Intra-canopy leaf trait variation facilitates high leaf area index and compensatory growth in a clonal woody-encroaching shrub

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Abstract

Leaf trait variation enables plants to utilize large gradients of light availability that exist across canopies of high leaf area index (LAI), allowing for greater net carbon gain while reducing light availability for understory competitors. While these canopy dynamics are well understood in forest ecosystems, studies of canopy structure of woody shrubs in grasslands are lacking. To evaluate the investment strategy used by these shrubs, we investigated the vertical distribution of leaf traits and physiology across canopies of *Cornus drummondii*, the predominant woody encroaching shrub in the Kansas tallgrass prairie. We also examined the impact of disturbance by browsing and grazing on these factors. Our results reveal that leaf mass per area (LMA) and leaf nitrogen per area (N_a) varied ~3-fold across canopies of *C. drummondii,* resulting in major differences in the physiological functioning of leaves. High LMA leaves had high photosynthetic capacity, while low LMA leaves hada novel strategy for maintaining light compensation points (LCP) below ambient light levels. The vertical allocation of leaf traits in *C. drummondii* canopies were also modified in response to browsing, which increased light availability at deeper canopy depths. As a result, LMA and N_a increased at lower canopy depths, leading to a greater photosynthetic capacity deeper in browsed canopies compared to control canopies. This response, along with increased light availability, facilitated greater photosynthesis and resource-use efficiency deeper in browsed canopies compared to control canopies. Our results illustrate how *C. drummondii* facilitates high LAI canopies and a compensatory growth response to browsing—both of which are key factors contributing to the success of *C. drummondii* and other species responsible for grassland woody encroachment.

Key Words: *Cornus drummondii*, tallgrass prairie; canopy structure; vertical light gradient; bush encroachment; browsing; plasticity; leaf area index, leaf mass per area; photosynthesis; gas exchange

Introduction

Over the past century, woody plants have expanded throughout grasslands worldwide in a phenomenon known as "woody plant encroachment" (Archer 1995, Stevens et al. 2017, Archer et al. 2017). In many cases, woody encroaching plants coexist with grasses, forming grass-tree mosaics, and have little impact on the structure and function of grasslands (Eldridge et al. 2011). In other cases, the dense canopies of some woody encroaching species can alter the light environment, resulting in a displacement of shade-intolerant grassland species and a gradual grassland-to-woodland transition (Ratajczak et al. 2012, Knapp et al. 2008, Brantley & Young 2007, Ratajczak et al. 2014). In mesic grasslands, many of the most abundant woody encroaching species have canopies with leaf area index (LAI) values exceeding that of many temperate deciduous forest communities (Brantley and Young 2007, Brantley and Young 2009). For example, *Cornus drummondii*, the predominant woody encroaching shrub in the tallgrass prairie of eastern Kansas (Ratajczak et al. 2011), and *Morella cerifera* in the coastal grasslands of Virginia, have mean LAI values of 8 and 10, respectively (**Figure 1A**; Knapp et al. 2008, Brantley & Young 2007). These values exceed the mean LAI of many temperate deciduous forests (~6.5; Norby et al. 2003, Asner et al. 2003), despite having canopies that are less than 1/10th the height (1-5 m vs. >20 m) for most temperate deciduous forests (Anderson et al. 2006, McGregor et al. 2020).

While a greater LAI increases total light capture and reduces light availability for shorter competitors, it also reduces light availability for successive leaf layers in the plant's own lower canopy (Monsi and Saeki 1953, Monsi and Saeki 2005). Moreover, self-shading is greater when LAI is concentrated across a small vertical distance due to reduced penumbral effects on light penetration (Smith et al. 1989, Kramer et al. 2014, Van Pelt et al. 2016). As a result, short canopies experience greater self-shading and lower light availability compared to taller canopies (Smith et al. 1989, Van Pelt et al. 2016). Self-shading becomes detrimental to a plant when the cost of producing and maintaining more leaves (a greater LAI) outweighs the benefits of additional light capture (Saeki 1960, Reich et al. 2009). For this reason, an optimal LAI exists for maximizing canopy photosynthesis relative to the physiology of the plant (Saeki 1960, Hikosaka 2005, Waring 1983).

Gradients of light availability across canopies have cascading impacts on leaf-level physiology and whole plant carbon dynamics (Lambers 2008, Niinemets 2010). Woody shrubs and trees have evolved high intra-canopy variation in leaf morphology and physiology under varying light conditions to maximize light harvesting and net carbon fixation while avoiding over-excitation and damage to photosynthetic apparatuses (Long et al. 1994, Legner et al. 2014, Niinements 2007, 1998). Leaves in the upper canopy typically exhibit higher leaf mass per area (LMA) (Poorter et al. 2009, Ellsworth and Reich 1993) and more nitrogen per unit leaf area (N_a) compared to leaves in the lower canopy (Ellsworth and Reich 1993). Much of this nitrogen is allocated to RuBisCO (RuBP carboxylase-oxygenase), chlorophyll, and other photosynthetic proteins (Hikosaka and Terashima 1996, Evans 1989). Therefore, high LMA leaves typically contain greater amounts of RuBisCO per unit area, which facilitates greater maximum rates of carboxylation (Vc_{max}), as well as increased chlorophyll per unit area, which facilitates greater maximum rates of electron transport (J_{max}) (Niinemets et al. 1998, Poorter and Evans 1998, Ripullone et al. 2003, Niinemets 2007, Carswell et al. 1999, Mendes et al. 2001). Together, J_{max} and Vc_{max} are rate-limiting steps to increasing maximum photosynthetic rates (A_{max}). (Powles 1984, Walker et al. 2014, Chen et al. 1993). These traits are beneficial in the upper canopy where photosynthetically active radiation (PAR) is high, but they are also associated with higher dark respiration rates (R_d) due to greater leaf energy demands (Ryan 1991, Amthor 2000, Bouma 2005, Givnish 1988).

