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Recommended Citation

Nick A. Varvel, Christina J. Hilt, Loretta C. Johnson, Matthew Galliart, Sara G. Baer, Brian R. Maricle, Genetic and environmental influences on stomates of big bluestem (Andropogon gerardii), Environmental and Experimental Botany, Volume 155, 2018, Pages 477-487, ISSN 0098-8472, https://doi.org/10.1016/j.envexpbot.2018.07.018. (https://www.sciencedirect.com/science/article/pii/S0098847218300807)

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Environmental and Experimental Botany 155 (2018) 477–487

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Genetic and environmental influences on stomates of big bluestem (*Andropogon gerardii*)

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ARTICLE INFO

Keywords:
Big bluestem
Common gardens
Ecotypes
Gas exchange
Sand bluestem
Stomata

ABSTRACT

Big bluestem (*Andropogon gerardii*) is a dominant C_4 prairie grass that has wide distribution and several genetically distinct ecotypes. Many of the ecotypic adaptations are related to water availability in the native environment. Stomates facilitate photosynthetic gas exchange and regulate water loss from the plant. As such, stomatal size and density represent possible adaptations to conserve water. We hypothesized drought-tolerant ecotypes of big bluestem would have fewer or smaller stomates compared to more mesic ecotypes. Five ecotypes of big bluestem were planted in four common gardens from western Kansas to southern Illinois, USA to determine genetic and environmental influences on stomates. Leaves of all ecotypes of *A. gerardii* were largely hypostomatous and genetics was a greater influence than environment for stomatal size and density. The drought-tolerant Sand bluestem had larger stomates on abaxial surfaces of leaves, but a lower density compared to most other ecotypes. The most mesic Illinois ecotype and the Kaw cultivar had the greatest density of stomates on abaxial surfaces of leaves. Sand Bluestem had a greater density of stomates on adaxial surfaces of leaves compared to all other ecotypes. Gas exchange measures followed patterns of stomate distribution, where abaxial CO_2 uptake rates were greater than adaxial CO_2 uptake rates, although differences between leaf surfaces was more pronounced in stomatal density than in CO_2 uptake. There were minor differences in size and density of stomates among sites that corresponded with precipitation, although these differences were minor, illustrating the genetic underpinnings of stomates in big bluestem. There is a genetic predisposition for drought-tolerant ecotypes to have fewer stomates, illustrating an evolutionary adaptation to drought tolerance in an important prairie species.

1. Introduction

Stomates are epidermal pores on leaves that regulate gas exchange processes in plants (Willmer and Fricker, 1996). Stomates thus influence photosynthetic CO_2 uptake and water loss through transpiration (Maricle et al., 2009). Density and size of stomates vary greatly between plants and represent potential adaptations to dry climates (Redmann, 1985; Willmer and Fricker, 1996).

Understanding responses of plants to dry conditions is of considerable interest when studying plant evolution and evolutionary response to environmental conditions can be studied at intraspecific levels by studying ecotypes (Lowry et al., 2015). An ecotype is a genetically distinct geographic population within a species that is adapted to specific environmental conditions (Lowry, 2012). Ecotypic variation allows us to understand the role genetics play in morphology, anatomy, and physiology of a species (Liu et al., 2012). Many plant species have variable ecotypes, including *Arabis fecunda* Rollins (Brassicaceae) that has ecotypes separated by elevation differences (Lesica and Shelly, 1995) and *Pinus sylvestris* L. (Pinaceae), which has numerous ecotypes within its extensive range (Oleksyn et al., 1992). Ecotypes have also been documented in numerous grass (Poaceae) species, including *Spartina alterniflora* Loisel. (Daehler et al., 1999), *Panicum virgatum* L. (Hartman et al., 2012), and *Andropogon gerardii* Vitman (Gray et al., 2014).

Andropogon gerardii (big bluestem) is a dominant grass species that has wide distribution across North America, in a range of grassland, savanna, and woodland ecosystems (Knapp, 1985; Shiflet, 1994). *Andropogon gerardii* has numerous genetically distinct ecotypes within the

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https://doi.org/10.1016/j.envexpbot.2018.07.018
Received 16 January 2018; Received in revised form 20 July 2018; Accepted 20 July 2018
Available online 27 July 2018
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species (Gray et al., 2014) and a great deal of variation across its native range (McMillan, 1959, 1964, 1965). Many of the ecotypic adaptations in *A. gerardii* are related to water availability in the native environment (Olsen et al., 2013; Caudle et al., 2014; Johnson et al., 2015; Mendola et al., 2015; Maricle et al., 2017). Considering the role of stomates in regulating water loss from plants (Redmann, 1985), it is reasonable to expect stomatal variations to relate to environmental conditions in the native range.

Stomatal size and density are variable across species, within a species, and within an individual (Martin et al., 1983). Much of this variation is dependent on development or other genetic factors (Willmer and Fricker, 1996; Sugano et al., 2010; Palchetti et al., 2014). Within a plant, stomates are often smaller and more numerous on leaves of higher nodes (Penfound, 1931; Ciha and Brun, 1975). Stomatal density varies along the leaf length (Miranda et al., 1981) and size varies with proximity to the leaf margin (Smith et al., 1989). Stomatal density can also vary among genotypes within a species (Ciha and Brun, 1975), and stomate size increases with ploidy level (Willmer and Fricker, 1996; Mishra, 1997), while density decreases with increased ploidy level (Mishra, 1997). Commonly, xeromorphic plants have greater density of smaller stomates compared to more mesic relatives (Kramer, 1969; Fahn, 1974; Slavík, 1974), typically with a lower total pore area per unit leaf area (Hameed et al., 2013). In other cases, xeromorphic plants have fewer stomates compared to more mesic plants (Duggar, 1927; Ristic and Cass, 1991; Willmer and Fricker, 1996). Other studies have found different strategies between succulent and nonsucculent xeromorphic plants regarding stomatal density (Sundberg, 1985).

The environment can also influence stomatal size or density, particularly in response to light and CO_2. Stomatal density is commonly higher in sun than in shade adapted leaves (Penfound, 1931; Friend and Pomeroy, 1970; Miskin and Rasmusson, 1970) and decreases with increase in CO_2 concentration in the atmosphere (Woodward, 1987; Woodward and Bazzaz, 1988). Many aspects of stomatal size or density are also influenced by water availability, with stomates commonly becoming smaller in size and greater in density with water stress relative to well-watered conditions (Baloch et al., 2013). Smaller stomates are quicker to close compared to larger stomates (Wang et al., 2007), potentially providing an advantage in dry conditions. Indeed, stomatal size can increase with irrigation (Penfound, 1931; Baloch et al., 2013) and decrease with a water deficit (Baloch et al., 2013). Stomatal density decreases with irrigation (Gindel, 1969) and increases with drier soils (Penfound, 1931; Ciha and Brun, 1975; Quarrie and Jones, 1977; Park et al., 2016). In other cases, stomatal density has been shown to increase with moderate drought, but decrease in more severe drought conditions (Xu and Zhou, 2008), illustrating how environmental influences on stomatal density are sometimes complex in nature.

Environmentally-influenced differences in leaf morphology can also alter the density and size of stomates. Often total leaf area is smaller in water-stressed plants, resulting in fewer total stomates (Ciha and Brun, 1975; Willmer and Fricker, 1996; Kramer et al., 2018). Stomatal density also decreases with larger epidermal cells, retaining the same stomate to epidermal cell ratio, as well as decreasing with increased stomatal guard cell length (Mishra, 1997). In some cases, however, genetic differences are more influential than environmental differences when considering stomatal size and density (Palchetti et al., 2014). This apparent variability indicates there is much to learn about how stomatal density is influenced by dry conditions, both within the lifetime of an individual and over evolutionary time.

