Drivers of trembling aspen secondary chemistry and impacts on plant communities in the aspen parkland.

by

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Abstract

Plant secondary chemicals are fundamental mechanisms by which plants interact with their environments, including defending against herbivores, attracting pollinators, altering neighbouring plant fitness or facilitating plant-plant communication. Yet our understanding of the extent to which secondary chemicals can impact herbivory has greatly outpaced our understanding of the role of chemistry in impacting other processes. In this thesis, I use trembling aspen (*Populus tremuloides* Michx.) as a model species to test the hypothesis that variations in the factors that drive plant secondary chemistry may also have the potential to indirectly act as drivers of plant community level processes through the mechanism of plant secondary chemistry. I first asked whether the drivers of aspen chemistry were consistent across the whole plant. I determined that the concentrations of aspen secondary chemicals vary as a function of tissue type, suggesting that drivers of aspen chemistry differ above and belowground. Second, using a regional survey of aspen chemistry, I tested hypotheses about the roles of spatial variation, climate and stand structure as drivers of aspen chemistry. I found that aspen chemistry was more locally variable than would be expected if genetics had the strongest control of chemical concentrations, and that light availability or location within the stand were most associated with changes to aspen chemistry. In my last two chapters, I tested whether these changes to aspen chemistry feedback into, and ultimately impact, understory community assembly and woody encroachment. I found evidence that aspen secondary chemistry might be acting as an ecological filter, as overstory secondary chemical concentrations were associated with changes in understory species richness and evenness. Further, I found evidence that aspen secondary chemistry might facilitate aspen encroachment into invaded habitats, as once a seedling had established, its growth was positively associated with the amount of aspen litter added to a plot. In contrast greater aspen litter inputs were associated with decreased growth in native grasslands.

Combined these findings provide evidence that aspen chemistry is variable, susceptible to changes in the local environment, and that it can directly impact ecological processes. Overall, this suggests that changes in the primary drivers of aspen secondary chemistry could have cascading impacts on an ecological system. Therefore, any changes in the local environment due to climate change or human modifications could have greater impacts on ecosystem processes than expected, as indirect impacts on aspen secondary chemistry are not always accounted for in current ecological models. Broadly, my findings emphasize the importance of including secondary chemical traits and the factors that influence them into trait-based models of community ecology.

Preface

Chapter 2 of this thesis has been published as "Dettlaff, MA, V Marshall, N Erbilgin and JF Cahill (2018) Root condensed tannins vary over time but are unrelated to leaf tannins: at *AOB Plants*. I contributed to the development of the basic concept, experimental design, data collection and processing, statistical analysis and writing. V Marshall contributed to sample collection and processing. JF Cahill and N Erbilgin were the supervising authors and assisted in concept development and manuscript editing

Chapter 3 of this thesis is intended for publication as "Dettlaff, MA, JA Cale, JF Cahill and N Erbilgin. Patterns in aspen secondary chemical phenotypes suggest local plasticity". I contributed to the development of the basic concept, experimental design, data collection, analysis and writing. JA Cale assisted with data collection and manuscript editing. JF Cahill and N Erbilgin were the supervising authors and assisted in concept development and manuscript editing

Chapter 4 of this thesis is intended for publication as "Dettlaff, MA, Inderjit, N Erbilgin and JF Cahill. Chemical inputs from overstory trees influence understory plant community assembly". I contributed to the development of the basic concept, experimental design, data collection, analysis and writing. Inderjit contributed to concept development and manuscript editing. JF Cahill and N Erbilgin were the supervising authors and assisted in concept development and manuscript editing

Chapter 5 of this thesis is intended for publication as "Dettlaff, MA, N Erbilgin and JF Cahill. An invasive grass and litter impact native tree establishment into a native grassland." I contributed to the development of the basic concept, experimental design, data collection, analysis and manuscript writing. JF Cahill and N Erbilgin were the supervising authors and assisted in concept development and manuscript editing

For my family, who make all things possible,

and especially

for Bruno Dettlaff
(1928 – 2018)

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Chapter 1: Introduction

Due to their sessile nature, plants have developed adaptation strategies to address the variability of conditions that they experience. This adaptation can be measured in physiological, phenological and chemical traits (Palacio-López et al. 2015, Siefert et al. 2015). Plant chemical traits are among the most plastic (Siefert et al. 2015); since the isolation and identification of the first plant secondary metabolite, morphine, in the early 1800s (Sertuerner 1817, Hartmann 2007) more than 200,000 secondary chemicals have been described (Kessler and Kalske 2018). Plant secondary metabolites are products of a plants metabolism that are not directly involved in the main processes of life such as respiration or photosynthesis. Early research considered them "waste products" of a plant's metabolism, and focused on the utility of plant secondary chemicals to humans, as dyes, medicines, and drugs (Hartmann 2007). Beginning in the late 1950s, research began to focus on their importance to plants as defences against herbivory (Fraenkel 1959). As chemical analytical techniques improved, more and more secondary chemicals were identified (Rosenthal and Janzen 1979, Karban and Baldwin 1997), their metabolic pathways were identified (Ganem 1978, Hashimoto and Yasuyuki 1994), and their importance as drivers of diversity and co-existence was established (Ehrlich and Raven 1964, Harbone 1972, Swain 1977).

Although much of this research has remained focused on the impact of individual chemicals on specific herbivores, there is increasing recognition for broader ecological and evolutionary roles for plant secondary chemicals. For instance, research has demonstrated that plant secondary chemicals can inhibit or facilitate neighbouring plants (Olson and Wallander 2002, Hierro and Callaway 2003, Hovstad and Ohlson 2008), increase tolerance to abiotic factors (Lavola 1998, Mellway and Constabel 2009, Koyama et al. 2014), attract pollinators (Stevenson et al. 2017), or facilitate plant-plant signaling (Karban et al. 2000, Hussain et al. 2019). Plant secondary chemicals are also highly variable across space and time (Seigler 1998), between different plant tissues (Erbilgin and Colgan 2012) and in response to biotic and abiotic conditions (Karban and Baldwin 1997). These variations in plant chemistry caused by external factors could in turn have impacts on ecological processes such as community assembly or susceptibility to invasion through the mechanisms outlined above. In this thesis, I will try to test the broad hypothesis that variations in the factors that drive plant secondary chemistry may also have the potential to indirectly act as drivers of ecological processes through the mechanism of plant secondary chemistry (e.g. Fig. 1-1).

Trembling aspen as a model species

In this thesis, I use trembling aspen (*Populus tremuloides* Michx.) and the aspen parkland of central Canada to test my hypothesis. Trembling aspen is the most widely distributed tree species in North America (Dickmann and Stuart 1983), found from the mixed-wood boreal forests of northern Canada to high mountains in central Mexico. The aspen parkland is a savannah ecosystem located at the boundary between the Great Plains to the south and the boreal forest to the north. As it is a transitional zone, it has attributes of both neighbouring ecosystems; it is characterized by small stands of trembling aspen, interspersed within a grassland matrix (Bird 1961). As aspen frequently reproduces via suckering, these small stands may actually only represent one or two genetic individuals (Mitton and Grant 1996). Precipitation in the aspen parkland is generally less than potential evapotranspiration (Hogg and Hurdle 1995), and so aspen there are typically smaller and slower growing compared to aspen in other, moister, ecosystems (Hogg and Hurdle 1995). Inter-annual variation in moisture levels (Bailey and Wroe

1974), along with disturbances (e.g. fire or grazing)(Campbell et al. 1994) contribute to frequent shifts in the boundaries between forested and grassland areas.

The unique attributes of the aspen parkland, relative to other, more traditional forest ecosystems, makes it an excellent setting to examine drivers of aspen chemistry and the impact of chemistry on ecological processes. The natural gradient of suitability from the southern edge of the parkland northwards should result in greater variation in naturally occurring levels of aspen secondary chemicals, as aspen can respond to stress or differing abiotic conditions by altering the concentrations of secondary chemicals in their tissues (Karban and Baldwin 1997, Kanaga et al. 2009, Jamieson et al. 2015). The coexistence of both forest- and grassland-dominated community types in a small area results in a regional pool of species with a large variety of evolutionary histories and ecological niches, and the frequent fluctuation of areas between forested and grassland dominated communities should increase the importance of any ecological filters on plant community composition.

Trembling aspen produces relatively high concentrations of two major classes of secondary chemicals; condensed tannins and phenolic glycosides (Lindroth and Hwang 1995, Barbehenn and Constabel 2011). The concentrations of both these chemicals may be heterogeneous across time or space as both genotype (Osier and Lindroth 2001) and environmental factors (McDonald et al. 1999, Osier et al. 2000a, Lindroth and St. Clair 2013, Najar et al. 2014, Gourlay and Constabel 2019) can impact their concentrations. Despite aspen's broad range, much of the research on trembling aspen chemistry has been done on populations from only a few ecosystem types, specifically the Rocky Mountain or Great Lakes regions of the Central United States (e.g. Lindroth and Hwang 1996, Lindroth et al. 1999, Bailey et al. 2007, Lamit et al. 2011). These studies also typically use aspen grown and studied in human-controlled conditions (e.g. Schweitzer et al. 2004, Keefover-Ring et al. 2014, Najar et al. 2014, Stevens et al. 2014, Madritch and Lindroth 2015). This has left a gap in our understanding of aspen chemistry. For example, we do not know if information about aspen's secondary chemical responses to external factors gained from one area of its range are applicable to other habitats, or to what extent greenhouse or common-garden studies will translate to trees into natural conditions. Clarifying these issues is needed, as it may be difficult to accurately predict how variation in environmental and phenological factors will impact ecological processes in the aspen parkland if we do not have a clear understanding of the drivers of aspen chemistry in this system.

Impacts of external factors on aspen secondary chemistry

In chapter two of this thesis, I address two aspects of impacts of external factors on aspen secondary chemistry (light green lines, Fig 1-1). Despite previous literature that has documented concentrations of secondary chemicals in leaves and roots of trembling aspen, no study has yet examined the concentrations of secondary chemicals in both tissues at the same time. The concentrations of secondary chemicals in roots and leaves should be correlated, as the evolutionary drivers that shape plant defence are similar across the whole plant (Kaplan et al. 2008, Parker et al. 2012). Both tissue types are also similarly vital to plant function and survival (Blossey and Hunt-Joshi 2003, Wurst et al. 2008), and potentially equally vulnerable to stress (Kaplan et al. 2008), so they should be similarly defended (Feeny 1976); however these hypotheses have not been tested. Secondly, it is not clear how seasonal changes may impact this relationship. Roots and leaves may not be equally vulnerable across the growing season, as priorities for growth, or lifecycles of enemies may alter the relative vulnerability or value of roots versus leaves over time. In this chapter, I test these hypotheses using a survey of condensed tannin concentrations in the roots and leaves of individual aspen stems across three time points.

In chapter three, I will address the issue of drivers of variation in aspen chemistry at the landscape scale (dark green lines, Fig 1-1). Previous studies have suggested that concentrations of both condensed tannins and phenolic glycosides in aspen are strongly controlled by genetic factors (Hwang and Lindroth 1997, Osier and Lindroth 2001, Rubert-Nason et al. 2015). Coupled with the clonal nature of aspen (Kemperman and Barnes 1976) and the existence of discreet aspen stands in the aspen parkland (Bird 1961) this suggests that the concentrations of phenolic glycosides and condensed tannins should be relatively similar between trees close together/within the same stand, as they are most likely clones. Research has also indicated that concentrations of trembling aspen secondary chemicals can change in response to factors such as water availability (Kanaga et al. 2009), ambient temperature (Jamieson et al. 2015), light availability (Osier and Lindroth 2006), and age (Donaldson et al. 2006b). Accordingly, the naturally occurring variation in these factors across a landscape should also be reflected in aspen secondary chemical concentrations. However, it is difficult to make predictions about how various environmental factors might interact to influence aspen chemical patterns within and among populations of aspen trees that may be single clonal organisms or groups of clones. In this chapter, I use a chemotyping approach, where secondary chemical concentrations in individuals are used to cluster those individuals into groups with similar chemical profiles. This approach results in a single response variable (chemotype) that represents an individual's chemistry that can be used to test my earlier hypotheses: to what extent do geographic variation or environmental factors impact aspen secondary metabolites? If one is more influential than the other, this will help guide broader understanding of the relative importance of genetic or environmental factors in impacting aspen secondary chemistry under natural conditions.

Impacts of plant chemistry on ecological processes

In chapter four, I test for a relationship between aspen secondary chemistry and plant community composition (light blue dashed line, Fig 1-1). Both the concept of biotic filters (Götzenberger et al. 2012, HilleRisLambers et al. 2012) and the extended phenotype (Dawkins 1982, Whitham et al. 2003) suggest that if we know how a dominant plant's traits might impact a community, and we know how those traits vary, we should be able to predict community outcomes. Both of aspen's major secondary chemical groups, condensed tannins and phenolic glycosides, have the potential to impact community composition through their impacts on the mineralization of soil nitrogen. These chemicals may enter the soil solution through the breakdown of senesced tissues (especially litter) or leaching (McClaugherty 1983, Hättenschwiler and Vitousek 2000). Once in the soil system; condensed tannins may reduce nitrogen mineralization rates (Basaraba and Starkey 1966, Rice and Pancholy 1973, Northup et al. 1995, Hättenschwiler et al. 2003, Schweitzer et al. 2004) by negatively impacting the activity of soil micro-organisms (Hättenschwiler and Vitousek 2000). Phenolic glycosides stimulate microbial activity, which may also result in a net loss of available nitrogen (Schimel et al. 1996). Lowered soil nitrogen availability should increase species richness and evenness (Thomas et al. 1999, Gilliam 2006), and greater variability in aspen chemistry could lead to greater heterogeneity, which is also associated with increased richness and evenness (MacArthur 1965, Violle et al. 2012). In this chapter, I will use a survey of aspen secondary chemistry and associated understory community composition to test if changes in overstory chemistry have an impact on understory community composition, and if those changes are in the direction expected given what we know about aspen chemical impacts on soil nitrogen.

In chapter five, I address the role of trembling aspen chemistry in impacting aspen encroachment into grassland habitats (dark blue dashed line, Fig. 1-1). The encroachment of woody plants into grasslands is increasing worldwide (Knapp et al. 2008, Wigley et al. 2010, O'Connor et al. 2014, Venter et al. 2018), including in the aspen parkland (Bailey and Wroe 1974, Scheffler 1976, Young et al. 2006). Encroachment often results in changes to plant species composition (Lett and Knapp 2005), loss of habitat for endangered species (Pringle et al. 2009, Albrecht et al. 2016), changes in carbon dynamics (Jackson et al. 2002, Knapp et al. 2008, Barger et al. 2011), and loss of plant species diversity (Ratajczak et al. 2012). It also reduces available pasture for cattle, which is of concern to landowners (Jones 1983). Invasive species are also increasingly common (Vilà et al. 2011, Pyšek et al. 2012), and may facilitate or hinder woody encroachment by altering biotic and abiotic conditions (solid dark blue line, Fig. 1-1) (Simberloff and Von Holle 1999, Liao et al. 2008, Pyšek et al. 2012) . In this chapter, I use smooth brome (*Bromus inermis* Leyss.), an invasive grass that is increasingly widespread across the aspen parkland (Otfinowski et al. 2007), to test for the impact of invasive species on woody plant encroachment. In the parkland, aspen encroachment may be further facilitated by a positive plant-soil feedback from the secondary chemicals present in aspen litter. The reduction in nitrogen mineralization caused by aspen secondary chemistry could provide a pool of organic nitrogen that only aspen can access (Schweitzer et al. 2008). As aspen stands are interspersed with grassland areas in the parkland, litter falling on adjacent grasslands may facilitate the expansion of existing aspen stands. However, the amount of litter that falls outside aspen stands, and its ability to impact aspen seedling survival and growth, in invaded or native habitats are unknown. In this chapter, I use a randomized block design to test this hypothesis by adding aspen litter to aspen seedlings planted in invaded and uninvaded habitats.

Together these chapters address two separate components of my overarching hypothesis; that variations in the factors that drive plant secondary chemistry may also have the potential to indirectly act as drivers of ecological processes through the mechanism of plant secondary chemistry (brown dotted lines, Fig. 1-1). This research is important as the frequency or intensity of many of the generally accepted drivers of plant secondary chemical variation, such as insect outbreaks, drought, or extreme temperature are expected to change due to increasing climate change (Dale et al. 2001). Restoration efforts that involve widespread species plantings may alter the natural patterns of genetic diversity in these systems (Gibson et al. 2017, Gibson and Nelson 2017). These changes may have hidden, cascading impacts on ecosystem processes through the mechanism of plant chemistry, however this issue is rarely addressed in the literature. In this thesis I hope to address that gap, which may enable land managers to better protect or restore natural ecosystems.

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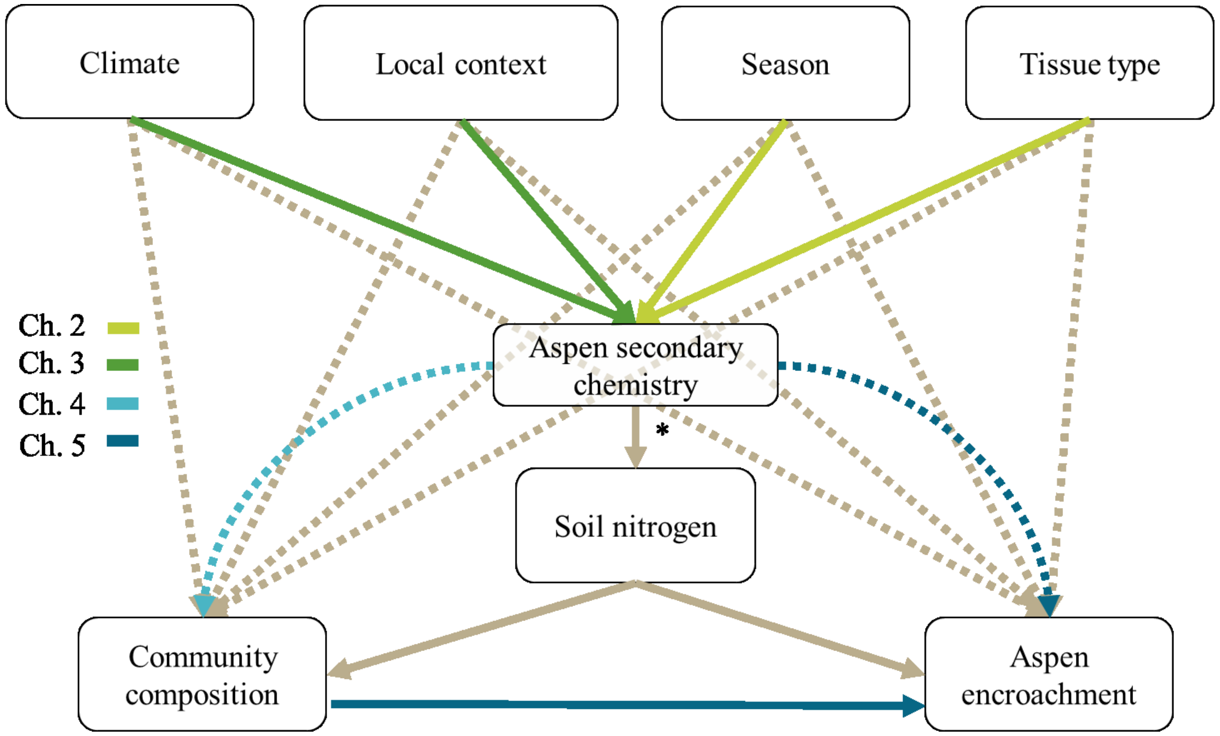

* Schweitzer et al, 2004, Schimel, 1999 and others

Figure 1-1: Conceptual diagram showing linkages between the elements of this thesis. Solid lines indicate direct impacts, broken lines indicate indirect impacts. Coloured lines indicate linkages tested in this thesis, colour coded by chapter. Brown lines indicate linkages discussed in this thesis, but not explicitly tested.

Chapter 2: Root condensed tannins vary over time, but are unrelated to leaf tannins

Abstract

Although the negative effects of root herbivores on plant fitness are expected to be similar to those of aboveground herbivores, the study of belowground plant defences is limited compared to the rich literature on aboveground defences. Current theory predicts that concentrations of defensive chemicals above and belowground should be correlated, as the evolutionary drivers that shape plant defence are similar across the whole plant. We conducted a field study to measure condensed tannin concentrations in *Populus tremuloides* roots, and determine how they relate to condensed tannin concentrations in leaves, position within the stand (edge vs. interior), tree size, or throughout the growing season. Overall, tannin concentrations were substantially lower in roots than leaves. At individual sampling periods, root and leaf tannin concentrations were not correlated, and did not vary with stand position or size. Across the growing season, both root and leaf tannin concentrations showed similar trends, with high concentrations early summer that declined through mid-summer and fall. These results suggest that the mechanisms that influence leaf and root tannin levels in aspen are independent within individual stems, possibly due to different evolutionary pressures experienced by the different tissue types or in response to localized (roots vs. foliage) stressors. However across individual stems, the similar patterns in chemical defence over time, independent of plant size or stand position indicate that larger scale processes can have consistent effects across individuals within a population, such as the relative investment in defence of tissues in the spring versus the fall. Overall, we conclude that using theories based on aboveground defence to predict belowground defences may not be possible without further studies examining belowground defence.

Introduction

The question of how plants defend aboveground tissues has received substantial research attention (Agrawal and Fishbein 2006, Chen 2008, Mithöfer and Boland 2012, Fürstenberg-Hägg et al. 2013), grounded in a well-developed body of theory (Loomis 1932, McKey 1979, Rhoades 1979, Bryant et al. 1983). However, our understanding of how plants defend themselves belowground is limited more limited than our understanding of aboveground defenses (Moore and Johnson 2017) and additional studies are needed (Barbehenn and Constabel 2011). We have only begun to apply theories of aboveground plant chemical defence to belowground tissues (Kaplan et al. 2008, Rasmann and Agrawal 2008), and the extent to which belowground defence may require new or modified theory is poorly understood, particularly in the context of belowground chemical defences (Rasmann and Agrawal 2008). This is a striking gap, as the few studies that have compared the effects of above- and belowground herbivory on plants indicate that they can have equivalent fitness consequences for the entire plant (Blossey and Hunt-Joshi 2003, Wurst et al. 2008), and are similarly vulnerable to stress (Kaplan et al. 2008).

Key to developing a theory to understand whole-plant chemical defence strategies will be more empirical data characterizing root and shoot defensive traits within individual plants. A first prediction would suggest that since plants in theory experience similar evolutionary pressures above- and belowground, chemical defence allocation would then be similar across the whole plant (Kaplan et al. 2008, Parker et al. 2012). However, evidence from non-defence related root and leaf traits suggests that there is no general pattern in plants; some are similar across the whole plant, and some are tissue specific (Craine et al. 2001, 2005; Ryser 2006; Kembel and Cahill 2011; Fujii et al. 2016). Complicating the issue is that many chemical defences within species are highly variable due to prior encounters with natural enemies (Chen

2008), plant age (Bowers and Stamp 1993, Donaldson et al. 2006b), local conditions (Veteli et al. 2002, Najar et al. 2014), and seasonality (Osier et al. 2000a). Whether root and shoot defensive chemistry changes in concert or independently in response to these basic ecological factors is unclear (Wurst et al. 2008).

The defensive chemistry of members of the genus *Populus* has been very well studied, in particular trembling aspen, *Populus tremuloides* (Michx.), provides a robust foundation upon which to test ideas related to leaf-root defence integration (Lindroth and St. Clair 2013). Trembling aspen produces two major groups of secondary chemicals, phenolic glycosides and condensed tannins (Lindroth and Hwang 1995, Barbehenn and Constabel 2011), concentrations of both can be quite high, though neither is consistently more abundant than the other. Condensed tannins are class of carbon based anti-nutritive defensive chemicals that despite their high concentrations and up-regulation following defoliation events are not considered to significantly affect the performance of lepidopteran herbivores in aspen (Lindroth and St. Clair 2013), though they have been indicated to reduce food quality (Lindroth et al. 1999). Condensed tannins also negatively impact fungal endophytes in *Populus* hybrids (Bailey et al. 2005) and reduce protein digestion in mammalian herbivores of various plant species (Barbehenn and Constabel 2011). Condensed tannins may also play a role in nutrient cycling in aspen forests, although there have been few empirical tests (Schweitzer et al. 2008, Madritch and Lindroth 2015).

Despite our mixed understanding of their ecological significance, aspen allocate a substantial portion of resources to the production of condensed tannins in leaves; up to 20% of dry leaf weight (Lindroth and Hwang 1995). Aspen leaf chemistry is also highly plastic, and is affected by many factors including plant age (Donaldson et al. 2006b), leaf age (Osier et al.

