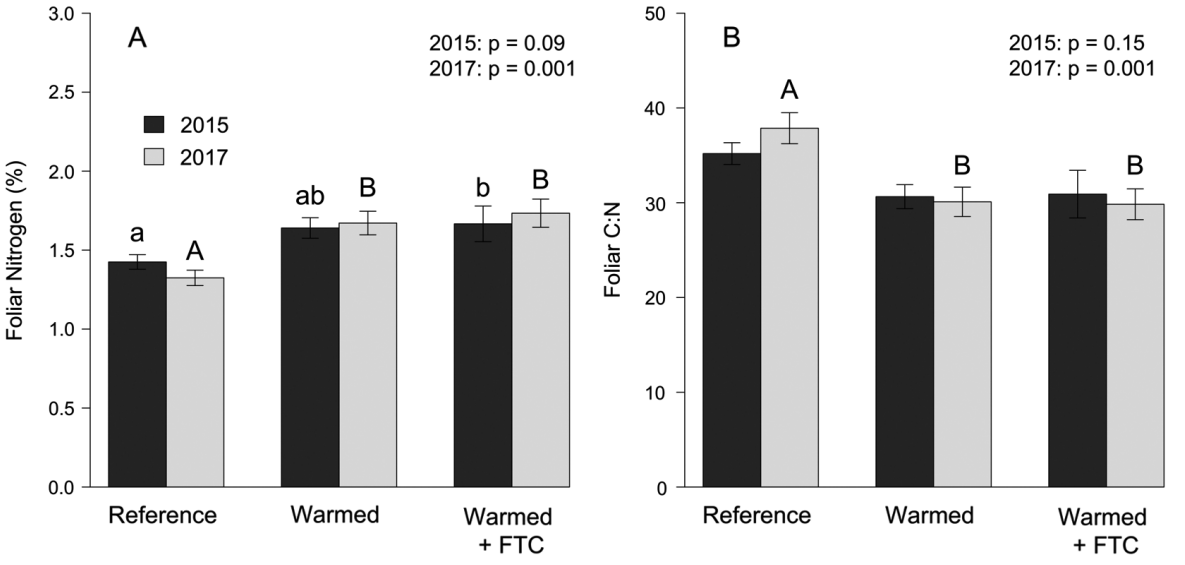

Fig. 1. Foliar N (A) and C:N (B) by treatment in August of 2015 and 2017. Data are means ± SEs ($n = 8$). Small letters (2015) and capital letters (2017) represent significant differences among treatments ($P \leq 0.1$).

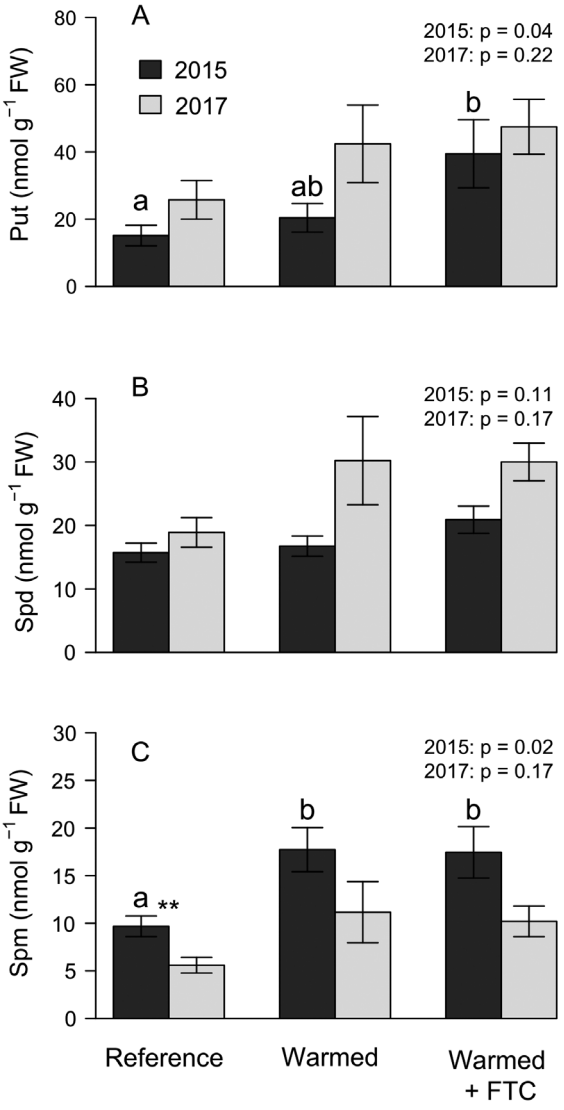

Fig. 2. Foliar free polyamines (Put (A), Spd (B), and Spm (C)) by treatment in August of 2015 and 2017. Data are means ± SEs (n = 8). Small letters (2015) and capital letters (2017) represent significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical differences in reference treatment between years ($^{**}P \leq 0.05$).