While there has been much research in the stomatal characteristics of plants (e.g., Miskin and Rasmusson, 1970; Ciha and Brun, 1975; Wang et al., 2007; Xu and Zhou, 2008; Baloch et al., 2013), how this relates to drought resistance at intraspecific levels in grasses is not well characterized. Further, the genetic by environment interactions that govern stomatal density or size remain an open question. This can be addressed with common garden experiments, which involve moving multiple plant populations or ecotypes from their native environments into a common environment (Clausen et al., 1948; Johnson et al., 2015). Often common gardens are arranged across a gradient to test for the effects of specific environmental factors, including elevation (Cordell et al., 1998), temperature (Oleksyn et al., 1998), or precipitation (Johnson et al., 2015).

In this experiment, five ecotypes of *A. gerardii* were planted in four common gardens across a precipitation gradient in the Great Plains of North America (Johnson et al., 2015). It was sought to determine the genetic by environment interactions on stomate size and density in *A. gerardii*. Therefore, it was hypothesized that *A. gerardii* ecotypes native to drier regions would have xeromorphic stomatal characteristics, mostly a reduction in total stomatal pore area per unit leaf area. Specifically, this can be manifested as fewer stomates or smaller stomates in drought-adapted ecotypes than those native to wetter regions. It was also hypothesized that differences between sites or years would be minimal compared to ecotypic differences, and thus the differences in stomatal size and density would be primarily genetic (Palchetti et al., 2014).

2. Materials and methods

2.1. Common gardens and sampling

Five ecotypes of *A. gerardii* were planted in four common gardens across a precipitation gradient of the Great Plains of North America (Gray et al., 2014; Johnson et al., 2015) (Fig. 1). There were four populations of an Illinois ecotype of *A. gerardii*, four populations of an Eastern Kansas ecotype, four populations of a Central Kansas ecotype, the Kaw cultivar of *A. gerardii* from eastern Kansas, and the Sand bluestem (*A. gerardii* ssp. *hallii*, or sometimes *A. hallii* Hack.) cultivar

Fig. 1. *Andropogon gerardii* ecotypes were collected from 4 populations from each of 3 sites (triangles) across 1050 km of the Great Plains region of North America. All ecotypes were grown in four common gardens (circles) across a precipitation gradient ranging from 50.5 cm to 116.7 cm average annual precipitation. In addition, two cultivars of *A. gerardii* were grown at all common gardens.

Table 1
Three ecotypes of *Andropogon gerardii* were collected from a precipitation gradient that spanned 1050 km across the Great Plains region of North America. Each ecotype was collected from four local populations; geographical location is provided for each population. Two cultivars of *Andropogon gerardii* were obtained from the USDA Plant Materials Center program; cultivars were treated as separate ecotypes for statistical comparisons.

Ecotype of *A. gerardii*	Population (Collection Site)	County	Latitude (N)	Longitude (W)
Central Kansas	Relict Prairie	Ellis, KS	38°51’	99°22’
	Webster Reservoir	Rooks, KS	39°24’	99°32’
	Saline Expt. Range	Ellis, KS	39°02’	99°14’
	Cedar Bluff Reservoir	Trego, KS	38°45’	99°46’
Eastern Kansas	Carnahan Cove St. Pk.	Pottawatomie, KS	39°20’	96°38’
	Konza Prairie	Riley/Geary, KS	39°05’	96°36’
	Tallgrass Prairie Nat. Pres.	Chase, KS	38°25’	96°33’
	Top of the World Pk.	Riley, KS	39°13’	96°37’
Illinois	Desoto Railroad Prairie	Jackson, IL	37°51’	89°14’
	Twelve Mile Railroad Prairie	Effingham/Fayette/Marion, IL	38°46’	88°50’
	Fults Hill Prairie	Monroe, IL	37°58’	89°48’
	Walters Prairie	Jasper, IL	38°59’	88°09’
Kaw	‘Kaw’ cultivar of *A. gerardii*	Riley, KS	USDA Plant Materials Center Accession 421276	
Sand Bluestem	‘Garden’ cultivar of *A. gerardii* var. hallii	Garden, NE	USDA Plant Materials Center Accession 421277	

Table 2
Andropogon gerardii ecotypes were grown in four common gardens across the Great Plains of North America, with mean annual precipitation (MAP) ranging from 50.5 to 116.7 cm annually. Mean annual potential evapotranspiration (PET) is also presented. Data are from Johnson et al. (2015). Leaf collection dates are indicated and precipitation from 1 January until the collection date are indicated for each site and year.

Common Garden Site	County	MAP (since 1961) (cm)	PET (since 1961) (cm)	2013 Collection Date; ppt. from 1 January to collection date	2014 Collection Date; ppt. from 1 January to collection date	2016 Collection Date; ppt. from 1 January to collection date
Colby, KS	Thomas, KS	50.47 (± 11.77)	144	8 July; 15.6 cm	30 June; 23.3 cm	21 July; 35.0 cm
Hays, KS	Ellis, KS	58.22 (± 13.13)	139	5 July; 22.1 cm	20 June; 25.1 cm	12 July; 46.5 cm
Manhattan, KS	Riley, KS	87.15 (± 20.04)	127	2 July; 38.3 cm	24 June; 49.3 cm	6 July; 48.0 cm
Carbondale, IL	Jackson, IL	116.73 (± 24.76)	99	16 July; 68.9 cm	17 June; 58.2 cm	27 June; 55.8 cm

Table 3
Weather data for the source populations of *Andropogon gerardii* ecotypes. Temperature severity index is number of days over 35 °C (95 °F)/total number of days. Number of precipitation events > 1.25 cm per year.

Region	Prairie Name	Prairie	County, State (Weather Site)	Number of Pcp Events > 1.25 cm	Pcp Driest Year (cm)	Mean Annual rainfall (cm)	Seasonal Mean Rainfall (cm)	Annual Diurnal Temp °C	Seasonal Diurnal Temp °C	Annual Mean Temp °C	Seasonal Mean Temp °C	Temp Severity Index	MAP/ MAT
CKS	Webster Reservoir	WEB	Rooks, KS (Webster Dam)	17	25.96	58.70	39.35	15	15.4	12.4	19.8	0.100	4.72
CKS	Saline Experimental Range	SAL	Ellis, KS (Plainville)	16	36.32	61.7	31.0	14.6	15.5	11.8	20.7	0.088	5.22
CKS	Cedar Bluffs Reservoir	CDB	Trego, KS (Cedar Bluffs Dam)	16	32.18	53.31	35.97	14.4	14.5	11.2	19.5	0.091	4.72
CKS	Relict Prairie	REL	Ellis, KS (Hays 1S)	16	32.61	58.0	37.7	14.6	15.5	12.0	20.6	0.088	4.82
EKS	Konza Prairie	KON	Riley/Geary, KS (Manhattan 6SW)	22	68.89	88.47	56.54	12.8	12.4	12.8	21.0	0.048	6.92
EKS	Tallgrass National Park	TAL	Chase, KS (Tallgrass Nat Park)	21	59.77	82.82	49.19	12.7	12.2	12.7	20.8	0.066	6.54
EKS	Carnahan Cove	CAR	Pottawatomie, KS (Wamego)	23	52.35	87.20	53.34	13.0	13.1	13.0	21.4	0.057	6.72
EKS	Top of the World Park	TOW	Riley, KS (Tuttle Dam)	21	45.11	81.12	50.70	13.1	13.2	11.7	20.3	0.057	6.95
IL	Desoto Prairie	DES	Jackson, IL (Carbondale, Il)	33	67.41	115.92	53.53	12.3	12.6	13.2	21.1	0.027	8.78
IL	Twelve Mile Prairie	TM	Effingham, IL, (Monroe, Fayette, Salem)	25	70.01	107.57	51.83	11.7	12.4	12.3	18.7	0.026	8.72
IL	Walters Prairie	WAL	Jasper, IL (Newton/ Charlesto)	27	69.18	104.04	50.80	10.8	11.7	13.4	21.8	0.014	8.11
IL	Fults Hill Prairie	FUL	Monroe, IL (Sparta)	31	69.38	111.27	55.14	11.9	12.6	13.2	21.0	0.031	8.38

