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Plasticity tradeoffs in salt tolerance mechanisms among desert *Distichlis spicata* genotypes

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Abstract. We investigated genetic differences in salinity tolerance among 20 saltgrass (*Distichlis spicata* (L.) Greene) genotypes, including constitutive, gender-based and phenotypic plasticity traits, to better understand the basis of adaptation and acclimation by saltgrass in diverse environments. On average, the plants survived NaCl treatments up to ~1 M, with reductions in growth and health that varied with genotype. For these 20 genotypes in a greenhouse study, we showed that greater plasticity in one salt tolerance mechanism was physiologically linked to lesser plasticity in another. Under various levels of constant salinity stress, genotypes employing a strategy of greater plasticity in foliar Na and lesser plasticity in both foliar K : Na and Na turnover rate were better able to substitute Na for K in some cellular functions, especially osmotic adjustment, leading to increased salinity tolerance. Although we observed gender segregation with salinity in the Owens (Dry) Lake Playa (Inyo County, CA, USA) population planted for dust control, from which the genotypes were collected, we did not observe gender differences in salinity tolerance in the greenhouse. Significant physiological plasticity tradeoffs among genotypes, however, did affect overall salinity tolerance and may be important for this species survival in diverse managed and natural habitats.

Additional keywords: dioecy, dust mitigation, osmotic adjustment, potassium, restoration, salinity, saltgrass, sex ratio, sodium.

Introduction

As sessile organisms, plants must deal with environmental heterogeneity either through adaptation, which occurs over multiple generations, or through acclimation – changes in physiology of an individual. Acclimation is also referred to as phenotypic plasticity: the capacity of a given genotype to express different phenotypes in different environments (Sultan 2000). Degree of phenotypic plasticity, or acclimation potential, in a trait is itself inherited and hence subject to selective forces (DeWitt and Scheiner 2004). Environmental stresses that are constant (e.g. high soil heavy metal concentration) should theoretically select for constitutively expressed stress tolerance mechanisms (i.e. expressed in all environments, even in control plants that have not been exposed to the stressor). In contrast, temporally or spatially-varying stress (e.g. pathogen attack) should select for greater plasticity in stress tolerance responses (i.e. response altered by environmental influences; Bradshaw and Hardwick 1989). However, multiple traits are involved in stress responses, and greater plasticity in one trait may be physiologically linked to lesser plasticity in another trait. Overall assessment of biomass production or reproductive output does not by itself provide insight into the physiological tradeoffs that must be understood to determine the effects of selection on traits or trait plasticity. This is because physiological plasticity may underlie morphological stability (Bradshaw 1965) and *vice versa*.

Saltgrass (*Distichlis spicata* (L.) Greene) is a dioecious, C_4 halophytic grass native to inland habitats (mesic to desert) from Saskatchewan, Canada to Patagonia, Argentina and also native to tidal marshes of the East, South and West Coasts of North America (Ram *et al*. 2004). Though morphological differences occur among coastal and inland accessions, genetic evidence indicates that they should not be classified as different species (Ram *et al*. 2004). Given its broad geographic distribution and the wide range in daily, seasonal and annual fluctuations in salinity and evaporative demand in its native range, saltgrass may be expected to display a great deal of local adaptation and/or a high degree of phenotypic plasticity. Indeed, it has both significant genetic variation and phenotypic plasticity in various traits (e.g. Ram *et al*. 2004; Marcum *et al*. 2007; Rukavina *et al*. 2008; Christman *et al*. 2009).

Known mechanisms of salinity tolerance in saltgrass include sodium (Na) exclusion at the root, selectivity for potassium (K) over Na uptake (Smart and Barko 1980), osmotic adjustment (lowering vacuolar water potential with Na and other solutes and producing compatible solutes such as

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glycinebetaine to lower cytoplasm water potential an equivalent amount, Marcum 1999), and Na exudation through foliar salt glands (Hansen *et al*. 1976; Marcum 1999). The degree to which phenotypic plasticity of salt tolerance mechanisms varies among genotypes and genetic variation in physiological tradeoffs among these mechanisms has not been studied.

Saltgrass segregates by gender along environmental gradients (Freeman *et al*. 1976; Eppley 2001). Consistent with the results by Freeman *et al*. (1976) for natural populations, the saltgrass genotypes that we randomly selected for this study appear to be segregated with salinity at the Owens (Dry) Lake Playa[1] (Inyo County, CA, USA) dust mitigation planting site (Dickey *et al*. 2005), with males being more prevalent on more stressful areas and the opposite being the case for females (see 'Results'). Thus, we hypothesised that male genotypes would exhibit greater salinity stress tolerance than female genotypes in a controlled-environment study. Although gender differences in salt tolerance have not been studied for saltgrass, gender differences in physiology have been demonstrated in other dioecious plants that segregate by gender along environmental gradients (Dawson and Ehleringer 1993; Correia and Barradas 2000; Dudley and Galen 2007).

Because saltgrass is increasingly planted for restoration and management (Dickey *et al*. 2005; Rukavina *et al*. 2008; Sargeant *et al*. 2008), it is important to understand relative differences in genetic variability and phenotypic plasticity of source material stress tolerance. Further, understanding how mechanisms of salinity tolerance correlate across genotypes and during acclimation provides insight into the physiological links among the various mechanisms and helps explain the species' success in diverse environments. The objectives of this study were to (1) quantify differences in known physiological mechanisms of salinity tolerance between the two genders and among 20 genotypes (10 of each gender) from the population planted for dust control on the Owens Lake Playa and (2) investigate the effect of genetic differences in plasticity (genotype by environment interactions) of these mechanisms on salinity tolerance under conditions of relatively constant salinity stress. It was hypothesised that saltgrass genotypes vary in salt tolerance and that this variation is underlain by differences in plasticity in various tolerance mechanisms. We wished to identify which mechanisms varied most among genotypes and elucidate how these related to salt tolerance under the conditions imposed. Theory predicts that genotypes with less plastic/more constant stress response mechanisms should show the greatest salt tolerance under relatively constant salt stress. We hoped to introduce more nuance to this theory by showing physiological linkages between greater plasticity in some salt tolerance mechanisms and lesser plasticity in others, with greater fitness linked more to overall strategies than to plasticity or lack thereof. An understanding of how plasticity in stress response relates to fitness of individuals and populations is critical for understanding genetic variation in natural saltgrass populations as well as for use and management of saltgrass in restoration or mitigation applications.