2000a), drought (Greitner et al. 1994, Roth et al. 1997, Kanaga et al. 2009), herbivory (Lindroth and Hwang 1995, Major and Constabel 2007, Lindroth and St. Clair 2013), shading (Lindroth and Hwang 1995, Hemming and Lindroth 1999, McDonald et al. 1999, Agrell et al. 2000, Osier and Lindroth 2006), competition (Donaldson et al. 2006a), and nutrient availability (Donaldson et al. 2006a, Osier and Lindroth 2006, Najar et al. 2014). In contrast, aspen root chemistry is less well understood; only two studies have been conducted, both on young aspen, showing that condensed tannin levels occur at levels around 4-5% of dry root weight, and decrease in response to increased nutrient availability (King et al. 2005, Stevens et al. 2014). Studies investigating condensed tannin concentrations in aspen roots in either naturally occurring or mature trees are as far as we can tell, absent from the literature.

In this study we use leaf and fine root tissue from mature, naturally occurring aspen to answer the following three questions; 1. Within a single aspen stem, are root tannin levels correlated with leaf tannin levels? 2. How do root and leaf tannins vary with time, stand position (edge vs interior), and stem diameter? 3. Is the ratio of root to leaf tannins within a single stem consistent over time?

Methods

Location and Sampling Design

This study took place at the Roy Berg Kinsella Research Ranch in central Alberta, Canada (53.08532 N, 111.5636 W). The ranch is located in the aspen parkland ecoregion, and is made up of a mixture stands of trembling aspen and rough fescue grassland (Lamb et al. 2009). The aspen parkland is at the edge of suitability for aspen, and these stands are small and fairly uniform, with similar levels of light penetration at the edge and interior of a stand.

In June 2015, we identified three stands of trembling aspen a minimum of 35m apart with no evidence of aspen suckering in the grassland areas between the stands. We sampled from several stands as aspen is a clonal species that propagates through root suckering, and many of the individual aspen “trees” in an stand could be clonal ramets of the same genet, which can cover large areas and live for thousands of years (Mitton and Grant 1996). This study focuses on the relationship between leaf and root tannins in naturally occurring aspen, so the frequent approach of using an artificially created common garden with propagated clones would not be feasible, despite the advantage of controlling for genetic relatedness between individuals. Using naturally occurring aspen, despite our best efforts to sample aspen in different stands, and different parts of stands, we do not know how the aspen we sampled are related. Therefore, we will use the term stem to refer to our sample individuals throughout this paper, as opposed to tree (which suggests no genetic relationship) or ramet (which suggests a clonal relationship). Within each of our identified stands, we selected six aspen stems, stratified into three pairs, spread around the perimeter of the stand. These pairs consisted of one stem from the edge of the stand and one from the interior. This allowed us to examine the effects of stand position, which may have differing light levels, on tree chemistry. We defined edge as stems within one metre of the adjacent grassland, with the interior as a minimum of six metres from the edge stem towards the centre of the stand. All 18 stems were between 3.5 and 9cm diameter at breast height.

To compare condensed tannin levels in different tissue types, we collected both leaf and fine root tissue from each individual stem. For the leaves, 5-10 mature leaves with no visible evidence of disease or damage were collected from the mid-crown of the stem using a pole pruner. For roots, approximately 5g of fine roots were collected by digging around the base of the focal stem until we found a lateral root, and then following this until we located fine root tissue to ensure the collected root tissue originated from the focal stem.

To examine the effect of season on tannin concentrations, leaf and root tissue from the 18 focal stems were re-sampled on August 13 and October 13 in the same manner as described above. Including the initial June sampling, these three sample periods were selected to align with the end of the early growing season (post leaf flush), mid growing season, and leaf senescence, respectively. To control for induction of secondary chemicals due to previous samplings, a new neighbouring stem, nearest in proximity and size to each focal stem, was also selected at each subsequent sampling as a control tree, bringing the total number of stems sampled to 54.

Across all sampling, stems were visually surveyed for health and stems with visible foliar or trunk damage were excluded from the study.

Chemical Analysis

Samples were placed on ice and transported to -80°C storage the same day as harvest. They were lyophilized for 48h, and ground using 2mm beads in a Qiagen TissueLyser II (Qiagen, Hilden, Germany) with bead mill at 30 rpm. The HCL-butanol method (Hagerman and Butler 1980, Porter et al. 1985) was used to analyse both leaf and root samples for condensed tannins. Ground tissue was weighed to within 5% of 30mg and the exact weight was recorded. This ground tissue was then extracted twice using 70% acetone with ascorbic acid. For aspen leaf tissue, initial tests showed that the concentration of condensed tannins in the supernatant was above the detection limit of the spectrophotometer, so the leaf tissue supernatant was diluted to 20%. Sample extracts were then reacted with the butanol-HCL reagent (5% 12M HCl in 95% butanol) as well as a solution of 2g ferric ammonium sulfate dissolved in 100ml of 2M HCl as recommended by Porter *et al.* (1985). The ratio of reagents was 5:30:1 (sample supernatant:

butanol-HCL: ferric solution). This sample was left to react for 50 min at 95°C, then absorbance at 550nm was measured using a Bio-Tek PowerWave XS Spectrophotometer (BioTek Instruments, Inc., Winooski,VT, USA). Condensed tannins were purified from leaves of natural trembling aspen collected on the University of Alberta campus (approximately 150 km from the study site) following the method of Hagerman and Butler (1980) and used as standards. The spectrophotometer readings were converted into milligrams of tannins per gram of tissue using a standard curve, with each sample value adjusted for initial sample weight and any dilution.

Statistical Analysis

To determine if previous sampling affected current chemistry we compared the focal stems at each time point to the paired sampling control stems using an ANOVA. To determine if leaf and root condensed tannin levels were correlated, and if time, stand position, and stem size influenced root chemistry, a linear mixed model was run with root condensed tannin concentrations as the response variable, while fixed factors were leaf tannin concentrations, season (as a repeated measure), diameter and stand position (edge or interior). Edge and interior pairs, nested within stand, served as a random effect. To determine if time, stand position, and stem diameter influenced leaf chemistry, a linear mixed model was run with leaf condensed tannins as the response variable, while fixed factors were season (as a repeated measure), size and stand position, with edge and interior pairs nested within stand as a random effect. To determine if leaf and root tannins co-varied over time, the ratio of root:leaf (R/L) tannins was calculated and analysed using a mixed effect model, with season (repeated measure), position in stand and size as fixed effects and pairs nested within stands as a random effect. Statistics were done in SPSS v. 25 (IBM Corp. 2016), graphs were created in Sigma Plot v. 11 (Systat Software Inc 2008).

Results

The sampled control stems were not significantly different from paired focal stems (df=1, F=0.062, p=0.980), which indicated that intervals between our repeated sampling points were long enough to avoid capturing any effects of induction due to previous sampling in our later time points.

Root tannin levels were not correlated with leaf tannin levels within a stem (Table 2-1, Fig. 2-1). Stem diameter and stand position did not affect root or leaf tannin concentrations (Table 2-1, see also Fig. 2-4 for root and leaf tannin levels graphed by time, stand and stand position). Time altered both root and leaf condensed tannin concentrations, with the highest concentrations of both occurring in June, dropping in August, and then rising slightly in October for leaves though tannin concentrations in roots stayed consistent from August to October (Table 2-1, Fig. 2-2).

The ratio of root to leaf tannins in a single stem was not consistent over time, with more allocation to roots relative to leaves in June as compared to August or October (Table 2-2, Fig. 2-3).

Discussion

We found that absolute concentrations of leaf and root tannins were different within sampled aspen stems (roots contained approximately 85% less tannins than leaves), which is consistent with existing literature (Kosola et al. 2004, 2006, King et al. 2005, Stevens et al. 2014). We also found that relative concentrations of condensed tannins in roots were not correlated with leaf concentrations from the same stem, and that the ratio of root to leaf tannins within a stem was not consistent over time. Root tannin concentrations were highest relative to leaves in June, and lower in August and October.

These results address a longstanding hypothesis in ecology that root and leaf defensive traits should be similar as both tissue types experience similar levels of herbivory and are similarly crucial the survival of a plant (Kaplan et al. 2008; Kembel and Cahill 2011; Parker et al. 2012). Our finding that condensed tannin concentrations were not linked between leaves and fine roots, and that relative concentrations of condensed tannins in leaves and roots may change over time disagrees with the hypothesis outlined above. Parker et al. (2012) suggested that comparing patterns of plant chemistry in above- and belowground tissues can also indicate the strength of forces that drive natural selection of plant chemical traits. Our results indicate that the evolutionary pressures experienced belowground may be weaker, as they have not selected for the same high concentrations of condensed tannins found aboveground.

The predictions made by Kaplan et al. (2008) are based on the optimal defence theory (McKey 1979, Rhoades 1979), however despite our results this theory might still apply belowground if tissues are differently vulnerable than aboveground tissues, or that the defences in aboveground tissues may not function in the same way to impact belowground herbivores. Therefore, we suggest additional research to determine if belowground tissues are less vulnerable to herbivory, or to show that defence chemicals work differently to negatively impact belowground herbivores.

We also found that there was as much variation in condensed tannin production within a stand, which is possibly one large clonal organism, as there was between stands. If a stand represents one large clonal organism, then this result contrasts previous data that shows that variation between clones is higher than variation within clones (Donaldson and Lindroth 2007). It is also possible that the variation we see within stands suggests that the stands are not in fact single clones, but several individuals together, which would put this finding more in line with existing research.

Our finding that, across sampled stems, leaf condensed tannins are highest in the early summer with lower levels later in the season agrees with the published results on aspen leaf chemistry (Lindroth and Hwang 1995, Roth et al. 1998, Osier et al. 2000a). Stem diameter and stand position were not significantly related to leaf tannins of aspen sampled in this study. Osier et al. (2000a), also following the rationale of optimal defence theory, suggested that a slight decrease in food quality that comes with elevated aspen condensed tannin concentrations could account for higher observed levels of condensed tannins during June, when population densities for aspen herbivores are at their peak (Parry et al. 1998). Our observed pattern could also be driven by changes in resource availability from stored resources which can influence poplar defensive chemistry (Najar et al. 2014) or changes in seasonal allocations patterns between growth and defence. The slight increase in leaf tannins in October also agrees with work suggesting that autumn cold was related to an increase in the concentration of tannins in poplars (Li et al. 2011), as well as observations that intense frost-defoliation can cause increased levels of condensed tannins in aspen foliage (St. Clair et al. 2009), which can be explained by studies indicating that phenolic compounds can aid in cold tolerance (Koyama et al. 2014).

While previous studies have indicated that aspen leaf tannins can vary over a growing season, to our knowledge none have examined how root tannin levels vary across the same time period. Our observed pattern that across all stems, fine root condensed tannins concentrations changed over the growing season, with highest levels in June and lower levels in August and October suggests that there might be some population level factor that is the driver of this pattern. Of the major belowground herbivores of aspen present in Alberta, ghost moth (*Sthenopis* *purpurascens*, Lepidoptera: Hepialidae), aspen root girdler (*Agrilus horni*, Coleoptera: Buprestidae) and poplar-butt borer (*Xylotrechus obliteratus*, Coleoptera: Cerambycidae) only poplar-butt borer exhibits feeding belowground during the early part of the growing season (Nord et al. 1965, Flaim and Platt 1999, Steed and Burton 2015), however these herbivores are severely understudied. Of the three, only ghost moth was observed at our site during this experiment, though not on any focal stems. There are also root pathogens of aspen, such as *Armillaria* sp., however these were not observed at the study site. Similar to aboveground tissues, resource availability could also play a role in the observed belowground seasonal pattern (Najar et al. 2014).

Conclusion

Our observation that root condensed tannin concentrations were not related to leaf condensed tannins within individual aspen trees indicates that aspen leaf and root defensive traits may not be strongly related in individual aspen. This suggests separate evolutionary drivers above and belowground. Similar patterns in roots and shoots over time, however, with highest levels in June and lower levels in August and October suggest that at the population level, there may be similar environmental drivers for population-level patterns. Overall more studies of leaf and root defensive traits are needed to clarify the extent to which selection acts at the whole plant level for these traits, or independently for different tissues. Our general finding that fine root condensed tannins were different from leaf condensed tannins suggests that the theories currently in use for aboveground plant defence may need some tweaking before they can be widely used to predict belowground traits.

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Table 2-1: Leaf and root condensed tannin levels as a function of time, stand position and tree diameter. GLMM models were used to test for the effects of listed factors on leaf and root condensed tannins**.**

	Root Condensed Tannins			Leaf Condensed Tannins		
Factor	df	F	p	df	F	p
Leaf Condensed Tannins	1	0.111	0.741	-	-	-
Time	2	12.726	0.0001	2	4.59	0.016
Stand Position	1	0.328	0.569	1	0.280	0.599
Tree Diameter	1	0.126	0.724	1	0.133	0.717

Table: 2-2: Ratio of root:leaf condensed tannins as a function of time, stand position and tree diameter. A GLMM model was used to test for the effects of the listed factors on root:leaf ratio.

	Root:Leaf Ratio of Condensed Tannins		
Source	df	F	p
Time	2	6.904	0.002
Stand Position	1	0.111	0.597
Tree Diameter	1	0.481	0.972

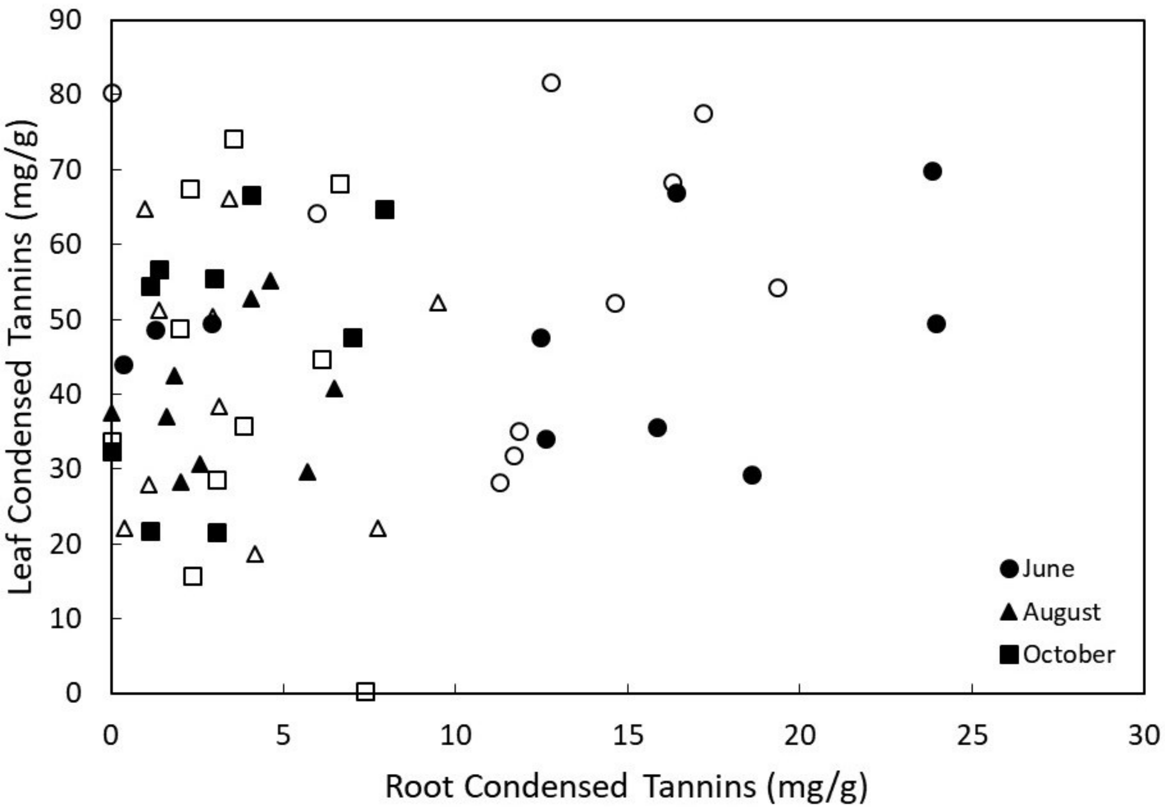

Figure 2-1: Root versus leaf condensed tannins (mg/g tissue) for individual *Populus tremuloides* stems. Symbol shape denotes the time-period during which the tissue was sampled, filled symbols (●) indicate stems in the interior of the stand and open symbols (○) stems in the edge of the stand.

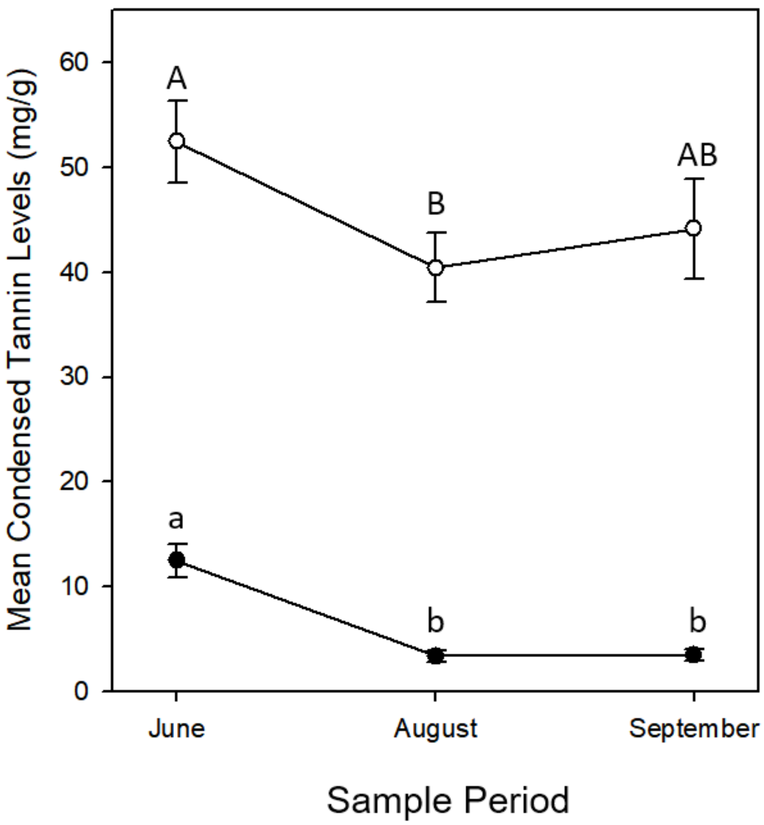

Figure 2-2: Average condensed tannin levels (mg/g) for roots (●) and leaves (○) across three time points in *Populus tremuloides* (means ±SE). Capital letters indicate significant differences ($p<0.05$) between leaf values, and lowercase letters differences between root values.

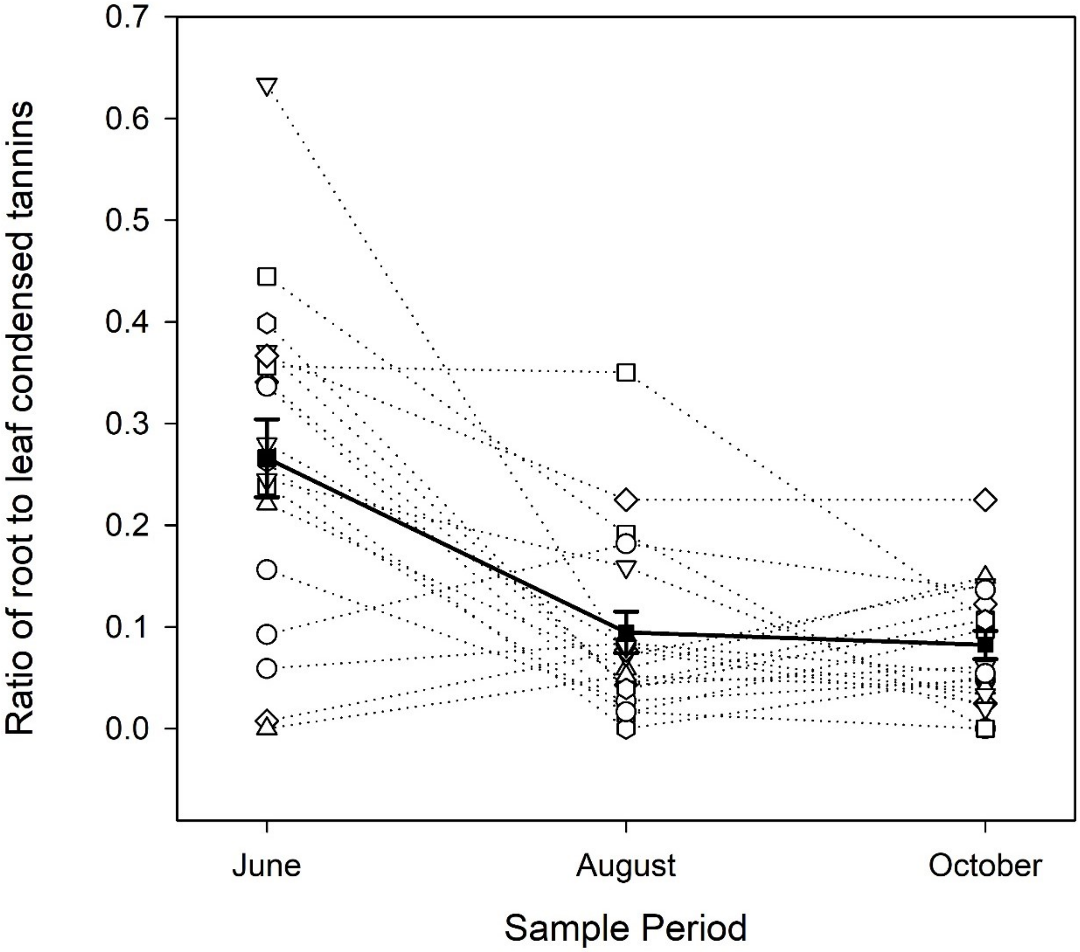

Figure 2-3: Ratio of fine root condensed tannins (mg/g tissue) to leaf condensed tannins (mg/g tissue) in *Populus tremuloides* collected over three time points. The unfilled points represent individual stems, with samples from the same stem connected with dashed lines. The filled markers (■) and solid line represent the mean ratio for each time point, with error bars indicating ±SE.

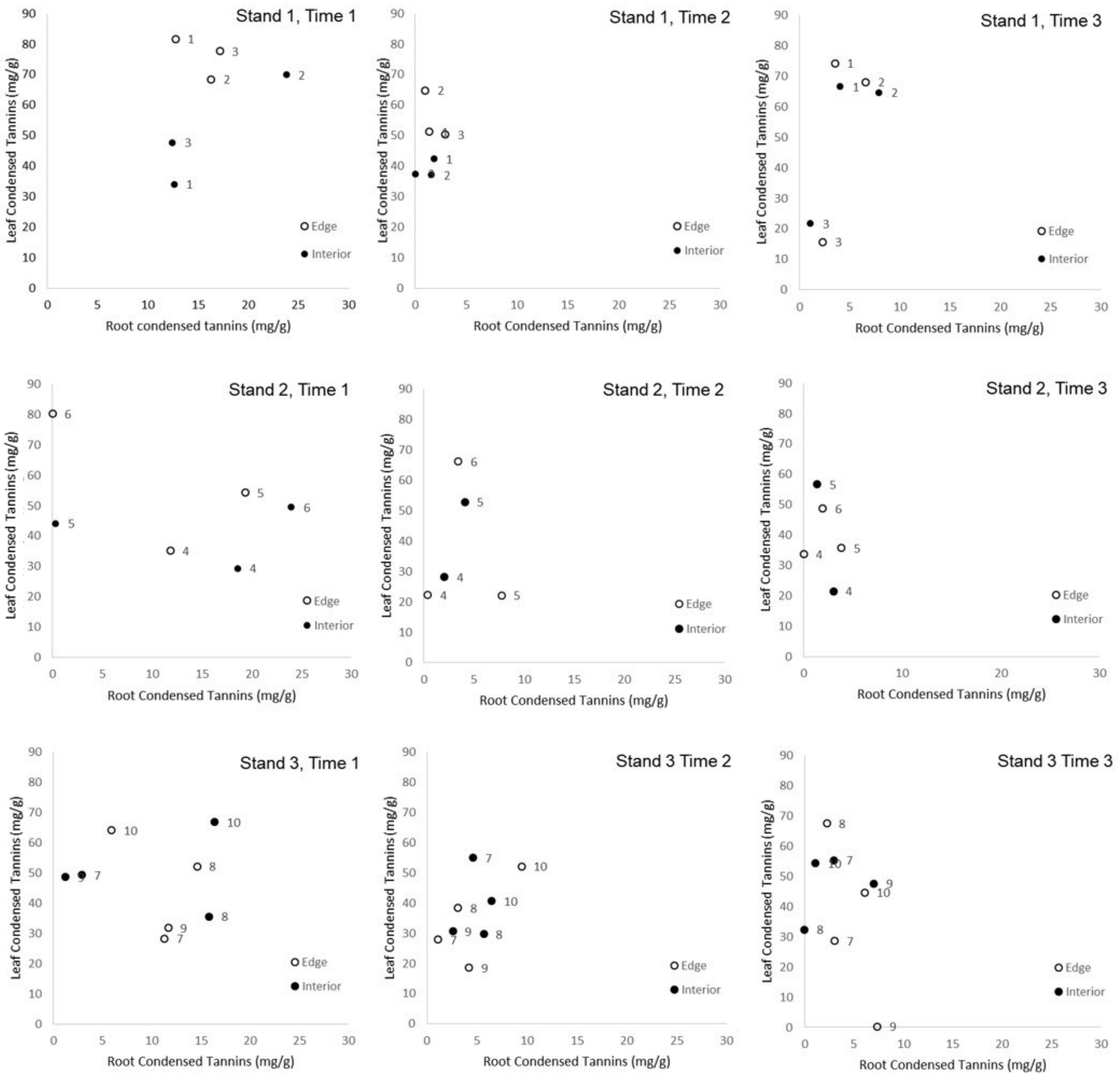

Figure 2-4: Fine root condensed tannins versus leaf condensed tannins for each stand and time period, with edge and interior trees separated by colour. The numbers next to each point indicate matching pairs of edge and interior trees.

