

## Leaf-level physiological responses of *Tamarix ramosissima* to increasing salinity

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### ABSTRACT

Over the past century, the invasive halophytic shrub *Tamarix ramosissima* Ledeb. has increased in abundance and distribution in riparian ecosystems of western North America. These increases coincide with anthropogenic modification of river systems, which decrease the rate of periodic overbank flooding, leading to an increase in soil salinity. Increased soil salinity negatively impacts the physiology of native riparian tree species, but the impact of increased soil salinity on *T. ramosissima* physiology is incompletely known. To measure the impacts of soil salinity on *T. ramosissima*, we measured leaf-level responses across a broad range of surface-soil salinity concentrations at two sites in western Kansas. Photosynthesis at 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  ( $A_{2000}$ ), stomatal conductance to water ( $g_s$ ), intercellular  $\text{CO}_2$  concentration ( $C_i$ ), and leaf  $\delta^{13}\text{C}$  showed little change over surface-soil salinities from 0.5 to 17.65 mmhos/cm. The small variation in leaf-level physiological responses suggests robust functioning of *T. ramosissima* across a broad range of surface-soil salinities. Leaf-level physiology and  $\delta^{13}\text{C}$  responses were assessed by canopy position, but responses were not significantly different. These results are among the first to show broad acclimation and robust physiological functioning for many leaf-level processes measured on mature trees grown across a wide surface-soil salinity gradient in the field.

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### 1. Introduction

Over the past century, major river modifications including damming, flow alterations, and diversions for water use have led to decreased periodic overbank flooding in semi-arid and arid riparian ecosystems (DiTomaso, 1998; Everitt, 1980). These alterations have decreased soil moisture content and increased surface-soil salinity, both of which influence community composition in riparian ecosystems (Glenn and Nagler, 2005; Pan, 2001; Ruan et al., 2009; Smith et al., 1998; Stromberg et al., 2007). Reduction of habitat quality in riparian ecosystems has contributed to the decline of native mesic tree species and opened a niche for invasion by *Tamarix ramosissima* Ledeb. in the western United States (Busch and Smith, 1995; Ladenburger et al., 2006; Ruan et al., 2009; Stromberg et al., 2007).

*T. ramosissima* is a Eurasian shrub or tree that is common around ephemeral waters of semi-arid and arid climates (Baum, 1967; Chew, 2009). *T. ramosissima* is halophytic (salt-loving plant) and a facultative phreatophyte (water-loving plant) (Busch et al., 1992;

Sala et al., 1996). The halophytic nature of mature *T. ramosissima* trees is one mechanism hypothesized to explain increased abundance in altered riparian ecosystems (Busch and Smith, 1995; Cui et al., 2010; Glenn and Nagler, 2005; Sala et al., 1996; Vandersande et al., 2001). *T. ramosissima* is reportedly tolerant of high salinities (Busch and Smith, 1995; Vandersande et al., 2001). However, increased saline conditions can impart metabolic stress even for halophytes (Khan et al., 2000; Moghaieb et al., 2004; Tal et al., 1979). Salt stress (e.g., NaCl) impacts plant physiology through a decline in leaf-level gas exchange, suppressed growth, osmotic effects, and the creation of reactive oxygen species (Parida and Das, 2005).

Plants have developed biochemical and molecular mechanisms to tolerate salt stress (Parida and Das, 2005). Examples of these mechanisms include exclusion of ions, compartmentalization of ions, and synthesis of compatible solutes (Tester and Davenport, 2003). *T. ramosissima* shows non-selectivity in ion exclusion from salt glands, which is hypothesized as one mechanism by which *T. ramosissima* maintains an acceptable salt balance (Berry, 1970). The tolerance of *T. ramosissima* to saline soils might be a result of the synthesis of compatible solutes to protect enzymatic activity and cellular osmotic potential (Ding et al., 2009; Ruan et al., 2007, 2009; Solomon et al., 1994). Solomon et al. (1994) showed that *Tamarix jordanis* Boiss. synthesizes N-methyl-L-proline (MP) and N-methyl-trans-4-hydroxy-L-proline (MHP) in the presence of high NaCl

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content. The two solutes are effective for maintaining the carboxylating activity of Rubisco in *T. jordanis*. Studies conducted along the Tarim River, China, showed *T. ramosissima* accumulated soluble sugars under salt stress which might contribute to the tolerance to high salinity in the species (Ruan et al., 2009). However, compatible solutes are energetically expensive to synthesize and may reduce plant growth or impact other physiological processes (Ding et al., 2009; Kleinkopf and Wallace, 1974; Tester and Davenport, 2003).

Few studies have reported how increasing salinity impacts physiological responses in *T. ramosissima*. Glenn et al. (1998) grew a mix of shrubs and trees, including *T. ramosissima*, in a greenhouse over a salinity gradient from 0 to 32 g l<sup>-1</sup> NaCl. *T. ramosissima* had a minor 2% reduction in relative growth rate, but transpiration markedly decreased between 16 and 32 g l<sup>-1</sup> NaCl (Glenn et al., 1998). Leaf-level processes such as transpiration, photosynthesis, and stomatal closure are sensitive to salinity stress (Parida and Das, 2005). Busch and Smith (1995) investigated how hydrologic variation and varying salinity in floodplain environments affects ecophysiological responses of dominant woody taxa including *T. ramosissima*. Physical site differences were subtle, and soil salinity did not vary significantly in areas sampled. Kleinkopf and Wallace (1974) found increasing salinity had a small effect on leaf-level gas exchange. Growth decreased in *T. ramosissima* at higher salt levels, which the authors attributed to a greater energy demand to transport salt to leaf salt glands.

