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Physiological Effects of Pathogen and Herbivore Risks Encountered by Quaking Aspen

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Master of Science

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ABSTRACT

Physiological Effects of Pathogen and Herbivore Risks
Encountered by Quaking Aspen

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Master of Science

Quaking aspen (*Populus tremuloides*) is the most widely distributed tree in North America (Lindroth and St Clair 2013), and a keystone species in our western montane forests (Worrall et al. 2015). Aspen has become a model organism for studies of genetics and physiology in woody plants (Bradshaw et al. 2000, Taylor 2002). Aspen is also economically important (Worrall et al. 2015) – wood is harvested for various uses, its scenic beauty helps sustain the tourism economy in many areas, and it has recently been studied as a possible source of biofuel (Sannigrahi et al. 2010).

Aspen is also a species of conservation concern, due to recent large-scale deterioration and decline of many aspen forests in the last two decades (Worrall et al. 2013). Several causal factors have been identified: fire suppression (Calder et al. 2011, Smith et al. 2011), increased ungulate herbivory (Kay and Bartos 2000), disease (Marchetti et al. 2011), and climate change (Worrall et al. 2013). My thesis focuses on two different biotic stressors of aspen: a fungal pathogen and ungulate herbivory. Understanding the relationship between aspen and their biotic stressors adds to our knowledge of aspen ecology and helps manage the increasing risk of decline in our aspen forests.

Chapter 1 is a study of the relationship between aspen and a necrotrophic fungal pathogen (*Drepanopeziza* sp.) during a major disease outbreak in 2015. I quantified the relationship between *Drepanopeziza* infection severity and aspen leaf functional traits, including morphological, chemical and phenological traits. I found that severe *Drepanopeziza* infection was associated with low concentrations of a key class of herbivore defense compounds (phenolic glycosides), and strongly associated with early budbreak and leaf-out in aspen stands. The association between infection and early budbreak was likely caused by unusually rainy conditions in May of 2015, which may have exposed leaf tissue to wet conditions that favor the dispersal of *Drepanopeziza* spores.

Chapter 2 is an experiment designed to determine whether the mode and timing of herbivory can influence aspen's defensive response. I specifically asked whether removing leaves, twigs and meristems together and removing leaves alone had unique effects on aspen sucker growth, survival, and phytochemistry. Additionally, I applied these simulated herbivory treatments to suckers on different dates to see whether early- or late-summer herbivory had greater effects on suckers. I found strong mode and timing effects on growth and survival, but not foliar chemistry.

Keywords: herbivory, plant defense, pathogen, ungulate, *Populus tremuloides, Drepanopeziza,* aspen, forest ecology

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CHAPTER 1

Outbreak of *Drepanopeziza* Fungus in Aspen Forests and Variation in Stand Susceptibility: Leaf Functional Traits, Compensatory Growth, and Phenology

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ABSTRACT

In the spring of 2015, a severe outbreak of the necrotrophic pathogen *Drepanopeziza* (also known as *Marssonina*) spread across large portions of aspen (*Populus tremuloides*) forests in the western United States. Among adjacent stands, some were diseased and others were not. *Drepanopeziza* infection in diseased aspen stands stimulated compensatory growth of second-flush leaves at the top of the canopy. These patterns of infection provided an opportunity to characterize associations of pathogen infection and leaf functional traits. Eight pairs of adjacent healthy and diseased aspen stands were identified across a forest landscape in northern Utah. Leaf size, specific leaf area (SLA), photosynthesis, starch concentration, and defense chemistry expression (phenolic glycosides and condensed tannins) were measured on original, first-flush leaves in the lower portion of the tree canopy of healthy and diseased stands and compensatory, second-flush leaves produced in the canopy top of diseased stands. Only first-flush leaves of diseased stands showed high levels of *Drepanopeziza* infection. Leaf area of second flush leaves of diseased stands was 3-fold larger than all other leaf types in healthy or diseased stands. Lower canopy leaves of healthy stands had the highest SLA. Photosynthesis was lowest in infected first-flush leaves, highest in second-flush leaves of diseased stands and intermediate in leaves of healthy stands. Foliar starch concentrations were lower in leaves of diseased stands than leaves from healthy stands. Condensed tannins were greater in second-flush leaves than first-flush leaves in both healthy and diseased stands. Phenolic glycoside concentrations were lowest in

infected leaves of diseased stands. Diseased stands leafed out a week earlier in the spring than healthy stands, which may have exposed their emerging leaves to rainy conditions that promote *Drepanopeziza* infection. Compensatory leaf re-growth of diseased stands appears to offset some of the functional loss (i.e. photosynthetic capacity) of infected leaves.

INTRODUCTION

Plant-pathogen interactions shape plant community assembly and ecosystem function (Gilbert 2002; Mordecai 2011). The pathogen life cycle involves spore dispersal, finding a suitable plant host, germination, infection, and reproduction (French and Manion 1975; Tack et al. 2012). Co-evolution has resulted in plant defense strategies that target and interfere with the pathogen's life cycle (Tack et al. 2012). The ability of plants to defend themselves from pathogen infection is determined by the efficacy of their defense traits (Anderson et al. 2004; Brown and Tellier 2011) and ecological conditions (Burdon et al. 2006). Environmental factors that influence patterns of pathogenicity include spatial and temporal conditions of disease outbreaks (Alexander and Holt 1998), competition (Alexander and Holt 1998), herbivory (Stephenson et al. 2004; Strauss et al. 2002) disturbance (Gilbert 2002) and weather conditions (Bjerke et al. 2014; Hewitt et al. 2016; Sturrock et al. 2011).

Weather patterns can have strong effects on the frequency and severity of pathogen outbreaks (Anderson et al. 2004; Garrett et al. 2011). Precipitation, humidity and temperature influence host susceptibility, pathogen virulence (Anderson et al. 2004), and cause shifts in phenology that alter pathogen-host interactions (Dantec et al. 2015; Dodd et al. 2008). Both direct and indirect effects of weather on fungal pathogenicity have been reported; rainfall tends to aid sporulation and dispersal, but also may improve the host plant's vigor and defense (Desprez-Loustau et al. 2006). Pathogen outbreaks that occur during unusual weather events

provide rare opportunities to better understand how these relationships work under natural conditions at the landscape scale (Bjerke et al. 2014).

Plant defense against pathogens or herbivores can be classified in three categories: resistance, tolerance, and escape. Several theories have been developed to explain how developmental, genetic, and environmental factors influence the relative importance of each defensive strategy (Stamp 2003). Resistance traits enable a plant to prevent or limit the extent and damage of pathogen infection. For example, genetic variation in glucosinolate production controlled the mycelial growth of the root pathogen *Verticillium longisporum* in Arabidopsis (Witzel et al. 2013). Tolerance reflects the ability of a plant to maintain fitness despite tissue infection. This may include the capacity for compensatory leaf growth after leaf damage or defoliation events (St Clair et al. 2009). Measuring the carbohydrate status and photosynthesis rates of original- and compensatory-flush leaf tissue identifies energy source-sink relationships, and the importance of compensatory reflushing as a tolerance strategy after leaf damage has occurred (St Clair et al. 2009). Escape reflects the ability of a plant to avoid pathogens by altering their phenology. Phenology has been recognized as an important factor regulating the distribution of insect folivores around the globe (Ayres and Lombardero 2000; Pureswaran et al. 2015), but the importance of phenology in plant pathogen escape has received less attention (but see Dodd et al., 2008; Dantec et al., 2015).

Aspen (*Populus tremuloides*) is a long-lived, clonal tree species that is widely distributed and ecologically important in forests of North America. Aspen forests are subject to a wide variety of pathogens and experience large inter- and intra-annual fluctuations in weather patterns across their range. Aspen also exhibit considerable variation in plant defense traits and budbreak phenology in the spring. For example, variation in condensed tannin concentrations was correlated with resistance to the fungal pathogen *Venturia moreletti* (Holeski et al. 2009b) and

variation in foliar phenolic glycosides and budbreak phenology can determine susceptibility to insect folivores (Donaldson and Lindroth 2007; Donaldson and Lindroth 2008; Osier and Lindroth 2006; Uelmen et al. 2016). Aspen also exhibit varying levels of tolerance to defoliation (Stevens et al. 2008), and can produce second-flush leaves following leaf damage and defoliation (Donaldson and Lindroth 2008; Harniss and Nelson 1984; St Clair et al. 2009). Defense chemistry expression has been used to assess the resistance potential of aspen to pathogens (Holeski et al. 2009b), and photosynthesis and foliar starch concentrations have been used to characterize their capacity for tolerance to leaf damage and stress (Rhodes et al. 2016; St Clair et al. 2009).

Drepanopeziza is a genus of necrotrophic fungal pathogens that infect the leaves of many *Populus* species (Spiers and Hopcroft 1998). Aspen have shown considerable genetic variation in their ability to resist *Drepanopeziza* infection (Busby et al. 2015; Sinclair and Lyon 2005). *Drepanopeziza* is dependent on rainfall to spread its spores to young, emerging aspen leaves (Ostry 1987), so the coincidence of aspen budbreak and rainfall could be a critical driver of this pathogen-host relationship. In 1981 and 1982, a widespread *Drepanopeziza* outbreak was documented across the western US (Harniss and Nelson 1984). In the spring of 2015, another widespread and severe outbreak of *Drepanopeziza* leaf spot occurred on aspen in several states in the Western US (personal observations: Sam St. Clair, John Guyon, Liz Hebertson, Joel McMillan). In many stands, severe necrosis of the original leaves spurred compensatory growth resulting in the production of a second flush of leaves at the tops of the aspen trees in mid-summer, as also observed by Harniss and Nelson (1984) in the 1981-1982 outbreak; this pattern has also been observed in insect- and frost-defoliated aspen (Donaldson and Lindroth 2008; St Clair et al. 2009). This compensatory regrowth appeared to produce much larger leaves, and showed none of the necrosis observed in the original leaves. In many cases, stands adjacent to

affected stands seemed to resist or avoid infection altogether, and while they appeared to produce new growth near the canopy top, leaf sizes were much more typical. Within the study area, healthy and diseased stands occupied the same environments and were often adjacent to each other, suggesting that differences were due to genotype rather than environmental factors.

The purpose of this study was to identify the relationships between patterns of infection, compensatory growth and functional traits of aspen leaves. We predicted: 1) that *Drepanopeziza* infection patterns would vary strongly between stands, and be greater in original first flush leaves (lower canopy) than compensatory reflush leaves (upper canopy) of infected stands; 2) shifts in leaf anatomy (area, SLA) primary metabolism (gas exchange, starch concentrations), and defense chemistry expression (phenolic glycosides and tannins) between diseased and healthy stands, and original and compensatory leaf growth; 3) differences in susceptibility to infection between stands would be related to defense chemistry expression and budbreak timing.

MATERIALS AND METHODS

Study Location

The study was conducted on Wolf Creek Ranch in the Wasatch Mountains of Utah, USA (40°31'30.91"N 111°15'30.45"W, elevation 2430 m). The ranch is dominated by an Aspen parkland-type landscape, with large stands of *Populus tremuloides* interspersed with open meadows. All measurements and leaf sample collections occurred on August 14, 2015.

Experimental Design

To characterize the effects of stand variation in relation to *Drepanopeziza* infection, we identified eight sites with a healthy and diseased stand adjacent to each other. Healthy stands were initially defined as stands that had very little or no visible leaf blight. In contrast, diseased stands had high incidence of leaf blight throughout the mid- and lower canopy. Paired healthy

and diseased stands were separated by 40 meters or less at each site. The study sites were approximately evenly spread across a landscape area of 30km^2. At each site, we collected data and leaf samples from three trees each in a healthy and diseased stand. In each case, we aimed to select trees of similar height (mean: 6.49 m SE: ± 0.176 m) and DBH (mean: 8.89 cm SE: ± 5.73 cm), using 10 cm in base trunk diameter as a selection target. Differences in height and DBH of trees between healthy and diseased stands were not statistically significant.

We used pole pruners to collect branch segments from both the lower- and upper-canopy of each tree. This sampling strategy ensured that original leaves produced at the end of spring in the lower canopy and compensatory-second flush leaves produced at the top of the canopy of diseased trees in the early to mid-summer were measured separately. Even though healthy trees had leaves that were not visibly different between the lower- and upper-canopies, we measured and sampled from the same canopy positions to match the sampling pattern for diseased trees, and to account for known ontological and phytochemical differences between leaves from different canopy locations (Holeski et al. 2009a). To minimize the confounding effects of leaf age, we only selected mature, fully-expanded leaves for analysis. Fully-expanded leaves are easily distinguished from immature, partially-expanded leaves by color, texture, and distance from apical meristems. We measured gas exchange immediately after branch collection (see below), then removed leaves from each branch segment and placed them on dry ice. Leaves were stored in the laboratory at -80° C until freeze-drying to preserve the integrity of phytochemical compounds (Lindroth and Koss 1996). After freeze-drying, leaf samples from the three trees sampled in each stand were pooled together for analysis.

Pathogen Identification and Quantification of Disease Severity

Fungal samples were isolated from infected leaves collected from each of our 8 stand pairs. Two distinctive types of lesions were observed on infected leaves, punctate and dendritic.

An isolation series was conducted on 15 percent V8 agar (Spiers 1989), and the cultures were placed in a growth chamber at 20° C for 4 weeks. The isolated fungi colonies were identified as *Drepanopeziza spp*. based on the distinctive morphology of the two celled macroconidia. The punctate lesions consistently yielded cultured colonies with microconidial morphology consistent with *Marssonina brunnea* (sexual stage *Drepanopeziza tremulae*) and the dendritic lesions yielded cultures consistent with *Marssonina populi* (*Drepanopeziza tremulae*) based on descriptions of *Marssonina* spp. (Spiers 1984; Spiers 1990).

To quantify the extent of *Drepanopeziza* infection, we scanned leaf samples using image analysis software to determine the relative amounts of healthy and infected leaf tissue. Leaves were laid on a flatbed scanner (Epson Expression 10000XL, Epson America, Inc., Long Beach, CA) and scanned using WinRHIZO software (WinRHIZO 2009, Regent Instruments Canada, Inc., Québec, Canada). WinRHIZO color analysis was used to determine the proportions of infected and healthy leaf tissue in each scanned image. This software uses the RGB color value of each pixel to classify pixels according to user-defined groups. For example, pixels that are colored black or brown were classified as "necrotic," while pixels in shades of green are classified as "healthy." The number of pixels in each group was used to calculate the areas of healthy and necrotic tissue on each leaf surface. Similar pixel classification tools have successfully been used to quantify pathogen-caused foliar damage in previous studies (*i.e.* Giertych and Suszka 2010). Based on visual comparisons between the original scans and processed images, it was clear that the pixel-classification tool was able to accurately distinguish between areas of healthy and necrotic leaf tissue. These areas were used to calculate the percent area infected for each set of pooled leaf samples.

