Species-specific combined effects of heatwaves, drought, and elevated [CO_2] on cellular metabolism in the foliage *Picea abies* and *Betula papyrifera*

Maegan Gagne[1], Rakesh Minocha[2], Stephanie Long[3], and Katherine McCulloh[4]

[1]University of Wisconsin- Madison
[2]Affiliation not available
[3]USDA Forest Service Northern Research Station
[4]University of Wisconsin-Madison

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Abstract

In recent decades, the rising severity of summer heatwaves has increased the co-occurrence of heat and drought stress leading to forest mortality and to reductions in crop yield. Plant responses to this combined stress can be unique from the response to either independent stress, yet few studies have investigated these responses in tree species. Our work examines adjustment of several primary metabolites and polyamine (stress indicating secondary metabolites) in paper birch and white spruce subjected to two seasons of repeated heatwaves, drought, and elevated CO_2. Our objectives were to determine if the metabolic adjustments in response to heatwave+drought stress are: 1) unique or shared with either individual stress; 2) greater in birch compared to spruce; and 3) carried over into the following season. Our data show that white spruce displayed many metabolic responses that were unique to the combined stress, especially in the first year, while paper birch displayed few. Further, the unique responses in spruce seen in the first season stress exposure did not carry into the following season indicating possible stress memory. Our data highlights the importance of considering species-specific responses to multiple stressors when making predictions about forest response to future climate scenarios.

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Maegan A. Gagne[1], Rakesh Minocha[2], Stephanie Long[2], Katherine A. McCulloh[1]

[1]Department of Botany, University of Wisconsin-Madison, Madison, Wisconsin USA

[2]US Forest Service, Northern Research Station, Durham, NH, USA

Correspondence to:magagne@wisc.edu, Forest Service Northern Research Station 271 Mast Road Durham, NH 03824

Abstract

In recent decades, the rising severity of summer heatwaves has increased the co-occurrence of heat and drought stress leading to forest mortality and to reductions in crop yield. Plant responses to this combined stress can be unique from the response to either independent stress, yet few studies have investigated these responses in tree species. Our work examines adjustment of several primary metabolites and polyamine (stress indicating secondary metabolites) in paper birch and white spruce subjected to two seasons of repeated heatwaves, drought, and elevated CO_2. Our objectives were to determine if the metabolic adjustments in

response to heatwave+drought stress are: 1) unique or shared with either individual stress; 2) greater in birch compared to spruce; and 3) carried over into the following season. Our data show that white spruce displayed many metabolic responses that were unique to the combined stress, especially in the first year, while paper birch displayed few. Further, the unique responses in spruce seen in the first season stress exposure did not carry into the following season indicating possible stress memory. Our data highlights the importance of considering species-specific responses to multiple stressors when making predictions about forest response to future climate scenarios.

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Introduction

The increasing severity of extreme heat events (*i.e* . heatwaves) has threatened the survival of many species worldwide. These frequent heatwaves have contributed to increased mortality across various forest types (Allen *et al.* 2010; McDowell & Allen 2015; McDowell*et al.* 2016; Adams *et al.* 2017; Ogaya, Liu, Barbeta & Peñuelas 2020) and to major reductions in crop yield (Siebers *et al.* 2015; Zampieri, Ceglar, Dentener & Toreti 2017; Ingvordsen*et al.* 2018). It has long been recognized that high temperatures and drought that occur during heatwaves, are the two major environmental factors that will challenge future plant productivity (Mittler 2006; Brodribb, Powers, Cochard & Choat 2020). Yet our understanding of how plants respond metabolically to the combination of high temperatures and drought in a climate with increasing CO_2 concentrations is far from complete.

Genomic, metabolomic, and proteomic studies on molecular responses to the combination of high temperatures and drought stress have largely been focused on model plant species (e.g. Vile *et al.* 2012, Killi *et al.* 2017, Zinta*et al.* 2018) and herbaceous crops (Jagadish *et al.* 2011; Obata *et al.* 2015; Perdomo, Conesa, Medrano & Ribas-carbó 2015; Templer *et al.* 2017; Li *et al.* 2019; Zhou *et al.*2019; Alhaithloul, Soliman, Ameta, El-Esawi & Elkelish 2020). These studies have shown that responses to multiple stressors tend to be unique and cannot be inferred from the response to either stress experienced independently (Rizhsky *et al.* 2002, 2004a, Prasad *et al.* 2011, Vile *et al.* 2012, Suzuki *et al.* 2014, Zhao *et al.* 2016, Lawas *et al.*2018, Sewelam *et al.* 2020). For example, when Arabidopsis plants were exposed to heat+drought, the molecular responses were dominated by drought-specific transcriptomic changes (Rizhsky *et al.* 2004a), but in wheat and sorghum, molecular responses were dominated by heat-specific transcriptomic changes (Aprile *et al.* 2013; Johnson*et al.* 2014). To date, little progress has been made characterizing molecular responses to the combination of heat and drought in woody species. In recent studies on eucalyptus and citrus trees, increases in antioxidants, amino acids, citrate, and cinnamate were found in response to heat+drought that were not found in response to either independent stress (Zandalinas, Balfagón, Arbona & Gómez-Cadenas 2017; Correia *et al.* 2018). On the other hand, Berini et al. (2018) showed that the levels of other metabolites (catechin and two unspecified resin acids) in paper birch and balsam fir were similar among plants treated with heat, drought, and heat+drought. These few examples suggest the degree to which woody plants respond to the combination of heat and drought is metabolically complex and species-specific.