To elaborate on the responses of *T. ramosissima* to soil salinity, we measured several leaf-level physiological responses over a wide surface-soil salinity gradient in western Kansas. High soil salinity lowers soil water potential disrupting plant water uptake, which causes water stress (Mahajan and Tuteja, 2005), and increased water stress can decrease leaf-level gas exchange (Chen et al., 2010). Furthermore, Na<sup>+</sup> is highly toxic and can disrupt enzymatic functioning reducing leaf-level photosynthesis (Parida and Das, 2005). For these reasons, we predicted that increasing surface-soil salinity would decrease leaf-level gas exchange as well as leaf-level water potential, with alterations in the stable isotopic signature of leaf <sup>13</sup>C and <sup>15</sup>N reflecting altered water-use efficiency and differences in soil pH across a salinity gradient. Similarly, salinity stress is exacerbated in shaded leaves and these leaves tend to show the first signs of salt stress (Parida and Das, 2005). Therefore, shaded leaves with lower canopy position should differentially show lower gas exchange rates and water potentials than sun leaves higher in the canopy. Thus, we predicted that leaf-level physiological responses would vary according to canopy structure across a salinity gradient.

## 2. Materials and methods

### 2.1. Study area

This research was performed at two sites in western Kansas (Fig. A-1). The Ashland research site is a Kansas Geological Survey and Kansas State University research site located adjacent to the Cimarron River, Ashland, Kansas, USA (37°11'19"). *T. ramosissima* is the predominant species at this site, but other herbaceous species are intermixed among the *T. ramosissima* and include *Sporobolus airoides* (Torr.), *Panicum virgatum* L., and *Schizachyrium scoparium* (Michx.) (Nippert et al., 2010). Soil textures at this site consist of coarse silts through medium sands. Cedar Bluff State Park is near Ellis, Kansas, USA (38°48'N and 99°43'W) and managed by the Kansas Department of Wildlife and Parks (KDWP). The size of Cedar Bluffs Reservoir varies year by year and receives intermittent flow from the Smoky Hill River in eastern Colorado. Riparian areas are dominated by juvenile and adult *T. ramosissima* as well as other

vegetation including *Sporobolus compositus* (Michx.), *S. scoparium*, and *Populus deltoides* (Bartr.).

### 2.2. Salinity analysis

In May 2009, four 10 m×5 m plots were established at each site. Four or five soil core samples were collected from each plot to 15 cm depth in May and September, 2009. All soil cores were homogenized into a single sample per plot. Analyses were conducted at the Kansas State University Soil Testing Center. Samples were sieved, dried, made into a soil paste, and the electrical conductivity (EC) of the soil paste was measured in mmhos/cm. Electrical conductivity (EC) serves as a proxy of soil salinity. Thus, EC and soil salinity are positively correlated (Rhoades et al., 1990).

### 2.3. Plant physiology

Five *T. ramosissima* individuals, each approximately 1.5 m in height, were randomly selected in each plot and the same individuals were measured during June, July, August, and September, 2009. Individuals were of similar size in each plot and at both sites. Physiological measurements were conducted at three canopy locations that were categorized as bottom of the canopy, middle of the canopy, and top of the canopy for each replicate. One leaf was measured per canopy location for a total of 15 leaves measured per plot per date. On each sampling date, gas exchange measurements were taken using a LiCor-6400 infra-red gas analyzer with a red/blue light source and a CO<sub>2</sub> injector (LiCor, Lincoln, Nebraska, USA). Irradiance inside the cuvette was 2000 μmol m<sup>-2</sup> s<sup>-1</sup>, CO<sub>2</sub> concentration was 400 ppm and relative humidity was maintained at ambient. Gas exchange measurements were made on new, mature leaves growing in full sunlight between 0800 and 1700 h Central Daylight Time (CDT). Measurements included photosynthetic rate at 2000 μmol m<sup>-2</sup> s<sup>-1</sup> (*A*<sub>2000</sub>), stomatal conductance to water (*g*<sub>s</sub>), and intercellular CO<sub>2</sub> concentration (*C*<sub>i</sub>). Dark respiration data were not collected. Measurements occurred on clear days and projected leaf area within the gas exchange cuvette was estimated using a LiCor 3100 leaf area meter (LiCor, Lincoln, Nebraska, USA). Water potential measurements were conducted at both pre-dawn (0300–0600 h CDT) and mid-day (1300–1500 h CDT) using a Scholander pressure bomb (PMS instruments, Albany, Oregon, USA). One leaf sample per individual per canopy position per plot was measured from June to September. Data were analyzed using a mixed effects model ANOVA in SAS 9.1 (Cary, North Carolina, USA), where site, plot nested within site, and canopy location were fixed effects, whereas sampling date was a random effect to account for repeated measures in the design. Gas exchange measurements were not recorded during September at either site and water potential data were not collected for the Ashland research site in September due to inclement weather.

### 2.4. Stable isotopic Analysis

Leaf samples were collected from each individual at each canopy position for each sampling period except for the Ashland research site in September, 2009. Samples were dried at 60 °C for 48 h and ground to a fine powder. Samples were analyzed for δ<sup>13</sup>C and δ<sup>15</sup>N stable isotopic signature using a Finnigan Delta-plus continuous flow isotope ratio mass spectrometer connected to an elemental analyzer. Isotopic analyses were performed at the Kansas State University Stable Isotope Mass Spectrometry Lab (SIMSL). The within run precision was <0.15‰ for δ<sup>15</sup>N and <0.05‰ for δ<sup>13</sup>C. Between run variation was <0.2‰ for δ<sup>15</sup>N and <0.08‰ for δ<sup>13</sup>C. C:N values were obtained from an elemental analyzer.

