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Plasticity, Not Adaptation to Salt Level, Explains Variation Along a Salinity Gradient in a Salt Marsh Perennial

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Abstract Evolutionary ecologists have long been intrigued by the fact that many plant species can inhabit a broad range of environmental conditions and that plants often exhibit dramatic differences in phenotype across environmental gradients. We investigated responses to salinity treatments in the salt marsh plant *Borrichia frutescens* to determine if the species is responding to variation in edaphic salt content through phenotypic plasticity or specialized trait response. We grew seedlings from fruits collected in high- and low-salt microhabitats, assigned seedlings to high- and low-salt treatments in a greenhouse, and measured traits related to salt tolerance. All traits were highly plastic in response to salinity. Plants from the two microhabitats did not differ in trait means or respond differently to the treatments. These results suggest that environmental differences between the two microhabitats are not creating genotypes adapted to high and low salt levels. In addition, despite evidence for variation in allozyme markers in this population, there was no significant genotypic variation (family effect) in any of the trait means measured across microhabitats. There was variation in plasticity for only leaf Na and leaf B concentration. The high degree of plasticity for all traits and the lack of differences among microhabitats across the salinity gradient suggest plasticity in many traits may be fixed for this species.

Keywords Boron · Sodium · Adaptive plasticity · Local adaptation · Optimal reaction norm · Phenotypic plasticity · Salt-tolerance traits · Specialization

Introduction

Evolutionary ecologists have long been intrigued by the fact that many plant species can inhabit a broad range of environmental conditions and that plants often exhibit dramatic differences in phenotype across environmental gradients. Researchers working to understand this relation-

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ship have typically focused on two strategies to account for phenotypic differences: specialization of traits that are adapted to local conditions (Turesson 1922; Clausen et al. 1948) and phenotypic plasticity, where different morphologies are produced from the same genotypes in different environments (Schmalhausen 1949; Bradshaw 1965). Although studies often present these as two opposing strategies, even individuals with highly specialized traits have at least a limited ability to adjust to local conditions through phenotypic plasticity. Moreover, a high degree of trait plasticity can also be favored by natural selection and could be adaptive if it allows an individual to maintain fitness in multiple habitats and overcome the passive limitations that are imposed in resource-limited environments (Pigliucci 2001; Sultan 2003; van Kleunen and Fischer 2005; Richards et al. 2006).

Tests of specialization and plasticity can be conducted through reciprocal transplant studies in the field or through studies under controlled conditions such as in the greenhouse (Kawecki and Ebert 2004). Reciprocal transplant experiments are well suited to test for local adaptation, but they cannot identify the specific environmental agent responsible for any differences. While greenhouse studies dramatically simplify the environmental factors of interest, they can provide evidence for specialization and local adaptation when individuals from a given habitat have higher overall fitness than individuals from other habitats in response to the appropriate level of an environmental factor (Pigliucci 2001, but see Valladares et al. 2007). For example, a plant adapted to a low light environment would have higher fitness than a plant from a high light environment under a low light treatment. This type of study will also estimate the significance of plasticity of traits as indicated by an environmental effect in an analysis of variance. Plasticity is visualized graphically as a "reaction norm" of the trait responses across different levels of the environmental factor. Genetic variation in plasticity (genotype by environment interaction or G by E) can be acted upon by natural selection, and a plastic response can itself be an important adaptation (Pigliucci 2001; Richards et al. 2006). G by E for traits known to help organisms adjust to environmental variation (inducible defenses in environments with and without predators, Relyea and Auld 2004; shoot elongation response in light and shaded environments, Dudley and Schmitt 1996; Schmitt et al. 1999) provides evidence that there is the potential for selection to act on plasticity in these traits to produce an adaptive plastic response.

Many salt marsh plant species are considered halophytes and are equipped with a variety of traits that allow them to survive and reproduce under the toxic and osmotic effects of substrate salinity. These traits include maintaining nutrient uptake (nitrogen, cations such as potassium, calcium, magnesium, and manganese), conservative water use to reduce the water demands of the plant, increasing succulence and use of cations, or manufacture of N-rich compatible solutes for osmotic adjustment (Flowers et al. 1977; Cavalieri and Huang 1979; Antlfinger and Dunn 1983; Glenn and O'Leary 1984; Donovan et al. 1996, 1997; Moon and Stiling 2000; Rosenthal et al. 2002). Combined, these traits allow for favorable water status, photosynthetic carbon gain, and growth of halophytes in saline habitats.

Given this variety of traits, salt marsh plants are well suited to investigate how specialization and phenotypic plasticity contribute to phenotypic variation in natural populations. The tidal cycle combined with the topography of the marsh and differential evaporation results in relatively predictable patterns of salt accumulation across the marsh landscape. Many studies have examined how this environmental gradient results in predictable species zonation and community structure in salt marshes (reviewed in Pennings and Bertness 2001). Most of the salt marsh plant species have a high degree of phenotypic variation and occupy a broad range of environmental conditions (Pennings and Richards 1998; Pennings and Bertness 2001; Richards et al. 2005). *Borrichia frutescens* L. (Asteraceae; all nomenclature follows Radford et al. 1968) is one of these species that have among the widest environmental breadth and produce a range in phenotypes (Richards et al. 2005). In a study of natural field populations, we found that the height of mature individuals in this species ranged on a gradual cline from 12.5 to 99.7 cm (95% confidence interval), and number of leaves ranged from five to 147 among individuals found 20–50 m apart. The salinity of the soil occupied by these individuals was the strongest predictor of all of the traits measured, ranging from approximately 4 to 127 ppt. These natural populations were also made up of a diversity of genotypes (0.87–0.98 Simpson's index) with an average of 46% polymorphic allozyme loci and an average expected heterozygosity of 0.09 (Richards et al. 2004). We found no association of diversity or allele frequency differences with high- and low-salt microhabitats, and a common garden study showed that this species flowers in both microhabitats from mid-May to mid-July, indicating the potential for gene flow (Richards, unpublished data).

