

# Ecophysiological responses of native invasive woody *Juniperus virginiana* L. to resource availability and stand characteristics in the semiarid grasslands of the Nebraska Sandhills

J. MSANNE\*, T. AWADA<sup>\*,+</sup>, N.M. BRYAN\*, W. SCHACHT<sup>\*,\*\*</sup>, R. DRIJBER<sup>\*\*</sup>, Y. LI<sup>\*\*\*</sup>, X. ZHOU<sup>\*,#</sup>, J. OKALEBO\*, D. WEDIN\*, J. BRANDLE\*, and J. HILLER\*

*School of Natural Resources\*, Department of Agronomy and Horticulture\*\*, University of Nebraska, Lincoln, Nebraska, 68583, USA*

*Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou 730000, China\*\*\**

*Campbell Scientific Inc., Logan, Utah, 84341, USA#*

## Abstract

Vegetation in grasslands is changing at an unprecedented rate. In the Nebraska Sandhills, this shift is attributed in part to encroachment of the woody species *Juniperus virginiana*. We investigated changes in resource availability and their feedback on seasonal trends in photosynthetic characteristics of *J. virginiana* trees scattered in open grasslands vs. a dense 57-year-old stand. Dense stand exhibited lower volumetric soil water content,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  $\delta^{13}\text{C}$ , as well as foliage  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and N content, compared to grasslands. Water potential was higher in trees in grasslands compared to dense stand. *J. virginiana* in dense stand exhibited similar trends to trees in grasslands for net photosynthetic rate ( $P_N$ ), stomatal conductance, transpiration, maximum photochemical efficiency of PSII, maximum carboxylation velocity, and maximum rate of electron transport.  $P_N$  peaked early summer and declined in the fall, with trees in open grasslands lagging behind those in dense stand. Plasticity of this species may place it at a competitive advantage in the Sandhills, further altering grasslands vegetation and ecosystem processes.

*Additional key words:* carboxylation velocity; eastern red cedar; electron transport; fluorescence; gas exchange; isotope ratio; soil nutrients.

## Introduction

Vegetation cover in grasslands and savannas and the resulting ecosystem processes are changing at an unprecedented rate across the globe (Archer 1994, Wilcox 2010, Twidwell *et al.* 2013, Ganguli *et al.* 2016). This phenomenon has been attributed to climate variability and change, increased carbon dioxide concentrations in the atmosphere, and changes in disturbance fire regimes and

anthropogenic management (Wilcox 2010, Diez *et al.* 2012). Globally, these ecosystems contribute to around 35% of the terrestrial net primary production (NPP, Chapin *et al.* 2011), and provide significant agricultural (e.g., livestock) and ecological services (Briggs *et al.* 2005). Over the past century, grasslands and savannas worldwide have experienced directional and sometimes persistent

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<sup>+</sup>Corresponding author; e-mail: [tawada@unl.edu](mailto:tawada@unl.edu)

**Abbreviations:** C – carbon;  $C_i$  – intercellular  $\text{CO}_2$  concentration; D – vapor pressure deficit between the leaf and air; DBH – diameter at breast height; DOY – day of year;  $E$  – transpiration;  $F_0$  – minimal fluorescence yield of the dark-adapted state;  $F_m$  – maximal fluorescent yield of the dark-adapted state;  $F_v$  – variable fluorescence;  $F_v/F_m$  – maximum quantum efficiency of PSII;  $g_s$  – stomatal conductance;  $J_{\max}$  – rate of electron transport; N – nitrogen; NNF – Nebraska National Forest; NPP – net primary production;  $P_N$  – net photosynthetic rate; PNUE – photosynthetic nitrogen-use efficiency;  $T_A$  – temperature of the air;  $T_L$  – temperature of the leaf;  $V_{\text{cmax}}$  – maximum carboxylation velocity; VSWC – volumetric soil water content; WUE – water-use efficiency;  $\delta^{13}\text{C}$  – carbon isotope ratio;  $\delta^{15}\text{N}$  – nitrogen isotope ratio;  $\Psi_{\text{pre}}$  – predawn water potential;  $\Psi_{\text{mid}}$  – midday water potential;  $\Psi_w$  – water potential.

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shifts from grass-dominated communities to open and, in some instances, closed-canopy woodlands. This trend has accelerated in recent decades (Wilcox 2010, Twidwell *et al.* 2013) modifying and in some cases threatening key ecosystem services (Norris *et al.* 2001, Throop *et al.* 2012, Nippert *et al.* 2013), including biodiversity (Pierce and Reich 2010), species distribution and composition (Dobson *et al.* 1997, Chapin *et al.* 1998, Norris *et al.* 2007), ecosystem NPP (Wilcox 2010), herbaceous (Ganguli *et al.* 2008) and livestock production (Throop *et al.* 2012), nutrient cycling (Archer 2010, Pierce and Reich 2010), and system ecohydrology (Eggemeyer *et al.* 2009, Awada *et al.* 2013). There is much to be learned about the implications of shifts from open grasslands and savannas to closed-canopy woodlands on water and biogeochemical cycles (Wilcox 2010, Nippert *et al.* 2013, Starks *et al.* 2014), and the feedback of these cycle and resource availability changes on plant ecophysiological performance. Furthermore, investigating the seasonal and spatial physiological activity are necessary for modeling efforts including the estimation and forecasting of energy, water and carbon exchange, as well as NPP of a plant community and its projected dynamics in the face of climate variability and change (Hamada *et al.* 2016).

Conversion of C<sub>4</sub>-dominated grasslands with relatively high photosynthetic capacity, high nitrogen- and water-use efficiencies and short growing season to C<sub>3</sub> woodlands with lower photosynthetic rates, longer growing season, and lower nitrogen- and water-use efficiencies in low to mid-latitude regions of the Great Plains of the continental U.S. (Eggemeyer *et al.* 2006, Eggemeyer *et al.* 2009, Awada *et al.* 2013) has implications on NPP, biogeochemical and ecohydrological cycles (McKinley and Blair 2008, Wilcox 2010), and fire regime (D'Antonio and Vitousek 1992, Brooks *et al.* 2004). With respect to ecohydrology, woody species encroachment has been found to modify water distribution in soils through shifts in transpiration rates, more precipitation interception, and less water infiltration, potentially resulting in drier soils (Liao *et al.* 2008, Rout and Callaway 2009, Boutton and Liao 2010, Awada *et al.* 2013). These changes have negative impacts on grasses and resilience of the these ecosystems (Diez *et al.* 2012). Woody plant expansion or encroachment in semiarid grasslands also has been found to increase below- (Jackson *et al.* 2002) and aboveground C pools (Pacala *et al.* 2001, McCulley and Jackson 2012). McKinley and Blair (2008) reported an increase in total ecosystem N and C pools in *Juniperus virginiana* forests compared to grasslands, likely because the absence of fire in *J. virginiana* stands allowed substantial accrual of C and N in aboveground biomass and litter layers. In another study, Billings (2006) found that plant litterfall from forested sites (*Ulmus* sp. and *Quercus* sp.) had greater N concentration than that in smooth brome (*Bromus inermis* Leyss) grass sites.