### 3. Results

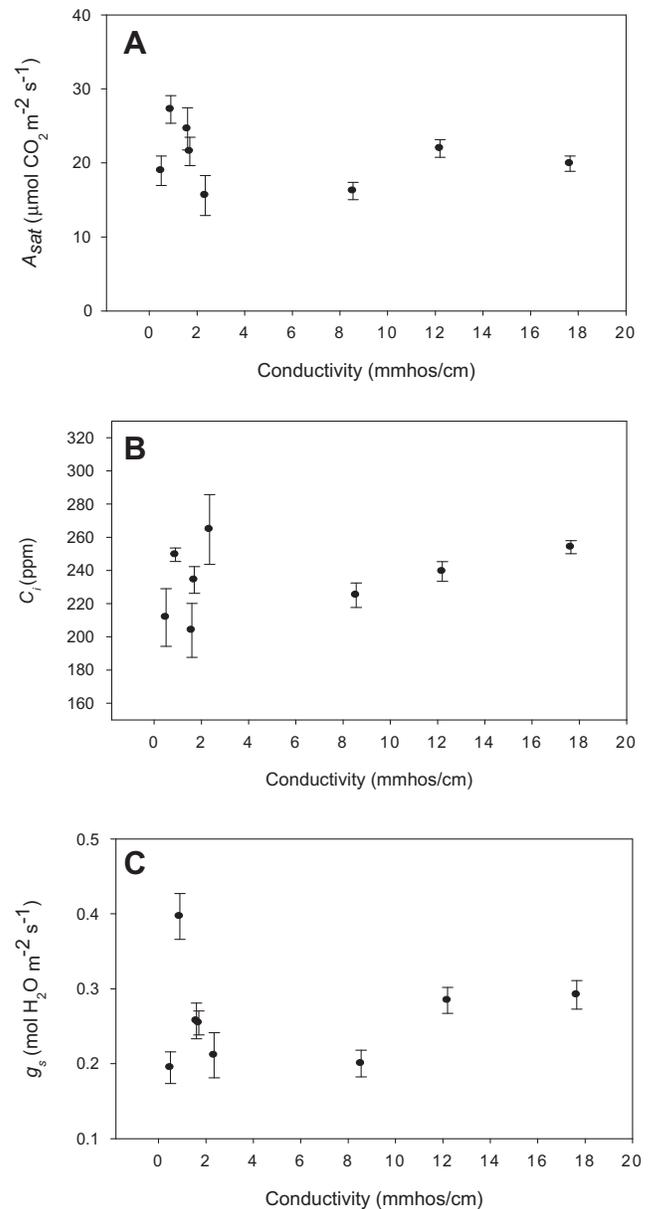
Electrical conductivity (EC) of soils at 15cm depth varied between and within both study sites with a range of 0.5–17.65 mmhos/cm (Table A-1). No trends were evident across sites or across plots nested within sites for leaf-level gas exchange responses and therefore, data is presented undifferentiated by site (Fig. 1A–C).  $A_{2000}$  values significantly varied by plot nested within site and by canopy position ( $p < 0.05$ ), but not across sites ( $p > 0.05$ ; Table A-2). Photosynthetic rates ranged from 15 to 27  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  across all plots (Fig. 1A; Table A-4).  $C_i$  values were not significantly different between sites ( $p > 0.05$ ), but did vary significantly among plots nested within sites ( $p < 0.05$ ) and by canopy position ( $p < 0.05$ ).  $C_i$  values ranged from 203 parts per million (ppm) to 264 ppm across all plots. Stomatal conductance to water ( $g_s$ ) rates ranged from 0.19 to 0.4  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  and did not vary significantly between sites ( $p > 0.05$ ), but did vary significantly across plots nested within site ( $p < 0.05$ ) and by canopy position ( $p < 0.05$ ). Photosynthesis, intercellular  $\text{CO}_2$  concentration, and stomatal conductance to water significantly varied by canopy position, but no trends were evident across canopy positions (Figs. 3A–C and 4A–C) and there was not a significant salinity\*canopy interaction ( $p > 0.05$ ).

Pre-dawn water potentials ranged from  $-0.9$  to  $-1.3$  MPa and mid-day water potentials ranged from  $-1.5$  to  $-2$  MPa (Fig. 2A; Table A-4). Pre-dawn water potentials did not vary significantly by canopy position ( $p > 0.05$ ) or between sites ( $p > 0.05$ ), but did vary significantly across plots nested within site ( $p < 0.05$ ). Mid-day water potentials did not vary significantly between sites ( $p > 0.05$ ), but did vary significantly across plots nested within site ( $p < 0.05$ ) and by canopy position ( $p < 0.05$ ). C:N values varied significantly between sites ( $p < 0.05$ ). At Cedar Bluffs Reservoir, C:N varied significantly between plots ( $p < 0.05$ ), but did not vary significantly by canopy position ( $p > 0.05$ ). At the Ashland research site, C:N values significantly varied across plots ( $p < 0.05$ ) and by canopy position ( $p < 0.05$ ). C:N values ranged from 16:1 to 31:1 across all plots.

Leaf samples had the heaviest  $\delta^{13}\text{C}$  signatures at the Ashland research site as compared to Cedar Bluffs Reservoir (Fig. 2B; Table A-4). Leaf  $\delta^{13}\text{C}$  values varied significantly between sites ( $p < 0.05$ ). Leaf  $\delta^{13}\text{C}$  varied significantly by canopy position at the Ashland research site, but not between plots. At Cedar Bluffs Reservoir, leaf  $\delta^{13}\text{C}$  values significantly varied between plots ( $p < 0.05$ ) and by canopy position ( $p < 0.05$ ). Leaf  $\delta^{15}\text{N}$  values significantly varied between sites ( $p > 0.05$ ) with heavier  $\delta^{15}\text{N}$  signatures at Cedar Bluffs Reservoir (Fig. 2D). Leaf  $\delta^{15}\text{N}$  values significantly varied between plots at the Ashland research site and Cedar Bluffs Reservoir ( $p > 0.05$ ). Leaf  $\delta^{15}\text{N}$  did not significantly vary by canopy position at either site ( $p > 0.05$ ).