Leaf Morphology

After freeze-drying the leaf samples, area measurements were obtained using a leaf area meter (LI-3000, LI-COR Environmental Inc., Lincoln, NE). Specific leaf area was calculated by dividing the total area of all leaves in each pooled sample by leaf mass measured on an analytical balance (Sartorius Analytical Balance CPA224, Sartorius AG, Göttingen, Germany).

Leaf Gas Exchange

Photosynthesis (assimilation maximum) and stomatal conductance were measured using a leaf chamber and portable gas analyzer (LI-COR 6400, LI-COR Environmental Inc., Lincoln, NE). Gas exchange was measured immediately after branch harvesting with the pole pruner. Measurements were made on the youngest fully expanded leaf of each harvested branch segment at ambient temperature and humidity. Baseline CO2 concentrations were maintained at 395 ppm using a CO2 mixer. Constant photosynthetic photon flux density (PPFD) of 1200 µmol m-2s-1 was achieved using a blue-red LED light source within the leaf chamber. Measurements were initiated by sealing the leaf in the chamber. After allowing CO2 and water vapor concentrations to stabilize (60-90 s), we logged rates of photosynthesis and stomatal conductance. We have previously determined that stomates do not begin to close until ~6 min after branch harvesting in aspen (St Clair et al. 2010). All measurements were taken between 10:00 and 15:00 h to avoid diurnal biases.

Phytochemical Analyses

Pooled leaf samples collected from each tree were freeze-dried, ground and homogenized using a mixer mill with a #10 mesh screen (Wiley Mill, Thomas Scientific, Swedesboro, NJ). Starch, phenolic glycosides (salicortin and tremulacin), and condensed tannins were extracted from the freeze-dried leaf samples in preparation for analysis.

Samples for starch analysis were prepared by removing sucrose and glucose by homogenizing the leaf samples for 5 minutes in 80% ethanol using a vortex, spinning the samples down in a centrifuge and removing the supernatant and repeating the extraction two more times (Hendrix 1993)). We added 1 ml DI water to the remaining plant tissue and autoclaved these samples for 1 hr at 275°C. After autoclaving, samples were vortexed for 2 minutes then centrifuged at 16.1g for 10 minutes. The supernatant was transferred to another tube and 1 ml of alpha-amylase solution (Megazyme, Wicklow, Ireland) was added to each sample. The samples were then incubated for 20 minutes in a boiling water bath. During incubation, samples were inverted every 5 minutes to ensure adequate mixing. After cooling, 15 µl of amyloglucosidase (Megazyme) was added to each sample and the samples were incubated in a heated vortex at 50°C for 45 minutes. Next, 20 µl of sample was pippeted into microplate wells. Finally, 200 µl of GOPOD reaction mix (Megazyme) were added to each sample well. After 15 min of incubation at room temperature, A¬550 absorbance was read using a spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). A standard curve, generated from purified starch standard (Megazyme), was used to quantify the unknown starch concentrations of the samples.

Phenolic glycosides were extracted from 40 mg of ground leaf tissue in 0.66 ml of methanol. Leaf tissue and methanol were combined in a 2 ml vial and vortexed for 1.5 minutes. Then, vials were centrifuged at 16.1 g for 1 minute. The supernatant was pippeted into a separate vial. This procedure was repeated twice more to produce a total of 2 ml supernatant for each extracted sample. Phenolic glycoside concentrations were quantified using high-performance liquid chromatography (Agilent 110 Series, Santa Clara, CA) with a Luna 2, C18 column (150 x 4.6 mm, 5 um) at a flow rate of 1 $ml \cdot min^{-1}$. Compound peaks were detected using a UV lamp at a

wavelength of 280 nm using purified salicortin and tremulacin standards isolated from aspen leaves (Lindroth et al. 1993).

Condensed tannins were extracted from 40 mg of ground leaf tissue. Leaf tissue was combined with 1 ml of 70% acetone-10 Mm ascorbic acid solution. Samples were vortexed for 30 minutes at 4°C, then centrifuged at 16.1g for 10 minutes. The resulting supernatant was pipetted into a separate vial. This process was repeated to generate 2 ml of supernatant for each sample. 100 µl of this supernatant were combined with 150 µl acetone-ascorbic acid solution, 1 ml acid butanol, and 50 µl of iron reagent, and then incubated in a boiling water bath as described in Porter et al. (1985). Condensed tannin concentrations were then quantified using a spectrophotometer (SpectraMax Plus 384). Purified condensed tannins isolated from aspen leaves were used as a measurement standard (Hagerman and Butler 1989).

Leaf Budbreak Survey

To explore whether the timing of budbreak was related to the incidence of *Drepanopeziza* infection, we monitored leaf out dates in our stands during the spring of 2016. We visited each site approximately every three days and recorded the first observation of leaf budbreak. To improve the temporal resolution of our survey, we assessed photos taken at each observation, and estimated the exact day of budbreak by comparing the size and development of leaves in each photo.

Statistical Analysis

Linear mixed-effects models (lmerTest package in R, Kuznetsova et al. 2015) were used to test whether differences in infection status (healthy vs. diseased) and leaf type (lower canopy original growth vs. upper canopy compensatory growth) influenced leaf function. Data exploration was conducted per the methods of Zuur et al. (2010) to verify that model assumptions were met. Leaf infection rate data was log-transformed prior to analysis to satisfy

equal variance assumptions. For each model, stand health and canopy position (lower canopy leaves vs. upper canopy leaves) were designated as fixed effects, and site was specified as a random effect. Response variables were log(leaf size), photosynthesis rate, stomatal conductance, tannin concentration, and total phenolic glycoside concentration. The estimated coefficients, along with *p*-values and confidence intervals, were calculated to determine whether stand health, leaf type, or their interaction was associated with changes in the response variables. Alpha was specified as 0.05. Least squared means were used to estimate the magnitude of the differences between upper and lower canopy leaves in diseased stands and leaf tissue from the corresponding canopy heights in healthy stands.

To test whether timing of budbreak was related to the incidence of infection, we created a linear mixed-effects model, with the average Julian day of leaf out in 2016 in each stand as a function of 2015 stand health (fixed effect) and site (random effect). The estimated coefficient for stand heath was used to determine whether this variable was correlated to the average day of leaf out. All statistical tests were performed in R version 3.3.1 (r-project.org).

RESULTS

Leaf Infection Rates

Only the original, first-flush leaves of diseased stands had high proportions of infection and necrotic tissue (43%). In contrast, second flush leaves of diseased stands and all leaves from healthy stands had very low levels of leaf infection and necrotic lesions (< 3%) (Fig. 1-1).

Leaf Morphology

Stand health condition and canopy position both significantly impacted leaf morphology. Upper canopy leaves were larger than lower canopy leaves in both healthy stands and diseased stands (Fig. 1-2a) ($p = 0.0001$). The mean leaf area of all upper canopy leaf samples was 39.3

(SE±3.2); the mean leaf area of all lower canopy leaf samples was only 13.2 (SE±3.2). However, the magnitude of the difference was much greater in diseased stands than healthy stands (4.5-fold vs. 1.6-fold, Fig. 1-2a).

Stand health condition and the interaction between stand health and canopy position both significantly affected SLA (Fig. 1-2b) ($p = 0.0128$ and $p = 0.0132$, respectively). These effects were primarily driven by high SLA in the lower canopy leaves of healthy stands. The average SLA of these leaves was 107, while averages for all other leaf types ranged from 78 to 83.

Starch

Starch was 62% lower ($p = 0.01$) in the first-flush leaves of diseased stands, compared to the lower canopy leaves of healthy stands. Starch concentrations in upper canopy leaves of diseased stands were not significantly different from the upper canopy leaves in healthy stands ($p = 0.5$) (Figure 1-4). Starch concentrations did not vary significantly between upper and lower canopy leaves overall (upper canopy mean = 2.42, SE±0.59; lower canopy mean = 2.30, SE±0.59; $p = 0.83$), and there was no significant interaction of stand health condition and canopy position for foliar starch ($p = 0.17$) (Fig. 1-4).

Leaf Gas Exchange

Upper canopy leaves had higher rates of photosynthesis than lower canopy leaves in both healthy and diseased stands ($p < 0.0001$). The mean rates of photosynthesis in all upper canopy measurements was 19.7 (SE±1.0); the mean rate of all lower canopy measurements was 9.2 (SE±1.0). However, the magnitude of the difference was much greater in diseased stands compared to healthy stands (4.3-fold vs. 1.2-fold, Fig. 1-3a). Stomatal conductance was higher in upper canopy leaves in both healthy and diseased stands (upper canopy mean = 0.225, SE±0.015; lower canopy mean =0.099, SE±0.015; $p < 0.0001$). However, the effect of canopy position was also much greater in diseased stands (3.5-fold vs. 1.4-fold, $p < 0.0001$) (Fig. 1-3b).

Defense Chemistry

Stand health condition did not significantly influence foliar tannin concentrations, but leaves at the top of the canopy had on average 54% higher tannin concentrations than leaves lower in the canopy (upper canopy mean = 8.64, SE±0.68; lower canopy mean = 5.62, SE±0.68; $p = 0.009$) (Fig. 1-5a). Phenolic glycoside concentrations varied significantly due to stand health condition, canopy position and their interaction (Fig. 1-5b). This was largely driven by significantly lower phenolic glycoside concentrations in lower canopy leaves of diseased stands, which were 50-60% lower than compensatory reflush leaves produced in the upper canopy of the same trees and leaves from healthy trees ($p < 0.001$). Phenolic glycoside concentrations in the upper canopy leaves of diseased trees were not significantly different from leaves in healthy trees ($p = 0.52$) (Fig. 1-5b).

Timing of Budbreak

The average day of leaf out for all stands in spring of 2016 was May 29th (Julian day 145). Diseased stands leafed out approximately 6.5 days earlier than healthy stands ($p = 0.008$) (Fig. 1-6).

DISCUSSION

The purpose of this study was to identify the relationships between patterns of *Drepanopeziza* infection, compensatory growth and functional traits of aspen leaves. Our results strongly supported our first prediction that infection patterns would vary between stands and be greater in original first flush leaves than compensatory reflush leaves of infected stands (Fig. 1-1). Our second prediction was also largely supported as leaf traits related to leaf anatomy, primary metabolism and defense chemistry expression all varied significantly in response to either health condition, canopy position related to compensatory regrowth of leaves, or both

(Figs. 1-2 through 1-5). Our third prediction was partially supported by the data; there was less evidence that defense chemistry expression may contribute to differences in stand susceptibility to *Drepanopeziza* infection and stronger evidence that budbreak timing may be involved.

Drepanopeziza infection was associated with both physical and chemical changes in original, lower canopy leaves and compensatory-flush, upper canopy leaves that created a clear pattern of infection on the landscape. Adjacent pairs of stands were extremely variable in their susceptibility to the fungus (Fig. 1-1). This pattern is consistent with studies of *Drepanopeziza* in other *Populus* species (Busby et al. 2013). These results suggest that drivers of infection are scale-dependent. Although the *Drepanopeziza* outbreak was likely triggered by regional weather patterns, it appears that infection rates were also strongly influenced by phenotypic variation, which is highly variable among aspen clones (Smith et al. 2011).

The most obvious effects of *Drepanopeziza* infection were severe necrosis of leaves produced in the late spring and the subsequent flushing of compensatory regrowth leaves later in the summer. Compensatory leaf production in aspen has previously been documented in response to frost damage (St Clair et al. 2009) and insect defoliation (Donaldson and Lindroth 2008). However, the dramatic enlargement of second-flush leaves we observed in this study was only noted in response to frost defoliation. Pictures in a publication by Harniss and Nelson (1984) from the large-scale Drepanopeziza outbreak in Utah in 1981-1982 show the same dramatic production of large, compensatory flush leaves that we observed.

Drepanopeziza infection was associated with lower SLA (thicker leaves) in diseased trees. This pattern contrasts with a positive relationship observed between SLA and pathogen infection severity in *Salix* sp. (Toome et al. 2010). An important question is whether native differences in SLA create variable susceptibilities to fungal infection, or if differences in SLA develop in response to fungal infection? The difference in the relationship between SLA and

infection in this study and Toome et al. (2010) may be related to differences in species of trees and pathogens involved. Whether reduced SLA is a cause or effect of *Drepanopeziza* infection is unknown. In either case, this is the first report of an association between SLA and pathogen infection in aspen.

Drepanopeziza infection had strong impacts on leaf gas exchange of the infected stands in this study. Infection directly decreased photosynthetic capacity of the original leaves to approximately 30% of that in uninfected leaves, but indirectly triggered compensatory leaf growth with rates of photosynthesis that were dramatically higher than is typical for aspen (Fig. 1-3a) (St Clair et al. 2010). Due to the high percentage of necrotic lesions covering the surface of infected leaves (42%) it is surprising that these leaves functioned at even 30% of their photosynthetic capacity. It would appear that the potential metabolic cost of dropping the infected leaves and replacing them is higher than just maintaining the leaves at lower rates of photosynthesis and producing compensatory growth leaves that partially compensate for their loss in function. The anatomy of the compensatory re-flush leaves had much higher photosynthetic capacity by producing more total leaf tissue per leaf and thicker leaves (Reich et al. 1998). These changes in leaf morphology and increased photosynthetic capacity in compensatory reflush leaves is nearly identical to that observed in aspen trees that experience frost damage (St Clair et al. 2009).

Foliar starch reserves have been used as a biomarker of leaf vigor and tree health (Wargo et al. 2002). Starch in original and reflush leaves of infected trees were lower than leaves in healthy trees (Fig. 1-4). This may suggest that compensatory regrowth leaves with high photosynthetic capacity can only partially offset losses of leaf function and the cost of regrowth.

Defense chemistry also varied with *Drepanopeziza* infection. Infected stands had reduced concentrations of phenolic glycosides in original leaves, while regrowth leaves had the same

level of phenolic glycosides as leaves from healthy trees (Fig. 1-5). Phenolic glycosides are known to be important deterrents of foliar herbivory in aspen forests (Lindroth and St Clair 2013), but their effect on fungal pathogens is not well understood (Holeski et al. 2009b). The reduced phenolic glycoside concentrations in the infected leaves could be either a cause or effect of *Drepanopeziza* infection. If phenolic glycosides confer resistance to *Drepanopeziza*, stands that naturally produce low concentrations of phenolic glycosides would be more susceptible to infection. This view is supported by the fact that constitutive levels of phenolic glycosides are known to vary among genotypes (Hwang and Lindroth 1997; Lindroth et al. 2002; Osier and Lindroth 2006). Also, some evidence suggests that phenolic glycosides have direct negative effects on fungal pathogens (Hubbes 1969). Alternatively, carbon limitation of infected leaves could constrain the expression of phenolic glycosides (Hale et al. 2005), or phenolic glycosides were metabolized by the fungus or otherwise degraded in the large areas of necrosis in these leaves.