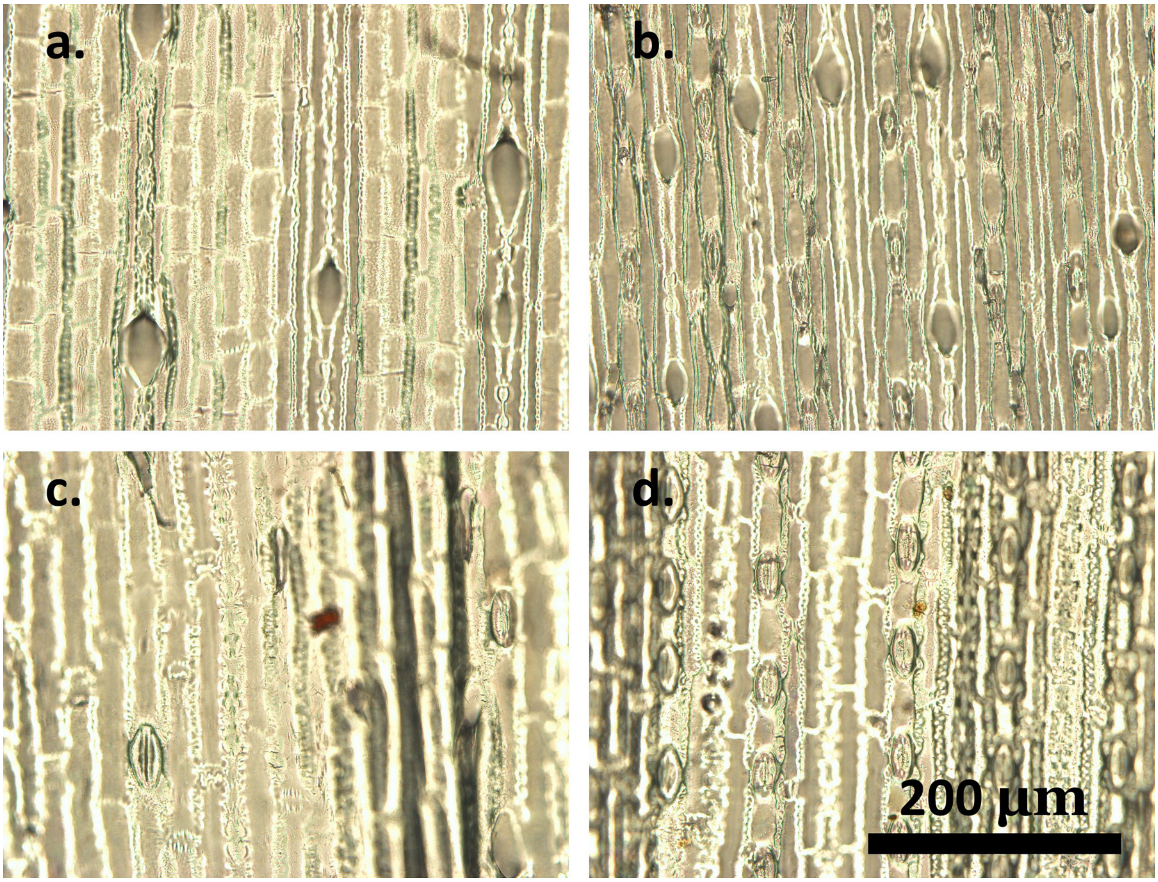

Fig. 2. Light micrographs of representative epidermal imprints of the Illinois ecotype (a, b) and the Sand bluestem cultivar (c, d) on adaxial (a, c) and abaxial (b, d) leaf surfaces of *A. gerardii* grown in the Carbondale, Illinois common garden site. Leaves of all ecotypes were largely hypostomatous, with most stomates on abaxial surfaces. The Sand bluestem cultivar had a greater number of stomates on the adaxial surface compared to all other ecotypes.

from western Nebraska and South Dakota (Table 1) (Caudle et al., 2014).

Seeds were collected in autumn of 2008 from field locations (Table 1) and were planted in potting soil in 10 cm × 10 cm pots to grow under greenhouse conditions. Potted plants were planted in four common gardens in August of 2009 (Caudle et al., 2014). The four common gardens were located across a precipitation gradient of 505 mm–1167 mm mean annual precipitation, in Colby, Hays, and Manhattan, Kansas, and Carbondale, Illinois, USA (Johnson et al., 2015) (Table 2). Plants were spaced 50 cm apart in rows, with landscape fabric placed around plants to prevent growth of other plants (Olsen et al., 2013; Caudle et al., 2014). Each common garden had plants in rows (blocks) that were 50 cm apart, with one individual of each population in each row, randomly arranged. Precipitation data were also collected at each site from local climatological sources (Table 2).

Leaves were collected from six replicate plants from each of the populations and cultivars at each common garden during peak growing season in 2013, 2014, and 2016 (Table 2). Young, fully expanded leaves were clipped from plants, frozen in the field on dry ice, and transported to the lab for later analysis. In separate analyses, we analyzed all plants in all sites for ploidy level using flow cytometry and found only slight variation in genome size among our populations. Average DNA by ecotype in gardens from home site only ranged from 5.6 to 5.9 pg, based on 40 plants per site (Galliart, unpublished data).

2.2. Stomate measures

Size and density of stomates were measured with epidermal imprints (Maricle et al., 2009). Clear nail polish was brushed on adaxial (top) and abaxial (bottom) surfaces of leaves, allowed to dry, and then peeled to make a detailed imprint of the epidermal cells (Horanic and Gardner, 1967). A section near the middle of the leaf length and width was measured to account for variation in stomate density across the leaf (Miranda et al., 1981). Then, at 400× magnification, stomates were counted within the field of view under the microscope. Area of the field of view was calculated from measures with a stage micrometer. For each side of each leaf, three random areas were counted and calculated to an average stomatal density per mm^2 of both adaxial and abaxial leaf surfaces. Also under 400× magnification, size of stomates was measured with an ocular micrometer. The stomatal pores are flanked by two guard cells, creating an elliptical-shaped pore. Stomates were measured at their maximum length, from one end of the entire structure where the guard cells come together to the same point on the other end of the structure.

From each of the four sites, a minimum of two replicate leaves (from different plants) of each population were measured, adding up to eight leaves from each ecotype per site. Six leaves from both cultivars at each site were also measured. Thus, measurements were made on stomates from 36 plants for each site, making a minimum 144 measurements for each year. The total experiment was 482 individual plants, representing each of the 5 ecotypes and cultivars at the 4 sites over 3 years, sampled from across an 1150 km precipitation gradient.

2.3. Gas exchange measures

Gas exchange measurements were made on representative *A. gerardii* individuals with an LI-6400 XT IRGA system (Li-Cor Biosciences, Inc., Lincoln, Nebraska, USA) to assess stomatal function. Measures were made on greenhouse-grown plants from the same 2008 seed

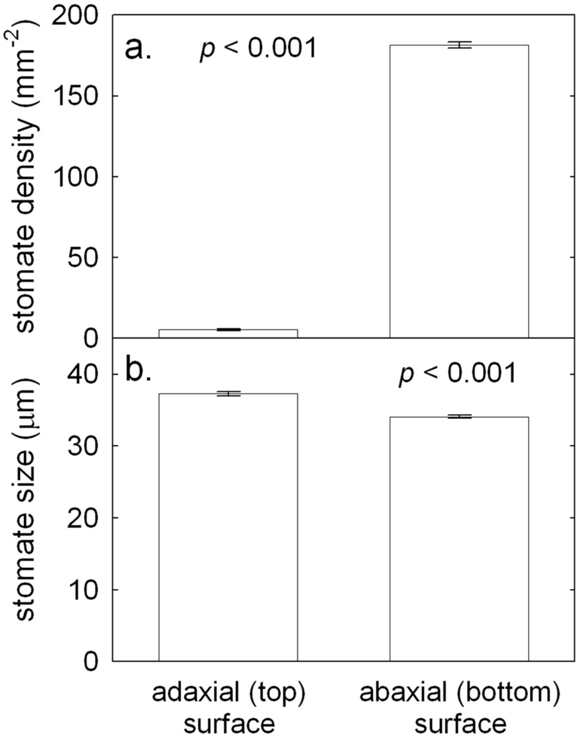

Fig. 3. (a) Stomate density (mm^{-2}) and (b) stomate size (µm) in five ecotypes of *A. gerardii* leaves. Bars are means ± standard errors of 482 replicates, as data were normally distributed. *P* values indicate comparisons between adaxial and abaxial leaf surfaces. *A. gerardii* leaves were hypostomatous and stomates on the adaxial surface were slightly larger than those on the abaxial surface.

collection. Plants were approximately four months old at the time of measurement.