Leaves in the lower canopy have lower LMA and N_a, resulting in decreased maximum photosynthetic rates and lower R_d rates (Poorter et al. 2009, Niinemets 2007, Ripullone et al. 2003, Ellsworth and Reich 1993). Leaves with lower R_d rates can reach the light compensation point (LCP) at lower PAR levels to achieve a net carbon gain from photosynthesis (Lewis et al. 2000, Walters et al. 1996, Moriwaki et al. 2019). LCP can also be minimized in shade leaves by allocating more nitrogen to thylakoids to increase apparent quantum yield (Φ) (Moriwaki et al. 2019), but this occurs at the expense of nitrogen allocation to RuBisCO which decreases the maximum carbon fixation rate of the leaf (Björkman 1981, Walters et al. 1996, Ögren and Evans 1993, Chen et al. 1993). Ecologically, species with a high capacity for intra-canopy variation in these traits can utilize leaves across a greater range of light conditions, maintain high LAI canopies, and maximize whole-canopy photosynthesis (Saeki 1960, Reich et al. 2009, Hikosaka et al. 2014, Niinemets et al. 2014, Chen et al. 1993).

The goal of our research was to determine the physiological mechanisms and traits that enable woody encroaching shrubs with dense canopies – specifically *Cornus drummondii* C.A. Mey. in tallgrass prairie – to utilize light efficiently while facilitating high LAI values across relatively short canopies. We examined the canopy structure, variation in leaf morphology and physiology, and allocation of nutrients across a vertical canopy gradient of intact *C. drummondii* shrub islands (**Figure 1**) in areas grazed by bison, ungrazed, and in response to simulated browsing (to mimic elk herbivory, sensu O'Connor et al. 2020). While the vertical distribution of leaf traits and physiology is well understood within the canopies of tree species in forest ecosystems (Meir et al. 2002, Sack et al. 2006, Ninnemets 2007, Poorter et al. 2009, Legner et al. 2014, Mullin et al. 2009, Wyka et al. 2012, Rozendaal et al. 2006, Markesteijn et al. 2007), similar investigations have not been conducted within the canopies of woody encroaching shrubs in grasslands. Throughout canopies of *C. drummondii,* we hypothesized that: (1) Plasticity in leaf morphology and physiology across a short (2-3 m) vertical gradient, facilitates high LAI canopies and maximizes carbon uptake, similar to larger vertical gradients previously reported for tree species, and (2) *C. drummondii* will rapidly adjust leaf morphology and physiology in response to disturbance (browsing and grazing) within a single growing season to maximize carbon gain.

Materials and Methods

Site Description: Research was conducted during the 2020 growing season (May-September) at the Konza Prairie Biological Station (KPBS), a 3,487-ha native tallgrass prairie in the northern Flint Hills ecoregion of Northeast Kansas, USA. The Flint Hills consists of the largest expanse of contiguous tallgrass prairie in North America. The region contains a heterogeneous landscape with varying topographic relief: shallow-soiled rocky uplands, steep slopes, and deep-soiled lowlands. KPBS is divided into experimental watersheds, each with a prescribed burn treatment (1, 2, 3, 4, or 20-year fire return intervals) and grazing treatment (bison, cattle, or no large grazers). Historically, the plant community of KPBS was dominated by C_4 grasses, including *Andropogon gerardii*, *Sorghastrum nutans*, *Schizachyrium scoparium*, and *Panicum virgatum,* and was devoid of woody vegetation with the exception of riparian areas (Knapp et al. 1998, Abrams 1986). Today however, woody plants are abundant across all watersheds with a burn frequency of three years or more (Heisler et al. 2003, Ratajczak et al. 2014a). The most abundant woody encroaching plants include *Cornus drummondii*, *Juniperus virginiana, Rhus glabra, Prunus americana,* and *Gleditsia triacanthos* (Briggs et al. 2002, Nippert et al. 2021).

Study Design: At KPBS, we measured eco-physiological responses of the clonal woody shrub *Cornus drummondii* CA Mey (roughleaf dogwood). The growth form of *C. drummondii* consists of dense clonal patches of interconnected ramets termed "islands" (Figure 1). We utilized a stratified random sampling for 15 shrub islands of similar height and island circumference. We did not sample locations where clonal islands had grown together, blurring the boundaries between one individual and another. Only distinct islands with maximum heights greater than 1.5 meters were considered during sampling. Shrub islands were selected from the lowlands of two watersheds, N4D and K4A, approximately 2.4 km apart. Both watersheds are burned every four years and were last burned in the spring (March/April) of 2017. Watershed N4D is grazed by native bison, while watershed K4A does not contain any large mammalian grazers.

The experiment consisted of three treatments: grazed, simulated browsing, and control. For the grazed treatment, five islands were randomly selected on watershed N4D with heights ranging from 2.03 to 2.85 meters. In this treatment, bison have been grazing continuously year-round since 1992 and did so throughout the course of the study. Bison forage on grasses which make up a majority of their diet (~80-90%), along with forbs to a lesser extent (~10-15%; Plumb and Dodd 1993, Hecker et al. 2021, Raynor et al. 2016). Bison do consume some browse items (~5%), but these species are usually less than one meter in height (Plumb and Dodd 1993). Bison do not typically consume a significant amount of leaves or stems of large woody trees and shrubs like *C. drummondii* (Knapp et al. 1999, Plumb and Dodd 1993, Coppedge et al. 1998, Raynor et al. 2016). However, grazing by bison has indirect impacts on woody plant distributions. Grass consumption by bison removes fine-fuels, leading to less intense fires and greater abundance of *C. drummondii* and other woody plants on grazed watersheds compared to ungrazed watersheds (Ratajczak et al. 2014, Briggs et al. 2002).