Tannin concentrations were not significantly affected by *Drepanopeziza* infection, but upper canopy leaves had higher levels of tannins than primary-lower-canopy leaves. The same positive relationship between canopy height and tannin concentration has been observed in *Populus angustifolia* (Holeski et al. 2012). This pattern may be related to greater light availability for canopy top leaves, which can drastically increase condensed tannin expression in aspen (Calder et al. 2011; Hemming and Lindroth 1999; Wan et al. 2014).

The timing of budbreak also varied with *Drepanopeziza* infection with diseased trees leafed out several days earlier than healthy trees. If these stands followed the same phenological patterns in 2015, their leaf tissues may have been exposed to *Drepanopeziza* spores in the early part of the growing season, which typically begins in the latter half of May, based on our phenological surveys. Because *Drepanopeziza* is dispersed during rainfall (Ostry 1987), early

budbreak that coincides with a rainstorm could dramatically increase the exposure risk relative to stands that delayed budbreak until after the rains had passed. May 2015 was exceptionally wet, with 2-4 times more rain than the monthly average across aspen's range in Utah where *Drepanopeziza* outbreak was reported (PRISM Climate Group, Oregon State University). We hypothesize that the heavy rains in May likely affected stands with early budbreak, while stands with late budbreak may have developed leaves after the rains had ceased.

CONCLUSIONS

Our study suggests that aspen may defend against *Drepanopeziza* using risk-tolerant and risk-averse strategies. Using a risk-tolerance strategy, stands leaf out early to maximize the length of the growing season. However, this increases their potential exposure to *Drepanopeziza*, because rainfall and spore dispersal are more likely to occur early in the growing season. If a stand becomes infected, compensatory leaf reflushing may partially offset losses in function of first-flush leaves. Using a risk-averse strategy, stands may leaf out later in the growing season. This could reduce their exposure and susceptibility to *Drepanopeziza* infection, avoiding the cost of compensatory growth but shortening the growing season due to delayed leaf out.

The evolution of these two strategies was likely determined by historical conditions, including weather regimes and the relative frequency and intensity of *Drepanopeziza* outbreaks. In years when *Drepanopeziza* spores are rare or when weather conditions reduce outbreak occurrence, stands with a risk-tolerant strategy have an advantage. When *Drepanopeziza* outbreaks are more frequent, the opposite pattern occurs. A long-term shift in the frequency and intensity of *Drepanopeziza* outbreaks could favor the persistence or expansion of some aspen genotypes and the demise of others. Because rainfall is so important in the life history of *Drepanopeziza* (Ostry 1987) and weather patterns strongly influence plant phenology, shifts in

precipitation patterns and warming temperature related to climate change may favor one strategy over the other. Warming temperatures will accelerate leaf out dates, and it is projected that more rain is likely in the spring period in large parts of aspen's range, including more extreme rain events (Dettinger et al. 2015). Together, these changes have the potential to increase the frequency and severity of *Drepanopeziza* outbreaks in aspen forests in the western United States. This study demonstrates the influence of leaf functional traits on susceptibility and response to *Drepanopeziza* and improves our understanding of the mechanisms of aspen's pathogen defense strategies and how these patterns may change in the future.

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FIGURES

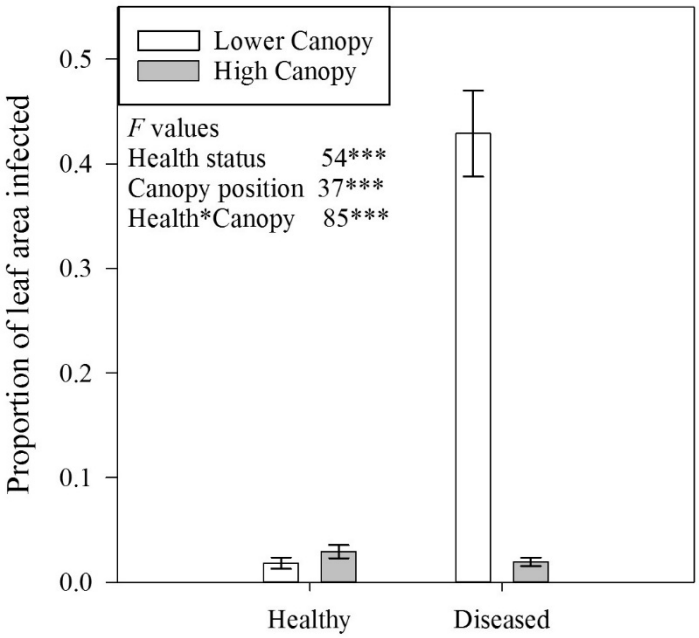

Figure 1-1. Main effects and interactions of stand health and canopy position, representing the differences in original and compensatory growth leaves on the proportion of leaf area infected by *Drepanopeziza*. *F*-values presented are for fixed-effect tests of log-transformed data. Asterisks indicate the level of significance for *P*-values: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Mean values presented with ± 1 SE.

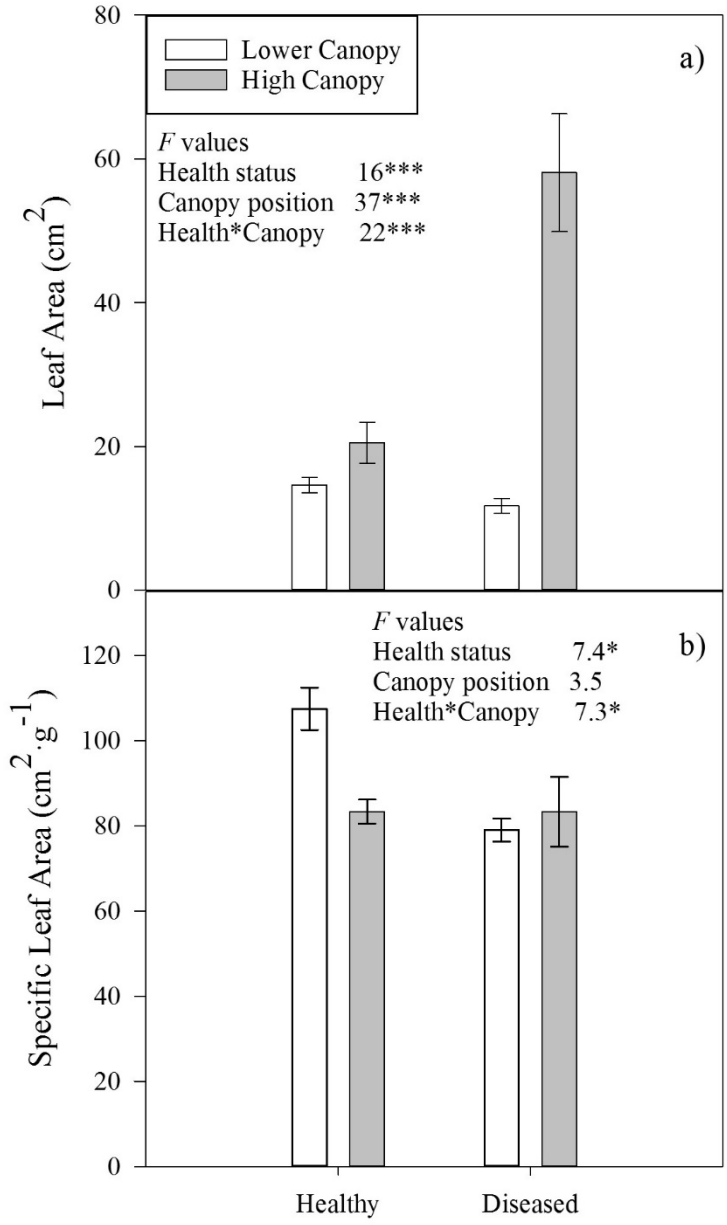

Figure 1-2. Main effects and interactions of stand health and canopy position on leaf area and SLA. Mean values presented with ± 1 SE.

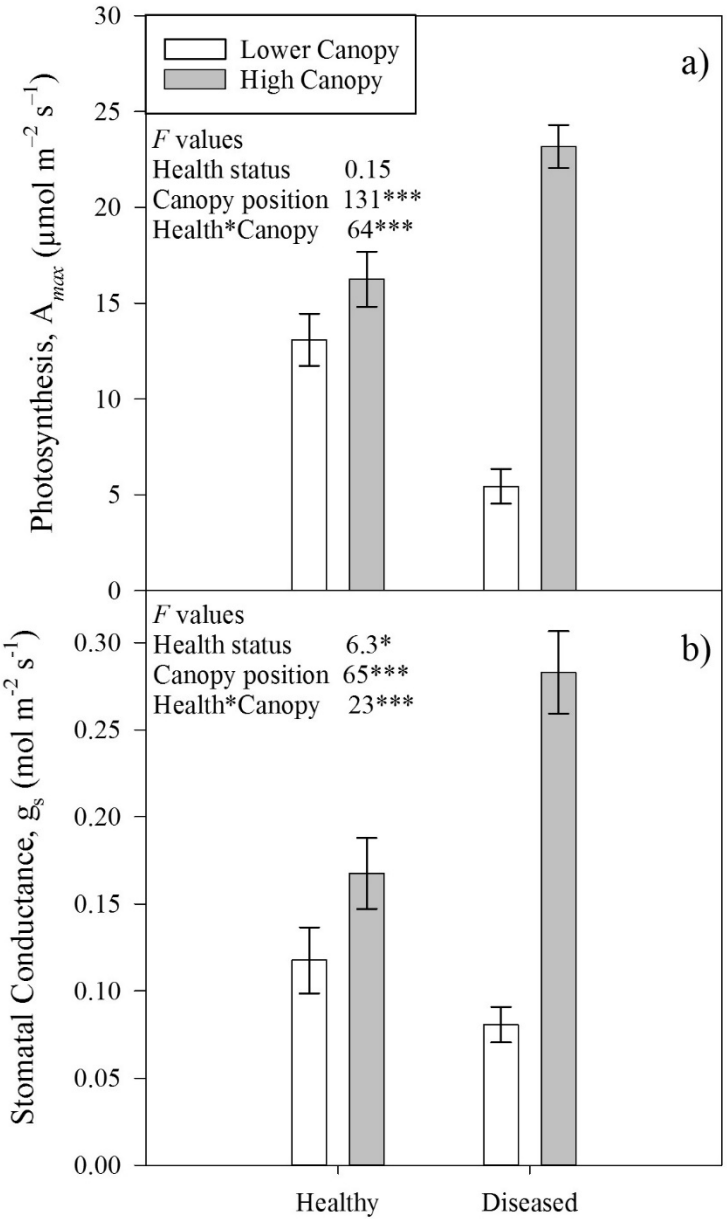

Figure 1-3. Main effects and interactions of stand health and canopy position on photosynthesis and stomatal conductance. Mean values presented with ± 1 SE.

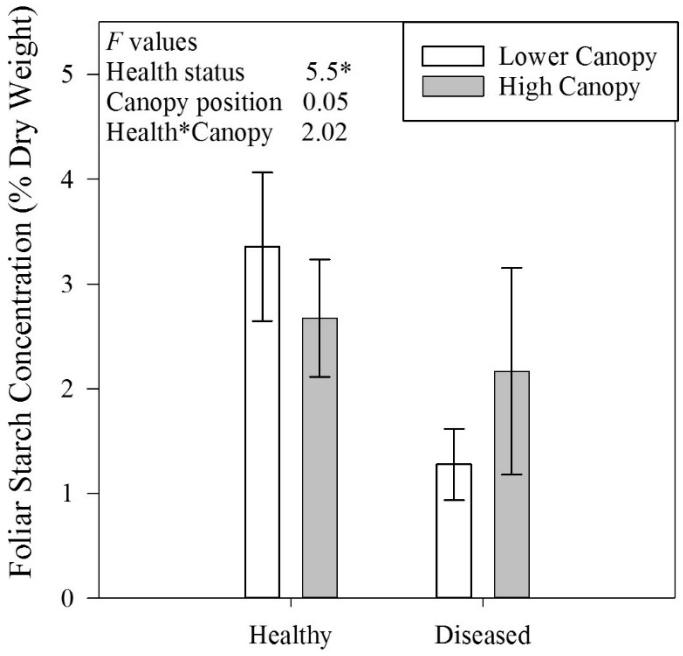

Figure 1-4. Main effects and interactions of stand health and canopy position on foliar starch concentration. Mean values presented with ± 1 SE.

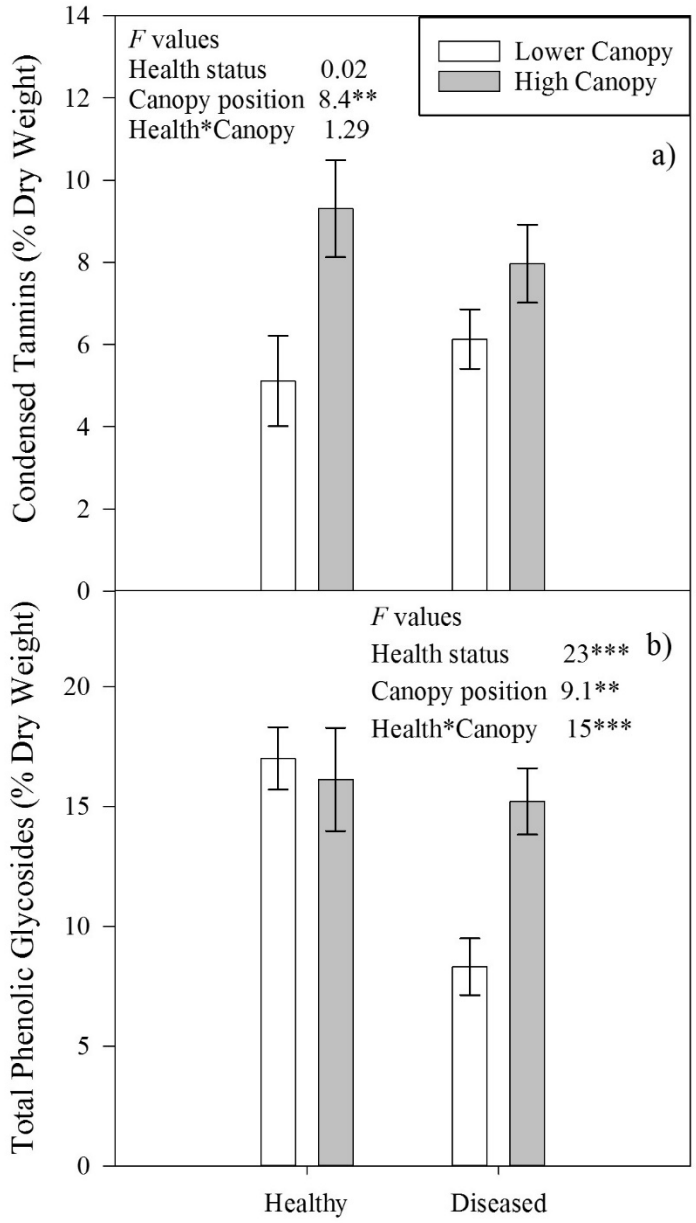

Figure 1-5. Main effects and interactions of stand health and canopy position on foliar defense chemistry. Total phenolic glycosides include salicortin and tremulacin. Mean values presented with ± 1 SE.