treatments in 2017 (Fig. 3I, J). Phenylalanine (Phe) was elevated in the *warmed* relative only to the *reference* treatment in 2017, but not in 2015 (Fig. 3K). Tryptophan (Trp) showed no changes with treatments (Fig. 3L).

Total foliar chlorophyll concentration was elevated in the *warmed + FTC* treatment relative to the *reference* plots in 2015 and elevated in the *warmed* treatment in 2017 (Fig. 4A). Chlorophyll a and b concentrations were elevated in the *warmed + FTC* treatment relative only to the *reference* treatment for both 2015 and 2017 (Fig. 4B, C), while the ratio of chlorophyll a:b was highest in the *reference* treatment in 2015 and similar among all treatments in 2017 (Fig. 4D). Total carotenoids were elevated with both the *warmed* and *warmed + FTC* treatments relative to the *reference* in 2017 but were not measured in 2015 (Fig. 4E). Soluble protein concentrations did not differ among treatments in either year (Fig. 4F) but were higher in all treatments in 2017. While there were no significant differences in fructose and glucose+galactose among treatments for either of the two years, sucrose was significantly higher with warming in 2015 (Table 3), but not different in 2017.

Relative to the *reference* plots, the *warmed* and *warmed + FTC* treatments both had elevated foliar P concentrations in 2015 and 2017 (Fig. 5B). In the *warmed* treatment, K was elevated relative to the *reference* treatment in 2015 and 2017, Zn was similarly elevated, but only in 2017 (Fig. 5A, C). Compared to the *reference* treatment, foliar Mg concentration was lower in both the *warmed* and *warmed + FTC* treatments, but only in 2017 (Fig. 5D). Foliar Ca and Mn were not different among treatments in either year (Fig. 5E, F).

Comparison of reference plots between 2015 and 2017

Though differences in foliar N and C:N occurred among treatments in both 2015 and 2017, neither was significantly different between years within the *reference* treatment (Fig. 1). However, PA concentrations differed between years in the *reference* plots. Spermine significantly decreased from 2015 to 2017, while Put and Spd exhibited a non-significant trend of increasing between years (Fig. 2). Some AAs increased from 2015 to 2017, including GABA, Pro, valine (Val), and tryptophan (Trp), while concentrations Glu and Phe decreased in *reference* foliage from 2015 to 2017 (Fig. 3). Though not significant, there were trends of increased concentrations of Ala, leucine (Leu), and isoleucine (Ile) from 2015 to 2017 (Figs. 2, 3). Total chlorophyll and chlorophyll a and b decreased significantly, while

