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Plant genome size influences stress tolerance of invasive and native plants via plasticity

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Abstract. Plant genome size influences the functional relationships between cellular and whole-plant physiology, but we know little about its importance to plant tolerance of environmental stressors and how it contributes to range limits and invasion success. We used native and invasive lineages of a wetland plant to provide the first experimental test of the Large Genome Constraint Hypothesis (LGCH)—that plants with large genomes are less tolerant of environmental stress and less plastic under stress gradients than plants with small genomes. We predicted that populations with larger genomes would have a lower tolerance and less plasticity to a stress gradient than populations with smaller genomes. In replicated experiments in northern and southern climates in the United States, we subjected plants from 35 populations varying in genome size and lineage to two salinity treatments. We measured traits associated with growth, physiology, nutrition, defense, and plasticity. Using AICc model selection, we found all plant traits, except stomatal conductance, were influenced by environmental stressors and genome size. Increasing salinity was stressful to plants and affected most plant traits. Notably, biomass in the high-salinity treatment was 3.0 and 4.9 times lower for the invasive and native lineages, respectively. Plants in the warmer southern greenhouse had higher biomass, stomate density, stomatal conductance, leaf toughness, and lower aboveground percentage of N and total phenolics than in the northern greenhouse. Moreover, responses to the salinity gradient were generally much stronger in the southern than northern greenhouse. Aboveground biomass increased significantly with genome size for the invasive lineage (43% across genome sizes) but not for the native. For 8 of 20 lineage trait comparisons, greenhouse location × genome size interaction was also significant. Interestingly, the slope of the relationship between genome size and trait means was in the opposite direction for some traits between the gardens providing mixed support for LGCH. Finally, for 30% of the comparisons, plasticity was significantly related to genome size—for some plant traits, the relationship was positive, and in others, it was negative. Overall, we found mixed support for LGCH and for the first time found that genome size is associated with plasticity, a trait widely regarded as important to invasion success.

Key words: climate change; genome size; invasive species; Phragmites australis; plant traits; plasticity.

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Introduction

Under global climate change, extreme environmental events and abiotic stress are increasing, likely favoring some plant species or particular genotypes while eliminating others (Anderson 2016). Although more and more studies are documenting the relationship between intraspecific genetic variation and ecological breadth in changing environments (Albert et al. 2011, Sides et al. 2014), the underlying mechanisms are not fully understood. The effects of environmental stressors associated with climate change (and their interactions), such as rising temperatures and increasing salinity in coastal ecosystems as sea level rises, are predicted to vary both among and within species depending on how plant genomic variation interacts with stressors and functional traits (Johnson et al. 2008, De Frenne et al. 2014, Suzuki et al. 2014). Genome size is thought to influence plant traits from the subcellular to organismal levels irrespective of the coded information (i.e., the nucleotype concept; Bennett 1971) and can interact with environmental stressors to affect both plant traits and responses (Bennett and Leitch 2005, Suda et al. 2015). Variations in plant genome size are generated by lineage-specific molecular mechanisms in both DNA amplification and DNA removal that alter the total amount of nuclear DNA content and thereby influence fitness-based selection (Bennetzen 2005). For example, a small genome may facilitate shorter cell cycles and faster rates of cell division translating to earlier germination, faster plant growth and development, and expression of traits such as higher photosynthetic rates and higher specific leaf area (Bennett 1987, Knight and Ackerly 2002). Conversely, the potentially higher costs associated with having a large genome, such as acquiring enough limiting nutrients like N and P for nucleic acid production (Šmarda and Bureš 2010, Guignard et al. 2016), may limit the conditions under which plants can grow either directly or indirectly (i.e., The Large Genome Constraint Hypothesis; Knight et al. 2005, Suda et al. 2015). Small genomes may therefore be advantageous relative to larger genomes when rapid growth can at least partially compensate for environmental constraints such as short growing seasons or unfavorable environmental conditions (Bennett 1987). Accordingly, the Large Genome Constraint Hypothesis (LGCH; Knight et al. 2005) predicts that plants with small genomes may have higher fitness under environmentally stressful (i.e., suboptimal) conditions than plants with larger genomes (Šmarda and Bureš 2010, Suda et al. 2015). Although the concept of the LGCH was originally framed using interspecific genome size comparisons, intraspecific comparisons better control for phylogenetic differences among taxonomic groups. Furthermore, the LGCH has not been explicitly tested using manipulative experiments (but see Kapralov and Filatov 2011, Carta and Peruzzi 2016 for correlative studies) and has never been tested at the intraspecific level.

Plasticity in plants, that is, the ability of a genotype to express different phenotypes under varying environmental conditions (Richards et al. 2006), has been a focus of researchers studying adaptation to changing environmental conditions for more than a century (Bradshaw 2006). One important implicit prediction of the LGCH is that plant populations with smaller genome sizes should be more plastic in their responses to environmental change (i.e., attain a wider array of trait states than populations with large-genome sizes; Knight et al. 2005). In fact, multiple studies have shown that species with small genomes are found across a wider range of environmental conditions (e.g., altitude, temperature, precipitation) than large-genome species which tend to be excluded by harsher environments (Knight et al. 2005, Pyšek et al. 2018).

A number of studies relating genome size to trait expression have used latitude of origin (and associated stressors at each latitude) as a proxy for abiotic selection pressures acting on genome size. Some studies found genome size positively correlated with latitude, others negatively correlated, while yet other studies found no relationship between genome size and latitude (reviewed in Knight et al. 2005). However, no studies that we are aware of have investigated how abiotic conditions and stress combinations at different latitudes interact with genome size to influence trait expression and plasticity.

Interestingly, naturalized and invasive plant species have disproportionately smaller genome sizes relative to all angiosperms, a trait that likely contributes to their success (Bennett et al. 1998, Kubesova et al. 2010, Suda et al. 2015). While

reliable data for holoploid (i.e., DNA content of the whole-chromosome complement) intraspecific plant genome size variation remain limited for most species (Šmarda and Bureš 2010), measurable significant variation exists for particular species, especially invasive grasses, even within ploidy levels (Lavergne et al. 2010, Meyerson et al. 2016*a*, Pyšek et al. 2018, 2019). Different holoploid genome sizes (which include a range of ploidy levels) within a species can function as de facto distinct species with variable responses to the same environmental conditions and interspecific interactions (Thompson et al. 1997, Thompson and Merg 2008, Tesitelova et al. 2013). However, nothing is known about how environmental stressors associated with climate change will interact with distinct intraspecific genome sizes of the same ploidy level to influence invasion and range expansion potential. One recent study of *Phragmites australis* found that small-genome size was associated with invasiveness and likely influenced drought tolerance (Pyšek et al. 2018), but studies relating plant genome size variation to environmental factors are scarce and correlative (Carta and Peruzzi 2016). Populations with small genomes may be better at expanding their ranges under some harsher environmental conditions associated with climate change (Suda et al. 2015), whereas plants with larger genomes may more often undergo range contractions under the same conditions. Plant genome size can affect the functional relationships between cellular and whole-plant physiology and therefore could also be an important factor in the successful colonization of new environments, but few studies have tested intraspecific genome size variants assembling under environmental stressors and invasions (Estrada et al. 2016).