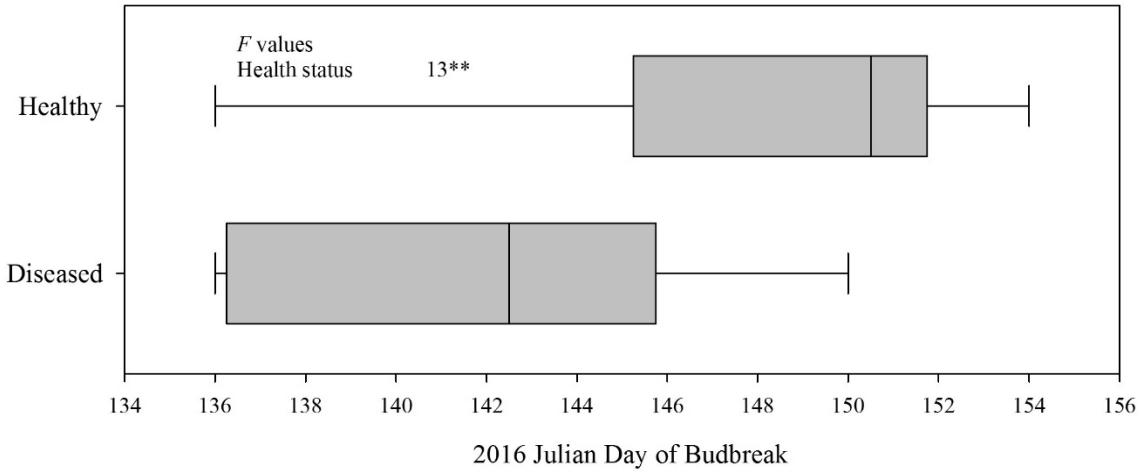

Figure 1-6. Boxplot of stand health effect on 2016 Julian day of budbreak. The mean day of budbreak for all stands was 145 (May 25th). Average day of budbreak in diseased stands was approximately 6.5 days earlier than healthy stands.

CHAPTER 2

Timing and Mode of Simulated Ungulate Herbivory Affect Aspen's Defensive Response

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ABSTRACT

One outstanding question in plant ecology is whether timing of herbivory or selection of specific plant tissues (mode of herbivory) by unique herbivore species can influence plant defense characteristics. In this experiment, we devised two different modes of simulated herbivory, representing a selective ungulate feeding strategy (leaf tissue removal only) and a bulk feeding strategy (leaves, twigs, and meristems taken together). We applied these treatments to juvenile aspen suckers in early summer, late summer, or at both times to determine the effects of herbivory mode, timing, and frequency on aspen's defensive response. We measured height, stem diameter, average leader length, foliar starch, foliar defense chemistry, survival, and aboveground biomass to characterize the effects on three key aspects of defense: resistance, tolerance, and escape. We found that mode, timing, and frequency had no effect on resistance traits. However, all three factors had palpable effects on aspen tolerance and escape. This experiment shows that unique herbivore species may potentially have disparate impacts on the plant community by selecting different tissues of the same plant, or browsing the plant at different times in the growing season.

INTRODUCTION

Herbivory structures plant communities (Augustine and McNaughton 1998) and is a driving force in plant evolution (Nunez-Farfan et al. 2007). The study of plant defense against

herbivores is central to the study of chemical ecology and invasion biology (Burkepile and Parker 2017). However, ecologists are still exploring how the diversity of herbivore species (Charles et al. 2017, Kafle et al. 2017) and the timing (Anderson and Frank 2003, Davis et al. 2014) and frequency (Wisdom et al. 2006) of herbivory events affect plant responses. Plants are often subject to numerous distinct species of herbivore that can cause different types of damage, with different effects on plant survival and compensatory response. For example, native ungulates and livestock may be grazers or browsers, and their different digestive morphologies may alter the ratio of leaves and twigs they consume (Bodmer 1990). Globally, many of these ungulates are currently experiencing shifts in abundance and distribution that alter their impact on plant community composition and structure (Spear and Chown 2009).

In response to herbivory, plants have developed a host of adaptations to guard against their natural enemies (Agrawal 2011). Broadly speaking, these adaptations can be classed into three categories: resistance, tolerance, and escape (Boege and Marquis 2005, Lindroth and St Clair 2013, Norghauer et al. 2014). Resistance allows plants to actively repel would-be herbivores, tolerance preserves plant fitness despite herbivory, and escape enables plants to minimize exposure by growing beyond their herbivore's reach or by altering their phenology. Studies in coevolution have revealed that different plants have developed unique resistance adaptations to defend themselves from specific herbivores or herbivore guilds. For example, many *Poaceae* lineages have developed silica-rich tissues in response to large herbivore grazing (Katz 2015). Plants can also use specific herbivory cues, including insect oral secretions, to signal systemic (Hui et al. 2003) or community-level responses (Kessler and Baldwin 2001) that reduce the negative impacts of herbivory. However, plants may be poorly adapted to novel herbivore introductions or changes in native herbivore density (for example, see Augustine and Frelich 1998, Rose et al. 2005, Bergstrom et al. 2009, and Relva et al. 2010). The Anthropocene

has been marked by global changes in ungulate communities through a litany of factors, including accidental and deliberate introductions, land use changes, predator control, hunting pressure, and livestock grazing (Spear and Chown 2009, Nuñez et al. 2010).

One outstanding question is whether novel ungulate herbivores can elicit unique defensive responses from affected plants by altering the timing and frequency of herbivory or by selecting specific plant tissues (Bork et al. 2013). Whether wild or domestic, large ungulate herbivores have significant economic value and have been profitably used by humans for millennia (Gordon et al. 2004, Spear and Chown 2009). However, recent introductions and changes in population size have modified natural ecosystems across the globe (Spear and Chown 2009). Understanding the trophic impacts of ungulate herbivores is key to maintaining diverse, resilient plant communities and habitat conditions as well as maximizing the economic and ecosystem services in these systems.

Many studies have shown that different species of ungulate herbivores are not functionally equivalent in most systems (i.e. Kay and Bartos 2000, Veblen et al. 2015, Scasta et al. 2016). However, most research has focused on differential space use and selection of different forage plant species. Few researchers have investigated whether different ungulates use a single plant species in different ways. Different ungulate species may select different tissues of the same plant or may prefer to consume the plant in different seasons. Optimal defense theory suggests that plants will strongly defend tissues that are consistently at risk of herbivory (Rhoades and Cates 1976, Rhoades 1979, Herms and Mattson 1992). Additionally, some theory suggests that herbivory of ephemeral versus persistent tissues may favor the evolution of unique plant defense chemistry (Rhoades and Cates 1976). Therefore, plants that have evolved with a late-season, leaf-eating herbivore may be maladapted to early-season herbivory of stems or twigs, and vice-versa. It is plausible that the type of tissues selected, along with the timing and

frequency of selection, could affect plant survival and defensive response – yet few studies have addressed this question.

Aspen (*Populus tremuloides*) forests of western North America provide a good study system to examine the disparate effects of multiple ungulate herbivores on a single plant species. Aspen support a wide variety of herbivores, and are exposed to as many as five different ungulate herbivore species in portions of its range: mule deer (*Odocoileus hemionus*), elk (*Cervus canadensis*), bison (*Bison bison*), domestic sheep (*Ovis aries*) and cattle (*Bos taurus*). Aspen is a widespread, economically- and ecologically-important species with well-characterized genetics and phytochemistry that typically regenerates via root suckering. Juvenile aspen suckers exhibit a combination of resistance, tolerance, and escape traits to defend against ungulate herbivores (Lindroth and St Clair 2013). Resistance mechanisms include the production of phenolic glycosides and tannins in stem and leaf tissues that reduce palatability and nutrition (Wooley et al. 2008). Tolerance mechanisms include the ability to translocate nutrients from overstory trees or belowground tissue through an extensive, clonally-integrated root system and to regrow after damage (Stevens et al. 2007). This type of tolerance can be quantified by measuring energy source and sink dynamics of non-structural carbohydrates in the leaf tissue (St Clair et al. 2009, Rhodes et al. 2016). Escape mechanisms include the ability to rapidly grow beyond the reach of ungulates, which typically have a vertical reach of about 1.5 meters (Bartos et al. 2014, Wan et al. 2014).

European colonization and the attendant land use changes have dramatically altered the range and population density of aspen's ungulate herbivores and created novel herbivory regimes in many areas (Fleischner 1994, Laliberte and Ripple 2004). Each one of these different herbivores has a unique digestive morphology, and may consume aspen suckers in different ways. Potential differences in herbivory patterns among these ungulate species include the

frequency, timing, and mode of herbivory, including patterns of tissue removal (leaf or twig consumption). Each factor could affect aspen's regeneration, recruitment success, and defense response. Understanding the unique effects of each ungulate species could help inform efforts to sustainably manage aspen forests.

The objective of this study is to examine how the timing, frequency, and mode of simulated ungulate herbivory affect aspen's resistance, tolerance, vertical escape, and survival. We hypothesize that: 1) the timing of herbivory will affect aspen tolerance and vertical escape, but not resistance. Early-season herbivory will be less tolerated, will reduce vertical growth, and will increase mortality more than late-season herbivory. 2) The frequency of herbivory will affect resistance, tolerance, and escape. Herbivory in both early- and late-summer will induce greater chemical resistance, will be less tolerated, will reduce sucker heights, and will increase mortality relative to herbivory in early- or late-summer alone. 3) The mode of herbivory will affect resistance, tolerance, and escape – defoliation will induce stronger chemical resistance and will be less tolerated than clipping, but clipping will reduce sucker height more than defoliation.

MATERIALS AND METHODS

Study Site

The study was conducted on Wolf Creek Ranch in the Wasatch Mountains of Utah, USA (40°31'30.91"N 111°15'30.45"W, elevation 2430 m). The ranch is dominated by an Aspen parkland-type landscape, with large aspen stands interspersed with open meadows. Root suckering occurs regularly in the understory of the aspen stands, but high levels of deer and elk herbivory prohibit the persistence of aspen suckers on the landscape. However, three years prior to our experiment, several large (~3 acre) ungulate exclosures were established within aspen

stands in different areas of Wolf Creek Ranch. This enabled the protected aspen stands to produce a large cohort of aspen suckers, all of a similar age and size.

Study Design

We selected 21 aspen suckers in each of five different exclosures on the ranch. Selection criteria included a height of roughly 115 cm (average was 114.6 cm, SE ±2.07; range = 80-152 cm), basal diameter between 8-18 mm, and minimal insect or pathogen damage, wilting, or stem breakage. Suckers were marked with aluminum tags at their base and GPS waypoints were recorded to facilitate relocation. Every sucker was randomly assigned to one of 7 different treatment conditions, each representing a unique combination of mode, timing and frequency of herbivory. The two modes of herbivory were a defoliation treatment and a meristem removal (clipping) treatment. The timing and frequency treatments involved imposing one of the mode treatments once either the last week of June (early summer) or the first week of August (late summer), or twice both early and late summer (repeated herbivory). A control group was left untreated, resulting in the 7 treatment combinations: 2 treatment modes * 3 treatment time schedules + 1 control group. Group assignments and initial treatments were applied in June of 2015. Treatments continued through the end of summer 2016.

In the defoliation treatment, 20 g of leaf tissue was carefully removed by hand plucking individual leaves at the distal end of the petiole. Leaves were removed from the top portion of the sucker. We began by plucking the newest leaves on the distal end of the terminal leader, then worked downwards toward the base of the tree until 20 g of tissue had been removed. In each case, we ensured that the terminal meristems on each branch were left intact. At the beginning of the experiment, 20 g of leaf tissue removal represented approximately 25-50% of the leaf canopy of each sucker. However, as the experiment progressed and suckers were repeatedly defoliated, some suckers were eventually stripped bare.

In the meristem removal (clipping) treatment, the upper branches of each sucker were pulled together by grasping the stem of the sucker and sliding the hands upwards. Then, garden shears were used to cut through both twig and leaf tissue to remove the top 20 g of biomass. To ensure that no more than 20 g were removed in a single treatment, we began by first clipping off a small amount of tissue and weighing it with a portable scale. If the amount of tissue removed was less than 20 g, we continued to clip biomass in small increments until the 20 g target was reached. At the beginning of the experiment, 20 g of biomass was approximately 10-20% of total aboveground biomass. However, as the experiment progressed and suckers were repeatedly clipped, some suckers were eventually clipped to the ground.

Both the defoliation and clipping treatments removed 20 g of biomass. However, the type of tissues removed were not the same. Defoliation treatments only removed leaf tissue, while the clipping treatment removed a combination of leaf, meristem, and twig tissues.

Field Measurements and Leaf Tissue Collection

Prior to every treatment period and in the second week of September (just prior to foliar pigment loss and the onset of fall senescence), the height and basal diameter of each sucker was recorded. Because suckers vary in growth form such that height is not always a good indicator of sucker size and vigor, we also recorded the length of the five tallest terminal branches. On each branch, we measured from terminal bud (or sometimes a clipped end, if a clipping treatment had been applied at an earlier period) to the bud scar. Bud scars are easily recognizable in juvenile aspen, and the distance between the bud scar and distal end of the leader represents the current season's growth. Survival was also recorded at each visit. After the 2015/2016 winter, 10 of our suckers were initially difficult to relocate. Three of these "lost" suckers were later recovered. They were typically broken at the base and were lying flat on the ground, sometimes a short distance from their corresponding GPS waypoint. Although dense understory vegetation

sometimes made searching difficult, we think it is unlikely that a healthy sucker would go missing unless it were broken off near the base. Therefore, we decided to count all missing suckers as dead.

Leaf tissue that removed during defoliation or clipping treatments was stored in a plastic bag and immediately placed on dry ice for transport to the lab. If a sucker was not scheduled to receive an herbivory treatment or was part of a control group, 5-7 young, yet fully expanded leaves (approximately 2 g) were plucked from several of the upper branches for analysis. We were careful to only remove the minimal amount of leaf tissue necessary for chemical analysis. This sampling occurred on the same dates that the herbivory treatments were administered to the other trees. Samples were immediately placed between blocks of dry ice in the field. Upon returning to the lab, all leaf tissues were stored at -80 C until freeze drying. Tissues were freeze dried for >48h using a Virtis Benchtop K lyophilizer (SP Scientific, Warminster, PA).