Gas exchange was measured separately on adaxial or abaxial sides of the leaf by applying Scotch tape to one side of the leaf (Maricle et al., 2009). Leaves were illuminated from the nontaped side at a PPFD of 1500 µmol m^{-2} s^{-1}, CO$_2$ was 400 ppm, flow rate through the chamber was 400 µmol s^{-1}, and temperature and humidity were maintained near ambient levels in the greenhouse. Measures were made during afternoon hours on three dates; during times of measurement, greenhouse temperatures ranged from 33.4 to 36.5 °C and relative humidity ranged from 34 to 45%. Photosynthetic CO$_2$ uptake (µmol CO$_2$ m^{-2} s^{-1}) and stomatal conductance (g_s, mol H$_2$O m^{-2} s^{-1}) were measured on both sides of each leaf. Intrinsic water use efficiency (WUE) was calculated as photosynthesis/g_s. Eight replicate individuals were measured from the Sand bluestem cultivar, 14 from the Central Kansas ecotype, and 18 from the Illinois ecotype. One representative leaf was measured from each plant; tape was applied to abaxial and adaxial sides in different locations on the same leaf to avoid any effects caused by the tape. The side of the leaf taped and measured first for each plant was alternated to further control for any impacts of the tape.

2.4. Data analysis

Stomate size and density data were analyzed by 12 Kruskal-Wallis and multiple comparison tests using R software (x64 3.3.1) to determine differences in medians between sites, ecotypes, and years. These 12 tests were performed over the three independent variables of sites, ecotypes, and years, and the four dependent variables of stomate size and stomate density on both adaxial and abaxial surfaces. Four two-way analysis of variance (ANOVA) tests were conducted across the adaxial and abaxial surfaces for stomatal density and sizeto verify there were no interactions among the independent variables. Due to the number of individual tests, a significance level of 0.004 was used to avoid inflation of Type I errors.

Photosynthesis data for the abaxial surface of leaves were analyzed with a one-way ANOVA across the three ecotypes, and adaxial surface of leaves were analyzed with a Kruskal-Wallis nonparametric test as the data were not normally distributed. Two paired *t*-tests were used to compare adaxial to abaxial photosynthesis data for the Illinois and Central Kansas ecotypes, while a Wilcoxon signed-rank nonparametric test was used for the Sand bluestem cultivar as the data were not normally distributed. For stomatal conductance data, two one-way ANOVAs were conducted to compare measurements between ecotypes on the adaxial and abaxial surfaces, while three paired *t*-tests were used to compare adaxial and abaxial conductance for each of the three ecotypes. No multiple comparison tests were conducted as ANOVAs did not detect any differences.

Principal component analysis (PCA) was used to reduce dimensionality of the stomatal data set and aid in data visualization using SAS JMP software (v13). For this analysis, the stomatal data for the 12 populations representing Central Kansas, Eastern Kansas, and Illinois ecotypes from each home site were used and cultivars were excluded because we did not have climate of population origin for the cultivars. PCA scores axis 1 and 2 were used in a stepwise regression in SAS v9.4 (GLMSELECT Procedure) using climate variables from the site of population origin (Table 3). In separate analyses, stepwise regression of stomatal morphology from each home site was used and climate of population of origin were used as explanatory variables.

3. Results

3.1. Stomatal size and density

In all *A. gerardii* ecotypes, stomates were arranged in parallel rows spanning the length of the leaves. Guard cells were of the graminaceous (grass-like) type (Martin et al., 1983), with two dumbbell-shaped guard cells that have bulbous ends accompanied by two paracytic subsidiary cells per stomate (Fig. 2). Stomates on *A. gerardii* leaves ranged from 30.2 to 45.1 µm in size and from 0.0 to 220.6 mm^{-2} in density. Leaves were largely hypostomatous, with a mean of 5.3 stomates mm^{-2} on adaxial surfaces, significantly less than the 182 stomates mm^{-2} mean on abaxial surfaces ($t = 95.529$, $df = 960$, $P < 0.001$) (Fig. 3; Supplementary Table 1). No adaxial stomates were found on 54% of the imprints examined. Adaxial stomates were 37.3 µm on average, significantly larger than abaxial stomates, which averaged 34.1 µm ($t = 9.383$, $df = 784$, $P < 0.001$) (Fig. 3; Supplementary Table 1).

Four two-way ANOVA tests demonstrated no significant interactions among any of the independent variables, so main effects are presented. Differences in stomatal size and density in *A. gerardii* were primarily driven by genetics, but with a small effect from environment. Overall, the more drought-adapted ecotypes had more adaxial stomates, fewer abaxial stomates, and larger stomates compared to more mesic ecotypes. The drought-adapted Sand bluestem cultivar had significantly more adaxial stomates than all other ecotypes, and the drought-adapted Central Kansas ecotype had more adaxial stomates than the mesic Kaw cultivar and Illinois ecotype ($H = 152.14$, $df = 4$, $P < 0.001$) (Fig. 4; Supplementary Table 2). Sand bluestem also had larger adaxial stomates than all other ecotypes ($H = 82.505$, $df = 4$, $P < 0.001$) (Fig. 4; Supplementary Table 2). Sand bluestem had fewer ($H = 50.119$, $df = 4$, $P < 0.001$) and larger ($H = 94.182$, $df = 4$, $P < 0.001$) (Fig. 4; Supplementary Table 2) abaxial stomates than all other ecotypes.

Stomatal size and density were mostly genetically controlled in *A. gerardii*, exhibiting some plasticity to environmental variability. Across years there was no significant difference in adaxial ($H = 1.539$, $df = 2$, $P = 0.463$) or abaxial ($H = 12.365$, $df = 2$, $P = 0.463$) (Fig. 5; Supplementary Table 3) stomate size. Minor differences in abaxial stomatal density were detected among years, but these differences did not correspond with precipitation. The 2014 leaves had significantly fewer abaxial stomates than the 2013 leaves ($H = 19.171$, $df = 2$, $P < 0.001$)