Materials and methods

Plant collection

The Owens (Dry) Lake Playa is located between the Sierra Nevada and Inyo Mountains in Inyo County, CA, USA (30°N, 118°W; 1083 m elevation; mean annual reference evapotranspiration (ET_0) of 1580 mm, mean annual precipitation is 137 mm; Breen and Richards 2010). This saline basin lake has almost completely dried following diversion of the Owens River to the Los Angeles Aqueduct in 1913. Because dust from the dry lake bed became a major source of airborne particulate matter, a massive dust control project was initiated in the late 1990s. This included 8.5 km^2 of saltgrass planted in numerous ~16 ha fields. Seed for this planting was collected from many (>80) saltgrass populations in the Owens Valley and on the margins of the dry lake playa. Seed was increased using open pollination of female plants by male plants in the collection through one generation on local seed farms. Harvested seed was grown in ~3 mL plugs for 11 weeks before planting on the playa in July 2002 (Dickey *et al*. 2005). About 30 million plugs were produced and planted. Although drainage and buried drip-irrigation systems were installed in the dust control fields to control salinity levels and provide nutrients, some fields initially fared better than others. The 20 saltgrass genotypes used in this study were 10 females and 10 males selected randomly within each gender from a larger group of 40 genotypes. This larger group was collected randomly, two per field, in March 2004, from 20 Owens Lake Playa fields showing either good establishment or heavy mortality of planted saltgrass. Gender was determined by observation of flowering tillers, either in the field or upon subsequent propagation in the greenhouse. Soil salinity (saturated paste electrical conductivity, EC_e) of the source fields was assessed monthly within 15 cm of drip-line emitters during June–August 2005 by Eathrworks Restoration (now NewFields Agricultural and Environmental Resources, LLC, Los Angeles, CA, USA) as a part of the ongoing monitoring associated with the dust control planting. Earthworks also assessed field condition as 'good' (>75% of plugs surviving), 'average' (25–50% of plugs surviving) or 'poor' (<25% of plugs surviving). Between collection (2004) and experiment initiation (2007), each genotype was vegetatively propagated to 24 individuals per genotype in a greenhouse at UC Davis using a 1 : 1 mixture of sand and heat-treated montmorillonite clay (Profile Products LLC, Buffalo Grove, IL, USA). Plants were held outside for the winter and early spring before beginning the experiments in March of 2007. All existing tillers were trimmed to 2 cm before experiment initiation.

NaCl treatments

In Experiment 1, 20 saltgrass genotypes (10 male, 10 female) were automatically watered twice daily with one of six NaCl concentrations (7, 150, 250, 350, 450 and 550 mM). Watering volume was adjusted as needed over the course of the experiment such that pots dripped profusely from below, indicating delivery of a volume sufficient to flush soil solution from pots and thereby prevent concentration of salts beyond

[1]Playa is a term for the flat-floored bottom of an undrained desert basin that periodically becomes a shallow lake.

treatment levels. Nutrient solution (a modified half-strength Hoagland's) was delivered separately three times per week between the two daily salt treatments. A control treatment of 7 mM NaCl rather than 0 mM NaCl was chosen to assure the micronutrient Na levels needed by some C_4 plants (Marschner 1995). To avoid potential salt shock, NaCl treatment solutions were not initially applied at full strength, but were gradually increased in 20% increments over a period of 18 days and then held steady for 6 weeks, mirroring conditions these saltgrass genotypes might experience in the field. This growing time reflects the length of time it took for many control plants to fill their 2.6 L pots (see Fig. 1). Plants were grown in a greenhouse at UC Davis, where they were arranged in a randomised complete block design with four blocks (six treatments × 20 genotypes × four blocks = 480 plants). Experiment 2 was a smaller follow-up study designed to test the limits of salinity tolerance. It used six of the initial 20 genotypes (three male, three female) that represented the full spectrum of salinity tolerance based on their performance in Experiment 1 (i.e. that showed low, average and high growth reduction at 550 mM NaCl relative to control). Plants were grown outside in Davis, CA in September and October of 2007. They were hand-watered twice daily with one of four NaCl concentrations (7, 550, 900 and 1100 mM) and arranged with four replicates in a split-plot design with replicate as the main plot and treatment as the subplot (four treatments × six genotypes × four replicates = 96 plants). Treatment solutions were gradually increased in 20% increments over 11 days and then held steady for 4 weeks. This shorter growing time reflected the need to harvest plants shortly after tiller death in the highest salinity treatments. Because of the somewhat different conditions of the two experiments, comparisons between them were limited and were made on values relative to controls for each experiment rather than on absolute values. Experiment 2 data were used only to determine limits of tolerance and are reported only in Fig. 2, because genotype and gender differences in physiology were better addressed by Experiment 1, which included many more genotypes.

Growth measurements

We measured leaf and stem lengths, recorded the number of live and dead leaves, and evaluated the condition of the youngest fully-expanded leaf of two tillers in every pot (three tillers in Experiment 2 owing to the smaller number of plants). Growth measurements were initiated on emergent tillers just after treatments reached full strength and repeated on the same tillers after 6 weeks of full strength treatments (4 weeks in Experiment 2). Growth during the full strength treatment period was determined by subtraction of initial from final measurements. Biomass of dried tillers collected after 6 weeks of full strength treatments in Experiment 1 was also measured. After Experiment 2 ended, all pots were watered with deionised water for 4 weeks to qualitatively observe potential recovery of 'dead' plants from belowground parts.

Water relations

Pre-dawn leaf total water potential (Ψ_w) and solute potential (Ψ_s) and estimated turgor (Ψ_p) and osmotic adjustment were measured after 5 weeks of full strength treatments for 10 randomly selected genotypes (five of each gender) for all four replicates of three treatments (7, 350 and 550 mM) in Experiment 1. On the evening before measurement, plants were washed thoroughly with deionised water to remove external salt and covered with plastic bags to assure maximum relative water content at the treatment soil water potential (Ψ_{soil}); because soil was watered twice daily to dripping, we calculated Ψ_{soil} based on solute concentration only. On the morning of measurement, leaves were harvested, wiped dry and placed in thermocouple psychrometer chambers. Chambers were sealed and suspended in an insulated water bath, and leaf Ψ_w measurements were logged every 30 min over a 24 h period. Average readings were taken when measurements stabilised. Sealed psychrometer chambers were then frozen twice in liquid nitrogen, causing cells to lyse, and replaced in the water bath, allowing Ψ_s to be measured over the following 24 h