Many studies have shown that most species harbor genetic diversity for eco-physiological traits (Arntz and Delph 2001; Geber and Griffen 2003; Caruso et al. 2005). In this study, we investigated whether divergent selection is acting on eco-physiological traits in the plants growing at the ends of the salinity gradient by comparing the genotypic responses of plants from a high-salt microhabitat (midsummer soil pore water salinities of 100–120 ppt) growing near a highly saline salt pan and plants from a low-salt microhabitat (25–30 ppt) in the upper area of the marsh,

approximately 20 m away. We used controlled salinity treatments in a greenhouse to assess physiological, nutrition, growth, and fitness trait responses. These data allowed us to determine the degree of plasticity in the traits and the degree of genetic variation for the traits and their plasticities. We expected that plants from both microhabitats would exhibit phenotypic plasticity in response to salt treatments, but we predicted that strong divergent selection on important traits would have led to differentiation of genotypes found in different microhabitats (Levene 1953; Hedrick 1976). This prediction would be supported if plants from high-salt microhabitats perform better under high-salt conditions than plants from low-salt microhabitats, with the rankings reversing under low-salt conditions. Alternatively, these salt marsh habitats may favor phenotypic plasticity such that genotypes from both high-salt and low-salt microhabitats are equally able to adjust their phenotypes appropriately.

Materials and Methods

Study Site and Species

Sapelo Island is located on the Atlantic coast of Georgia, USA (31°28′ N, 81°14′ W). The vegetation patterns in Sapelo Island marshes are typical of southeastern marshes in the USA, with lower elevations dominated by *Spartina alterniflora* (Poaceae; Pennings and Bertness 2001; Richards et al. 2005). Higher elevations of the marsh are flooded irregularly and are characterized by a gradient of environments which range from lush meadows (salinities of 20–40 ppt) to highly saline salt pans (in excess of 100 ppt) over distances of 20–50 m (Richards et al. 2005). Salinity tolerance in *B. frutescens* is associated with Na accumulation and dilution through succulence and synthesis of the N-rich compatible solutes proline and glycine-betaine (Cavalieri and Huang 1979; Antlfinger and Dunn 1983; Moon and Stiling 2000). *B. frutescens* reproduces both clonally and sexually, and the relative contribution of each type of reproduction is unknown. Selfing rates estimated from genetic studies of *B. frutescens* suggest that when the plant reproduces sexually, it is mainly by outcrossing (Antlfinger 1982).

Sampling Design

We collected a dry floral head (infructescence) from ten plants separated by at least 2 m spanning approximately a 20 m^2 area in each of two microhabitats in the Cabretta Marsh on Sapelo Island. In a study of allozyme diversity, we found that ramets spaced more than 2 m apart were unlikely to belong to the same clone and that this population consists of a diverse set of genotypes (0.98 Simpson's index; Richards et al. 2004). Each floral head constitutes a maternal family of seeds that, due to the predominately outcrossing mating system, are at least half-sibs (Antlfinger 1982). Because these seeds were not produced in a controlled environmental setting, we cannot completely isolate the genetic component of the phenotype from the environmental component. We therefore refer to the responses, which include maternal or other environmental effects, as "genotypic," not "genetic." We examined seed mass, number of seeds produced, and germination rates as an indicator of the importance of maternal effects or environmental effects in our sample.

All seeds were removed from each floral head, and seed mass was recorded to the nearest 0.01 mg. Seeds were cold stratified for 7 days at 4°C. After stratification, seeds were planted in a completely randomized block design in individual 12 cm^3 wells in 72 well flats. We used a 1:1 mixture of sterilized sand and organic potting medium (Fafard #3B, Agawam, MA, USA). The flats were placed in a temperature and light-controlled greenhouse under conditions approximating early summer conditions in the salt marsh. Photoperiod was controlled at 14-h days and 10-h nights. Day temperature was maintained at 30°C and night temperature at 25°C. Pots were watered daily and fertilized weekly with half-strength Hydro-Sol solution, containing a nitrogen (N)/phosporus (P)/potassium (K) ratio of 5:11:26 (Scotts-Sierra Horticultural Products Company, Maysville, OH, USA). Germination was recorded daily for the first 24 days and weekly thereafter.

Experimental Treatments

We randomly selected 16 seedlings, approximately 6 weeks old, per family for seven maternal families originating from low-salt microhabitats and seven maternal families originating from high-salt microhabitats. Seedlings were transplanted into 15 cm diameter (1.6 L) plastic pots, filled with the same 1:1 mixture of sterilized sand and organic potting medium (Fafard #3B, Agawam, MA, USA), and placed on greenhouse benches in a randomized complete block design. Each spatial block consisted of 28 seedlings representing one of each family × salinity treatment combination. Salinity treatments consisted of two levels of NaCl concentration in water: low salinity (L) at 4 ppt and high salinity (H) at 40 ppt (sea water is approximately 32 ppt). Although 40 ppt is less than the maximum salinity observed in field soils, it was chosen because when seedlings establish in the spring, conditions in the field are characterized by much lower salinities than the extremes seen in midsummer (Pennings, unpublished data). We recorded individual seedling height at 6 weeks and initiated treatments. Salinity treatments were applied every

other day to completely flush the soil with the appropriate salinity level. To avoid shock, treatments were gradually increased from 1 to 4 ppt (L) and 10 to 40 ppt (H), reaching final salinity levels 2 weeks after treatment initiation. Thereafter, final salinity treatment levels were applied three times per week. Daily watering and weekly fertilization continued throughout the course of the experiment. However, on a daily basis, only enough water to saturate the soil was applied to minimize salinity loss. Live plants were harvested after 6 months. Flowering occurred in only the low-salt treatment and in only six families: three from the high-salt and three from the low-salt microhabitats.