Using tetraploid populations from the native and invasive lineages of *Phragmites australis*, we tested whether genome size predicts how plants will respond to individual and combined environmental stressors, specifically whether plants with large genomes would be at a disadvantage under stress relative to plants with small genomes (i.e., the LGCH). We also asked whether these bottom-up effects could cascade through the community to affect both trophic (via defense traits) and competitive (via biomass) relationships. Specifically, we tested whether (1) salinity in climates associated with mid- and southern latitudes negatively affects plant growth, physiology, nutritional, and defense traits, (2) whether plants with small genomes are more stress-tolerant than plants with larger genomes (the Large Genome Constraint Hypothesis) and whether stressors might interact (heat × salinity) to strongly favor small-genome plants, (3) whether stress tolerance is highest for plants originating from high or low latitudes and is associated with smaller genome size, and (4) whether small-genome plants are more phenotypically plastic than large-genome plants.

Methods

Focal species and greenhouse locations

Phragmites australis (Cav) Trin. ex Steud. is a cosmopolitan species within Poaceae that is adapted to wide climatic and latitudinal ranges (±60°), including extreme environments. It exhibits a high genetic and genomic diversity (Saltonstall 2002, Meyerson et al. 2016*a*, Pyšek et al. 2018), intra- and interspecific hybridization (Meyerson et al. 2010, 2012, Lambertini et al. 2012, Saltonstall et al. 2014), and variations in ploidy (Clevering and Lissner 1999, Keller 2000, Lambertini et al. 2006, Meyerson et al. 2016*a*). *Phragmites australis* has a global distribution of diverse cytotypes (4*x*–12*x*, based on $x = 12$) and genome size variation of up to 22% within cytotypes (Suda et al. 2015), on par with intraspecific genome size variation found in another well-studied grass, *Zea mays* (Reviewed in Šmarda and Bureš 2010) but greater than found globally for *Arabidopsis thaliana* (Long et al. 2013).

We standardized reciprocal greenhouse experiments using *P. australis* as the focal species at the University of Rhode Island (URI; 41.48° N, 71.53° W) and Louisiana State University (LSU; 30.41° N, 91.19° W) allowing us to determine how greenhouse location, latitude of origin and associated climate, salinity, and genome size interact to influence plant fitness and defense. The greenhouses were not controlled for temperature and humidity meaning climatic conditions within each greenhouse covaried with the local climate. We took advantage of natural climatic differences between Rhode Island and Louisiana and manipulated salinity for identical clones in each greenhouse. The URI greenhouse location

represents a moderate temperate-zone climate whereas the LSU greenhouse location is subtropical, close to the southern range limit of both the native and invasive genotypes. Monthly temperatures during the experiment (mid-February to mid-June) were 9–14°C higher at LSU than URI.

Selection and planting of clones

We focused on the native and invasive lineages of *P. australis* in North America because they grow sympatrically in many places and have demonstrated intraspecific genome size diversity both within and between lineages (Pyšek et al. 2018, 2020). Because a previous study found trait differences for *P. australis* by ploidy level (Meyerson et al. 2016*a*), we used only tetraploids (4x) for this study, the most common and widespread cytotype in North America, regardless of lineage. We selected *P. australis* genotypes collected from the wild but grown in our garden collections for at least 5 years to minimize maternal effects. From these, we selected clones based on lineage (native or invasive), genome size range of 1.92–2.25 pg, and source locations collected from the East, Gulf, and West coasts of the United States that spanned more than 16° of latitude (Table 1). A total of 35 genotypes (12 native, 17 invasive; Table 1), each with 20 identical clonal replicates in each greenhouse, were propagated from rhizomes in early February 2016 at both URI and LSU. Rhizomes were harvested; all roots were removed and were carefully rinsed to remove all soil. Each greenhouse used 7–10 g of identical starting material harvested from the same clone. Rhizomes were planted in sand in 13.4 × 14.0 cm hard plastic rectangular pots in both greenhouses. Some planted clones did not survive and reduced replicates for some populations (Table 1).

Genetic and karyotype identification

We extracted DNA from dried plant tissue for each population using the Omega E.Z.N.A. SP Plant DNA Kit. To confirm the lineage for each population, we performed restriction fragment length polymorphisms (RFLP) analyses following previously published methods (Saltonstall 2003). Monoploid genome size and ploidy data for all plants were quantified using propidium iodide and DAPI flow cytometry following previously published methods, described in Pyšek et al. (2018). Monoploid genome sizes (i.e., the DNA content of the monoploid chromosome set, with chromosome number x; Cx value) were calculated as 2C-value/ploidy level.

Salinity

Pots were distributed among plastic pools (1.22 m diameter), with each of the 35 genotypes represented once per pool. Pools were then assigned at random to one of three levels of salinity: freshwater (0 ppt), mesohaline (10 ppt), and polyhaline (20 ppt). For the 10 ppt pools, we dissolved 290 g of Instant Ocean (Instant Ocean, Blacksburg, Virginia, USA) in 25 L of tap water. For 20 ppt pools, we dissolved 580 g of Instant Ocean in 25 L of tap water. Salinity levels were measured daily using a YSI meter and adjusted when necessary by adding tap water or Instant Ocean.

Plant traits

We measured total above- and belowground biomass for each pot at the end of the experiment in mid-May 2016 (≈3.5 months following planting) by harvesting the aboveground and belowground plant material separately, oven-drying at 70°C, and weighing to the nearest 0.01 g. We measured stem density for all pots and maximum stem height from the base of the stem at the soil surface to the tip of the uppermost leaf. For aboveground nutrient analysis (percentage of carbon, percentage of nitrogen, C:N ratio), the top three leaves were collected from a single plant per pot and all roots and rhizomes were washed clean of sand. Samples were processed and analyzed at the University of Rhode Island on a Carlos Erba CN analyzer according to the methods reported in Cronin et al. (2015). Leaf toughness was measured in May prior to harvest using a penetrometer (Itin Scale, Brooklyn, New York, USA) which measures the force (in kg) necessary to puncture the leaf tissue (Salgado and Pennings 2005). Also, in May, total phenolics (nM/g of dried leaf tissue) were estimated using a modified version of the Folin-Ciocalteu method (Waterman and Moles 1994). Further details are provided in Cronin et al. (2015).

Stomatal size and density affect plant water use and photosynthetic efficiency. To determine mean stomatal size and density, we used the epidermal leaf peel technique (Winn 1996) on both

Table 1. (a) Exotic and (b) native genotypes, number of surviving replicates after planting (REP), garden source (source), genome size (GS), ploidy, and location of origin (Lat, Long) for *Phragmites australis* populations used in reciprocal transplant experiment.