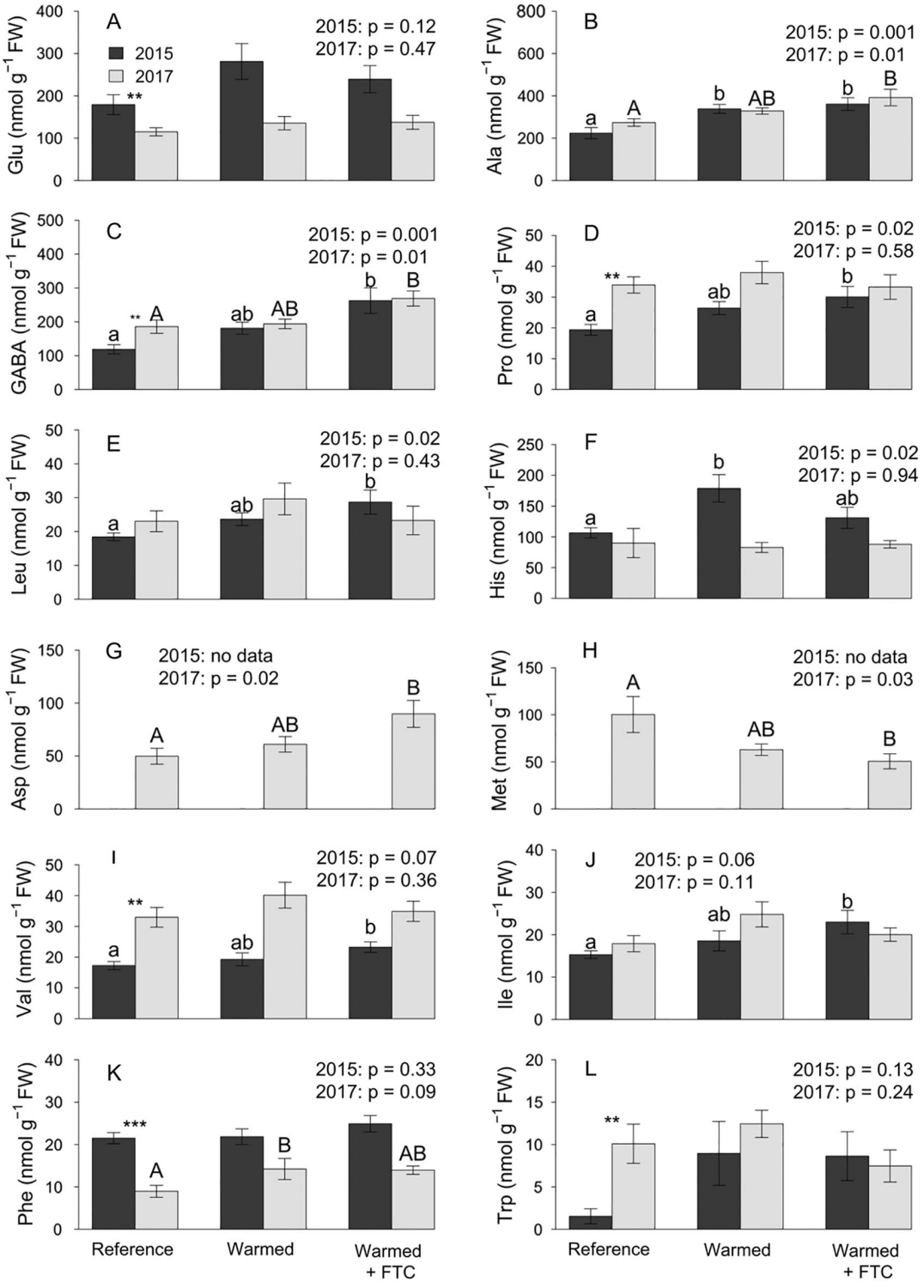

Fig. 3. Foliar free amino acids (Glu (A), Ala (B), GABA (C), Pro (D), Leu (E), His (F), Asp (G), Met (H), Val (I), Ile (J), Phe (K), and Trp (L)) by treatment in August of 2015 and 2017. Data are means ± SEs (n = 8). Small letters (2015) and capital letters (2017) represent significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical differences in reference treatment between years ($^{**}P \leq 0.05$, $^{***}P \leq 0.001$).