Traits Measured

We measured four physiological traits, eight leaf nutritional traits and five growth traits related to salt tolerance and overall performance for each plant. Physiological traits were photosynthetic rate (Li-Cor Model LI-6400 Portable gas exchange system: Li-Cor-Inc., Lincoln, NB, USA), midday shoot water potentials (Model 1000 Pressure Chamber Instrument: PMS Inc., Corvallis OR, USA), succulence (g water in all leaves/cm^2 total leaf area), and integrated photosynthetic water use efficiency (WUE) as determined from stable carbon isotope ratios, $\delta^{13}C$ (Farquhar et al. 1982; Donovan and Ehleringer 1994). Photosynthetic rate and midday shoot water potentials were measured at the final harvest between 10:30 A.M. and 3:00 P.M. on three consecutive clear sunny days. To control for the effect of light quality variation within and between days, all plants across blocks were randomly assigned a number to identify the order in which their photosynthetic rates were measured, and 50–75 plants were measured each day. Photosynthetic rates were measured on the uppermost fully expanded leaf. Standard conditions for measurements were 350 μmol/mol CO_2, saturating light level 1,500 μmol m^{-2} s^{-1} and leaf temperature 28.7±0.2°C. Measurements were taken once leaf parameters had stabilized (<1% total system coefficient of variation, change of CO_2 and H_2O signals over time) after 3–5 min. For each plant, all live leaf tissue at final harvest was used for calculating succulence, and after drying, a subset of these leaves was used for leaf carbon isotopic composition (^{13}C) and leaf nutritional trait analyses. Leaves that were shed due to salt loading were excluded from all morphological, physiological, and nutritional measurements. Leaf $\delta^{13}C$ was measured with a continuous flow mass spectrometry (Finnegan, continuous flow mass spectrometer, Bremen, Germany). During photosynthesis, a combination of diffusion and enzymatic properties result in a discrimination against the heavier ^{13}C that is incorporated into plant tissue (Farquhar et al. 1982). When stomates close and/or photosynthetic capacity increases, the concentration of intercellular CO_2 decreases (reflecting increasing WUE), and discrimination against ^{13}C decreases. Thus, leaf $\delta^{13}C$ provides an integrated measure of leaf intercellular CO_2 and WUE over the lifetime of the leaf. Less negative values of leaf $\delta^{13}C$ indicate more assimilation of the heavier ^{13}C and higher integrated WUE.

Leaf nutritional traits were: Na, N, P, K, boron (B), calcium (Ca), magnesium (Mg), and manganese (Mn) concentration. Leaf N was measured on dried leaf material (Carbo Erba NA 1500 elemental analyzer, Milan, Italy). Leaf acid extracts (Sah and Miller 1992) were analyzed for B, Ca, K, Mg, Mn, Na, and P concentration on an inductively coupled plasma-atomic emission spectrophotometer (Thermo Jarrell-Ash Enviro 36: Thermo Electron Corp. Woburn, MA, USA).

Growth traits were: height, shoot dry biomass, root dry biomass, total dry biomass, and total leaf area (Li-Cor Model LI-3100 Leaf area meter: Li-Cor, Inc., Lincoln, NE, USA) at final harvest. Plants were dried in a forced air oven at 60°C for 72 h to determine components of dry biomass. We used total biomass as our measure of fitness, which is the best indicator of performance for a perennial plant (de Kroon and van Groenendael 1997).

Data Analysis

We used the SAS statistical package (version 9.1.3 for Windows) for all data analyses (SAS Institute, Cary, NC, USA). To determine if there were any pre-existing differences in seed quality between the source populations, *t* tests were used to test for differences between high-salt and low-salt families for average seed mass, average number of seeds, and percent germination (n=10 families for each microhabitat). Linear regressions were performed for mean seed mass on number of seeds, percent germination on number of seeds, and percent germination on mean seed mass.

To test the hypothesis that the different source populations were adapted to local salinity conditions, we examined the effects of microhabitat source, treatment, family, and block on trait values. For these analyses, we used multivariate analyses of covariance (MANCOVA; Sokal and Rohlf 1995) in PROC GLM with initial height (measured at 6 weeks before treatment initiation) as a covariate, to test for significance of differences in traits, given the correlation structure. Microhabitat source and treatment were treated as fixed factors, family and block were treated as random factors. Because of limited degrees of freedom, responses were analyzed in three groups for MANCOVA: (1) physiological traits, (2) leaf nutritional traits, and (3) growth traits. Height, total biomass, leaf area, shoot biomass, and root biomass were $\log_e$-transformed to meet the assumptions of normality. To determine the appropriate model for analysis, we constructed MANCOVA

models including the effects of microhabitat source, salinity treatment, family nested within microhabitat source, and the interaction between microhabitat and salinity treatment (Table 1, full model). In every case, the main effect of microhabitat was insignificant. We removed the effect of microhabitat source ($P>0.05$) for the final MANCOVA model that showed consistent significant effects of salinity treatment, family, and interaction between salinity treatment and family (Table 1, family model), with the exception of the leaf nutritional traits ($P=0.06$).

For each response variable, we also performed a separate univariate mixed model analysis of covariance (ANCOVA) using the type III method of PROC MIXED (Littell et al. 2006). For these univariate analyses, treatment was a fixed factor and family, block, and family by treatment interaction were designated as random.

Results

To test for potential confounding maternal or environmental effects, we examined differences in seed mass, number of seeds produced, and germination rates between the high- and low-salt microhabitats. Average seed mass per seed head ($8.73\pm1.4\times10^{-4}$g and 9.64 ± 0.50 g$\times10^{-4}$g), average number of seeds per seed head (57.6 ± 3.8 and 64.3 ± 5.0), and average germination rates (37.6 ± 5.5 and 46.4 ± 5.5 days) were not significantly different between families from high-salt and low-salt microhabitats. Linear regression showed no relationship between the total number of seeds produced per maternal family and the mean seed mass ($P=0.822$, $r^2=0.05$). There was also no relationship between total number of seeds produced and percent germination ($P=0.139$, $r^2=0.069$). Mean seed mass was positively related to percent germination ($P=0.029$, $r^2=0.195$), but overall there was no evidence for maternal effects or any other differences in seed quality between the source populations.

Genotypic Variation and Specialization to Salt Level in *B. frutescens*

Plants grown from seed originating from low-salt microhabitats were not different from those originating from high-salt microhabitats, as indicated by the lack of significance of the microhabitat effect in the nested MANCOVA (Table 1, full model). In all cases, the general phenotypic patterns were similar, as indicated by comparison of the reaction norms for all plants from the high-salt and low-salt microhabitats (Fig. 1). In addition, flowering occurred in only six families (23 individuals): three from the high-salt and three from the low-salt microhabitats. Plants from both microhabitats suffered mortality only in

Table 1 Multivariate tests of plasticity and specialization

Multivariate *F*	Full model						Family model					
	Microhabitat		Salinity treatment		Family (microhabitat)	Microhabitat × salinity treatment	Salinity treatment		Family		Family × salinity treatment	
	ndf/ddf		ndf/ddf		ndf/ddf	ndf/ddf	ndf/ddf		ndf/ddf		ndf/ddf	
Physiological traits	4/17	0.48 NS	4/17	55.77***	48/67.5	1.23 NS	4/17	1.60 NS	4/8	40.11***	52/33.1	1.69*	40/32.2	1.75*
Leaf Nutrition traits	8/37	1.79 NS	8/37	115.81***	96/259.5	1.47**	8/37	2.80*	8/30	98.48***	104/217.6	1.49**	64/179.53	1.36 NS
Growth traits	5/155	1.01 NS	5/155	368.86***	60/729.6	1.72***	5/155	1.18 NS	5/143	349.99***	5/143	1.70***	65/679.7	1.74***

Shown are MANOVAs for: full model including main effect of microhabitat and family model without the main effect of microhabitat.Traits are grouped as: (1) physiological traits include succulence, photosynthesis, midday shoot water potential, and water use efficiency; (2) leaf nutrition traits include leaf nitrogen (N), boron (B), calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), and phosphorous (P); and (3) growth traits include leaf area, height, shoot biomass, root biomass, total biomass. Multivariate *F* statistics are presented with significance levels.

ndf numerator degrees of freedom, *ddf* denominator degrees of freedom

*$P<0.05$; **$P<0.01$; ***$P<0.001$; NS $P\geq0.05$.