In the Great Plains, substantial shifts in vegetation cover have been attributed to woody species expansion,

including mesquite (*Prosopis glandulosa* Torr.) in Texas (Archer 1995), ponderosa pine (*Pinus ponderosa* P.&C. Lawson) in the Black Hills of South Dakota (Shinneman and Baker 1997), and eastern red cedar (*Juniperus virginiana*, L.), which can be found in every state east of the 100<sup>th</sup> meridian (McKinley and Blair 2008, Starks *et al.* 2014), encroaching into 7 million ha of grasslands, shrublands, and riparian forests in the Great Plains, along a precipitation gradient ranging from semiarid in its western distribution (Eggemeyer *et al.* 2009) to mesic in its eastern distribution (Willson *et al.* 2008).

Similar to other areas of the Great Plains, the semiarid grasslands of the Nebraska Sandhills – which at 50,000 km<sup>2</sup> are the largest stabilized sand dune formation in the Western Hemisphere – are experiencing a nonlinear increase in the encroachment of *J. virginiana* [current rate exceeding 10,000 ha per year (Helmink 2012)]. Limited studies have addressed the ecophysiology of *J. virginiana* in the Nebraska Sandhills (*e.g.*, Eggemeyer *et al.* 2006, Eggemeyer *et al.* 2009, Awada *et al.* 2013), and these studies did not investigate the impact of density or changes in plant-soil processes and resource availability associated with encroachment. The Sandhills offer a unique ecosystem due to their economic (*i.e.*, livestock production) and ecological importance, and the potential implications of this encroachment on regional ecohydrology. The Nebraska Sandhills are a major recharge zone for the High Plains Ogallala Aquifer [36 and 65% of the aquifer area and water, respectively, are located under Nebraska (Bleed and Flowerday 1998)]. Thus, woody species encroachment has the potential to significantly alter groundwater recharge (Awada *et al.* 2013), which is estimated to be 6 to 14% of annual precipitation in the Sandhills (Szilagyi *et al.* 2005), as well as soil C and N pools and carbon storage (Mellor *et al.* 2013). Additionally, the Sandhills occupy the drier region of the northern Great Plains where several years of consecutive drought are not uncommon (Awada *et al.* 2013).

The focus of this study was therefore to investigate shifts in key plant-soil processes associated with expansion of *J. virginiana* into grasslands, and their feedback on the ecophysiology of this species when found in open grasslands or mature dense-canopy woodland, in the drought prone semiarid grasslands of the Nebraska Sandhills, where the species is found in its most western locations. This was accomplished by investigating *J. virginiana* seasonal variability in water relations, photosynthetic capacity [including seasonal maximum carboxylation velocity ( $V_{\text{cmax}}$ ) and rate of electron transport ( $J_{\text{max}}$ ), which to our knowledge have not been reported in the field for this species], and water- and nitrogen-use efficiencies as impacted by environmental variability and soil characteristics. Outcome from this study is also critical to developing ecophysiological predictive models for water, carbon and energy balance, and to forecasting the dynamics and performance of this species in grasslands of the Great Plains.

## Materials and methods

**Sites characteristics:** The study was conducted at the Nebraska National Forest (NNF), Halsey, Nebraska, USA (825 m a. s. l., 41°51'45"N, 100°22'06"W), between 2006 [starting day of year (DOY) 262] and 2007 over a 12-month period. The NNF is managed by the US Forest Service, University of Nebraska, Lincoln. Most of the NNF is Sandhills grassland with about 10,000 ha hand-planted coniferous species, including the invasive native *J. virginiana*. Climate is semiarid continental, with a mean annual precipitation of 570 mm, 75% of which falls during the growing season (April–September). Mean annual temperature is 8.4°C. The mean minimum temperature in January is –14°C, and the mean maximum temperature in July is 31°C. Soils are Valentine fine sand (mixed, mesic Typic Ustipsamments) with deep loose sandy well-drained soils.

Two sites were selected, a dense mature stand of *J. virginiana* (hereafter, dense), and an open grassland with young scattered individuals of *J. virginiana* (hereafter, open). Sites and trees characteristics are presented in the *text table* below. The dense *J. virginiana* site has little to no growth of understory species with the exception of occasional cacti (*Opuntia* spp.), and scattered individuals of sedges (*Carex* spp.). The open grassland site is composed of a low density overstory of *J. virginiana* and ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson), and a perennial grassland understory community of mostly C<sub>4</sub> species, including prairie sandreed (*Calamovilfa longifolia* (Hook.) Scribn.), little bluestem (*Schizachyrium scoparium*), and switchgrass (*Panicum virgatum*). Kentucky bluegrass (*Poa pratensis* L.), yucca (*Yucca* spp. L.), and pricklypear (*Opuntia* spp. P. Mill) can also be found on site.

| Parameter                                                     | Open         | Dense       |
|---------------------------------------------------------------|--------------|-------------|
| Site tree density [ha <sup>-1</sup> ]                         | 43           | 537         |
| Site basal area [m <sup>2</sup> ha <sup>-1</sup> ]            | 5.6          | 7.89        |
| Site average DBH [m]                                          | 0.39 ± 0.64  | 0.10 ± 0.26 |
| Age [yr] (*Planted even-aged 57-year-old stand.)              | 20–30        | 57 *        |
| Average DBH of trees in this study [m]                        | 0.39 ± 0.64  | 0.19 ± 0.83 |
| Average height of trees in this study [m]                     | 6.4 ± 0.55   | 9.1 ± 0.38  |
| Ratio of green canopy/total height of trees in this study [%] | 0.99 ± 0.004 | 0.7 ± 0.005 |
| Average crown volume of trees in this study [m <sup>3</sup> ] | 95.23 ± 24.8 | 21.45 ± 2.4 |

**Micrometeorological and soil measurements:** Micro-meteorological variables including precipitation, atmospheric relative humidity, and air and soil temperatures were continuously recorded with a weather station installed at each site during the study period. One set of soil moisture sensors (*ML2 Theta Probe*, *Dynamax Inc.*, TX, US) were installed at soil depths of 0.2, 0.4, and 1 m. Soil moisture sensors were installed in between the trees in the dense site, and in the open but close to the canopy in the open site. Measurements were taken every 15 s and stored as half-hourly means on a datalogger (*CR10X*, *Campbell Scientific Inc.*, UT, US). In the dense stand, soil moisture data interruption occurred during the spring due to power problems, and data between DOY 225 and 350 were questionable for the sensor placed at 0.2 m depth, possibly due to rodent damage; thus data from these two periods were not incorporated in this study. Additionally, three replicates of volumetric soil water content (VSWC) [%] in the upper 0.3 m and 2 m of the soil profile were collected regularly using the *TRIME FM (IMCO)*, Germany) system with access tubes. Weather data from the station on site were validated against a station at Halsey, NE (High Plains Regional Climate Center, HPRCC, University of Nebraska, UNL; <http://www.hprcc.unl.edu>).