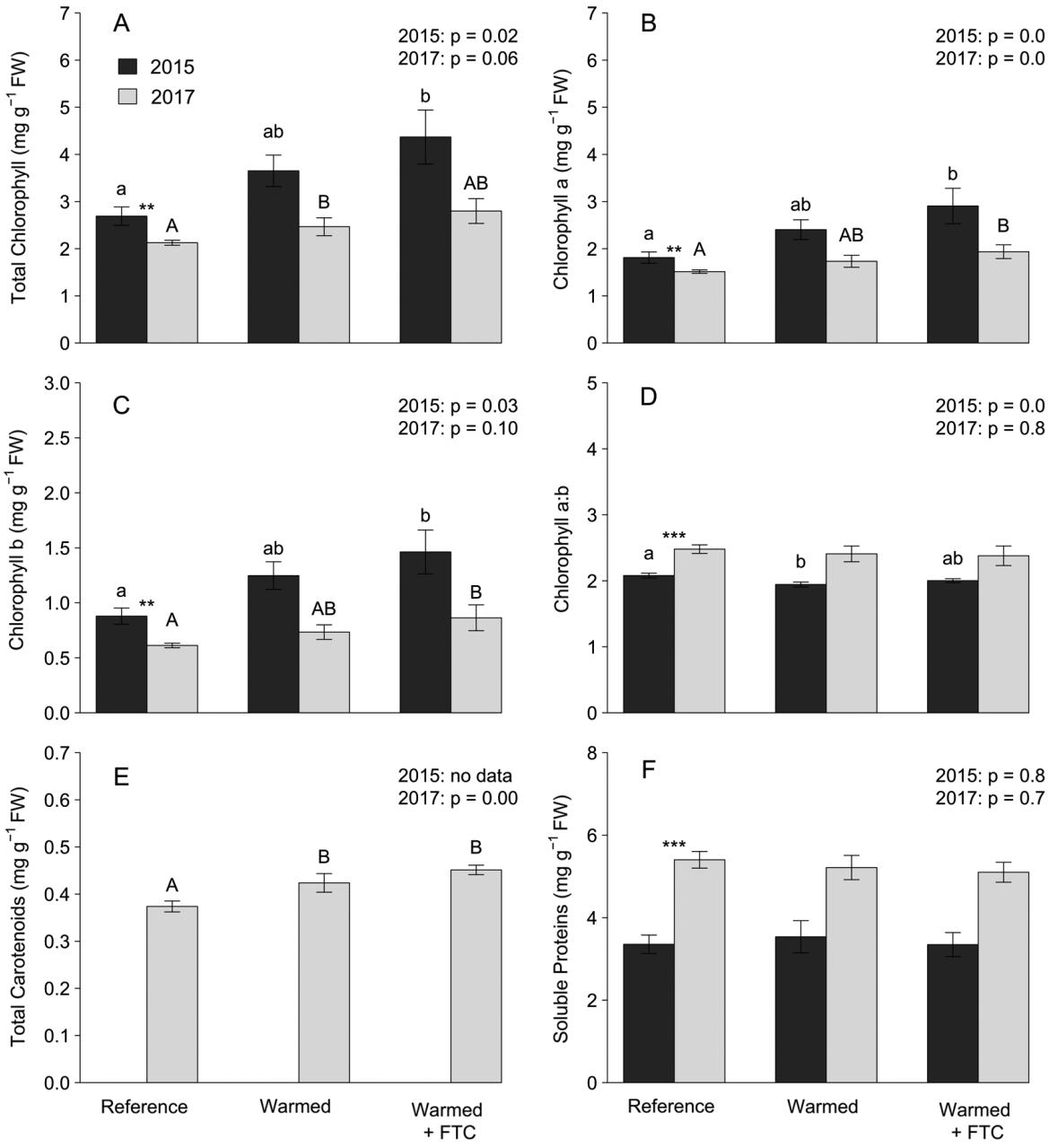

Fig. 4. Total foliar chlorophyll (A), chlorophyll a (B), chlorophyll b (C), chlorophyll a:b (D), carotenoids, (E) and soluble proteins (F) concentrations by treatment in August of 2015 and 2017. Data are means ± SEs (n = 8). Small letters (2015) and capital letters (2017) represent significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical differences in reference treatment between years ($^{**}P \leq 0.05$, $^{***}P \leq 0.001$).

chlorophyll a:b ratios and soluble protein concentrations increased from 2015 to 2017 in *reference* foliage (Fig. 4). Of the simple sugars, sucrose was significantly higher in 2015 relative to 2017 (Table 3). Foliar concentrations of exchangeable P, Zn, Mg, Ca, and Mn and increased in the *reference* treatment between 2015 and 2017 (Fig. 5).

Table 3. Soluble sugars (mg/g FW) by treatment for 2015 (P = 0.06, 0.44, and 0.55) and 2017 (P = 0.22, 0.17, and 0.19) for sucrose, fructose, and glucose + galactose, respectively.

Soluble sugars (mg/g FW)	Year	Control	Warmed	Warmed + FTC
Sucrose	2015	17.44 ± 3.68^{a**}	36.32 ± 7.46^{b}	23.09 ± 4.12^{a}
	2017	5.81 ± 1.84	15.70 ± 5.36	13.73 ± 4.56
Fructose	2015	128.41 ± 9.53	155.26 ± 16.71	148.39 ± 17.88
	2017	152.68 ± 15.10	149.28 ± 13.63	193.64 ± 22.69
Glucose+Galactose	2015	153.54 ± 12.80	179.86 ± 18.98	171.28 ± 18.56

Notes: These measurements were taken at the time of foliar sampling in August of each year. Distinct letters within a row represent statistically significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical differences in reference plots between years (**$P \leq 0.05$). Data are means ± SEs (n = 8 trees per treatment).