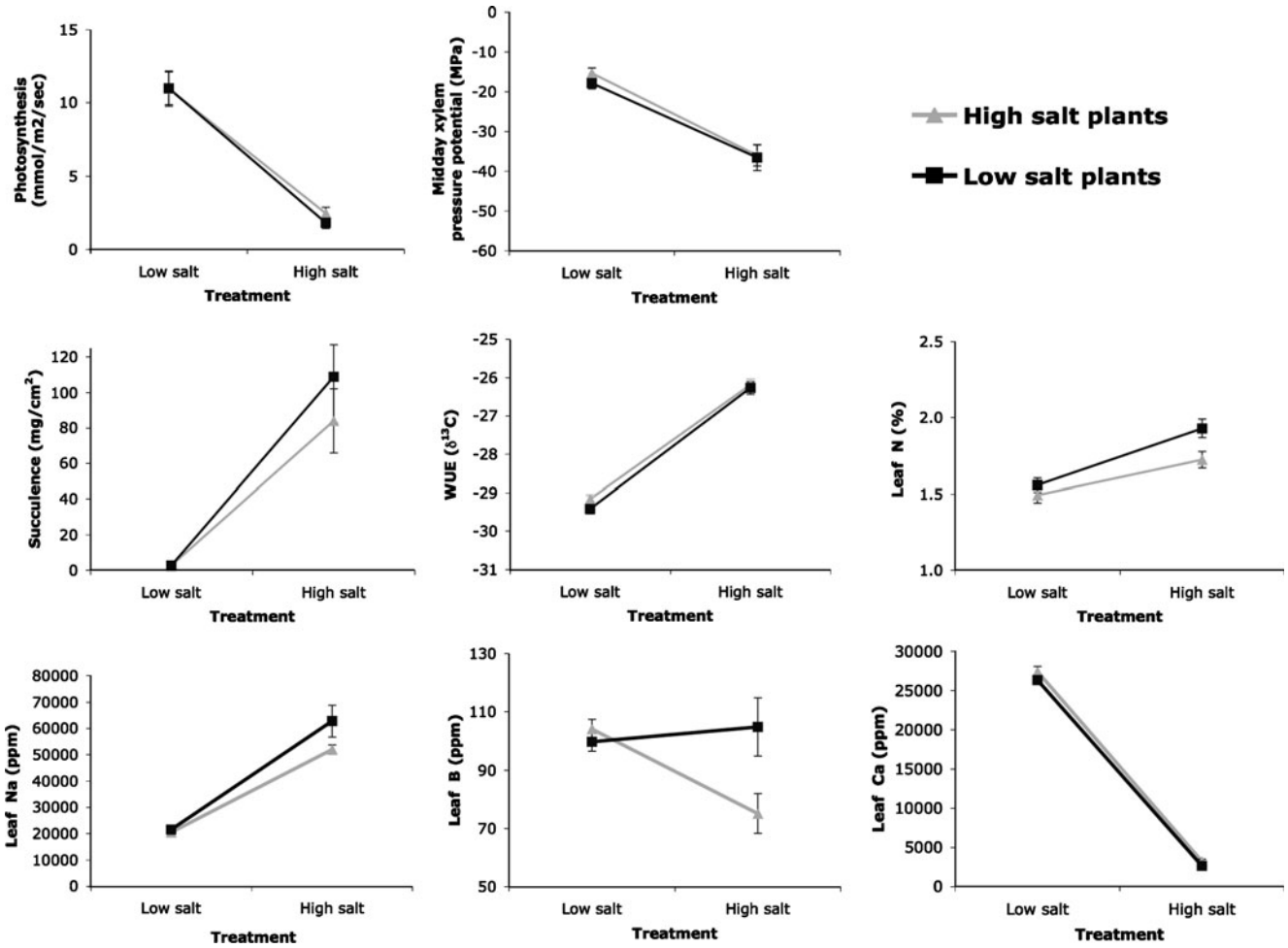

Fig. 1 Adaptation to high and low salt for 17 traits. Shown are reaction norms (means±1 standard error) for 17 traits measured in high-salt and low-salt source plants under high- and low-salt treatments. Sample sizes vary for each trait and in each source by treatment combination. For succulence, photosynthesis, WUE, %N, final height, total biomass, leaf area, shoot biomass and root biomass, *N* ranges from 28 to 56 in each source by treatment combination. For Midday XPP, *N* ranges from 15 to 21 for each source by treatment combination. For B, Ca, K, Mg, Mn, Na, and P, *N* ranges from 5 to 46. *Midday XPP* midday shoot water potential, *WUE* water use efficiency, *N* leaf nitrogen, *B* leaf boron, *Ca* leaf calcium, *K* leaf potassium, *Mg* leaf magnesium, *Mn* leaf manganese, *Na* leaf sodium, and *P* leaf phosphorous

the high-salinity treatment (15% and 21% from high-salt and low-salt microhabitats, respectively). Chi-square contingency tables showed no significant difference in mortality between plants from the high-salt and low-salt microhabitats (df=1, χ^2=0.811, P=0.368).

Across all families from both microhabitats, we did not find significant among-family variation in any trait means (no significant family effect, Table 2). Chi-square contingency tables also showed no significant difference in mortality between families across microhabitats (df=13 χ^2 =11.913, P=0.535). We found G by E interaction for only leaf Na (F=6.46, P<0.0001) and B (F=2.55, P=0.017; family × salinity, Table 2), which is reflected in little variation for the reaction norms of the high-salt and low-salt families for most of the other traits (Fig. 2).