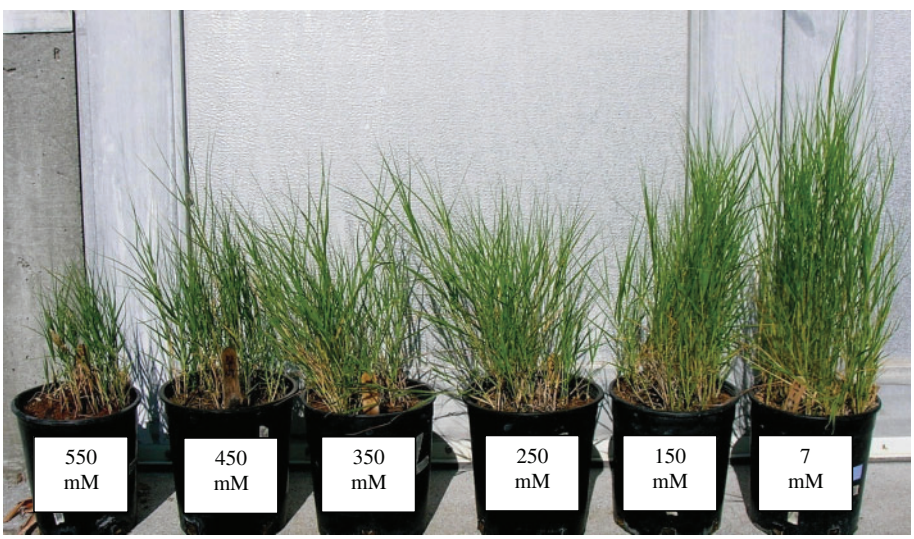


Fig. 1. Saltgrass plants of genotype 21 in 2.6 L pots at all Experiment 1 NaCl treatment levels, illustrating a typical response to treatments.

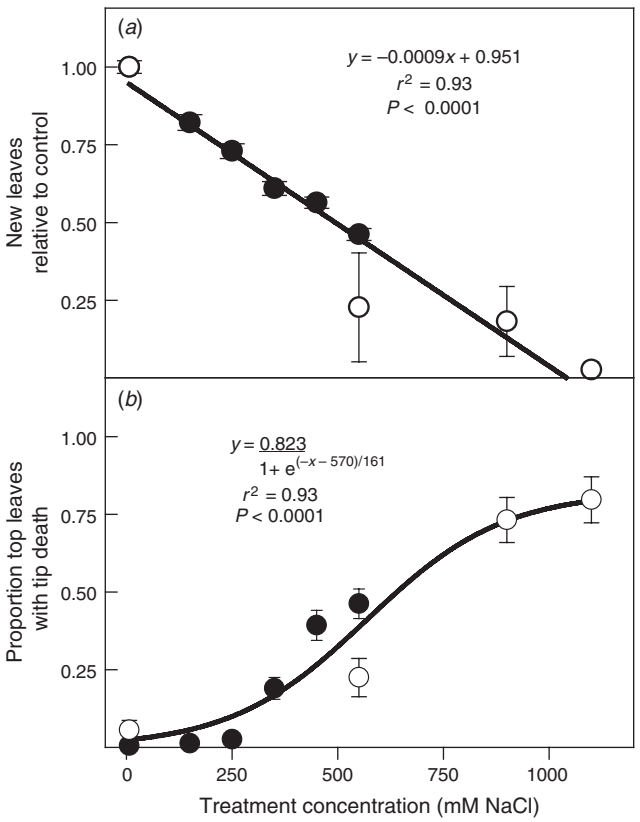


Fig. 2. Combined results for both experiments showing NaCl treatment effects on means and s.e. for saltgrass (*a*) new leaf production and (*b*) tip death. Closed circles represent Experiment 1 ($n = 80$), open circles represent Experiment 2 ($n = 24$) with fewer genotypes and higher salt concentrations. Note that the open circle in upper left of (*a*) covers a closed circle in the same plotted location.

period. Ψ_p was calculated as the difference between Ψ_w and Ψ_s, and osmotic adjustment was calculated as the sample Ψ_s subtracted from the mean Ψ_s of the four control plants for the same genotype.

Foliar Na exudation rates

Sodium exudation rates were determined concurrently for the same subset of plants by harvesting the youngest fully expanded leaf on a randomly-selected tiller 24 h after plant washing, swirling the harvested leaves in 45 mL of distilled demineralised water, and measuring Na concentration of the resulting solution by atomic emission spectroscopy (AAnalyst 200, Perkin-Elmer, Wellesley, MA, USA). Leaves were dried and weighed and exudation rate was calculated as mg Na kg^{-1} dry leaf s^{-1}.

Foliar Na and K concentrations

Leaves harvested concurrently with those harvested for exudation rate were washed, oven-dried, weighed and placed in crucibles in a muffle furnace at 450°C for 6 h. The remaining ash was dissolved in 25 mL of 0.1 M HCl. Na and K concentrations of these solutions were measured by emission spectroscopy. Leaf molality due to Na and K was calculated by dividing foliar elemental concentrations (converted to mol kg^{-1}) by a representative leaf water content ((FW – DW)/DW). The approximately linear relationship between NaCl or KCl molality and Ψ_s at 25°C (Campbell and Gardner 1971) was used to estimate leaf Ψ_s due to Na, K and counter-ions. This assumed that the counter-ion was chloride or dissociated from Na and K to the same degree as chloride. The proportion of osmotic adjustment due to Na for the 550 mM treatment was calculated as ((sample Ψ_s due to Na – mean control Ψ_s due to Na)/osmotic adjustment). Net K *v.* Na selectivity was calculated as the molar K : Na foliar ratio divided by the K : Na ratio of the soil solution (in this case, Na treatment/3 mM K treatment) (Flowers and Colmer 2008).

Foliar Na turnover rates

Turnover rate was calculated as Na exudation over 24 h divided by foliar Na concentration, yielding the number of times per day that an amount equivalent to all the foliar Na was exuded through the salt glands. Although the term 'turnover rate' may not be entirely accurate because it is not known which Na pools may be exuded versus retained, this term provided economy of expression in reference to a useful concept – the ratio of Na exuded to foliar Na.

Statistical analyses

Linear and non-linear correlations among salt tolerance indices and treatment concentrations in experiments 1 and 2 were calculated in SigmaPlot (Systat Software Inc., Chicago, IL, USA). Experiment 1 was analysed using mixed model ANOVAs in JMP version 5.0.1 (SAS Institute, Cary, NC, USA) with independent variables treatment, gender, genotype (random and nested within gender), block (random), all possible 2-way interactions, and one 3-way interaction (block × gender × treatment). The other 3-way interaction, block × genotype × treatment, comprised the residual error. Data for tiller biomass relative to control and stem length were square-root transformed to satisfy assumptions of normality and homogeneity of variance. When data were collected for all six treatments (tiller biomass relative to control, leaf length, stem length), treatment salinity was designated as a continuous variable. When data were collected for only three treatment concentrations (leaf water relations, Na exudation and turnover rates, foliar Na and K concentrations), treatment salinity was designated as a discrete variable. Tukey–Kramer pairwise comparisons were used to further evaluate significant treatment differences for these variables. We ran Spearman rank order correlations on genotype means for cation relations and biomass and performed linear regression on genotype means for biomass relative to control *v.* proportion of osmotic adjustment due to Na at 550 mM NaCl.