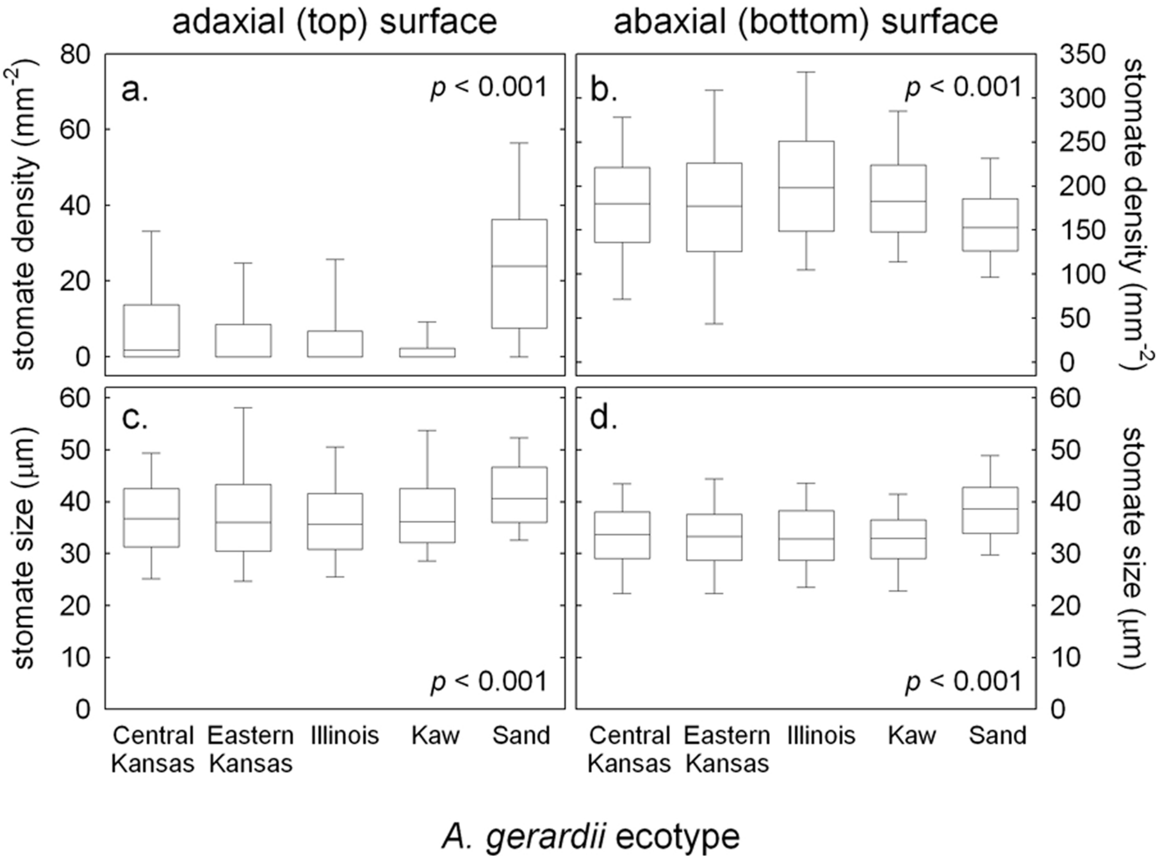

Fig. 4. Box plots showing stomatal density (a, b) and stomatal size (c, d) on adaxial (a, c) and abaxial (b, d) leaf surfaces among ecotypes of *A. gerardii*. Boxes show the median and the first and third quartiles. Error bars show maximum and minimum values. Box-and-whisker plots were used as the data were not normally distributed. Note the different scales on the Y axis of panels a and b. Sample sizes ranged from 63 to 66 replicates per cultivar and 113–119 replicates per ecotype. *P* values indicate comparisons among ecotypes within each panel on the figure. The more drought-tolerant ecotypes had more stomates on adaxial surfaces of leaves and fewer stomates on abaxial surfaces as well as larger stomates on both surfaces.

(Fig. 5; Supplementary Table 3), which does not correlate with precipitation differences between years. There was no difference in adaxial stomatal density among years ($H = 6.587$, $df = 2$, $P = 0.037$) (Fig. 5; Supplementary Table 3).

There was no significant difference in adaxial stomatal density ($H = 10.471$, $df = 3$, $P = 0.015$) or adaxial stomate size ($H = 1.808$, $df = 3$, $P = 0.613$) (Fig. 6; Supplementary Table 4) in *A. gerardii* among common garden sites. Plants growing in Hays had significantly more abaxial stomates than leaves from Carbondale or Manhattan ($H = 20.869$, $df = 3$, $P < 0.001$) (Fig. 6; Supplementary Table 4). There was no significant difference in abaxial stomate size among sites ($H = 8.857$, $df = 3$, $P = 0.031$) (Fig. 6; Supplementary Table 4).

PCA analyses of stomatal morphology for the 3 ecotypes (comprised of 12 populations) from their home sites showed that the first two axes is explained 74.4% of the variation (axis 1: 41.6%, axis 2: 32.8%) (Fig. 7). Only axes 1 and 2 significantly explained variation. The PCA showed abaxial size and abaxial density arrayed in opposite ends along axis 1, and adaxial size and adaxial density clustered together mainly along axis 2. The PCA showed separation of mean ecotype scores, with less distinction among Eastern Kansas and Illinois ecotypes, and more separation of Central Kansas from Illinois and Eastern Kansas ecotypes. In the loading matrix (Supplemental Table 5), principal component 1 was associated negatively with abaxial size (−0.83), and positively associated with both abaxial density (0.75) and adaxial density (0.57). Eastern Kansas and Illinois were moderately associated with axis 1 (0.38, −0.30). In the loading matrix, principal component 2 was associated with adaxial size (0.80) and adaxial density (0.62) and negatively associated with abaxial density (−0.43). Central Kansas was associated with axis 2 (0.52) (Supplementary Table 5).

Stepwise regression of PCA scores showed climate variables did not explain variation in axis 1, and seasonal mean precipitation explained axis 2 (Supplementary Table 6). When analyzing the stomatal data from home site as explained by climate variables, climate variables were only related to aspects of precipitation. Adaxial density was explained by precipitation of the driest year, adaxial size was explained by mean annual precipitation, and abaxial density was explained by seasonal mean precipitation. Abaxial size was not significantly explained by climate variables (Supplementary Table 6).

3.2. Gas exchange measures

Photosynthetic rates were measured on adaxial and abaxial leaf surfaces of representative *A. gerardii* ecotypes (Fig. 8). CO_2 uptake and stomatal conductance (g_s) correlated with stomatal density measures, although differences between leaf surfaces were more pronounced in stomatal density than in CO_2 uptake (Figs. 3 and 8).

CO_2 uptake was significantly higher on abaxial surfaces ($t = -5.205$, $df = 17$, $P < 0.001$), ($t = -5.124$, $df = 17$, $P < 0.001$), ($V = 19$, $n = 8$, $P = 0.035$) compared to adaxial leaf surfaces of all three ecotypes of *A. gerardii* (Fig. 8). Mean photosynthesis rates ranged from 11.2 to 13.5 µmol $CO_2\,m^{-2}\,s^{-1}$ on abaxial surfaces and from 4.5 to 6.4 µmol $CO_2\,m^{-2}\,s^{-1}$ on adaxial surfaces across ecotypes (Fig. 8). There was no difference in photosynthesis rates among ecotypes on either the adaxial ($H = 0.760$, $df = 2, 37$, $P = 0.684$) or abaxial ($F = 0.473$, $df = 2, 37$, $P = 0.627$) surfaces of the leaves (Fig. 8).