ID	Reps URI	Reps LSU	Source	Population	Genotype	GS	Ploid	Lat	Long
a) Exotic *P. australis*									
RRM	20	20	LSU	Rockefeller RD, Cameron, LA	Exotic	1.93	4x	29.68	−92.81
NYM	20	20	URI	Montezuma, NY	Exotic	1.94	4x	43	−76.7
ECM	20	20	LSU	East Cameron, Cameron, LA	Exotic	1.95	4x	29.77	−93.29
CRM	20	20	LSU	Creole 2, Cameron, LA	Exotic	1.95	4x	29.88	−93.07
GMI	20	19	LSU	I-40, AZ	Exotic	1.96	4x	34.72	−114.49
Kirkpatrick	20	20	URI	Baltimore, MD	Exotic	1.96	4x	39.24	−76.6
Severn River	20	20	URI	Severn River, MD	Exotic	1.97	4x	39.08	−76.62
NBM	20	20	URI	Moncton, NB	Exotic	1.98	4x	46.1	−64.8
BSCM	20	20	URI	Bath, ME	Exotic	2.03	4x	44.51	−70.35
TCM	20	20	URI	Choptank, MD	Exotic	2.05	4x	38.77	−75.95
SAML	20	20	LSU	Salinas River, CA	Exotic	2.09	4x	35.5	−120.65
GBM	20	20	URI	Great Bay, ME	Exotic	2.11	4x	43.05	−70.9
Death V	13	13	URI	Death Valley, CA	Exotic	2.12	4x	36.24	−116.83
FPM	20	20	URI	Falmouth, MA	Exotic	2.13	4x	41.55	−70.6
CHM	20	20	URI	Charlestown, RI	Exotic	2.21	4x	41.36	−71.64
RAPM	20	20	URI	Rappahannock, VA	Exotic	1.96	4x	37.94	−76.83
NIBM	20	20	URI	Naushon Island, MA	Exotic	2.03	4x	41.47	−70.76
b) Native *P. australis*									
RCN	20	20	BOTH	Rachel Carson, Wells, ME	Native	1.98	4x	43.36	−70.48
MDN	14	20	BOTH	Choptank, MD	Native	2.15	4x	38.77	−75.95
MEE	20	20	URI	Holt Forest, Arrowsic, ME	Native	2.19	4x	43.88	−69.78
Mackay	20	20	URI	Mackay Island, NC	Native	2.20	4x	36.51	−75.95
PORN	20	16	LSU	Port Orford, OR	Native	2.22	4x	42.76	−124.5
GBN	20	20	URI	Great Bay, ME	Native	2.22	4x	43.05	−70.9
NBS	20	20	URI	Moncton, NB	Native	2.22	4x	46.1	−64.8
JPN	17	17	URI	Jacobs Point, RI	Native	2.23	4x	41.71	−71.29
LCN	20	13	LSU	Little Caliente Hot Springs, CA	Native	2.24	4x	34.54	−119.62
NYE	20	20	URI	Montezuma, NY	Native	2.24	4x	43	−76.7
SCRN	20	15	LSU	Santa Clara River, CA	Native	2.25	4x	34.36	−119.01
USGN	17	14	URI	St. George, UT	Native	NA	4x	37.1	−113.57

Note: Abbreviations are AZ, Arizona; CA, California; LA, Louisiana; MD, Maryland; ME, Maine; NB, New Brunswick; NC, North Carolina; NY, New York; OR, Oregon; RI, Rhode Island; VA, Virginia; UT, Utah.

the adaxial and abaxial leaf surfaces. Briefly, we applied fingernail polish to the leaf surface and then used clear tape to affix the peel to a clear glass slide. Using a 20× objective of a Zeiss Axio Imager M2 Light Microscope on the bright field setting, we photographed a randomly selected area (0.15 mm^2) of each peel on both leaf sides. Using ImageJ (version 1.50, Schneider et al. 2012), we counted all stomata for each plant for all treatment combinations on the adaxial and abaxial leaves. We also measured the length and width of all stomates for all populations and treatments and recorded their averages for all treatments on the adaxial and abaxial leaves. Stomatal conductance (g_s) measures the exchange of water and CO_2 through the stomate of a leaf thereby directly regulating the plant water relations and photosynthesis (Urban et al. 2017). An index of stomatal conductance was measured as the product of stomatal density, mean stomate length, and mean stomate width (see Douhovnikoff et al. 2016 for an example with *P. australis*). For each treatment combination, we computed stomatal conductance for the abaxial and adaxial leaf surfaces and then took an average of the two.

Every *P. australis* source population in our study was subjected to all combinations of two different greenhouse conditions (*u*; LSU, URI) and three salinity levels (*s*; 0, 10, 20 ppt). For

each population (x) and garden-salinity combination, we calculated the population mean for a *P. australis* trait (T_{xus}) such that there were six mean values for each population x. Plasticity was calculated as the difference between the maximum and minimum trait value standardized by the mean among the six treatment combinations for that population (see Eller and Brix 2012, Bhattarai et al. 2017).

$$\text{Plasticity of population } x = [\text{MAX}(T_{xus}) - \text{MIN}(T_{xus})]/\text{T}_{xus-mean}.$$

Predictions

In our analysis, we planned to use Akaike's information criteria corrected for finite sample size (AIC_c) to select the most informative model (Burnham and Anderson 2010). A priori, we predicted that salinity and greenhouse location would be present in the AICc best models for each response variable and that their effects on each trait would be strong. Specifically, and based on the published literature (e.g., Lissner and Schierup 1997, Vasquez et al. 2005, Achenbach et al. 2013), we expected that increased salinity would negatively impact *P. australis* growth, nutritional condition, stomatal conductance, and plant defenses (i.e., the salinity gradient $\approx$ a stress gradient). The much warmer conditions at the Louisiana greenhouse were also predicted to impact plant traits because of interactions between high temperatures and salinity. Because an earlier study found that genome size was important to trait variation in all likely models for traits measured (Meyerson et al. 2016*a*), we also predicted that genome size would be an important factor in the AIC_c best models in this study. If plants with smaller genomes can better tolerate stress, then we expected to find genome size $\times$ salinity and/or genome size $\times$ greenhouse interactions in the best-supported models. For example, we predicted that under salt stress (20 ppt), there would be a strong negative relationship between genome size and biomass (i.e., plants with small-genome size grow largest) but under more benign conditions (0 ppt), the genome size–biomass relationship would be weaker. Because the introduced lineage has, on average, a smaller genome size, we also expected that the native populations would be more strongly affected by the salinity gradient than the invasive populations. Finally, because of the known latitudinal clines in *P. australis* traits (Cronin et al. 2015, Allen et al. 2017), we expected latitude to be a common predictor of trait variation among our source populations in these models.

Statistical methods

Strong correlations existed among some of our response variables (Appendix S1: Tables S1 and S2). Notably, stomate length and width were negatively correlated with density, adaxial and abaxial stomate densities were positively correlated, and above- and belowground biomasses were positively correlated (within or among treatments, $r \geq 0.60$, uncorrected $P \leq 0.05$). To avoid redundant analyses and inflated type I errors, we limited our analyses below to 10 traits that were not or were only weakly correlated with each other: stomate density on the adaxial leaf surface, stomatal conductance, aboveground biomass, leaf toughness, and above- and belowground percentage of carbon and percentage of nitrogen and total phenolics. Because genome size is confounded with lineage (i.e., the introduced lineage has a smaller genome size than the native lineage; Pyšek et al. 2018), separate analyses were conducted for each lineage.

We used separate generalized linear mixed models (GLMM) to test whether *P. australis* growth, physiological, nutritional, and herbivore defense traits were influenced by the greenhouse where the study was conducted (LSU, URI), salinity level (0, 10, 20 ppt), genome size, and latitude and longitude of origin of the *P. australis* populations. *Phragmites australis* population and pool number were included as random effects in the model to account for within-lineage variation and position effects within the greenhouse, respectively. All two- and three-way interactions among predictor variables were included in the models. To help normalize data distributions and homogenize variances among treatments, stomate density, leaf toughness, total biomass, and total phenolics were *ln*-transformed. Quantile–quantile plots and studentized residuals were used to identify potential outliers in the distribution of trait estimates. However, in no case did the removal of these data points qualitatively change the conclusions of the model. Finally, data were analyzed using SAS 9.3 Proc MIXED

with normally distributed errors (SAS Institute, Cary, North Carolina, USA).

For each of the 10 dependent variables and two lineages, we used Akaike's information criteria corrected for finite sample size (AIC_c) to select the most informative model (Burnham and Anderson 2014). Candidate models were constructed using all possible combinations of predictor variables. There were two restrictions to the possible combinations of variables. (1) The basic mixed-effects framework was retained in all models to account for the nonindependence among populations and pools (pots within pools). (2) Interaction terms could only be present in the model if their main effects were also present in the model. Candidate models were ranked by AIC_c from lowest to highest value and AIC_cs with a Δ_i value (= $AIC_{ci} - AIC_{cmin}$) of ≤2 were considered to have substantial support (Burnham and Anderson 2010). AIC_c weights (w_i) were reported which indicate the weight of evidence (as a proportion) in favor of model i being the best model given the set of candidate models. Goodness of fit of the AIC_c best model was computed in R (package MuMIn) using the method of Nakagawa and Schielzeth (2012). Here, the proportion of variance explained was divided into two components: (1) the marginal R^2 which measures the variance explained by all fixed effects combined and (2) the conditional R^2 which measures the variance explained by the model, that is, all the fixed and random effects combined.