Results

Field results

For the field saltgrass population from the Owens (Dry) Lake Playa dust mitigation site that we assessed, median soil salinity (saturated paste electrical conductivity (EC_e)) at locations where male genotypes were collected (4.2 dS m^{-1}) was significantly higher than at locations where female genotypes were

collected (median 3.3 dS m^{-1}; $P = 0.037$; Mann–Whitney U-test, $n = 10$ each gender). In addition, females represented 31% of the genotypes in areas that had poor vegetation development and 56% of the genotypes in areas that had good vegetation development.

Growth

Higher salinity treatments decreased growth and increased tip death on the most recently-formed leaves in Experiments 1 and 2 (Figs 1, 2). Combined data on new leaf production rate (one key index of salt tolerance; Munns and Termaat 1986) from both experiments suggest that leaf production fell to 90% of control at ~54 mM NaCl and reached 0% at ~1040 mM NaCl. There did not appear to be a threshold response, but rather a linear decrease as salinity rose above control values. Leaf tip death (another index of salt tolerance) did show a threshold response, with tip death occurring only at very low levels for treatments <300 mM and increasing rapidly for concentrations >400 mM.

There were significant genotype ($P < 0.0001$) but not gender ($P = 0.68$) differences in tiller biomass relative to control, another measure of salt tolerance (see Table S1 available as an Accessory Publication to this paper). We detected small but significant gender differences in average leaf length ($F > M$, Fig. 3*a*) and in average stem length (M > F), and the stem length difference decreased with increasing treatment salt concentrations (Fig. 3*b*).

Water relations

Increasingly concentrated salt treatments, which reduced Ψ_{soil} from –0.033 MPa at the control level to –2.54 MPa at 550 mM NaCl, significantly reduced leaf Ψ_w and Ψ_s and led to increased osmotic adjustment (Table 1). Ψ_p was not completely maintained but remained positive when Ψ_{soil} was –2.54 MPa in the 550 mM NaCl treatment. No significant gender ($P = 0.21$–0.53) or genotype ($P = 0.24$–0.87) differences were observed in any of these variables (Table S2), indicating that all genotypes were able to osmotically adjust to salt treatments.

Foliar ion relations

Foliar Na concentration, exudation rate and turnover rate all increased, and foliar K : Na decreased with increasing treatment salinity indicating phenotypic plasticity in these traits (Table S3; Fig. 4). In all cases, the 7 mM treatment mean for all genotypes combined was significantly different from the 350 and 550 mM treatments by Tukey–Kramer comparisons, but there were no significant differences between the 350 and 550 mM treatments. There were no significant

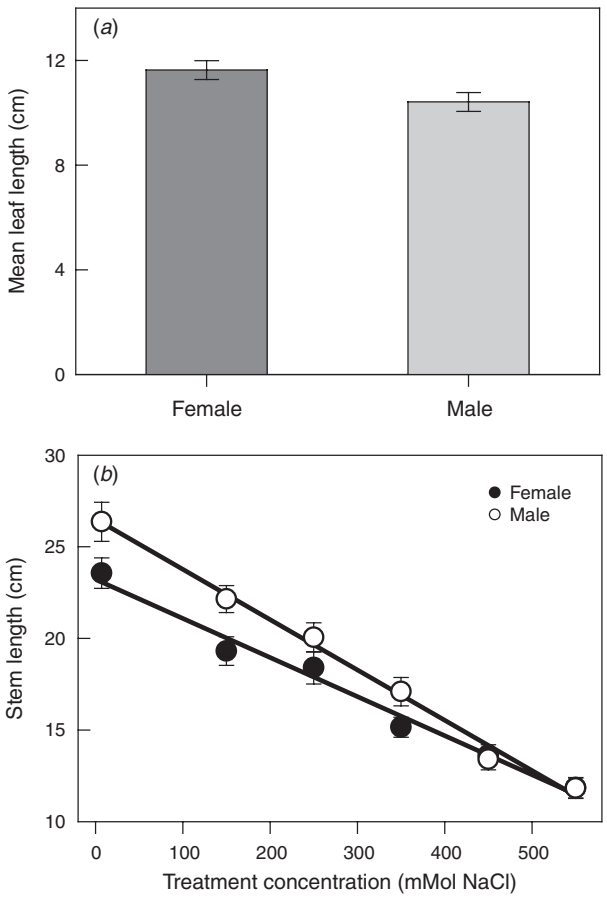
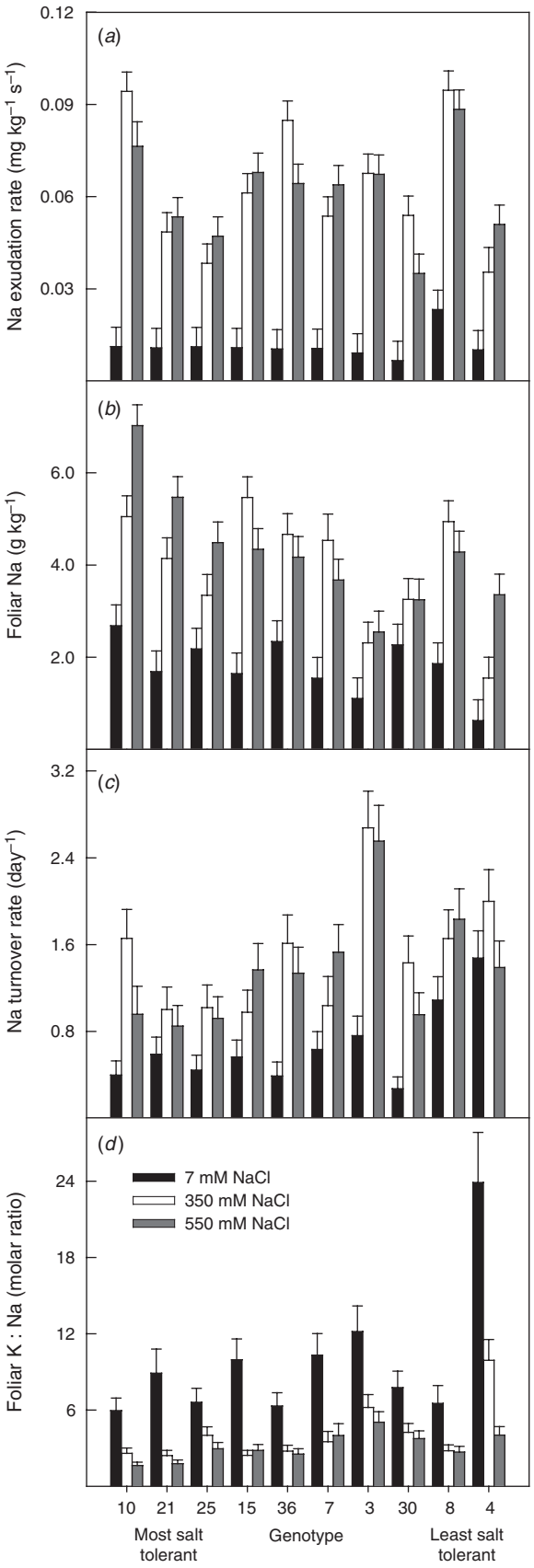


Fig. 3. Least-squares means and s.e. ($n = 240$) for saltgrass gender averages in (*a*) leaf length and means and s.e. ($n = 40$) for (*b*) gender averages in stem length by treatment in Experiment 1. Females had significantly longer leaves for all treatments, whereas males had longer stems, with the gender difference decreasing as treatment salinity increased.