Chapter 3: Patterns in aspen secondary chemical phenotypes suggest local plasticity

Abstract

Plants produce different classes of secondary chemicals simultaneously, and characterizing the production and distribution of these chemicals is crucial to our understanding of how plants respond to external processes over a heterogeneous landscape. However, it can be difficult to get a clear understanding of the entire chemical profile of plants. Thus, determining if these chemicals fall into broader chemotypes can address this issue, and measuring them on many individuals across a large area can allow us to determine whether regional or local processes drive chemotype patterns, which will impact local chemotype diversity. Using a survey of natural aspen stands across central Alberta we asked if aspen chemotypes were present, how they were distributed, and if they were associated with regional climate variables, stand structural characteristics, or individual tree measurements. We identified two aspen chemotypes; one characterized by high concentrations of condensed tannins and one with high phenolic glycosides. Despite predictions in the literature that aspen stands should have low chemotype diversity due to clonal growth patterns, more than 50% of stands exhibited both chemotypes. Neither regional climatic factors nor individual tree measurements were associated with particular chemotypes. In contrast, location of stems within the stand, as well as an interaction between location, canopy openness and stand height best explained the chemotype patterns observed. These results suggest that plasticity in response to local processes is likely the major driver of aspen chemotypes, though further research in field conditions is needed to confirm this link.

Introduction

Secondary chemicals are one of the key mechanisms through which plants mediate interactions with their environment (Seigler 1998), thus the composition and concentrations of these chemicals within plants can be used to infer much about a species' life history (Rhoades 1979). Accordingly, much of the research in plant secondary chemicals focuses on the identification of biologically relevant chemicals, the description of factors that influence their concentrations, and their roles in mediating interactions of plants with other organisms and the environment. This approach has provided us with a great breadth of understanding of plant secondary chemicals (Rosenthal and Janzen 1979, Karban and Baldwin 1997). Currently, we lack understanding of how different groups of chemicals a plant produces interact, and how they are distributed within and between populations of plants growing under natural conditions.

One common technique to address this is to identify chemically distinct parts of a population of morphologically similar plants (generally referred to as a chemical phenotype or "chemotype" (Santesson 1968, cited in Keefover-Ring et al. 2009)). By quantifying the concentrations of individual chemicals within and between populations, we can use a clustering approach to classify them into chemotypes, and therefore obtain a single response variable that reflects a plant's secondary chemistry. These chemotypes can then be used to understand complex chemical profiles that may have ecological implications different from the roles of individual chemicals that make up that chemotype (e.g., Taft et al. 2015). After chemotypes are identified, we can also map their spatial distribution and examine what factors have likely influenced this distribution. If the scale of chemotype diversity matches the structure of focal species' populations, including clonal properties, this could indicate genetic control of chemotypes. Alternatively, if plants show plasticity over a different spatial scale, then we would expect that they could vary due to a variety of environmental factors operating at different scales (e.g., regional such as climate, latitude or local such as resource availability or microclimate).

Despite the fact that the secondary chemistry of trembling aspen (*Populus tremuloides* Michx.), is well-characterized , and they produce different classes of secondary chemicals, chemotypes have rarely been used in studying poplars (but see Keefover-Ring et al. 2014). In general, aspen secondary chemicals fall into two major groups; condensed tannins and phenolic glycosides (Lindroth and St. Clair 2013). Condensed tannins can have variable structures (Schweitzer et al. 2008), but due to the difficulty of separately quantifying these structural types they are typically grouped together in aspen (but see Rubert-Nason and Lindroth 2019). Aspen primarily produce four phenolic glycosides; salicin, salicortin, tremuloidin, and tremulacin which can be quantified individually (Lindroth and Hwang 1995).

Research has suggested a variety of ecological roles for aspen's secondary chemicals. Historically condensed tannins have been considered as defence chemicals with an anti-nutritive effect on herbivores (Fraenkel 1959) due to their ability to bind with proteins and reduce digestibility. However evidence for these negative effects in experimental conditions is not clear, with studies showing opposing results (Osier and Lindroth 2001, Donaldson and Lindroth 2004) depending on the herbivore species. Condensed tannins have also been shown to contribute to frost hardiness by acting as anti-ice nucleating agents (Koyama et al. 2014) and to help limit UV damage (Lavola 1998, Mellway and Constabel 2009). Condensed tannins, particularly those produced by *Populus* species, have also been shown to impact soil nutrient cycling by reducing nitrogen mineralization in the soil (Hättenschwiler and Vitousek 2000, Schweitzer et al. 2004), potentially creating a pool of nitrogen available only to aspen (Schweitzer et al. 2008, Madritch and Lindroth 2015). Unlike condensed tannins, phenolic glycosides have a more consistently negative impact on insect herbivores (Hwang and Lindroth 1997, Osier et al. 2000b, Donaldson and Lindroth 2007), as well as inhibiting plant pathogens (Kruger and Manion 1994) and mammalian herbivores (Bailey et al. 2007). They can also impact soil nutrient availability by increasing the uptake of nitrogen by microorganisms, reducing the pool of available nitrogen to plants (Schimel et al. 1996).

Like most plants, the production of different classes of secondary chemicals in aspen is controlled by a variety of factors. There is consistent evidence for substantial genetic control of both phenolic glycosides and condensed tannins, though the genetic component of phenolic glycoside production is frequently found to be greater than that of tannins (Hwang and Lindroth 1997, Osier and Lindroth 2001, Rubert-Nason et al. 2015). Because aspen typically grows in clonal stands (Mitton and Grant 1996), the strong genetic component of their chemistry should lead to a homogeneity of chemotypes within naturally occurring aspen stands. However, like many other plants, concentrations of secondary chemicals in aspen foliage can also change in response to environmental factors such as water availability (Kanaga et al. 2009), ambient temperature (Jamieson et al. 2015), light availability (Osier and Lindroth 2006), and age (Donaldson et al. 2006b), which would suggest that aspen chemotypes should also vary in response to these factors. However, the majority of studies on aspen chemistry has been performed under common-garden or greenhouse conditions and there is little evidence for these factors being associated with variation in aspen chemical concentrations under field conditions. These environmental factors also vary at differing spatial scales under field conditions, thus if they do impact aspen chemistry, it is difficult to make predictions about how they might interact to influence aspen chemotype patterns within and among populations of trees that may be single clonal organisms or groups of clones.

In this study, we use a survey of aspen foliar chemistry throughout a landscape (36,200km^2) in central Alberta to answer three questions; 1) Do natural populations of aspen exhibit different chemotypes based on the concentrations of condensed tannins and phenolic glycosides? 2) What is the distribution of these chemotypes across spatial scales? 3) Is there evidence of chemotypic plasticity in response to environmental factors across spatial scales?

Methods

Experimental Design.

To investigate aspen chemistry at a landscape scale, we chose ten sites across central Alberta that covered the main portion of the aspen parkland within the province. The final site locations were distributed within a 36,200km^2 region, with sites a minimum of 48km apart; this distance makes it highly unlikely that single genets were found in multiple sites, for further site details see Appendix 3-1. At each site we identified two aspen stands a minimum of 40 m apart and established three to four 50m^2 circular (3.99m radius) plots in each stand (total of 10 sites, 20 stands, and 78 plots surveyed) to examine variation within and among stands and sites. Plots were located at the edge of the stand as well as in the interior to allow us to test for differences in chemical composition between edge and interior trees, which experience different environmental conditions such as light, soil moisture and ambient temperature. Edge plots were placed so they included the last trees within the stand before it transitioned to grasslands, interior plots were placed so there was a minimum of 5 meters between the boundaries of the plots, following a line into the stand perpendicular to the forested edge. To allow us to test for the influence of environmental variables on aspen chemotypes, we used Climate WNA (Wang et al. 2016) to calculate 25 year averages of mean annual temperature, mean annual precipitation, average summer temperatures, May-September precipitation, precipitation that fell as snow and climatic moisture deficit for each site (details of variable calculations are given by Wang et al. 2016)

As the focus of this study was to test for the presence of chemotypes in naturally occurring aspen stands, and to determine if they were related to environmental factors, we did not test for genetic relatedness of individuals sampled in this experiment. We acknowledge the uncertainty of the genetic relationships between individuals by referring hereafter to an individual sampled in this study as a stem, as opposed to a tree (which suggests no genetic relationship) or a ramet (which suggests a clonal relationship).

Sampling for Chemical Analysis and Data Collection.

To determine the chemical composition of each stem in our plots we haphazardly collected five fully expanded leaves from the mid-crown of each stem in each plot using pole pruners, or a slingshot in June 2015 (similar to the method outlined in Youngentob et al. 2016). Tissue samples were placed on ice in the field and stored at -80° C within 24 h of collection. To keep sample size constant across all plots, we randomly chose five stems per plot for all subsequent chemical analyses. Samples from the selected stems were then lyophilized and ground in a QIAGEN TissueLyser II (Hilden, GER). The freeze dried and ground tissue was stored at -80° C until chemical analysis.

Stand characteristics were also measured to allow us to determine if factors that vary at the stand level were associated with chemotype variation. We measured density of stems within our 50 m^2 plots as a measure of competition, and took hemispherical photographs using a digital camera with fisheye set 1.5m off the ground on a tripod at the plot center to determine canopy openness as a measure of light availability. Canopy openness was calculated from the photographs using Gap Light Analyzer software (Frazer et al. 1999). We also measured stem diameters as a proxy for age to allow us to determine if attributes of the individual stems also impacted chemotype distribution. While stem diameter is not always a perfect proxy for age (r^2 =

0.75 (Ripple and Larsen 2000), $r^2 = 0.66$ (Hessl and Graumlich 2002)) time constraints did not permit coring in this study. We acknowledge that this is not a perfect metric of age, and accordingly treat conclusions based on variation in stem diameter with caution.

Chemical Analysis.

Condensed tannins were measured using the same method as Dettlaff et al. (2018), originally outlined by Hagermann (1988) with the modifications suggested by Porter et al. (1985). Briefly, we extracted condensed tannins from the freeze-dried and ground tissue using a solution of 70% acetone and ascorbic acid. The supernatant was then reacted with a butanol-HCL reagent and a solution of ferric ammonium sulfate dissolved in HCL (for colour development) for 50 min at 95° C. We then measured absorbance at 550 nm using a Bio-Tek PowerWave XS Spectrophotometer (BioTek Inst, Inc., Winooski, VT, USA). Condensed tannin standards were purified from leaves of naturally growing aspen collected on the University of Alberta campus (located roughly in the centre of the study area) following the method of Hagerman and Butler (1980).

To measure phenolic glycosides, 30 mg freeze-dried and ground tissue was added to 1.5 ml methanol and placed in an ultrasonic bath at 4° C for 30 min and then centrifuged at 13,000 rpm for 15 min. The supernatant was collected, transferred to glass chromatography vials and stored at -20 ° C. Phenolic glycoside concentrations were determined using an ultra-high performance liquid chromatograph (1290 Infinity, Agilent Tech. Santa Clara, CA, USA) fitted with a Poroshell 120 EC-C18 column (2.1 x 150 mm, 2.7 µm; Agilent Tech.) and a diode array detector (Agilent Tech.). A binary mobile phase of (A) water plus 0.1 % formic acid (v/v) and (B) methanol plus 0.1% formic acid (v/v) was run at a 0.3 mL/min at a temperature of 50º C using a gradient of 5 – 60% B over 15 min, 60% B held until 16 min, 60 – 80% B over 16 – 18 min, 80 – 50% B over 18 – 21 min, 50 – 5% B over 21 – 22 min, 5% B held over 22 – 23 min. A 2 µL injection volume was used. Quantifications were performed using compound absorbance at a wavelength of 274 nm and a standard curve calculated using serial dilutions of analytical standards. Standards of salicin, tremulacin and salicortin were obtained from APIN chemicals Ltd (Oxfordshire, UK). Tremuloidin was provided by Dr. Richard Lindroth (University of Wisconsin-Madison). For both condensed tannins and phenolic glycosides, resulting concentrations were converted into mg/g of dried tissue.

Statistical Analysis.

Concentrations of each secondary metabolite analyses were used with functions available in the R package "fpc" version 2.1-11.1 (Hennig 2018) to cluster samples into chemotypes using a partitioning-around-medoids technique (Kaufman and Rousseeuw 1990), with the number of clusters estimated by optimum average silhouette width. The specific function we used (pamk) performs a Duda-Hart test (Duda and Hart 1973) to determine if there should be more than one cluster, and identifies the number of clusters in the data from a range of user-inputted clusters in an unsupervised manner. For this analysis, we allowed a range of clusters from 1-10. For a given cluster, the mean value of each secondary chemical for all trees within that cluster was subsequently calculated. Clusters were labeled based on the compound with the greatest mean value (concentrations) within a cluster. To confirm our clustering, we performed a non-metric multidimensional scaling (NMDS) ordination in PCORD 7 (McCune and Mefford 2016) on the same matrix of chemical values used in the clustering analysis, colour coded the results based on the chemotypes identified by the clustering analysis, and visually inspected the ordination to confirm separation of the two chemotypes.

To determine how environmental factors influence chemotypes at the site level, we used the results of the cluster analysis to calculate the proportion of trees in the tannin chemotype for each site (where 0 means all stems were the phenolic glycoside chemotype, and 1 means all stems were in the tannin chemotype). Using this as our response variable, we performed a binomial logistic regression with mean annual temperature, mean annual precipitation, Hargreaves climatic moisture deficit, average summer temperature, May-September precipitation, precipitation that fell as snow, elevation, latitude, and longitude. We also used backwards stepwise regression on the same model to determine which subset of these factors would give the best fit.

To determine if attributes of stand structure were associated with certain chemotypes, we again calculated the proportion of stems in each plot that were in the tannin chemotype (as above), and used a binomial logistic mixed model, with proportion as the response variable and stand density, stand height, canopy openness, and position in the stand as fixed effects and site as a random effect.

At the individual stem level, we used a logistic mixed model to determine if stem diameter and position in the stand (interior vs. edge) were related to chemotype (here coded 0 for the phenolic glycoside chemotype and 1 for the condensed tannin chemotype), with site and plot nested within site as random effects.

Due to a concern that the relative concentrations of the four phenolic glycosides may have been affected by placing the leaf tissue on ice after collection, as opposed to liquid nitrogen, which could cause salicortin to degrade into salicin and tremulacin to degrade into tremuloidin, we also performed a cluster analysis with the measured concentrations for salicin and salicortin, and tremulacin and tremuloidin in each tree pooled together, again followed by confirmation of chemotype separation using NMDS ordination. We then used the new clustering results to test for drivers of aspen chemistry using the same logistic regression modelling approach outlined above.

For all models, we subsequently calculated type II sum of squares values for each factor. All statistical analyses were performed in R (R Core Team 2018) and graphs were created in Microsoft Excel (2013).

Results

The cluster analysis indicated that the aspen fell out into two chemotypes (Table 3-1, Fig. 3-1) and the Duda-Hart test indicated that chemotype clusters were not homogenous ($p < 0.001$). Chemotype one was characterized by high concentrations of condensed tannins, and chemotype two had high concentrations of two phenolic glycosides, salicin and tremuloidin, as well as condensed tannins. Concentrations of salicortin and tremulacin were similar between the two chemotypes. Both chemotypes occurred at all sites, in varying proportions (Fig. 3-2). At the plot level, 40 of the 78 plots contained both chemotypes, 18 plots had only the tannin chemotype and 20 plots had only the phenolic glycoside chemotype.

The proportion of occurrence of the two chemotypes at the site level was not associated with any environmental factor measured (Table 3-2). Backwards stepwise regression indicated that the best combination of variables was a model with only average summer temperature, May-September precipitation, and climatic moisture deficit as explanatory variables. A reduced model with only these three factors still showed no significant relationship with chemotype (average summer temperature $p = 0.2924$, May-September precipitation $p = 0.1658$, and climatic moisture deficit $p = 0.1964$).

While we found no strong predictor of the proportion of occurrence of different chemotypes at the plot level, the best predictor was an interaction among location within the stand (edge/interior), canopy openness, and stand height (*p*=0.072) (Table 3-2). For plots on the edge of the stand, as stand height increases, canopy openness shifts from a positive association with the condensed tannin chemotype to a negative association (i.e., to shifts towards the phenolic glycoside chemotype) (Fig. 3-3). For plots in the interior of the stand, as stand height increases, canopy openness goes from no relationship with the condensed tannin chemotype, through a negative relationship in stands between 8-11m tall to a positive relationship with the condensed tannin chemotype in stands 11-18m tall (Fig. 3-3).

At the individual aspen stem level, location in the stand was significantly associated with chemotype at an alpha level of 0.1, but stem diameter was not (Table 3-2). Trees that occurred in the edge of the stand were more likely to exhibit the phenolic glycoside chemotype, while trees in the interior of the stand were more likely to exhibit the condensed tannin chemotype (Fig. 3-4).

Re-clustering using fewer individual phenolic glycosides to address the concern that the concentrations of individual chemicals might have degraded resulted in two chemotypes similar to the initial clustering. One chemotype was characterized by high levels of condensed tannins, the other by high levels of the two pooled phenolic glycosides (Appendix 3-2, Appendix 3-3). As with the initial clustering exercise, both of the new chemotypes occurred at all sites, and 36 of the 78 50 m^2 plots contained trees from both chemotypes. None of the site level climatic variables were associated with the new chemotypes (Appendix 3-4). At the plot level, we still found no strong predictor of chemotype occurrence. Location within stand was the best predictor ($p = 0.078$) (Appendix 3-4), interior trees tended to be the condensed tannin chemotype, and edge trees the new phenolic glycoside chemotype. At the tree level, we did find that stem diameter was significantly associated with the new chemotypes (p = 0.029) (Appendix 3-4); trees in the new condensed tannin chemotype had larger diameters than trees in the new phenolic glycoside chemotype. As these results are similar to those results obtained with our earlier clustering using all four phenolic glycosides independently, we will primarily discuss the earlier clustering since it incorporates more detail about trembling aspen's secondary chemical concentrations. We have included data on this second set of clustering as supplemental information.

Discussion

We identified two chemotypes of trembling aspen across central Alberta, a condensed tannin chemotype, and a phenolic glycoside chemotype. To our knowledge, this is the first analysis documenting chemotypes in trembling aspen in naturally occurring populations. The concentrations for aspen secondary chemistry in this study were not as high as some values reported, but were well within the reported range for condensed tannins (up to 25% dry leaf weight in Donaldson and Lindroth (2008), our highest 17%, and mean 4%) and total phenolic glycosides (also up to 25% dry leaf weight in Donaldson et al. (2006b), our highest 16%, mean 3.5%).

Our finding that the mean concentrations of salicin and tremuloidin were higher than those of salicortin or tremulacin contrasts most published work on aspen chemistry (Lindroth and Hwang 1996, Najar et al. 2014). The unusually high levels of salicin and tremuloidin could be caused by degradation of salicortin and tremulacin into salicin and tremuloidin respectively (Julkunen-Tiitto and Meier 1992). Our alternative clustering approach, which addressed this issue by pooling the phenolic glycosides with degradations products, did not find substantially different results. Thus even if sample degradation did occur in this study, it likely has not impacted our results. A re-analysis of leaf tissue collected and analyzed following our original procedure of keeping leaves on ice before freezing, versus leaf tissue from the same trees placed directly into liquid nitrogen and then frozen to see if that impacted the relative concentrations of individual phenolic glycosides would also help address this question of degradation.

We found co-occurrence of chemotypes at multiple scales, with no evidence of large scale drivers of chemotype occurrence. In contrast, local factors (position within stand, stand height, and canopy openness) were associated with the proportion of the two chemotypes. Since aspen typically grow in clonal stands (Mitton and Grant 1996), we expected that aspen stems close together would exhibit the same chemotype, as genetics is expected to play a strong role in controlling the production of condensed tannins and phenolic glycosides in aspen (Hwang and Lindroth 1997, Osier and Lindroth 2001, Rubert-Nason et al. 2015). Contrary to our expectations, 50% of all plots surveyed contained both chemotypes, suggesting that environmental factors and probably their interaction with tree genetics influences aspen chemical concentrations at this scale. For example, we found that aspen stems in plots on the edge of the stand were more likely to exhibit the phenolic glycoside chemotype than the condensed tannin chemotype (Fig. 3-4). If genotypes vary at a smaller scale than the stand level, these results could be explained by environmental filtering of genotypes. Stems on the edge of an aspen stand may be more apparent to herbivory (*sensu* Feeny 1975, 1976, Rhoades and Cates 1976) and thus it would be reasonable for these trees to have higher concentrations of phenolic glycosides to defend against herbivory. However, plant apparency and related herbivory pressure is difficult to measure reliably in field (Feeny 1991, Stamp 2003), and thus this hypothesis is difficult to support more concretely.

The lack of association between our site level factors and chemotype occurrence is contrary to our predictions. As increasing water limitation is usually associated with increasing concentrations of phenolic glycosides in aspen (Kanaga et al. 2009), we expected that sites with lower rainfall or climatic moisture index should be more likely to exhibit the phenolic glycoside chemotype. Likewise, we expected that aspen stems in warmer sites would be more likely to exhibit the phenolic glycoside chemotype as increasing temperatures have been associated with decreased condensed tannins (Jamieson et al. 2015) . Even though our backwards stepwise regression did highlight that a model with climatic moisture index, May-September precipitation and mean summer temperature was the most parsimonious for explaining chemotypes at the site level, none of the terms was significant.

We found evidence supporting phenotypic plasticity in response to smaller scale factors as the possible cause of chemotypic expression at this scale. Stems on the edge of an aspen stand were more likely to exhibit a higher proportion of the phenolic glycoside chemotype and stand height and canopy openness had differing impacts on aspen chemotypes depending on the location of the plots within a stand. However, the mechanism driving this difference is not clear. Shading can reduce condensed tannin concentrations (Lindroth and Hwang 1996, Hemming and Lindroth 1999, Agrell et al. 2000, Osier and Lindroth 2006), which agrees with our finding of increased condensed tannin chemotype occurrence in more open stands for edge plots between 2.5 and 11 m tall, and interior plots 11-18 m tall. But the opposite pattern was found for edge trees 11-18m tall and interior trees between 8 and 11m tall (Fig. 3-3), and we also did not find significant effects of light availability on aspen chemotypes. Earlier studies demonstrating impacts of light limitation on aspen chemistry were generally conducted in common garden settings and typically created substantial differences between high and low light treatments (High

= 70%, Low = 15% of ambient light in Osier and Lindroth (2006), and 100% and 44% in Agrell et al. (2000)). In the current study, the mean canopy openness in our plots was 31% (SD = 11%, min = 11%, max = 60%). This relatively low openness and low variation among plots may be insufficient to observe the reported changes in plant chemotypes or light impacts on aspen parkland may be of lesser importance. It also highlights the potential differences between field and greenhouse studies.

Alternatively, aspen stem ontogeny may explain the higher abundance of phenolic glycoside chemotype as younger aspen stems tend to produce more phenolic glycosides while older trees shift to producing more condensed tannins (Donaldson et al. 2006b). In the current study, we used tree diameters as a proxy for tree age because generally larger diameter has been associated with increasing age in aspen (Ripple and Larsen 2000); however stem diameter was not related to chemotype. In the aspen parkland, stands expand outward (Bailey and Wroe 1974), and thus edge trees tend to be younger than interior trees. Likewise, shorter, denser stands are overall younger than taller more open stands (Chen et al. 1998). This agrees with our observations of more stems with the high phenolic glycoside chemotype in edge plots. These results support our plasticity hypothesis.