For the control and simulated browsing treatments, ten islands were randomly selected on watershed K4A. Five islands were assigned to the control treatment, with no form of herbivory imposed, ranging from 2.02 to 2.45 meters in height; and five islands were assigned to the browsed treatment ranging from 1.83 to 2.47 meters in height. For islands in the simulated browsing treatment, leaf thinning by hand was implemented to mimic elk browsing as much as possible following the protocol of O'Connor et al. (2020). Leaves were ripped by hand, resulting in occasional terminal bud damage and stem fraying. Fifty percent of an island's leaves were removed, along with significant amounts of new, non-woody stems. This process was done as evenly as possible throughout the canopy on every ramet in the island above 10 cm from ground level. After removal, the leaves were deposited outside the study area. We acknowledge that this treatment is not a perfect mimic of elk herbivory, but only our best surrogate, as we were not able to mimic the bite pattern and head movements associated with actual elk herbivory. This treatment was completed once during the early growing season (May 27th - June 1st), just before the start of sampling, and once during the mid-growing season (July 10th), nine days after our second sampling period.

Leaf Area Index: Leaf area index (LAI) was measured indirectly using an ACCUPAR LP-80 ceptometer between the times of 12:00 and 15:00 in full sunlight on July 7th and July 8th. The ACCUPAR LP-80 ceptometer estimates LAI based on light transmission through the canopy (Bréda 2003). LAI measurements were made near the center of each shrub island across a vertical canopy gradient consisting of four depths: 0 cm (top of the canopy), 50 cm, 100 cm, and 150 cm depth from the top of the canopy (**Figure 1b**). For each LAI measurement, eight instantaneous measurements of PAR were taken and averaged directly outside the canopy facing four directions, and eight instantaneous measurements of

PAR were taken above a square meter area near the center of the shrub island at a given canopy depth facing four directions (two measurements per direction; 90-degree rotation between directions). LAI was calculated by the ceptometer from the averaged measurements.

Leaf-level Physiology: Leaf gas exchange was measured using a LI-6400XT open gas exchange system (LI-COR, Lincoln, Nebraska, USA). Instantaneous measures of net photosynthesis at ambient light intensity (A_{net}), stomatal conductance (g_s), and intrinsic water-use efficiency (iWUE; A_{net}/g_s) were measured at five positions within the canopy of each island of *C. drummondii* (see **Table 1** for a list of traits and their definitions). These positions consisted of a vertical canopy gradient in the center of the shrub island at four depths (**Figure 1b**): 0 cm (top of the canopy), 50 cm, 100 cm, and 150 cm depth from the top of the canopy. The fifth canopy position was on the outer perimeter ("out") of the island in full sunlight (**Figure 1b**). Ramets in the "out" position typically had leaves at similar heights to the surrounding grassy matrix, which contains 40-80 cm tall vegetative tillers. Instantaneous measurements were collected four times at regular intervals from early June to early September 2020. Measurements were taken from 9:00 to 15:00 on the newest, healthy, fully expanded leaves. At each canopy position, gas exchange was measured *in situ* for two leaves and averaged prior to further analysis. The reference chamber CO_2 concentration was set to 400 µmol CO_2 mol^{-1}, relative humidity was maintained between 40% and 60%, and the leaf chamber's PAR level was set to the ambient light level for each canopy position. An ACCUPAR LP-80 ceptometer was used to measure ambient PAR on a prior date with full sunlight. All PAR measurements were made within the same week as gas exchange measurements.

In addition to assessing instantaneous gas exchange, light response curves and A-c_i response curves were measured for all locations and canopy positions. Gas exchange measurements for both types of curves were made from July 3rd through July 17th. The light response curves were developed from *in situ* measurements at eight PAR intensities in the following order: 2000, 1200, 800, 400, 150, 50, 25, and 0 µmol m^{-2}-s^{-1}. For each of the light response curve measurements, the reference CO_2 level was set to 400 µmol CO_2 mol^{-1}. While vertical and temporal gradients in CO_2 can exist within canopies, 400 µmol CO_2 mol^{-1} was used to standardize all measurements. For each measurement, the leaf was given a minimum of

90 seconds and a maximum of 450 seconds to equilibrate between changes in light intensity. Light response curves were used to calculate apparent quantum yield (Φ), LCP, R_d, and A_{2000}. Φ was calculated as the slope of a line through the points at PAR values of 0, 25, and 50 μmol m^{-2}-s^{-1}. LCP was calculated by fitting a line of best fit through the first few PAR intensities and then solving for LCP at a photosynthetic rate of 0 μmol m^{-2}-s^{-1}. R_d was calculated as the rate of photosynthesis at a PAR of 0 μmol m^{-2}-s^{-1}. Light saturated rates of photosynthesis (A_{sat}) could not be calculated because many of the leaves at depths 0 cm, 50 cm, and the "out" position did not asymptote. Therefore, A_{2000} was used as a proxy. A_{2000} was calculated as the rate of photosynthesis at 2000 μmol m^{-2}-s^{-1} of PAR and was equal to A_{sat} for most leaves at the 50 cm, 100 cm, and 150 cm depths.