Surprisingly, many recent studies examining plant responses to future climate-change type scenarios such as high temperatures and drought, tend to overlook changes in atmospheric CO_2concentration and fail

to incorporate it into the experimental design. Rising atmospheric CO_2 and other greenhouse gases are driving global climate change (IPCC 2014), but few investigations have included elevated CO_2 (eCO_2) as an additional environmental factor when examining responses to multiple abiotic stressors (Li, Tiiva, Rinnan, Riitta, Julkunen-Tiitto, Anders & Rinnan 2020). Because there is limited data on the molecular responses of woody plants to the combination of high temperatures, drought and eCO_2, and because the few existing studies suggest plant responses to multiple stressors tend to be unique compared to responses to either independent stress, our first objective is to quantify a subset of metabolic compounds in two woody species exposed to repeated heatwaves, drought, and heat+drought stress under $eCO_2$to determine if their responses to the combined stress are unique or shared with either individual stress.

One of the major causes of cellular-level stress during unfavorable abiotic conditions is the overproduction of reactive oxygen species (ROS). ROS can be beneficial as signaling molecules under normal growing conditions, but under severe stress their quantities become toxic (Ślesak, Libik, Karpinska, Karpinski & Miszalski 2007; Foyer & Noctor 2009; Singh *et al.* 2016). During plant stress, some compounds that scavenge ROS activity can accumulate at high intracellular concentrations without hindering critical cellular metabolism (*e.g* . polyamines). Polyamines are biochemical indicators of environmental stress and their accumulation has been shown to improve tolerance to several types of stress. However, their functions under stress are still not fully understood. Polyamines can stabilize macromolecules, scavenge radicals, promote the production of antioxidant systems, and serve as compatible solutes and signaling molecules (review by Minocha *et al.* 2014). At the same time polyamines catabolism does produce ROS (Gupta, Sengupta, Chakraborty & Gupta 2016). To date, little is known about how polyamine metabolism is affected by multiple co-occurring stressors, especially the combination of heat and drought stress (Cvikrová, Gemperlová, Martincová & Vanková 2013).

Oxidation of large complex cellular components such as DNA, RNA, proteins, lipids, and smaller molecules such as free amino acids (AAs) with ROS alter cellular activity (Sharma, Jha, Dubey & Pessarakli 2012; Ahmad *et al.* 2017). When proteins or free AAs interact with ROS, the AA side chains are modified, causing both structural and functional changes to the compound (Stadtman & Levine 2003). Some of these oxidation reactions are reversible, such as those reactions with methionine and cysteine and may have an antioxidant function (Stadtman & Levine 2003; Kim 2020). Free AAs are also important for cell signaling and regulating plant responses to multiple stressors. For instance, during water stress some AAs serve as osmolytes to maintain turgor pressure prolonging cellular metabolism (Rai 2002; Sharma & Dietz 2006; Sharma *et al.* 2019). Given the central role AAs have in cellular protection and in nitrogen (N) and carbon (C) metabolism, and due to the lack of clarity on how AAs and their downstream products (*e.g* . polyamines and soluble proteins) are affected by multiple stressors, here we examine the impact multi-stress exposure has on AAs and their downstream products.

Carbon metabolism can be disrupted by high temperature or drought exposure, but the consequences can be most severe when both stressors co-occur (Rizhsky *et al.* 2002; Birami *et al.* 2018). These stressors can also have contrasting effects on C allocation. In beech saplings, Blessing, Werner, Siegwolf & Buchmann (2015) found that heat stress increased C allocation to roots Ruehr *et al.* (2009) found it decreased under drought. Because heat and drought stress can disrupt C uptake, mobilization, and utilization, the depletion of essential nonstructural carbohydrates (NSC; including starch and soluble sugars) is likely when both stressors co-occur (Birami *et al.* 2018). However, recent studies on the impacts of drought and high temperature stress on NSC dynamics have yielded mixed results. Some studies examining drought stress observed a depletion in NSC (*e.g.*Mitchell *et al.* 2013; Sevanto, Mcdowell, Dickman, Pangle & Pockman 2014; Maguire & Kobe 2015) while others have found an increase or no change (*e.g.* Anderegg *et al.* 2012; Gruber, Pirkebner, Florian & Oberhuber 2012). There is evidence that the accumulation of NSC, particularly soluble sugars, during drought may be linked to drought tolerance in some species (Piper 2011; O'Brien, Burslem, Caduff, Tay & Hector 2015). High temperature stress has also generated mixed responses in NSC content where declines or no changes have been observed (Wilson 1975; Rowland-Bamford, Baker, Allen Jr. & Bowes 1996; Zha, Ryyppö, Wang & Kellomäki 2001; Adams *et al.*2013). Although total NSC often decline in response to high temperatures, the accumulation of some soluble sugars may be an important trait related to heat tolerance (Niinemets 2010), especially the accumulation of sucrose and glucose (Liu & Huang 2000). Because the

accumulation of soluble sugars may improve drought and heat tolerance, and because eCO_2 has also been shown to have a positive effect on soluble sugar accumulation (Vu, Newman, Allen, Gallo-Meagher & Zhang 2002), here we examine changes in soluble sugar content in plants exposed to these three environmental factors.

The main goal of the study is to examine several primary metabolites involved in N and C metabolism in paper birch (*Betula papyrifera*) and white spruce (*Picea glauca*) subjected to repeated summer heatwaves, drought, and eCO_2. Paper birch and white spruce are functionally dissimilar species that occupy the same geographic region in the boreal and hemi-boreal zones. Paper birch is a broadleaf angiosperm with a rapid growth rate compared to white spruce, a slow growing conifer. Conifers invest heavily in C-rich protective resins that are used against herbivore and pathogen attack which for many angiosperms including birch, are only produced in low concentrations (Trapp & Croteau 2001). These two species are also on different ends of the leaf economic spectrum. White spruce is on the conservative end with a long leaf life span, low specific leaf area, and an overall more expensive energy investment for leaf construction as compared to paper birch that has a short leaf lifespan, high specific leaf area, and cheaper leaf construction costs. The structural and functional differences between white spruce and paper birch are critical for predicting how C and N metabolism may be impacted by multiple abiotic stressors. For instance, it has been suggested that resource conservative species, such as spruce, are more resistant to C loss during stress than less conservative species such as birch (Saura-Mas & Lloret 2007). Because conifers and angiosperms allocate C resources and energy differently to growth and secondary metabolism (*e.g.* leaf construction and defense compounds), the way in which C and N metabolism is altered by abiotic stress is expected to differ between them as well. These species have already demonstrated that recurrent heatwave stress affects their gas exchange, growth, and the ability to photosynthetically acclimate differently (Gagne, Smith, McCulloh, in press). Therefore, it may be predicted that shifts in metabolite pools will also occur in a species-specific manner.