To evaluate whether the invasive lineage is more phenotypically plastic than the invasive lineage, we computed Hedge's d for each of the ten plant traits:

$$d = \frac{\overline{X}^{inv} - \overline{X}^{nat}}{s} j$$

where $\overline{X}^{inv}$ and $\overline{X}^{nat}$ are the mean plasticities for trait X for the invasive and native lineage, respectively. s is the pooled standard deviation and j is a correction for small sample size. Bootstrapped 95% confidence intervals for d were computed in MetaWin 2.1 (Rosenberg 2007). A significant difference in plasticity between the two lineages would be evident if the confidence intervals did not include zero. For each trait and lineage, we also used least-squares regression to determine whether a relationship existed between plasticity level for each trait and genome size.

Results

Among the candidate models used to evaluate trait variation in *P. australis*, the most likely (i.e., the AIC_c best) models included the predictor variables greenhouse location (U), salinity level (S), genome size (G), and the interactions between greenhouse location and genome size (U × G) and salinity and genome size (S × G; Table 2). The lone exception was the trait stomatal conductance, for which only greenhouse location was an important predictor. The interaction between greenhouse and salinity (U × S) was also present in the AIC_c best model for eight of the ten traits for each lineage. Model goodness of fit was particularly strong for aboveground biomass: R^2 for fixed effects (i.e., marginal R^2) was 0.63 and 0.45 for the invasive and native lineage, respectively (Table 2). Averaged among traits and the two lineages (±SE), R^2 for fixed plus random effects (i.e., conditional R^2) was 0.42 ± 0.03.

As expected, salinity level (S) and greenhouse location (U) strongly affected plant traits (Table 2, Fig. 1). For the native and invasive lineages, aboveground biomass declined precipitously with increasing salinity. Relative to the no-salinity (control) treatment, biomass in the high-salinity treatment was 3.0 and 4.9 times lower for the invasive and native lineages, respectively (Fig. 1A). In fact, most plant traits were negatively impacted by salinity: Relative to the no-salinity treatment, invasive and native plants in the high-salinity treatment had leaves that were 7.6 and 2.4 times less tough (Fig. 1J), 21.2% and 31.5% lower in aboveground percent N (Fig. 1D), 4.0% and 5.9% lower in density of stomates (Fig. 1B), 2.5% and 4.5% lower in aboveground total phenolics (Fig. 1E), and 3.3–4.8% lower in aboveground and belowground percent C (Fig. 1F,G). In contrast, belowground total phenolics increased between the control and high-salinity treatment by 7.7% and 2.6% for the invasive and native lineage, respectively (Fig. 1I).

The southern LSU greenhouse was considerably warmer on average (~6°C) than the northern URI greenhouse (mean daily temperature:

Table 2. Summary of model-selection results for each plant trait.

Plant trait	AICc best model	AICc	ΔAIC	Akaike wt	R^2-fixed	R^2-model
a) Exotic genotypes						
Biomass (above)	U S G S × G U × S U × G	988.0	0	0.862	0.63	0.67
Stomate density	U S G N S × G U × S U × G	3466.3	0	0.557	0.30	0.41
Stomatal conductance	U	−7384.9	0	0.999	0.17	0.25
% Nitrogen (above)	U S G S × G U × S U × G	847.7	0	0.656	0.38	0.62
% Nitrogen (below)	U S G S × G U × S U × G	683.8	0	0.708	0.15	0.23
% Carbon (above)	U S G S × G U × S U × G	1608.1	0	0.890	0.26	0.37
% Carbon (below)	U S G S × G U × S U × G	2636.6	0	0.818	0.02	0.27
Leaf toughness	U S G S × G U × S U × G	688.8	0	0.874	0.27	0.36
Phenolics (above)	U S G N S × G U × S U × G	416.7	0	0.492	0.29	0.58
	U S G S × G U × S U × G	417.9	1.2	0.270		
Phenolics (below)	U S G U × G S × G	687.3	0	0.782	0.15	0.36
b) Native genotypes						
Biomass (above)	U S G S × G U × S U × G	709.5	0	0.562	0.45	0.69
	U S G N S × G U × S U × G	710.2	0.7	0.396		
Stomate density	U S G U × G S × G	204.3	0	0.749	0.16	0.60
Stomatal conductance	U	−4614.8	0	1.000	0.16	0.47
% Nitrogen (above)	U S G S × G U × S U × G	581.0	0	0.652	0.32	0.43
	U S G U × G S × G	582.8	1.8	0.265		
% Nitrogen (below)	U S G S × G U × S U × G	555.1	0	0.876	0.15	0.28
% Carbon (above)	U S G S × G U × S U × G	1043.6	0	0.823	0.11	0.28
% Carbon (below)	U S G N S × G U × S U × G	1561.7	0	0.808	0.03	0.35
Leaf toughness	U S G S × G U × S U × G	488.0	0	0.635	0.18	0.35
Phenolics (above)	U S G S × G U × S U × G	303.2	0	0.899	0.14	0.53
Phenolics (below)	U S G U × G S × G	428.5	0	0.548	0.07	0.24
	U S G S × G U × S U × G	429.7	1.2	0.301		

Notes: AIC_c best model is the model with the smallest AIC_c value and, in cases with ties, the model with the fewest parameters. All supported models are included (ΔAIC ≤ 2.0). Abbreviations are E, longitude; G, genome size; L, lineage (native or invasive); N, latitude; S, salinity treatment (0, 10, and 20 ppt); U, university greenhouse (LSU, URI). Goodness-of-fit statistics are computed using the method of Nakagawa and Schielzeth (2013) where R^2-fixed is the marginal R^2 and estimates the variance explained by the fixed effects, and R^2-model is the conditional R^2 and estimates the variance explained by the fixed effects plus random effects.

30.2° ± 0.7°C vs. 24.1° ± 0.9°C; mean ± SE). Associated with this warmer climate at LSU, invasive and native plants averaged 91.3% and 234.1% greater aboveground biomass than in the URI greenhouse (Fig. 1A). Notably, invasive and native plants in the southern greenhouse had 8.9 and 9.1 times tougher leaves (Fig. 1J), 26.5% and 18.7% lower aboveground percent N (Fig. 1D), 12.3% and 12.7% greater stomate density (Fig. 1B), 28.0% and 31.1% greater stomatal conductance (Fig. 1C), and 5.1% and 3.9% lower total phenolics aboveground (Fig. 1H), respectively, than in the north. Interestingly, invasive plants had the greatest percent N belowground at URI (4.3% greater) but the native plants had more belowground percent N at LSU (17.3%; Fig. 1E). Other traits exhibited small differences (<3%) between the two greenhouses.

Consistent among plant traits was a greenhouse × salinity ($U \times S$) interaction (Table 2, Fig. 1). Although the results are somewhat mixed, the general tendency was for the salinity gradient to have a stronger impact on plants in the southern than northern greenhouse. For example, the decline in invasive and native biomass from between the 0 ppt and 20 ppt salinity treatments at LSU was steeper than the decline at URI (the U × S interaction was significant in both cases; invasive $F_{2,555} = 26.5$, $P < 0.001$; native $F_{2,309} = 9.21$, $P < 0.001$; Fig. 1A). Similar patterns were observed for percent N aboveground (Fig. 1D), percent C aboveground (Fig. 1F), aboveground phenolics (Fig. 1H), and leaf toughness (Fig. 1J). Interestingly, percent N belowground increased at LSU and decreased at URI with increasing salinity (Fig. 1E).