Immediately following each light response curve, A-c_i response curves were collected by taking measurements at seven concentrations of CO_2 in the following order: 400, 250, 100, 50, 500, 800, and 1000 μmol CO_2 mol^{-1}. For all A-c_i curve measurements, the PAR intensity was set to 2000 μmol m^{-2} s^{-1}, and the leaf was given a minimum of 90 seconds and a maximum of 450 seconds to equilibrate between changes in [CO_2]. A-c_i response curves were developed using the Farquhar-Berry-von Caemmerer model of photosynthesis (Farquhar et al. 1980, von Caemmerer and Farquhar 1981). This was done using the "fitaci" function from the "plantecophys" package (Duursma 2015) in R version 4.0.2 (R Core Team 2020). J_{max} and Vc_{max} were then derived from the curve using the "coef" function.

Leaf Traits: Following each gas exchange measurement, the measured leaf was immediately harvested. For leaf area, the petiole was removed, and area was measured on fresh leaves using a Li-3100 leaf area meter (Li-Cor, Lincoln, NE, USA). Leaves were then dried at 60 °C for a minimum of 72 hours, and leaf dry mass was measured thereafter. Leaf mass per area (LMA) was calculated by dividing leaf dry mass by leaf area (Pérez-Harguindeguy et al. 2013).

The leaf elemental abundance and stable isotopic signatures for carbon were performed at the Stable Isotope Mass Spectrometry Laboratory at Kansas State University. Dried leaves were ground using a Wig-L-Bug amalgamator, and samples were packed in tin capsules prior to analysis. Carbon and nitrogen stable isotope ratios as well as percent carbon and nitrogen of homogenized leaf samples were measured following combustion using an Elementar vario Pyro cube coupled to an Elementar Vision mass spectrometer for isotope analysis. Isotopic abundance ratios were converted to δ notation using the following equation:

$$\delta = [\frac{R_{sample}}{R_{standard}} - 1] * 1000$$

where R is the ratio of heavy to light isotopes for the sample and standard, respectively. Working laboratory standards were annually calibrated against the internationally accepted standard, Vienna Pee-Dee Belemnite for $\delta^{13}C$. Within-run and across-run variability of the laboratory working standard was < 0.05 ‰ for $\delta^{13}C$.

C:N ratio was calculated by dividing leaf percent carbon (%C) by leaf percent nitrogen (%N) for each sample. Relative photosynthetic nitrogen-use efficiency (PNUE) was calculated by dividing A_{net} by N_a. N_a was calculated using the following equation:

$$N_a = \frac{(\%N) \times (leaf\ mass)}{(leaf\ area)}$$

Data Analysis: To make comparisons among treatments, depths and sampling periods, repeated measures linear mixed-effects models were developed using the software package 'nlme' (Pinheiro et al. 2020) in R version 4.0.2 (R Core Team 2020). For each model, the response variable (PAR, LAI, LMA, N_{Area}, C:N, %N, A_{net}, $\delta^{13}C$, iWUE, and PNUE) were fit with canopy position, herbivory treatment (control, browsed, and grazed), and sampling date as fixed effects and replicate as a random effect. Linear mixed-effects models were also developed for the response variables extracted from A-c_i and light response curves (J_{max}, Vc_{max}, LCP, A_{2000}, Φ, and R_d). Parameters were fit with canopy position and herbivory treatment as fixed effects and replicate as a random effect. For all models, significant main effects and interactions are summarized in **Table 2.** For each significant interaction ($\alpha < 0.05$), a Tukey's HSD test was performed to make pairwise comparisons. To further determine which response variables should be included in models, a best-fit model was created for each response variable using the model selection tool ‘dredge()’ from the MuMIn package (Barton 2015). ‘dredge()’ determines the best fit-model from all possible models based on the lowest AICc score. All global models, best fit models, and the AICc scores are summarized in Table S1.

In addition to the mixed-effects models, all variables were arranged in a Pearson correlations matrix using the ‘ggpairs’ function from the package ‘GGally’ in R. One matrix was generated for leaves sampled throughout the growing season (Figure S3) and another was made for the leaves sampled for the A-c_i and light response curves (Figure S4).

Results

Light availability and LAI: For all treatments, PAR decreased and LAI increased significantly with canopy depth (**Table 2, Figure 2**). These trends were least pronounced for the browsed treatment compared to the control and grazed treatments (**Figure 2**). In the control and grazed treatment canopies, mean LAI was 8.0 (control) and 6.6 (grazed), with a maximum LAI of 10.3 in the control treatment. Mean PAR was reduced by 97.5% and 92.1% between the top (0 cm) and bottom (150 cm) of control and grazed treatment canopies. In the browsed treatment canopies, the mean LAI was only 4.1 and PAR was reduced by 78.4% over the same vertical distance (**Figure 2**).

Leaf mass per area (LMA) and N per area (N_a): Both LMA and N_a varied significantly by treatment, depth, and across the growing season (**Table 2**). All two-way interactions were significant for LMA, as well as interactions for treatment*depth and date*depth for N_a. Overall, LMA and N_a decreased with depth for all treatments and at all time points during the growing season (**Figure 3**). LMA in upper canopy leaves (0 and 50 cm) and “out” position leaves increased throughout the growing season in all treatments (except the 50 cm depth in the control treatment; **Figure 3A**). LMA in lower canopy leaves in the control and grazed treatments remained relatively constant but increased throughout the growing season in the browsed treatment, resulting in greater LMA in lower canopy leaves in August and September compared to the control and grazed treatments (**Figure 3A**). Similarly, N_a in lower canopy leaves in the browsed treatment were higher in August and September compared to control and grazed treatments, but the differences were only marginal (**Figure 3B; Table 2**).