A total of 20 soil samples for each site, divided into litter and mineral soil (0–0.15 m), were collected within a

20 × 20 m plot. Ammonium (NH<sub>4</sub><sup>+</sup>) [mg(N) kg<sup>-1</sup>], nitrate (NO<sub>3</sub><sup>-</sup>) [mg(N) kg<sup>-1</sup>], elemental carbon (C) [%] and nitrogen (N) [%], total soluble nitrogen (includes NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and soluble organic N), and isotopic ratios of carbon (δ<sup>13</sup>C) [‰] and nitrogen (δ<sup>15</sup>N) [‰] were determined. For NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> determination, soil samples were extracted using a 1 M potassium chloride solution, shaken for 0.5 h, and then allowed to settle overnight at 4°C. The settled solution was then pipetted out and sent to the Ecosystems Analysis Laboratory at the University of Nebraska for NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> analysis using a *Lachat 8500 Series 2 Autoanalyzer* (*Lachat Instruments*, Loveland CO, USA). The <sup>15</sup>N and <sup>13</sup>C were determined separately on individual samples by combustion, and isotope ratio mass spectrometry using a *Eurovector E3000* elemental analyzer interfaced with an *Isoprime* continuous flow isotope ratio mass spectrometer (*GV Instruments*, Manchester, UK). The <sup>15</sup>N and <sup>13</sup>C were expressed relative to atmospheric nitrogen and PDB, respectively. Elemental composition of percent C and N was calculated using glutamic acid and ammonium sulfate, respectively. Sucrose, caffeine, and urea were analyzed to check for C, and methionine was used for N. Analyses were conducted at the Water Sciences Laboratory, Nebraska Water Center, University of Nebraska, Lincoln (<http://watercenter.unl.edu/waterscilab/Isotope.asp>).

**Foliage ecophysiological measurements:** Seasonal gas-exchange measurements started in the late summer of 2006 (DOY 262) and were concluded 12 months later in 2007. Several unshaded, south-facing needle-like scale foliage were selected for the measurement. The number of individual samples ( $n$ ) per tree varied between 1 in winter and 4 during the growing season, and values from each tree per sampling date were pooled together for analysis to avoid pseudoreplication. Ladders were used to access the upper canopy. Net photosynthesis ( $P_N$ ) [ $\mu\text{mol m}^{-2} \text{s}^{-1}$ ], stomatal conductance ( $g_s$ ) [ $\text{mol m}^{-2} \text{s}^{-1}$ ], and transpiration rates ( $E$ ) [ $\text{mmol m}^{-2} \text{s}^{-1}$ ] were determined with an infra-red gas analyzer, with a 6  $\text{cm}^2$  chamber mounted with a LED light source (LICOR 6400 – 02B; LICOR Inc., Lincoln NE, US). Measurements were conducted at light saturation – PAR was set at 1,500  $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  (Eggemeyer *et al.* 2006, Bihmidine *et al.* 2010) – and were conducted between 10:00 and 14:00 h solar time in winter, and 09:00 and 14:00 h solar time during the growing season. After completion of each measurement, and due to the scale-like foliage of *J. virginiana*, foliage material in the chamber was collected and measured with a leaf area meter (LICOR 3000; LICOR Inc., Lincoln NE, US) to correct for gas-exchange measurements. The response of photosynthesis to intercellular  $\text{CO}_2$  concentration ( $P_N/C_i$  curve) was determined at light saturation following the protocol described in Long and Bernacchi (2003), Bihmidine *et al.* (2010), and Bihmidine *et al.* (2014). Maximum carboxylation velocity ( $V_{\text{cmax}}$ ) [ $\mu\text{mol m}^{-2} \text{s}^{-1}$ ] and rate of electron transport ( $J_{\text{max}}$ ) [ $\mu\text{mol m}^{-2} \text{s}^{-1}$ ] were calculated for each  $P_N/C_i$  curve by nonlinear regression using the curve fitting utility (Sharkey *et al.* 2007). It was difficult to obtain reliable  $P_N/C_i$  curves under certain environmental conditions (e.g., low soil water and/or low/high air temperatures), curves were not included in the analysis if data points were outside the physiologically possible limits, or points did not transition between Rubisco-carboxylation

limited and RuBP-regeneration limited assimilation rate. Thus, figures for  $V_{\text{cmax}}$  and  $J_{\text{max}}$  have missing data points and/or lack of standard error bars.

Chlorophyll (Chl) fluorescence of dark-adapted foliage was measured with a leaf chamber (2  $\text{cm}^2$ ) fluorometer attached to the LI-6400 infrared gas analyzer (LICOR Inc., Lincoln NE, US). The variable fluorescence ( $F_v$ ) is the difference between  $F_0$  and  $F_m$ . When measured on dark-adapted needles,  $F_v/F_m$  is proportional to the maximum potential quantum yield of photosynthesis (Bihmidine *et al.* 2010). Predawn ( $\psi_{\text{pre}}$ ) [MPa] and midday ( $\psi_{\text{mid}}$ ) [MPa] water potentials were measured using a pressure chamber (PMS Instrument Co., Albany OR, US). Nitrogen concentration (N) [%], and isotopic ratios of carbon ( $\delta^{13}\text{C}$ ) [‰] and nitrogen ( $\delta^{15}\text{N}$ ) [‰] were determined on several needles from each individual tree, including those used for gas-exchange measurements (Water Sciences Laboratory, Nebraska Water Center, University of Nebraska, Lincoln, <http://watercenter.unl.edu/waterscilab/Isotope.asp>).

**Statistical analyses:** Data were analyzed using SAS statistical package (SAS Institute, Inc. 1998). Repeated measure analyses using the MIXED MODEL procedure in SAS was used with trees as random factor and treatments (open vs. closed) as fixed factor, as well as regressions and orthogonal contrasts to compare species responses and to determine the effects of environmental conditions on measured parameters within and between species. Means were separated ( $p < 0.05$ ) using the pairwise mean comparisons in SAS. Principle component analysis (PCA) was used to analyze both ecophysiological and morphological attributes associated with trees as impacted by density and resource availability. PCA was conducted using the R open source programming *ade4* multivariate data analysis package developed by Dray *et al.* (2007). The *factoextra* package supplemented the *ade4* package for the purposes of visualization of the multivariate outputs (Kassambara 2015).

## Results

**Micrometeorological and soil parameters:** Average annual air temperature ( $T_A$ ) was slightly higher in open grassland with scattered young trees (10.62°C) than in the dense mature stand of *J. virginiana* (9.6°C). Trends corresponded well with historical records, reaching maximum in months of July and August ( $\sim 30^\circ\text{C}$ , DOY 181–244), and a minimum in January and February ( $\sim -20^\circ\text{C}$ , DOY 1–60; Fig. 1A). Cumulative precipitation was 590 mm, slightly above the long-term average of  $\sim 570$  mm, with the lowest precipitation recorded in July and August (cumulative 81 mm) and in November and December (cumulative 34 mm, DOY 306–365, Fig. 1A), and the highest in May and June (cumulative 208 mm, DOY 122–182). Average volumetric soil water content (VSWC) was consistently and significantly greater in open grasslands with scattered *J. virginiana*, relative to the

dense stand (Fig. 1B–F), but differences became smaller with soil depth. On an annual basis, the VSWC in the top 2 m averaged 38% less in the dense stand of *J. virginiana* (6.7%) compared to open grassland (10.9%); these trends are consistent with Awada *et al.* (2013).

Ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and total inorganic N in the litter layer and mineral soil (0–0.15 m) were significantly greater by at least 25% in the open grassland with scattered young trees of *J. virginiana* than in the dense mature stand (Table 1). Available N declined by 20 and 27% in dense stand relative to open grassland, but total carbon and nitrogen increased by 18 and 37%, respectively. As expected, the mineral soil carbon isotopic ratio ( $\delta^{13}\text{C}$ ) was significantly higher (i.e., more enriched,  $-23\text{‰}$ ) in the open grassland with young *J. virginiana* trees than in the dense mature stand ( $-25.9\text{‰}$ ) reflecting a

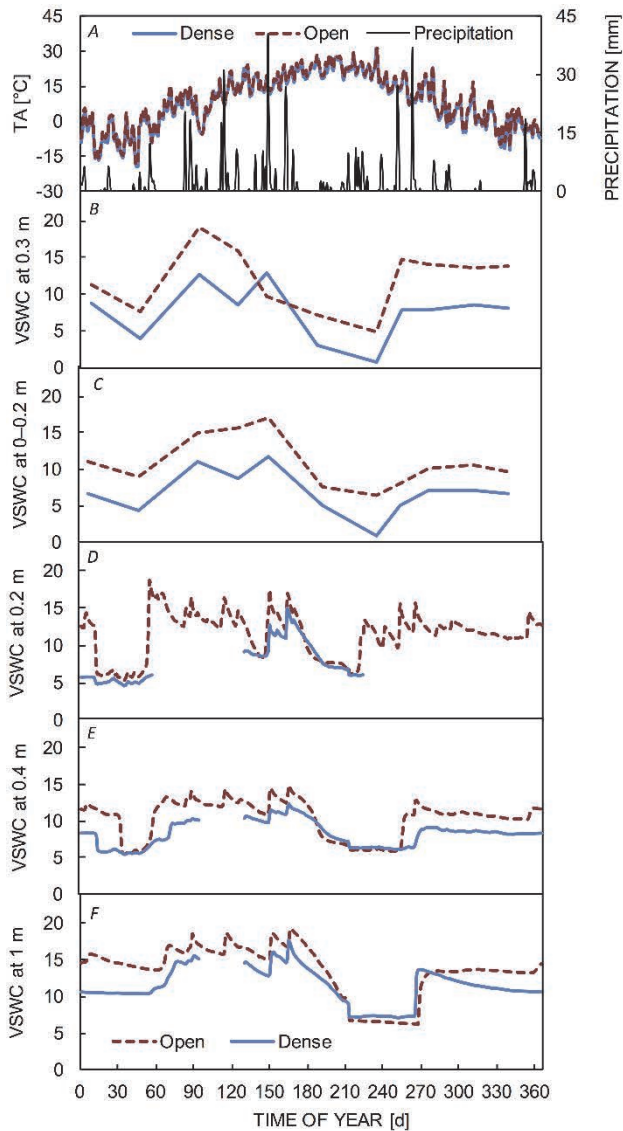


Fig. 1. Seasonal trends in average daily air temperature and precipitation (A), volumetric soil water content at 0–0.3 and 0–2 m using access tubes (B,C), VSWC at 0.2, 0.4, 1 m using continuous measurements (D,E,F) in open grasslands and dense stand of *Juniperus virginiana* in the semiarid grasslands of the Nebraska Sandhills between 2006–2007 (starting day of year 262 in 2006 and continuing for 12 months). Missing data points of VSWC in the dense stand were due to power problems in the spring, and sensor damage at the depth of 0.2 m between DOY 225 and 350 possibly due to rodent activity.

shift from  $C_4$ -dominated to a  $C_3$ -dominated community (Table 1). Isotopic N ratio ( $\delta^{15}\text{N}$ ) in the mineral soil was slightly more enriched in the dense mature *J. virginiana* stand (0.45‰) than in the open grassland with scattered young trees (0.35‰).

**Ecophysiological parameters:** Predawn ( $\Psi_{\text{pre}}$ ) and mid-day ( $\Psi_{\text{mid}}$ ) water potentials displayed significant seasonal variation that generally corresponded with VSWC in both

Table 1. Means and associated standard errors of measured soil and *Juniperus virginiana* foliage parameters in open grasslands with scattered *J. virginiana* and dense tree stand in the Nebraska Sandhills. \* – statistical significance between means at  $p < 0.05$ .

| Parameter                                                                | Open          | Dense          |
|--------------------------------------------------------------------------|---------------|----------------|
| <b>Soil</b>                                                              |               |                |
| $\text{NH}_4^+$ [mg N kg <sup>-1</sup> ]                                 |               |                |
| Litter                                                                   | 12.66 ± 2.3   | 6.5 ± 0.58*    |
| Mineral Soil                                                             | 0.64 ± 0.13   | 0.48 ± 0.04*   |
| $\text{NO}_3^-$ [mg N kg <sup>-1</sup> ]                                 |               |                |
| Litter                                                                   | 2.72 ± 0.6    | 1.5 ± 0.8*     |
| Mineral Soil                                                             | 0.30 ± 0.04   | 0.21 ± 0.03*   |
| Total Inorganic N [mg N kg <sup>-1</sup> ]                               |               |                |
| Litter                                                                   | 15.38 ± 2.7   | 8.08 ± 2.8*    |
| Mineral Soil                                                             | 0.94 ± 0.16   | 0.69 ± 0.04*   |
| Total Soluble N [mg N kg <sup>-1</sup> ]                                 |               |                |
| Litter                                                                   | 17.25 ± 2.5   | 13.3 ± 9.8     |
| Mineral Soil                                                             | 33.6 ± 3.02   | 24.5 ± 1.8*    |
| Carbon [%]                                                               | 1.18 ± 0.23   | 1.40 ± 3.10    |
| Nitrogen [%]                                                             | 0.53 ± 0.07   | 0.73 ± 0.11*   |
| $\delta^{13}\text{C}$ Mineral Soil [‰]                                   | -23.0 ± 0.30  | -25.9 ± 0.80*  |
| $\delta^{15}\text{N}$ Mineral Soil [‰]                                   | 0.35 ± 1.05   | 0.45 ± 0.81    |
| <b>Foliage</b>                                                           |               |                |
| $P_N$ [ $\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$ ]     | 8.55 ± 1.3    | 8.3 ± 1.33     |
| $g_s$ [ $\text{mol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{ s}^{-1}$ ] | 0.11 ± 0.018  | 0.11 ± 0.02    |
| $E$ [ $\text{mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{ s}^{-1}$ ]  | 2.26 ± 0.47   | 2.17 ± 0.48    |
| WUE [ $P_N/g_s$ ]                                                        | 76.8 ± 5.7    | 73.4 ± 6.88    |
| PNUE [ $P_N/N$ ]                                                         | 5.37 ± 0.77   | 8.78 ± 1.22*   |
| $\Psi_{\text{pre}}$ [MPa]                                                | -0.49 ± 0.05  | -0.72 ± 0.07*  |
| $\Psi_{\text{mid}}$ [MPa]                                                | -1.48 ± 0.13  | -1.59 ± 0.14   |
| $F_v/F_m$                                                                | 0.72 ± 0.045  | 0.78 ± 0.045   |
| $V_{\text{cmax}}$ [ $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ]              | 33.67 ± 1.52  | 31.50 ± 1.94   |
| $J_{\text{max}}$ [ $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ]               | 137.04 ± 3.25 | 130.3 ± 7.02   |
| C [%]                                                                    | 47.3 ± 1.16   | 48.3 ± 0.71    |
| N [%]                                                                    | 1.56 ± 0.036  | 0.99 ± 0.037*  |
| $\delta^{13}\text{C}$ [‰]                                                | -24.8 ± 0.16  | -26.6 ± 0.063* |
| $\delta^{15}\text{N}$ [‰]                                                | -2.48 ± 0.086 | -5.9 ± 0.095*  |