After the final measurements were recorded in September 2016, all suckers were clipped at ground level. Plant tissues were stored in the lab for < 7 days until they could be dried at 70 C to a stable mass (~3 days), and weighed using an analytical balance.

Foliar Chemistry

After freeze drying, leaf tissue samples were ground and homogenized using a mixer mill with a #10 mesh screen (Wiley Mill; Thomas Scientific, Swedesboro, NJ). Equal portions of leaf tissue from suckers in the same exclosure and treatment group were pooled together for analysis. If any of the 3 trees in the group had no leaf tissue available at the time of sample collection, equal portions of leaf tissue from the remaining trees were pooled. We analyzed these tissues to measure 3 key classes of phytochemicals: non-structural carbohydrates, phenolic glycosides (salicortin and tremulacin), and condensed tannins.

To quantify starch concentrations, sucrose and glucose were removed from 20 mg leaf tissue samples using an 80% ethanol solution (Hendrix 1993). The freeze-dried leaf tissue and 0.67 ml of ethanol solution were added to a 2ml microcentrifuge tube and vortexed for 20 minutes at 80°C. The supernatant was removed, and the process was repeated twice to produce 2 ml of extract. The remaining plant tissue was used to quantify foliar starch concentrations. We first added 1 ml DI water to these samples and autoclaved them for 1 hr at 275°C. After autoclaving, samples were vortexed for 2 minutes then centrifuged at 16.1g for 10 minutes. The supernatant was transferred to another tube and 1 ml of alpha-amylase solution (Megazyme) was added to each sample. The samples were then incubated for 20 minutes in a boiling water bath. During incubation, samples were inverted every 5 minutes to ensure adequate mixing. After cooling, 15 µl of amyloglucosidase (Megazyme) was added to each sample and the samples were incubated in a heated vortex at 50°C for 45 minutes. Next, 20 µl of sample was pippeted in duplicate into microplate wells. Finally, 200 µl of GOPOD reaction mix was added to each sample well. After 15 min of incubation at room temperature, A¬550 absorbance was read on the spectrophotometer. A standard curve, generated from purified starch standard (Megazyme), was used to calculate starch concentrations.

Phenolic glycosides were extracted from 40 mg of ground leaf tissue in 0.66 ml of methanol. Leaf tissue and methanol were combined in a 2ml vial and vortexed for 1.5 minutes. Then, vials were centrifuged at 16.1 g for 1 minute. The supernatant was pippeted into a separate vial. This procedure was repeated twice more to produce a total of 2 ml supernatant for each extracted sample. Phenolic glycoside concentrations were quantified using high-performance liquid chromatography (Agilent 110 Series, Santa Clara, CA) with a Luna 2, C18 column (150 x 4.6 mm, 5 um) at a flow rate of 1 ml min-1. Compound peaks were detected using a UV lamp at

a wavelength of 280 nm using purified salicortin and tremulacin standards isolated from aspen leaves (Lindroth et al. 1993).

Condensed tannins were extracted from 40 mg of ground leaf tissue. Leaf tissue was combined with 1 ml of 70% acetone-10 Mm ascorbic acid solution. Next, samples were vortexed for 30 minutes at 4℃, then centrifuged at 16.1g for 10 minutes. The resulting supernatant was pipetted into a separate vial. This process was repeated to generate 2 ml of supernatant for each sample. 100 µl of this supernatant were combined with 150 µl acetone-ascorbic acid solution, 1 ml acid butanol, and 50 µl of iron reagent, and then incubated in a boiling water bath as described in Porter et al. (1986). Condensed tannin concentrations were then quantified using a spectrophotometer (SpectraMax Plus 384, MDS, Toronto, Canada). Purified condensed tannins isolated from aspen leaves were used as a measurement standard (Hagerman and Butler 1989).

Statistical Analysis

Data exploration was conducted per the methods of Zuur et al. (2010). Response variables were recorded near the end of the growing season after 1 and 2 years of treatment (September 2015 and September 2016, respectively). All continuous response variables were analyzed using linear mixed effects ANCOVA models. The June 2015 values for each response variable were used as the covariate to control for initial differences between suckers. Sampling group was designated as a fixed effect with seven levels, each representing a unique combination of herbivory mode and treatment timing. Stand was specified as a random factor. Additionally, logistic regression was used to analyze two binary response variables: survival and vertical escape (escape was defined as having a height greater than 150). Mean height values alone may not be a reliable indicator of vertical escape – a small number of very small suckers could drive mean values downwards, masking the ability of most suckers to escape. Using logistic regression, we can directly estimate treatment effects on a sucker's probability of escape. Again,

sampling group was used as a fixed effect and stand was specified as a random effect in our generalized logistic regression models. Due to our modest sample size, no covariates were applied. For all analyses, Tukey's HSD was used to compare least squared means of the various treatment groups, and Alpha was set at 0.05.

To compare defoliation to clipping and June, August, and June+August treatments to each other, we created new mixed effects models with the control group excluded. This allowed for a simple, 2*3 model design with two levels of herbivory mode (defoliation and clipping) and three levels of herbivory timing (June, August, and June+August). Mode, timing, and the mode* timing interaction were specified as fixed effects, while June 2015 pre-treatment values and site were again used as covariates and a random effect, respectively. Alpha was specified as 0.05.

RESULTS

Growth Characteristics

After one year, both modes of simulated herbivory significantly reduced sucker height relative to the controls when applied in June or June+August. Additionally, the negative effect of August clipping was also significant (Fig. 2-1). After two years, only June and June+August clipping significantly reduced sucker heights. Clipping more negatively affected height than defoliation across both years (Table 1-1). Defoliated trees were 44% taller than clipped trees after one year, and 37% taller after two years (Table 2-2). Repeated clipping was more damaging than June- or August-only clipping, while repeated defoliation was roughly equal to June-only defoliation.

Both modes of herbivory negatively affected stem diameter, but again, this was dependent on the timing of the treatment (Fig. 2-2). After one year, only June defoliation and June+August clipping created significant negative effects. After two years, all June and

June+August treatment effects were significantly negative, regardless of herbivory mode. There were no significant differences between the two modes after the second year (Table 2-1). Repeated herbivory was no more damaging than June herbivory alone.

Only clipping treatments had significant negative effects on average leader length (Fig. 2-3). In the first year, June and June+August clipping reduced average leader length by more than half, relative to controls. August clipping reduced average leader length by roughly 1/3. At the same time, defoliation had small and insignificant negative effects on leader length. After the second year, only June and June+August clipping significantly affected average leader length, once again bringing the average length below 1/2 that of controls. August clipping once again reduced leader lengths by about 1/3 after the second year, but within-group variation was increased and the difference between this group and the control group was not significant. The negative effect of defoliation was increased in the second year, but within-group variation was large, and least squared means of the three defoliation treatment groups were not statistically distinguishable from the control group.

Final aboveground biomass followed the same general trend as the other metrics of growth (Fig. 2-7). Both modes of herbivory caused ~50% reductions in biomass in June and June+August, but the effect was only significantly different from the control group when applied in June+August. The effects of defoliation and clipping were nearly equal within each treatment time (Tables 2-1 and 2-2).

Escape

Across all treatment groups, the overall probability of vertical escape at the end of the experiment was 0.648 (log(odds) = -1.126, SE±0.235). Most suckers in the control group had reached escape height by August 2015, shortly after the experiment began (Fig. 2-9). All other treatment groups had a probability of escape <0.5 at all sampling periods, and the general trends

indicated that the prospects of escape were especially reduced in June or June+August treatments. However, our modest sample size made statistical analysis difficult, and only June and June+August defoliation treatment groups had escape probabilities that were significantly lower than the control group at the end of the experiment (Tukey HSD p=0.037 and 0.016, respectively).

Survival

Overall, mortality rates were less than 10% in each group in the first year (Fig. 2-8). There was no detectable difference in the odds of survival between any of the treatment groups after one year. However, there was considerable mortality over the 2015-2016 winter season, with continued mortality throughout the 2016 growing season (Fig. 2-8). By the end of the 2016 growing season, there were some large differences between groups. A Tukey HSD test did not confirm any statistically-significant differences, but our modest sample size made statistical analysis difficult. True effect sizes are uncertain, but the general patterns of mortality suggest that June treatments are more severe than August treatments, as mortality rates were consistently higher in groups receiving June treatment.

Foliar Chemistry

No significant mode, timing, or frequency effects were detected in any of our foliar chemistry analyses (Table 2-1, Figs. 2-4 through 2-6). Condensed tannin, total phenolic glycosides, and starch concentrations were highly variable within and between groups, and there was no consistent pattern from season to season.

Heavy defoliation in some treatment groups left little or no leaf tissue for collection at the end of each growing season. Thus, some treatment groups were poorly represented in the data. Particularly, phenolic glycoside data from September 2015 was inadequate for calculating the strength of the main and interactive effects of herbivory mode and timing (Table 2-1). Instead,

we have included data from the following June, when nearly all test subjects had sufficient leaf tissue available for sampling. However, the difference in timing should be noted**.**

DISCUSSION

The goals of our study were to assess how timing, frequency, and mode of herbivory affected aspen herbivore defense traits, including resistance, tolerance, vertical escape, and survival. We hypothesized that: 1) the timing of herbivory will affect aspen tolerance and vertical escape, but not resistance. Early-season herbivory will be less tolerated, will reduce vertical growth, and will increase mortality more than late-season herbivory. 2) The frequency of herbivory will affect resistance, tolerance, and escape. Herbivory in both early- and late-summer will induce greater chemical resistance, will be less tolerated, will reduce sucker heights, and will increase mortality relative to herbivory in early- or late-summer alone. 3) The mode of herbivory will affect resistance, tolerance, and escape – defoliation will induce stronger chemical resistance and will be less tolerated than clipping, but clipping will reduce sucker height more than defoliation.

Timing

In line with our first hypothesis, we found that the timing of herbivory had a strong effect on tolerance, a slight effect on vertical escape, and no effect on resistance. No significant associations between treatment date and foliar defense chemistry were found. Phenolic glycosides were higher in June and June+August treatments than in August treatments, but not significantly so. Induction of phenolic glycosides is known to occur in the new leaves produced by indeterminately growing branches following a defoliation event (Stevens and Lindroth 2005, St Clair et al. 2009, Call and St Clair *in press*). However, it is unknown whether this same type of induction can occur in leaves that are already fully-developed at the time of herbivory.

Therefore, it is possible that the observed differences between timing treatments was linked to the ratio of old and new leaf tissue at the time leaf samples were collected. Trees that were defoliated in June were often able to replace lost leaf tissue by the time of September sample collection, while trees that were defoliated in August trees often did not. If chemical induction occurs in leaves that develop after the initial herbivory event, the ratio of old and young leaf tissues in each sample could explain the weak trends in foliar defense that we observed.

Herbivory timing had pronounced effects on traits associated with tolerance (Table 1). Key traits that are associated with tolerance include height, stem diameter, average leader length, and biomass. Tolerance is typically defined as the ability of a plant to maintain fitness despite herbivore damage (Strauss and Agrawal 1999, Nunez-Farfan et al. 2007). In aspen, growth is a key component of fitness (Stevens et al. 2007). Thus, the ability to maintain high rates of growth despite damage indicates a high level of tolerance. As we predicted, early-season herbivory was less tolerated than late-season herbivory. Metrics of physical growth – a key aspect of aspen tolerance – generally showed a consistent response to timing: August treatments were highly tolerated and June treatments were more damaging (Figs. 2-1 through 2-3). In fact, August-only treatments rarely produced any detectable changes in growth; their mean values were statistically-indistinguishable from control groups, except for 2015 measures of height and average leader length in the August clipping treatment. This pattern is consistent with previous studies of herbivory timing that show herbivory during seasons of intense plant growth is more damaging (Cook and Stoddart 1963, Teague and Walker 1988, Ash and McIvor 1998).

We also quantified foliar starch concentrations, which are key measures of aspen vigor; high levels of foliar starch indicate high leaf tissue productivity (Rhodes et al. 2016). However, starch data did not confirm the observed trends in growth; the data were highly variable and the pattern was inconsistent across seasons. Nevertheless, our physical growth measurements clearly

show that early season herbivory is a greater hindrance to growth. One explanation is that June defoliation causes suckers to invest heavily in replacing lost leaf tissue, while suckers that are defoliated later in August may simply forgo photosynthesis for the remainder of the growing season. We did not record how frequently or how quickly leaves were replaced, but as mentioned above, it was clear that leaves were more likely to be replaced in the June treatment groups. These new leaves are likely resource sinks for the first several weeks following defoliation, as they grow and develop. The cost of developing new leaf tissues and the lost opportunity to photosynthesize in the peak of the growing season is likely greater than the cost of losing late-season photosynthesis alone. Additionally, mortality was highest in the June Foliar treatment group, and generally lower in the August-only treatment groups (Fig. 2-8). However, logistic regression did not reveal any significant differences between groups.

The third facet of aspen's defense against ungulate herbivory is vertical escape (Lindroth and St Clair 2013). We hypothesized that early-season herbivory would have a greater negative effect on vertical escape than late-season herbivory. This hypothesis was supported by height data from 2015 – for both modes of herbivory, June treatment groups had lower mean heights than August treatment groups (Fig. 2-1). This effect appeared to increase in magnitude in the second year, but within-group variation also increased, reducing the confidence of our estimates (Table 2). However, logistic regression revealed significant differences in the probability of escape between the control group and June or June+August defoliation groups in the second year (p-values = 0.037 and 0.016, respectively), and a general trend of low probability of escape in all June or June+August treatments (Fig. 2-9). Overall, the effect of timing was consistent across treatment modes, following the same pattern as its effect on tolerance. As discussed earlier, we suspect that greater metabolic costs are associated with early-season herbivory, and that these costs prevent suckers from reaching escape heights.

Ungulate preference for aspen is determined by individual plant quality and by the relative nutritional quality of aspen compared to other plants available on the landscape; both factors vary throughout the season (Osier et al. 2000, Lindroth et al. 2002, Beck and Peek 2005, Villalba et al. 2014). Preference for aspen may increase as the season progresses, because aspen retain their nutritional quality longer than most forb species (Tew 1970). However, tannin concentrations also increase throughout the spring and early summer (Osier et al. 2000). Different ungulates have shown unique responses to condensed tannins (Robbins et al. 1991, Robbins et al. 1995). Therefore, species that are negatively affected by tannins may prefer to consume aspen earlier in the season, when foliar tannin concentrations are lower, while species that are less affected by tannins may prefer to consume aspen later in the season, when its nutritional quality is high relative to other available forage. In the Intermountain West, domestic ungulates are typically only present in the mid- to late-summer, precluding the possibility of early-season herbivory. However, more studies are needed to determine the suite of conditions that make aspen an attractive source of forage for each unique ungulate species. If the season of aspen use varies between ungulate species, their effect on sucker growth and survival could be dramatically different, even if the total biomass consumed annually by each species is similar.