Regardless of the cause of their distribution, phenotypic plasticity or filtering of genotypes, the two aspen chemotypes may reflect aspen’s ability to survive in its rather large range in North America. Stems in the condensed tannin chemotype should be more susceptible to arthropod herbivores (Lindroth and St. Clair 2013) than stems in the phenolic glycoside chemotype, but more tolerant to other environmental factors such as nutrient stress (Madritch and Lindroth 2015), freezing (Koyama et al. 2014), and UV stress (Lavola 1998). However some literature suggests that compared to forest edges, temperature is more stable in the interior (Chen et al. 1999), and light availability (ie potential exposure to UV damage) is less (Matlack 1993), and thus the utility of these benefits seems slight. Stems in the phenolic glycoside chemotype are likely better defended against herbivory (Osier et al. 2000b, Wooley et al. 2008), and may be more exposed, but as noted elsewhere, apparency is difficult to measure. However, assuming the production of these chemicals must provide some kind of fitness benefit, and possibly to different stressors, the co-occurrence of both strategies at a small scale suggests that aspen stands may be resistant to a diversity of stressors at a small scale.

As the natural range of trembling aspen is extensive, more work is needed to determine if similar chemotypes in aspen chemistry exist across that range, and if the plasticity we observed is itself widespread or restricted to certain areas. The aspen parkland may not reflect the environmental conditions over much of aspen's range, as it is a transitional zone on the edge of suitability for aspen (Hogg and Hurdle 1995). This environmental variability might have selected for higher phenotypic plasticity in this area compared to others, and thus further studies examining the naturally occurring spatial distribution of aspen secondary chemicals are needed.

Conclusions.

Overall we provided evidence that the chemotype approach in naturally occurring aspen stands can allow greater understanding of the factors that influence aspen chemistry, as well as the impact of variation in that chemistry on possible aspen resistance. We found that aspen chemotypes varied in a way that did not fit with predictions based on strong genetic control of aspen chemistry and single-clone aspen stands. Since we did not directly measure the relatedness in our trees, further studies relating genetics to foliar chemistry in *in-situ* natural aspen stands would be needed to confirm this hypothesis. These findings also provide evidence that aspen chemistry is plastic, and responds to factors at a relatively small scale. Further research, involving genotyping and chemotyping of aspen in a variety of field conditions is needed to confirm this hypothesis, and to help understand how factors manipulated in lab conditions apply to natural aspen stands.

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Table 3-1: Mean values (±SE) of secondary chemicals in two chemotypes of trembling aspen (*Populus tremuloides*) identified in naturally growing aspen stems sampled across central Alberta, Canada

Chemotypes	Mean Concentrations (mg/g)				
	Condensed tannins	Phenolic Glycosides			
		Salicin	Salicortin	Tremuloidin	Tremulacin
Tannin	55.55 (± 1.77)	13.31 (± 0.79)	2.22 (± 0.24)	7.23 (± 0.59)	5.69 (± 0.68)
Phenolic glycoside	21.37 (±1.61)	23.04 (± 0.86)	1.79 (± 0.23)	15.48 (± 0.73)	4.97 (±0.69)

Table 3-2: Regression coefficients, X^2 values and *p* values for each variable tested in models that compared environmental and climate impacts on the relative frequency of occurrence of two chemotypes of trembling aspen (*Populus tremuloides*) sampled across central Alberta, Canada. Model terms where $p < 0.10$ are indicated in bold.

Predictors	**Regression coefficient ±SE**	X^2	***p***
Site Level Predictors			
Mean annual precipitation	-0.18 ± 0.96	0.036	0.848
Mean annual temperature	13.45 ± 96.59	0.019	0.889
Average summer temperature	-38.13 ± 134.69	0.081	0.775
May-September precipitation	0.85 ± 2.57	0.124	0.724
Precipitation as snow	0.43 ± 2.20	0.042	0.836
Climatic moisture deficit	0.69 ± 2.32	0.086	0.768
Elevation	-0.09 ± 0.32	0.096	0.755
Latitude	1.15 ± 27.75	0.040	0.839
Longitude	4.74 ± 23.33	0.086	0.768
Plot Level Predictors			
Stand Height (SH)	0.76 ± 0.97	0.013	0.906
Stand Density (SD)	0.11 ± 0.26	0.070	0.790
Location within stand (L)	20.80 ± 18.58	1.309	0.252
Canopy openness (CO)	0.15 ± 0.16	0.801	0.370
H x D	-0.01 ± 0.04	0.851	0.356
H x L	-1.58 ± 1.63	0.104	0.745
D x L	-0.42 ± 0.76	0.155	0.693
H x CO	0.02 ± 0.03	0.002	0.967
D x CO	-6.64E-4 ± 0.01	0.003	0.956
L x CO	-0.65 ± -0.58	0.004	0.952

H x D x L	0.02 ± 0.08	0.101	0.750
H x D x CO	-7.08E-5 ± 1.67E-3	0.012	0.911
H x L x CO	**0.05 ± 0.05**	**3.217**	**0.072**
D x L x CO	0.01 ± 0.02	0.983	0.321
H x D x L x CO	2.61E-4 ± 2.74E-3	0.009	0.924
Stem Level Predictors			
Stem diameter	-0.03 ± 0.11	1.145	0.227
Location within stand	**0.37 ± 0.66**	**3.052**	**0.080**
Stem diameter x Location	0.04 ± 0.06	0.422	0.515

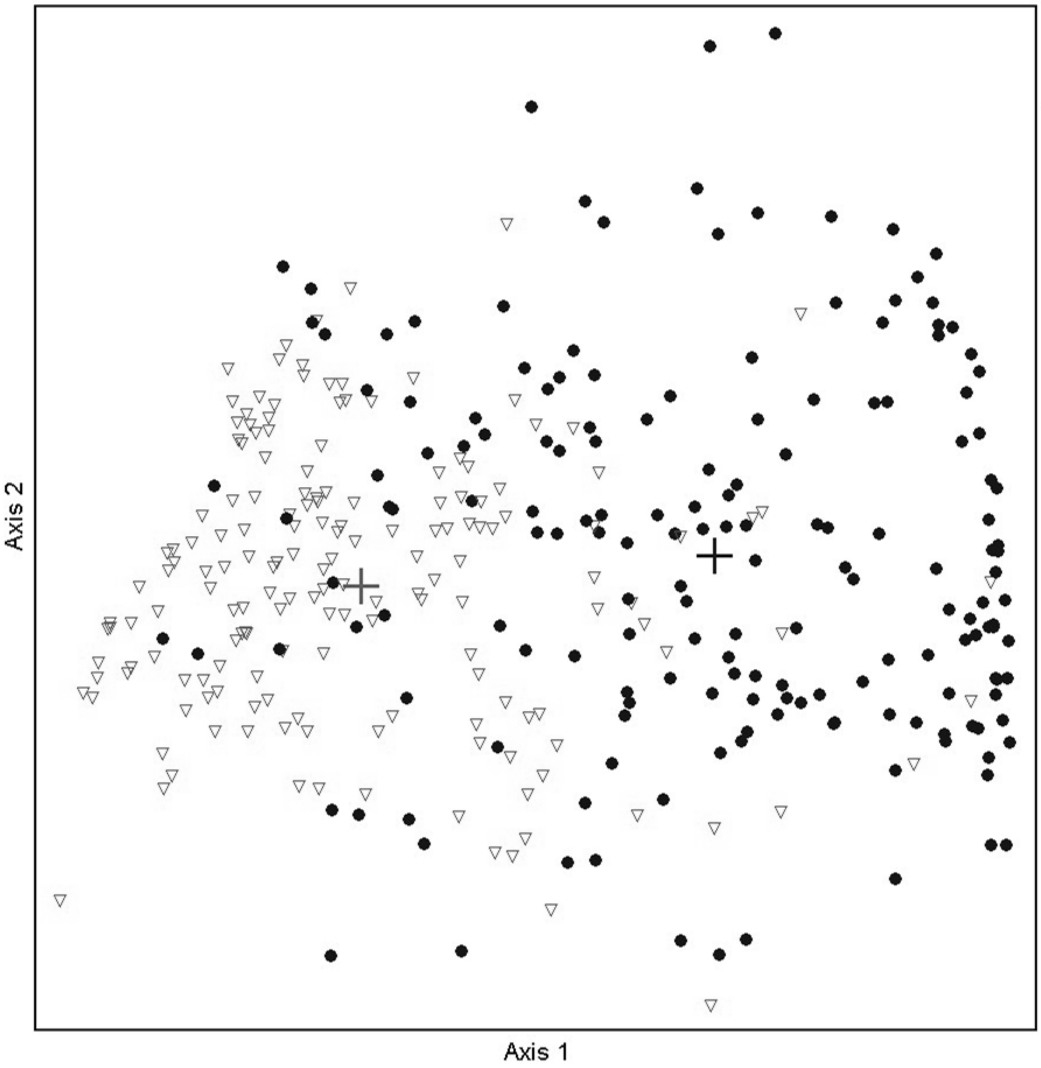

Figure 3-1: NMS ordination of trembling aspen foliar chemistry using the concentrations of five trembling aspen secondary chemicals, colour-coded according to the groups described by our pam clustering procedure. Black circles indicate the phenolic glycoside chemotype and inverted green triangles the condensed tannins chemotype. The coloured crosses represent the centroids for each group. Axis one explained 72% of the variation in the data, and axis two 20%. Axis one represents a shift from high levels of condensed tannins (left) to high levels of salicin and tremuloidin (right). Axis two represents a gradient of trees with overall higher secondary chemical values at the bottom to lower values towards the top.

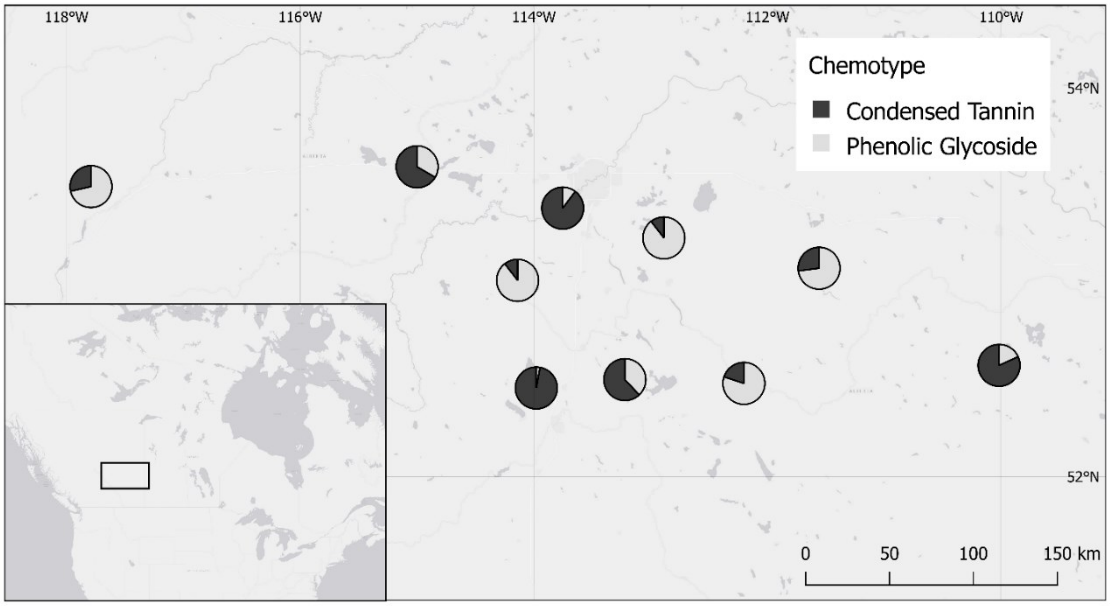

Figure 3-2: Trembling aspen *(Populus tremuloides*) survey site locations in central Alberta, Canada coloured proportionally by the frequency of occurrence of stems belonging to each identified chemotype.

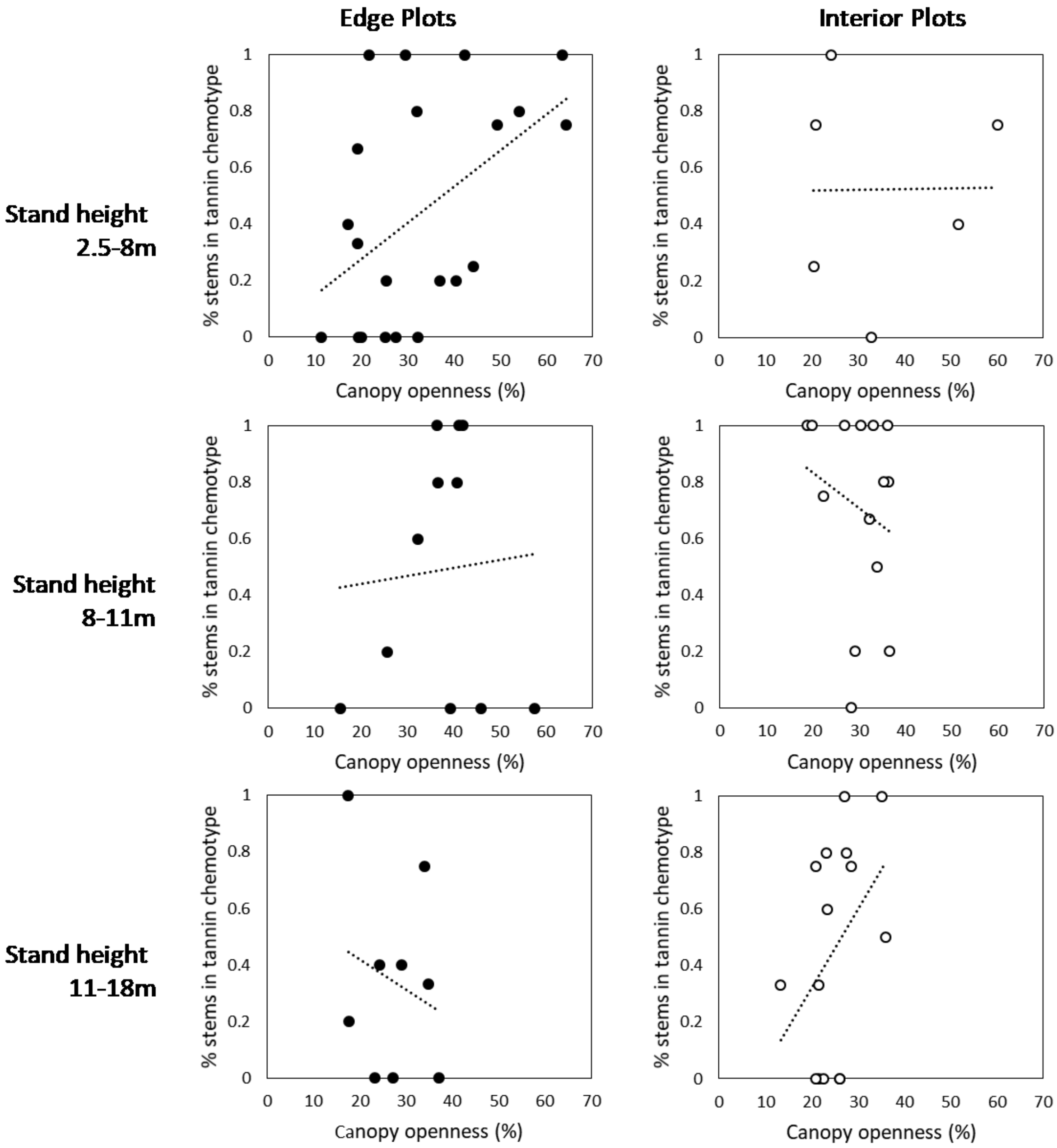

Figure 3-3: Plots showing the three way interaction between canopy openness, stand height and location versus chemotype in trembling aspen (*Populus tremuloides)* (p=0.07). Plots on the left with closed circles show plots on the edge of the stand, plots on the right with open circles show plots in the interior of the stand. The plots were divided into thirds based on stand height, the top row has plots between 2.5-8m tall, the middle with 8-11m tall and bottom row with 11-18m.

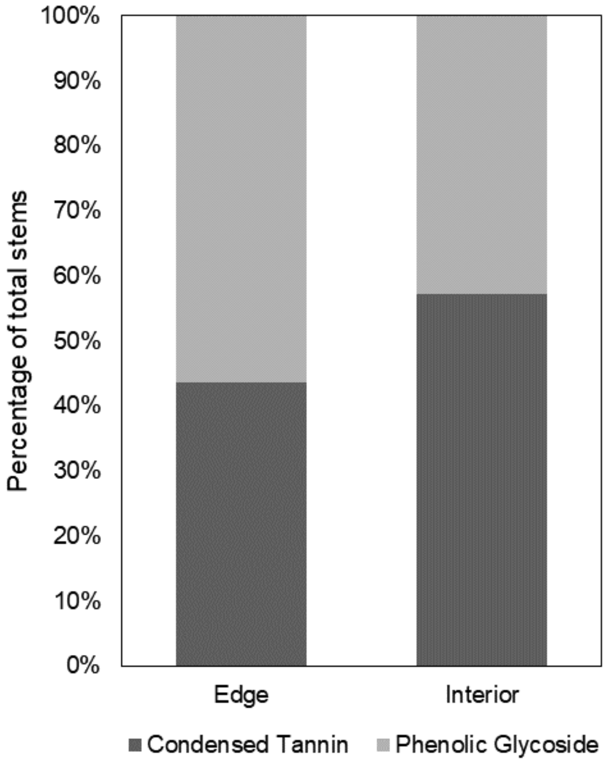

Figure 3-4: Percentage of trembling aspen (*Populus tremuloides*) stems in edge and interior plots that were either in the high phenolic glycoside or tannin chemotypes. (*p* = 0.0299)

Chapter 4: Chemical inputs from overstory trees influence understory plant community assembly

Abstract

Traits of dominant plants can impact subordinate species, as described in both the biotic filters and extended/community phenotype frameworks. Investigations of trait-assembly processes have typically focused on morphological traits, and effects of plant chemical traits on community assembly have been generally overlooked. Using trembling aspen, a dominant tree species whose secondary chemicals (mainly condensed tannins and phenolic glycosides) can reduce the availability of mineral nitrogen in soil, we tested the hypothesis that secondary chemicals of dominant plants can influence understory community composition and compared their effectiveness in explaining understory community composition to stand structural characteristics. Across ten sampled sites over 36,200 km^2, we found that variation in understory community composition was related to overstory leaf chemistry, with the effects varying with type and concentration of chemicals. Specifically, not all understory species were associated with the same concentrations of condensed tannins in the overstory. Higher condensed tannin concentrations in the overstory leaves were also associated with increased understory species richness, whereas higher phenolic glycoside concentrations were associated with decreased understory richness and the occurrence of grasses. We also found that increased variation in condensed tannin concentrations among trembling aspen trees within a site was associated with decreased understory species evenness and changes in community composition. Condensed tannin concentrations were, overall, more influential in influencing community composition than phenolic glycosides and at least as effective as stand structural characteristics (canopy openness, stand density) at predicting understory community composition; stand height was associated with

some aspects of understory community composition, but not species richness or evenness. Combined, these findings highlight the importance of plant chemical traits as drivers of community assembly.

Introduction

There is growing recognition that the traits of dominant organisms can drive community assembly. The concept of biotic filters states that one individual or one species, particularly one that is dominant, can create conditions that affect the ability of other species in the same community to establish and grow (Götzenberger et al. 2012, HilleRisLambers et al. 2012). The extended phenotype theory suggests that the effect of the genes of an individual can be measured at a level of organization beyond the individual, such as that of population, community, or ecosystem (Dawkins 1982, Whitham et al. 2003). Despite their separate theoretical development, both theories can lead to the same conclusion; given a thorough understanding of the biology of dominant organisms, we should be able to predict specific subordinate community outcomes that occur as a consequence of specific trait values in a dominant organism.

When extending these theories to plant communities, the concept of biotic filters typically does not consider plant chemical traits such as primary and secondary chemicals, instead focusing primarily on aspects of competition (Laughlin and Abella 2007, Violle et al. 2012). While the extended phenotype has been applied to plant secondary chemical traits, its impacts have typically not been measured in the context of plant community assembly. Instead the extended phenotype typically is applied to impacts of dominant organisms on communities of other taxa such as arthropods (LeRoy et al. 2006, Bálint et al. 2016). This historical focus of biotic filters on physical traits and extended phenotypes on non-plant taxa has left a theoretical gap between these two theories, as neither has addressed the question of how plant secondary chemicals can impact the subordinate species in that community. This question has been addressed to some extent by the allelopathy literature (Zeng 2014), though the focus of impacts on community assembly are usually in the context of invasive species with novel weapons (Callaway et al. 2005) or on examples of direct impacts on neighbouring plant communities (Ehlers et al. 2014). Taken together, it is apparent that there is a gap in our understanding of the influence of secondary chemicals of dominant plant species on subordinate plant communities.

Trembling aspen (*Populus tremuloides* Michx.) is an excellent model system to use to address this gap as it produces high levels of two types of secondary chemicals, condensed tannins and phenolic glycosides, in its leaves (Lindroth and Hwang 1995, Barbehenn and Constabel 2011). However, the concentrations of both can be heterogeneous across time or space, as both genotype (Osier and Lindroth 2001) and environmental (see McDonald et al. 1999, Osier et al. 2000a, Kanaga et al. 2009, Lindroth and St. Clair 2013, Najar et al. 2014, Dettlaff et al. 2018) factors can impact their concentrations.

Condensed tannins and phenolic glycosides enter the soil through litter-fall or leaching into through-fall rainwater, with the former likely making up a larger percentage of inputs (McClaugherty 1983). In fallen litter, water-soluble condensed tannins and phenolic glycosides leach out of the leaves and enter the soil system, while insoluble chemicals are released through decomposition (Hättenschwiler and Vitousek 2000). Once in the soil, aspen condensed tannins can reduce nitrogen mineralization rates (Basaraba and Starkey 1966, Rice and Pancholy 1973, Northup et al. 1995, Hättenschwiler et al. 2003, Schweitzer et al. 2004) by negatively impacting the activity of soil microbes (Hättenschwiler and Vitousek 2000). Phenolic glycosides influence soil microbial communities by stimulating microbial activity, which subsequently also reduces nitrogen availability (Schimel et al. 1996).

These chemically created changes in resource availability (particularly nitrogen) have the potential to change understory community composition in aspen forests. For example, increased soil nutrient availability is typically associated with decreased species richness (Thomas et al. 1999, Gilliam 2006), and thus increased concentrations of condensed tannins and phenolic glycosides in aspen leaves should lead to increased species richness via their negative impact on soil nitrogen availability. To our knowledge, this relationship has not been investigated directly (but see Lamit et al. 2011). Other resources such as light availability are also known to impact understory species (Ehrenreich and Crosby 1960, Klinka et al. 1996, Jennings et al. 1999, Barbier et al. 2008). By comparing the impact of light availability on understory community composition to that of chemical concentrations, we can place our hypothesized chemically mediated changes into a broader context of relative importance to understory community assembly processes.

There are other theories or approaches often used to guide hypotheses on impacts of traits and trait variation that can also be examined in the context of the impact of aspen chemical traits on plant communities. The fact that not all organisms contribute equally to a community trait value is frequently considered in the context of weighted mean traits (Grime 1998). Weighted values are predicted to be more accurate when estimating the impacts of traits than those that are not weighted (Vile et al. 2006), but this is not commonly tested in chemical traits. Some chemical impacts may not scale linearly with concentration (Belz et al. 2005), and therefore weighting would not have an impact. However, by comparing weighted and arithmetic mean values of chemical traits, we can determine the applicability of the mass ratio hypothesis to investigations of chemical traits. When predicting the impact of chemical trait variation on community composition, increased heterogeneity typically results in greater species diversity

(MacArthur 1965, Violle et al. 2012), and thus greater heterogeneity of chemical impacts may result in greater species diversity. However, wind often causes mixing of fallen leaves (Staelens et al. 2003), meaning that patterns of foliar chemical heterogeneity may not translate into increased diversity. This can be tested by comparing variability in *P. tremuloides* chemical concentrations with species diversity.

In this study, we use a survey of *P. tremuloides* foliar chemistry, stand structural characteristics, and understory community composition to conduct a test of the above predictions about the impact of *P. tremuloides* traits and other physical parameters on understory community composition. Broadly, we are asking three questions. 1. Do *P. tremuloides* chemical traits and their variation impact understory communities as predicted by literature on the impacts of nitrogen on community composition, the mass ratio hypothesis, or the diversity-heterogeneity hypothesis? 2. Do stand structural characteristics predict community composition? 3. What is the relative importance of this chemically driven impact compared to stand structural characteristics in predicting understory community composition?