Discussion

We found that growing season warming generally increased concentrations of foliar sucrose, N, K, Mg, P, and chlorophyll along with rates of photosynthesis relative to *reference* plots, but that the effect of soil warming on these responses was not consistent across years for the same treatment and sometimes between treatments as well. These data suggest significant shifts in foliar primary (amino acids, sucrose) and secondary (polyamines, carotenoids) metabolism in northern hardwood forests as a result of projected climate change for this region. The simultaneous changes in foliar nutrition, cellular C and N metabolites, and critical processes such as photosynthesis caused by exposure to environmental stress as demonstrated in this study or via genetic manipulation once again points to the well-known facts about the interdependence of these variables for maintaining essential cellular functions; stress-induced change in one central metabolite may cause an imbalance in all its connected pathways eventually affect the overall functionality and growth rates of the organism. For example, in transgenic hybrid poplar cultures, the overexpression of a single gene involved in the synthesis of putrescine resulted in changes in nutrient levels, C and N metabolism that included changes in organic acids, sugars, amino acids, polyamines, and overall stress responses including oxidative stress (Bhatnagar et al. 2002, Page et al. 2007, Mohapatra et al. 2009, 2010*a*, *b*). Though the long-term functional consequences of these metabolic shifts for ecosystems are yet to be identified, our results indicate that growing season warming and increasing winter soil FTCs will have distinct effects on foliage that would not be evident from examining climate change in one season alone.

We acknowledge that the trees might have expressed a response to the stress experienced during the first year of exposure to treatments in the winter of 2013/2014 that we did not directly measure and that these trees possibly also gained partial ecological stress memory for these specific treatments (Walter et al. 2013, Hilker and Schmülling 2019). Environmentally induced epigenetic changes have been shown to enable plants to remember past environmental interactions (Latzel et al. 2016). Epigenetic changes are the basis for changes in gene expression and metabolism leading to plasticity in forest trees that help them adjust rapidly to a constantly changing environment (Madhusudhan 2015, Gömöry et al. 2017). Therefore, we do realize that the observations in 2015 and 2017 may not be the over-compensated responses similar to those that may have occurred earlier in the study (2014), but rather are adaptive responses to the continued treatments. Since these questions cannot be answered with the present study, especially with its short duration, the continued follow-up and additional longer term studies can be illuminating. Also, many other climate change experiments such as ours aim to examine the long-term effects of changes in temperature, including those in temperate forests of central Massachusetts (Melillo et al. 2017, Chen et al. 2019).

Effects of treatments on foliar metabolism and nutrition

The observed similarities in foliar N concentrations between plots experiencing growing season warming with and without winter soil FTCs may suggest that increased soil N availability with