### 4. Discussion

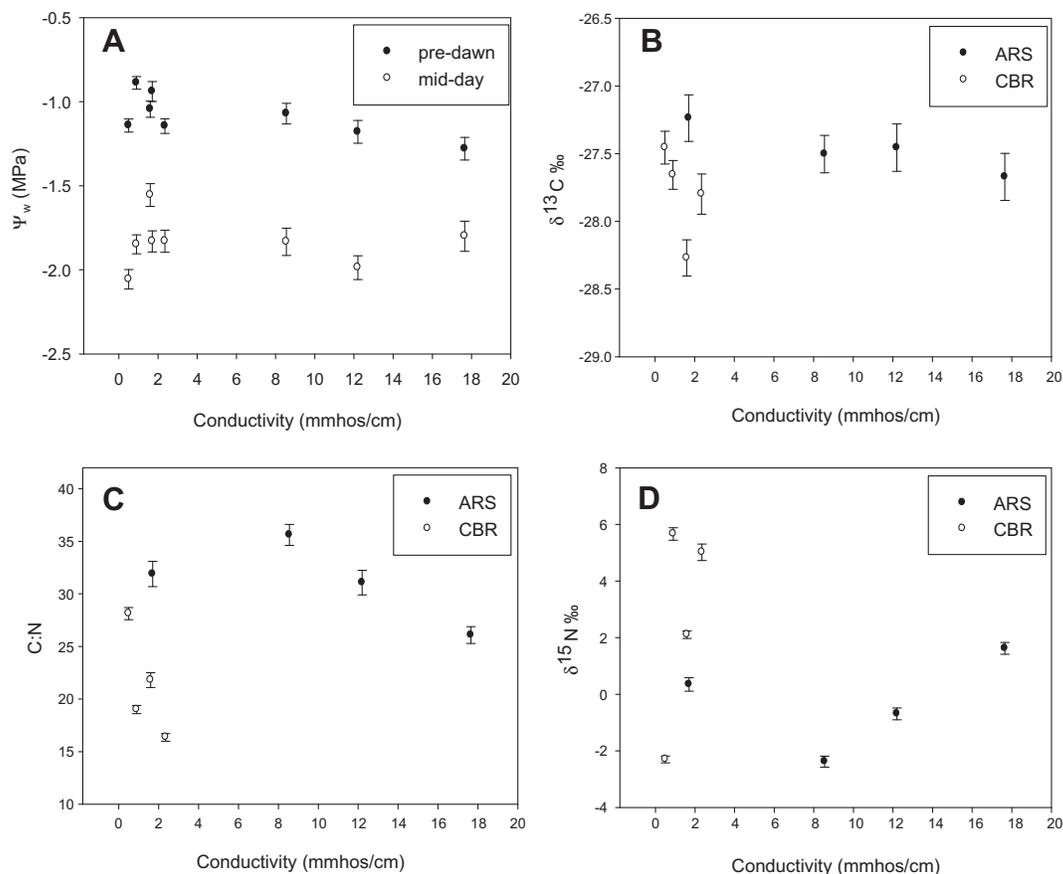
Increasing salinity causes salt stress in most plants and this stress is reflected in leaf-level physiological measurements (Khan et al., 2000; Leport et al., 2006; Tester and Davenport, 2003). Salt stress inhibits photosynthesis, suppresses growth, affects protein synthesis, and alters energy and lipid metabolism (Parida and Das, 2005). In this study, soil electrical conductivity (EC) varied broadly across both study sites at 15 cm soil depth. *T. ramosissima* is a facultative phreatophyte and is known to utilize the water table, especially during droughts (Sala et al., 1996; Devitt et al., 1997; Glenn and Nagler, 2005). At both Cedar Bluff State Park and the Ashland Research Site, previous measurements of groundwater salinity were comparable to our measurements of soil salinity at 15 cm depth (Fig. 1A). For example, the EC of groundwater at the



**Fig. 1.** *Tamarix ramosissima* mean ( $\pm 1\text{SE}$ ) A) photosynthetic rate at 2000  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  ( $A_{2000}$ ), B) intercellular  $\text{CO}_2$  concentration ( $C_i$ ), and C) stomatal conductance to water ( $g_s$ ) sampled across a wide range of salinity concentrations as expressed by electrical conductivities (EC). Gas exchange measurements were not recorded on September, 2009 at either site due to inclement weather. Data presented are averages over the sampling period for each plot(site).

Ashland Research Site was 10 mmhos/cm (Butler et al., 2005) and the EC of groundwater at Cedar Bluff State Park was 5 mmhos/cm (Polacik, 2010). Therefore, a gradient of surface-soil salinity exists at each of these sites, and water uptake from the unsaturated rhizosphere (top 15 cm), groundwater, or both (similar to Nippert et al., 2010) results in the uptake of saline water. However, because source-water uptake over time was not specifically measured in this analysis, the possibility exists that some unrecognized water source may have been utilized by *T. ramosissima* to avoid high concentrations of salts.

We expected leaf-level physiological measurements to decline as surface-soil EC increased (Gulzar et al., 2003; Parida et al., 2004; Parida and Das, 2005). However, we found no support that leaf-