Stomatal conductance measures showed similar patterns as

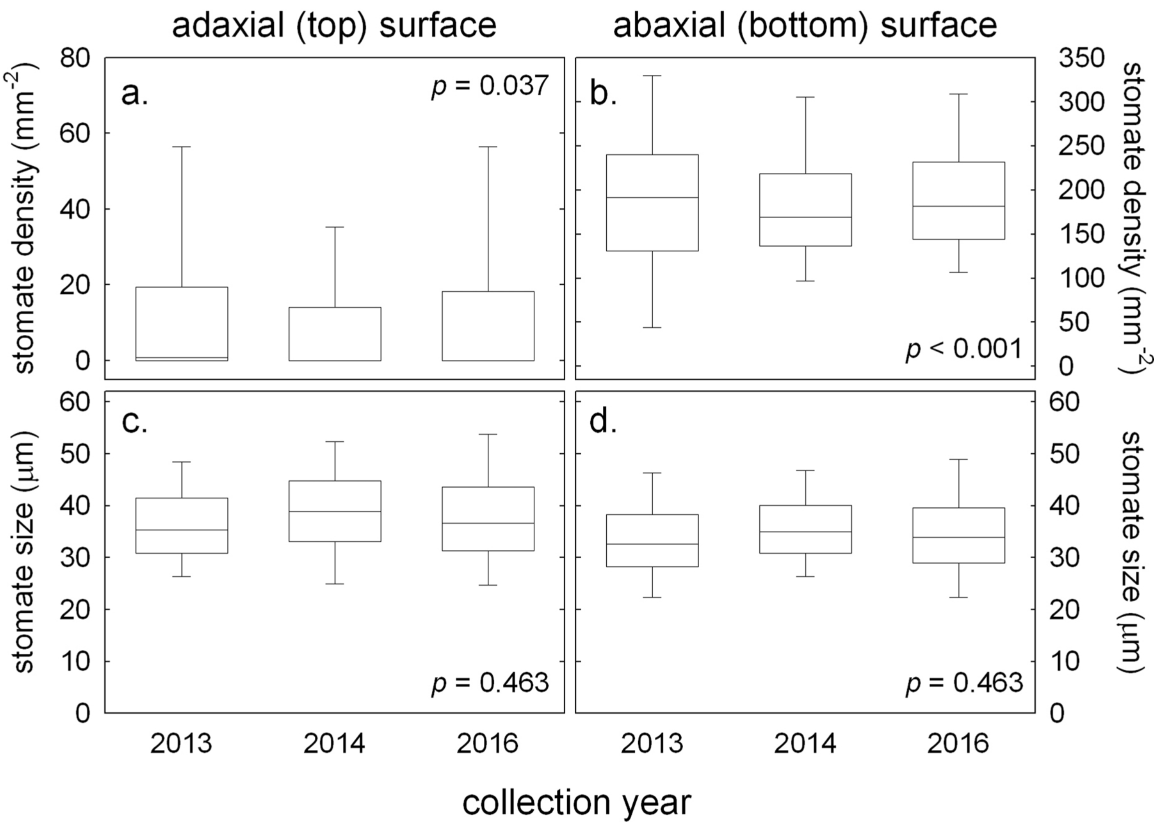

Fig. 5. Box plots showing stomatal density (a, b) and stomatal size (c, d) on adaxial (a, c) and abaxial (b, d) leaf surfaces of *A. gerardii* among collection years. Boxes show the median and the first and third quartiles. Error bars show maximum and minimum values. Box-and-whisker plots were used as the data were not normally distributed. Note the different scales on the Y axis of panels a and b. Sample sizes ranged from 141 to 195 replicates per year. *P* values indicate comparisons among years within each panel on the figure. On the abaxial surface of leaves of *A. gerardii*, the leaves collected in 2014 had significantly fewer stomates than the leaves collected in 2013.

photosynthesis across adaxial and abaxial surfaces as well as across ecotypes. Stomatal conductance was significantly higher on abaxial surfaces of leaves than on adaxial ($t = -7.185$, $df = 13$, $P < 0.001$), ($t = -3.685$, $df = 7$, $P = 0.008$), ($t = -2.885$, $df = 7$, $P = 0.024$) (Fig. 8) across all three ecotypes. Mean stomatal conductance ranged from 0.095 to 0.120 mol H_2O m^{-2} s^{-1} on abaxial surfaces and from 0.050 to 0.068 mol H_2O m^{-2} s^{-1} on adaxial surfaces across ecotypes (Fig. 8). There was no difference in stomatal conductance among ecotypes on either adaxial ($F = 1.519$, $df = 2, 37$, $P = 0.232$) or abaxial ($F = 1.112$, $df = 2, 37$, $P = 0.340$) surfaces of the leaves (Fig. 8).

Intrinsic water use efficiency (WUE), calculated as photosynthesis divided by stomatal conductance, showed similar patterns as photosynthesis when making comparisons across plants (data not shown). Mean WUE ranged from 107.7 to 120.2 µmol CO_2 mol^{-1} H_2O on abaxial surfaces and from 76.7 to 99.0 µmol CO_2 mol^{-1} H_2O on adaxial surfaces across ecotypes. WUE was significantly higher on abaxial than adaxial surfaces of leaves ($t = 2.023$, $df = 39$, $P = 0.003$) across all three ecotypes. There was no difference in WUE among ecotypes on either adaxial ($F = 1.062$, $df = 2, 37$, $P = 0.356$) or abaxial ($F = 0.310$, $df = 2, 37$, $P = 0.735$) surfaces of the leaves (data not shown).

4. Discussion

In this study, stomatal size and density were compared among five ecotypes of *A. gerardii* grown in four common gardens across an 1150 km precipitation gradient. Effects of genetics (ecotype) and environment (sites, years) were compared. Stomates in *A. gerardii* were primarily influenced by genetics, but also with some influences from the environment.

4.1. Stomates in A. gerardii and other grasses

There is great diversity across grasses regarding size of stomates; sizes of 17 µm have been reported in *Phragmites communis* Trin. (Liu et al., 2012), 50 µm in *Leymus chinensis* (Trin.) Tzvel. (Xu and Zhou, 2008), 56 µm in barley (Miskin and Rasmusson, 1970), 70 µm in oat (Eckerson, 1908), and 84 µm in wheat (Eckerson, 1908). In contrast to these cool-season grasses, stomatal size of *A. gerardii* ecotypes in this study was lower, ranging from 30.2 to 45.1 µm. These were similar to measures of 34–45 µm in *A. gerardii* (Knapp et al., 1998) and slightly smaller than measurements of 45–50 µm in maize (Eckerson, 1908; Miranda et al., 1981), a close relative to *A. gerardii*. *A. gerardii* appears to have smaller stomates compared to many other grasses, perhaps an adaptation to life in drought-prone habitats (Duggar, 1927; Ristic and Cass, 1991; Willmer and Fricker, 1996).

There is also great diversity across grasses regarding stomatal density; stomatal densities of 25 mm^{-2} have been reported in oat (Eckerson, 1908), 80 to 129 mm^{-2} in *Leymus chinensis* (Xu and Zhou, 2008; Chen and Wang, 2009), 90 mm^{-2} in *Cynodon dactylon* (L.) Pers. (Hameed et al., 2013), 98 mm^{-2} in barley (Miskin and Rasmusson, 1970), 386 mm^{-2} in *Sesleria caerulea* (L.) Ard. (Lloyd and Woolhouse, 1978), and 850 mm^{-2} in *Phragmites communis* (Liu et al., 2012). Among closer relatives, stomatal densities of 72 mm^{-2} and 125 mm^{-2} have been reported in maize (Eckerson, 1908; Miranda et al., 1981), 76 mm^{-2} in Sand bluestem (Awada et al., 2002), and 190 mm^{-2} in *A. gerardii* (Knapp et al., 1994). In the present study, stomatal density of *A. gerardii* ecotypes ranged from 0.0 to 220.6 mm^{-2}, similar to values presented by Knapp et al. (1994) for *A. gerardii*, slightly higher than numbers presented by Knapp et al. (1998) for *A. gerardii* and Awada et al. (2002) for *A. hallii* and other prairie grasses, and substantially