Leaf stoichiometry: Overall, C:N increased significantly through the growing season (**Figure 4B, Table 2**). This increase was primarily driven by a decline in leaf %N (**Figure 4D, Table 2**). C:N also varied significantly by canopy depth, whereby values were greater in the "out" position compared to the 50, 100, and 150 cm depths (**Figure 4A, Table 2**). This response was primarily influenced by changes in %N by depth, which were greatest at the 50 and 100 cm depths and lowest in the 150 cm depth and "out" position (**Figure 4A, 4C, Table 2**). %N also varied by treatment with significantly greater values in the browsed treatment compared to the control and grazed treatments (see **Figure S1B** available as Supplementary Data at Tree Physiology Online**; Table 2**).

A_{net}, PNUE, iWUE, and leaf $\delta^{13}C$: Instantaneous photosynthetic rates at ambient PAR (A_{net}) varied significantly by treatment, depth, date, and all two-way interactions (**Table 2**). Overall, photosynthetic rates were highest at the top of the canopy and on the outside of the island and decreased with canopy depth (**Figure 5a**). In the browsed treatment, photosynthetic rates in lower canopy leaves were significantly higher compared to the control and grazed treatments (**Figure 5a; Table 2**). Control and grazed treatments were similar at all depths until the last sampling period in September, where photosynthetic rates decreased in upper canopy leaves and outside of islands in the grazed treatment, resulting in smaller differences in photosynthetic rates between the top (0 cm) and bottom (150 cm) of the canopy compared to the control treatment (**Figure 5a**). Photosynthetic rates in the "out" position were similar to rates at the top (0 cm) of the canopy.

PNUE varied by all main effects and significant interactions between treatment*depth and date*depth (**Table 2**). However, the best-fit model based on the lowest AICc did not include the date*depth interaction (Table S1). Overall, PNUE declined with canopy depth in the control and grazed treatments (**Figure 5b**), PNUE did not show the same declining trend with canopy depth in the browsed treatment, where values stayed relatively constant through the canopy (**Figure 5b**).

All main effects and interactions between treatment*date and treatment*depth varied significantly for iWUE (**Table 2**). Overall, iWUE values were highest during the last sampling period, and this trend was particularly pronounced in the grazed and control treatments (**Figure 5c**). A significant decline in iWUE by canopy depth was found in the control treatment during the July and September sampling periods, but otherwise iWUE stayed relatively constant throughout the canopy in the browsed and grazed treatments (**Figure 5c**).

Leaf $\delta^{13}C$ was lowest in the browsed treatment compared to the control and grazed treatments (see **Figure S1D** available as Supplementary Data at Tree Physiology Online, **Table 2**). There was also a significant interaction between sampling date and canopy depth (**Table 2**). Leaf $\delta^{13}C$ was highest in the upper canopy and "out" position and declined with depth. At the 150cm depth, Leaf $\delta^{13}C$ decreased throughout the growing season, leading to significantly greater values in the first sampling period compared to the final sampling period. (p=0.027; see **Figure S2** available as Supplementary Data at Tree Physiology Online; **Table 2**).

***A*-*c*$_i$ response curves:** J_{max} and Vc_{max}, derived from *A*-c_i response curves, varied significantly by canopy depth (**Table 3**). J_{max} and Vc_{max} were highest at the top of the canopy and declined with depth (**Table 4**), and both variables showed a nearly two-fold difference between the top (0 cm) and bottom (150 cm) of canopies. J_{max} and Vc_{max} in the "out" position were greater than the bottom of the canopy (100 and 150 cm depths), and had similar values to the top (0 and 50 cm depths) of the canopy (**Table 4**). J_{max} also varied significantly by treatment and Vc_{max} varied marginally by treatment (p=0.055; **Table 3**), with greater values in browsed treatment canopies compared to the control and grazed treatments for both variables (**Table 3, 4**).

Light response curves: For all treatment types, both A_{2000} and Φ varied significantly by depth (**Table 3**). A_{2000} and Φ values were greatest at the top of the canopy (0 cm) and "out" position and decreased with depth in the canopy (**Table 4)**. The best-fit model for A_{2000} based on AICc also included treatment (**Table S1**), and while treatment did not have a significant effect on A_{2000} ($p = 0.128$; **Table 3**), values for A_{2000} were marginally greater in the browsed treatment compared to the grazed treatment (**Table 4**).

R_d and LCP both varied significantly by depth and showed a significant treatment*depth interaction (**Table 3**). R_d declined with canopy depth in the control and grazed treatments but stayed relatively constant throughout the canopy in the browsed treatment (**Table 4**). LCP declined with canopy depth in the grazed treatment and insignificantly in the control treatment but stayed relatively constant in the browsed treatment (**Table 4**). In the "out" position, both R_d and LCP were greater in the control treatment compared to the grazed treatment.

Pearson's correlation coefficients: For both LMA and N_a, significant positive correlations existed with the physiological parameters Φ, R_d, A_{2000}, J_{max}, and Vc_{max} ($r= 0.62$-0.87; see **Figure S3** available as Supplementary Data at Tree Physiology Online). C:N ratio was not significantly correlated with any of the physiological parameters, and %N was only weakly correlated with Vc_{max} and J_{max}. Between physiological parameters, strong positive correlations existed between J_{max} and Vc_{max} ($r=0.81$), Vc_{max} and A_{2000} ($r=0.91$), and J_{max} and A_{2000} ($r=0.73$; **Figure S3**). Φ was correlated with J_{max} ($r = 0.74$) and Vc_{max} ($r= 0.60$), but only weakly correlated with A_{2000}. For R_d, the strongest correlations occurred with J_{max} ($r = 0.64$) and Φ ($r=0.72$; **Figure S3**).