In the current study conducted under eCO_2 conditions, we sought to answer the following questions: Are changes in N and C metabolism in response to heat+drought stress unique or shared with either individual stress? Will shifts in N and C metabolite pools be greater in birch compared to spruce under combined stresses? Will the changes observed after one year of heat+drought stress be carried over into the following season? We hypothesize that: 1) plant responses to combined heat+drought stress will be unique from either independent stress, 2) that N and C metabolite pools in birch will show a greater response to heat+drought stress than spruce, and 3) changes observed in N and C metabolism after one year of stress will be carried over into the following growing season.

Materials and methods

Plant material and growth conditions

A detailed description of the plant material and growth conditions is described in Gagne, Smith, McCulloh, in press). Briefly, 1 to 2-year-old paper birch and 3-year-old native Wisconsin white spruce saplings were grown in the Biotron Laboratory and Controlled Environment Research Center on the University of Wisconsin campus for two growing seasons (June-Oct 2016, April-Sept 2017). Daily temperature and water availability were altered to test their effects on survival and growth of the saplings. Additionally, the atmospheric CO_2concentration in the rooms was elevated from ambient conditions to 700 μmol CO_2 mol^{-1} to simulate a future intermediate emission projection scenario. Two greenhouse rooms were established in 2016; one received monthly heatwaves while the other served as the control non-heatwave room. In the second year, we doubled the number of rooms and split the plants from 2016 accordingly into their respective rooms. Temperatures in the non-heatwave rooms were based on 30-year weekly averages from Hayward, WI, USA, a location central to the distribution of these species in northern WI. All greenhouse rooms received the same temperature regime (daily temperature flux: June ˜12-27*C, July ˜13-28*C, August ˜13-27*C) except for heatwave days, where heatwave rooms were raised by 10*C for 8 continuous days in the middle of June, July, and August. To alter the watering regime, half of the plants in all rooms were well-watered, and the other half received less water. In 2016, the well-watered birch received ˜1.3 L wk^{-1} and the reduced-water birch received ˜0.65 L wk^{-1}; the well-watered spruce received 300 mL wk^{-1} and the reduced-water received

150 mL wk^{-1}. In 2017, the volumes increased to account for the increase in plant size to ˜4.8 L wk^{-1} and ˜2.4 L $wk^{-1}$for the birch and 600 mL wk^{-1} and 300 mL wk^{-1} for the spruce. From this point forward, the four treatment groups are referred to as: control (*C* ; well-watered, no heatwave); drought (*D* ; reduced-watered, no heatwave); heat (*H* ; well-watered, heatwave) and heat+drought (*HD* ; reduced-watered, heatwave).

Metabolite tissue collection and processing

Leaf samples were harvested in mid-June one week prior to the first heatwave event of 2016 and 2017. Late-August samples were harvested three days after the final heatwave of 2016 and 2017 for metabolite analysis. Fresh tissue of 2-4 healthy mature birch leaves or one spruce branchlet from the current year's growth was removed from each plant (n = 4-6 plants per treatment) using a razor blade. The foliage was immediately put into liquid nitrogen and then transferred to a -20*C freezer to await processing. The samples were freeze-dried, ground using a mini Wiley Mill (Thomas Scientific, Swedesboro, NJ) and then passed through a 0.85 mm sieve (#20 mesh). The dried samples were stored at -20*C until analysis.

Quantification of free amino acids, free polyamines, and soluble inorganic ions

Freeze-dried leaf tissue (25 mg) was subjected to 3 freeze-thaw cycles in 5% perchloric acid (PCA). PAs and AAs were simultaneously dansylated and quantified via reverse-phase HPLC per Minocha & Long (2004) with minor modifications described in (Majumdar *et al.* 2018). Briefly, PCA extracts were incubated at 60*C for 30 min containing 2.694 M sodium carbonate solution and dansyl chloride (dissolved in HPLC grade acetone). After the incubation period, the reaction was terminated using acetic acid. Sample tubes were kept open under a flow hood to allow CO_2 to escape. The acetone was then removed under vacuum. Filtered HPLC-grade methanol was used to resuspend the dansylated compounds. The solution was then filtered using 0.45 µm nylon syringe filter. Gradient elution of mobile phase A (acetonitrile) and mobile phase B (25mM sodium acetate buffer (pH 5.94) containing 3% 1-propanol and 10% acetonitrile) was used. PAs and AAs were analyzed, and the data were processed using Perkin Elmer TotalChrom software (version 6.2.1). PAs were quantified using an internal standard, AAs with external standard curves. To quantitate those AAs which did not separate, the areas and concentrations of each were added together to create a combined standard curve (i.e. Arg+Thr, Arg+Thr+Gly).

The 5% PCA extracts analyzed for AAs and PAs were also analyzed for inorganic ions and P via simultaneous axial inductively coupled plasma optical emission spectrophotometer (ICP-OES, Vista CCD, Varian, Palo Alto, CA). The extract was diluted 100x in ddH_2O. For each sample, triplicate readings were taken, and the spectral data were analyzed with Vista Pro software (version 4.0) using a set of 6 standards of the elements of interest. ICP analysis was done in accordance with EPA SW-846 compendium, method 6010.