Plasticity of Salt-Tolerance Traits

The response of seedlings to salinity treatment indicated that all of the traits considered are highly plastic (P<0.001, Table 2). Photosynthetic rate decreased, and midday shoot water potentials became more negative in response to higher salinity (81% and 115%, respectively, Fig. 2). However, many of the other physiological traits increased at higher salinity. Leaf succulence increased by over 3,500%, WUE increased (indicated by 10% increase in δ^{13}C values), leaf N increased by 26%, and leaf Na levels increased by 163% (Fig. 2). Leaf nutrient concentrations (B, Ca, K, Mg, Mn, and P) were reduced in the high-salt treatment (total amount of reduction ranged from 18% in B to 89% in Ca, Fig. 2). All growth characters (final height,

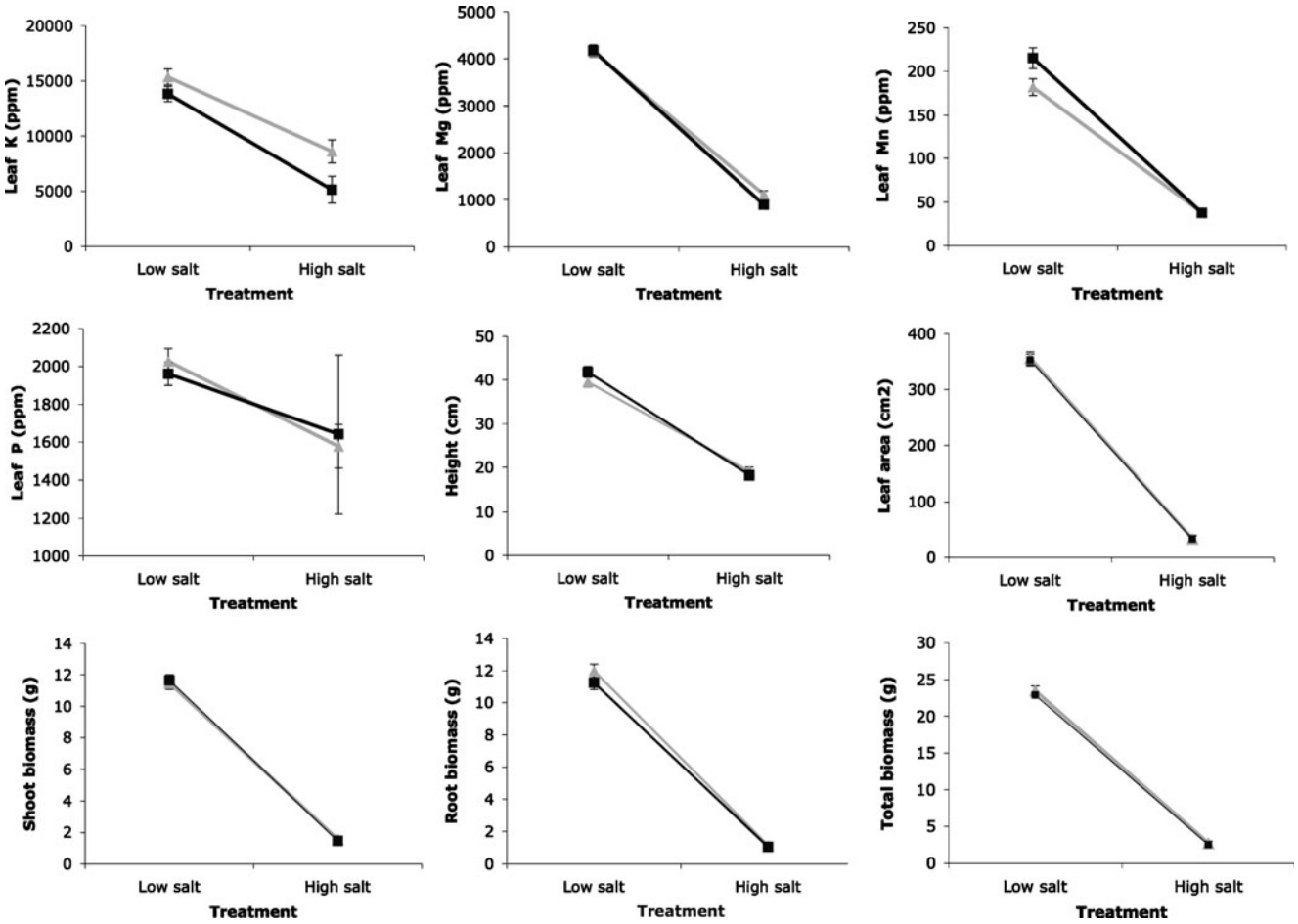

Fig. 1 continued.

total leaf area, shoot biomass, root biomass, and total biomass) decreased in response to higher salinity (Fig. 2), although the magnitude of the change varied among traits. Total height was reduced by 53% and total leaf area by 90%. Total biomass was reduced by 89% in the high-salt treatment, which reflected an 87% reduction in shoot biomass and a 91% reduction in root biomass.

Discussion

Because *B. frutescens* demonstrates substantial phenotypic variation across environments (Richards et al. 2005), has average levels of expected heterozygosity at allozyme loci and high clonal diversity in natural populations (Richards et al. 2004), we predicted that differential selection pressures would have led to adaptation to high- or low-salt microhabitats and variation in plasticity (G by E). Instead, we found that traits were extremely plastic in response to controlled salinity treatments but that there was no genotypic variation in trait means, and variation in trait plasticity for only two out of 17 traits. Our study confirms that the traits we measured were responsive to different salt environments and potentially important for salt tolerance, but we did not find that seedlings from the two microhabitats had genotypically based differences in these traits. The results do not support the hypothesis of specialization or adaptation to salt level, but suggest instead that highly plastic reaction norms for these traits allow plants to live across a broad range of salinity.