Methods

Study species and aspen parkland

Trembling aspen is the most widely distributed tree species in North America (Dickmann and Stuart 1983) occurring in many ecotypes across its range. However, research on ecological aspects of the species' chemistry has typically focused on populations from the middle of its geographic range, particularly the Rocky Mountain or Great Lakes regions in the Central United States (Lindroth and Hwang 1996, Lindroth et al. 1999, Stevens and Lindroth 2005, Lamit et al. 2011). In contrast, the aspen parkland ecotype of western Canada parkland occurs at the transition between the boreal forests and Great Plains at the edge of suitability for *P. tremuloides* (Hogg and Hurdle 1995).

Site selection

We selected ten sites across Alberta, separated by at least 48 km (Appendix 4-1). This distance was chosen in part to reduce the likelihood that genets were shared among sites as 48 km is a greater distance than any reported genet size (0.42 km^2 (Grant 1993)), and sites were separated by a matrix of grassland, cropland, and isolated forest patches, rather than continuous aspen forest.

At each of our ten sites, we identified two stands of predominantly aspen forest that were a minimum of 40 m apart. The individual "trees" measured within or between stands may or may not be related, and thus we refer to them then as stems in this study. At eight of our ten sites, we established four 50 m^2 circular plots (3.99 m radius) in each stand, for a total of eight plots per site (Fig. 4-1). At the other two sites, we could not find enough suitable plot locations, resulting in seven plots per site, giving us a total of 78 plots. All stand and chemical measurements were taken in these plots. Other work in this system suggests that litter deposition decreases to less than 10% of what is found under the main part of the canopy at ~ 3m from the forest edge (M. Dettlaff, unpublished data). To ensure that the understory communities we surveyed were influenced primarily by the stems measured in the plots, we sampled plant percent cover in a 1 m x 1 m quadrat located at the plot centre.

Chemical analysis

To determine the concentrations of condensed tannins and phenolic glycosides for each trembling aspen stem in our plots in the first two weeks in June 2015, we collected five to ten fully expanded leaves haphazardly from the mid-crown of each stem in each plot using pole pruners and/or a slingshot (similar to the method outlined in Youngentrob et al. (2016)). Leaf tissue was kept cold in the field and stored at -80 °C within 24 h of collection. The number of stems in each plot varied from five to eighty, so to keep sample size constant, five individual stems (mean diameter 8.73cm (±0.31 SE)) were chosen in each plot at random and used for all further analyses. Samples from the selected stems were freeze dried for 48 h and ground into a fine powder using a Qiagen Tissuelyser II (Qiagen, Hilden, GER). The freeze-dried and ground tissue was stored at -80°C until analysis.

We measured condensed tannins using the Butanol-HCL method of Hagerman and Butler (1980) with the modifications suggested by Porter et al. (1985). We extracted the ground tissue twice with 70% acetone with ascorbic acid. Sample extracts were reacted with a butanol-HCL reagent (Hagerman and Butler 1980) as well as with a solution of ferric ammonium sulfate dissolved in HCL (Porter et al. 1985). Samples were left to react for 50 min at 95 °C, then absorbance at 550 nm was measured using a Bio-Tek PowerWave XS Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Specific details are reported in Dettlaff et al. (2018). Condensed tannin standards were purified from leaves of natural trembling aspen collected on the University of Alberta campus (located roughly in the centre of the study area) following the method of Hagerman (1988).

To measure total phenolic glycosides, the freeze-dried and ground tissue was added to 1.5 ml methanol and placed in an ultrasonic bath at 4 °C for 30 min and then centrifuged at 13,000 rpm for 15 mins. The concentrations of the four most abundant phenolic glycosides in *P. tremuloides*, salicin, salicortin, tremuloidin, and tremulacin, were determined using an ultra-performance liquid chromatograph (1290 Infinity, Agilent Tech., Santa Clara, CA, USA) fitted with a Poroshell 120 EC-C18 column (2.1 x 150 mm, 2.7 µm; Agilent Tech.) and a diode array detector (Agilent Tech.). A binary mobile phase of (A) water plus 0.1% formic acid (v/v) and (B) methanol plus 0.1% formic acid (v/v) was run at 0.3 mL/min and a temperature of 50 ºC using a gradient of 5 – 60% B over 15 min, 60% B held until 16 min, 60 – 80% B over 16 – 18 min, 80 – 50% B over 18 – 21 min, 50 – 5% B over 21 – 22 min, 5% B held over 22 – 23 min. A 2 μL injection volume was used. Quantifications were performed using compound absorbance at a wavelength of 274 nm and a standard curve calculated using serial dilutions of analytical standards. Standards of salicin, tremulacin, and salicortin were obtained from APIN chemicals Ltd (Oxfordshire, UK). Tremuloidin was provided by Dr. R.L. Lindroth (University of Wisconsin-Madison). Information about the impacts of trembling aspen phenolic glycosides on ecosystem processes is typically presented in terms of total phenolics, as opposed to each individual chemical. Since we could not make predictions about the impacts of each individual chemical, we calculated total phenolics for each tree by taking the sum of salicin, salicortin, tremuloidin, and tremulacin measured in leaves from each trembling aspen stem. All chemicals are recorded as milligrams of compound per gram of dried leaf tissue.

Calculation of chemical metrics

To test whether there is a dose-dependent (mass ratio) impact of secondary chemical concentrations, we calculated weighted means of both condensed tannins and phenolic glycosides for each plot in addition to arithmetic means. We calculated weighted means two ways: by using the area of each of our five stems, relative to (1) the total area of the other five stems in our plot that were sampled for foliar chemistry or (2) the total area of all trees in the plot, to weight each individual tree's foliar chemical values. The first approach assumes that the selected trees are a random subset of all other trees, while with the second, we are trying to better relate what we found to the overall stand. Of the two methods of calculating weighted chemical values, weighting individuals versus only the five other trees in the plot that had chemical measures had a better fit (lower AIC) in seven of ten comparisons, and so we primarily discuss weighted means in the context of the five-tree weighting. The results for weighting using all trees are included as Appendix 4-2.

To calculate a metric of variability in chemical concentrations that could be used to test the applicability of the diversity-heterogeneity hypothesis to chemical traits, we calculated the coefficient of variation (CV) for both chemical groups in each plot. CV was calculated as the standard deviation of condensed tannin or total phenolic glycoside values in each plot divided by the arithmetic mean value of condensed tannins or phenolic glycosides and multiplied by 100.

Calculation of understory plant community structure and stand characteristics

We used richness, evenness, and ordination methods to quantify different aspects of understory community composition for each of our plots. Species richness was calculated as the total number of species observed in each 1m x 1m quadrat. We calculated species evenness using Pielou's evenness, as $E=H'/\ln S$. Using a non-metric multidimensional scaling (NMDS) ordination, we generated axis scores for the community composition of each of the plots. We also calculated Pearson and Kendall r^2 values for each species and axis pair to determine which species were most associated with each axis.

To determine the relative importance of trembling aspen chemistry compared to other plant traits that influence light availability, we measured three aspects of aspen stand structure: canopy openness, stand density, and stand height. For canopy openness, we took a hemispherical photograph of the canopy above understory vegetation at each plot centre and calculated canopy openness using the Gap Light Analyser (Frazer et al. 1999) software package. Stem density was the number of stems in each plot, and stand height was measured as the height of the tree canopy using a TruPulse 200 laser range finder (Laser Technology, Inc., Centennial, CO, USA).

Statistical methods

Impact of aspen chemical traits on understory communities

To see if any of the chemical metrics were associated with understory community composition as well as with the mass ratio hypothesis, we used linear mixed models (LMM). In each model, we compared metrics of community composition (species richness, species evenness, and ordination axis scores for each plot for all three axes from the NMDS ordination) to the arithmetic and weighted condensed tannin and phenolic glycoside means. Each community composition metric was run in an LMM with site as a random effect, and one of the four condensed tannin or phenolic glycoside means (arithmetic and weighted) as a fixed effect. We used p-values for each model to determine which chemical metrics were significantly associated with community composition and AIC values to determine if arithmetic or weighted means gave a better fit.

To test our prediction that increased heterogeneity in aspen foliar chemical concentrations is associated with increased diversity, we used LMMs with CV of condensed tannins and phenolic glycosides as fixed effects, site as a random effect, and our five understory metrics (species richness, species evenness, and ordination axis scores from all three axes) as predictor variables.

To see if individual species or growth forms were associated with particular concentrations of aspen chemicals, we calculated mean values (± SE) of overstory condensed tannins and phenolic glycosides in the plots where each species was identified in our understory community survey (species that were found in only one plot were excluded). This gave us an estimate of the average value of overstory chemistry with which each species was associated. We also classified species into three growth forms: shrubs, forbs, and graminoids. We then performed ANOVAs to determine if there was a significant difference in mean condensed tannins and phenolic glycosides among species and growth forms. Where our data was not normally distributed, repeated transformations did not result in a normal distribution, so we performed a Kruskal-Wallis one-way analysis of variance on ranks.

Impact and relative importance of stand characteristics on understory communities

To determine the impact of secondary chemicals on understory community composition to stand characteristics, we ran additional LMM with three stand metrics (canopy openness, stem density, and stand height) as fixed effects and site as a random effect. To determine the relative importance of chemical metrics, we used AIC values for each model to see which was the most parsimonious. We also used the technique of Nakagawa and Schielzeth (2014) to calculate r^2 values for all our models (a null model which included only site as a random effect, as well as the already-described models with chemical means, chemical variation, and stand characteristics as fixed effects for a total of ten models per community compositional metric, including the null). The r^2 calculation gives two r^2 values for each model: the marginal r^2, which represents the percentage of variation explained by the fixed effect in the model, and the conditional r^2, which represents the percentage of variation explained by the whole model. We used both r^2 values to determine which of our six chemical metrics and three stand metrics explained the most variation for each community composition metric.

LMM and associated calculations were performed in R (R Core Team 2018). The ordination was performed in PCORD 7 (McCune and Mefford 2016). ANOVAs and all graphing were conducted using Sigma Plot v. 11 (Systat Software Inc 2008).

Results

The most commonly occurring shrubs in our understory plots were *Symphoricarpos occidentalis* Hook.*,* *Rosa acicularis* Lindl.*,* and *Amelanchier alnifolia* (Nutt.) Nutt. The most common forbs across all our plots were *Aralia nudicalis* L.*, Galium boreale L*. Many plots were also invaded by *Bromus inermis* Leyss. an escaped pasture crop that is a common invader in this system. The overall values of condensed tannins (mean = 38.56 $mg^{-1}g$, min = 1.33 $mg^{-1}g$, max = 113.00 $mg^{-1}g$) and phenolic glycosides (mean = 36.48 $mg^{-1}g$, min = 3.48 $mg^{-1}g$, max = 115.30 $mg^{-1}g$) at the plot level were similar. However, there was a negative relationship between mean condensed tannins and phenolic glycosides at the plot level (condensed tannins = 48.535 – (0.273 * phenolic glycosides), $F_{1,76} = 6.406$, $p = 0.013$).

Our NMDS returned a three-axis solution with each axis accounting for approximately 20% of the variation in the data (NMDS 1 $r^2 = 0.207$, NMDS 2 $r^2 = 0.184$, NMDS 3 $r^2 = 0.202$). Axis one was positively associated with a shrub-dominated understory, axis two with the presence of birch seedlings, and axis three with a gradient of dominant grasses (Table 4-1).

Impact of aspen chemical traits on understory communities

Both classes of trembling aspen leaf chemicals were associated with understory plant diversity but in opposite ways (Fig. 4-2, Table 4-2). Increased phenolic glycosides (both weighted and unweighted) were significantly associated with declines in species richness and evenness, and with changes in our NMDS axes two and three. Meanwhile, mean condensed tannins were significantly associated with increased species evenness, and weighted condensed tannins were significantly associated with both species richness and evenness.

Weighted means were not uniformly better than un-weighted means at predicting understory community composition. Weighted means had lower AIC values compared to unweighted means for tannins and phenolic glycosides versus richness, as well as phenolic glycosides versus axes two and three. Unweighted means had lower AIC values for tannins and phenolic glycosides versus evenness and condensed tannins versus axes two and three (Table 4-2).

Increased variation in condensed tannin concentrations (condensed tannin CV) was associated with increased species evenness and with NMDS axis two (Table 4-2, Fig. 4-3). Variation in total phenolic glycosides was not significantly associated with any of our community measures (Table 4-2, Fig. 4-3).

We found significant differences in the mean concentrations of condensed tannins that individual species were associated with, both overall as well as within growth forms. There was no significant difference between the mean concentrations of phenolic glycosides associated with individual species, but when comparing growth forms, graminoides were associated with higher mean foliar phenolic glycosides than forbs or shrubs (Table 4-3, Fig. 4-4).

Impact and relative importance of stand characteristics on understory communities

In contrast to the chemical traits, two of the three stand metrics, canopy openness and stand density, were not associated with any of our community compositional measures (Table 4-2). Stand height was significantly associated with NMDS axis 3 (Table 2, Fig. 4-5). For three of the five community compositional metrics, a trembling aspen chemical measure had the lowest AIC value of all tested variables: weighted phenolic glycosides for richness and condensed tannin coefficient of variation for evenness and NMDS axis 2 (Table 4-2). Stand height had the lowest AIC value for NMDS axis 3 (Table 4-2). While no variables were significantly associated with NMDS axis 1, canopy openness had the lowest AIC value (Table 4-2).

Discussion

Impact of aspen chemical traits on understory communities

We expected that increases in both condensed tannins and phenolic glycosides would be associated with increased species richness and evenness, as both are associated with reduced soil nitrogen availability (Basaraba and Starkey 1966, Rice and Pancholy 1973, Schimel et al. 1996, Hättenschwiler and Vitousek 2000, Hättenschwiler et al. 2003). In general, increased tannins (mean and weighted mean) were associated with increased richness and evenness, which agrees with work demonstrating similar impacts on understory communities in a common garden experiment (Lamit et al. 2011). However, phenolic glycosides showed a negative relationship with richness and evenness, contrary to our predictions. This is potentially due to differences in the mechanism by which they impact soil nitrogen availability. Condensed tannins bind nitrogen into polyphenol-protein complexes, while phenolic glycosides facilitate microbial activity, and thus all available mineral nitrogen is converted into microbial growth over a short time period (Schimel et al. 1996). Over longer time periods, these microbes should die, releasing the nitrogen back into the soil, ultimately causing a delay in nitrogen availability, not a reduction, which should not impact community composition in the same way that a reduction would. This prediction is supported by our results, as condensed tannins fit our expected pattern of increased richness and evenness with increasing condensed tannins based on previous results showing increased diversity with reduced nitrogen inputs (Tilman 1993, Thomas et al. 1999, Gilliam 2006). Under this model, condensed tannins may be functioning in this system as a co-existence mechanism for understory vegetation, though experiments that test the impact of these two chemicals independently and a greater understanding of the impacts of phenolic glycosides on soil chemistry are both needed to confirm this.

We also predicted that using weighted means (Grime 1998) of secondary chemicals of trembling aspen would give a better fit than arithmetic means when compared to understory community composition, as they would better account for the true inputs of these chemicals to the understory. However, the studies of mass ratio typically do not use chemical traits (e.g. Vile et al. 2006, Pakeman et al. 2011), and literature on the impacts of chemicals on other plant species suggests they do not necessarily scale linearly with dosage (Belz et al. 2005). Thus, despite greater accuracy in estimating leaf chemistry impacts, weighting may not result in greater predictive ability, at least not in all study systems. Our mixed results (weighted means were better in four comparisons, unweighted were better in five, and results were not consistent across response variables or chemical classes) indicate that caution should be used when trying to estimate the impacts of plant chemical traits on ecosystem processes, and that analyses should allow for both the possibility of dosage thresholds as well as the improved predictive power of weighted means.

Surprisingly, increased heterogeneity in chemical concentrations was not associated with increased species richness in the current study. In contrast, increased condensed tannin heterogeneity was unrelated to species richness and was associated with reduced species evenness and with aspects of community composition explained by NMS axis 2. Variation in phenolic glycoside concentrations was unrelated to species richness, evenness, or community composition. We expected that overstory chemical heterogeneity would create patchiness in understory impacts, which should lead to greater plant diversity. However, despite the variability in overstory chemistry, the inputs may not be so heterogeneous. Litter fall is not simply related to the location of original trees and can be difficult to predict accurately (Staelens et al. 2004), as it can be influenced by small variations in ephemeral factors such as wind (Staelens et al. 2003).

Since we did not measure condensed tannins in the soil solution, we cannot predict what degree of heterogeneity of inputs that understory plants are subjected to. The lack of a relationship between heterogeneity and aspects of understory communities may also be explained by potential non-linear dosage responses of chemical impacts (Belz et al. 2005) as outlined above. Additionally, interpretation is complicated by an inverse relationship between condensed tannin variation and mean concentrations of condensed tannins, and thus the pattern may be an artifact of the data, driven by the underlying association between the coefficient of variation and mean values. To properly tease apart this issue, an experimental setup where concentration and variation of condensed tannin inputs are varied independently is needed.

The changes we found in community composition may be driven by the significant associations between individual species or growth forms and overstory chemistry. Not all species were associated with the same range of mean overstory condensed tannins. This association does not appear to be driven by one particular plant family or their relative abundance, as the species with the highest associated condensed tannin concentrations belong to four different plant families (Santalaceae, Poaceae, Elaganaceae, Juncaceae), and members of the most common families found in our sampling (Asteraceae, Rosaceae) appear near the top and bottom of the list. In addition, we found that there were significant differences in mean associated condensed tannins between species within shrubs and within forbs, suggesting that this pattern is also not driven by one particular growth form. However, the responses of individual species did not appear to be driven by these plants taking advantage of the impacts of condensed tannins on soil mineral nitrogen availability (Northup et al. 1995). Plants with mycorrhizal associations are hypothesized to benefit from this shift in nitrogen (Jones et al. 2005); however, the species with the highest associated condensed tannin values (*Commandra umbellata* (L.) Nutt) shows low rates of mycorrhizal infection (Lesica and Antibus 1986), while the species with the lowest associated condensed tannin values (*Spiraea alba* Du roi) is more frequently considered to be mycorrhizal (Chagnon et al. 2015). We also found that growth forms were associated with different concentrations of phenolic glycosides, with graminoids associated with significantly higher concentrations than shrubs or forbs. Some graminoid species produce phenolic glycosides (Vicari and Bazely 1993), which provides an argument for broad-scale tolerance of these chemicals. However, the phenolic glycosides produced by grasses are different to the ones commonly produced by trembling aspen (Castells et al. 2002). Previous research has also indicated that germination and growth of some graminoids can be reduced by condensed tannins (Lodhi and Killingbeck 1982), but we believe this is the first report on a positive association between external phenolic glycosides and graminoid abundance, and as such, further investigation into this topic is required.

Impact and relative importance of stand characteristics on understory communities

In this study, we also sought to assess the relative importance of chemically driven impacts compared to other factors that are reported to be influential on understory communities. Our results suggest that trembling aspen chemical traits are better than stand metrics at explaining understory community composition, as only stand height was a significant predictor of any aspect of understory community composition. This finding is consistent with work showing that increasing stand height is positively associated with percent cover (Légaré et al. 2002). While unexpected, canopy openness was not associated with species richness, evenness, or aspects of understory community composition, agreeing with other findings showing that current under-canopy light conditions are not a strong predictor of aspects of understory community composition (Messier et al. 1998, Bartemucci et al. 2006, Bartels and Chen 2013).

Additionally, the aspen parkland is located at the edge of suitability for trembling aspen. As such, our overall canopy openness (31%) is higher than reported light values for pure trembling aspen stands in other ecotypes (Messier et al. 1998, Bartemucci et al. 2006), and light limitation may therefore not be as important in parkland aspen stands as other resources, potentially increasing the relative importance of chemically mediated impacts on nitrogen availability in this system.

Conclusion

Overall, our results support our hypothesis that trembling aspen chemistry may influence understory community composition, and more generally, that chemical traits can impact understory community composition. However, the effects depend on the chemical in question and its relative concentrations. It appears that, as suggested by other works (Schweitzer et al. 2004, 2008), condensed tannin concentrations have the potential for complex impacts on ecosystem function beyond their role as a defence chemical. We also found significant relationships between phenolic glycosides and understory community composition, though the direction of the results was not what we expected; thus, the mechanism is not clear. Trembling aspen chemical metrics in general were better predictors of understory community composition than stand structural characteristics. Accordingly, we strongly encourage future studies investigating the possible impacts of plant secondary chemicals in contexts other than plant-insect interactions, as this information is needed to guide our emerging understanding of plant secondary chemicals as drivers of community assembly.

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Table 4-1: Species with Pearson and Kendall r value >0.35 for each axis in our NMS ordination.

Axis	**Species**	**Pearson and Kendall r**
NMS 1	*Symphoricarpos occidentalis*	0.629
	Rubus idaeus	0.539
	Carex spp.	-0.389
	Festuca hallii	-0.358
	Lonicera diocia	-0.356
NMS 2	*Betula papyrifera*	0.486
	Carex spp	-0.430
	Bromus inermis	-0.408
NMS 3	*Poa pratensis*	0.617
	Bromus inermis	-0.518
	Equisetum arvense	0.426
	Cornus canadensis	0.380

Table 4-2: p values, r^2 values and AIC values from LMM models comparing a null model with site as a random effect (first section), measures of *Populus tremuloides* foliar chemistry (second section), foliar chemical variation (third section), and measures of stand structure (fourth section) to five measures of understory plant community composition (columns). All models had site as a random effect. Models with significant p values ($p<0.05$) are highlighted in bold.

Variable	**Richness**			**Evenness**			**Axis 1**			**Axis 2**			**Axis 3**		
	p value	r^2m r^2c	AIC	p value	r^2m r^2c	AIC	p value	r^2m	AIC	p value	r^2m r^2c	AIC	p value	r^2m r^2c	AIC
Null (site)	-	- 0.407	141.2	-	- 0.440	-72.2	-	- 0.735	85.4	-	- 0.406	116.3	-	- 0.263	117.8
Mean condensed Tannins	0.119	0.041 0.369	141.1	**0.005**	**0.115** **0.484**	**-77.6**	0.131	0.201 0.727	85.2	0.117	0.042 0.370	116.1	0.523	0.007 0.288	119.4
Weighted condensed tannins	**0.054**	**0.055** **0.391**	**139.8**	**0.007**	**0.095** **0.492**	**-77.1**	0.169	0.015 0.726	85.6	0.238	0.021 0.382	117.0	0.988	0.000 0.263	119.8
Mean phenolic glycosides	**0.041**	**0.038** **0.419**	**139.1**	**0.005**	**0.063** **0.492**	**-77.6**	0.877	<0.001 0.735	87.4	**0.025**	**0.046** **0.419**	**113.4**	**0.011**	**0.068** **0.314**	**113.7**
Weighted phenolic glycosides	**0.019**	**0.049** **0.425**	**137.9**	**0.011**	**0.052** **0.482**	**-76.4**	0.807	<0.001 0.735	87.4	**0.016**	**0.052** **0.421**	**112.7**	**0.006**	**0.079** **0.320**	**112.5**
Condensed tannin CV	0.127	0.039 0.342	141.4	**<0.001**	**0.168** **0.482**	**-81.2**	0.233	0.012 0.735	86.0	**<0.001**	**0.212** **0.379**	**107.1**	0.846	0.001 0.267	119.8
Phenolic glycoside CV	0.142	0.021 0.403	141.1	0.310	0.010 0.437	-71.2	0.071	0.015 0.742	84.2	0.268	0.012 0.401	117.1	0.957	<0.001 0.264	119.8
Canopy openness	0.735	0.001 0.411	143.1	0.687	0.002 0.430	-70.3	0.058	0.023 0.713	84.0	0.779	0.001 0.404	118.2	0.572	0.004 0.265	119.5
Stand density	0.582	0.003 0.426	142.9	0.520	0.005 0.440	-70.6	0.098	0.013 0.763	84.4	0.397	0.008 0.397	115.8	0.161	0.025 0.321	118.0
Stand height	0.353	0.009 0.411	148.8	0.404	0.007 0.441	-67.5	0.116	0.012 0.743	84.3	0.728	0.001 0.420	117.6	**0.002**	**0.107** **0.342**	**109.3**

Table 4-3: Relationship between individual species and growth forms and mean foliar condensed tannins and phenolic glycosides of overstory trembling aspen (*Populus tremuloides*). Where data were not normally distributed, Kruskal-Wallis analysis of variance on ranks tests were used to test for differences between groups; otherwise, ANOVA (indicated in italics) was used. Significant results are highlighted in bold.