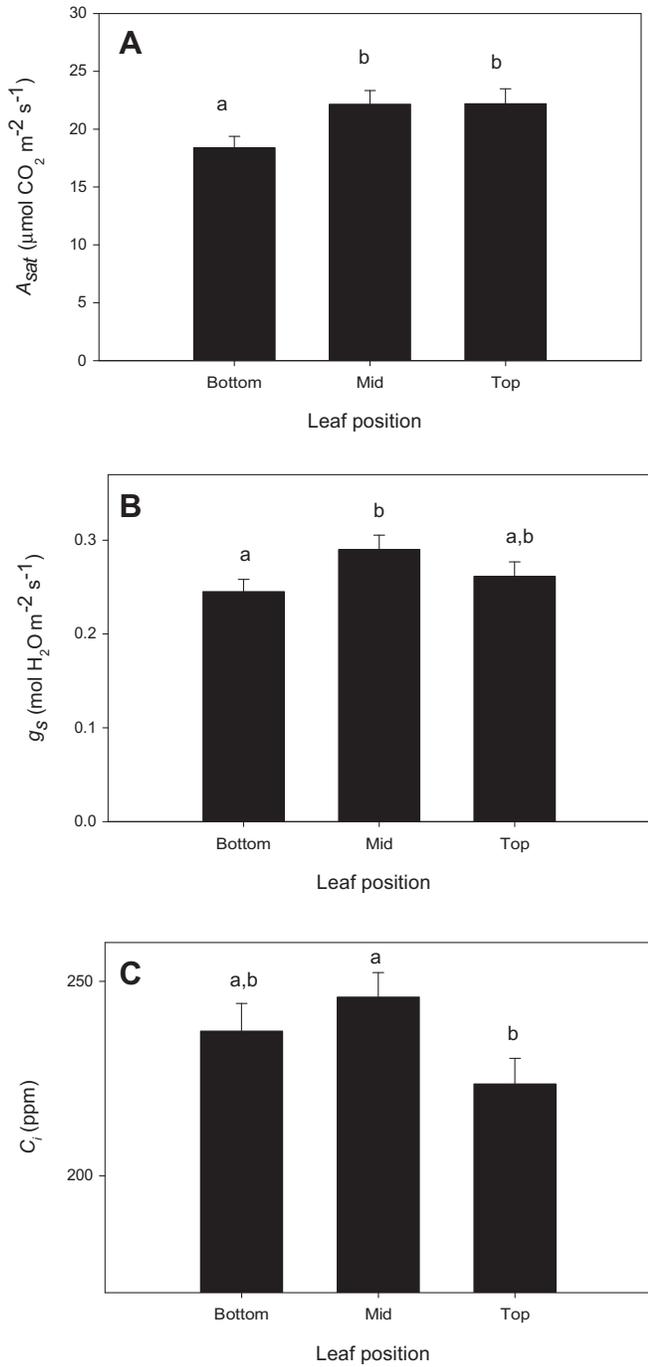


**Fig. 2.** *Tamarix ramosissima* mean ( $\pm 1$ SE) A) pre-dawn (black circles) and mid-day (white circles) water potential, B) stable carbon isotopic signature ( $\delta^{13}\text{C}$ ) for the Ashland Research Site (ARS) and Cedar Bluffs Reservoir (CBR), C) C:N for both ARS and CBR, and D) stable nitrogen isotopic signature ( $\delta^{15}\text{N}$ ) for ARS and CBR. ARS data are denoted by black circles and CBR data are denoted by white circles. ARS was not sampled in September, 2009 because of inclement weather.

level physiological responses of *T. ramosissima* varied as a function of surface-soil EC over the salinity gradient measured. *T. ramosissima* physiological functioning was maintained across all surface-soil EC values, suggesting that *T. ramosissima* is able to accommodate a broad range of salinities, which is consistent with other studies (Brotherson and Winkel, 1986; Busch and Smith, 1995; Ruan et al., 2009). As surface-soil salinity increased among all plots between sites, water potential did not change significantly. Soil salinity disrupts the soil-plant-atmosphere-continuum by which plants obtain water (Mahajan and Tuteja, 2005). We predicted that leaf-level water potentials would decrease as surface-soil salinity increased. As plants become water stressed, leaf-level gas exchange is typically reduced and  $\delta^{13}\text{C}$  values become heavier (Parida and Das, 2005; Tester and Davenport, 2003). Photosynthesis, stomatal conductance to water, and intercellular  $\text{CO}_2$  concentration did not significantly change as surface-soil salinity increased. It may be hypothesized, then, that the driver of physiological responses in *T. ramosissima* was available soil moisture, not surface-soil salinity. However, it is also possible that the threshold surface-soil salinity to elicit a physiological decline from *T. ramosissima* was not reached. Previous results from a greenhouse study by Glenn et al. (1998) suggest that *T. ramosissima* leaf-level physiology exhibited marginal decreases until 29 mmhos/cm (20,000 ppm) EC. When tested under field conditions, our results are consistent with Kleinkopf and Wallace (1974), who showed there were only marginal effects on *T. ramosissima* leaf-level gas exchange over a salinity gradient from 0 to  $\sim 17.5$  mmhos/cm.

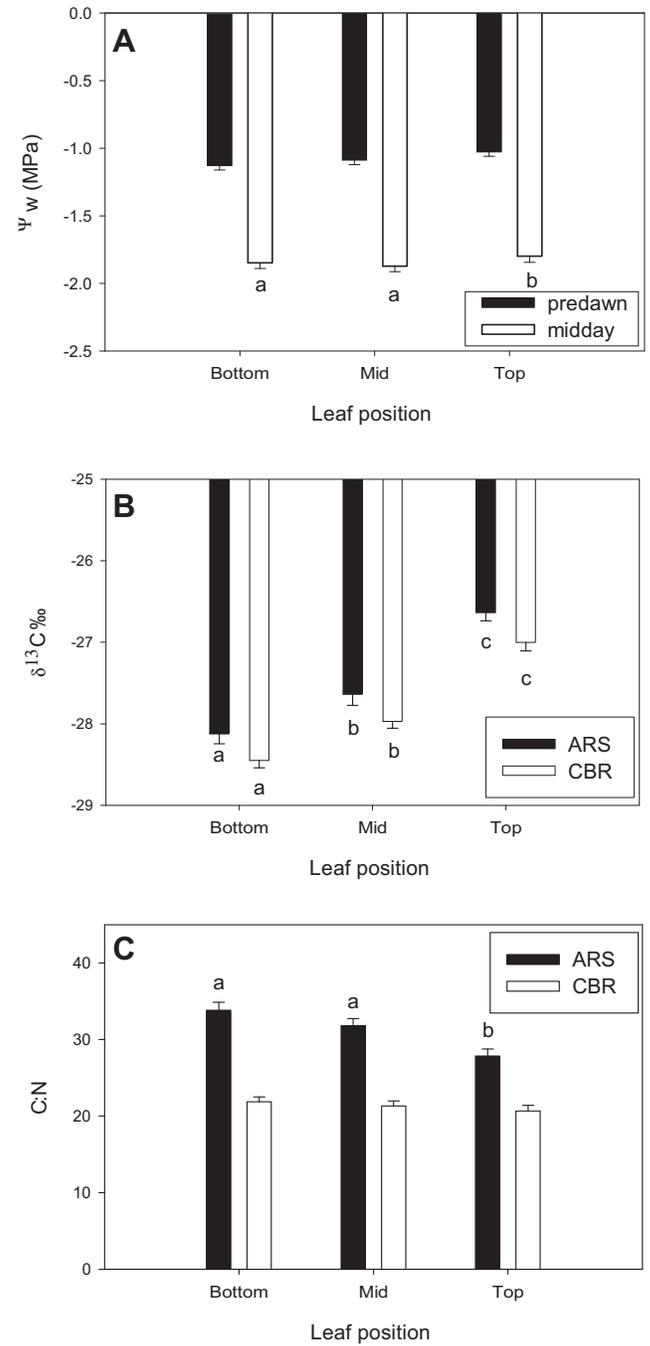
Kleinkopf and Wallace (1974) did observe a reduction in *T. ramosissima* growth as salinity increased. The authors attributed this growth decline to diversion of energy for use in salt pumping and energy production through respiration. Indeed, salt is exuded through salt glands of *Tamarix* species via an apoplastic xylem pathway (Campbell et al., 1974; Arndt et al., 2004). Since regulation of salinity is an energy-requiring process, we expected to see declines in leaf-level physiology for *T. ramosissima* trees by canopy position.