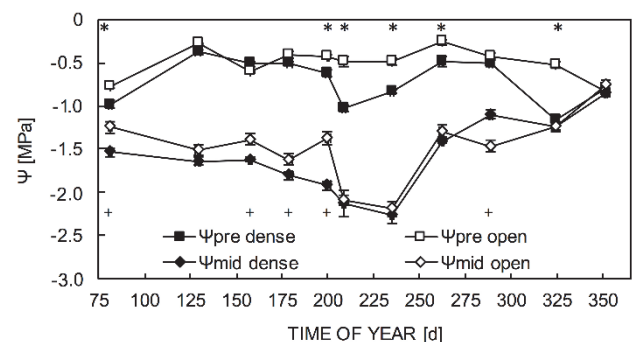


Fig. 2. Seasonal trends in average predawn ( $\Psi_{\text{pre}}$ ) and midday ( $\Psi_{\text{mid}}$ ) water potential with standard error bars, in open grasslands (open symbols) and dense stand (full symbols) of *Juniperus virginiana* in the semiarid grasslands of the Nebraska Sandhills between 2006–2007 (starting day of year 262 in 2006 and continuing over 12 months). \* – significant differences between open and dense within a sampling date ( $p < 0.05$ ,  $n = 5$  trees).

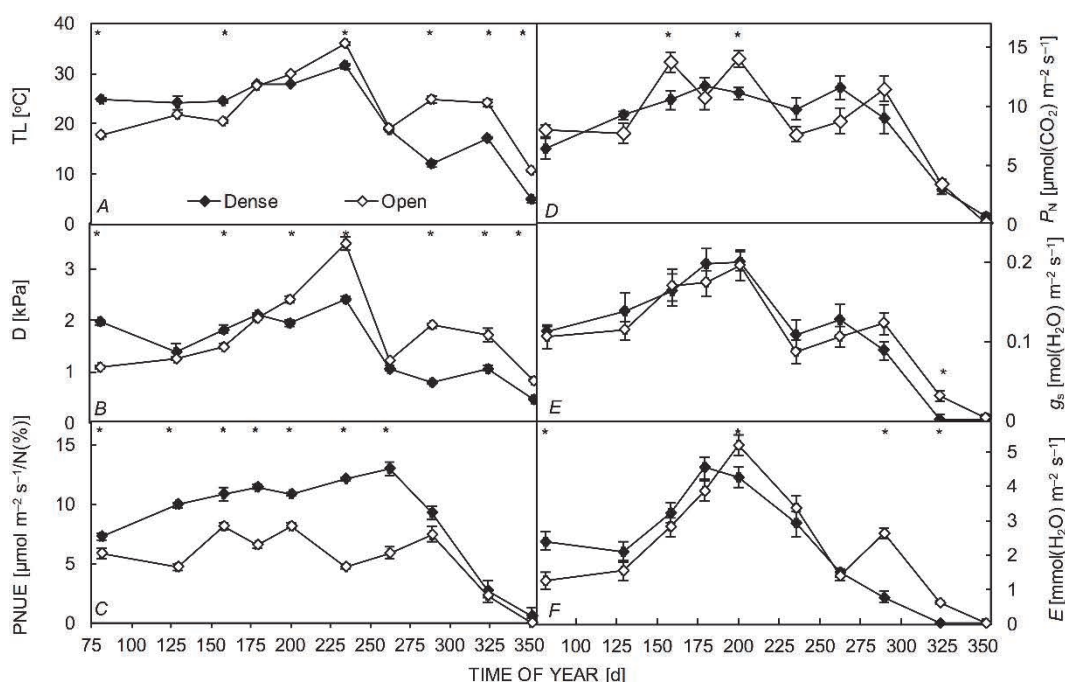


Fig. 3. Seasonal trends in average leaf temperature (A), vapor pressure deficit (B), photosynthetic nitrogen-use efficiency (C), net photosynthesis at light saturation (D), stomatal conductance (E), and transpiration rates (F), with standard error bars, in open grasslands (open symbols) and dense stand (full symbols) of *Juniperus virginiana* in the semiarid grasslands of the Nebraska Sandhills (between 2006–2007, starting day of year 262 in 2006 and continuing over 12 months). \* – significant differences between open and dense within a sampling date ( $p < 0.05$ ,  $n = 5$  trees).

open and dense *J. virginiana* sites (Fig. 2). Although significantly lower  $\Psi_{\text{pre}}$  were not observed in the dense mature stand relative to open grassland with young scattered *J. virginiana* trees until mid-July to mid-September (DOY 200–262),  $\Psi_{\text{mid}}$  in the spring and summer (DOY 75–200) was significantly lower on the dense mature site than that of the open grassland.  $\Psi_{\text{mid}}$  reached its lowest values at both sites toward the end of July (DOY 209), and remained low until the end of August (DOY 235) with an average  $-2.2$  MPa, before recovering with increased water availability.

Gas-exchange measurements indicated that  $P_N$ ,  $g_s$ , and  $E$  of young and mature trees of *J. virginiana* trees growing in open grassland and dense sites, respectively, followed expected seasonal trends, being low in the fall, winter and late summer, and increasing with the improvement in weather conditions in the spring reaching highest values during the summer (Fig. 3D–F). Maximal values of  $P_N$  were observed in young trees in open grassland and were attained in June (DOY 158) and July (DOY 200), averaging  $14.03 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Trees in the dense mature site had lower  $P_N$ , averaging  $10.9 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the same period.  $P_N$  did not differ during the remainder of the study period, and rates continued to vary a little throughout the growing season. The significant drop of  $P_N$  in the open grassland on DOY 235 was related to significantly higher leaf temperature ( $T_L$ ) and vapor pressure deficit (D) on that sampling date relative to dense mature site. This impacted  $g_s$  and  $E$ , and potentially increased photorespiration

leading to a reduction in  $P_N$ . By DOY 200 (July),  $g_s$  and  $E$  had peaked for both sites before declining for the remainder of the season. While  $g_s$  showed little variability between the two sites,  $E$  was generally higher in *J. virginiana* in open grassland than that in dense canopy (Fig. 3E,F).