Quantification of simple sugars

Using 25 mg of freeze-dried leaf tissue, soluble sugars were extracted and analyzed by the method described here (detailed method to be published elsewhere). Briefly, soluble sugars were extracted in 80% EtOH at 65*C for 30 minutes. The extract was then filtered using 0.45 µm nylon syringe filter. The sugar profiles were determined using reverse-phase high performance liquid chromatography paired with a refractive index detector (HPLC-RID, Shimadzu Scientific Instruments Inc, Columbia MD). For sugar separation, an isocratic mobile phase of 80% acetonitrile at a 2 ml min^{-1} flow rate and a Luna NH_2 column (250×4.6 mm, 5 µm, Phenomenex Inc, Torrance, CA) was used. Each sugar was quantified using a 6-point external standard curve (0.0625- 2 mg ml^{-1}). The chromatographs were analyzed, and the data were processed using Perkin Elmer TotalChrom software (version 6.2.1). To quantitate those sugars which did not separate, the areas and concentrations of each were added together to create a combined standard curve (i.e. xylose+arabinose, glucose+galactose, maltose+trehalose).

Quantification of total soluble proteins and chlorophylls

Using 25 mg of ground freeze-dried leaf tissue, total soluble proteins (TSP) were extracted in 500 µl (birch) or 250 µl (spruce) of Tris buffer (100 mM Tris-HCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, and 10% (v/v) glycerol, pH 8.0) by 3 freeze-thaw cycles (Jones, Hare & Compton 1989). The supernatant

(centrifugation 13,000 x g for 5 min) was analyzed for total soluble protein content per Bradford (1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Absorbances were 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 analyzed with Hitachi UV Solutions software version 2.0.

Using 3-5 mg of ground freeze-dried leaf tissue, chlorophyll a +b were extracted in 1 ml of 95% EtOH at 65°C for 16 hours and detected as described in Minocha et al. (2009). Chlorophyll a +b were analyzed 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 absorbances in the range of 350-710 nm. Equations from Lichtenthaler (1987) were used to calculate chlorophyll a +b concentrations in the leaf tissue.

Total N and C analyses

Total foliar N and C content was determined in foliage collected in late August 2016, and early June and late August 2017 (n= 5-6). The tissue was dried for 72 h at 70°C, ground using a mini Wiley Mill (Thomas Scientific, Swedesboro, NJ) and passed through a 0.85 mm sieve (#20 mesh). The samples were dried at 70°C for additional 16 h before aliquots of 3 to 5 mg dry weight were sealed in tin capsules. The material was sent to the Stable Isotope Mass Spectrometry Laboratory at Kansas State University in 2016 and to the Stable Isotope Core Laboratory at Washington State University in 2017 for analysis following the methods of Nippert *et al.* (2013) and Révész *et al.*(2012), respectively. Briefly, the samples were run through an elemental analyzer using glutamic acid and acetanilide as standards, respectively, to determine N and C content.

Statistics

The data were analyzed using XLstat 2019: Data Analysis and Statistical Solution for Microsoft Excel (Addinsoft, Paris, France). One-way analysis of variance (2016 data) or linear mixed-effects models (2017 data) were performed on the data for amino acids, polyamines, inorganic ions, soluble sugars, chlorophyll a +b , total soluble protein, and leaf nitrogen followed by Tukey's HSD post hoc test to determine if differences existed among treatment groups on a given sampling date. The fixed factor was *treatment* for all sampling dates and the random factor was *greenhouse room* for only the 2017 sampling dates. This statistical approach was used instead of analyzing a 2x2 factorial design to avoid potential issues of pseudoreplication in the first year when there was only one greenhouse room per temperature treatment.

Results

Impacts of heatwaves and drought on foliar N-containing metabolites

Of the 20 common proteinogenic AAs, 18 were quantifiable by our HPLC system. In addition, we quantified γ-aminobutyric acid (GABA) and ornithine (Orn), two of the common non-protein AAs in plants (Fig. 1, 2, S1, S2). Before the first heatwave event in 2016, AA concentrations were similar among the treatment groups apart from Glu and Gln in spruce and Val in birch (Fig. 1, 2, S1, S2). The response of the spruce plants to the combined heat+drought stress was vastly different from the response of the birch (Fig 1, 2). The *HD* spruce displayed several novel changes in the concentration of 10 AAs (Gln, Ser, Pro, GABA, Val, Ile, Trp, Phe, Cys, and His) in August at the end of the 2016 season (Fig. 1). There were no statistically significant differences in AAs between the H plants and the C plants, or the D plants and the C plants in late August 2016. By the start of the 2017 season, all previously elevated AAs had reverted back to levels similar to the C plants, with the exclusion of Asp (increased in *HD* plants) and Glu and Val (decreased in *HD* plants; Fig. 1a, b, h). By the end of the second summer of heatwave stress, the *HD* spruce tended to respond similar to the D plants (Asp, Ala, His; Fig. 1a, e, o) or the response was between that of the D and H response (Phe, Orn; Fig. 1l, n). There were a few statistically significant differences found between the D ,H , and *HD* spruce and the C plants after two years of repeated stress (Glu, H vs C ; Ala, *HD* vs C ; and Orn, D vs C).

The birch plants did not exhibit the same extreme responses of AA metabolism to the *HD* treatment as

the spruce. At the end of the first season of stress, Phe and Trp were the only AA to accumulate under the combined stress (Fig. 2j, k; Phe, $p < 0.05$*HD* vs *C* ; Trp, $p < 0.05$ *HD* vs*C*). Compared to the *H* treatment, Cys was reduced in the*D* and *HD* treatments at this time as well (Fig. 2k, l). At the start of the second season, only few differences in AAs were found among treatment groups and none were unique to the *HD* treatment (Ser, Leu, Orn; Fig. 2b, i, m). By the end of the second summer of heatwave stress, unlike the spruce plants, the *HD* birch did not display unique responses from either independent stress. Instead the*HD* birch tended to respond more similarly to the *D* plants than the *H* plants in several AAs (Ala, Pro, GABA, Val, Ile, Orn; Fig. 2d, e, f, g, h, m).