From the seasonal measurements, PAR was strongly correlated with LMA ($r=0.773$) and N_a ($r=0.790$) of leaves as well as A_{net} ($r=0.776$) and $\delta^{13}C$ ($r=0.728$; see **Figure S4** available as Supplementary Data at Tree Physiology Online), but PAR was not correlated with %N or C:N ratio of leaves. Within sampling periods, the morphological parameters LMA and N_a were strongly correlated to leaf A_{net} ($r = 0.752$ - 0.864), and $\delta^{13}C$ ($r = 0.753$ – 0821; **Figure S4**). Between physiological parameters, correlations existed between A_{net} and $\delta^{13}C$, but both parameters were only weakly correlated to iWUE (**Figure S4**).

Discussion

The mechanisms enabling *C. drummondii* and other woody encroaching shrubs with dense canopies to facilitate large light reductions across a small canopy distance is not well understood. This project investigated leaf morphological and physiological responses within discrete layers of *C. drummondii* canopies and the influence of simulated browsing (removing leaves to mimic elk herbivory) and bison grazing (reducing competition from grasses) on these factors. Overall, our results indicated that: (1) Leaf morphology of *C. drummondii* varied greatly across a small vertical distance in response to light availability, resulting in major differences in the physiological functioning of leaves. High LMA leaves had high photosynthetic capacity, while low LMA leaves used a novel strategy for maintaining light compensation points (LCP) below ambient light levels. (2) *C. drummondii* leaf morphology and physiology were modified in response to disturbance by simulated browsing, but not grazing, within a single growing season, resulting in a compensatory growth response that facilitated greater photosynthetic capacity and resource-use efficiency in the lower-canopies of browsed *C. drummondii* islands.

Vertical variation in leaf morphology and resource allocation in *C. drummondii*.

Species with a high capacity to vary leaf morphology in response to light availability can utilize leaves across a greater range of light conditions to achieve greater LAI (Saeki 1960, Reich et al. 2009, Hikosaka et al. 2014, Niinemets et al. 2014, Chen et al. 1993). We found that *C. drummondii* canopies had LAI greater than most temperate deciduous forests and reduced mean PAR by 97.5% despite having heights of only 1.5-3.0 m. Therefore, we hypothesized that *C. drummondii* must be capable of high plasticity in leaf morphology and physiology. Supporting this hypothesis, we found that LMA and N_a varied ~3-fold across canopies of *C. drummondii*. Both parameters decreased with canopy depth and were strongly correlated with the ambient light conditions of the leaf. This is consistent with changes in LMA and N_a found across most forest canopies in response to light availability (Poorter et al. 2009). However, differences in LMA across a canopy are typically greater in tall species compared to shorter species (Cavaleri et al. 2010, Porter et al. 2009, Koch et al. 2004, Oldham et al. 2010) due to hydrostatic constraints on the canopy from increasing height (Niinemets 1997, Ishii et al. 2008). On average, LMA varies 4-fold across 100 m *Sequoia* canopies, which can attain LAI values of ~15, but only 2-fold across canopies of most tree species (Koch et al. 2004, Oldham et al. 2010, Sack et al. 2006, Poorter et al. 2009, Legner et al. 2014, Carswell et al. 1999, Gratani et al. 2006, Gratani et al. 2014, Rozendaal et al. 2006, Markesteijn et al. 2007, Wyka et al. 2012, Van Pelt et al. 2016). While substantially shorter in stature, LMA varied more across *C. drummondii* canopies than the canopies of most tree species. Leaf C:N was constant across canopies despite the large variation in N_a indicating that changes in LMA resulted equally from changes in nitrogen and carbon. However, across the growing season, leaf C:N increased and %N decreased indicating that carbon accumulation contributed more to increases in LMA in upper canopy leaves across the growing season. This is likely due to thickening and enhanced lignification of cell walls in response to increased water limitation, and possibly the result of increased storage of starch and other non-structural carbohydrates—a product of high photosynthetic rates (Niinemets 1997, Edwards et al. 2010, Poorter et al. 2009, Moore et al. 1998, Paul and Foyer 2001). Leaf C:N was greatest on the outer edges of islands and could possibly act to deter herbivores, which tend to target more palatable species containing lower leaf C:N (Diaz et al. 2007, Schädler 2003, McDowell et al. 2011). Overall, the high capacity of *C. drummondii* to vary leaf morphology, both across canopies and throughout the growing season, enables it to allocate nitrogen and carbon advantageously in response to the large intra-canopy gradients of light found within high LAI canopies.

Influence of leaf morphology on leaf physiology and plant performance.

Variation in leaf morphology led to substantial differences in physiological functioning throughout the canopy that maximized leaf photosynthesis. Most physiological parameters were strongly correlated to leaf LMA and N_a (**Figure S3, Figure S4**). Photosynthetic parameters J_{max} and Vc_{max} were highest at the top of the canopy, leading to a greater A_{2000} compared to lower canopy leaves. This is beneficial at the top of the canopy where light availability is high. To maximize carbon gain under low-light conditions, very low LCP values were achieved by leaves at the 100 and 150 cm canopy depths. This is critical to maintaining a high LAI since leaves that do not receive the minimum light required to reach photosynthetic compensation negatively impacts net canopy photosynthesis of the plant (Larcher 2003).