The maximum efficiency of PSII ( $F_v/F_m$ ) followed a similar trend in young and mature *J. virginiana* trees growing in open grassland and the dense sites, respectively, with efficiency peaking in early summer and declining gradually in the fall and into the winter (value of  $\sim 0.4$  in December, DOY 352). Younger trees growing in the open grassland seemed to recover faster in March (DOY 82), exhibiting significantly higher values than their counterparts in the dense mature stand (Fig. 4A), associated possibly with faster snow melt and warming of soils in grasslands relative to the dense stand, as well as higher nitrogen content in needles. The maximum carboxylation velocity ( $V_{\text{cmax}}$ ) and maximum rate of electron transport ( $J_{\text{max}}$ ) did not differ between *J. virginiana* trees from open and dense stands during the growing season (Fig. 4B,C); however,  $V_{\text{cmax}}$  and  $J_{\text{max}}$  in the mature dense stand declined faster in mid-October (DOY 290), and exhibited slower recovery in early spring (end of March, DOY 82) compared to younger trees in the open grassland. This was consistent with  $F_v/F_m$  recovery and could be associated with more favorable climatic and light conditions during these periods and higher foliage N in open grassland vs. dense stand (Hamada *et al.* 2016).

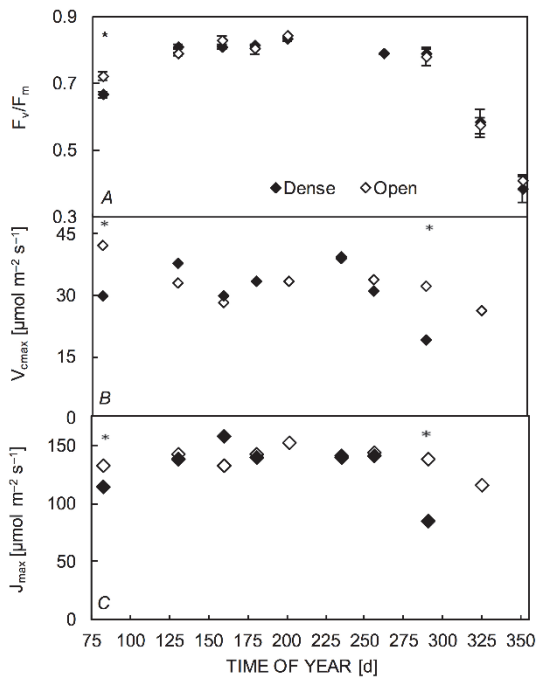


Fig. 4. Seasonal trends in average photosynthetic efficiency of photosystem II in dark-adapted leaves (A), maximum carboxylation velocity (B), and rate of electron transport (C), in open grasslands (open symbols) and dense stand (full symbols) of *Juniperus virginiana* in the semiarid grasslands of the Nebraska Sandhills. It was difficult to obtain enough samples for reliable measurements of  $V_{cmax}$  and  $J_{max}$  during periods of low or high temperatures and when water was limiting, thus the absence of data points and standard error bars on some dates. \* – significant differences between open and dense within a sampling date ( $p < 0.05$ ).

**Photosynthetic N-use efficiency and needle C and N isotopic signatures:** Carbon content of *J. virginiana* foliage did not differ between sites and showed very little seasonal variability, averaging around 50% C (Fig. 1S, *supplement available online*). Foliage N concentration was higher in young trees in open grassland relative to mature trees in dense site (average 1.5% vs. 1%), reflecting the decline in N availability in the later (Fig. 1S). Foliage N exhibited seasonal variability; however, this variability was not statistically significant because of the relatively large standard error that was observed on select sampling

dates (May–June, DOY 12–200 in open site, and November–December, DOY 234–352 in dense site; Fig. 1S). This resulted in higher N-use efficiency (PNUE) in the dense mature stand for most of the growing season (Fig. 3C; March–September, DOY 81–262), with seasonal means of 10.77 vs. 6.28 in the dense and open sites, respectively. PNUE decreased sharply in November and December (DOY 324–352), mainly associated with the seasonal decline in photosynthetic activity.

Needle  $\delta^{15}N$  and  $\delta^{13}C$  isotopic ratios differed significantly between sites (Fig. 1S).  $\delta^{15}N$  was more enriched in *J. virginiana* needles of young trees in open grassland and showed no seasonal variation (values ranged from  $-2$  to  $-3\text{‰}$ ), compared to those from dense mature stand (values ranged from  $-5$  to  $-6.5\text{‰}$ ; Fig. 1S). Foliage  $\delta^{13}C$  ratio was similarly less enriched in dense stands and showed little seasonal change (values ranged from  $-26$  to  $-27\text{‰}$ ), compared to those sampled from open grassland ( $-24$  to  $-25.5\text{‰}$ ), indicating a significantly greater intrinsic water-use efficiency (WUE) of *J. virginiana* trees in open grassland compared to dense tree site (Fig. 1S, Fig. 5).  $\delta^{13}C$  ratio was positively related to N content and but insignificantly negatively related to PNUE (Fig. 5), however, when data were averaged across all sampling dates (Table 1), results showed the predicted significantly higher PNUE and lower  $\delta^{13}C$  ratio or WUE in dense stand compared to open grassland site.

**Principle component analysis (PCA):** The first two axis of the PCA explained a total of 70.8% of the variability between *J. virginiana* trees in open grasslands and dense forest (PC1 and PC2 explained 43.3 and 27.5% of the variance, respectively).  $g_s$ ,  $F_v/F_m$ ,  $P_N$ ,  $E$ ,  $\Psi_{pre}$ , and PNUE were strongly correlated to the first principal component (PC1) (Fig. 6). These attributes changed as expected in a directional way over seasons, and based on the strong correlations of  $g_s$  and  $E$  with PC1 (0.88 and 0.82, respectively), we can conclude that these two variable are good predictors in PC1 over the seasons, in both open grassland and dense stand. PC2 was strongly correlated to N (0.93),  $\delta^{15}N$  (0.94), and  $\delta^{13}C$  (0.84), suggesting that these variables are key in separating the *J. virginiana* in open grasslands from mature individuals in dense forests, and reflect the changes in resource availability that grassland ecosystems undergo in response to vegetation shift.

## Discussion

Woody plant encroachment in grassland and savanna ecosystems is a global phenomenon (Archer 1994, Binggeli 1996, Ganguli *et al.* 2008). In the Great Plains of the US, woody species expansion is one of the greatest contemporary threats (Briggs *et al.* 2005, Twidwell *et al.* 2013), converting millions of hectares of grasslands and shrub lands to woodlands and forests (McKinley and Blair 2008), and resulting in shifts in resource availability,

ecosystem processes, productivity and the services they provide. Our results demonstrated significant shifts in ecosystem services within 60 years of land conversion, *i.e.*, from a diverse  $C_4$ -dominated grassland to an even-aged (57 yrs.) stand of *J. virginiana* with sparse understory. The shift to a dense stand of *J. virginiana* resulted in drier soils, in the top 0.3 m for most of the year, which may impact the ecohydrological cycle (Huxman *et al.* 2005, Bond

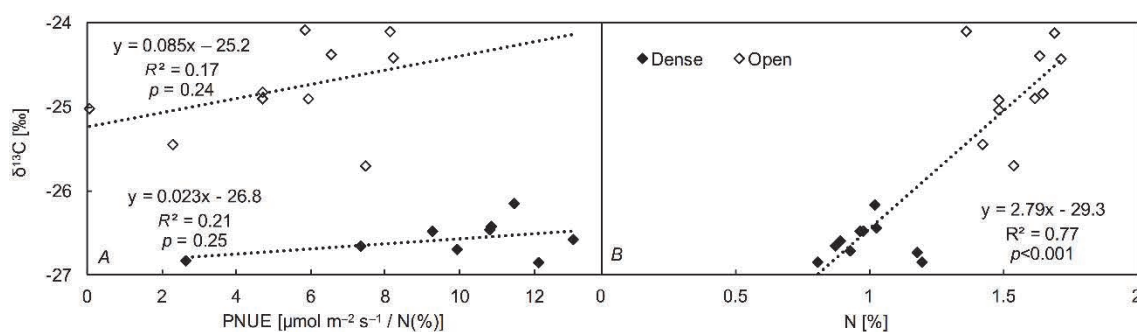


Fig. 5. Relationship between carbon isotopic ratio ( $\delta^{13}\text{C}$ ) and photosynthetic nitrogen-use efficiency (PNUE) or foliage nitrogen content (N), in open grasslands (*open symbols*) and dense stand (*full symbols*) of *Juniperus virginiana* in the semiarid grasslands of the Nebraska Sandhills.