Of the three PAs quantified, putrescine (Put) was the most abundant in spruce tissue whereas spermidine (Spd) or spermine (Spm) was the most abundant in the birch (Table 1). Prior to the first heatwave in 2016, no differences were found among treatment groups of either species. At the end of the 2016 season, the *HD* spruce produced significantly less Put ($p < 0.05$ *HD* vs *C*) and Spd ($p< 0.05$ *HD* vs *C*) than the *C* plants where concentrations of these PAs were $< 50\%$ of that of the*C* plants (Table 1). Overall, Put concentrations were higher in the spruce foliage in the second year compared to the first year. In the beginning of the second season, all treatment groups of spruce had similar foliar PA concentrations. A decrease in Put and Spd was observed in the *HD* spruce at the end of each season. Although PA quantities in the *D* and *H* spruce were not impacted by one season of stress, two seasons of recurrent stress did result in decreased PA metabolism. In late August of 2017, the *D, H,*and *HD* spruce had lower concentrations of Put (Table 1; $p < 0.01$ *D* vs *C* ; $p < 0.01$*H* vs *C;* $p < 0.01$ *HD* vs *C*) and Spd than the *C* plants (Table 1; $p < 0.01$ *D* vs*C* ; $p < 0.001$ *H* vs *C;* $p <0.01$ *HD* vs *C*). At this time, the *H* and *HD*plants also had higher concentrations of Spm compared to the *C*plants ($p < 0.001$ *H* vs *C;* $p <0.05$ *HD* vs *C*).

In birch, the greater quantities of Spd and Spm where found in the first year. Very few changes in foliar PA concentration were found in the stress-treated birch across both years (Table 1). By the end of the first year, differences among treatment groups were only found in Spd where *H* plants had a significantly higher concentration than the*C* , *D* , and *HD* plants, and the *D* and *HD*plants concentrations were 74% lower than the *C* plants (Table 1;$p < 0.01$ *D* vs *C* ; $p < 0.01$ *HD* vs *C*). For birch, the second year of treatments did not result in statistical differences among treatment groups at the beginning or end of this season except for higher Spm concentration in the *H* -treated plants than the *D* and *HD* plants (Table 1; $p < 0.05$ *H* vs *D* ; $p < 0.05$ *H* vs*HD*).

In June 2016, no differences were found in total soluble protein (TSP) among treatment groups for either species. The unique response observed in the *HD* spruce in TSP and total chlorophyll concentration in late August 2016 was analogous to the response of several AAs at this time. The *HD* spruce produced significantly more TSP than the*C* , *D* , and *H* treatments where these plants produced 140% more TSP than the *C* plants (Fig. 3a, S3a; $p< 0.001$ *HD* vs *C* , *D* , *H*). Chlorophyll $a + b$ concentration decline by nearly 70% in the *HD* plants as well (Fig. 3b; $p < 0.001$*HD* vs *C*). By the following season, TSP and Chlorophyll a+b content in the *HD* spruce no longer differed from the*C* plants. At the end of the first season, no statistical differences in total leaf N or C were found among any of the treatment groups in spruce (Fig. 3, S3). At the start of the second year, the only significant differences among treatment groups were found in C content where the *H* and *HD* plants 3-4% more total C than the*C* plants (Fig. 3d, S3d). By the end of the second season of recurrent stress, only the *D* -treated spruce exhibited a reduction in total leaf N by $>30\%$ compared to the *C*plants ($p < 0.05$ *D* vs *C*).

Total soluble protein in birch was not affected by treatment at any of the sampling dates (Fig. 3e, S3e). Unlike the unique response in chlorophyll $a + b$ observed in spruce after one season of*HD* stress, all birch treatment groups had 28-33% less foliar chlorophyll $a + b$ than the *C* plants (Fig. 3f S3f;$p < 0.05$ *D* vs *C* ; $p < 0.05$ *H* vs *C* ; $p < 0.05$ *HD* vs*C*), but total N and C in these plants was not affected (Fig. 3g, h, S3g, S3h). At the start of the second season, all treatments had similar TSP, Chlorophyll $a + b$, total N, and total C. By the end of year two, only total leaf N differed among treatment groups where the *D* and *HD* birch had lower N than the *H* plants (Fig. 3g, S3g; $p < 0.05$ *D* vs *H* ; $p< 0.05$ *HD* vs *H*).

Impacts of heatwaves and drought on foliar soluble sugars and inorganic ions

Using an HPLC-RID system, we quantified the monosaccharides: xylose, arabinose, rhamnose, fructose, mannose, galactose, and glucose; the disaccharides: sucrose, maltose, and trehalose; and the oligosaccharide raffinose. However, co-elution of xylose and arabinose, glucose and galactose, and maltose and trehalose resulted in unresolved peaks and therefore, data for these sugars are presented as xylose+arabinose, glucose+galactose, and maltose+trehalose. In both species, maltose+trehalose was not detected at any of the sampling dates and raffinose (and rhamnose in spruce) was only detected in trace amounts in August 2017 (Fig. 4, 5, S4, S5). Additionally, mannose was only present in the birch tissue. A major difference between the two species was in the type of sugar they accumulated in greatest concentration.

Prior to the first heatwave in 2016, there were no statistical differences among treatment groups of either species in soluble leaf sugars. Soluble sugars in spruce were minimally affected by one season of heatwave and drought stress where statistical differences in sugar concentration were only between the *HD* and *H* plants in xylose+arabinose content (Fig. 4b, S4b; $p < 0.05$*HD* vs *H*). In June 2017, all treatment groups had similar foliar sugar concentrations. Fructose and glucose+galactose concentrations were highest at this time whereas sucrose content was relatively low compared to other sampling dates (Fig. S4e). Two seasons of *HD* stress resulted in an $> 70\%$ increase in fructose compared to the *C* and *H* treatments (Fig. 4g;$p < 0.05$ *HD* vs *C* ; $p < 0.05$ *HD* vs *H*) and less xylose+arabinose than the *H*treatment (Fig. 4h, S4h; $p < 0.05$ *HD* vs*H*). In 2017, shifts in foliar sugar concentrations occurred from fructose and glucose+galactose as the dominant sugars early in the season to sucrose being most abundant later in the season for all treatment groups (Fig. S4g). Xylose+arabinose also accumulated in all the treatment groups by the end of 2017 season (Fig. S4f).