Sun and shade leaves have varying leaf morphology and physiology (McClendon, 1962; Oberbauer and Strain, 1986; Wylie, 1951). Shaded leaves tend to be less photosynthetically efficient than sun leaves and typically show signs of salt stress first (Oberbauer and Strain, 1986; Stephens et al., 2009). Therefore, we expected less energy to be contributed to leaf maintenance in shaded leaves and thus, a larger decline in leaf-level photosynthesis, stomatal conductance, and intercellular  $\text{CO}_2$  concentration in shaded leaves. Leaf-level gas exchange,  $\delta^{13}\text{C}$ , and mid-day water potential varied significantly ( $p < 0.05$ ) by canopy position, but a significant canopy\*salinity interaction did not exist. Over the range of surface-soil salinities measured, physiological responses to increasing surface-soil salinity did not impact shaded leaves in the bottom of the canopy proportionally more than leaves in the top of the canopy. It is possible that a surface-soil salinity concentration to elucidate significant changes by canopy position was not reached. It is also possible that salt tolerance strategies (i.e. leaf-level proline concentration, number of salt glands on leaf, rate of NaCl exudation



**Fig. 3.** *Tamarix ramosissima* mean ( $\pm 1SE$ ) A) photosynthetic rate at 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  ( $A_{2000}$ ), B) intercellular  $\text{CO}_2$  concentration ( $C_i$ ), and C) stomatal conductance to water ( $g_s$ ) response by canopy position.

or compartmentalization) of *T. ramosissima* do not vary by canopy position. As noted previously, soil moisture may be the predominant resource regulating leaf-level physiological responses, and not high surface-soil salinity. While the response did not vary as a function of surface-soil salinity concentration, leaves at the bottom of the canopy had reduced photosynthesis and increased intercellular  $\text{CO}_2$  concentration. The higher C:N of leaves at the bottom of the canopy suggests reduced allocation of N to photosynthesis. Low nitrogen content can cause lower photosynthetic rates regardless of irradiance (Cai et al., 2008). Furthermore, shaded



**Fig. 4.** *Tamarix ramosissima* mean ( $\pm 1SE$ ) A) pre-dawn (black bars) and mid-day (white bars) water potential, B) stable carbon isotopic signature ( $\delta^{13}\text{C}$ ) for the Ashland research site (ARS) (black bars) and Cedar Bluffs Reservoir (CBR) (white bars), and C) C:N for both ARS (black bars) and CBR (white bars).

leaves tend to have lower nitrogen concentrations than sun leaves (Evans, 1993; Evans and Poorter, 2001).

C:N varied significantly between the Ashland research site and Cedar Bluffs Reservoir. Cedar Bluffs Reservoir had much lower C:N values suggesting *T. ramosissima* leaves had a higher foliar nitrogen content at this site. Drivers of  $\delta^{15}\text{N}$  likely varied between sites.  $\delta^{15}\text{N}$  increased between soil conductivities of 8.55 and 17.65 mmhos/cm, which corresponded to an increase in soil pH from 7.3 to 8.5 (Fig. 2D). Pataki et al. (2005) showed  $\delta^{15}\text{N}$  increased significantly in saline *T. ramosissima* leaves compared to

non-saline *Populus* leaves. The response of  $\delta^{15}\text{N}$  was attributed to increased soil pH associated with saline soils. High soil pH results in the volatilization and loss of  $\text{NH}_3$  which enriches the remaining substrate in  $\delta^{15}\text{N}$ . At Cedar Bluffs Reservoir,  $\delta^{15}\text{N}$  values were much higher than the Ashland research site, but showed no trends over the surface-soil salinity gradient. High  $\delta^{15}\text{N}$  and high C:N values at Cedar Bluffs Reservoir likely reflect higher nitrogen availability. Craine et al. (2009) showed a correlation between  $\delta^{15}\text{N}$  and nitrogen availability, suggesting that  $\delta^{15}\text{N}$  increases as soil nitrogen availability increases. The results at Cedar Bluffs Reservoir do not suggest that high salinity resulted in higher  $\delta^{15}\text{N}$  responses because the surface-soil salinity gradient at this site was much narrower than the Ashland research site. Alternate explanations for the carbon and nitrogen dynamics could be changes in soil textures between sites (McLauchlan, 2006; McInerney and Bolger, 2000) or differences in precipitation (Austin and Sala, 2002; Knapp and Smith, 2001). However, the Ashland research site and Cedar Bluffs Reservoir received similar precipitation amounts for 2009 (~450 mm) and soil textures were also similar, consisting of coarse silts through medium sands.

The primary objective of this study was to assess leaf-level physiological responses of *T. ramosissima* to increasing salinity. Our results illustrate robust physiological responses for many leaf-level variables measured on mature *T. ramosissima* trees grown across a wide surface-soil salinity gradient in the field. Leaf-level physiological responses reflect the mechanisms of salt tolerance by *T. ramosissima* (salt glands, accumulation of proline, etc.), which were robust at sites with high salinities in surface-soils. These responses support previous research that has shown high salinity might contribute to the competitive advantage of *T. ramosissima* compared to salt-sensitive riparian species (Busch and Smith, 1995; Glenn et al., 1998; Ruan et al., 2009). Arid and semi-arid environments are predicted to become more saline (Jolly et al., 2008), but these results suggest increasing surface-soil salinity

will not be a major barrier for *T. ramosissima* persistence and range expansion.