In theory, LCP decreases as a function of decreasing R_d and increasing Φ, but previous work suggests that Φ is relatively constant across canopies and that vertical variation in LCP is primarily driven by changes in R_d (Bond et al. 1999, Posada et al. 2009, Valladares et al. 1997, Avalos et al. 2007). Nonetheless, a few species such as *Pseudotsuga menziesii* var. glauca (Beissn.) Franco., *Abies grandis* (Dougl.) Lindl., and *Acer rubrum* L. have been found to have greater Φ in shade leaves compared to sun leaves, enabling shade leaves to further decrease LCP (Nippert and Marshall 2003, Kubiske and Pregitzer 1996, Oberbauer and Strain 1986, Langenheim et al. 1984). Decreased Φ may also result from increased leaf reflectance in upper canopy leaves to prevent damage to photosynthetic apparatus under high light conditions (Langenheim et al. 1984). Contrary to expectation, Φ in canopies of *C. drummondii* decreased with increasing canopy depth. A similar finding has been reported by Dusenge et al. (2015) in tropical montane tree species with greater Φ in sun leaves compared to shade leaves, but this strategy has been rarely observed in other woody species. While decreased Φ negatively impacted the LCP of lower canopy leaves of *C. drummondii*, LCP was still maintained below ambient light levels, due to more than a 3-fold and 5-fold decrease in R_d in the control and grazed treatments. This may indicate a novel strategy for lowering LCP in *C. drummondii* canopies. Increasing Φ can require greater nutrient allocation to chloroplasts to increase the density of thylakoids, protein complexes, and concentrations of photosynthetic pigments (Moriwaki et al. 2019, Hikosaka and Terashima 1995). However, large reductions in R_d, enable *C. drummondii* to maintain LCP below ambient light conditions while theoretically allocating less nitrogen and nutrients to leaves compared to plants with a greater Φ (Moriwaki et al. 2019). While this strategy has benefits, a potential tradeoff exists. Lower canopy leaves of *C. drummondii* cannot utilize light efficiently in the presence of sunflecks compared to species with greater Φ values. However, sunflecks contribute less to carbon gain within canopies of species that concentrate LAI due to reduced penumbral effects (Smith et al. 1989, Stenberg et al. 1998, Van Pelt et al. 2016, Chazdon and Pearcy 1991). Brantley and Young (2009) found that sunflecks in mesic woody encroaching shrub canopies were smaller, shorter in duration, and less intense than those in deciduous forest canopies and contributed to only 5% of the total light below canopies compared to 32% below deciduous forest canopies. The scarcity of light from sunflecks in *C. drummondii* canopies may favor this strategy of reducing LCP, resulting in greater resource-use efficiency than otherwise possible.

Influence of simulated browsing and grazing on *C. drummondii* canopy dynamics.

Previous research has shown that many species have a compensatory growth response that minimizes the impact of herbivory (Maschinski and Whitham 1989, McNaughton 1983). O'Connor et al. (2020) found that ramet density of *C. drummondii* islands had not decreased after two consecutive years of high intensity simulated browsing and maintained similar levels of nonstructural carbohydrates (glucose, sucrose, and starch) as un-browsed islands. These parameters did not decrease until fire and browsing were present in combination. In 2018 and 2019, Wedel et al. (2021) found that after four and five years of high intensity simulated browsing, *C. drummondii* islands still maintained similar recruitment and ramet mortality rates as un-browsed islands, and relative growth rates were similar between browsed and un-browsed islands during a droughted growing season in 2018. These results indicate that *C. drummondii* has a compensatory growth response to herbivory. However, the mechanisms supporting this response have not been described.

The results of this study provide a mechanistic explanation for the compensatory growth response of *C. drummondii*. Mechanisms leading to compensatory growth can be divided into intrinsic mechanisms involving changes in physiology and morphology/development, and extrinsic mechanisms involving modifications of the environment (McNaughton 1983). In *C. drummondii,* defoliation from browsing altered the canopy light environment, resulting in increased PAR values at deeper canopy depths compared to control islands. Over time, leaf morphology and resource allocation throughout canopies of *C. drummondii* were modified in response to browsing, which resulted in greater LMA and N_a in lower canopies compared to the control treatment. This increase in LMA and N_a corresponded with the increase in light intensity at those depths. Leaves with increased LMA and N_a had a higher photosynthetic capacity due to increased J_{max}, Vc_{max}, and A_{2000}, and could reach greater photosynthetic rates at moderate light intensities (400-600) due to increased Φ. The resulting physiology in conjunction with higher PAR levels led to higher photosynthetic rates, increased iWUE, and increased PNUE in lower canopy leaves of the browsed treatment compared to the control treatment and explains the compensatory growth response seen in *C. drummondii*. Improved iWUE may also explain why Wedel et al. (2021) found that browsed islands could maintain similar relative growth rates to un-browsed islands during a drought in 2018. The ability of *C. drummondii* to change its investment strategy within a single growing season is beneficial in grassland ecosystems where changes to the canopy light environment can occur as a result of frequent disturbance.

For the grazed treatment, we hypothesized that the distribution of LAI and leaf morphology of islands of *C. drummondii* in watersheds grazed by bison would differ from that in un-grazed (control) watersheds, and that these differences would lead to higher rates of whole canopy photosynthesis in the grazed treatment. Grazers such as cattle and bison do not directly consume *C. drummondii* or other woody shrubs, but previous work has shown that grazing by bison decreases grass abundance and reduces fire intensity, which facilitates positive feedbacks that drive the survival and spread of *C. drummondii* (Ratajczak et al. 2014, Briggs et al. 2002, Lett and Knapp 2003). However, our results indicate that grazing did not impact *C. drummondii* canopy dynamics. The distribution of LAI and PAR were similar to control canopies at all depths. Leaf morphology and physiology across canopies and in the "out" position of the grazed treatment also had very few differences from the control treatment. It is possible that the similarities between the control and grazed treatments are due to the absence of fire preceding sampling for this study, or it may also be that canopy dynamics are similar between the control and grazed treatments even when fire is present and increased abundance of *C. drummondii* in grazed watersheds results entirely from increased ramet survival in the presence of less intense fires. However, more research is needed to determine whether differences exist during years immediately following a fire.