2008, Volder *et al.* 2010, Awada *et al.* 2013, Zou *et al.* 2014). Our previous work on *J. virginiana* in the Sandhills of Nebraska has shown that *J. virginiana* trees compete for soil moisture with grasses in top layers when water is available during spring and early summer, and shift water uptake to deeper layers with decrease in surface moisture (Eggemeier *et al.* 2009, Awada *et al.* 2013). In addition, the shift in allocation of biomass in trees associated C and N pools to above ground can alter the biogeochemical cycles and impact nutrient availability (Li *et al.* 2012). Litter decomposition of *J. virginiana* is much slower than that of grassland herbaceous plants because of its chemical composition (Mellor *et al.* 2013); and despite organic N accumulation in litter of *J. virginiana* needles, it appears to contribute little to the inorganic N in the mineral soils in the short term [ $< 2$  years, McKinley (2007)]. *J. virginiana* has also a longer growing season (most of the year) than grasses, and is capable of depleting soil nutrients and sequestering them in aboveground biomass which exacerbates the problem of nutrient limitations (McKinley *et al.* 2008). Mineral soil (0–0.15 m) analyses showed a slight but insignificant increase of  $\delta^{15}\text{N}$  (more enriched) in dense site compared to open grassland (Table 1), while plant tissue (needles)  $\delta^{15}\text{N}$  showed that tree foliage in open grassland was significantly more enriched in  $^{15}\text{N}$  than in dense *J. virginiana* stand (Fig. 1S). The  $\delta^{15}\text{N}$  has been used to evaluate N cycling status and responses to environmental changes in an ecosystem by providing a measure that integrates present and past N cycling processes (Pardo *et al.* 2002). Microbes discriminate against the heavier  $^{15}\text{N}$ , therefore microbial nitrification produces  $^{15}\text{N}$ -depleted  $\text{NO}_3^-$  and  $^{15}\text{N}$ -enriched  $\text{NH}_4^+$ . Nitrification increases when N availability exceeds uptake (van der Sleen *et al.* 2015), and if not taken up by plants, depleted  $\text{NO}_3^-$  is leached, contrary to the enriched  $\text{NH}_4^+$ , which is retained on the soil exchange surface. Thus, increased nitrification results in gradual  $^{15}\text{N}$  enrichment over time of the soil profile, plants, and of the available  $\text{NH}_4^+$  pool (Pardo *et al.* 2002). We did not measure soil layers below 0.15 m, and plants do not fractionate upon N uptake (*i.e.*, if soils are enriched so would be the plants),

therefore, plants generally should have a  $\delta^{15}\text{N}$  that is reflective of the inorganic N source. *J. virginiana* was found to have roots that extend below 7 m of the soil surface and is able to uptake water from deep in the soil profile (Eggemeier *et al.* 2009). Mycorrhizal associations (Liang *et al.* 2008), like those found in *J. virginiana* (Williams *et al.* 2013), and the lower soil nutrients in the dense stand relative to the open, seems to have altered microbial N cycling, to become tighter in dense stands, leading to a greater depletion of foliar  $\delta^{15}\text{N}$  (van der Sleen *et al.* 2015).

#### Ecophysiological responses to changes in the environment:

Surface soils in the dense *J. virginiana* site were consistently drier than those of open grasslands for most of the year; differences declined with soil depth. Drier surface soils, and significantly lower  $\Psi_{\text{pre}}$  and  $\Psi_{\text{mid}}$  in trees in the dense site during the summer months relative to open site (Fig. 2) did not seem to have a negative feedback on the performance of *J. virginiana* trees growing in dense-canopy site, which maintained physiological activity and generally exhibited similar trends for  $P_{\text{N}}$ ,  $g_{\text{s}}$ ,  $E$ ,  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ ,  $F_{\text{v}}/F_{\text{m}}$  to trees growing in the grassland site during the growing season, however  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ ,  $F_{\text{v}}/F_{\text{m}}$  declined faster in the fall and recovered at a later date in the spring in dense compared to open grasslands trees (Figs. 3,4). The lower  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ ,  $F_{\text{v}}/F_{\text{m}}$  in trees in dense vs. open canopy in the fall and spring could be related to lower Rubisco concentration (Wilson *et al.* 2000), redistribution of limited resources during the winter acclimation process which results in slight increase in N (also observed in this study, Muller *et al.* 2009) and/or photoinhibition which shade trees are more susceptible to (Miyazawa *et al.* 2007). Understanding the dynamics and peak of these parameters are critical for accurately modeling and forecasting energy budgets and NPP (Hamada *et al.* 2016). *J. virginiana* is considered a drought tolerant species (Bihmidine *et al.* 2010) and has the ability to maintain stomatal opening and photosynthetic activity at relatively low water potentials (Eggemeier *et al.* 2006, Willson *et al.* 2008), reaching xylem cavitation and 50% loss of conductivity at