	Growth form	Species	Species within shrubs	Species within forbs	Species within graminoids
Condensed tannins	H = 0.127 df = 2 p = 0.939	**H = 98.392** **df = 47** **p < 0.001**	***F = 3.890*** ***df = 10*** ***p = <0.001***	**H = 56.787** **df = 29** **<0.001**	*F = 2.031* *df = 6* *p = 0.072*
Phenolic glycosides	**H = 6.061** **df = 2** **p = 0.048**	H = 56.721 df = 47 p = 0.157	H = 8.849 df = 10 p = 0.546	H = 34.780 df = 29 p = 0.212	H = 7.083 df = 6 p = 317

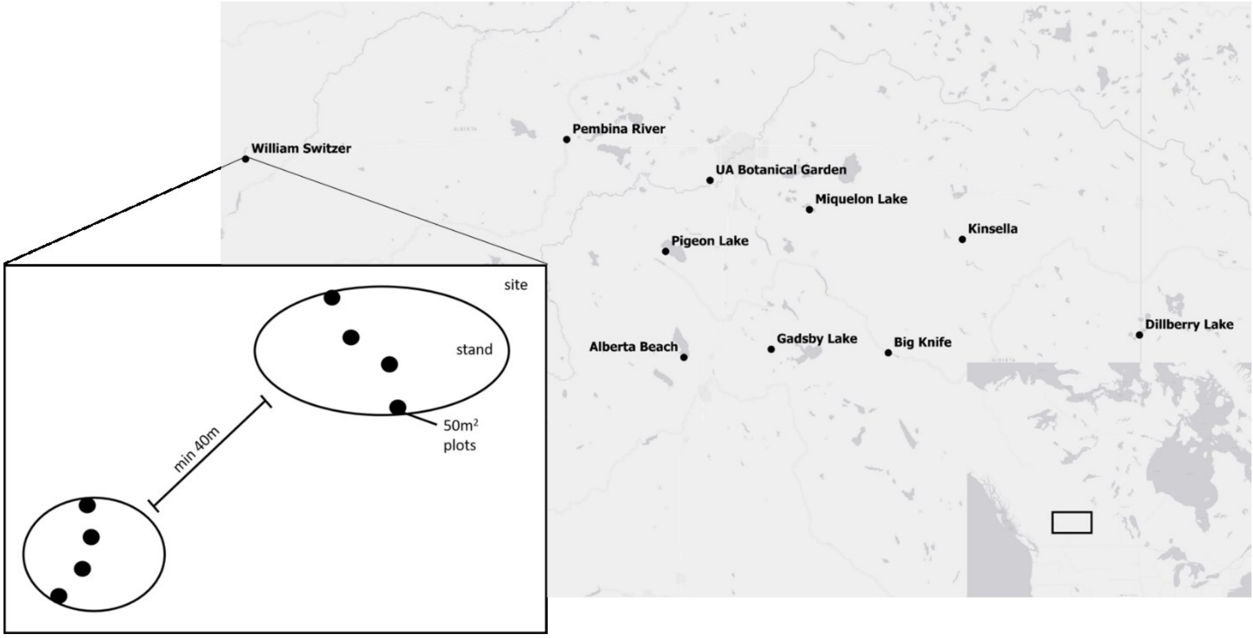

Figure 4-1: Locations of the ten study sites within Central Alberta, where aspen (*Populus tremuloides*) foliar chemistry was sampled and understory community composition was measured, and a schematic of sampling design within each site. Additional details about each site are given in Appendix 4-1.

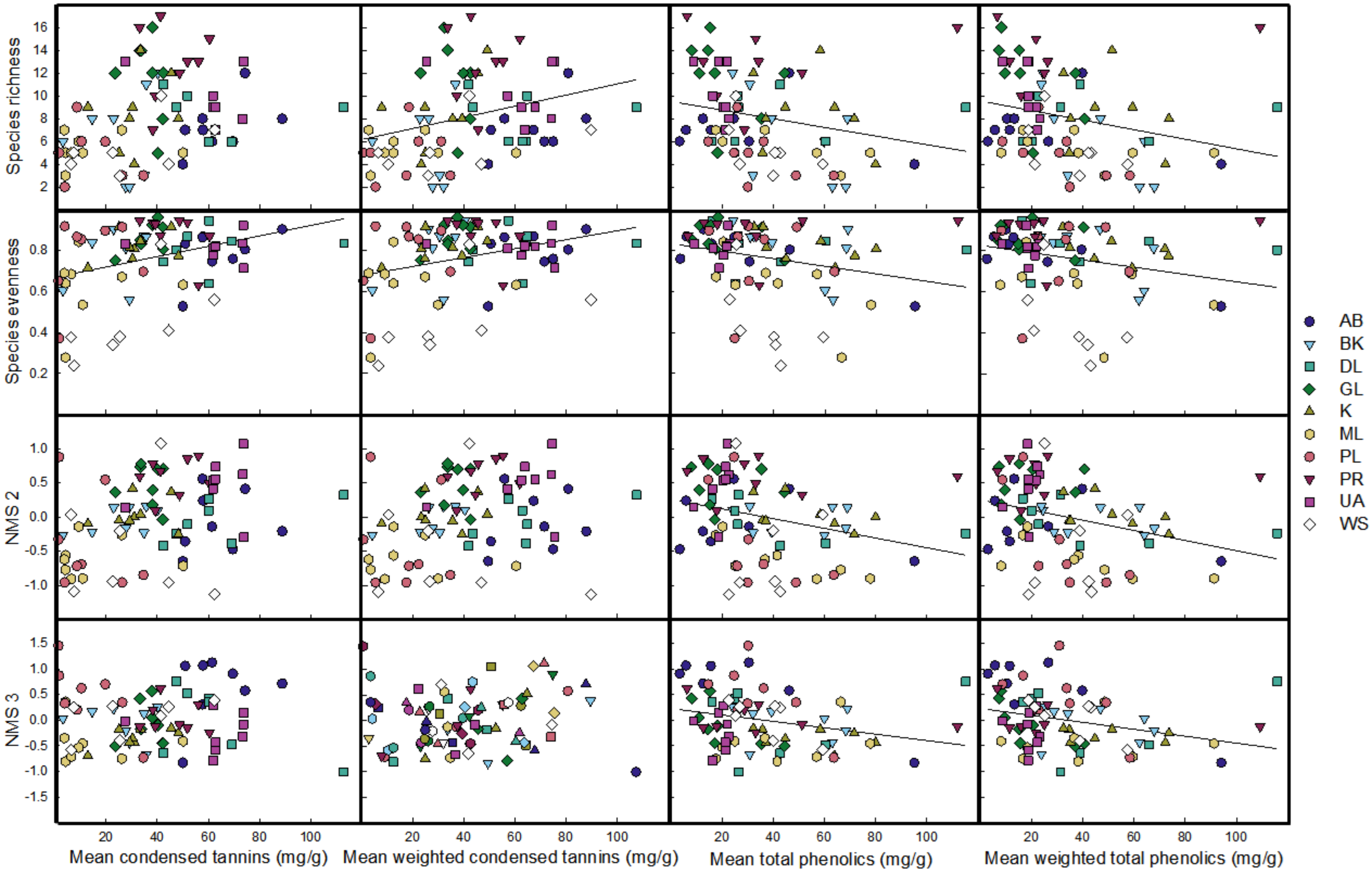

Figure 4-2: The relationship between *Populus tremuloides* foliar condensed tannin concentrations (mean condensed tannins, community-weighted mean condensed tannins) and foliar total phenolics (mean and weighted) versus four measures of understory community composition (species richness, species evenness, and the axis scores from two NMS ordination axes), measured in 78 plots at 10 sites across the aspen parkland of Alberta. Trend lines indicate significant relationships (see Table 4-3 for p values). Symbol colour and shape indicate site; for site abbreviations and details, see Appendix 4-1

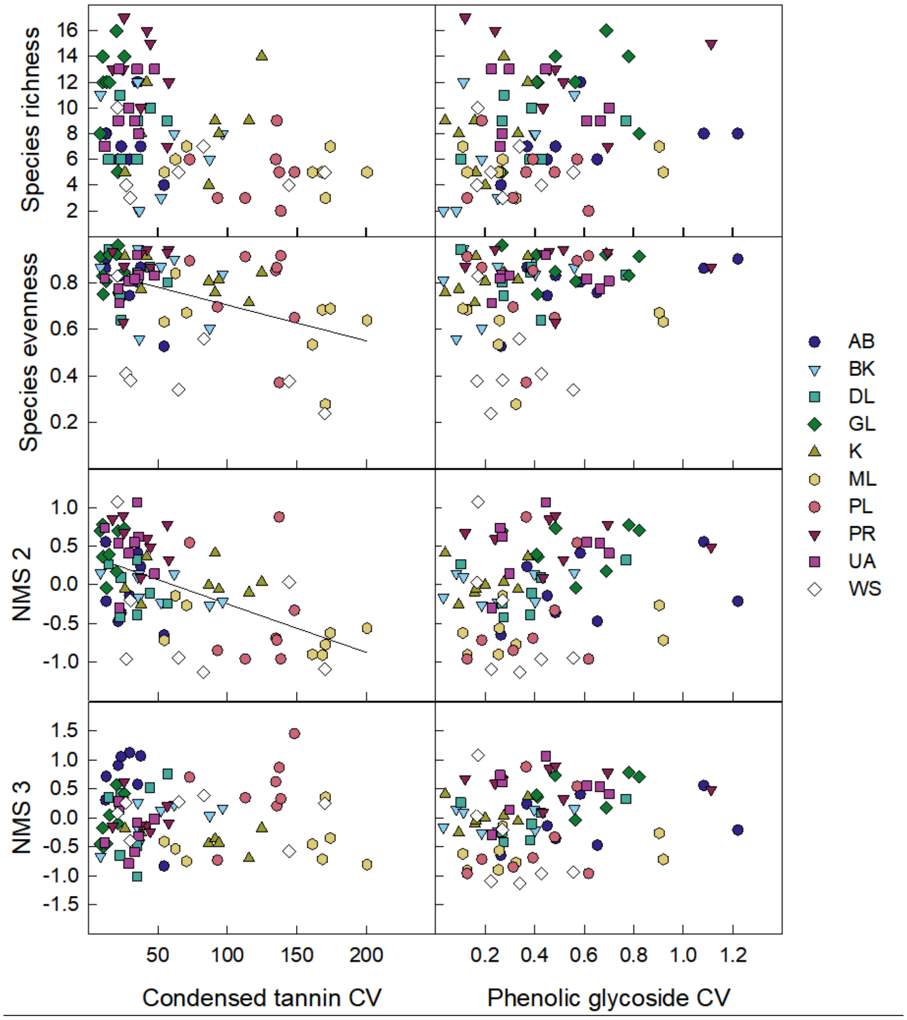

Figure 4-3: The relationship between *Populus tremuloides* foliar condensed tannin and total phenolic glycoside coefficient of variation versus four measures of understory community composition (species richness, species evenness, and the axis scores from two NMS ordination axes), measured in 78 plots at 10 sites across the aspen parkland of Alberta. Trend lines indicate significant relationships (see Table 4-3 for p values). Symbol colour and shape indicate site; for site abbreviations and details, see Appendix 4-1.

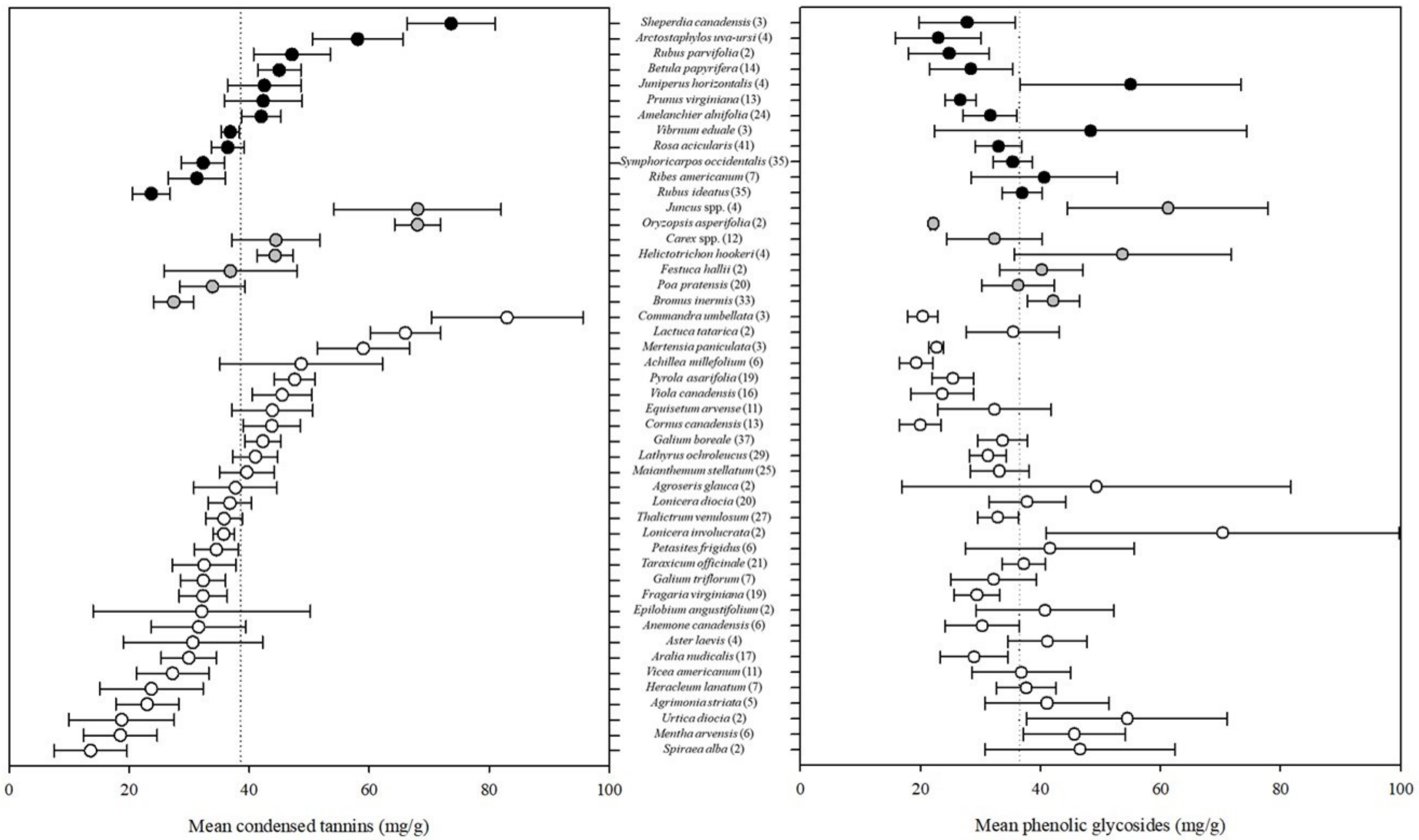

Figure 4-4: The mean (± 1 SE) condensed tannins and phenolic glycosides of only plots where each species identified in our understory community was present, sorted by growth form (shrubs, grasses, forbs) and then by mean condensed tannins. Black markers denote shrubs, grey markers graminoids, and unfilled markers forbs. Numbers in brackets indicate the number of plots each species was found in. Species found in only one plot were excluded. Vertical lines represent the mean value of each secondary chemical across all plots.

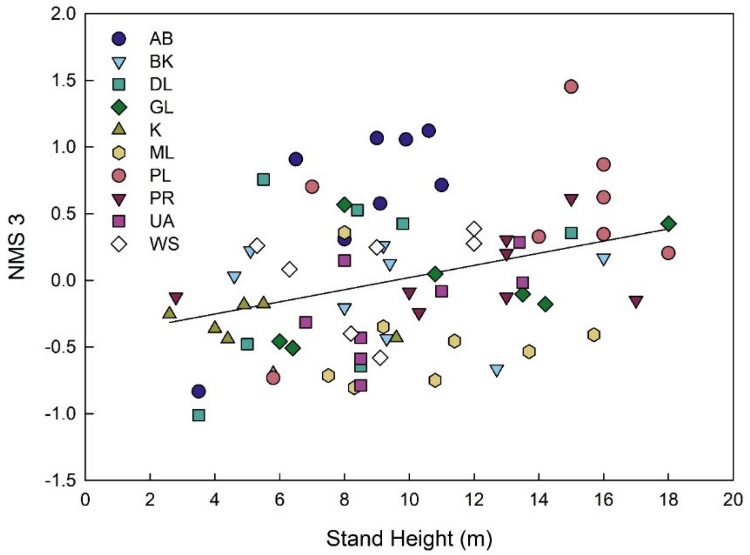

Figure 4-5: The relationship between stand height (m) of pure *Populus tremuloides* stands versus axis scores from an NMS ordination on understory community composition data measured in those same stands, in 78 plots at 10 sites across the aspen parkland of Alberta. Symbol colour and shape indicate site. Trend line indicates a significant relationship; see Table 4-3 for statistical details. For site abbreviations and details, see Appendix 4-1

Chapter 5: An invasive grass and litter impact native tree establishment into a native grassland

Abstract

Woody plant encroachment and invasive plant species are two critical factors that negatively impact grassland ecosystems globally. While studies have extensively investigated these two factors individually, research on how invasive species impact the susceptibility of a habitat to tree encroachment is less common. Focusing on the dynamics of the Aspen Parkland, a highly endangered savannah ecosystem in western Canada, we conducted a manipulative experiment to determine the effect of a widespread invasive grass, smooth brome (*Bromus inermis*), in North America on the growth and survival of trembling aspen (*Populus tremuloides*) seedlings. We also used litter manipulation to determine if plant litter was a potential mechanism of inhibition (as has been proposed for brome litter) or co-existence (as has been hypothesized for aspen litter) in this system. We found that the invasive grass reduced aspen survival by 57% compared to un-invaded habitats, likely mediated by reduced soil moisture availability, while litter manipulation had no effect. Of the surviving seedlings, local context had strong and complex impacts on growth; the addition of aspen litter onto invaded habitats resulted in larger seedlings while aspen litter additions onto native habitats resulted in decreased growth. The mechanisms underlying this may include previously identified effects of brome on soil nutrient availability and aspen litter impacts on soil microbial activity. These results suggest that invasion by smooth brome will alter the long-standing dynamics of tree establishment in this mesic grassland. Though smooth brome may serve as an initial barrier to aspen seedling establishment, accumulation of aspen litter from nearby stands should lead to more robust growth of seedlings in brome patches at the edge of existing aspen stands. Overall, the increasing impacts of invasive species in this system

will slow the rate of aspen regeneration; coupled with climate change-induced drought, this could lead to significant changes to this already endangered landscape.

Introduction

Grasslands make up approximately 40% of the land area worldwide (Anderson 2005) and they are subject to a diversity of anthropogenic and climate-related threats. A globally increasing concern is encroachment of woody plants into grasslands (Archer 1995, Briggs et al. 2005, Wigley et al. 2010, O'Connor et al. 2014, Venter et al. 2018). This can result in changes to plant species composition (Lett and Knapp 2005), loss of habitat for endangered species (Pringle et al. 2009, Albrecht et al. 2016), changes in carbon dynamics (Jackson et al. 2002, Knapp et al. 2008, Barger et al. 2011), and particularly in North America, loss of plant species diversity (Ratajczak et al. 2012). Although the initial drivers of encroachment of woody plants may be different; such as human-mediated changes in disturbance regimes (Roques et al. 2001) or climate (D'Odorico et al. 2010), once encroachment begins, positive feedbacks between already established woody plants and juveniles may accelerate the process (Ratajczak et al. 2011). Among susceptible areas, savannah systems may be even more at risk (Stevens et al. 2017). They are among the most human-impacted ecosystem types (Scholes and Archer 1997) and changes in biotic or abiotic conditions may have significant impacts on woody plant establishment (Roques et al. 2001, Ward 2005). Furthermore, the increasing incidence of invasive species in savannahs worldwide may also affect the establishment of woody plants in these sensitive habitats, however exactly how is still uncertain (Davis et al. 2005, Hoffmann and Haridasan 2008).

Invasive plant species have the potential to modify woody plant encroachment in several ways; previously invaded systems may be susceptible to further habitat degradation (Simberloff and Von Holle 1999, Pyšek et al. 2012) due to lowered biotic resistance. Invasive species may also alter biotic conditions through increased primary productivity (Liao et al. 2008) and subsequent litter deposition (Farrer and Goldberg 2009), which can reduce evapotranspiration (Larson and Whitman 1942), ameliorate soil temperatures (MacKinney 1929), decrease light (Standish et al. 2001) and nutrient (Rodgers et al. 2008) availability, or through the release of allelopathic chemicals (Hierro and Callaway 2003). In this study, we investigate the impact of the invasive grass smooth brome (*Bromus inermis* Leyss.) on encroachment by trembling aspen (*Populus tremuloides* Michx.) in the aspen parklands of western North America.

The aspen parkland is an endangered (Rowe 1987, Gunning 2001, Schieck et al. 2014) savannah ecosystem located at the boundary between the Great Plains to the south and the boreal forest to the north. As a transitional zone, the landscape is characterized by a mixture of small aspen stands interspersed within a grassland matrix (Bird 1961). Despite declines in aspen elsewhere in North America, the cover of these aspen patches has increased over time in the central parkland (Bailey and Wroe 1974, Scheffler 1976, Young et al. 2006) likely due to the extirpation of plains bison (*Bison bison* subsp. *bison*) (Campbell et al. 1994, Bork et al. 2013) and fire suppression (Strong 1977). In addition to the conservation consequences of a loss of native grassland habitat, this encroachment by trembling aspen is of concern to landowners due to loss of pasture for cattle (Jones 1983).

The primary abiotic factors that control aspen seedling establishment, and thus the establishment of new aspen stands, are the simultaneous availability of moisture (McDonough 1979, Fechner et al. 1981, van den Driessche et al. 2003) and light (Kaelke et al. 2001), and thus any factor that alters the availability of these two resources could drastically impact aspen encroachment. Aspen seedling survival and growth may also be facilitated by positive plant-soil feedbacks based on aspen litter deposition (Schweitzer et al. 2008). Aspen litter contains secondary chemicals which can alter soil nitrogen mineralization rates (Basaraba and Starkey 1966, Rice and Pancholy 1973, Northup et al. 1995, Hättenschwiler and Vitousek 2000, Schweitzer et al. 2004), which is hypothesized to benefit aspen while inhibiting other species (Schweitzer et al. 2008). As trembling aspen stands in the parkland are interspersed within the grasslands, aspen litter falling on nearby grassland areas may be an additional factor in facilitating aspen encroachment in this system. However, the amount of litter that falls outside aspen stands, and the maximum distance from a stand where enough litter falls to create potential feedbacks are not well established.

There are several non-native species present in the aspen parkland, including smooth brome which is simultaneously a widely-planted forage crop in western North America (Otfinowski et al. 2007), as well as one of the highest priority invasive species in Canada (Catling and Mitrow 2005). Smooth brome has the potential to impact aspen encroachment as it can influence the biotic and abiotic conditions of invaded habitats; areas invaded by smooth brome have lower native plant species richness (Stotz et al. 2017), which has been found to have opposing impacts on the susceptibility of communities to invasion (Levine and D'Antonio 1999, Bennett et al. 2014). Brome invaded areas may have higher nitrogen availability (Piper et al. 2014, 2015), which can improve aspen seedling growth and survival (DesRochers et al. 2003). Brome invaded habitats also have greater aboveground primary productivity, reduced light availability, and increased litter deposition (Vinton and Goergen 2006), while the effect of smooth brome invasion on soil moisture is still unclear (Willson and Stubbendieck 1995, Carrigy et al. 2016). Smooth brome is also associated with negative plant-soil feedbacks on native species, likely through alteration to soil microorganism communities (Jordan et al. 2008, Chagnon et al. 2018) which could further negatively impact aspen encroachment. Taken together, it is clear that smooth brome may impact aspen encroachment through changes in niche conditions, but the direction of that impact is difficult to predict.

To determine how trembling aspen encroachment in the aspen parkland might be impacted by prior invasion by smooth brome and by litter-driven feedbacks, we used an experimental planting of aspen seedlings in both brome and native grassland, along with litter manipulation to answer the following research questions: 1. To what extent does litter fall on grassland habitats outside aspen stands? 2. Does the presence of invasive smooth brome or aspen litter impact the survival and growth of aspen seedlings? 3. Do changes to biotic and abiotic conditions associated with smooth brome or litter manipulation treatments drive the impacts of treatments on aspen growth and survival?

