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Consumer control of the establishment of marsh foundation plants in intertidal mudflats

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ABSTRACT: The establishment of foundation plants in bare mudflats is a critical process. While consumers are increasingly recognized to exert strong top-down control of plant performance in salt marshes, studies to date have focused on the effects of consumers on mature stands rather than on plants that are recolonizing after disturbance or where restoration has occurred. Furthermore, whether consumer-facilitated fungal infection differentially affects newly establishing plants in mudflats compared to mature stands remains poorly understood. In a salt marsh in southern Brazil, we examined the effects of herbivory by the crab *Neohelice granulata* and fungal infection on the survival and growth of *Spartina alterniflora* transplanted into mudflats. We additionally tested the effects of herbivory and fungi on newly established versus well-established stands of *S. alterniflora.* Highly intensive natural crab herbivory significantly reduced the development of *S. alterniflora* and increased its fungal infection by 50%. Light herbivory, removing only small areas of plant leaves, reduced the height growth and leaf production of directly affected tillers by about 14 to 18%, and both newly and well-established, clonally integrated stands of *S. alterniflora* allocated energy towards the formation of new tillers. While herbivory facilitated fungal infection and subsequent fungal damage in leaves, no significant effects of fungicide treatment or its interactions with crab grazing on *S. alterniflora* growth were detected, suggesting a saprophytic rather than a pathogenic role of fungi in this 3-species interaction. Here, we found that marsh grasses transplanted for restoration or those colonizing disturbance-generated mudflats may be facilitated by protection against consumers.

KEY WORDS: Top-down control · Herbivory · *Spartina alterniflora* · Fungal infection

INTRODUCTION

Over the past century, coastal ecosystems have been degrading at a global scale due to anthropogenic impacts and climate-related stressors (oysters: Beck et al. 2011; seagrass: Waycott et al. 2009; coral reefs: Wilkinson 2008; mangroves: MEA 2005). The decline of North American salt marshes has also been dramatic, with losses of marsh habitat reaching up to 90% over the past century in some regions (Gedan & Silliman 2009, Silliman et al. 2009). The loss of salt marsh habitats has led to subsequent reductions in ecosystem functioning and provisioning of valuable services such as fisheries production, carbon storage, and shoreline protection (MEA 2005, Silliman et al. 2009). To combat these losses to marsh-associated species and local human communities, coastal managers are increasingly turning

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towards restoration of salt marshes on bare substrate as a primary conservation measure (Teal & Peterson 2009, Silliman et al. 2015). Understanding the ecological factors that limit marsh grass survival and productivity in mudflats or sandflats will be key for increasing the success of these restoration efforts.

In salt marshes, the establishment of habitat-forming marsh grasses is a critical stage in the initial development of intertidal communities and the recovery of habitats after disturbance, as these plants ameliorate stressful environmental conditions for other less stress tolerant species, promoting colonization and development of diverse communities (Semchenko et al. 2012). Past research on plant establishment in mudflats has largely focused on physiological tolerances to physical stresses, such as flooding, anoxia, and high salinities (Chung 2006). While salt marshes are also often strongly controlled by consumers (Silliman & Bertness 2002, Silliman et al. 2005), it is unclear whether newly establishing or colonizing plants are affected by consumers in these critical stages (e.g. Costa et al. 2003, Alberti et al. 2010). Further, while grazers limit marsh plant survival and growth directly due to the removal of plant biomass and reduced photosynthetic capacity (Costa et al. 2003, Alberti et al. 2007, 2010, Lewis & Boyer 2014, He et al. 2015), herbivores can also decrease marsh plant growth through the indirect facilitation of fungal infection in grazing wounds (Silliman & Newell 2003, Daleo et al. 2009). Whether consumers facilitate fungal infection in grazing wounds and indirectly affect the growth of newly establishing plants in mudflats, however, remains largely untested.