Frequency

Our second hypothesis was generally unsupported by the data – the frequency of herbivory had no effect on resistance or tolerance, and weak and inconsistent effects on vertical escape. We suspected that more frequent herbivory would induce strong changes in foliar defense chemistry, but extreme within-group variability precluded the detection of any treatment effects. Surprisingly, tolerance was also unaffected by the frequency of herbivory. In almost all metrics of growth, June+August treatment group means were not significantly different from June-only group means. The one exception was September 2015 height: June+August clipping

reduced mean sucker height slightly more than June clipping alone (Fig. 2-1). As discussed above, June treatments were poorly tolerated, and August treatments were well-tolerated. Our results suggest that June herbivory does not modify the effect of subsequent August herbivory; even previously-damaged suckers still tolerate August herbivory extremely well. The frequency of herbivory also had very little effect on vertical escape. Although the average height of twice-clipped suckers was reduced in 2015, this effect disappeared in 2016, and logistic regression revealed no significant differences in escape probability between June-only and June+August treatments groups in either mode treatments (p-values > 0.9 in both cases). Although mortality rates were generally high in June+August treatment groups, there were no significant differences between June+August treatments and any other treatment groups (Fig. 2-9).

Mode

Our third hypothesis was only partially supported by the data. Herbivory mode had almost no effect on resistance and tolerance traits and very little effect on vertical escape. Clipping and defoliation did not seem to trigger unique chemical defense responses (Table 2-1, Figs. 2-4 through 2-6). This was contrary to our expectations, as foliar defense chemistry induction is well-documented in aspen (Mattson and Palmer 1988, Osier and Lindroth 2004, Stevens and Lindroth 2005) and the severity of our simulated herbivory treatments seemed more than sufficient to trigger this induction. Additionally, domestic sheep are deterred by high tannin concentrations (Min et al. 2003), and have demonstrated a preference for aspen with low phenolic glycoside content (Villalba et al. 2014). Other ungulates may respond in a similar manner – differences in deer herbivory have been linked to aspen chemical phenotype (Lindroth and St Clair 2013).

One possible explanation for the lack of treatment effects is the way leaf tissue was removed: in our defoliation treatment, leaves were carefully plucked at the base of the petiole.

There is some evidence that this method of defoliation does not induce a significant phenolic glycoside response in the remaining leaves – wounding or partial consumption of individual leaves may be necessary to trigger a systemic response throughout the sucker (Mattson and Palmer 1988). The clipping treatment was similar – individual leaves were usually wholly removed; few individual leaves were cut into fragments during the leaf and twig harvesting.

Our herbivory treatments also lacked the chemical elicitors that are present in the saliva of some of aspen's insect herbivores (Havill and Raffa 1999, Stevens and Lindroth 2005). The role of ungulate saliva in defense chemistry induction is less understood. However, one simulated herbivory experiment showed that deer saliva applied at the wound site actually caused a small decrease in foliar tannin concentrations (Keefover-Ring et al. 2016). Further research is needed to determine whether natural herbivory from ungulate herbivores can induce defense chemistry, and whether different ungulate species have different effects.

In our experiment, the specific mode of herbivory had little impact aspen tolerance. The mean biomass and basal diameter measures were roughly equal in both treatments, suggesting that the two treatments were tolerated equally well. The effects on height and average leader length were stronger in clipping treatments than in defoliation treatments (Table 1). The mean height of clipped trees was 31% lower than defoliated trees after one year, and 28% lower after 2 years (Table 2). However, because height and average leader length are both directly affected by clipping (which physically removes height and length) and only indirectly affected by defoliation (which reduces photosystem capacity), these metrics are poor indicators of a ramet's ability to maintain positive growth rates. We did not measure the direct effect of treatments on sucker height – we only recorded the mass of the tissue removed, not the vertical length. Defoliation treatments had minimal effects on sucker height, while clipping treatments often dramatically reduced sucker height. However, the effect was dependent on the density and arrangement of

terminal leaders – suckers with a single, long terminal leader were clipped shorter than suckers with a dense grouping of terminal leaders, in order to remove the same amount of biomass from each sucker. Therefore, it is difficult to determine how much of the observed reductions in mean height are caused by reduced growth rates versus the direct effects of clipping. The notion that trees were less tolerant of meristem herbivory is also refuted by our observed mortality rates – generally speaking, defoliation treatments caused higher rates of mortality (see Fig. 2-8), although no significant differences between groups were found. Stem diameter and final biomass measurements both indicate that the mode of herbivory had no real effect on tolerance.

The mode of herbivory clearly affected aspen sucker height, but it's effect on escape is less clear. As discussed earlier, the mode of herbivory strongly influenced average sucker height and leader length (Figs. 2-1 and 2-2, Tables 2-1 and 2-2). However, when escape was considered as a binary variable, the effects of the two different herbivory modes were nearly indistinguishable (Fig. 2-9). This is likely because the negative effects of clipping on sucker height were not evenly distributed among all test subjects. Some suckers were dramatically shortened, with several eventually being clipped to the ground. This had a strong effect on the mean heights of clipped treatment groups. However, most suckers were only mildly shortened, and many of these were still able to reach escape height. Thus, the effect of mode on vertical escape was probably dependent on the morphology of the individual sucker, especially the density and arrangement of the terminal leaders (as mentioned above). Logistic regression indicates that overall, clipping was no worse than defoliation, as the total number of escaping suckers in both treatments was comparable.

Our two contrasting modes of simulated herbivory were specifically designed to test whether different feeding strategies had the potential to alter aspen's defensive response. Although we found no evidence that differential tissue selection could alter chemical resistance

or tolerance traits, we found some evidence that, gram for gram, clipping of leaves, twigs, and meristems can result in greater height suppression than defoliation alone. However, this effect is probably dependent on sucker morphology. High levels of ungulate browsing have been shown to reduce aspen sucker heights and can prohibit post-disturbance regeneration (Lindroth and St Clair 2013). No study of which we are aware has attempted to determine whether ungulate species possess unique preferences for specific aspen tissues. However, elk are known to feed on aspen twigs during the winter months (Baker et al. 1997). Our results indicate that this type of feeding, which removes only meristems and twigs, could be particularly harmful to aspen suckers. This type of winter foraging is linked to regeneration failure in some areas (Baker et al. 1997, Suzuki et al. 1999, McCain et al. 2003). Additionally, differences in grazer and browser feeding habits and oral dexterity are well-known (Robbins et al. 1995, Beck and Peek 2005). This may enable a browser, such as deer, to take only leaf tissues, while elk or cattle may be more likely to take leaves and twigs together. Further studies are needed to experimentally test this prediction.

CONCLUSIONS

This experiment shows that all herbivory is not equal: the mode, timing, and frequency, of herbivory are all important factors in determining aspen's defensive response. Herbivory timing affected sucker height, stem diameter, and leader length: June treatments were more damaging than August treatments. Repeated herbivory caused higher mortality rates and reduced sucker heights, compared to single herbivory events. Herbivory mode affected sucker height and leader length: clipping had greater negative effects than defoliation. These traits contribute to the tolerance and vertical escape aspects of aspen's defensive phenotype. Foliar chemistry and aspen resistance traits were unaffected by our experimental factors.

With multiple stressors threatening the health of aspen forests in the Intermountain West (Worrall et al. 2015), understanding those factors that influence aspen forests' spatial distribution and persistence is a major focus of current research (Rogers et al. 2013, Dudley et al. 2015, Hansen et al. 2016). Ungulate herbivory has emerged as a key factor influencing aspen's establishment and persistence (Seager et al. 2013, Rogers and Mittanck 2014, Rogers et al. 2015). The next step towards effective management hinges on the ability to determine the specific impacts of each unique ungulate species. Several studies have attempted to evaluate the functional similarity of different ungulate species in aspen ecosystems (Kay and Bartos 2000, Beck and Peek 2005, Bork et al. 2013, Clark et al. 2017) and on western rangelands in general (Veblen et al. 2015, Scasta et al. 2016). However, these studies typically focus on the degree to which each ungulate species utilizes aspen as a forage resource, and do not address whether ungulate species can use aspen in unique ways. Our experiment shows the potential for unique effects of distinct herbivore species on aspen suckers, even if microhistological fecal studies show equal use of aspen (i.e. Beck and Peek 2005). Aspen researchers should recognize that ungulate herbivores may have species-specific effects on aspen. Even after accounting for differences in aspen preference, potentially disparate tissue selection and timing of use could result in different levels of damage to aspen suckers. Studies that manipulate herbivore access often lack the spatial and temporal resolution to determine when aspen is most heavily used by the animal, and reliable techniques for determining the proportions of leaves, twigs, and meristems consumed have not yet been developed. A key next step will be to determine the specific feeding habits of each species, including the timing of aspen use and the specific tissues selected by the animal.

We know that changes in herbivore populations can alter community structure through plant *species* selection, and we can now add the possibility that they alter community structure

through plant *tissue* selection and the *timing* of that selection. Now that timing and mode effects have been demonstrated with experimental herbivory, the stage is set for future studies to assess the strength of these factors in natural ungulate communities.

ACKNOWLEDGEMENTS

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FIGURES

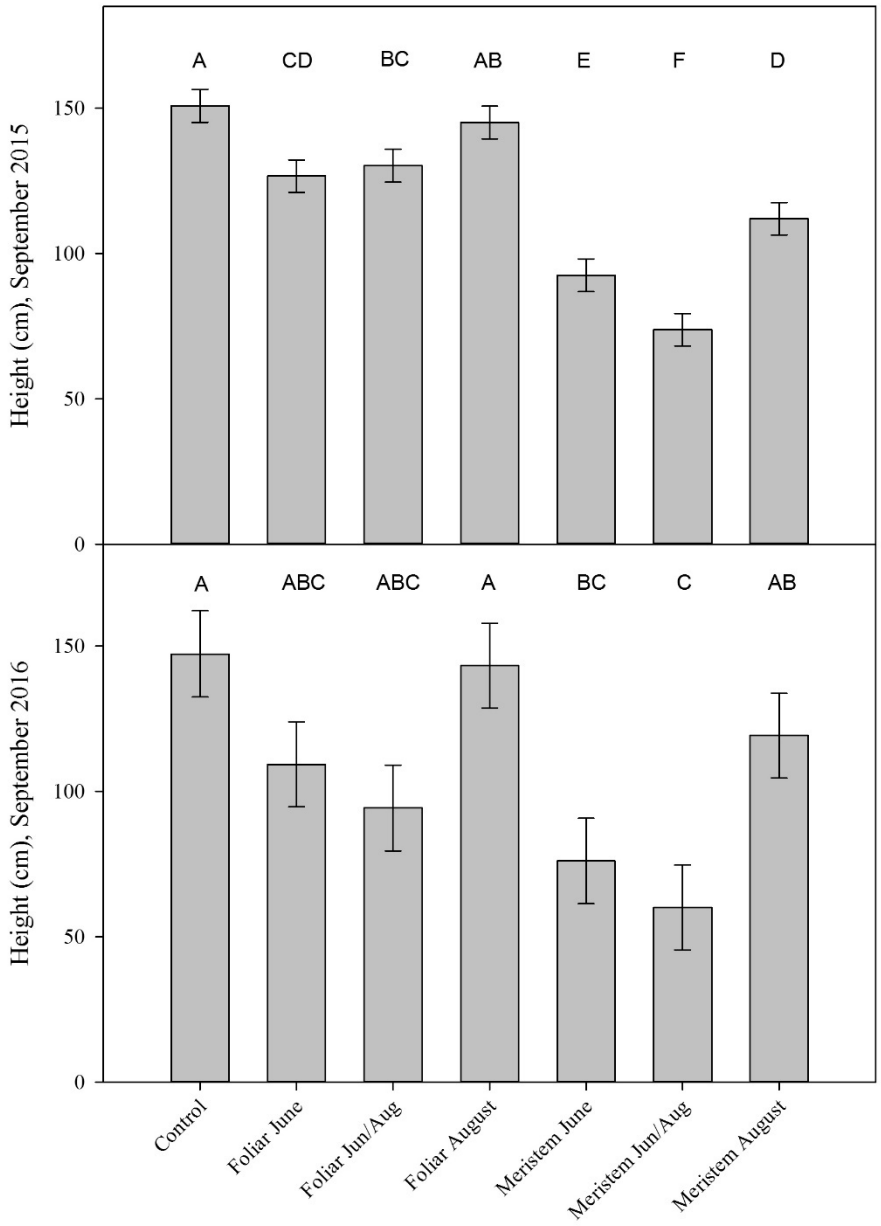

Figure 2-1. Least squared mean height of suckers in each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). Error bars ± 1 SE. Treatment groups not connected by the same letter are significantly different (Tukey HSD, $\alpha = 0.05$)