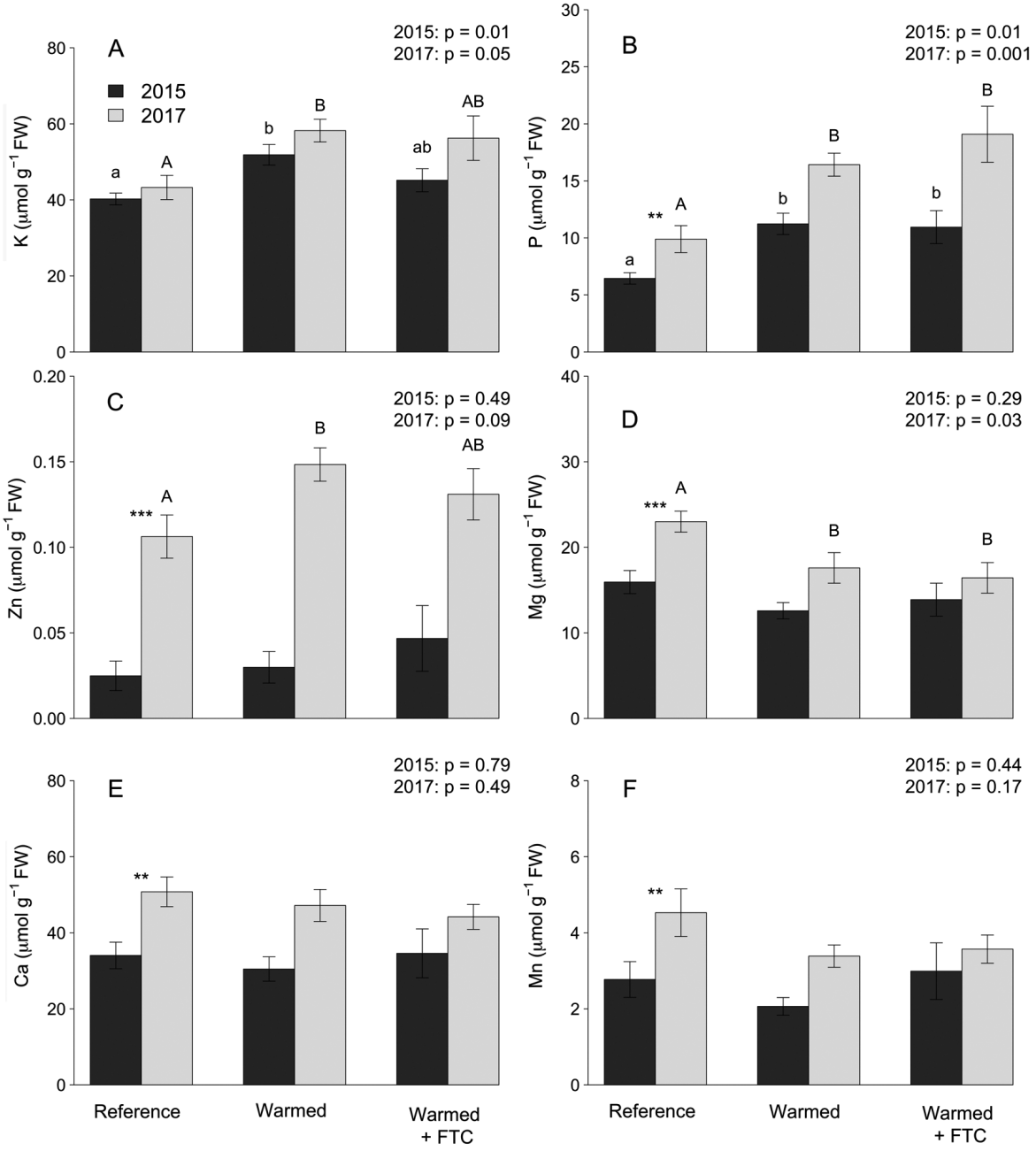

Fig. 5. Foliar soluble element (K (A), P (B), Zn (C), Mg (D), Ca (E), and Mn (F)) concentrations by treatment in August of 2015 and 2017. Data are means ± SEs (n = 8). Small letters (2015) and capital letters (2017) represent significant differences among treatments ($P \leq 0.1$). Asterisks represent statistical treatment between years ($^{**}P \leq 0.05$, $^{***}P \leq 0.001$).

growing season soil warming had a larger impact on foliar N concentrations than winter soil FTCs (as reported from this experiment by Harrison et al. 2020*b*). However, an alternative explanation for similar foliar N in the *warmed* plots with and without FTCs is that foliar N is differentially

allocated by trees when exposed to growing season warming and winter soil FTCs. For example, higher accumulation of several N salvaging AAs and PAs in the *warmed + FTC* treatment relative to *warmed* treatment alone may make up for decreased root N uptake in the *warmed + FTC* treatment relative to both the *warmed* and *reference* treatments (Sanders-DeMott et al. 2018). Nitrogenous compounds (PAs and AAs) are commonly formed in response to increased soil N availability (Minocha et al. 2014, 2015, 2019) and maybe a metabolically simple way for trees to store excess N that can then be directed toward C assimilation activities and/or protection from stress, depending upon the local conditions in which trees are growing.

The effects of growing season warming with or without winter FTCs on foliar Spm reaffirms the role of PAs in tree response to changing soil temperatures. Since Spm is an indicator of stress tolerance (Seifi and Shelp 2019 and references therein), elevated levels of endogenous Spm in foliage in the *warmed* plots indicate stress tolerance was induced in those plots by assimilation of toxic ammonia into spermine. On the other hand, a significant increase in foliar Put within the *warmed + FTC* treatment in 2015 may have been caused by soil FTC-induced stress. Thus, an increase in Spm concentration in the *warmed* treatment, and additionally, PAs Put and Spm in the *warmed + FTC* treatment indicates the dual functions of PAs where increased N availability due to growing season warming led to possible positive effects on tree growth, while stress from soil FTCs led to an accumulation of Put and Spm for stress tolerance.