Newly establishing plants in mudflats, such as those often targeted for restoration projects, are likely to be susceptible to consumer grazing and fungal infection. Vegetative propagation by rhizomes is a major mechanism of spatial occupation and local population maintenance of salt marsh plants including *Spartina*, which are often highly clonal (Pennings & Callaway 2000, Brewer 2011). The physiological integration of plant tillers not only allows the sharing of resources, such as water, photo-assimilates, and nutrients, but is also responsible for tolerance against herbivore damage through resource remobilization from storage organs, spreading of chemical defenses, and compensatory growth (Schmid et al. 1988). Thus, newly establishing plants with few integrated tillers may be susceptible not only to grazing due to reduced resources but also to fungal infection induced by grazer wounds. An experimental test is necessary to resolve this question and will have important implications for the success of marsh restoration.

To evaluate the direct (i.e. consumption) and indirect (i.e. fungal infection) effects of consumer grazing on newly colonizing *Spartina alterniflora*, we conducted 2 transplant experiments in a salt marsh in southern Brazil, where we manipulated the presence of crab grazing and fungi. In this region, the burrowing crab *Neohelice granulata* (Dana 1851) is one of the most abundant herbivores of the genus *Spartina* (Costa et al. 2003, Alberti et al. 2007, Daleo et al. 2009). *N. granulata* decreases the biomass of *S. densiflora* and *S. alterniflora* plants (Alberti et al. 2007, Daleo et al. 2009, Freitas et al. 2015), restricting the spatial distribution of these species (Costa et al. 2003). Additionally, experiments conducted in Argentina (Daleo et al. 2009) and southern Brazil (Freitas et al. 2015) have demonstrated that wounds generated by *N. granulata* herbivory can facilitate fungal infection in leaves of *S. alterniflora* and *S. densiflora*. Here, we specifically test the hypothesis that *N. granulata* herbivory affects establishing *S. alterniflora* plants through both direct consumption of tissues and indirect facilitation of fungal infection.

MATERIALS AND METHODS

Study site and abiotic data

Two experiments were carried out on an intertidal mudflat 23 cm below the mean water level of the Patos Lagoon estuary, which is located on Pólvora Island (Rio Grande, Brazil, 32° 01′ S, 52° 06′ W). Salt marshes cover 45 ha of the island but increase only 60 cm in elevation. This site is characterized by a warm temperate climate and by a microtidal regime (0.5 m) with an irregular flooding pattern driven primarily by winds and freshwater runoff from a 200 000 km^2 watershed (Costa et al. 2003, Marangoni & Costa 2012).

Throughout the experimental duration, air temperature was monitored by the National Institute of Meteorology weather station located in the city of Rio Grande. Water level (to the nearest centimeter on a graduated ruler) and salinity were determined daily, between 10:00 and 11:00 h, at a fixed station located 2 km north of the study site. Simultaneous measurements of the water level at the fixed station and the study site were made, allowing for an estimation of flooding frequency (percentage of time flooded) at the study site over the experimental periods. Additionally, pore water salinity of the surface sediment (top 10 cm) was measured weekly using a refractometer at 3 points within the study site.

Fig. 1. Exclusion and partially enclosed cages with *Spartina alterniflora* clumps transplanted in a mudflat at Pólvora Island, southern Brazil

Expt 1: Effects of crab grazing and fungal infection on *Spartina alterniflora* transplants into mudflats

To test whether crab herbivory affects *Spartina alterniflora* survival and growth through direct consumption and/or indirect fungal infection, we conducted a fully factorial field experiment manipulating crab grazing (3 levels: no grazing, simulated grazing, and crab grazing) and fungal presence (2 levels: with and without fungi). We used clumps of *S. alterniflora* transplanted into mudflats, as is often used in *S. alterniflora* restoration projects. The 6 treatment combinations were (1) crab grazing + with fungi, (2) crab grazing + without fungi, (3) simulated grazing + with fungi, (4) simulated grazing + without fungi, (5) no grazing + with fungi, and (6) no grazing + without fungi. Each treatment combination was replicated 10 times.