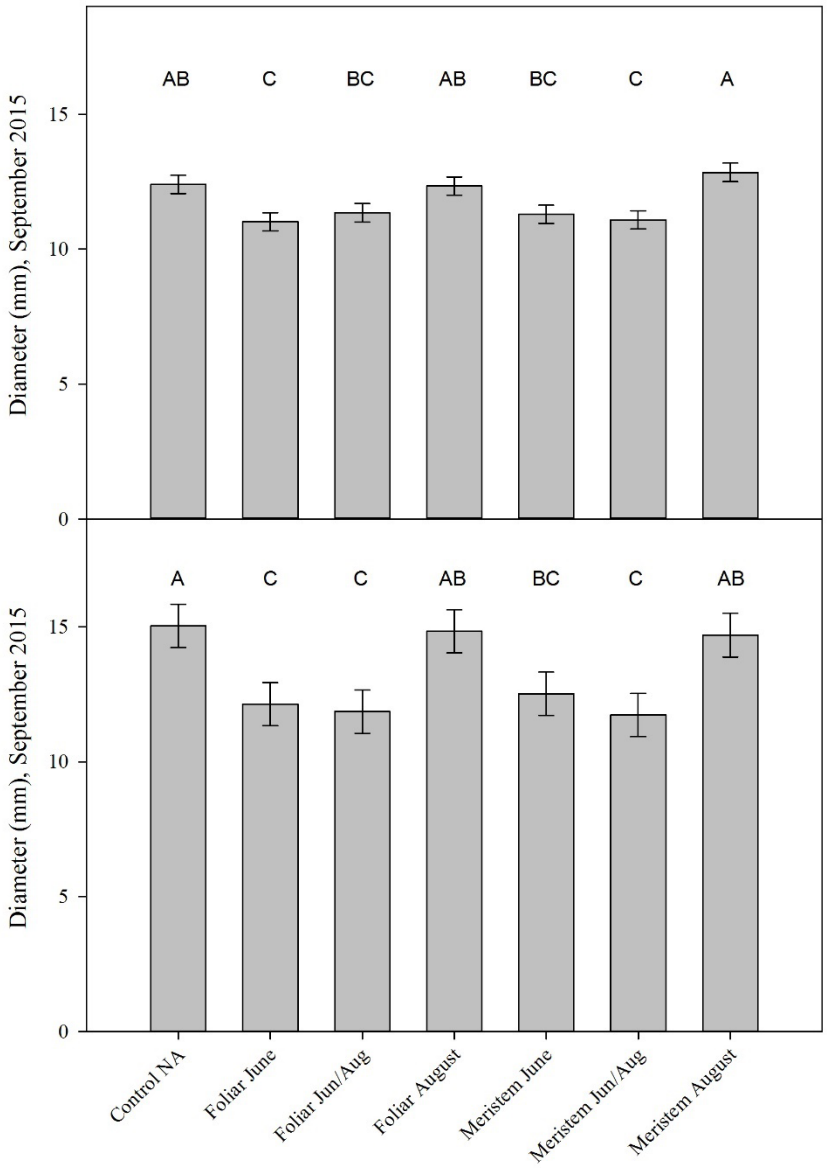

Figure 2-2. Least squared mean basal diameter of suckers in each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). Error bars ± 1 SE. Treatment groups not connected by the same letter are significantly different (Tukey HSD, $\alpha = 0.05$)

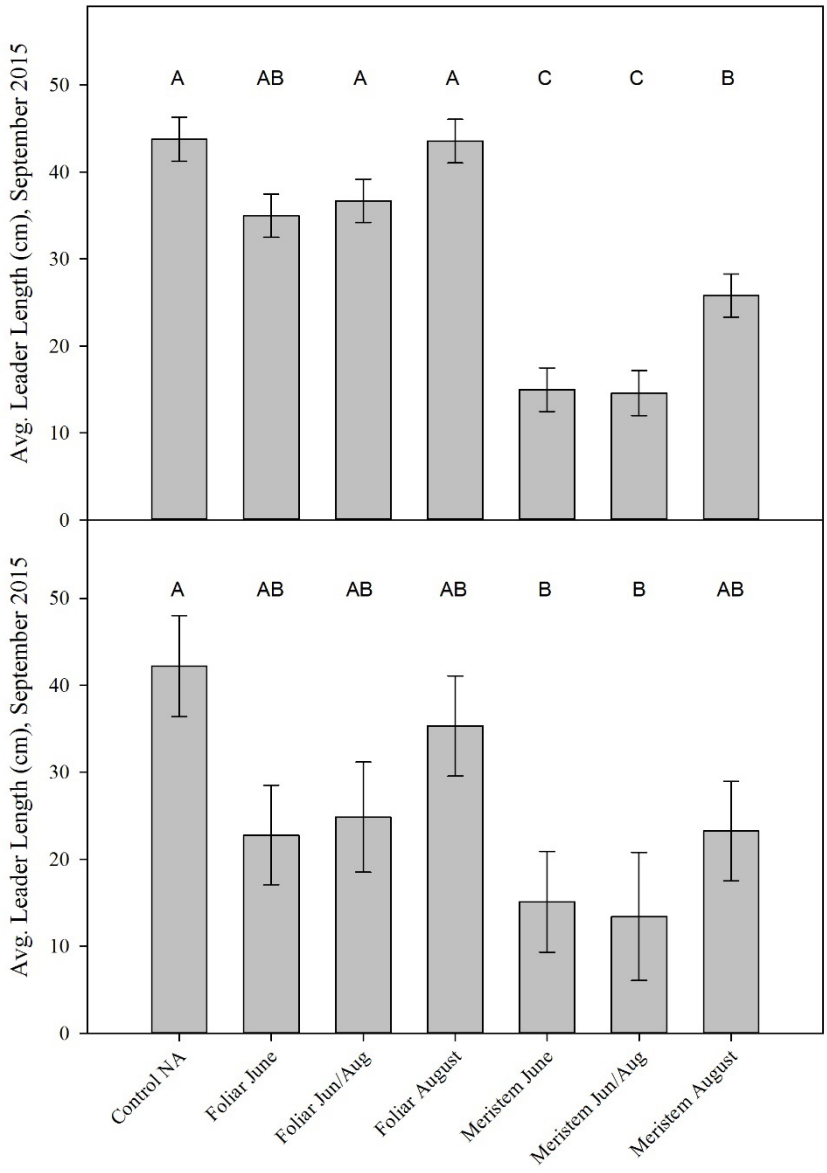

Figure 2-3. Average leader length (least squared means) of suckers in each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). This measurement is calculated by averaging the length of the five tallest leaders on each sucker, from apical bud to bud scale scar. Error bars ± 1 SE. Treatment groups not connected by the same letter are significantly different (Tukey HSD, $\alpha = 0.05$)

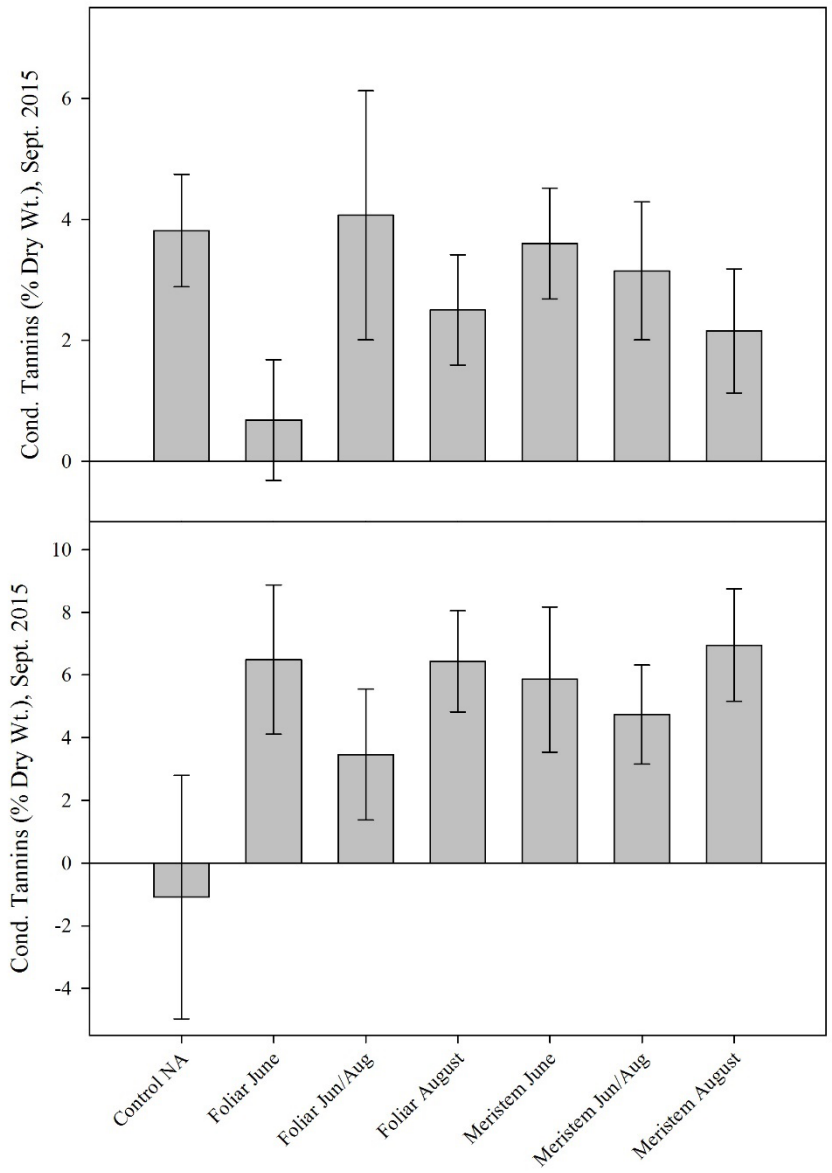

Figure 2-4. Foliar condensed tannin concentrations (least squared means) for each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). Error bars ± 1 SE. Tannin concentrations were quite variable within treatment groups, and no treatment groups were significantly different from any other (Tukey HSD, $\alpha = 0.05$).

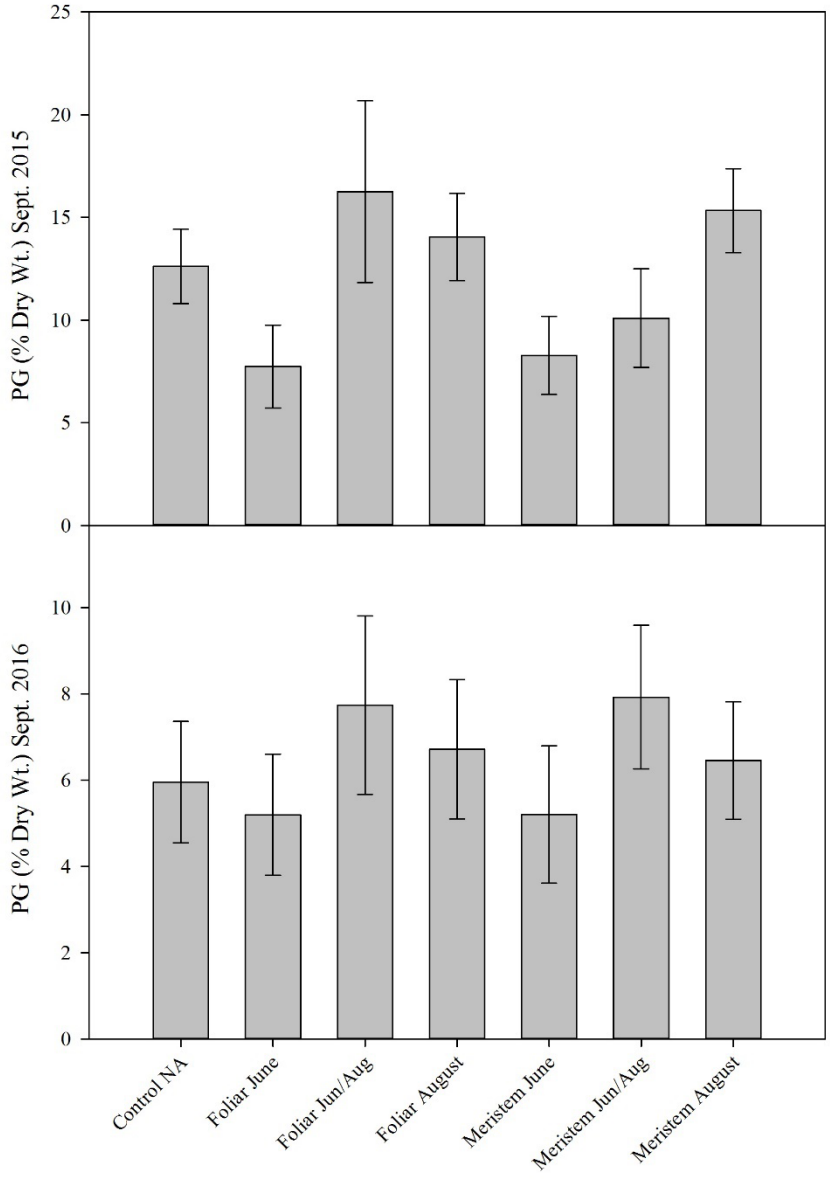

Figure 2-5. Foliar phenolic glycoside concentrations (combined salicortin and tremulacin, least squared means) for each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). Error bars ± 1 SE. No treatment groups were significantly different from any other (Tukey HSD, $\alpha = 0.05$).

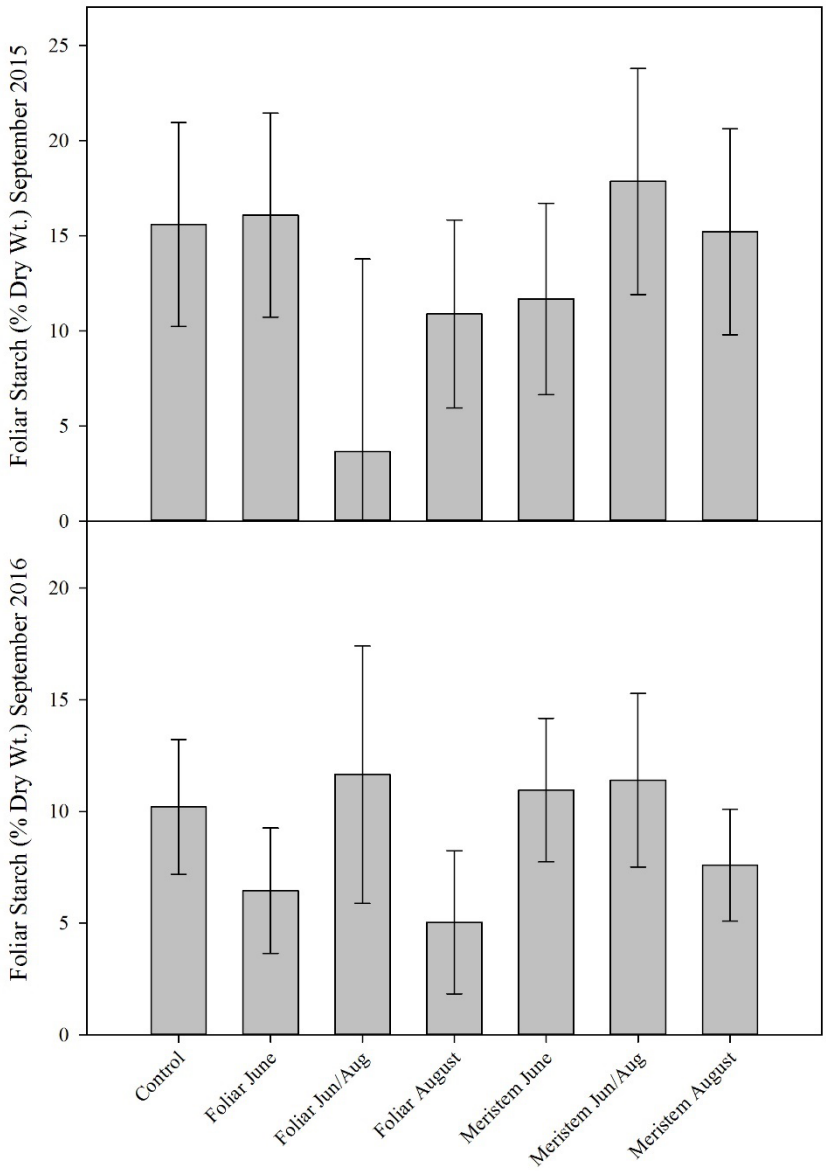

Figure 2-6. Foliar starch concentrations (least squared means) for each of the seven unique treatment groups near the end of the growing season in 2015 and 2016 (after 1 and 2 years of treatment, respectively). Error bars ± 1 SE. No treatment groups were significantly different from any other (Tukey HSD, $\alpha = 0.05$).