Table 1. Mean Ψ_{soil} calculated from treatment concentrations and least-squares means and s.e. ($n = 40$) across all genotypes for leaf Ψ_w, Ψ_p, Ψ_s, osmotic adjustment, and for Ψ_s due to Na, K, and counter-ions, Experiment 1

Letters denote Tukey–Kramer comparisons made within columns. Treatments not connected by the same letter are significantly different ($P < 0.05$)

Treatment (mM NaCl)	Ψ_{soil} (MPa)	Leaf Ψ_w (MPa)	Leaf Ψ_p (MPa)	Leaf Ψ_s (MPa)	Osmotic adjustment (MPa)	Ψ_s due to Na and counter-ion (MPa)	Ψ_s due to K and counter-ion (MPa)	Ψ_s due to Na + K and counter-ions (MPa)
7	–0.03	–1.44a ±0.08	0.35a ±0.05	–1.77a ±0.05	0.00a ±0.05	–0.22a ±0.03	–1.82a ±0.03	–2.04a ±0.04
350	–1.61	–2.19b ±0.07	0.09a ±0.04	–2.30b ±0.04	–0.50b ±0.05	–0.49b ±0.03	–1.60ab ±0.03	–2.09a ±0.04
550	–2.54	–2.64c ±0.06	0.12a ±0.04	–2.76c ±0.04	–0.97c ±0.04	–0.53b ±0.03	–1.43b ±0.03	–1.96a ±0.04



gender differences in any of these variables ($P = 0.44$–0.93, Table S3). However, they all showed significant genotype differences ($P = 0.0005$–0.010) and genotype × treatment interactions ($P = 0.001$–0.024). This indicates that genotypes responded differently to treatments in how much they increased foliar Na levels and exudation and turnover rates or decreased K : Na ratios in response to increasing salinity (Table S3; Fig. 4). These significant interactions indicate genetic differences in phenotypic plasticity.

Although there was no correlation between genotype averages for exudation rate and foliar Na content at 550 mM ($r = 0.29$; $P = 0.40$), genotypes with higher Na turnover rates had lower foliar Na levels (Fig. 5*a*). Genotypes with lower turnover rate, higher foliar Na levels, and lower K : Na had the least growth reduction (and hence the greatest salt tolerance) at 550 mM, as evidenced by significant rank correlations between these variables and relative biomass (Fig. 5*b–d*). There was no relationship between genotype average osmotic adjustment at 550 mM and genotype average foliar Na concentrations at 550 mM ($r = 0.31$, $P = 0.39$, also recall that there were no significant genotype differences in osmotic adjustment). This indicates that greater osmotic adjustment did not explain better growth in genotypes with higher foliar Na concentrations. Although Ψ_s due to Na and counter-ion(s) became more negative and Ψ_s due to K and counter-ion(s) became less negative with increasing treatment salinity, Ψ_s due to combined Na and K remained relatively constant among treatments (Table 1). Genotypes for which Na provided a larger proportion of the measured osmotic adjustment at 550 mM showed greater salt tolerance, as shown by biomass at 550 mM NaCl relative to control biomass (Fig. 6). Although greater net K *v.* Na selectivity among genotypes correlated negatively with salt tolerance (Fig. 5*d*), overall average K *v.* Na selectivity was very high, particularly at the higher treatment concentrations (Table 2).

Discussion

The salt treatments in Experiment 1 reduced saltgrass growth substantially, although they did not push the plants to their limits of tolerance, as evidenced by their continued growth, greenness, and Ψ_w well above the –4 MPa level measured in highly stressed plants in the field by Alpert (1990; see also Fig. 1). The results of Experiment 2 agree well with other greenhouse studies (Marcum *et al.* 2005, 2007) showing limits of tolerance close to 1 M NaCl (approximately twice the concentration of sea water) for at least some accessions of this species. Nevertheless, all of the plants in Experiment 2, including those treated with 1100 mM NaCl that appeared ‘dead’ by the end of the experiment, grew new tillers with regular watering after salt treatments ceased. This indicates that some underground parts survived for 4 weeks even at these very high NaCl concentrations.

Fig. 4. Least-squares means and s.e. ($n = 4$) for significant treatment × genotype interactions in saltgrass (*a*) exudation rate, (*b*) foliar Na, (*c*) Na turnover rate and (*d*) foliar K : Na in Experiment 1. Genotypes are arranged in order of greatest to least salt tolerance (mean tiller biomass in the 550 mM NaCl treatment relative to control biomass).