Methods

Location and experimental design

We conducted a manipulative field experiment at the Roy Berg Kinsella Research Ranch in central Alberta, Canada (53.08523 N, 111.5636 W). The ranch is in the aspen parkland ecoregion, and consists of a mixture of trembling aspen stands set into a matrix of rough fescue grassland (Lamb et al. 2009). Areas of both aspen forest and native grassland have been invaded by smooth brome, a perennial grass introduced to North America as a forage crop (Otfinowski et al. 2007). Smooth brome is still actively planted across Alberta; however, the fields where this experiment was carried out have never been seeded. Thus, the brome infested areas in this study are a consequence of passive invasion, with seed most likely spreading from the surrounding pastures. In brome-invaded areas, brome forms dense, visually distinct patches; at the edges, brome cover drops from 60% to zero in less than one meter (Bennett et al. 2014), and so invaded areas are easily distinguished from uninvaded areas.

In May 2016, we established 15, 3 m x 2 m blocks inside brome patches and 15 (unpaired) similarly sized blocks within the native grassland matrix. All 30 blocks, were located within a five ha area and were a minimum of 20 meters apart. Due to space constraints, we placed two brome blocks, within the same large patch, (but with 20 m between them), all others were in independent patches. Within each block, we placed 12 plots (each 50 cm x 50 cm) in a four by three grid, with a 15 cm buffer between plots (Fig. 5-1).

Litter fall estimation

To determine the natural rates of aspen litter fall, we established a litter trap transect at ten aspen stand-grassland transitions within the study site in August 2016. Each transect consisted of four litter traps; one in the grassland 5 m from the edge of the aspen stand, one in the grassland 1m from the edge, one at the edge of a stand, and one inside the aspen stand. In October of 2016, we emptied the traps, removed non-aspen litter and measured the field weight of litter that fell into each trap. Our experimental design called for adding fresh litter to our treatment plots, as drying may have affected the secondary chemical concentrations. Thus, we calculated the mean litter inputs as field weight values to allow us to more accurately match our litter addition treatments to the natural litter fall.

Litter treatments

We imposed our litter treatments in October of 2016 and 2017 (see Table 5-1 and Fig. 5-1 for details). Of the 12 plots in each block, we randomly assigned each as one of two controls or one of ten litter manipulation treatments. Litter in the two control plots was not manipulated or disturbed in any way. The ten treatment plots had all existing litter raked off and had differing amounts, and types of litter added back to the plots. To determine the impacts of trembling aspen litter on our aspen seedlings, four treatment plots received freshly fallen aspen litter in the amounts we calculated each position along from the litter trap transects. The litter we added was collected from aspen stands at the field site using additional 1m x 1m litter traps placed under aspen forest patches in August of each year to ensure we were adding current-year litter, then homogenized across all traps before being added to our plots. As a comparison for the aspen litter, to see if its effects were different from the litter that naturally falls on a plot, four plots received the same amounts of litter as the aspen treatments but using the grassland or brome-dominated (self) litter that was already present on the plot. Of the remaining two plots, one had the total amount of litter that was raked off it returned, and one had no litter replaced (full litter removal). Together, these treatments allow us to determine different impacts of aspen litter versus existing litter and litter amount in influencing tree establishment.

In the growing season following litter addition (June 2017), we planted two one-year-old aspen seedlings purchased from a commercial nursery (Tree Time, Edmonton, Alberta) into each plot (Fig. 5-1), for a total of 720 seedlings. While seed germination may be critical factor in aspen encroachment, prior challenges with highly variable germination and establishment in this field site necessitated the use of seedlings.

Sampling

To determine how the aspen seedlings responded to our treatments, in August 2018 we counted surviving trees and collected all living aboveground biomass for each seedling. Since the root systems were well integrated into the grassland community by the time of harvest, we did not collect belowground biomass.

To determine how the biotic and abiotic conditions in each plot were impacted by litter manipulations or if they varied between smooth brome and native grassland communities, we measured soil moisture, the biomass of neighbouring plants, litter biomass, and available photosynthetically active radiation (PAR) in each plot. We measured percent soil moisture using an ML3 Theta Probe (Delta-T Devices, Cambridge, UK) in August 2017 (two months after planting) and in June 2018 (one year after planting). We measured neighbour biomass and litter biomass by clipping the live vegetation and raking up any litter in our 20 cm x 40 cm sub-plots in August of 2018. Photosynthetically active radiation (PAR) was measured above and below the canopy using a handheld light meter (AccuPAR model Par-80, Decagon Devices, Pullmann WA, USA), and we calculated available PAR as the percentage of light that passed through the vegetation (under canopy/over canopy * 100). We used under-canopy light availability as it best estimates the light available to the aspen seedlings we planted in each plot.

Statistical Analysis

Analysis of litter fall patterns.

To determine if the amounts of litter that fell at each point of our litter trap transect were significantly different from each other we used an ANOVA.

Analysis of tree survival and growth

To determine if raking, independent of our litter manipulations altered aspen seedling performance, we conducted two analyses on subsets of the data. First, we used binomial regression to compare the survival of trees in unraked plots versus plots that were raked and had 100% of the litter returned (Table 5-1– control" vs. "self–background" respectively). We made the same comparison for aspen biomass using a linear model with final biomass as the response. In all cases, the impact of raking was not significant (survival: model estimate = -0.811, F = 16.715, p = 0.153, biomass: model estimate = 0.153, F = 0.090, p = 0.765), and thus we combined the two plot types in subsequent analyses.

To determine if our litter manipulation treatments or the presence of smooth brome impacted aspen seedling survival, we conducted a logistic mixed-effects model with the proportion of aspen which survived until August 2018 in a given plot as our response variable.

Litter type (aspen vs. self litter), litter amount (the amount of litter added to each plot), and community type (brome vs. native) were included as fixed effects, and block as a random effect. To determine if these factors impacted aboveground biomass we used a linear mixed-effects model with final weight as the response variable, litter type, litter amount, and community type as fixed effects and block as a random effect.

Impact of treatments on biotic and abiotic conditions

To determine if the biotic or abiotic conditions were different between smooth brome and grassland plots, or were impacted by our litter manipulation treatments, we used mixed-effects models with soil moisture (measured in August 2017 and June 2018), final neighbour biomass, final litter biomass, or available PAR (2017 and 2018) as response variables. We used each of these variables as the response in an individual model that had community type, litter amount, and litter type as well as their interactions as fixed effects, and block as a random effect.

Impact of biotic and abiotic conditions on aspen growth and survival

To determine if any of these biotic or abiotic factors influenced aspen survival or growth we used a stepwise regression approach to determine which factors were most associated with survival and final weight of aspen seedlings. At each step, factors with $p < 0.05$ were added to the model and factors with $p > 0.10$ were removed.

We performed analyses in SPSS v.25 and used Sigma Plot v.11 to produce graphs.

Results

Aspen litterfall was significantly different among the four positions along the transect ($F = 29.13$, $p < 0.0001$, Supp. Fig. 1). There was a sharp gradient of aspen litterfall moving from within the stand to within the grasslands. On average interior traps received 173 g/m^2 of litter, edge traps received 99.62 g/m^2, traps one meters from the edge received 51 g/m^2 and traps five meters from the edge received 15 g/m^2.

Across all plots, the mortality rate of aspen seedlings was 75% over the 14 months of this study. Further, at harvest, many of the surviving seedlings had few (<10) leaves and had suffered die-back of the main stem.

Aspen seedling survival and growth

Aspen seedling survival was significantly impacted by community type (Table 5-2, Fig. 5-2), with 35% survival in native grassland patches and 15% survival in brome patches. Community type impacts on survival were consistent across all litter amount and type treatments, as indicated by a lack of main effects or interaction terms, including the litter factors (Table 5-2).

In contrast to the effects of community type on seedling survival, the final biomass of surviving seedlings was impacted by an interaction among community type, litter type, and litter amount (Table 5-2). In general, seedling biomass increased with increasing additions of aspen litter in brome patches, but decreased with increasing additions of aspen litter in native grassland habitats (Fig. 5-3).

Impacts of litter manipulation treatments and smooth brome on biotic and abiotic conditions

Soil moisture was significantly lower in brome than in native grassland patches at both time periods (Table 5-3) and was not influenced by litter manipulations (Table 5-3). There were no significant differences in neighbour plant biomass or litter biomass between community types (Table 5-3); neighbour biomass was not affected by either of our litter treatments. Litter biomass was significantly positively related to litter amount and an interaction between litter amount and litter type (Table 5-3). There was no significant difference in available PAR between community types or litter treatments at either time period (Table 5-3).

Impacts of biotic and abiotic conditions on seedling growth and survival

Survival was significantly positively associated with both neighbour plant biomass and soil moisture in August 2017 (model estimate = 0.072, t = 9.703, p <0.001) Aspen seedling final weight was significantly positively associated with neighbour plant biomass (model estimate = 0.034, t = 1.994, p = 0.048)

Discussion

Impact of smooth brome invasion

Habitats invaded by invasive grass in the aspen parkland may be less susceptible to initial establishment by trembling aspen, as aspen survival was greatly reduced by the presence of smooth brome. These findings agree with other research, suggesting that invaded grasslands may be initially resistant to woody species establishment (Davis et al. 2005, Hoffmann and Haridasan 2008), but is in disagreement with the biotic resistance theory, which suggests that intact communities should be more resistant to invasion (Levine and D'Antonio 1999). Previous studies have also found that competition from another native grass, *Calamagrostis canadensis* (Michx), inhibits aspen growth, which was primarily attributed to *C. canadensis* acting as a physical barrier to aspen sprouting (Landhäusser and Lieffers 1998, Landhäusser et al. 2007). Our results indicate that reduced aspen seedling survival was likely a function of soil moisture, which was significantly lower in brome invaded areas than in native grasslands, making it the most likely driver of the observed changes in aspen seedling survival. This finding agrees with the substantial body of literature that documents the importance of soil moisture availability to aspen seedling establishment (McDonough 1979, Fechner et al. 1981, van den Driessche et al. 2003).

Impacts of litter

We did not find evidence that litter influenced trembling aspen survival. There was no relationship between survival in smooth brome patches and the amount of litter present in the plot, nor did our litter manipulations impact light availability or soil moisture, despite evidence of such impacts in the literature (Larson and Whitman 1942, Vinton and Goergen 2006, Farrer and Goldberg 2009). While we did find that appreciable amounts of aspen litter fell outside aspen stands, we did not find any evidence to support theories of a positive impact of natural inputs of trembling aspen litter on aspen survival (Schweitzer et al. 2008), however we only applied litter in amounts that was natural in this system. It is possible that a greater inputs, or inputs over a longer period might have an impact.

Aspen litter additions increased final biomass of aspen seedlings in brome patches but slightly decreased it in uninvaded habitats. This suggests that there may be some benefit to aspen growth of having its own litter present, but that the benefit is dependent on the neighbouring community. The observed increase in growth in brome invaded areas could be explained by greater soil nitrogen availability in smooth brome patches (Piper et al. 2014, 2015). This increase was only present with the addition of trembling aspen litter, which could be due to the suppression ability of aspen secondary chemicals on the soil microorganisms (Schimel et al. 1996, Hättenschwiler and Vitousek 2000). The compounds associated with brome-conditioned soils have negative impacts on other plant species (Jordan et al. 2008, Piper et al. 2014, Chagnon et al. 2018). If chemicals in aspen litter can ameliorate the negative impact on the microorganism community in brome patches, then aspen seedlings would have more access to soils with higher nitrogen than those habitats without the litter addition. Suppression of micro-organisms could also explain the slight decrease in final biomass in uninvaded areas as there may be beneficial micro-organisms in uninvaded soils that are suppressed by aspen litter. Further research to determine the exact impact of aspen litter on soil microorganism communities is needed to clarify the mechanism behind this unexpected result.

Management implications

The rate of aspen encroachment into tame pastures may initially decrease as pasture lands are increasingly converted from intact native prairie to mixes of introduced grasses (Schieck et al. 2014). This could be beneficial to land managers concerned about aspen encroachment (Jones 1983), as they may already be actively planting smooth brome (Otfinowski et al. 2007). Accumulation of aspen litter in invaded habitats at the edge of aspen stands may facilitate aspen growth, but inputs of aspen litter large enough to impact seedling growth only occurred very close to existing stands, which could further contribute to slow rates of encroachment and aspen regeneration, as the zone where young aspen can become successfully established will be relatively narrow. This may lead to a wholesale change of the landscape, as existing aspen stands reach the end of their lifespans or are removed by land managers to increase available forage for livestock (Fitzgerald and Bailey 1984), and are unable to regenerate effectively in increasingly anthropogenic landscapes. Additionally, climate change scenarios for the aspen parkland predict lower rainfall (Hogg and Hurdle 1995), which given the importance of moisture availability in this study and others will also negatively impact aspen seedling regeneration, regardless of the presence of invasive species. Together, our results emphasize the importance of conservation of intact parkland areas to preserve conditions where aspen seedlings may successfully regenerate, and prevent the loss of the characteristic parkland mosaic of trembling aspen stands and native fescue grasslands.

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Table 5-1: Litter manipulation treatment details. Aspen litter was collected from the forest floor of nearby aspen forest stands. Only current year litter was used. Amounts of litter were calculated using litter traps placed inside aspen stands, at the edges and 1 and 5 meters into the grassland. See Fig. 5-1 for a diagrammatical description.

Treatment	**Raked?**	**Litter amount added**	**Litter type added**
Control (2 per block, all others 1 per block)	No	N/A	N/A
Aspen – Inside	Yes	45 g	Aspen litter
Aspen – Edge	Yes	25 g	Aspen litter
Aspen – 1m	Yes	10 g	Aspen litter
Aspen – 5m	Yes	1 g	Aspen litter
Self - Inside	Yes	45 g	Existing litter that was raked off the plots
Self - Edge	Yes	25 g	Existing litter that was raked off the plots
Self – 1m	Yes	10 g	Existing litter that was raked off the plots
Self – 5m	Yes	1 g	Existing litter that was raked off the plots
Self – Background	Yes	~ 110 g	Existing litter that was raked off the plots
Litter Removed	Yes	0 g	N/A

Table 5-2: The impacts of community type (smooth brome versus native grassland), litter type (aspen versus grass), litter amount, and interactions on the survival and growth of trembling aspen (*Populus tremuloides*). Model coefficents (C), f and p values were calculated using mixed model regressions, p values < 0.05 highlighted in bold.

	Survival			**Final Biomass**		
	C	**$F_{1,352}$**	**p**	**C**	**$F_{1,172}$**	**p**
Community Type (CT)	**-0.91**	**5.26**	**0.022**	1.02	0.71	0.402
Litter Type (LT)	0.75	0.17	0.681	1.01	0.74	0.390
Litter Amount (LA)	0.01	0.05	0.820	-0.01	0.05	0.832
CT * LT	-1.16	2.14	0.144	**-2.89**	**8.14**	**0.005**
CT * LA	-0.01	0.03	0.836	**-0.00**	**5.55**	**0.020**
LT * LA	-0.01	0.16	0.693	-0.03	0.62	0.431
CT * LT * LA	0.02	0.50	0.480	**0.08**	**5.58**	**0.019**

Table 5-3: The impacts of community type (smooth brome versus native grassland), litter type (aspen versus grass), litter amount, and interactions on soil moisture measured in August 2017 (SM 17) and June 2018 (SM 18), standing biomass of neighbours (Neigh. bio), litter biomass, and photosynthetically active radiation (PAR) measured in August 2017 and 2018. Model coefficients (C), f and p values were calculated using mixed model regressions, p values < 0.05 highlighted in bold.

	SM Aug 2017			**SM Jun 2018**			**Neigh. biomass**			**Litter biomass**			**PAR 2017**			**PAR 2018**		
	C	**F**	**p**	**C**	**F**	**p**	**C**	**F**	**p**	**C**	**F**	**p**	**C**	**F**	**p**	**C**	**F**	**p**
Community type (CT)	**-1.6**	**5.9**	**0.02**	**-2.6**	**6.6**	**0.01**	1.2	0.1	0.71	1.5	0.1	0.78	1.8	1.1	0.29	-7.4	1.5	0.22
Litter type (LT)	0.1	0.1	0.76	-0.9	1.6	0.20	0.7	0.0	0.95	-3.2	3.7	0.05	-3.2	1.3	0.26	-3.2	0.7	0.40
Litter amount (LA)	-0.0	0.1	0.83	0.0	0.0	0.93	-0.0	0.8	0.37	**0.1**	**67.7**	**0.00**	-0.0	1.3	0.26	0.0	0.4	0.55
CT*LT	-0.1	0.0	0.88	0.7	0.7	0.39	-1.3	0.3	0.57	-1.3	0.1	0.76	2.5	0.5	0.46	1.7	0.1	0.76
CT*LA	-0.0	0.5	0.47	0.0	0.0	0.99	0.0	0.2	0.65	-0.0	0.0	0.99	0.0	0.0	0.94	-0.0	1.8	0.18
LA*LT	-0.0	0.0	0.87	0.0	0.0	0.93	-0.0	0.8	0.38	**0.5**	**55.9**	**0.00**	0.1	1.9	0.17	0.2	0.4	0.54
CT*LT*LA	0.1	0.9	0.33	-0.0	0.0	0.92	-0.1	0.5	0.49	0.0	0.0	0.91	0.0	0.0	0.95	-0.2	1.7	0.19

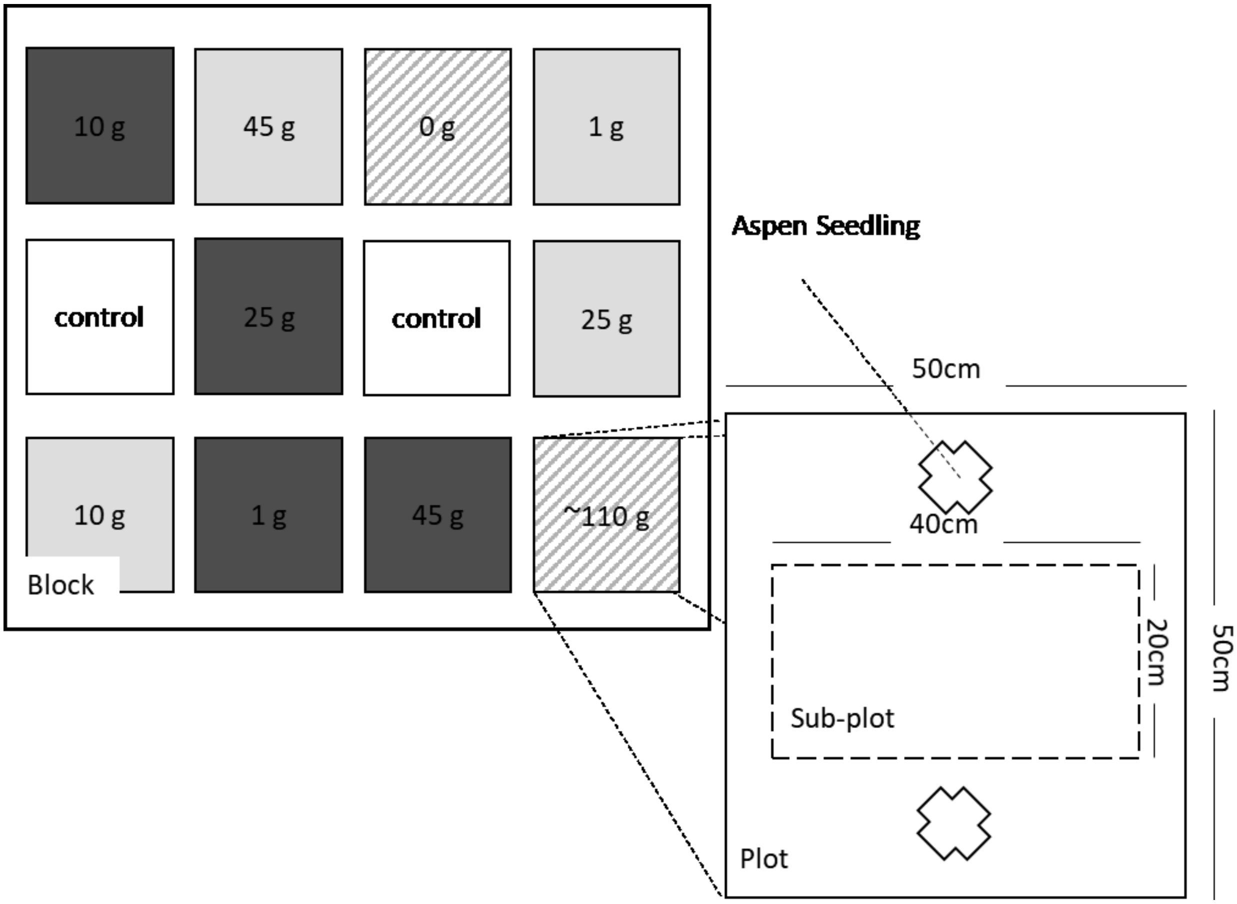

Figure 5-1: Experimental and planting design showing the layout of plots, subplots and planting locations for aspen seedlings. Thirty total blocks were established, fifteen in native rough fescue grassland and fifteen in areas invaded by smooth brome. Within each block, we established twelve plots and implemented randomly assigned a litter manipulation treatment over the whole 50cm x 50cm area (plot assignments from block one are used for illustration).Two plots were assigned as controls (white background), where the litter was not manipulated, the other ten were raked. Of those ten, four had aspen litter added back, in amounts calculated from litter trap transects (45 g, 25 g, 10 g, and 1 g, dark grey background). Four had the same amounts, but using the existing litter added back (45 g, 25 g, 10 g, and 1 g light grey background). One plot had all the raked off litter added back (~110 g striped background), and one had no litter was added back (0 g, striped background)

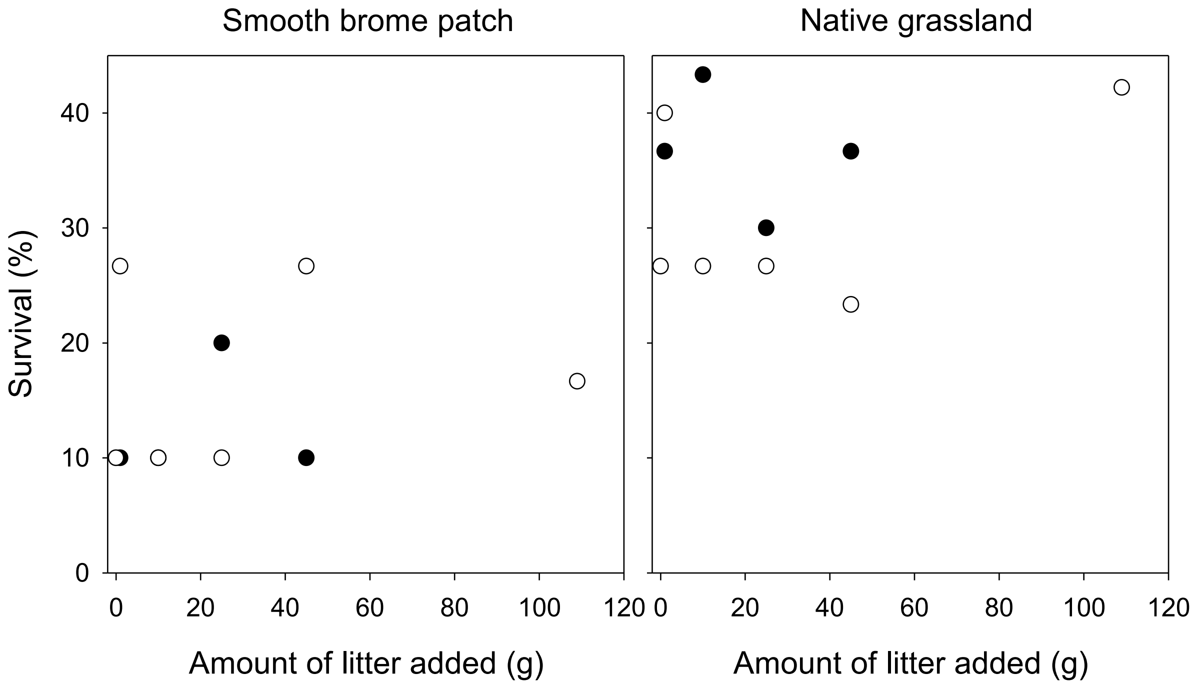

Figure 5-2: Impacts of smooth brome (*Bromus inermis)* presence and amount and type of litter on the survival of trembling aspen (*Populus tremuloides*). Symbol colour indicates type of litter, with filled circles (●) indicating aspen litter, and open circles (○) indicating self litter; litter that was raked from the each plot and then added back. The difference between smooth brome and native grassland is significant ($p = 0.022$), while litter type and litter amount were not significant (see Table 5-2).