Conclusions and Implications

Overall, our results have important implications for understanding the growth investment strategy of *C. drummondii* and other woody shrubs which enables them to achieve dense canopies, respond positively to periodic grassland disturbance, and ultimately facilitate successful encroachment in grassland ecosystems. This study revealed that these characteristics are driven by the capacity of *C. drummondii* to dramatically alter leaf traits in response to light gradients—both spatially to achieve dense canopies, and temporally to achieve compensatory growth.

Future research is needed to determine whether high intra-canopy variation in leaf traits exist in other woody encroaching species and whether this is a major characteristic differentiating woody encroaching species that cause large disruptions to grassland structure and function from those that coexist with grasses (Eldridge et al. 2011, Ratajczak et al. 2012). In *C. drummondii*, large leaf trait variation across canopies enables high-LAI values which lower light availability, displacing understory grasses, and resulting in fire suppression (Ratajczak et al. 2011, Lett and Knapp 2003). Once *C. drummondii* escapes fire it spreads rapidly across grasslands (Ratajczak et al. 2011). While leaf trait variation across a canopy is a major factor determining the optimal LAI to maximize whole-canopy photosynthesis (Saeki 1960, Hikosaka 2005), LAI is also limited by other factors such as water and nutrient availability (Asner et al. 2003). Future research is needed to determine the potential drivers of LAI in other woody encroaching shrubs and in other grasslands to better understand the extent to which intra-canopy variability in leaf traits (morphological and physiological) drives LAI of woody encroaching plants under different abiotic conditions. This relationship, along with a better understanding of canopy architectural traits, are central to determining why certain woody species become dominant encroachers of grasslands, while most other woody species do not change in abundance over time.

Data and Materials Availability

Data will be made publicly available at the Konza Prairie LTER data repository (http://lter.konza.ksu.edu/data).

Conflict of Interest

None Declared

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Authors Contributions

All authors contributed to the ideas and methodologies of the paper; ET, SB, JN, and RK collected the data; ET analyzed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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<u>Tables</u>

Table 1: List of measured canopy and leaf traits with a brief description and units accompanying each variable.

Table 2: Summary for the mixed effects models analysis of variance. Table contains all variables that were measured at multiple periods throughout the growing season. All significant effects ($p<0.05$) are bold font and insignificant effects are normal font ($p>0.05$). Abbreviations: PAR = photosynthetically active radiation; LMA = leaf mass per area; N_a = leaf nitrogen per unit area; %N = percent leaf nitrogen; A_{net} = instantaneous photosynthetic rate at ambient light intensity; iWUE = intrinsic water-use efficiency; PNUE = photosynthetic nitrogen-use efficiency.

Table 3: Summary for the mixed effects models analysis of variance. Table summarizes response variables that were only measured during one period of the growing season. All significant effects ($p<0.05$) are bold font and insignificant effects are normal font ($p>0.05$). An asterisk is placed next to effects with marginal differences. Abbreviations: Vc_{max} = maximum velocity of carboxylation; J_{max} = maximum velocity of electron transport; LCP = light compensation point; R_d = dark respiration; Φ = apparent quantum yield; A_{2000} = photosynthetic rate at 2000 μmol m^{-2}-s^{-1}; LAI = leaf area index.

Table 4: Summary of means and standard errors for parameters extracted from the A-c_i and light response curves. See **Table 1** for variable units. Abbreviations: Vc_{max} = maximum velocity of carboxylation; J_{max} = maximum velocity of electron transport; LCP = light compensation point; R_d = dark respiration; Φ = apparent quantum yield; A_{2000} = photosynthetic rate at 2000 μmol m^{-2}-s^{-1}.

Figures

Figure 1: (A) A large island of *C. drummondii* with a dense canopy on watershed K4A at Konza Prairie Biological Station, Manhattan, Kansas. The growth form of *C. drummondii* consists of dense clonal patches of interconnected ramets termed “islands”. (B) Diagram showing a cross section through the center of a *C. drummondii* island illustrating its growth form and our sampling locations. Black circles represent the measurement location for each canopy depth. Diagram credit: *C. drummondii* island animation by Emily Wedel.

Figure 2: (A) LAI and (B) PAR measured in *C. drummondii* canopies at varying canopy depths (out, 0 cm, 50 cm, 100 cm, and 150 cm) and herbivory treatments (browsed, control, grazed). Point and whiskers represent the mean ± standard error of the one sampling period for LAI, and the mean ± standard error for all four sampling periods for PAR.

Figure 3: (A) LMA and (B) N_a of leaves in *C. drummondii* canopies varying by canopy position (out, 0 cm, 50 cm, 100 cm, and 150 cm), herbivory treatment (browsed, control, and grazed), and sampling period (6/4/, 7/1, 8/1, 9/5). Point and whiskers represent the mean ± standard error.

Figure 4: C:N ratio of leaves varying by depth (A) and date (B), and %N of leaves varying by depth (C) and date (D). Point and whiskers represent the mean ± standard error.

Figure 5: (A) Ambient photosynthetic rates, (B) photosynthetic nitrogen-use efficiency, and (C) intrinsic water-use efficiency of leaves in *C. drummondii* canopies varying by canopy position (out, 0 cm, 50 cm, 100 cm, and 150 cm), herbivory treatment (browsed, control, and grazed), and sampling period (6/4, 7/1, 8/1, 9/5). Point and whiskers represent the mean ± standard error.