The increase in foliar AAs in the *warmed* plots (Ala, His, and Phe) and *warmed + FTC* plots (Asp, Ala, Pro, GABA, Val, Ile, Iso) indicated that the addition of FTCs had a differential effect on the partitioning of N within foliar cells. Amino acids have been reported to increase with both increased soil N availability (Minocha et al. 2003, 2015, 2019) and stress (Rai 2002, Mekonnen et al. 2016). The additional increase in the concentrations of AAs along with an increase in PAs (Put and Spm) with *warmed + FTC* treatment relative to *warmed* alone is compelling evidence of elevated foliar N metabolism because of winter soil FTC-caused root damage (Sanders-DeMott et al. 2018).

Cells can adapt and prime themselves after previous exposure to stressors (Freitas et al. 2019), thereby trees in *warmed + FTC* treatment may be adapting to damage caused by previous winter soil FTCs (stress memory), so later sampling (in 2017) yielded less dramatic treatment effects compared to 2015. It is also possible that the reduced effects of FTCs on foliar AAs in the winter of 2017 compared to 2015 are due to the milder temperatures in the 2017 winter or a combination of both factors.

The increase in chlorophyll, carotenoid, and sucrose concentrations together with elevated rates of photosynthesis observed in both the *warmed* and *warmed + FTC* treatments indicate increased photosynthetic capacity in both warmed treatments. Li et al. (2020) also reported an increase in rates of photosynthesis with warming in four subtropical montane tree species. Similar to our findings, total chlorophyll concentrations were shown to increase alongside foliar N in maple, chestnut, and beech trees (Kira et al. 2015) and with increased soil N availability in seedlings of various Mediterranean forest tree species (Oliet et al. 2013). The overall lower concentrations of soluble sugars in 2017 when the photosynthetic rate was higher due to warmer conditions also indicated higher metabolic activity this year (Table 3). Hartmann and Trumbore (2016) rightly emphasized that concentrations of soluble sugars in tree organs cannot be simply interpreted as indicators of C storage capacity, rather they are the difference between the supply and demand for C currency compounds at a given moment.

Temperate forests have been shown to have N and P colimitation (Vadeboncoeur 2010), but growing season warming increases rates of net mineralization and availability of N for trees that are not offset by soil FTCs in winter (Harrison et al. 2020*b*). The increased foliar P concentrations with soil warming may indicate that warming either increased soil P availability or root uptake of P or P accumulation in leaves, or a combination thereof. Sanders-DeMott et al. (2018) found that the *warmed + FTC* treatment increased root damage and decreased root N uptake relative to both the *reference* and *warmed* treatments, yet we observed that even with fine root damage, trees in both treatments had greater foliar P concentrations than trees in the *reference* plots. The positive effect of soil N availability on foliar P resorption has recently been

reported by See et al. (2015). Phosphorus is one of the main drivers of energy transfer via adenosine triphosphate (ATP) for enzymatic reactions. Being a constituent of DNA and RNA that are needed for protein synthesis and reproduction (Burke and Lupták 2018). However, the long-term effects of these changes on productivity at this site are yet to be seen.

It can be speculated that the increased concentrations of foliar K in the *warmed* treatment may have contributed to the greater rates of photosynthesis since K plays a critical role in plant growth and metabolism by affecting most of the biochemical and physiological processes (Wang et al. 2013). In addition, the decrease in foliar Mg in both of the *warmed* treatments impaired may have impaired the export of sucrose from cells. This might have led to limited cellular metabolism causing an accumulation of AAs similar to the earlier report by Fischer et al. (1998) revealing Mg deficiency caused accumulation of AAs and sugars in cells mainly due to the lack of transport/utilization of metabolites downstream. Because foliar Mg concentrations can be modulated in response to the changing demands on soil Mg (Ruan et al. 2012), it is possible that increased microbial metabolic activity with soil warming in both the *warmed* and *warmed + FTC* treatments (Schindlbacher et al. 2011) simultaneously increased microbial demand for some nutrients, which in turn decreased their amount available in the soil and uptake by the trees.