Soluble sugar concentrations in birch were impacted more by the stress treatments than they were in spruce, especially after the first season (Fig. 5, S5). Although there were differences among treatment groups in fructose, glucose+galactose, sucrose, and xylose+arabinose, none of the stress treatments significantly differed from the *C* plants (Fig. 5c-d, S5c-d). Instead, differences were found only between the *D*and *H* plants where the *D* plants produced more fructose and glucose+galactose (fructose, $p < 0.05$ *D* vs*H* ; glucose+galactose, $p < 0.05$ *D* vs*H*), but less sucrose and xylose+arabinose than the *H*plants (sucrose, $p < 0.05$ *D* vs *H* ; xylose+arabinose, $p < 0.01$ *D* vs *H*). At this time, the combined *HD* plants exhibited concentrations between that of the *D* and *H* plants (Fig. 5c, d). Similar to the spruce, the only statistical difference in sugar concentration at the end of the second season was in xylose+arabinose where the *HD*plants produced 21% less than the *C* plants (Fig. 5h; $p< 0.05$ *D* vs *H*). Similar to spruce, in birch also, shifts in foliar sugar concentrations occurred from fructose and glucose+galactose as the dominant sugars early in the season to sucrose being most abundant later in the season for all treatment groups in 2017 (Fig. S4g).

The most abundant soluble (in 5% PCA) inorganic ions in both spruce and birch foliage tended to be Ca and K (Fig. S6). At the start of 2016, there were no differences in the concentration of soluble inorganic ions among treatment groups apart from K in the birch. By the end of the first season in spruce, only few statistical differences in inorganic ion concentration were found among treatment groups. Specifically, the*HD* plants had nearly 50% more Mg than the *H* plants (Fig. 6b; $p < 0.05$ *HD* vs *H*), but no differences from the *C* plants were found. However, at the start of the second season, several statistical differences were found among the spruce treatment groups, but the only unique response observed in the *HD*plants was in a 20% reduction in P compared to the *C* plants (Fig. 6c; $p < 0.05$ *HD* vs *C*). Calcium was reduced in the *D* plants compared to the *H* plants ($p< 0.05$ *D* vs *H*), Mg was 13% lower in *D*plants compared to *C* ($p < 0.05$ *D* vs*C*), K was elevated in the *D* plants compared to both the*H* and *HD* plants ($p < 0.01$ *D* vs*H; p* < 0.01 *D* vs *HD*), and Fe was reduced by 32-36% in the *D* , *H* , and *HD* plants compared to the *C* plants ($p < 0.05$ *D* vs *C* ;$p < 0.05$ *H* vs *C* ; $p < 0.05$ *HD* vs *C*). By the end of the second growing season, Ca, Mg, and Fe concentrations no longer differed among treatment groups (Fig. 6d). However, K was elevated by 42% in the *H* plants relative to the *C* and *D* ($p < 0.05$*H* vs *C; p* < 0.05 *H* vs *D*), P was elevated in *H* plants relative to the *D* and *HD*plants ($p < 0.05$ *D* vs *H; p* < 0.05 *D* vs *HD*), and Mn was nearly doubled in the *D*plants relative to the *C* and *H* plants (Fig. 6d, S6d;$p < 0.01$ *D* vs *C; p* < 0.05*D* vs *H*).

Inorganic ion concentrations in birch were largely unaffected by recurrent heatwave and drought stress (Fig.

6, S6). After the first season of stress, the D treatment resulted in >150% increase in Fe compared to the C and H treatment (Fig. 6f;$p < 0.01$ D vs C; $p < 0.05 D$ vs H) and 50-150% increase in Mn compared to the$C$, H , and HD treatments (Fig. 6f; $p< 0.001$ D vs C; $p < 0.001$ D vs$H$; $p < 0.01$ D vs HD). Potassium concentrations were reduced by 18% in the H plants and 20% in the HD plants relative to the C plants at the start of the second season (Fig. 6g; $p < 0.05$ H vs C; $p < 0.01$ HD vs C). No other differences among treatment groups were found at this time or at the end of the season as well (Fig. 6g, h).

Discussion

Species-specific metabolic response to combined heat+drought stress

The differences observed in metabolic response of young white spruce and paper birch trees to the combination of heatwave and drought stress versus the response to either independent stress is indicative of the problem of generalizing plant responses across species to multiple stressors acting simultaneously in the growth environment. White spruce showed many unique metabolic responses to the combined stress, while paper birch displayed few. The HD birch tended to share responses with the D plants (*e.g.* total leaf N, Spd, Spm, and several AAs) or the response was between that of the D and$H$ plants (*e.g.* soluble sugars). These findings partially support our first hypothesis that plant responses to the combination of heatwave and drought stress will be unique from either independent stress. Furthermore, the unique responses in spruce did not remain constant over time, but instead were mainly limited to the first year of heatwave exposure. A potential explanation for this is that "ecological stress memory" is responsible for the adjustments between the first and second year of treatment (Walter, Beierkuhnlein, Jentsch & Kreyling 2013). The idea behind ecological stress memory is that individual plants will respond to a stress event differently if they have previously been exposed to that stress.