Plasticity of Ecologically Important Traits

Our study demonstrated that several salt-tolerance traits are highly plastic. In addition to growth reduction, reduced photosynthetic rate, and reduced uptake of nutrients (B, Ca, K, Mg, Mn, P), *B. frutescens* responded to salt by increasing WUE, N, Na, and succulence and decreasing midday xylem pressure potential. We predicted these general responses in *B. frutescens* based on the literature of salt tolerance in halophytes (Antlfinger and Dunn 1983; Glenn and O'Leary 1984; Donovan et al. 1996, 1997; Moon and Stiling 2000; Rosenthal et al. 2002). Our data is consistent with the concept that the low osmotic potential of

Table 2 Plasticity and genetic variation in salt-tolerance traits

	Salinity treatment			Family			Salinity treatment × family			Residual	
	ndf/ddf	MS	*F*	ndf/ddf	MS	*F*	ndf/ddf	MS	*F*	DF	MS
Succulence	1/14.9	319,803	25.82***	13/13.2	12,425	0.97 NS	13/145	12,879	1.59 NS	145	8,122.92
Photosynthesis	1/19.5	2,293.47	52.98***	13/13.1	34.05	0.78 NS	13/127	43.69	1.05 NS	127	41.60
Midday XPP	1/18.2	4,767.96	48.8***	13/11	74.07	0.72 NS	12/32	101.65	1.21 NS	32	84.35
WUE	1/14.6	214.79	321.01***	13/12.8	0.57	0.84 NS	13/83	0.68	1.29 NS	83	0.53
N	1/15.3	4.16	49.87***	13/12.7	0.14	1.65 NS	13/83	0.08	0.9 NS	83	0.09
B	1/9	2,013.27	2.48 NS	13/5.7	510.44	0.42 NS	8/71	884.19	2.55*	71	346.84
Ca	1/12.4	4,968,015,626	676.77***	13/1	12,353,212	3.09 NS	8/71	6,759,316	0.61 NS	71	11,149,845
K	1/10.7	378,876,222	21.56***	13/2.8	24,619,760	1.44 NS	8/71	17,478,386	0.96 NS	71	18,175,867
Mg	1/12	93,558,959	363.88***	13/1.4	1,109,145	6.68 NS	8/71	241,246	0.67 NS	71	361,105
Mn	1/13.8	246,422	110***	13/0.3	7,736.78	12.52 NS	8/71	1,957.70	0.48 NS	71	4,090.92
Na	1/8.4	11,140,305,828	136.5***	13/7.1	51,530,275	0.37 NS	8/71	91,902,061	6.46***	71	14,220,812
P	1/9.5	962,358	3.99 NS	13/4.7	342,607	1.08 NS	8/71	254,469	1.66 NS	71	153,224
Leaf area	1/15.4	349.16	396.44***	13/13.2	1.13	1.26 NS	13/147	0.90	1.31 NS	147	0.69
Final height	1/15.8	25.01	365.39***	13/13.2	0.11	1.55 NS	13/153	0.07	1.24 NS	153	0.06
Shoot	1/15.7	192.47	752.88***	13/13.2	0.36	1.39 NS	13/149	0.26	1.23 NS	149	0.21
Root	1/16.4	236.06	175.45***	13/13.3	0.17	1.2 NS	13/149	0.14	0.99 NS	149	0.14
Total biomass	1/16.9	212.84	1,574.8***	13/13.4	0.20	1.5 NS	13/149	0.13	0.86 NS	149	0.15

Shown is a two-way mixed model ANCOVA based on type III sum of squares for physiological traits, leaf nutrition traits and growth traits. Mean square, *F* statistics, and significance are presented for the main effects of salinity treatment, family (random), and treatment by family interaction (random)

Midday XPP midday shoot water potential, *WUE* water use efficiency, *N* leaf nitrogen, *B* leaf boron, *Ca* leaf calcium, *K* leaf potassium, *Mg* leaf magnesium, *Mn* leaf manganese, *Na* leaf sodium, *P* leaf phosphorous, *ndf* numerator degrees of freedom, *ddf* denominator degrees of freedom

*$P<0.05$; **$P<0.01$; ***$P<0.001$; NS $P>0.05$.