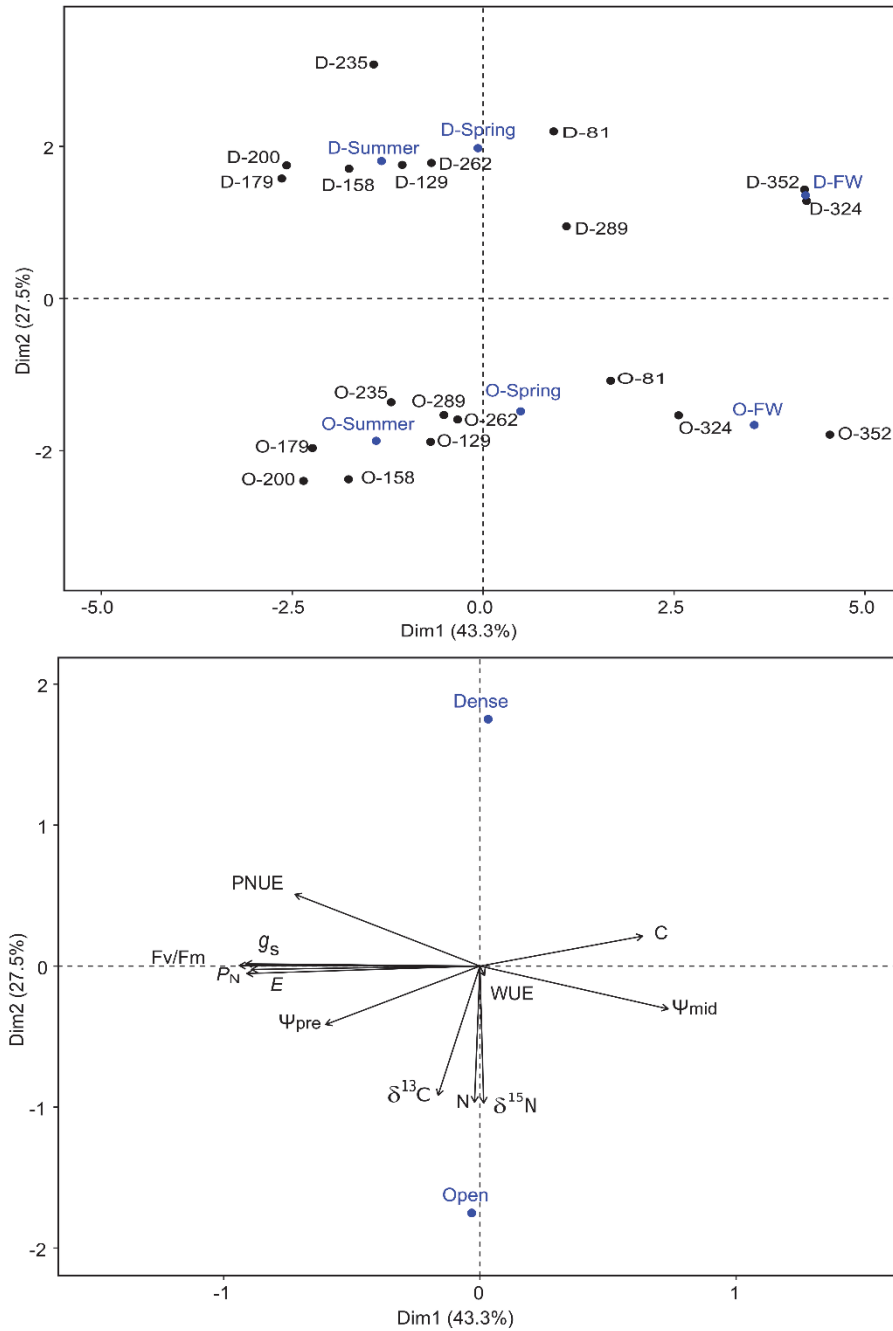


Fig. 6. Principal Component Analysis scores for PC1 (Dim1) and PC2 (Dim2) represent in a biplot for *Juniperus virginiana* in open grasslands (O) and dense forest stand (D) (A). The number following the letter represents the day of year measurements were collected. O/D-Spring, O/D-Summer, and O/D-FW represent the average scores of trees in the spring, summer, and fall-winter seasons in open grasslands (O) or dense forest (D). Principal Component Analysis correlation circle of 12 variables measured on all sampled days (B): net photosynthesis ( $P_N$ ), transpiration ( $E$ ), quantum efficiency of photosystem II ( $F_v/F_m$ ), stomatal conductance ( $g_s$ ),  $\Psi_{pre}$  and  $\Psi_{mid}$  water potentials, carbon and nitrogen isotopic ratio ( $\delta^{13}C$  and  $\delta^{15}N$ ), nitrogen content (N), water-use efficiency (WUE), carbon (C), and photosynthetic nitrogen-use efficiency (PNUE).  $V_{cmax}$  and  $J_{max}$  have missing data points and thus were not incorporated in the analysis. O–Open and D–Dense represent average scores for both PC1 (Dim1) and PC2 (Dim2) across the entire study period for all parameters.

$\Psi_w = -5.8$  [MPa] in stems and  $\Psi_w = -4.9$  [MPa] in roots (Willson *et al.* 2008), values that were not reached in this study. *J. virginiana* expands and survives in arid and

semiarid environments because it has significant plasticity in water uptake (Eggemeyer *et al.* 2009, Caterina *et al.* 2014) and access to deeper soil layers (roots can reach 7 m

deep) where water is available during the periods of low water availability (Walker and Noy-Meir 1982). That said, tree growth does not only depend on energy capture and photosynthetic rates, but also on the efficiency and effectiveness of carbohydrate integration, as well as the costs of maintaining and producing the photosynthetic system (Givnish 1988). *J. virginiana* in the dense stand maintained photosynthetic activity during the growing season and dealt with water stress by maintaining  $\Psi_w$  above a threshold (Willson *et al.* 2008), due in part to the significantly smaller trees and canopy size in dense vs. open grassland despite the age difference between trees in the two sites, resulting from competition for resources, as well as self-pruning under low light conditions – height and crown volume in dense canopy were 30 and 77%, respectively, lower than in the open grassland. Awada *et al.* (2013) and Caterina *et al.* (2014) reported a positive functional relationship between sap wood area, tree diameter, canopy size, foliage and water use in *J. virginiana*. The higher water-use efficiency expressed by  $\delta^{13}\text{C}$  values in trees in the open grasslands relative to dense forest could be attributed to internal  $\text{CO}_2$  availability, irradiance intensity, and/or the source of  $\text{CO}_2$  isotopic composition in trees from dense vs. open canopy (Ehleringer *et al.* 1986).

**Conclusion:** Woody plant encroachment in grassland and savanna ecosystems continues to be a significant threat, resulting in major and persistent shifts in ecosystem structure, function, and composition, and the services provided (Archer 1994, Binggeli 1996, Ganguli *et al.* 2008, Liu *et al.* 2013, Archer and Predick 2014, Bestelmeyer *et al.* 2015). Our results made new contribution to the mounting literature on woody species expansion into grassland ecosystems, and showed the importance of

understanding the dynamics of ecophysiological traits and their responses to changes in the environment, to improve estimations of ecosystem services components including NNP, energy balance, and dynamic of vegetation in response to environmental variability.

We demonstrated in this study that *J. virginiana* in its most western location led to a shift in vegetation composition, water availability, and soil characteristics, with significant plasticity in response to resource availability (*i.e.*, nutrients, light, and water) and limitation. This species with other drought tolerant species in the ecosystem may play a dual role in the face of climate change, by maintaining ecosystem functioning in the face of drought providing resilience, and/or encroached areas with low biological and functional diversity may negatively impact resilience. Better understanding of the mechanisms of vegetation shift, physiological resistance strategies of species in grasslands in response to abiotic and biotic environmental stresses associated with climate variability and change, and thresholds in the face of climate extremes, are important for predicting the directional changes of these ecosystems in the face of climate change (Craine *et al.* 2013). Additionally, they are also important for the development of adaptive management plans that can be implemented to either slow or control the encroachment and progression of *J. virginiana* and other woody species into grassland and savanna ecosystems, assist in the restoration of these ecosystems (depending on the state and degree of alteration), or concentrate on the new *status quo* (Bestelmeyer *et al.* 2015, Yahdjian *et al.* 2015). In the end, management decisions will depend on the ecological and economic service values that managers and society places on these new altered and persistent ecosystems (Bestelmeyer *et al.* 2015, Yahdjian *et al.* 2015).

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