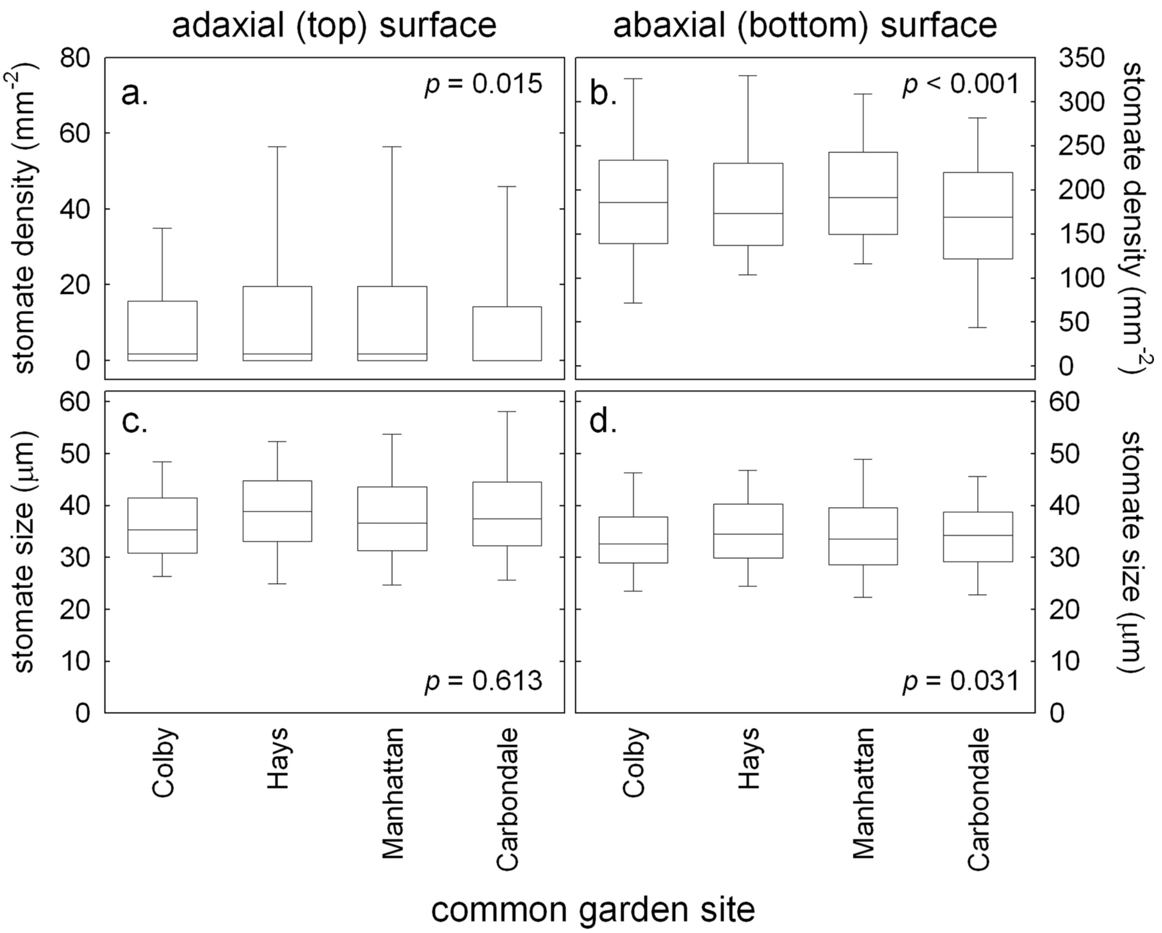

Fig. 6. Box plots showing stomatal density (a, b) and stomatal size (c, d) on adaxial (a, c) and abaxial (b, d) leaf surfaces of *A. gerardii* among common garden sites. Boxes show the median and the first and third quartiles. Error bars show maximum and minimum values. Box-and-whisker plots were used as the data were not normally distributed. Note the different scales on the Y axis of panels a and b. Sample sizes ranged from 111 to 126 replicates per site. *P* values indicate comparisons among sites within each panel on the figure. On the abaxial surface of leaves of *Andropogon gerardii*, the leaves collected in Manhattan had significantly more stomates than leaves collected in Hays and Carbondale.

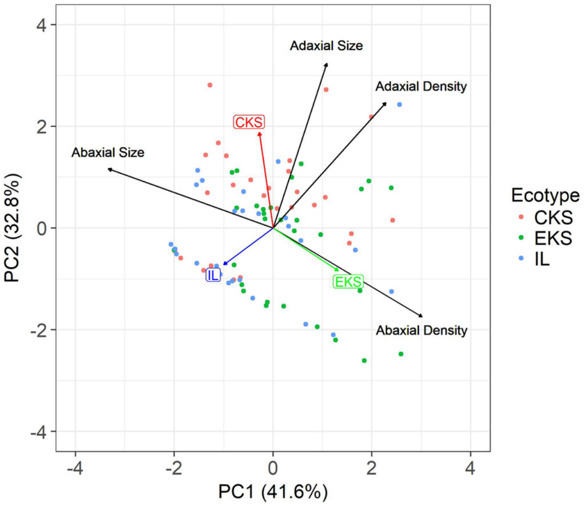

Fig. 7. Principle component analysis biplot of stomatal size and density characteristics of *A. gerardii* ecotypes grown in their home site.

higher than stomatal densities presented by Naz et al. (2010) for desert grasses.

Stomates represent a point for water to exit the leaf, so reductions of stomatal pore area represent a possible adaptation to conserve water (Redmann, 1985; Willmer and Fricker, 1996). Grasses typically respond to water stress by reducing the total pore area per leaf area (Hameed et al., 2013). Mathematically, reduced total pore area can be accomplished by decreasing size or density of stomates (Campbell and Norman, 1998). Most drought-adapted plants have low stomatal densities (Duggar, 1927; Ristic and Cass, 1991; Willmer and Fricker, 1996; Naz et al., 2010), but this is typically accompanied by increased stomatal size (Slavík, 1974). In any case, total pore area per leaf area is important. In the case of *A. gerardii*, general shape of stomates was relatively constant so length and density measures are good indicators of overall pore area.

4.2. Genetic influences on stomate size and density

Andropogon gerardii is a drought-adapted species (Knapp, 1985; Johnson et al., 2015) and it displays stomate patterns consistent with other xeromorphic species (Kramer, 1969; Fahn, 1974; Naz et al., 2010). The more drought-tolerant ecotypes of *A. gerardii* had fewer and larger stomates, whereas the more mesic ecotypes had more numerous and smaller stomates. Sand bluestem, the most drought-tolerant ecotype in this study, had more adaxial and fewer abaxial stomates, as well as larger stomates on both surfaces compared to more mesic ecotypes. The more mesic ecotype from Illinois and the Kaw cultivar had fewer adaxial and more abaxial stomates than Sand bluestem, as well as smaller stomates on both surfaces. This is similar to many previous studies on stomates in numerous plant species, which regularly report

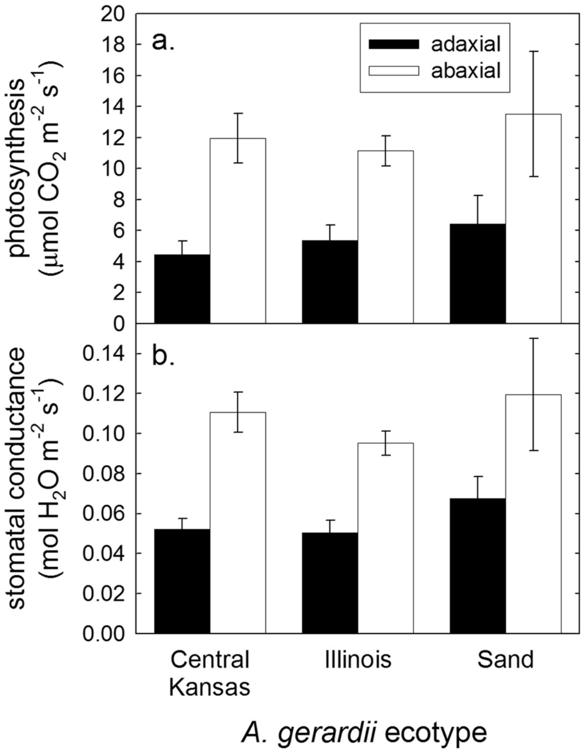

Fig. 8. (a) Photosynthesis rates (μmol $CO_2\,m^{-2}\,s^{-1}$) and (b) stomatal conductance (mol $H_2O\ m^{-2}\ s^{-1}$) on the adaxial and abaxial leaf surfaces of *A. gerardii* across three ecotypes. Bars are means ± standard errors of 8–18 replicates, as data were normally distributed. *A. gerardii* showed significantly higher photosynthesis and stomatal conductance on the abaxial surfaces of leaves than the adaxial surfaces and there was no significant difference among ecotypes on either surface of the leaves.

an inverse relationship between stomatal size and stomatal density (Miskin and Rasmusson, 1970; Kramer and Boyer, 1995).

There is generally an inverse relationship between size and density of stomates (Miskin and Rasmusson, 1970; Willmer and Fricker, 1996). Drought-tolerant lines of the grasses *Triticum aestivum* L. (Baloch et al., 2013) and *Zea mays* L. (Ristic and Cass, 1991) had greater density and smaller size of stomates compared to drought-sensitive lines. Populations of *Cynodon dactylon* (L.) Pers. adapted to a salt range had higher stomatal density compared to a more mesic population (Hameed et al., 2013). By contrast, the more drought-tolerant ecotypes of *A. gerardii* had fewer and larger stomates compared to more mesic ecotypes. A larger stomate would have a larger boundary layer compared to a smaller pore (Campbell and Norman, 1998), which could be an adaptation to conserve water. Clearly there are different strategies involved regarding stomates and drought tolerance in grasses.