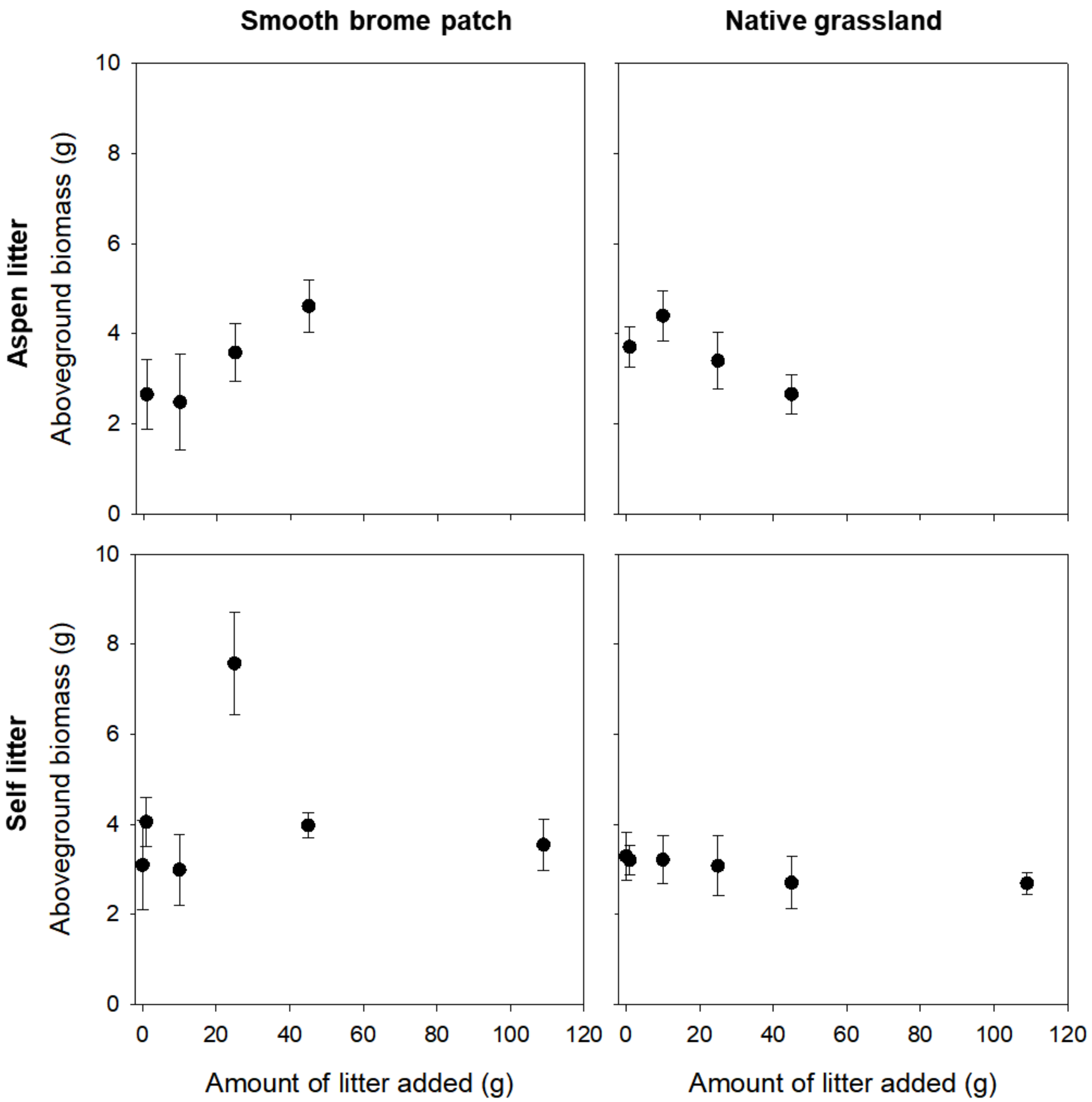

Figure 5-3: Impacts of smooth brome (*Bromus inermis)* presence and amount and type of litter on aboveground biomass of trembling aspen (*Populus tremuloides*). There is a significant three way interaction between litter type, community type and litter amount (p = 0.019).

Chapter 6: Conclusions and future directions

The overarching objective of this thesis is to test whether drivers of plant chemistry indirectly influence plant community dynamics by affecting plant secondary chemistry. I tested this hypothesis in two parts: first I investigated the relative influence of several drivers on aspen secondary chemistry (phenolic glycosides and condensed tannins) at various spatial scales; second, I tested for the effects of aspen secondary chemistry on ecological processes. Dynamics of these interactions are illustrated in Fig. 6-1.

Drivers of aspen secondary chemistry in the aspen parkland

I found that the concentration of secondary chemicals in trembling aspen fine roots was substantially lower than what is typically found in leaves (Fig. 6-1), which is generally supported by the literature (Stevens et al. 2014). I also found that fine root and leaf chemistry were not correlated within individual trees. This finding suggests that there are likely separate evolutionary drivers for above and belowground defences, which corresponds to earlier research showing independent drivers for above and belowground traits in this system (Kembel and Cahill 2011). These results indicate that the chemical ecology of belowground tissues may not follow the same patterns as has been outlined for aboveground tissues, necessitating more work clarifying the differences between the two.

I found evidence that the drivers of aspen foliar chemistry are likely acting at a relatively fine-scale (Fig. 6-1). Due to the clonal nature of trembling aspen, aspen stands likely represent many ramets of a single clone, as opposed to many genetically different individuals (Mitton and Grant 1996). Earlier studies have repeatedly reported that genetics has a strong influence on aspen secondary chemical concentrations (Lindroth and Hwang 1996, Schweitzer et al. 2004, Rubert-Nason et al. 2015). Therefore, I expected aspen chemistry to be similar between trees in the same aspen stands. My results contradict this expectation, but the reasons underlying this difference are not clear. It may be that stands in the aspen parkland sampled are not many ramets of the same genet, but instead unique genetically different individuals, thus the greater local diversity. However, recent work investigating aspen clonal structure across the parkland shows it had lower effective clonal diversity than other subregions, and that the effective number of unique clones in a 50 m^2 area (like our measured plots) would be one (Latutrie et al. 2019).

I also expected to find changes in landscape-scale climatic factors like moisture or temperature to be strong drivers of aspen secondary chemistry, but we could find no evidence for this. Instead, I found that local factors like position in the stand or stem diameter were the best predictors of aspen secondary chemical concentrations, though we did find evidence that season has a widespread impact on aspen chemistry (Fig. 6-1). These results added to my observation of unexpectedly high local variation, suggesting either that the results from greenhouse and growth chamber experiments on aspen chemistry may not be applicable to trees grown in field conditions, or that there are geographical variations in drivers across aspen's range. However, I showed that aspen chemistry was variable across the aspen parkland and that it was impacted by local factors, suggesting that if aspen chemistry can impact ecological processes, those impacts should be detectable in the differences between communities receiving relatively high or low inputs of different aspen secondary chemicals.

Impacts of trembling aspen chemistry on ecological processes

I found evidence that aspen secondary chemicals may influence two ecological processes, community assembly and woody encroachment (Fig. 6-1). Increased condensed tannin concentrations were associated with increased species richness and evenness, and additions of aspen litter improved aspen growth, supporting previous research that suggests that condensed tannins can alter understory community composition through changes to nitrogen availability (Northup et al. 1998) and that these changes can increase aspen fitness (Schweitzer et al. 2008). However, the relationship between phenolic glycosides and understory community composition did not fit with existing hypotheses, as increased phenolic glycosides were associated with lowered species richness and evenness. Furthermore, aspen litter only increased aspen seedling growth in areas invaded by invasive grass, which also does not fit with existing hypotheses. Further research clarifying the mechanisms of aspen's chemical impacts on community composition and seedling growth is needed.

Shortcomings and future directions

Contrary to my expectations, the major drivers of aspen chemistry in this system were not broad-scale climatic factors. It may be that the natural range of variation in these factors was too low between my study sites to establish this relationship, as all sites occurred within one ecosystem type. Alternatively, more samples might be needed to adequately detect patterns at the landscape-level. I also base some of my interpretations, especially in chapters 2 and 3 on the expected pattern of genetic diversity in this system, but I did not quantify it. Further research explicitly including genetic sampling would do much to address this issue.

Likewise, I draw on links, established in earlier works, between aspen chemical inputs and changes to soil nitrogen availability (Northup et al. 1995, 1998, Schimel et al. 1996, Schweitzer et al. 2004) without actually measuring soil nitrogen mineralization rates to determine if they varied with overstory chemical concentrations or were affected by the addition of aspen litter in the current studies. Further studies clarifying these impacts may also help to explain the unexpected pattern of reduced species richness and evenness that was associated with increased phenolic glycoside concentrations.

I found local drivers of aspen chemistry such as light availability, stand density, and location within stand that have the potential for indirect impacts on community processes in this system. When modelling the distribution of plant communities in aspen systems, the direct impacts of forest stand dynamics are frequently included (e.g. Chipman and Johnson 2002, Kreyling et al. 2012), but to my knowledge, the indirect impacts of changing stand dynamics on community composition through changes to plant secondary chemistry are not. This oversight may lead to models that underestimate the impacts of climate change or human use, which could have negative consequences for the conservation of rare species or make restoration efforts more difficult.

The growing understanding that plant traits can drive ecosystem processes (Diaz et al. 2004) has changed our understanding of plant community ecology (Carmona et al. 2016); however these studies typically do not consider plant secondary chemical traits in their investigations. This may be due to the relative difficulty of sampling and analyses, unfamiliarity with their importance, or their high diversity and plasticity, which may make accurate characterization difficult. However, plant chemical traits have the potential to be just as influential as other traits, perhaps more, as they can also impact community processes beyond the bodies of associated plants (Whitham et al. 2003, Schweitzer et al. 2004). Therefore, I broadly recommend that secondary chemical traits and the factors that influence them should be more widely incorporated into trait-based models of community ecology.

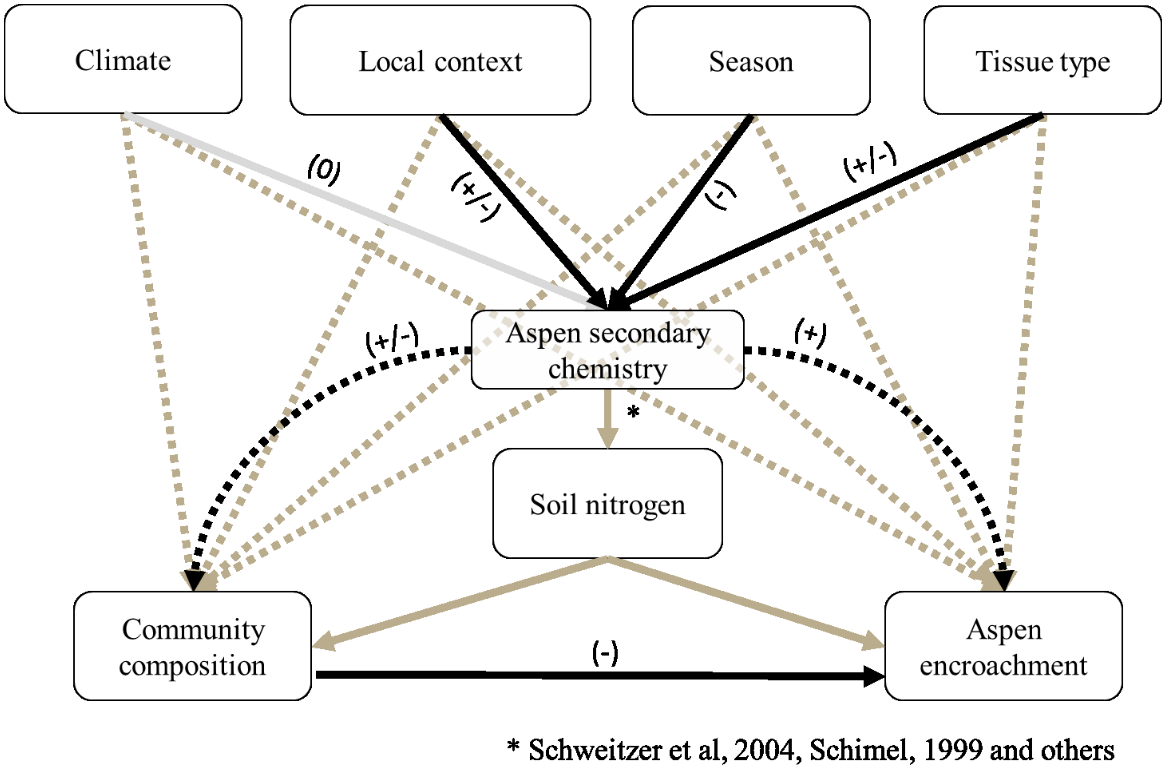

Figure 6-1. Conceptual diagram showing linkages between aspen chemistry, its drivers and ecosystem processes investigated in this thesis. Black linkages were investigated and showed significant impacts. The direction of the impact is indicated in brackets, with +/- indicating either mixed effects or that the relationship between effects was dependent on variation in categorical rather than continuous factors. Grey linkages were investigated, and no relationship was found. Brown linkages are discussed in this thesis, but not explicitly tested.

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Appendices

Appendix 3-1 Full name, abbreviations, latitude and longitude coordinates, and elevation for each of the ten sites surveyed.

Name	Location	Elevation (m)
Aspen Beach Provincial Park	52.464, -113.979	900
Big Knife Provincial Park	52.488, -112.202	680
Dillberry Lake Provincial Park	52.582, -110.018	625
Gadsby Lake Natural Area	52.507, -113.22	880
Roy Berg Kinsella Research Ranch	53.084, -111.558	690
Miquelon Lake Provincial Park	53.239, -112.887	780
Pigeon Lake Provincial Park	53.021, -114.138	875
Pembina River Provincial Park	53.602, -114.998	775
University of Alberta Botanic Garden	53.391, -113.752	700
William Switzer Provincial Park	53.501, -114.998	1150

Appendix 3-2: Mean values (±SE) of secondary chemicals in two chemotypes of trembling aspen (Populus tremuloides) identified in naturally growing aspen stems sampled across central Alberta, Canada

Chemotype	Mean concentrations (mg/g)		
	Condensed tannins	Salicin + salicortin	Tremuloidin + tremulacin
Condensed Tannin	58.09 (± 1.53)	13.92 (± 0.73)	11.14 (± 0.83)
Phenolic glycoside	14.56 (±1.03)	27.81 (± 1.00)	23.44 (± 1.19)

Appendix 3-3

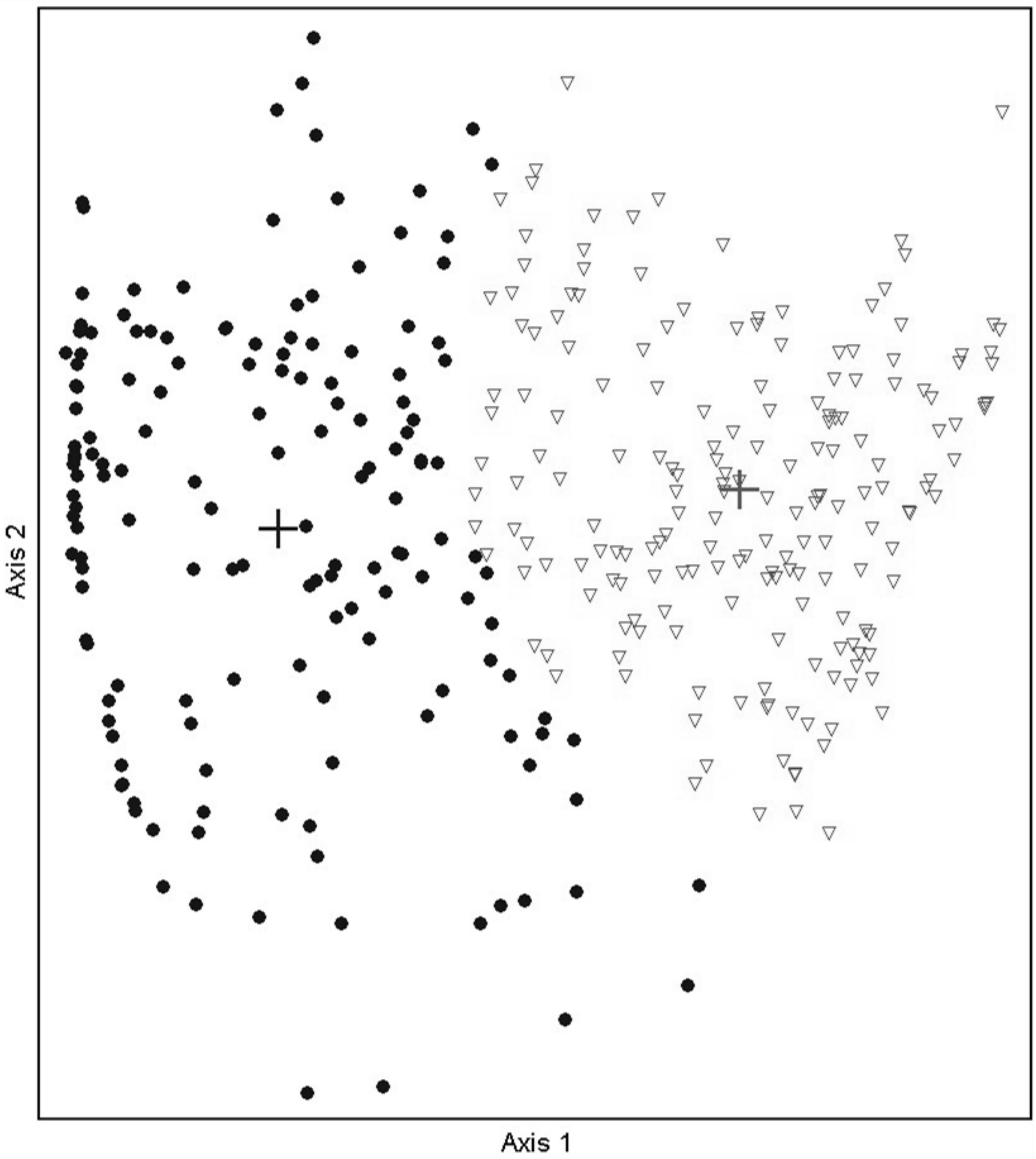

Figure S3-3: NMS ordination of trembling aspen leaf chemistry using the concentrations of three groups of secondary chemicals, colour-coded according to the groups described by our pam clustering procedure. Black circles indicate the phenolic glycoside chemotype and inverted green triangles the condensed tannins chemotype. The coloured crosses represent the centroids for each group. Axis 1 explained 69.8 percent of the variation in the data, axis 2 explained 22.3 percent. Axis one represents a shift from high levels of phenolic glycosides (left) to high levels of condensed tannins (right). Axis two represents a gradient of trees with overall higher secondary chemical values at the top to lower values towards the bottom.

Appendix 3-4: Regression coefficients, X^2 values and *p* values for each variable tested in models that compared environmental and climate impacts on the relative frequency of occurrence of two chemotypes of trembling aspen (*Populus tremuloides*) sampled across central Alberta, Canada. Model terms where $p < 0.10$ are indicated in bold. Site level predictors were run in individual logistic regression models, with proportion of trees in chemotype 1 as the response variable and each climate factors as the predictor. Plot and stem level predictors were run in combined logistic mixed effects models, with proportion of trees in chemotype one/chemotype identity as the response variables. Site was included as a random effect for the plot model and plot nested within site as a random effect for the stem model.

Predictors	**Regression coefficient**	**F**	**p**
Site level predictors			
Mean annual precipitation	0.001	0.090	0.772
Mean annual temperature	-0.178	0.219	0.652
Average summer temperature	0.030	0.074	0.793
May-September precipitation	0.001	0.201	0.666
Precipitation as snow	-0.001	0.046	0.835
Climatic moisture deficit	0.001	0.049	0.830
Elevation	-0.000	0.095	0.766
Latitude	-0.127	0.256	0.652
Longitude	0.010	0.033	0.859
Plot level predictors			
Stand Height	0.013	0.043	0.837
Stand Density	-0.015	0.611	0.438
Location within stand	-0.831	3.218	0.078
Canopy openness	0.026	1.394	0.242
Stem level predictors			
Stem diameter	**-0.059**	**4.834**	**0.029**
Location within stand	0.908	1.487	0.233
Stem diameter x location	-0.019	0.094	0.759

Appendix 4-1: Full name, abbreviations, latitude and longitude coordinates, elevation and mean annual precipitation from 2008 to 2015, and mean foliar chemical values for each of the ten sites used in this survey. Mean annual precipitation was obtained from the Alberta Climate Information Service (ACIS).

Name	Location	Elevation (m)	Mean CT (SE) (mg/g)	Mean PG (SE) (mg/g)	Species Richness
Aspen Beach Provincial Park (AB)	52.464, -113.979	900	61.35 (4.81)	29.27 (10.63)	26
Big Knife Provincial Park (BK)	52.488, -112.202	680	23.66 (3.77)	48.39 (6.57)	25
Dillberry Lake Provincial Park (DL)	52.582, -110.018	625	63.24 (8.94)	44.59 (13.02)	26
Gadsby Lake Natural Area (GL)	52.507, -113.22	880	36.18 (2.57)	20.54 (4.45)	41
Roy Berg Kinsella Research Ranch (K)	53.084, -111.558	690	35.52 (5.36)	53.01 (6.37)	23
Miquelon Lake Provincial Park (ML)	53.239, -112.887	780	14.27 (5.74)	42.85 (7.91)	19
Pigeon Lake Provincial Park (PL)	53.021, -114.138	875	15.69 (4.13)	34.33 (5.46)	20
Pembina River Provincial Park (PR)	53.602, -114.998	775	46.66 (3.48)	37.06 (11.82)	31
University of Alberta Botanic Garden (UA)	53.391, -113.752	700	62.13 (5.35)	19.00 (1.61)	28
William Switzer Provincial Park (WS)	53.501, -114.998	1150	28.64 (8.09)	36.84 (4.86)	38

Appendix 4-2: p values, r^2 values, and AIC values from LMM models that used an alternate method to calculate weighted values of condensed tannins and phenolic glycosides. In this calculation, individual tree areas were compared to the total stem area of all trees observed in the plot, not just the 5 trees that were sampled for foliar chemistry. Models had site as a random effect. Models with significant p values ($p<0.05$) are highlighted in bold.

Variable	**Richness**			**Evenness**			**Axis 1**			**Axis 2**			**Axis 3**		
	p value	r^2m r^2c	AIC	p value	r^2m r^2c	AIC	p value	r^2m	AIC	p value	r^2m r^2c	AIC	p value	r^2m r^2c	AIC
Weighted Condensed Tannins	0.592	0.003 0.401	142.9	0.218	0.016 0.444	-71.7	0.705	0.001 0.734	87.3	0.445	0.007 0.399	117.7	0.527	0.005 0.260	119.4
Weighted Phenolic Glycosides	**0.003**	**0.076** **0.435**	**135.1**	**0.002**	**0.077** **0.460**	**-78.7**	0.377	0.003 0.737	86.7	0.152	0.019 0.392	116.3	0.131	0.026 0.248	117.7

Appendix 5-1

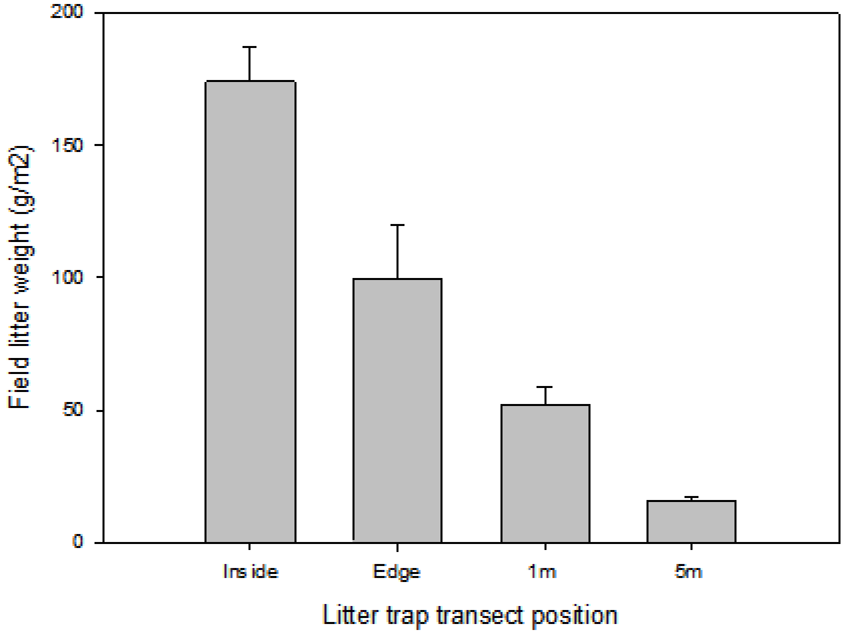

Figure S5-1 Mean litter fall of trembling aspen (*Populus tremuloides*) litter at each transect position in grams/m^2. The inside position was approximately 5 m from the edge of the stand.