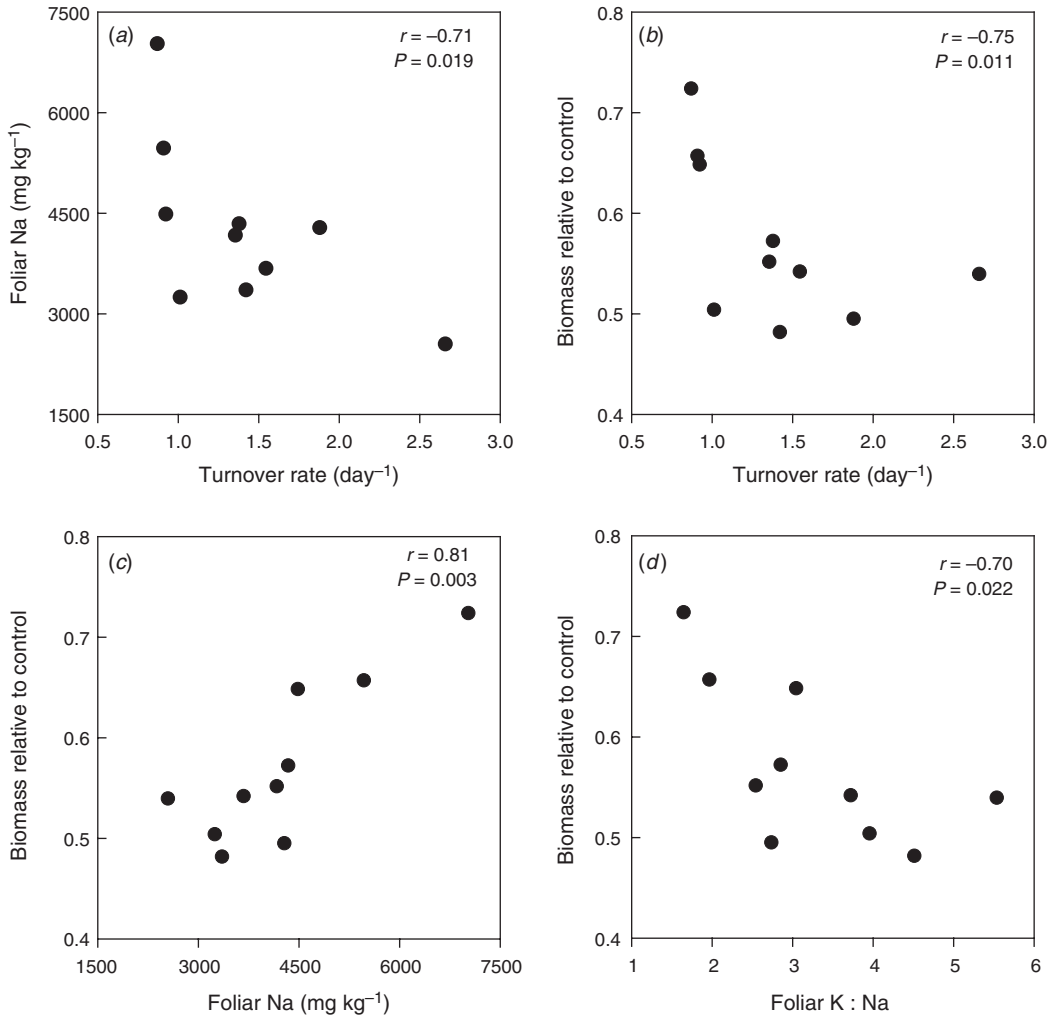


Fig. 5. Spearman rank correlations among saltgrass genotype averages ($n = 4$) at 550 mM NaCl for foliar Na, turnover rate, foliar K:Na, and biomass relative to control, Experiment 1. Biomass at 550 mM relative to control biomass is used as a measure of relative salt tolerance among genotypes.

Overall patterns in salinity tolerance mechanisms

Although the most salt tolerant genotypes continued to increase in foliar Na concentration as treatments increased from 350 to 550 mM NaCl (Fig. 4), the average foliar Na concentrations across all genotypes appeared to reach a limit and did not increase further with increasing salinity treatment between 350 and 550 mM (Table 1). The idea that Na reaches a limit in its contribution to Ψ_s is present in the literature (Glenn *et al*. 1996), and may be related to the maximum capacity of tonoplast and plasmalemma transporters to move Na leaking passively into the cytoplasm from the vacuole and apoplast back into these compartments (Jeschke and Hartung 2000).

Because Ψ_s attributed to the combined effect of K + Na ions remained relatively constant across treatments, whereas Ψ_s due to K became less negative with increasing Na treatment concentrations (Table 1), it appears that saltgrass substitutes Na for K in some cellular functions, especially osmotic adjustment. This substitution likely frees up K for other plant functions and allows for lower energy expenditure on Na exclusion and on selective uptake and transport of K. Na substituting for K in cellular functions, most commonly as an osmoticum in the vacuole but also as a counter-ion in long distance nitrate transport, has been observed in many other species (Subbarao *et al*. 2003). Genetic differences in substitution rates relating to improved plant performance under low K relative to Na availability (Marschner *et al*. 1981; Figdore *et al*. 1987, 1989) have also been observed in other species. This study is the first to our knowledge to document that such substitution occurs in saltgrass. It is also the first study we know of to relate genetic differences in substitution to genetic differences in salinity tolerance in any grass species (with greater substitution related to greater salinity tolerance). Salinity tolerance among genotypes in cultivated wheat species and some wild *Triticeae* tends to show the opposite pattern, with better salt tolerance correlating positively with higher K and lower Na (Colmer *et al*. 2006). Nevertheless, although greater K *v.* Na selectivity among saltgrass gentoypes was correlated negatively with salt tolerance (Fig. 5*d*), overall net K *v.* Na

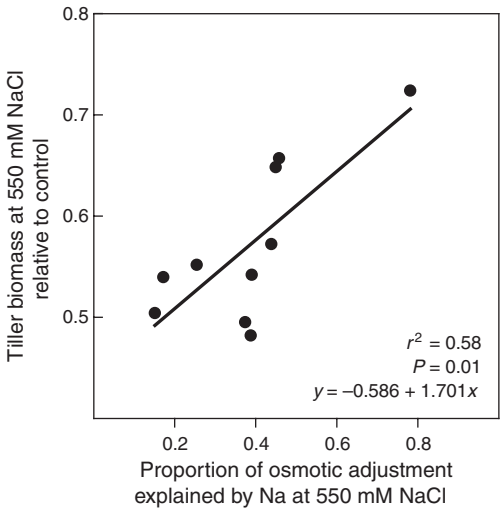


Fig. 6. Regression of saltgrass genotype average biomass ($n = 4$) relative to control on proportion of osmotic adjustment from Na at 550 mM NaCl, Experiment 1. Genotypes using more Na for osmotic adjustment had greater salt tolerance (measured as biomass at the highest treatment concentration, 550 mM NaCl, relative to control biomass).

Table 2. Leaf Na and K molality and net K:Na selectivity, Experiment 1
Least-squares means and s.e. ($n = 40$) for foliar Na and K osmolality (calculated from elemental concentrations and water content) and net K : Na selectivity averaged across all genotypes at three NaCl treatment concentrations. Note that K concentration of nutrient solution was 3 mM and foliage concentrations are for youngest fully-expanded leaves

Treatment (mM NaCl)	Leaf Na molality ($mM\,kg^{-1}$)	Leaf K molality ($mM\,kg^{-1}$)	K : Na selectivity
7	49 ± 6	406 ± 7	19 ± 45
350	109 ± 6	357 ± 8	472 ± 46
550	118 ± 6	320 ± 7	592 ± 45

selectivity was very high relative to other halophytes (Flowers and Colmer 2008; Flowers *et al*. 2010), particularly at the higher treatment concentrations (Table 2). This indicates an important role for this mechanism allowing saltgrass to tolerate high salinity in the growth medium. In addition, the sampling of only the youngest fully-expanded leaf for this study likely contributed to the very high net K : Na selectivity levels measured here relative to those of other halophytes. This is because younger foliage tends to have higher K and lower Na than more mature foliage (Marschner 1995).