The spruce exhibited many distinctive metabolic responses to the combined stress, especially in the first season (*e.g.* AAs, PAs, TSP, chlorophyll $a + b$) whereas the birch did not. These findings do not support our second or third hypotheses that N and C metabolite pools in birch will show a greater response to heat+drought stress than the spruce, and that the changes observed in N and C metabolism after one year of stress will be carried over into the following growing season. In the first season of prolonged HDstress, relatively more metabolic adjustment occurred through the accumulation of several AAs and TSP whereas in the second season ofHD stress, adjustment occurred through changes in PAs and fructose. The major adjustments to individual AAs from the HDtreatment in spruce show how carbon and nitrogen assimilation can become completely disrupted under multiple stressors. For example, Ser, Gly, and Gln are involved in the recycling of carbon from photorespiration (Blackwell, Murray & Lea 1990). Photorespiration is known to increase during both drought and high temperatures (Jordan & Ogren 1984; Wingler*et al.* 1999) and it is very likely that the activity of this pathway was elevated in the HD spruce, as indicated by the 5-fold increase in Ser and 2-fold increase in Gln. Unfortunately, during the analysis Gly did not separate from Arg and Thr, therefore changes in Gly content in 2016 could not be assessed. Furthermore, the elevated levels of Phe and Trp suggest a greater proportion of carbon may have been allocated to protective compounds, *e.g.* phenolics. Such response was observed in *Eucalyptus* exposed to heat+drought triggering a novel accumulation of cinnamate, a substrate central to phenylpropanoid biosynthesis derived from Phe (Correia *et al.* 2018). Proline is a well-known indicator of osmotic stress and both Pro and GABA have been shown to provide protection from a number of environmental stress factors (Bouché & Fromm 2004; Liang, Zhang, Natarajan & Becker 2013b). The substantial increases in Pro and GABA indicate the combination of heat and drought may have drastically increased osmotic stress in these plants that was not experienced under either independent stress. Two decades ago, it was suggested that until recent climate warming, drought may have been the only factor limiting growth of white spruce (Barber, Juday & Finney 2000). If elevated temperatures had not been a selective pressure in white spruce's recent life history (*i.e.* no transgenerational epigenetic inheritance), these plants may have responded by overcompensating in AA accumulation when experiencing both heat and drought stress for the first time. But by the second season, the plants had already experienced the combined stress and therefore did not have the same over-compensatory response due to ecological stress memory. The first year may have primed their stress-response systems for a more

prepared response the following year (Hilker & Schmülling 2019). This may also explain why no carry-over effect was observed at the start of the second season. Our findings highlight the importance of not inferring plant responses to multiple stressors based on the responses to either independent stress for these data show that the addition of high temperatures during a prolonged drought can have major consequences on primary metabolism in spruce that are not experienced when either stress is applied independently.

The only unique responses in the *HD* birch plants from either of the individual stressor was the accumulation of Trp and Phe in late August 2016. Phenylalanine and Trp are aromatic AAs that are precursors to a wide variety of secondary metabolites, the phenylpropanoids derived from Phe, and auxin, phytoalexins, glucosinolates, and alkaloids derived from Trp (Radwanski & Last 1995; Tzin & Galili 2010). All of these compounds have significant roles in protection against abiotic and biotic stress and their production is stimulated by stress (Dixon & Paiva 1995). The novel accumulation of Phe and Trp in the *HD*plants suggests there were likely accumulations of other phenolic compounds involved in the catabolism of Phe and Trp. It was surprising that the *HD* birch did not accumulate sucrose in either year in response to the combined stress. These plants also did not accumulate Pro in either year which is why sucrose may may accumulated since it often replaces Pro as the dominant osmolyte under the combination of high temperatures and drought (Rizhsky *et al.* 2004b). Also, to our surprise, the combined stress did not uniquely alter soluble inorganic ion concentration or other soluble sugar concentrations (aside from xylose+arabinose in late 2017).

Metabolic responses to individual heat and drought stress

High temperatures alone had a minimal impact on spruce in both years of treatment. Polyamines (and to a lesser extent potassium) were the only compounds affected by the heatwaves which occurred only at the end of the second year. The decline in Put and Spd observed with *H*and/or *HD* at the end of second growing seasons may have been due to their oxidation and consumption into Spm. Elevated levels of Spm were also observed in the *H* birch at this time signifying its involvement in cellular stress response. Spermine plays a major role in protecting plants from heat stress through its involvement in elevating transcript levels of heat shock-related genes (Sagor, Berberich, Takahashi, Niitsu & Kusano 2013). Alternatively, the decline of Put and Spd may also point to a fatigued defensive system at the end of the two-year experiment (Sgobba, Paradiso, Dipierro, de Gara & de Pinto 2015). Polyamine accumulation increases tolerance to a variety of stressors (reviewed by Rhee, Kim & Lee (2007); Shi & Chan 2014), but due to the production of H_2O_2 via PA catabolism, their long-term accumulations may have toxic effects (Bhattacharjee 2005; Mohapatra, Minocha, Long & Minocha 2010; Minocha*et al.* 2014). Additionally, the *H* spruce showed a moderate increase in K at the end of second growing season that was not observed in the water limited *D* or *HD* plants which may suggest the*H* plants had higher rates of evaporative cooling which also increased water and nutrient uptake (Pregitzer & King 2005).

In general, the lack of response in birch to the heatwave only treatment suggests that the birch may have been better equipped to tolerate high temperatures than drought. Even so, we expected to detect some metabolic adjustment in response to air temperatures near 40^{*}C. The few metabolic adjustments observed were contrasting and only occurred at the end of the 2016 season where the *H* birch produced nearly twice as much Spd and 30% less chlorophyll $a + b$ as the *C* plants. Elevated levels of Spd suggests these plants may have had greater heat tolerance (Shao, Wang & Yu-Fen 2015), while lower chlorophyll $a + b$ indicates the plants were experiencing oxidative stress (Havaux & Tardy 1999). During the heatwave events, these plants likely had increased rates of daytime respiration (Liang, Xia, Liu & Wan 2013a) which would have led to the consumption of large quantities of carbohydrates and other compounds such as proteins (Araujo, Tohge, Ishizaki, Leaver & Fernie 2011). Alternatively, the lack of apparent response may have been due to the complexity of plant responses to high temperature, and we may not have measured the compounds that were most impacted. We did not, for example, measure heat shock proteins and dehydrins (Hanin *et al.* 2011; Jacob, Hirt & Bendahmane 2017; Aspinwall *et al.* 2019).