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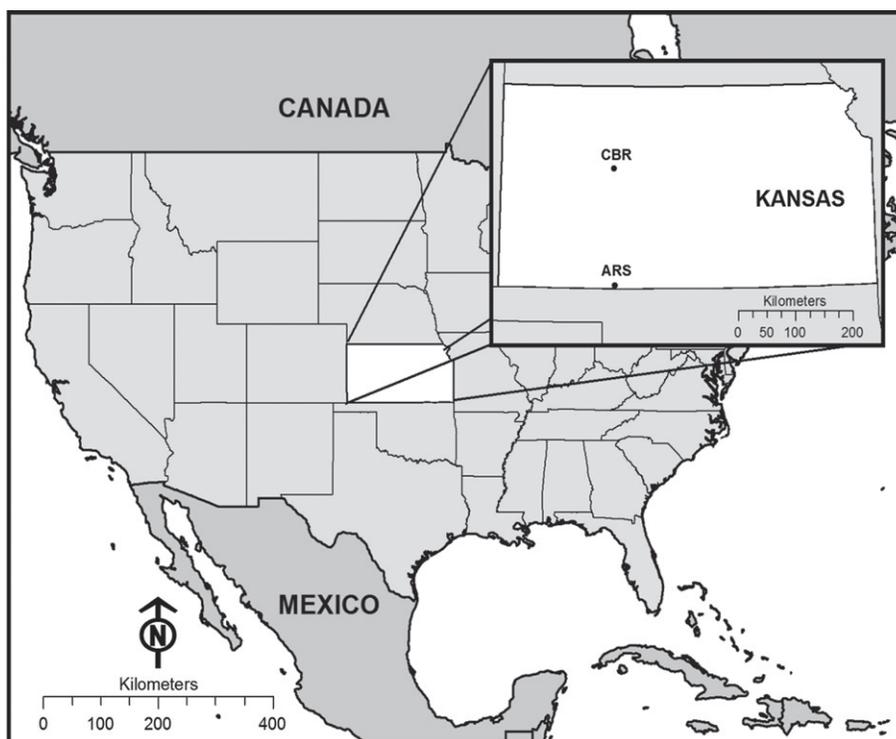
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### Appendix

**Table A-1**

The electrical conductivity ( $\pm 1\text{SE}$ ,  $n = 2$ ), soluble  $\text{Na}^+$  paste, pH, and estimated CEC among plots between the Ashland Research Site (ARS) and Cedar Bluffs Reservoir (CBR).

Plot	Electrical conductivity (mmhos/cm)	Soluble Na paste (meq/100g)	pH	Estimated CEC (meq/100g)
ARS (A)	1.65 $\pm$ 0.45	1.3	7.3	21
ARS (B)	12.2 $\pm$ 0.1	3.4	7.7	13
ARS (C)	17.65 $\pm$ 0.025	1.67	8.5	7
ARS (D)	8.55 $\pm$ 0.5	2.02	7.4	8
CBR (E)	2.35 $\pm$ 0.05	0.09	7.3	21
CBR (F)	1.6 $\pm$ 0.6	0.12	7.3	17
CBR (G)	0.9 $\pm$ 0.3	0.05	4.6	17
CBR (H)	0.5 $\pm$ 0	0.06	6.9	14



**Fig. A-1.** Locations of the Ashland research site (ARS) and Cedar Bluffs Reservoir (CBR) in Kansas, USA.

**Table A-2**

Statistical summary table for all response variables using a mixed model ANOVA.

Response Variables	Canopy Position			Plot(Site)			Site		
	F	d.f	P	F	d.f	P	F	d.f	P
$A_{2000}$	7.22	141	0.0010	5.95	316	<0.0001	0.35	316	0.55
$g_s$	4.04	141	0.0197	10.85	316	<0.0001	0.03	316	0.87
$C_i$	3.80	141	0.0247	4.65	316	0.0001	0.00	316	0.96
$\psi_w$ pre-dawn	1.67	406	0.1395	8.01	406	<0.0001	0.34	406	0.56
$\psi_w$ mid-day	4.69	409	0.001	6.79	409	<0.0001	2.67	409	0.10
$\delta^{13}C$	44.1	407	<0.0001	3.73	407	<0.0013	10.84	407	0.001
$\delta^{15}N$	0.47	407	0.628	1.99	407	<0.0001	3.78	407	<0.0001
C:N	50.8	407	<0.0001	36	407	<0.0001	322	407	<0.0001

**Table A-3**Total precipitation (mm), mean daily air temperature ( $^{\circ}C$ ), and average mid-day water potential by month for the Ashland research site (ARS) and Cedar Bluffs Reservoir (CBR).

	Ashland Research Site					Cedar Bluffs Reservoir				
	May	June	July	August	September	May	June	July	August	September
Mean Daily Air Temperature ( $^{\circ}C$ )	17	24	27	26	20	17	23	25	24	18
Total Precipitation (mm)	34	112	48	87	9	60	29	67	82	60
Mid-Day Water Potential (MPa)	N/A	-1.6	-1.8	-2.18	N/A	N/A	-1.5	-2.0	-2.1	-1.8

**Table A-4**Mean values ( $\pm$ 1SE) for  $A_{sat}$ ,  $C_i$ ,  $g_s$ , pre-dawn and mid-day  $\psi$ ,  $\delta^{13}C$ ,  $\delta^{15}N$ , and C:N presented by plot within site, sampling period, and canopy position.