Average Ψ_s attributable to K + Na remained relatively constant among treatments, although overall average Ψ_s continued to decrease with increasing treatment solution salinity (Table 1). This indicates that as Na levels reached their tolerable limits and K became increasingly difficult to take up due to intensifying scarcity relative to Na, plants manufactured organic compatible solutes to continue decreasing Ψ_s sufficiently to take up water from soils of low Ψ_{soil} and maintain Ψ_p (Flowers 1985). This phenomenon has been previously documented in saltgrass (Marcum 1999).

Genotypic differences in phenotypic plasticity of salt tolerance mechanisms

Foliar Na, foliar Na turnover rates and foliar K : Na ratios are a suite of mechanistically linked traits that showed (1) plasticity (varied with salt concentration of medium), (2) inheritance (significant differences among genotypes), (3) inherited plasticity (significant treatment (environment) × genotype interactions) and (4) an influence on salinity tolerance under the experimental conditions. Genotypes with higher foliar Na, lower foliar Na turnover rates and lower foliar K : Na ratios at 550 mM NaCl achieved the greatest salt tolerance at this concentration by substituting Na for K and thus completing a greater proportion of the necessary osmotic adjustment (which did not vary among genotypes) with Na. We hypothesise that this is the most energetically favourable strategy for osmotic adjustment under the conditions imposed. Stated in terms of plasticity, genotypes that showed greater plasticity in foliar Na and lesser plasticity in both Na turnover rates and K : Na selectivity were the most salt tolerant in this study. Nevertheless, it is possible that these results are dependent upon the relatively constant salt stress imposed. For example, it seems possible that if similar salinity levels were imposed in a widely fluctuating pattern, genotypes with higher turnover rates might have been better able to continuously adjust to that kind of rapidly changing environment. This alternative strategy might be selected for in locations with variable salinity levels, unlike the consistently high salinity of the Owens Playa. The multigenic nature of salinity tolerance (Flowers *et al*. 2010) might facilitate selection for different relative uses of the various tolerance mechanisms in different environments.

Another related trait – Na exudation – showed phenotypic plasticity (significant genotype × environment interaction) but was not correlated with salinity tolerance in this study ($r = 0.13$, $P = 0.71$, Spearman rank order correlation). This is in contrast to results obtained for salt-excreting grasses *Cynodon* and *Zoysia* (*Poaceae*), which had positive relationships between Na exudation rates and salinity tolerance (Marcum *et al*. 1998; Marcum and Pessarakli 2006). In addition, Na exudation rate was positively correlated with salt tolerance among Chloridoid grass genera (Marcum 1999).

Comparison with other saltgrass studies

The Na exudation rates measured for plants exposed to 550 mM were comparable to those of other saltgrass studies (Marcum *et al*. 2007; Christman *et al*. 2009). However, the measured foliar Na concentrations were lower and K levels higher than those studies. Foliar K : Na ratios were also higher than those measured in other studies (Hansen *et al*. 1976; Marcum *et al*. 2007). This may have occurred because younger leaves have lower Na and higher K levels than older leaves (Marschner 1995), and only the youngest fully-expanded leaf on a given tiller was sampled in this study, whereas the previously-cited studies sampled more mature foliage. More perplexing is the difference in pattern of K : Na ratio. Although the K : Na ratio was negatively correlated with salinity tolerance among the 10 genotypes sampled for this trait, the most salt tolerant genotype among eight genotypes in Marcum *et al*.'s (2007) study had the highest K : Na ratio (significantly higher than

the other seven, which did not vary significantly from each other), maintained low Na levels and had high Na exudation rates. This unique genotype in Marcum *et al*.'s (2007) study appears to possess a suite of traits that is opposite those we found to confer the greatest salt tolerance in Owens Lake Playa saltgrass (high foliar Na, low foliar K : Na and low turnover rate). We note that the unique genotype was the only one collected from a coastal estuary (San Diego, CA, USA). All others were collected in inland areas (Idaho or Utah, USA), though specific habitat types for these or the one commercially available cultivar were not specified (Marcum *et al*. 2007). These comparisons may indicate a possible difference in coastal and inland salt tolerance strategies or may simply highlight the difference in degree of genetic variability that can occur when collections are made at narrow versus broad spatial scales.

Na turnover rate

The term 'turnover rate' is used for economy of expression to refer to the biologically significant ratio between exuded Na and foliar Na, though it is not known specifically which pools of Na are retained *v.* exuded. Marcum *et al*. (2007) performed the same calculation, though they did not use this term (see below). Others have calculated % Na exuded (also referred to as excreted, secreted, or recreted), though calculation methods vary depending on the type of measurements made. Studies that measured or calculated fluxes of Na entering leaves through the transpiration stream showed 40% of Na uptake was exuded through salt glands for *Avicennia marina* (*Acanthaceae*; Waisel *et al*. 1986) and 50% for *Spartina alterniflora* (*Poaceae*; Bradley and Morris 1991). Taleisnik and Anton (1988) found that exudation accounted for 11% of radioactively-labelled Na introduced in a study of *Pappophorum philippianum* (*Poaceae*). Warwick and Halloran (1992) calculated % Na exuded as excreted Na/total Na (excreted + foliar) over the course of the study (6, 8, 13, 18 days) and found values ranging from 50 to 80% for *Diplachne fusca* (*Poaceae*), though they acknowledge that salt crystals were likely lost from leaf surfaces for longer experimental runs. Using Warwick and Halloran's calculation method, saltgrass genotypes in this study treated with 550 mM NaCl exuded 45–70% of Na over a 24 h period, with a mean of 55%.

The fastest Na turnover rates measured by Marcum *et al*. (2007) were 0.33 day^{-1}, whereas ours were >2.5 day^{-1}. This is due to the lower Na concentrations but similar exudation rates measured in this study relative to Marcum *et al*.'s (2007) and it suggests that although foliar Na may differ among leaves of different ages, exudation rate is similar among age classes, perhaps allowing younger leaves to adjust more rapidly to salinity driven changes in Ψ_{soil}. This may be part of the reason for the common pattern of senescence of older leaves before younger leaves with increasing salt stress (Munns and Termaat 1986). These high turnover rates also suggest that the proportion of osmotic adjustment due to Na storage in vacuoles can adjust rapidly to changing conditions.

Pre-dawn disequilibrium and K dissociation

This species showed substantial pre-dawn disequilibrium with leaf $\Psi_w = -1.44$ MPa in the low salt control treatment (7 mM NaCl; $\Psi_{soil} = -0.033$ MPa; Table 1) and no water stress. This low pre-dawn leaf Ψ_w was associated with leaves that had relatively high solute concentrations (49 mM Na and 406 mM K; Table 2). It seems unlikely that these solutes would be functioning in osmotic adjustment, and although K has many cellular functions, Na is needed only in micronutrient amounts for C_4 processes (Marschner 1995). Pre-dawn disequilibrium is a well documented phenomenon, particularly in halophytes (Donovan *et al*. 2001), that can be attributed in part to high concentrations of apoplastic solutes, particularly K and Na (James *et al*. 2006). Solutes needed for osmotic adjustment during the day may move into the apoplast at night to prevent cell damage from water uptake that might occur with high Ψ_{soil}, and the apoplastic solutes could account in part for the large pre-dawn disequilibrium.