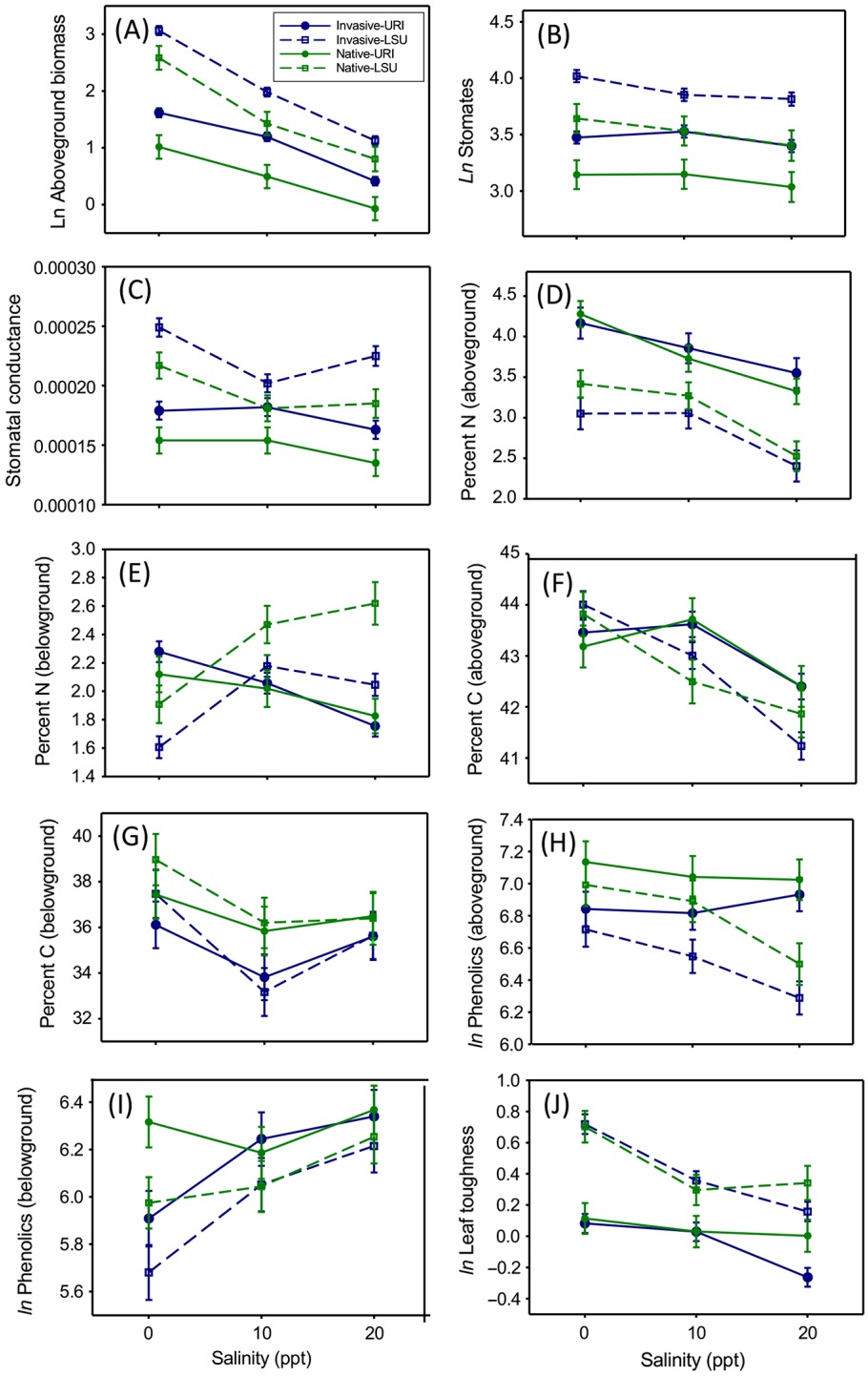

Fig. 1. The effects of salinity and greenhouse (LSU, URI) on (A) aboveground biomass, (B) stomate density on the adaxial leaf surface leaf toughness, (C) stomatal conductance, percent N in (D) aboveground and (E) belowground tissues, percent C in (F) aboveground and (G) belowground tissues, total phenolics (H) aboveground and (I) belowground, and (J) leaf toughness. Statistical results for the fixed effects from the AIC_c best models are reported in Appendix S1: Table S2. Separate analyses were conducted for the native and invasive lineages.

Among the *P. australis* populations, monoploid genome size was 0.505 ± 0.005 (n = 17) for the invasive lineage and 0.548 ± 0.006 (n = 12) for the native lineage. The difference in genome size between lineages was significant (t_{26} = 5.69, P < 0.001). Genome size and its interaction with greenhouse location (U × G) and salinity ($S \times G$) were included in all of the models for the traits measured except for the model for stomatal conductance (g_s; Table 2). Aboveground biomass increased with genome size: For the invasive lineage, biomass increased by 43% across the range of genome sizes (irrespective of garden, Fig. 2A). Similar results were found for biomass of the native lineage, although the proportional increase in biomass was greater at URI than LSU (Fig. 2B). For 8 of 20 comparisons (2 lineages × 10 traits), the U × G interaction was significant (uncorrected $P \leq 0.05$, Fig. 2; Appendix S1: Table S3). One of the strongest interactions involved leaf toughness. For the native lineage, leaf toughness increased by 76% over the range of genome size in the LSU garden (0.89–1.57 kg of force) while it increased by only 11% over the same genome size range at the URI garden (0.92–1.03 kg). For the invasive lineage, leaf toughness increased by 18% (0.87–1.03 kg) and decreased by 32% (1.52–1.33 kg) across the genome size range in the URI and LSU gardens, respectively (Fig. 2M). Interestingly, there were a number of other cases in which the U × G interaction was significant and the slope of the relationship between genome size and the trait was in the opposite direction between the LSU and URI gardens: percent N belowground and percent C belowground for the invasive lineage (Fig. 2G, K) and stomate density, above- and belowground phenolics for the native lineage (Fig. 2D, P, R). Effect sizes (i.e., proportional change in the trait across the range of genome sizes) were ≤0.18. Although a genome size × salinity interaction (G × S) was present in all models, the effect was not significant in any of the AIC_c best models and the differences in the trait–genome size relationship among salinity treatments were generally quite small (Appendix S1: Fig. S1).

Latitude of origin (N) was only important in the model for stomate density and aboveground phenolics for the invasive lineage and percent belowground C for the native lineage (Table 2; Appendix S1: Table S3). Only for percent belowground carbon was there a statistically significant relationship ($F_{1,7.73}$ = 13.6, P = 0.006). Here, percent C increased with the latitude of origin of the native plants. Longitude of origin (W) was not included in any of the best models for any of the traits (Table 2).

We found no evidence that trait plasticity was greater for the invasive than native lineage. The mean effect size (Hedge's d) was 0.036 with a 95% bootstrapped confidence interval that overlapped zero (−0.313 to 0.311). For the invasive lineage, there was a significant relationship between genome size and plasticity for four of the ten traits (Fig. 3; Appendix S1: Table S4). Plasticity in stomatal conductance and aboveground phenolics decreased (Fig. 3A, F) and plasticity in belowground percent C and N increased with increasing genome size. There were also two cases where plasticity in the native lineage was linearly related to genome size. Similar to the invasive lineage, one relationship was positive (percent C aboveground, Fig. 3D) and the other was negative (leaf toughness, Fig. 3B).