S. alterniflora clumps were obtained by spade digging natural monospecific stands which were transported to an unheated greenhouse, where tillers with fragments of rhizomes and roots were individualized and cultivated for 40 d in trays with a 1:1 ratio of beach sand and organic compost mixture. This procedure was done to ensure that there was no prior grazing in these tillers and to allow for the cultivation and use of vegetated propagules, a standard propagation unit used in many local marsh restoration projects (Costa 2011). At the end of November 2012, 60 *S. alterniflora* clumps were transplanted into plots in a mudflat at the experimental site. Each plot contained 1 clump (average height of 47.8 ± 1.5 cm) with 2 uprooted tillers (tagged with a plastic-coated wire; each tiller had 3 leaves). The plots were spaced 1 m apart in 5 rows and were randomly assigned to 1 of the 6 treatments listed in the previous paragraph.

In all no-grazing and simulated-grazing treatments (with and without fungi), *S. alterniflora* clumps were protected with cylindrical cages (60 cm height, 20 cm diameter) constructed of galvanized steel mesh (3 mm mesh size) (Fig. 1). In both crab grazing treatments, clumps were surrounded by partially enclosed cages, where plants remained open to natural grazing by *Neohelice granulata*. No visual differences were observed between plants surrounded by partially enclosed cages and plants in nearby newly colonized natural patches. In both simulated-grazing treatments, we wounded a 4 cm^2 area of 1 leaf in each tiller in each clump with a scalpel to create 3 longitudinal incisions (scratches) parallel to leaf veins, as is commonly observed in natural *N. granulata* grazing (Costa et al. 2003, Freitas et al. 2015). As plants had an average of 3 leaves per tiller, we simulated grazing wounds on 1 leaf per tiller, a grazing intensity (33% of the leaves) comparable to natural grazing (Alberti et al. 2007). The utilization of a

simulated-grazing treatment with plants protected by cages from natural crab grazing created a distinctive level of herbivore impact (discrete) compared with natural crab grazing (continuous) and also served to control for a standard grazing intensity to evaluate fungal infection and the effects of fungi on plants. For the without-fungi treatments, all tillers were sprayed with 1.5 g l^{-1} Daconil BR fungicide (dissolved in estuarine water, applied during low tide) on a weekly basis to remove fungi from leaves. Previous studies using similar concentrations of Daconil showed no effects on *S. alterniflora* growth (Silliman & Newell 2003, Sala et al. 2008).

On the day of transplantation, the density of *N. granulata* crab burrows in a 1 m^2 quadrat around each *S. alterniflora* clump was quantified as a proxy of crab density (Alberti et al. 2007), and the tiller height and leaf number of each artificially wounded tiller in each transplanted clump were measured. Then, 21 d later, when plants in natural crab grazing treatments (partially enclosed cages) had been substantially grazed and their leaf blades were extensively destroyed, we measured the height and number of leaves per surviving tiller and total number of tillers per clump. All leaves in each clump were collected by clipping at ground level with scissors. For plants inside enclosed cages (no-grazing and simulated-grazing treatments), 12 leaves from wounded tillers in each treatment were randomly collected (total leaves: n = 36) and transported (in the dark and refrigerated) to the laboratory for ergosterol analysis (see 'Quantification of fungal infection' for details). The clipped plants were oven dried for 72 h at 60°C to quantify biomass (g m^{-2} dry weight).

Chi-square tests (χ^2) were used to analyze differences in the survival frequency of transplanted tillers among treatments. The separate and interactive effects of crab grazing and fungicide on the height, leaf number, and aboveground biomass from survived clumps of *S. alterniflora* were examined using unbalanced 2-way ANOVAs, followed by Tukey's HSD multiple comparisons at the 5% significance level (Zar 2010). Height was log(x + 1) transformed to fit the assumptions of ANOVA. All statistical analyses were conducted with SYSTAT 5.0 (Systat Software).