At 7 mM NaCl, mean calculated leaf Ψ_s due to Na + K was –2.04 MPa, which was lower than the mean measured leaf Ψ_s of –1.79 MPa (Table 1). The measurement techniques used combine both apoplastic and symplastic solutes. However, 95% confidence intervals around each of these means nearly overlap (–1.95 to –2.13 and –1.67 to –1.91, $n = 10$ genotypes, $t = 2.228$). In addition, although most Na in plant cells is dissociated whether in symplast or apoplast, it is unlikely that all the measured K was simultaneously dissociated. As little as 12% of foliar K remaining undissociated (i.e. bound to sites on proteins, etc., and thus fulfilling other functions in the cell) would be sufficient to align calculated Ψ_s due to Na + K with measured Ψ_s.

Gender differences in morphology but not salinity tolerance

No gender differences in salinity tolerance were detected based on relative biomass (Table S1) or leaf production rate. This is unexpected given earlier findings of gender segregation along salinity gradients (Freeman *et al*. 1976) and our finding that males occurred on more saline sites than females in the planted population on the Owens Lake Playa. One possible explanation is that females and males differ in their tolerance of some other stress that co-occurs with high salinity under some environmental conditions. Low nutrients, drought, boron toxicity and sodicity (high ratio of Na to other available cations) are all possibilities. There is some very interesting evidence in the literature supporting the possibility of differences in low nutrient tolerance. In two different salt marshes, female majorities occurred on sites with higher soil nutrient content (Eppley 2000; Eppley *et al*. 2009). Females were tough neighbours, greatly reducing competitor height when grown in soils collected from female majority areas in one of these salt marshes (Eppley 2006). Higher rates of arbuscular mycorrhizal fungi (AMF) colonisation occurred in females than in males in field-collected roots from both salt marshes as well as in greenhouse-grown plants (Eppley *et al*. 2009), although the abundance of AMF spores was equal in male and female majority soils. Taken together, these studies suggest that females may compete better on 'good' sites despite higher nutrient requirements because of their cooperation with AMF fungi, whereas males, with their lower nutrient requirement, comprise the majority on poorer sites. Specific investigations

of gender differences in low nutrient tolerance in saltgrass and soil nutrient measurements in field locations showing gender segregation along salinity gradients are needed to support or refute this explanation. Although a more recent field study (Mercer and Eppley 2010) did not find evidence of physiological differences between genders in juvenile saltgrass, sample size for this comparison was low and mature plants were not tested, and so the possibility of physiological differences between genders was not ruled out.

We detected slight gender differences in leaf length (females > males, Fig. 3*a*). The stem length of males was greater than that of females at lower salt concentrations, and this difference diminished with increasing salt concentration (Fig. 3*b*). Morphological differences in stem length may be explained by different reproductive functions, as taller stems may be better positioned for wind distribution of pollen. Differences in leaf size may be related to leaf energy budgets. Eppley (2001) found that females grew at lower elevations than males in a salt marsh. Maricle *et al*. (2007) investigated eight C_4 salt marsh grass species and found that higher elevation marsh species had narrower leaves than lower elevation marsh species. Narrower leaves allow higher elevation marsh species to regulate leaf temperature with less access to water for evaporative cooling because a reduced dimension leads to reduced boundary layer resistance to convective cooling.

Restoration and revegetation implications

Although gender differences in salinity tolerance were not detected, it is very interesting that a degree of gender segregation was encountered in the Owens Lake Playa population, which was planted in ratio of ~50 : 50, without regard for or knowledge of propagule gender. This, along with evidence of saltgrass gender segregation in natural populations (Freeman *et al*. 1976; Eppley 2001), suggests that for long-term viability of planted populations of this perennial grass, it might be advisable to monitor spatial patterns in gender to ensure individuals of both sexes are present. It might even be useful to facilitate sufficiently close physical proximity of genders to allow for pollination by creating patchy conditions in soil properties (e.g. salinity, elevation, nutrient levels). Although saltgrass is a prodigious vegetative propagator, sexual reproduction is needed for rapid evolutionary change (Ridley 2004), and segregation of sexes has been shown to decrease fecundity in some saltgrass populations (Eppley 2005).

These results also suggest caution when using foliar Na levels to monitor health in restoration plantings, because these varied not only with external environment salinity, but also with genotype. Plants with higher foliar Na may in fact be less stressed than those with lower foliar Na where external salt concentrations are high (Fig. 4*b*). If attempting to use foliar Na levels as a measure of plant stress in the field, it would be wise to sample from a large number of individuals to characterise a given area. Leaf Ψ_w measurements varied only with environmental salinity and not with genotype (Table S2), and might provide a more reliable measure of overall plant stress in the field.

Although a suite of traits that appear to confer the greatest salt tolerance under fairly constant salinity stress was identified in this study, it would be premature for managers to screen genotypes for planting based only on this set of traits. This is because plants in the field are subject to an array of stresses (e.g. salinity, boron toxicity, drought, flooding, low nutrients, heat, cold). These stresses may fluctuate in space or time (e.g. with seasonal patterns or management regime changes) and could favour different sets of traits than those which confer the best salinity tolerance under relatively constant salinity stress.

Conclusion

We found genetic variation in physiologically linked salt tolerance mechanisms, showing that a strategy of greater plasticity in foliar Na along with lesser plasticity in both foliar K : Na and Na turnover rate were related to an ability to substitute Na for K in some cellular functions, especially osmotic adjustment, leading to increased salinity tolerance under relatively constant salinity stress. To our knowledge, no previous study has shown Na substitution for K in saltgrass, and we also believe that no previous study with grasses has shown an improvement in salt tolerance with greater Na substitution for K. This finding also adds nuance to the theory that there should be lesser plasticity in response to a constant stress and greater plasticity in response to fluctuating stress, showing that greater plasticity in one stress response may in fact be physiologically linked with lesser plasticity in another. This finding makes distinctions on degree of plasticity in different environments difficult to substantiate. Although gender segregation with salinity in the planted Owens (Dry) Lake Playa population was observed in this study, and others have observed such segregation in natural populations, no gender difference in salinity tolerance was observed. We hypothesise that physiological differences between genders may instead occur in tolerance of another factor (e.g. low nutrient stress) that is often correlated with salinity in the field.

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