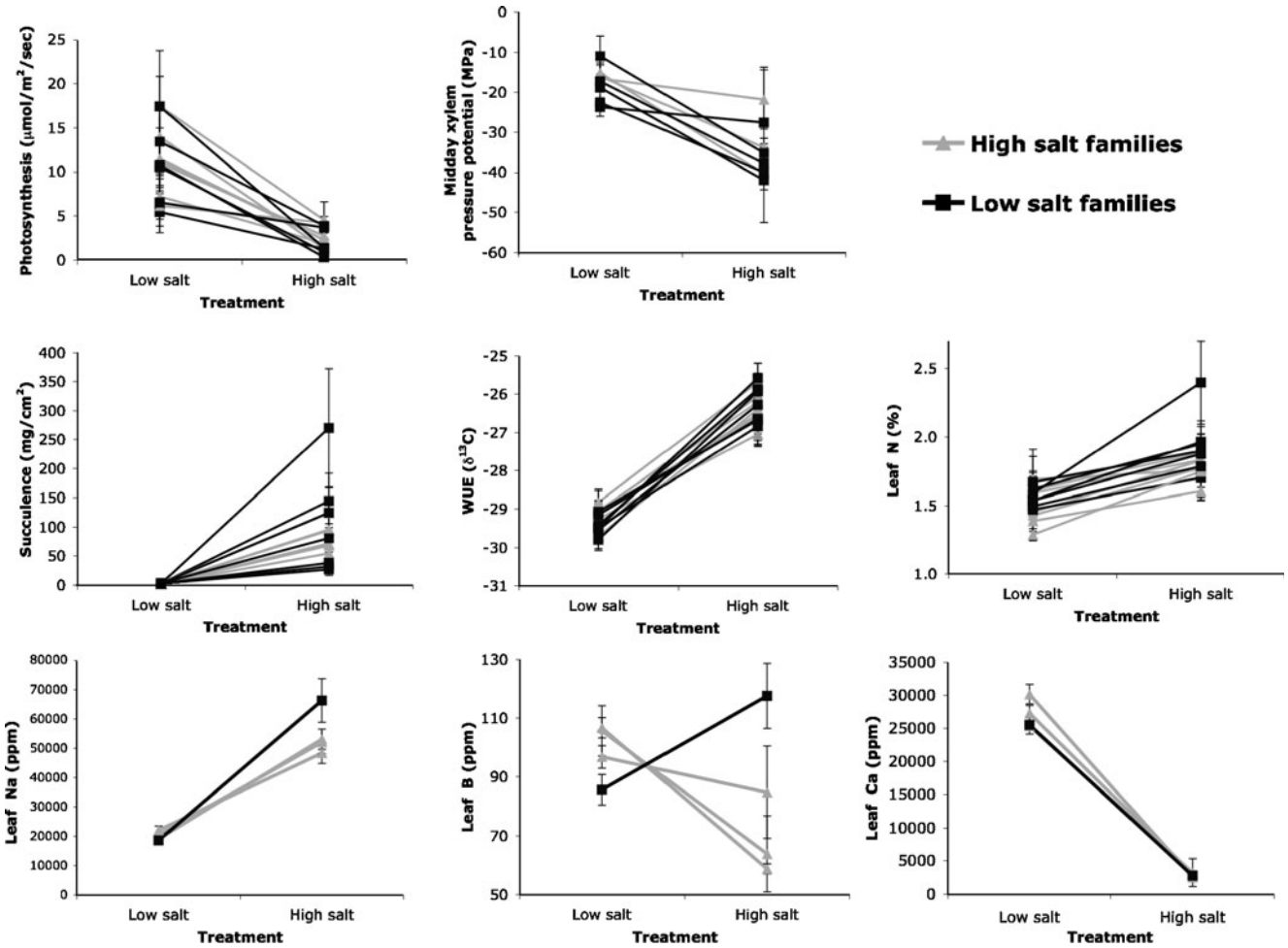

Fig. 2 Plasticity of 17 traits. Shown are reaction norms (means±1 standard error) for 17 traits measured under high- and low-salt treatments. For succulence, WUE, %N, final height, total biomass, leaf area, shoot biomass, root biomass, N=14 families (seven high-salt and seven low-salt families). For photosynthesis, N=13 families (seven high salt and six low salt). For Midday XPP, N=8 families (three high salt and five low salt). For B, Ca, K, Mg, Mn, Na, P, N=4 families (three high salt and one low salt). *Midday XPP* midday shoot water potential, *WUE* water use efficiency, *N* leaf nitrogen, *B* leaf boron, *Ca* leaf calcium, *K* leaf potassium, *Mg* leaf magnesium, *Mn* leaf manganese, *Na* leaf sodium and *P* leaf phosphorous

the soil in the high-salt treatment triggered the plants to close their stomates to conserve water and therefore increase WUE. Our data also suggests that the plants used more N (to produce compatible solutes) and absorbed more Na to adjust to the low osmotic potential of the soil. Increased succulence was also expected to maintain turgor and dilute the toxic effects of stored salts.

The reduced performance in high salt for all traits is inevitable to some degree, but these patterns of plasticity may be considered adaptive because they allow plants to tolerate a broad range of salinity and concentrations that would have been lethal to nonhalophytes (Pigliucci 2001; Sultan 2003; van Kleunen and Fischer 2005). The importance of this plasticity is reflected in the natural standing populations, where plants in high-salt microhabitats are similarly limited in stature but still manage to reproduce clonally as well as through an abundance of viable seed. Half of the seeds used in this study, for example, came from plants growing in one of these high-salt microhabitats.

No Genotypic Differentiation or Specialization to Salt Level in *Borrichia*

Previous studies have found that strong differential selection can cause genetic differences and lead to local adaptation even if populations are close together and experience high levels of gene flow (Levene 1953; Jain and Bradshaw 1966; Hedrick 1976; Schmidt and Rand 1999). Accordingly, we predicted that selection could have resulted in a substantial amount of genetic differentiation for salt-tolerance traits across microhabitats. However, we found that genotypes from both microhabitats showed similar responses to variation in salinity and across both

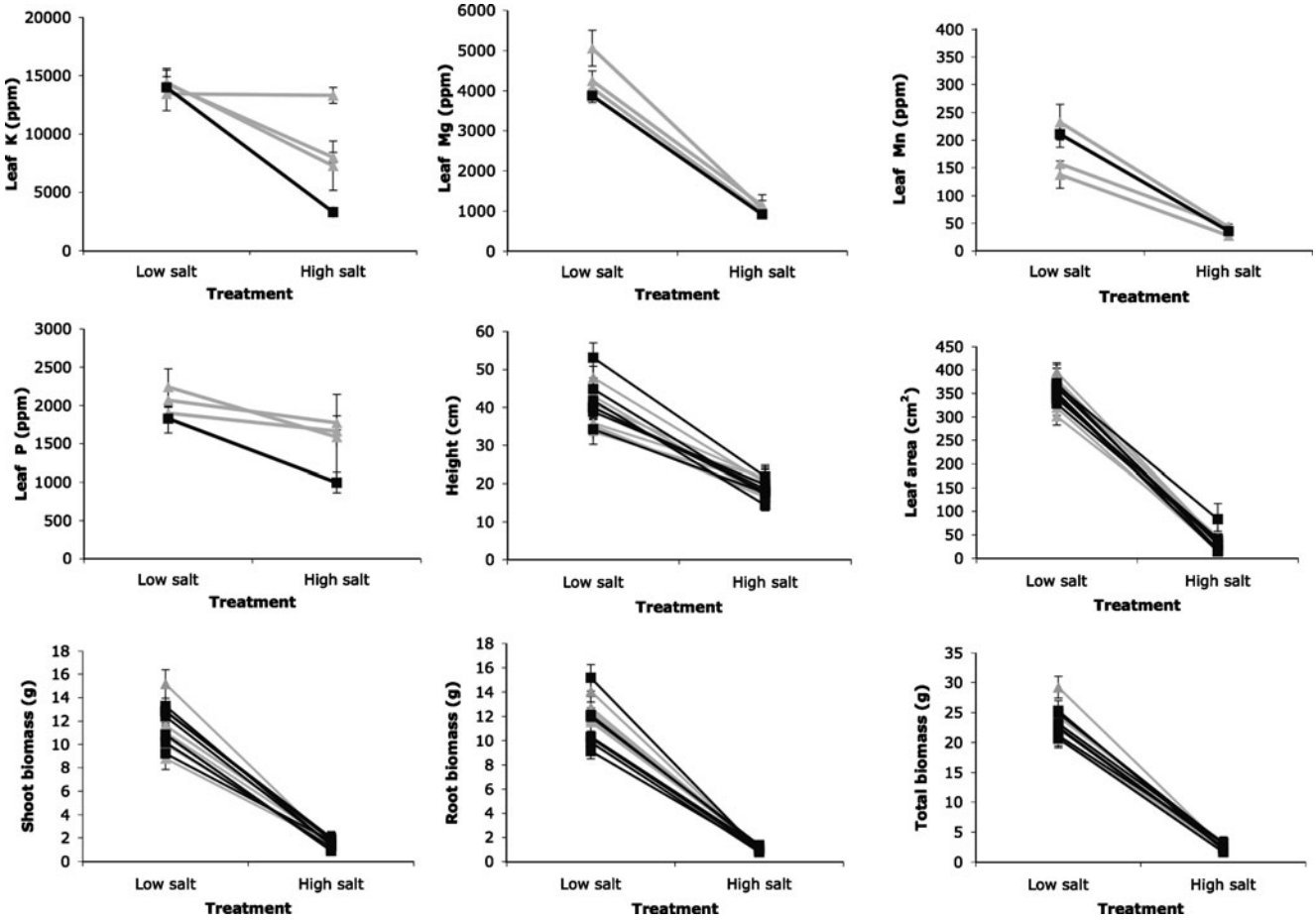

Fig. 2 continued.

habitats; there were no family differences in trait means and G by E for only Na and B. This was true despite the fact that this population consists of a diversity of genotypes, and we used naturally outcrossed seeds. This sample of plants should contain the maximum amount of genetically based variation compared to mature individuals in the field that have experienced selection.