Expt 2: Effects of *Spartina alterniflora* establishment duration on fungal facilitation by crab grazing wounds

To test whether the extent of fungal infection facilitated by crab grazing wounds and plant growth are dependent on the establishment length of *S. alterniflora* (e.g. newly established versus well-established stands), we conducted a second factorial experiment in March 2013 with the following treatments: establishment time (0 vs. 3 mo) and fungal presence (with vs. without fungicide). The growth of marsh grass clumps through tillering may increase the resistance of individual tillers to grazing and/or fungal infection because of clonal integration and shared resources. Thus, this second experiment seeks to evaluate this response of individual tillers from grasses of different sizes due to establishment time.

S. alterniflora within the 3 mo establishment treatment were sprouted from the basal meristems of the plants established in the mudflat the previous year (n = 20; from crab removal treatments of Expt 1), while 0 mo marsh grass clumps (n = 20) were obtained by taking plants from mature stands to an unheated greenhouse, where tillers were individualized and cultivated for 40 d before being transplanted into crab exclusion cages installed in the mudflat. The number of tillers and leaves of the clumps, planting distance, and exclusion cages for the newly transplanted *S. alterniflora* clumps were all the same as in Expt 1. All establishment month 3 clumps had been previously protected against crab grazing in exclusion cages for 3 mo, after cutting *S. alterniflora* tillers at the end of Expt 1 (December 2012). Each of these clumps had on average 9 ± 4 (standard error) tillers at the beginning of Expt 2, and no plant exhibited sign of grazing damage. In all fungicide treatments, half of the clumps of each age were sprayed with the fungicide Daconil BR (1.5 g l^{-1}) every 5 d, and the other half were left unmanipulated. Clumps of the 0 mo treatments were randomly assigned to a fungal presence treatment (with vs. without fungi); however, clumps of the 3 mo establishment treatment previously treated with fungicide were also assigned to this treatment. Overall, we had the following 4 treatments (10 replicate clumps per treatment): (1) 0 mo, fungi present; (2) 0 mo, fungi absent; (3) 3 mo, fungi present; and (4) 3 mo, fungi absent.

In all treatments, 1 pair of tillers per clump of similar size and leaf number was marked with a colored electric wire, with 1 tiller randomly assigned to the simulated grazing (wounds in 2 leaves) treatment and 1 tiller randomly assigned to the control treatment. Considering that the 2 clump types had a different initial number of tillers per clump, we created simulated wounds on 33% (of 6 leaves, 2 leaves were wounded) and 7.4% (of 27 leaves, 2 leaves were wounded) of establishment month 0 and month 3

clumps, respectively. At the beginning of the experiment and 35 d later, the total number of tillers and the height and number of leaves of the damaged tillers (with and without simulated grazing) in each clump were quantified. Differences between these 2 measurements were used to estimate tiller production per clump, as well as height growth (cm) and leaf production of damaged and undamaged tillers. Six leaves were randomly selected from undamaged tillers and damaged tillers with and without fungicide application, for both the 0 mo and 3 mo establishment length treatments, and collected for the quantification of fungal biomass. Only up to 1 leaf was taken from each of the 10 clumps per treatment. These leaves were placed in individual plastic bags and transported (in the dark and refrigerated) to the laboratory for ergosterol analysis.

To examine the effects of establishment length and fungicide application on tiller production, we evaluated both the absolute number of new tillers produced per clump and the birth rate of tillers (in tillers per tiller per day). This second estimate of tiller production takes into account the uneven initial number of tillers between replicates in the 3 mo clumps and also clump size-related differences in tiller production. Additionally, tiller birth rate is assumed to decline with increasing density and competition between plant modules as net resource supply decreases (Costa & Seeliger 1988, Brewer 2011). Thus, differences in tiller birth rate per capita among treatment levels may indicate changes in soil resource supply or plant ability to obtain local resources. Two-way ANOVA followed by Tukey's HSD post hoc multiple comparison test were used to evaluate the absolute number and birth rate of new tillers.