Discussion

Few studies have investigated how genome size interacts with environmental variation. One recent example of such as study is a survey of mangrove plant communities growing in intertidal regions characterized by high salinity, high temperatures, and saturated soils demonstrated that reduced genome size in mangroves was an adaption to environmental stress (Gaut 2018, Lyu et al. 2018). Other recent observational studies have focused on genome size selection within species. For example, Díez et al.'s (2013) study on teosinte, a wild progenitor of maize, found that genome size variation correlated with temperature and precipitation across two altitudinal gradients. Likewise, the predictable geographic distribution of intraspecific genome sizes in *Arabidopsis thaliana* suggests that plant genome size is a trait that adapts to local conditions (Long et al. 2013). Although plant stress is hypothesized to cause genome rearrangements leading to adaptation (i.e., The Genome Shock Hypothesis; McClintock 1984), Gaut (2018) suggests that the above examples "provide compelling evidence"

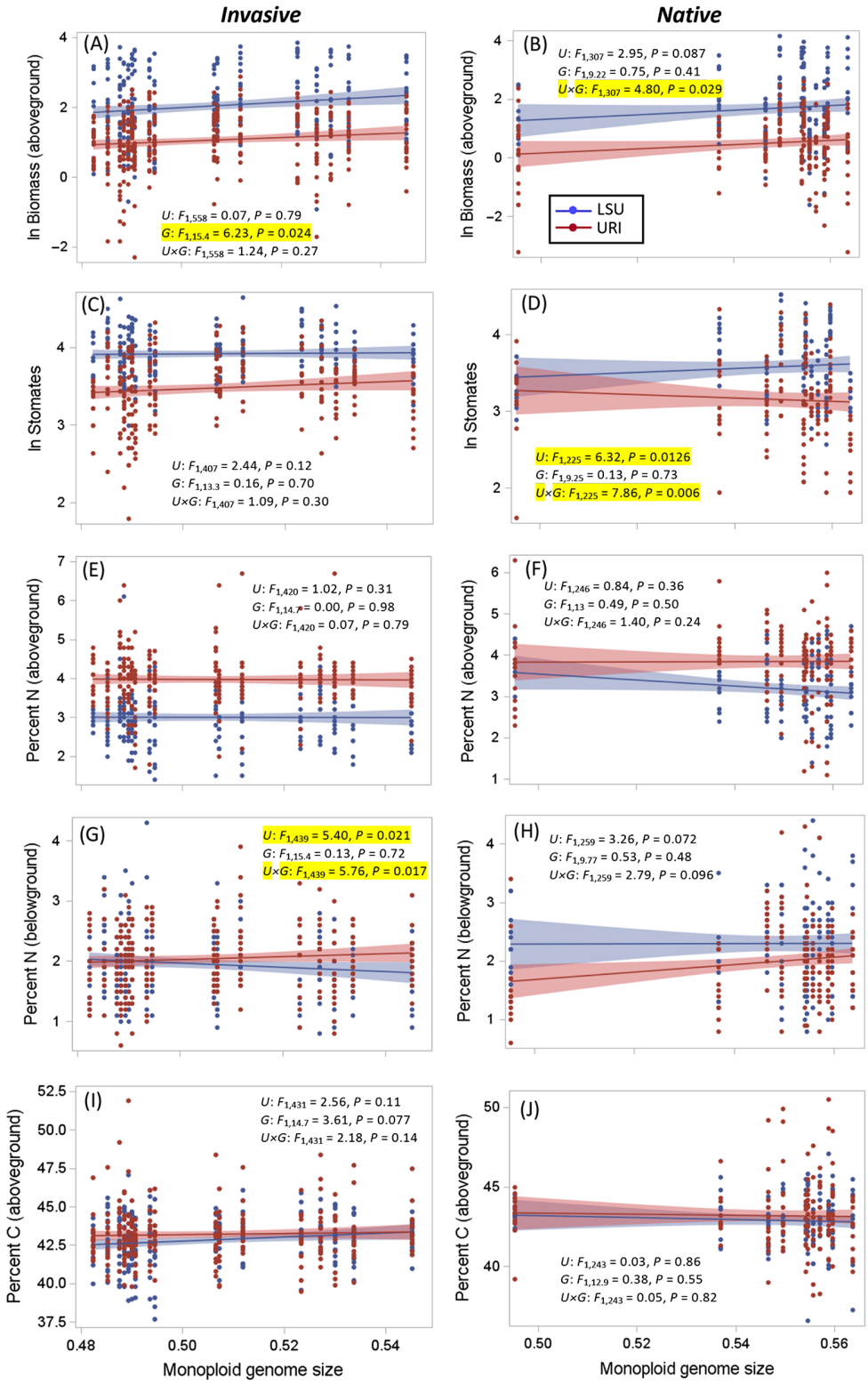

Fig. 2. Relationship between genome size and plant trait value for each *Phragmites australis* lineage (invasive,

(Fig. 2. *Continued*)
native) and nine plant traits (stomatal conductance was excluded because genome size was not a factor in the AIC_c best model; Table 2). Raw data are plotted along with the least-squares regression lines and 95% confidence bands for each greenhouse location. Mixed-effects model results for the fixed factors U = greenhouse, G = genome size, and U × G = greenhouse × genome size interaction are also provided and those effects with an uncorrected *P*-value ≤0.05 are highlighted in yellow.

that genome size shifts in plants are likely adaptive and due to selection on phenotypic traits correlated with genome size. However, whether such selection is a result of constraints against large genomes is not yet known (e.g., Lynch 2007, Gaut 2018).

Multiple studies have found correlations between genome size and abiotic field conditions (e.g., Knight and Ackerly 2002, Dušková et al. 2010, Carta and Peruzzi 2016). Drought tolerance and various growth, nutritional, and herbivore defense traits are also correlated with genome size for *P. australis* (Meyerson et al. 2016*a*, Pyšek et al. 2018). However, this study is the first that we know of that explicitly tests the Large Genome Constraint Hypothesis in an experimental setting by measuring plant trait responses to environmental stressors. Many of the traits that