Comparison of reference plots in 2015 and 2017

Several changes in foliar nutrition and metabolism occurred between 2015 and 2017 in the *reference* plots, which likely were the result of differences in environmental conditions between the two years. Leaf metabolic processes are dynamic and highly responsive to micro-environmental changes (Minocha et al. 2014). The winter of 2017 was milder than 2015, with warmer air and soil temperatures, a smaller snowpack, and fewer experimentally induced soil FTCs. Annual precipitation was greater in 2017 (1528 mm/yr) than in 2015 (1299 mm/yr) (Hubbard Brook Watershed Ecosystem Record 2021). These differences in temperature and precipitation between the two years likely explain some of the differences observed in the *reference* trees across the two years. Out of the 27 variables analyzed in 2015 and again in 2017, 18 were significantly different for the *reference* treatments between the two years. While some metabolites (Spm, Glu, Phe, total chlorophyll, chlorophyll a and b, and sucrose) were lower in 2017 compared to 2015, others (Pro, GABA, Val, ratio of chlorophyll a:b, soluble proteins, rate of photosynthesis) were higher. Inorganic elements (Ca, Mg, Mn, Zn, and P) were also higher in the foliage of 2017. These differences across years demonstrate the need to conduct long-term studies in order to effectively evaluate the impacts of climate change on forest health and functions.

Conclusions

Our results indicate that growing season soil warming had multiple positive effects on foliar nutrients and cellular metabolism and that winter FTCs, in addition to growing season soil warming, created additional responses leading to changes in foliar C and N partitioning. While we found positive effects of growing season warming on K, Mg, P, and rates of photosynthesis in red maple, we also observed increased stress-associated AAs and reduced photosynthetic capacity without further change in N following the winter with more FTCs. The direct implications of nutritional and metabolic shifts with climate change are not yet understood, though previously published literature has shown that these changes have the potential to significantly alter forest nutrient cycling and C sequestration in seasonally snow-covered ecosystems, which are increasingly experiencing the effects of winter climate change. More widespread monitoring of the impacts of climate change on foliar metabolites may help identify subtle changes in tree functions that may have important implications for forest nutrient, N and C cycling but are difficult to capture with coarser estimates of foliar chemistry. Continued observations for a longer period and an increase in sampling times per growing season might help deepen our understanding of the metabolic processes that control tree functions under these conditions.

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PHT conceived of and established the experiment. RSD, JLH, SL, and RM collected samples in the field. SL,

JLH, and RSD analyzed samples in the laboratory. JLH, MB, SL, and RSD analyzed the data. All authors contributed to the writing of the manuscript. We thank Frank Bowles, Stephanie Juice, and Andrew Reinmann for their invaluable contribution to the establishment and execution of the CCASE experiment. Laura Sofen, Amy Werner, Laura Clerx, Jonathan Gewirtzman, Brendan Leonardi, and Gabe Winant helped to maintain CCASE. We thank Cam McIntire, Adam Wild, Maroua Jabouri, and Katie Jennings for their help collecting foliage. Laura Sofen, and Jonathan Gewirtzman helped to maintain CCASE and assisted with laboratory and fieldwork. We are grateful to the staff at the Hubbard Brook Experimental Forest including Amey Bailey, Scott Bailey, Nick Grant, Ian Halm, Brendan Leonardi, Mary Martin, and Tammy Wooster who aided with site establishment, data collection, and data management. We also thank USDA Forest Service, NRS for providing support for all the metabolic analyses conducted in Durham, NH. This research was supported by an NSF Long Term Ecological Research (LTER) Grant to Hubbard Brook (NSF 1114804 and 1637685) and an NSF CAREER grant to PHT (NSF DEB1149929). RSD was supported by NSF DGE0947950, a Boston University (BU) Dean's Fellowship, and the BU Program in Biogeoscience. Jamie Harrison was supported by a BU Dean's Fellowship. Megan Blagden was supported by a BU Undergraduate Research Opportunity Program fellowship. This manuscript is a contribution to the Hubbard Brook Ecosystem Study. Hubbard Brook is part of the LTER network, which is supported by the NSF. The Hubbard Brook Experimental Forest is operated and maintained by the USDA Forest Service, Northern Research Station, Madison, WI. The authors declare no conflict of interest.

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Data Availability

Data are available from the Environmental Data Initiative: https://doi.org/10.6073/pasta/f1c686538b00ca5a42f748dfe9ec2d0d