Site			$A_{2000}$ ( $\mu\text{mol CO}_2$ $\text{m}^{-2} \text{s}^{-1}$ )	$C_i$ (ppm)	$g_s$ (mol $\text{H}_2\text{O}$ $\text{m}^{-2} \text{s}^{-1}$ )	$\psi_{pre}$ (MPa)	$\psi_{mid}$ (MPa)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C:N
ARS	Plot	A	21.50 $\pm$ 1.9	234.25 $\pm$ 8.1	0.25 $\pm$ 0.016	-0.94 $\pm$ 0.059	-1.83 $\pm$ 0.062	-27.23 $\pm$ 0.17	0.35 $\pm$ 0.24	31.88 $\pm$ 1.2
		B	21.95 $\pm$ 1.2	239.36 $\pm$ 5.8	0.28 $\pm$ 0.017	-1.18 $\pm$ 0.067	-1.99 $\pm$ 0.071	-27.45 $\pm$ 0.18	-0.69 $\pm$ 0.21	31.07 $\pm$ 1.2
		C	19.91 $\pm$ 1.0	253.98 $\pm$ 3.9	0.29 $\pm$ 0.019	-1.28 $\pm$ 0.067	-1.80 $\pm$ 0.089	-27.67 $\pm$ 0.17	1.62 $\pm$ 0.21	26.07 $\pm$ 0.80
		D	16.21 $\pm$ 1.2	225.11 $\pm$ 7.4	0.20 $\pm$ 0.018	-1.07 $\pm$ 0.061	-1.83 $\pm$ 0.081	-27.50 $\pm$ 0.14	-2.38 $\pm$ 0.19	35.61 $\pm$ 1.0
	Month	June	17.15 $\pm$ 0.79	237.68 $\pm$ 5.5	0.22 $\pm$ 0.011	-1.03 $\pm$ 0.036	-1.59 $\pm$ 0.041	-27.39 $\pm$ 0.17	0.20 $\pm$ 0.21	29.07 $\pm$ 0.76
		July	27.05 $\pm$ 1.4	221.66 $\pm$ 7.4	0.31 $\pm$ 0.017	-0.78 $\pm$ 0.042	-1.83 $\pm$ 0.085	-27.50 $\pm$ 0.14	0.05 $\pm$ 0.22	29.70 $\pm$ 0.81
		August	15.60 $\pm$ 0.86	254.65 $\pm$ 2.8	0.24 $\pm$ 0.016	-1.55 $\pm$ 0.041	-2.18 $\pm$ 0.038	-27.52 $\pm$ 0.13	-1.08 $\pm$ 0.32	34.71 $\pm$ 1.2
	Canopy	Bottom	18.56 $\pm$ 1.0	240.75 $\pm$ 5.9	0.25 $\pm$ 0.015	-1.12 $\pm$ 0.055	-1.80 $\pm$ 0.064	-28.12 $\pm$ 0.12	-0.21 $\pm$ 0.26	33.82 $\pm$ 1.0
		Middle	20.45 $\pm$ 1.1	239.38 $\pm$ 4.4	0.27 $\pm$ 0.016	-1.13 $\pm$ 0.055	-1.97 $\pm$ 0.062	-27.63 $\pm$ 0.14	-0.47 $\pm$ 0.26	31.80 $\pm$ 0.92
		Top	20.64 $\pm$ 1.5	234.17 $\pm$ 6.8	0.25 $\pm$ 0.016	-1.10 $\pm$ 0.062	-1.82 $\pm$ 0.072	-26.63 $\pm$ 0.10	-0.14 $\pm$ 0.27	27.85 $\pm$ 0.90
CBR	Plot	E	15.60 $\pm$ 2.7	264.70 $\pm$ 21	0.21 $\pm$ 0.030	-1.15 $\pm$ 0.043	-1.83 $\pm$ 0.065	-27.79 $\pm$ 0.15	5.01 $\pm$ 0.29	16.36 $\pm$ 0.36
		F	24.60 $\pm$ 2.8	203.90 $\pm$ 16	0.26 $\pm$ 0.024	-1.05 $\pm$ 0.047	-1.56 $\pm$ 0.067	-28.27 $\pm$ 0.13	2.10 $\pm$ 0.14	21.80 $\pm$ 0.70
		G	27.23 $\pm$ 1.9	249.47 $\pm$ 4.0	0.40 $\pm$ 0.031	-0.89 $\pm$ 0.037	-1.85 $\pm$ 0.057	-27.65 $\pm$ 0.11	5.67 $\pm$ 0.22	19.00 $\pm$ 0.39
		H	18.95 $\pm$ 2.0	211.71 $\pm$ 17	0.19 $\pm$ 0.021	-1.14 $\pm$ 0.039	-2.06 $\pm$ 0.057	-27.49 $\pm$ 0.12	-2.30 $\pm$ 0.12	28.12 $\pm$ 0.58
	Month	June	30.83 $\pm$ 1.6	190.89 $\pm$ 8.7	0.33 $\pm$ 0.025	-0.85 $\pm$ 0.034	-1.47 $\pm$ 0.043	-27.57 $\pm$ 0.12	3.60 $\pm$ 0.47	20.11 $\pm$ 0.65
		July	31.58 $\pm$ 1.9	189.27 $\pm$ 15	0.36 $\pm$ 0.040	-0.99 $\pm$ 0.037	-1.97 $\pm$ 0.054	-28.09 $\pm$ 0.11	3.09 $\pm$ 0.45	22.87 $\pm$ 0.85
		August	11.06 $\pm$ 1.0	293.96 $\pm$ 11	0.18 $\pm$ 0.012	-1.28 $\pm$ 0.044	-2.09 $\pm$ 0.064	-28.00 $\pm$ 0.12	2.72 $\pm$ 0.47	20.67 $\pm$ 0.71
	Canopy	September	–	–	–	-1.11 $\pm$ 0.039	-1.75 $\pm$ 0.070	-27.56 $\pm$ 0.16	1.17 $\pm$ 0.37	21.49 $\pm$ 0.82
		Bottom	18.19 $\pm$ 1.8	232.95 $\pm$ 14	0.24 $\pm$ 0.022	-1.14 $\pm$ 0.038	-1.89 $\pm$ 0.057	-28.45 $\pm$ 0.09	2.63 $\pm$ 0.40	21.88 $\pm$ 0.61
		Middle	24.30 $\pm$ 2.2	254.16 $\pm$ 13	0.32 $\pm$ 0.027	-1.05 $\pm$ 0.041	-1.80 $\pm$ 0.054	-27.97 $\pm$ 0.08	2.44 $\pm$ 0.39	21.31 $\pm$ 0.66
Top	24.03 $\pm$ 2.2	211.20 $\pm$ 12	0.27 $\pm$ 0.028	-0.98 $\pm$ 0.032	-1.78 $\pm$ 0.059	-27.00 $\pm$ 0.10	2.82 $\pm$ 0.40	20.68 $\pm$ 0.73		

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