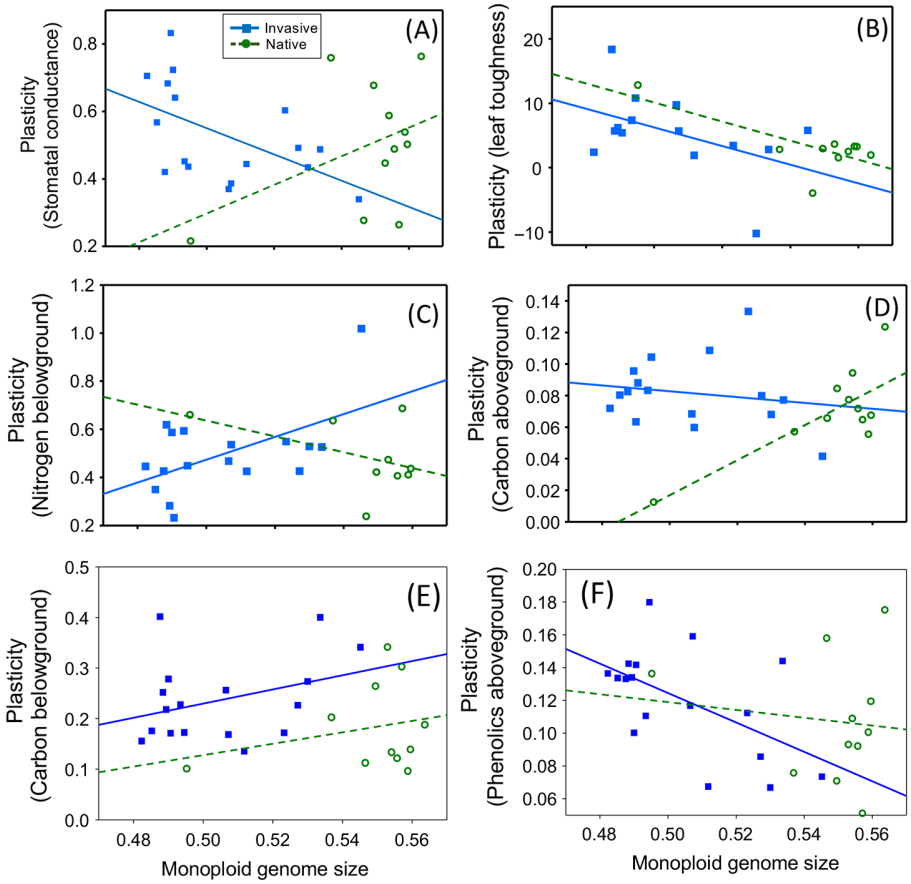

Fig. 3. Relationship between genome size, lineage and trait plasticity for (A) stomatal conductance, (B) leaf toughness, (C) percent N belowground, (D) percent C aboveground, (E) percent C belowground, and (F) total phenolics belowground. Lines are fit by least-squares regression, and separate analyses were performed on each lineage. Statistical results are in Appendix S1: Table S4.

we measured were influenced by salinity, genome size, and greenhouse location (Table 2; Appendix S1: Table S3), but the relationships of plant traits to genome size were sometimes indirect. For example, plasticity has been shown to increase invasion success when it increases plant fitness but no link between plasticity and genome size has previously been made in invasive species (Richards et al. 2006, Chun 2011, Davidson et al. 2011). Our study found that genome size influences trait plasticity for traits related to plant fitness (e.g., stomatal conductance; Fig. 3A, C, E, F), indicating that genome size may indirectly influence invasion success via plasticity (Hypothesis 3, below).

High salinity negatively affects plant growth, physiology, nutritional, and defense traits

There has been a significant amount of research on how plants respond to and tolerate individual stressors, but less work has focused on the effects of stressor combinations on plant performance and plant traits (Suzuki et al. 2014, Ramegowda and Senthil-Kumar 2015). Research to date indicates plant responses to multiple simultaneous stressors are complex and varied. How a plant responds to stress combinations depends on the particular plant species, plant growth stage, and the particular stress combination, stress intensity, and duration (reviewed in Suzuki et al. 2014). Furthermore, the combined effects of stressors can be additive or synergistic worsening the overall impact of environmental stress, but combined stressors also can have a positive interaction with one stressor mitigating the effects of the other; some traits may increase under the stress (e.g., biomass) while others decrease (e.g., reproductive output; Suzuki et al. 2014).

Our predictions for the stress gradient manipulations for both salinity and greenhouse location (mid- and southern latitudes) were supported: Across both lineages, plants in the freshwater treatment outperformed plants in the high-salinity treatments for most traits in both greenhouses (Fig. 1). Most notably, biomass decreased for both lineages of *P. australis* as salinity increased as several other studies have reported (Lissner and Schierup 1997, Vasquez et al. 2005, Achenbach et al. 2013). Consistent with an earlier study (Bhattarai et al. 2017), we found a strong greenhouse location effect (plasticity), with the plants growing in the southern warmer garden outperforming the plants grown in the cooler northern garden for most traits including biomass, leaf toughness, and stomate density, even under conditions of increasing salinity (Fig. 1). However, we also found the reverse for leaf N and aboveground phenolics; these traits were greater for plants grown in Rhode Island than those in Louisiana.

Some traits measured at the southern LSU greenhouse were greater such as biomass and leaf toughness, while others, such as aboveground phenolics and percent nitrogen, were greater at the northern URI greenhouse. Interestingly, plants in the southern greenhouse generally appeared to be more sensitive to the salinity gradient, showing a greater change in trait values from fresh to saltwater than the northern plants as shown for all of the traits in Fig. 1. This result is not surprising given that under heat stress, plants increase stomatal conductance to cool leave via transpiration. Heat-enhanced transpiration can increase plant salt uptake thereby exacerbating salinity stress (Keleş and Öncel 2002, Suzuki et al. 2014). Similarly, another study found that at higher temperature, germination in *P. australis* was more sensitive to a salinity gradient (Greenwood and Macfarlane 2006). The variable results between greenhouses and among traits that we found emphasize the importance of context and stress combination (here greenhouse location, salinity) as well as the particular traits measured. We proposed four hypotheses to test the LGCH and investigated whether genome size interacts with environmental stressors to affect plant traits associated with fitness and plant defense.

Hypothesis 1: Small-genome plants are more stress-tolerant than plants with larger genomes

Plant populations with smaller genomes are hypothesized to exhibit greater stress tolerance than those with larger genomes, and indeed, Pyšek et al. (2018) found evidence that *P. australis* populations with smaller genomes had greater drought tolerance than those with larger genomes. However, empirical studies testing the relationship between genome size and stress tolerance are rare (Knight et al. 2005, Suda et al. 2015, Carta and Peruzzi 2016), and, prior to this study, correlative.

This study provided a strong test of plant stress tolerance as it relates to genome size by minimizing potentially confounding phylogenetic effects by focusing on a single species at the intraspecific level and by using genetically identical clones for each population in both gardens. In fact, *Phragmites australis* has been suggested as in ideal model system for investigating the role of genome size in invasion success (Suda et al. 2015, Meyerson et al. 2016*b*). The variation in *P. australis* monoploid genome size is greater than is known for other genera that include invasive species, thereby facilitating its use to investigate associations between genome size, environmental stressors, and functional traits (Suda et al. 2015), and the difference in genome size between the invasive (smaller) and native (larger) lineages was significant.

Despite establishing salinity and thermal stress gradients that impacted a broad range of plant traits, we did not find that smaller genome size directly ameliorated plant stress, at least for the stressors that we tested—salinity, high temperature, and their interaction. We did find a number of stress gradient × genome size interactions that were significant (Fig. 2; Appendix S1: Fig. S1) but there was no clear indication that plants with smaller genomes outperformed plants with larger genomes when conditions were more stressful; that is, the slope of the relationship between genome size and a trait should be more steeply negative in a more stressful (e.g., saline) than benign (e.g., freshwater) environment. Such relationships were not evident in Fig. 2 and Appendix S1: Fig. S1. It is possible that despite the influence of genome size on other plant traits, genome size may not be important for stress tolerance at the intraspecific level (but see discussion below under *Hypothesis 3: Small-genome plants have greater plasticity than large-genome plants*). Alternatively, fully exploiting a wider range of available genome size variation within *P. australis* (i.e., verified Cx range is 0.42–0.833 pg for nearly 600 *P. australis* plants collected throughout its global range and analyzed by the authors, L. A. Meyerson, P. Pyšek, J. Suda, *unpublished data*) in another similar experiment could reveal stress effects related to genome size at the intraspecific level. However, it is also possible the genome size could be significant for other stressors not measured in this study such as drought, flooding, high CO_2, herbivory, and pathogens since it has been established that the ways in which plants respond depend on the particular stressor (Suzuki et al. 2014, Ramegowda and Senthil-Kumar 2015).