The general lack of G by E could signify the importance of plasticity in this species because if selection favors plasticity, there should be less variation in plasticity (Pigliucci 2001). In this study, the significance of the G by E for Na uptake was due to the fact that one family from the low-salt microhabitat took up significantly more Na in the high-salt treatment but obtained the same biomass as the other families. The mechanisms employed by *B. frutescens* to tolerate high substrate salinity are not completely understood and may vary by genotype. We cannot say with certainty that maintaining lower levels or taking up greater levels of Na is adaptive for this species without further investigation.

The interaction for B uptake was similarly driven by this same family, which displayed a pattern opposite to the rest of the families. B is an essential nutrient for normal growth, but the role of B is not completely understood, and the difference between levels that are deficient and levels that are toxic in soils is relatively small for most plants (Rozema et al. 1992; Camacho-Cristóbal et al. 2008). The importance of B in the salt marsh system has been suggested since B concentration in sea water is 15 to 20 times higher than what is considered optimal for nonhalophytes (Rozema et al. 1992). Rozema et al. (1992) found that halophytes took up less B compared to nonhalophytes, growth in halophytes was not reduced at B concentrations found in sea water (0.35 mol m^{-3}), and most species took up less Na with increased B. This study did not include *B. frutescens* but found a lot of variation between six halophyte species in response to B. Similarly, Rosenthal et al. (2002) report that the halophyte *Helianthus paradoxus* and desert-adapted nonhalophyte *Helianthus deserticola* had genotypically based reduction of B uptake compared to a suite of closely related *Helianthus* species in controlled greenhouse experiments, suggesting that this may be a general adaptation to water-limited habitats. The G by E pattern found in this study indicated that B uptake could be important for some genotypes to maintain fitness in response to increased salt content.

Is Plasticity Adaptive?

Determining whether plasticity is adaptive has become a major goal of ecological genetics and is important for determining the evolutionary trajectory of plastic traits. Previous work has investigated the adaptive value of plasticity by arguments from design (Pigliucci 2001) or by manipulating traits (Schmitt et al. 1999; Pigliucci and Schmitt 2004). More recently, several studies have used selection analyses (Lande and Arnold 1983) to test the hypothesis of adaptive plasticity by comparing the relationship between fitness and trait values in different environments or the relationship between fitness and trait plasticity across environments (Pigliucci 2001; Callahan and Pigliucci 2002; Stinchcombe et al. 2004). However, the power to detect a significant relationship between fitness and traits or trait plasticity depends on the presence of variation for those traits or plasticities. It is possible that adaptive plasticity could have been detected using more genetic families, other genetic variants such as crosses between the high-salt and low-salt individuals, or phenotypic manipulations to increase phenotypic variance (Sinervo and Basolo 1996; Schmitt et al. 1999; Pigliucci and Schmitt 2004).

Still, we did find evidence of selection against succulence (β=−0.233±0.10, P=0.05) and for increased levels of plasticity in succulence (β=10.38±4.60, P=0.05). For this analysis, we regressed the cross environment plasticity and the grand mean of the trait for each family as estimated within each treatment against the grand relative mean fitness (sensu Stinchcombe et al. 2004). The regression coefficient (β) indicates the strength and direction (positive or negative) of selection. Succulence could be an important trait for *B. frutescens* to tolerate high salt because this species does not have salt glands or salt bladders to export salt, and the majority of the Na that are taken up are probably stored in the vacuole of the cell. Succulence has been identified in several studies as an important trait differentiating closely related species or subspecies that live in environments of different salt content. For example, Reimann and Breckle (1995) found that the salt-tolerant subspecies *Salsola kali traga* was able to increase succulence more than the nonsalt-tolerant subspecies *Salsola kali ruthenica*. Similarly, studies on *H. paradoxus* suggest that the evolution of increased succulence may have been an important adaptation to allow *H. paradoxus* to survive salty habitats (Rosenthal et al. 2002; Karrenberg et al. 2006).

We expected that strong selection by substrate salinity could lead to adaptation to microhabitats in the salt marsh, but given sufficient genetic variation, modest gene flow, and depending on the frequency of the different microhabitats, theory predicts that phenotypic plasticity should be favored in heterogeneous environments (van Tienderen 1991; Pigliucci 2001; Sultan and Spencer 2002; Kawecki and Ebert 2004). Relatively predictable edaphic factors result in strong species zonation patterns across environmental gradients in the salt marsh, (Pennings and Bertness 2001; Richards et al. 2005). Still, conditions vary daily, and storms, human disturbance, and shifts in barrier island geomorphology can alter community-level distribution patterns in a matter of years (Clark 1986, 1990). It is likely that these environmental changes reduce the benefits of adaptation to local conditions, especially for a long-lived species like *B. frutescens* (Clark 1990). Several studies have demonstrated that organisms overcome this type of environmental heterogeneity through the plasticity of purportedly adaptive traits which respond to light availability (Schmitt 1993; Dudley and Schmitt 1996; Sultan 2003), water availability (Sultan 2003; Dudley 1996), nutrient availability (Crick and Grime 1987; Sultan 2003), salt content (Hester et al. 1996; Florin and Hoglund 2007), and predators (van Buskirk and Relyea 1998; Lively 1986). The consistency of the trait responses for families across microhabitats support the hypothesis that plasticity has been selected in *B. frutescens* such that these plants display a fixed reaction norm for important salt-tolerance traits across experimental salt treatments. Since salt marsh habitats typically consist of a high degree of environmental variation, a strategy of high phenotypic plasticity could be more the rule than the exception in this system.

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