Further, stomatal density typically differs between leaf surfaces, and these differences can be confirmed with direct stomate counts and with gas exchange measurements. Like many drought-adapted grasses, leaves in *A. gerardii* are primarily hypostomatous. In the current study, abaxial density in Sand bluestem ranged from 144 to 181 mm^{-2} whereas adaxial density ranged from 0 to 31 mm^{-2}. Abaxial density in the other ecotypes of *A. gerardii* ranged from 158 to 216 mm^{-2} whereas adaxial density ranged from 0 to 5 mm^{-2}, similar to distributions reported by Knapp et al. (1994) for *A. gerardii*.

Functional measures of gas exchange follow similar patterns as stomatal size and density when comparing leaf surfaces. Photosynthetic rates and stomatal conductance were higher on abaxial surfaces than adaxial surfaces in all *A. gerardii* ecotypes. However, factors other than stomatal conductance can influence gas exchange rates (Campbell and Norman, 1998). Whereas abaxial to adaxial ratios of stomatal density in *A. gerardii* were on the order of 100:1 (Fig. 3), abaxial to adaxial ratios of photosynthesis rates were closer to 2:1 (Fig. 8). Other biophysical parameters are clearly important when considering gas exchange in *A. gerardii*, including path length for H_2O versus CO_2, responses of the different leaf surfaces to light, and how long the tape was on the leaf (Wang et al., 2008). Leaf shape or leaf rolling might also influence gas exchange (Barnes, 1985; Campbell and Norman, 1998; Maricle et al., 2009); how these factors influence gas exchange or drought tolerance in *A. gerardii* remain an interesting area for further investigation.

Stomatal densities in *A. gerardii* in this study are somewhat lower than previously-published densities of adaxial stomates, but similar to abaxial stomates in *Seslaria caerulea* (Lloyd and Woolhouse, 1978). The drought-tolerant *Schizachyrium scoparium* (Michx.) Nash also has hypostomatous leaves (Awada et al., 2002). Mean density for stomates of *S. scoparium* is 11.9 mm^{-2} for the adaxial surface and 112.2 mm^{-2} for the abaxial surface and *A. gerardii* ssp. *hallii* has 19.4 mm^{-2} for the adaxial surface and 75.9 mm^{-2} for the abaxial surface. Similarly, *A. gerardii* is also known to have hypostomatous leaves (Knapp et al., 1994), and desert grasses measured by Naz et al. (2010) had hypostomatous leaves. Although there is not universal consistency regarding placement of stomates on leaf surfaces (Redmann, 1985; Awada et al., 2002), one strategy of drought-adapted grasses is to have hypostomatous leaves with smaller or fewer stomates.

There are other genetic factors that can influence stomatal density in grasses like *A. gerardii*. As ploidy level increases, stomatal density decreases due to larger epidermal cells (Mishra, 1997). There are many 9*x* populations of *A. gerardii* in Eastern Kansas (McAllister et al., 2015), but our Eastern Kansas ecotype did not have larger stomates than other ecotypes of *A. gerardii*. In other cases, genetic differences in stomates among populations of *Sesleria caerulea* (L.) Ard. were attributed to maintaining leaf temperature (Lloyd and Woolhouse, 1978). A number of genes have been implicated in stomatal size, density, and development (Wang et al., 2007; Chen et al., 2017), and some of these genes also respond to the environment.

4.3. Environmental influences on stomate size and density

Andropogon gerardii has some ability to acclimate to environmental conditions through its stomates. Ecotypes of *A. gerardii* showed minor differences among sites or years, with stomatal characteristics related to precipitation attributes in their home site (Supplementary Table 6). PCA axis 2 relates to precipitation (Fig. 7), which had positive associations with adaxial size and adaxial density of stomates and a negative association with abaxial density of stomates. The Central Kansas ecotype of *A. gerardii* had a positive association with PCA axis 2 whereas the Eastern Kansas and Illinois ecotypes did not, suggesting the Central Kansas ecotype might be most responsive to precipitation when considering stomatal size and density. The site and year differences detected in the present study were possibly caused by factors like soil moisture, vapor pressure deficit, temperature, light, or CO_2 availability, potentially owing to microclimate differences (Casson and Gray, 2008). Many grasses are responsive to water stress regarding stomatal size or density (e.g., Xu and Zhou, 2008; Naz et al., 2010; Baloch et al., 2013), and *A. gerardii* responds to some degree in this manner. Increased CO_2 has been shown to decrease stomatal density in *A. gerardii* (Knapp et al., 1994), but drought apparently has a lesser influence.

Stomatal size can decrease (Xu and Zhou, 2008; Baloch et al., 2013) and density can increase (Taft, 1950; Gindel, 1969) in some plants when there is a water deficit or salinity (Hameed et al., 2013). Leaf morphology is known to be affected by water availability (Ciha and Brun, 1975; Kramer et al., 2018), and reduced total leaf surface can reduce total numbers of stomates. Other environmental factors can influence stomates, for example higher CO_2 concentrations decrease stomatal density (Knapp et al., 1994; Casson and Gray, 2008). More light availability increases stomatal density (Penfound, 1931), which is commonly more influential than water availability (Taft, 1950).

However, stomatal size and density are known to be more heavily influenced by genetics than by environmental conditions (Palchetti et al., 2014). Indeed, there were fewer effects of environment on stomates in *A. gerardii* compared to genetic influences.

4.4. Conclusions

Ecotypes of *A. gerardii* had different sizes and densities of stomates, with the more drought-tolerant ecotypes of *A. gerardii* having fewer and larger stomates, whereas the more mesic ecotypes had more and smaller stomates. These patterns are consistent with numerous other plants in some aspects, with an inverse relationship between stomate size and density. In other ways, these results were different from other plants, as the drought tolerant ecotypes of *A. gerardii* had fewer and larger stomates compared to more mesic ecotypes. Genetics was the primary influence on stomates in *A. gerardii*, with little effect of environment. Tolerance to water stress is commonly accompanied by a reduction in total pore area (Hameed et al., 2013), potentially consistent with results in *A. gerardii*. An ability to adjust stomatal size or density via plasticity could allow a plant an ability to regulate water loss during times of water stress (Wang et al., 2007; Casson and Gray, 2008; Xu and Zhou, 2008; Naz et al., 2010; Baloch et al., 2013). However, drought-adapted grasses like *A. gerardii* might employ different strategies, like altering stomatal size or density over evolutionary time. Alternatively, drought tolerance in C_4 plants like *A. gerardii* might come from mechanisms other than stomates (Ghannoum, 2009), for example from adjustments to photosynthesis (Johnson et al., 2015), water potential (Maricle et al., 2017), other aspects of leaf anatomy (Olsen et al., 2013), smaller evaporative surface area (Kramer et al., 2018), or nitrogen metabolism (Heckathorn and DeLucia, 1996). Measures of similar photosynthesis rates among ecotypes despite differences in stomatal size and density suggest that photosynthesis in *A. gerardii* is more heavily influenced by factors other than stomates. However, stomatal size and density appear to be related to drought tolerance in *A. gerardii*. Commonly drought tolerance is a result of many structural and functional components (Tucker et al., 2011) in addition to stomates. As this method of drought tolerance is genetically-controlled, it could present an evolutionary response to changing climatic conditions and projected increased aridity in its native range (Cook et al., 2015).

Author statement

Nick Varvel: Writing, Investigation, Methodology. **Christina Hilt**: Investigation. **Loretta Johnson**: Conceptualization, Funding Acquisition. **Matthew Galliart**: Formal Analysis. **Sara Baer**: Conceptualization, Funding Acquisition. **Brian Maricle**: Writing, Methodology.

Acknowledgements

We thank Keri Maricle, Adam Urban, Wenkai Shu, and Cera Smart for assistance in collecting leaves and making epidermal imprints. This project was funded by the National Research Initiative competitive grant no. 2008-35100-04545 from the USDA National Institute of Food and Agriculture. Additional support was provided by the Kansas Academy of Science and the FHSU Department of Biological Sciences.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.07.018.

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