Hypothesis 2: Stress tolerance is highest for plants originating from high and low latitudes and is associated with smaller genome size

A recent common garden study that included a global collection of *P. australis* populations in Aarhus, Denmark, found that both monoploid genome size and latitude of plant origin were significant predictors of almost all of the plant traits studied (Meyerson et al. 2016*a*). A quantile regression analysis of a different global data set analyzed for the relationship between plant genome size and latitude revealed that within the upper 20–35% of *P. australis* genome sizes, there was a significant negative relationship between latitude and genome size, with the smallest and largest genome sizes found at the highest and lowest latitudes, respectively. The same analysis also found the greatest variability in genome sizes occurred at mid-latitudes (i.e., 25°–50°; Meyerson et al., *unpublished data*), whereas in the present study on *P. australis* populations in North America, latitude was only important for stomate density and phenolics in the invasive lineage and percent belowground carbon in the native lineage. Other environmental factors had more important effects. This was a surprising result given that earlier studies in the field and in common gardens found latitude of origin to also influence multiple traits of *P. australis* such as stem height, stem density, plant biomass, nutritional condition, plant defense, and herbivory (Cronin et al. 2015, Mozdzer et al. 2016). For example, Cronin et al. (2015) conducted a field study which found that environmental factors and genetic factors combined to influence plant traits. Allen et al. (2017) found a strong latitudinal gradient in the field in terms of galling rates, but a gradient was not evident in the complementary greenhouse experiment. A possibility is that latitudinal effects are primarily plastic responses to the environment. Another explanation for the disparity between this study and previous ones is that this study did not include as wide a latitudinal of origin range as the other studies cited (e.g., ~15° this study vs. ~22° in

Cronin et al. 2015). However, this study was designed to minimize maternal effects by using plant material grown under greenhouse conditions and used identical clones from a single source for all populations in both gardens so that any differences detected among plant populations are genetically based unlike studies conducted in the field. In addition, while we were able to include populations from the southern end of the native range (Mackay Island, NC, southern California, Table 1), we did not have populations from the northernmost edge of the North American native *P. australis* range due to importation restrictions of foreign plant materials from Canada. It may also be that although studies have shown that species with small genomes are found across a wider range of environmental conditions (e.g., altitude, temperature, precipitation) than with those with large genomes (Knight et al. 2005, Pyšek et al. 2018), salinity may interact differently with genome size than other stressors not included in this study.

Hypothesis 3: Small-genome plants have greater plasticity than large-genome plants

Increased plasticity may facilitate plant invasions if high fitness can be maintained across a wide range of abiotic conditions or if plasticity causes the plant to exploit resources more efficiently at one end of an environmental gradient (Richards et al. 2006, Chun 2011, Davidson et al. 2011). Trait plasticity is also thought to be critical for stress tolerance in plants because it can help plants mitigate the consequences of environmental variability via trait adjustment (reviewed in Scheepens et al. 2018). We predicted that plasticity would be negatively correlated with genome size (see Knight et al. 2005), but we found that these relationships were variable among plant traits and between the native and invasive *P. australis* lineages. Plant genome size influences plasticity for 6 out of 10 traits (4 for the invasive lineage, 2 for the native lineage), with positive or negative slopes depending on the lineage and trait. However, in contrast to other studies (Douhovnikoff et al. 2016, Bhattarai et al. 2017), we did not find evidence that trait plasticity was greater for the invasive lineage relative to the native. Importantly, these are the first data we know of that show that genome size is related to plant trait plasticity.

We also found a negative relationship for two key defensive traits, leaf toughness (significant only for the native) and total phenolics aboveground (significant only for the invasive), suggesting that the smaller genomes can allocate resources toward defense when environmental conditions warrant it and otherwise toward growth and reproduction. Interestingly, for 6 out of 10 of the cases for the invasive lineage (stomate density, stomatal conductance, leaf toughness, percentage of C aboveground, phenolics aboveground and phenolics belowground), we found support for a negative relationship between genome size and plasticity though only total aboveground phenolics and stomatal conductance were significant (Appendix S1: Table S4). The native lineage also demonstrated a negative relationship for genome size and plasticity in half the cases but only leaf toughness was significant (Fig. 3A, F; Appendix S1: Table S4). On the other hand, half the cases with a significant relationship between genome size and plasticity had positive slopes (Fig. 3; Appendix S1: Table S4). While the negative relationships between plasticity and genome size that we identified support the LGCH, potentially contributing to the success of small-genome size plants under some conditions, the traits with positive relationships do not. The mix of negative and positive relationships between plasticity and genome size appear to be trait dependent necessitating further experiments to examine additional traits and abiotic conditions to shed light on these mixed results.

Douhovnikoff et al. (2016) suggested that plasticity in stomatal morphology could facilitate the adaptation of a genotype to changing environmental conditions. They found that stomatal conductance had a greater mean and higher plasticity in the invasive than native lineage of *P. australis*. While plants in both of our greenhouses had no water limitations, the southern garden had a mean daily temperature that was on average >6°C higher than the northern greenhouse over the length of this experiment. The plants of both lineages grown in Louisiana had greater stomate density and stomatal conductance than those grown in Rhode Island, presumably due to the higher temperatures and greater solar radiation relative to those grown in Rhode Island (Fig. 1B, C). Since global temperatures

have been steadily rising over the last several decades and are already exerting stress on some plants (Dusenge et al. 2019), the plasticity exhibited by both lineages of *P. australis* for stomatal conductance could be advantageous given that greater stomatal conductance can result in higher evaporative cooling that helps plants tolerate higher temperatures under climate change.

Previous studies have found that *P. australis* demonstrates lineage-specific responses to increases in temperature that include morphology, growth, and some traits related to photosynthesis (reviewed in Packer et al. 2016, Eller et al. 2017). Others have noted plasticity in some *P. australis* traits both in its native and introduce ranges, but particularly for the invasive lineage in North America (Mozdzer et al. 2013, Douhovnikoff et al. 2016, Bhattarai et al. 2017, Eller et al. 2017). Because, on average, the invasive lineage in North America has a smaller genome size than the native lineage (Suda et al. 2015, Meyerson et al. 2016, Pyšek et al. 2018), it could be that plasticity in stomatal conductance for the invasive lineage is an important mechanism for its success under stress.

Confirming results from other studies, we found that the relationship of plant traits to genome size can be positive, negative or neutral—even within the same study—depending on the traits under investigation (Suda et al. 2015, Meyerson et al. 2016*a*) and, as demonstrated by this study, the conditions under which plants are grown. Our results highlight the need for additional studies on more species and traits under varying environmental conditions and suggest that as environmental stress increases, changes in plant population genomic structure could follow under climate change. This may be particularly true at species range margins where abiotic conditions tend to be more stressful.

Conclusions

More explicit tests of the Large Genome Constraint Hypothesis are needed to determine whether the interaction of genome size with environmental stressors will influence future plant distributions, particularly for other species. As the rate of species introductions continues to accelerate (Levine and D'Antonio 2003, Seebens et al. 2017, Seebens et al. 2018) and species shift their ranges due to stressors, we need to better understand how novel webs of interacting species organize under a changing environment in order to more accurately predict habitat shifts and trophic interactions across large spatial scales (Thompson 2009). Plant fitness, phenology, morphology, and resistance to pathogens and herbivores also vary with genome size for some species (Thompson et al. 1997, Nuismer and Gandon 2008, Henery et al. 2010, Hahn et al. 2012, Meyerson et al. 2016*a*), but this has been rarely studied. Therefore, a better understanding how plant trait responses vary with genome size under emerging stress gradients may help to inform predictions as new species arrive and as plant ranges expand and contract (van der Putten 2012, Wardle et al. 2013, Violle et al. 2014), and including genome size in explanatory models may improve their power to predict invasiveness at least for some traits (Pandit et al. 2014) and more fully test the Large Genome Constraint Hypothesis.

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