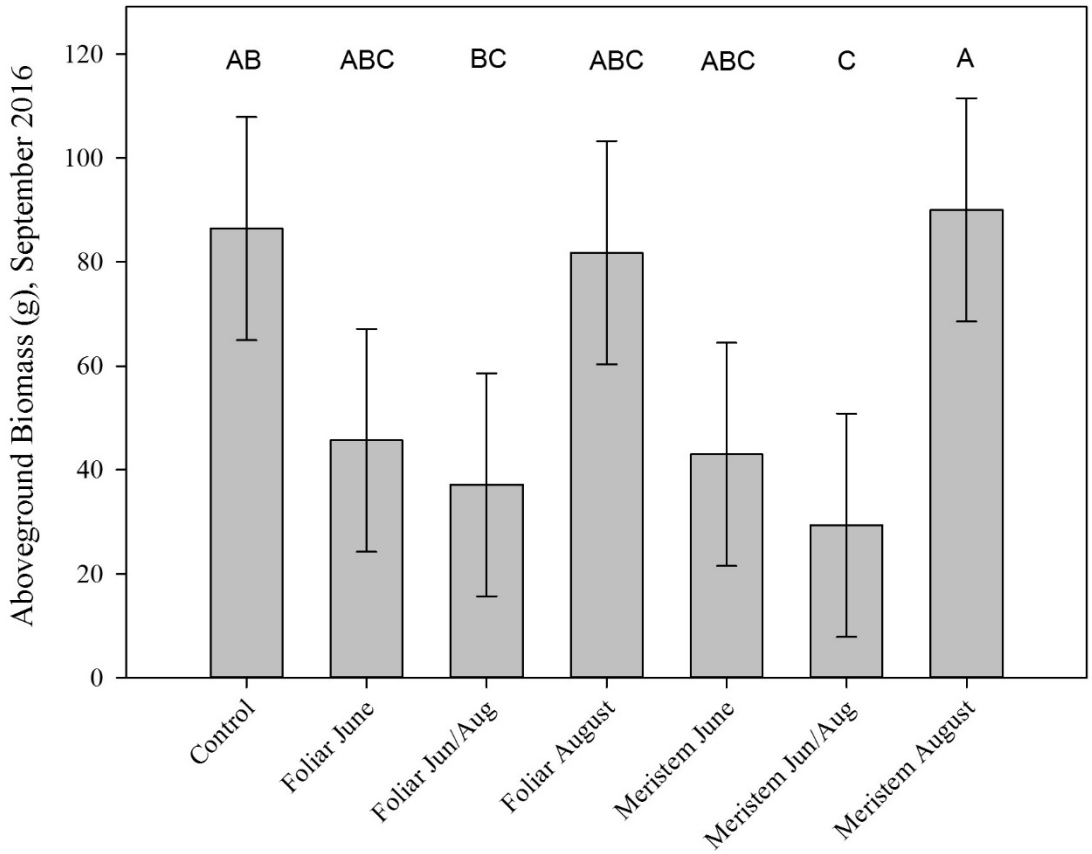

Figure 2-7. Aboveground biomass (dry weight, least squared means) for each of the seven unique treatment groups near the end of the growing season in 2016. Error bars ± 1 SE. Treatment groups not connected by the same letter are significantly different (Tukey HSD, $\alpha = 0.05$)

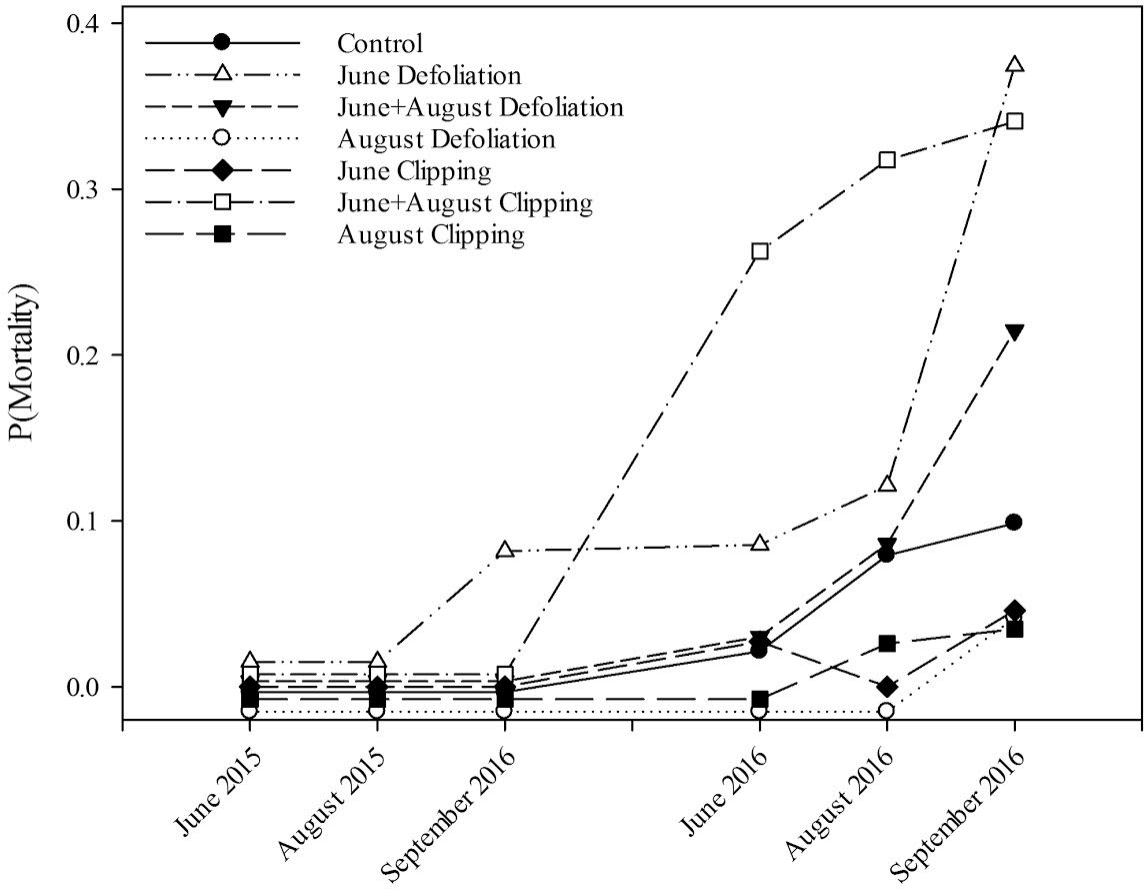

Figure 2-8. Probability of mortality in each treatment group at each sampling period. Data has been manually jittered by ±0.015, and error bars omitted to improve readability. Due to our modest sample size, probability estimates are highly uncertain, and no treatment groups are significantly different from any other (Tukey HSD).

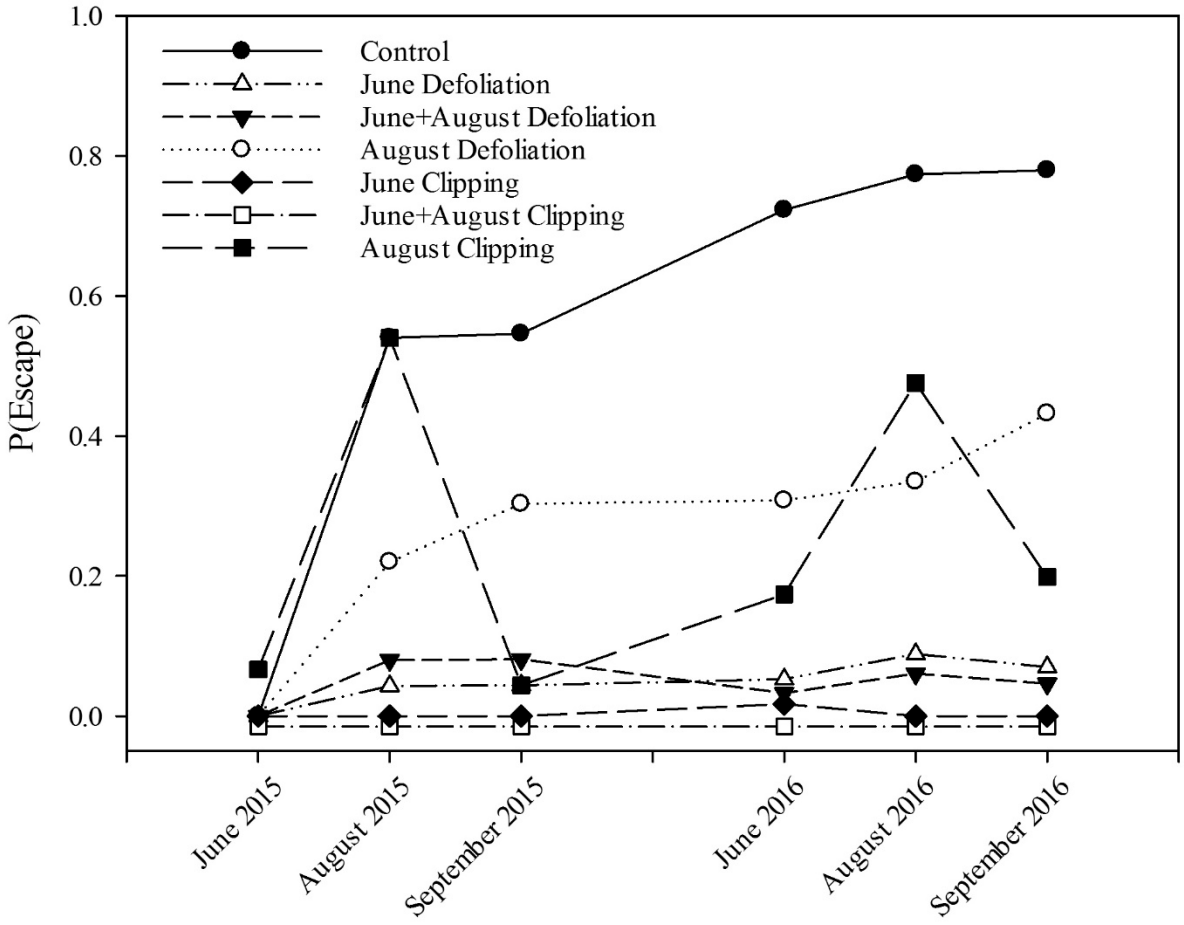

Figure 2-9. Probability of escape in each treatment group at each sampling period. The critical height threshold was set at 150cm. Measurements were recorded immediately prior to treatment. Thus, June and August values represent the probability of escape prior to the implementation of simulated herbivory treatments, and trees that had once "escaped" were often brought below the critical height threshold by subsequent treatments. This effect is particularly evident in the August Clipping treatment group. Data has been manually jittered by ±0.015 and error bars have been omitted to improve readability.

TABLES

Table 2-1. Fixed-effect tests of herbivory mode, herbivory timing, and their interaction for seven response variables at the end of each growing season. For foliar phenolic glycosides, September 2015 data has been replaced with June 2016 data.

Response	Season	Herbivory Mode		Herbivory Timing		Mode*Timing	
		F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Height	September 2015	216	<.0001	32.7	<.0001	7.75	0.0035
	September 2016	9.712	0.0056	10.128	0.001	.1152	0.8918
Basal Diameter	September 2015	0.6394	0.4341	18.83	<.0001	1.175	0.3308
	September 2016	0.00027	0.959	21.576	<.0001	0.2383	0.7903
Avg. Leader Length	September 2015	88.71	<.0001	9.255	0.0014	0.3822	0.6873
	September 2016	10.445	0.0045	4.865	0.0209	0.1897	0.8289
Foliar Tannin Concentration	September 2015	0.497	0.494	0.482	0.629	3.627	0.0594
	September 2016	0.0771	0.7864	1.3502	0.2985	0.1198	0.8883
Foliar PG Concentration	June 2016	0.0173	0.8966	1.962	0.1662	0.4398	0.6503
	September 2016	0.0088	0.9279	1.9057	0.2111	0.008	0.9921
Foliar Starch Concentration	September 2015	2.0725	0.1756	0.3954	0.6817	2.3134	0.141
	September 2016	0.936	0.359	1	0.404	0.1877	0.8319
Aboveground Biomass	September 2016	0.0006	0.9411	10.245	0.0009	0.225	0.8

Table 2-2. Least squared means and standard errors of treatment categories.

Response	Season	Defoliation Treatments		Clipping Treatments		June Treatments		June+August Treatments		August Treatments	
		LSM	*SE*	*LSM*	*SE*	*LSM*	*SE*	*LSM*	*SE*	*LSM*	*SE*
Height	September 2015	133.807	4.763	92.56	4.763	109.36	4.95	101.865	4.96	128.325	5.947
	September 2016	114.922	10.527	83.35	10.526	91.063	11.599	76.633	11.658	129.712	11.587
Basal Diameter	September 2015	11.573	0.237	11.737	0.237	11.162	0.258	11.232	0.259	12.572	0.261
	September 2016	12.951	0.523	12.97	0.523	12.34	0.556	11.811	0.557	14.732	0.56
Avg. Leader Length	September 2015	38.675	1.538	18.7	1.538	25.242	1.823	25.872	1.84	34.949	1.836
	September 2016	28.197	2.764	16.584	2.951	19.075	3.088	18.329	3.712	29.767	3.1
Foliar Tannin Concentration	September 2015	2.184	0.954	2.735	0.796	2.109	0.829	3.102	1.188	2.168	0.826
	September 2016	5.303	1.416	5.688	1.4	6.016	1.734	3.935	1.553	6.536	1.407
Foliar PG Concentration	June 2016	20.332	1.557	20.002	1.644	23.651	20.045	19.548	2.175	17.304	2.181
	September 2016	6.64	1.493	6.54	1.358	5.109	1.474	7.951	1.748	6.71	1.492
Foliar Starch Concentration	September 2015	8.313	4.546	14.2981	3.5301	13.274	3.756	8.29	5.94	12.353	3.685
	September 2016	7.883	2.919	10.429	2.399	9.057	2.556	11.737	3.979	6.674	2.379
Aboveground Biomass	September 2016	54.872	18.318	54.123	18.318	44.335	18.99	33.255	18.99	85.902	18.99

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