Unexpectedly, the drought treatment had a minimal impact on metabolism across the two seasons in both species. Drought stress is known to induce the production of various osmoprotectants including compatible solutes that help maintain osmotic balance and protect cells during dehydration. Proline is a well-studied

compatible solute and has been shown to have many protective functions during plant stress (Kemble & Macpherson 1954; Demiral & Turkan 2005; Kaushal, Gupta & Bhandhari 2011; Per *et al.* 2017). Glycine and GABA also act as important compatible solutes that alleviate osmotic stress (Di Martino, Delfine, Pizzuto, Loreto & Fuggi 2003; Renault *et al.* 2010). Unlike the*HD* spruce, the *D* -treated spruce showed a lack of Pro, GABA, and Gly accumulation suggesting other osmolytes, such as betaines or polyols, may be responsible for maintaining cell turgidity and restoring osmotic homeostasis during drought. This lack of Pro accumulation is a somewhat uncommon response among species under drought (Hossain & Hoque 2014; Zandalinas *et al.* 2017). Although neither the *D* or HD birch showed evidence of Pro, Gly, or GABA accumulation, Spd, a compound with anti-senescent properties (Nambeesan*et al.* 2010), was reduced in the water-limited birch (*D*and *HD*) at the end of the first season. Spermine was not elevated at this time which indicates Spd may have been oxidized to form 1,3-diaminopropane as opposed to Spm, a response indicative of increased oxidative stress (Cvikrova *et al.* 2013).

During drought conditions soluble sugars such as hexoses (e.g. glucose, fructose), can also accumulate in conjunction with Pro and polyamines to act as osmoprotectants (Sengupta, Chakraborty, Saha, Gupta & Gupta 2016; Templer *et al.* 2017). Although not significantly different from *C,* the *D* birch did have elevated levels of foliar fructose and glucose+galactose at the end of the first season compared to the *H* birch, and the *D* spruce had elevated levels of fructose at the end of the second season compared to the *H* spruce suggesting that these sugars may be facilitating osmotic homeostasis under water stress. Still, the mechanisms utilized to cope with prolonged drought stress in these species is still not clear. We can speculate that the dominant osmolytes may be various soluble sugars under water stress (including those not quantified here, *e.g.*sorbitol, (Lo Bianco, Rieger & Sung 2000), but due to the prolonged nature of the drought treatment ($>$ 3 months) which continuously suppressed photosynthesis (Gagne, Smith, McCulloh, in press), carbohydrate reserves may have been depleted by the end of August. It is also likely that photosynthates were translocated from the leaves to the stem and roots for post-drought recovery (O'Brien *et al.* 2015; Tomasella, Petrussa, Petruzzellis, Nardini & Casolo 2020).

Overall, we found that white spruce exhibited many unique molecular responses to the combined stress, while paper birch displayed few and tended to share responses with the *D* plants. The differences in metabolic response of young white spruce and paper birch trees to the combination of heatwave and drought stress under eCO_2versus the response to either independent stress shows how generalizing plant responses to multiple stressors is problematic. Our data highlights the species-specific nature of metabolic adjustment to multiple stressors which should be considered when making predictions about forest response to future climate scenarios. The data also suggest that paper birch may lack the ability to metabolically adjust to extreme heat events in the future, which may limit their future distribution within boreal forests.

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Table 1. Soluble foliar polyamines in the current year's foliage of white spruce (top) and paper birch (bottom) collected in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Polyamine content is expressed as nmol g^{-1} DW for putrescine, spermidine, and spermine. Data are mean (n = 5-8) ± SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test.

Figure legends

Figure 1. Foliar amino acid content in the current year's foliage of white spruce in June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Amino acid content is expressed as nmol g^{-1} DW for aspartic acid (a), glutamic acid (b), glutamine (c), serine (d), alanine (e), proline (f), GABA (γ-aminobutyric acid, g), valine (h), methionine (i), isoleucine (j), tryptophan (k), phenylalanine (l), cysteine (m), ornithine (n), and histidine (o). Data are mean (n = 5-8) ± SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test.

Figure 2. Foliar amino acid content in paper birch foliage in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Amino acid content is expressed as nmol g^{-1} DW for glutamic acid (a), serine (b), glycine (c), alanine (d), proline (e), GABA (γ-aminobutyric acid, f), valine (g), isoleucine (h), leucine (i), tryptophan (j), phenylalanine (k), cysteine (l), and ornithine (m). Data are mean (n = 5-8) ± SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test.

Figure 3. Foliar total soluble proteins (TSP), chlorophyll$a$ +b , total N, and total C expressed as percent change from control values in white spruce (left column) and paper birch (right column). Data was collected in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Data are mean (n = 5-8) +- SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date using the actual data values based on Tukey's HSD post hoc test. No Data was collected for total N and C in June of 2016.

Figure 4. Simple soluble sugars in the current year's foliage of white spruce in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Values are expressed as a percent of the control. Data are mean (n = 4-8) +- SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test. Asterisks indicate quantities below the detection limit.

Figure 5. Simple soluble sugars in the foliage of paper birch in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Values are expressed as a percent of the control. Data are mean (n = 4-8)

+- SE. Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test. Asterisks indicate quantities below the detection limit.

Figure 6. Foliar inorganic ions and P concentration in white spruce (left column) and paper birch (right column) in early June (pre-heatwave) and late August (post-heatwave) of 2016 and 2017. Values are expressed as a percent of the control values for 5% PCA soluble calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), iron (Fe), and phosphorus (P). Data are mean (n = 4-8). Letters indicate statistical differences (p [?] 0.05) among treatment groups on a given date based on Tukey's HSD post hoc test.

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