Simulated grazing was conducted as in Expt 1 on clumps of all treatments. Additionally, the direct effect of crab grazing over wounded tillers was evaluated by comparing them with non-wounded tillers within each clump type and fungal presence treatment combination. Two-way repeated measures ANOVA (SYSTAT GLM procedure) was used to compare height growth and leaf production of wounded and non-wounded tiller pairs, where the repeated (within-subject) factor was simulated-grazing, and the fixed between-subject factors were transplant clump age and fungal presence. Values of height growth, tiller production, and tiller birth rate were $\log_{10}(x)$ transformed to fit the assumptions of ANOVA. These analyses compare the height and leaf response of integrated pairs of tillers, i.e. 1 tiller of the pair wounded and the other not. Both individual tillers of the pair were connected with each other and with their surrounding sister tillers throughout the experiment. Paired tillers were considered a repeated (within-subject) factor to examine the effects of simulated grazing. Repeated measures ANOVAs have been previously applied to compare the performance and induced systemic resistance to herbivory of interconnected individuals (e.g. ramets or vegetative modules), since it takes into account the interdependence of shoots/tillers of a clonal clump (Gómez et al. 2008).

Quantification of fungal infection

In the laboratory, we examined the surface of leaves collected from each of the 2 experiments (described earlier in this section) with a stereomicroscope (10× magnification), and the proportion of damaged leaf area was estimated using a damage rating scale of 6 levels: 0, 20, 40, 60, 80, and 100%. Damaged areas in leaves from the treatments of crab removal and without simulated crab wounds were always related to senescent tissues. The leaves were individually kept in amber flasks with 100% methanol in a freezer (–20°C) before quantification of fungal infection (Newell et al. 1988).

Fungal biomass was quantified by measuring leaf ergosterol content. Ergosterol is a fatty acid that is well correlated with metabolically active fungal biomass and has been widely used to estimate fungal biomass on the leaves of marsh plants (Silliman & Newell 2003, Sieg et al. 2013). Ergosterol content (µg erg g^{-1} fresh weight) was determined at the Micotoxinas e Ciência de Alimentos Laboratory, using a method adapted from Newell et al. (1988). To do this, 0.10 g fresh weight of each leaf sample was used, and the ergosterol content in 100% methanol extracts of *S. alterniflora* leaves was quantified against a standard curve of commercial 95% ergosterol (Sigma-Aldrich), with UV absorption peak characteristics of ergosterol at 282 nm in a HPLC/UV (Shimadzu). For both experiments, leaf ergosterol content was not quantified for plants inside partially enclosed cages (crab grazing treatments) since leaf blades subjected to natural crab grazing were extensively destroyed. Additionally, leaves of plants in no-grazing + without-fungi treatments did not have their ergosterol content quantified, due to restriction of funding for chemical analysis. Previous studies have found no differences in fungal biomass of intact green *S. alterniflora* leaves (i.e. with no grazing scars), whether fungicide was applied or not (Silliman & Newell 2003).

Table 1. Summary of 2-way ANOVA results testing the effects of grazing in open and exclusion cages and fungicide treatments on the height and leaf number of marked tillers, number of tillers, and aboveground biomass of survived *Spartina alterniflora* clumps transplanted into the tidal flat. Significant results are shown in **bold** (ANOVAs were unbalanced, n = 53; p < 0.05). Height was log(x + 1) transformed for the analysis

Factor	df	Height		Leaf number		Tiller number		Biomass	
		F	p	*F*	p	*F*	p	*F*	p
Grazing (G)	2	4.81	**0.0126**	33.52	**≪0.001**	58.50	**≪0.001**	4.17	**0.0216**
Fungicide (F)	1	0.23	0.63	0.00	0.97	1.03	0.31	0.10	0.76
F × G	2	0.14	0.87	0.21	0.81	0.37	0.69	0.20	0.85
Residual	47								

To examine the effects of grazing and fungicide application on the mean leaf fungal biomass and percentage of leaf area damaged in Expt 1, we compared ergosterol content among 3 treatments (simulated grazing + with fungi, simulated grazing + without fungi, and no grazing + with fungi), using 1-way ANOVAs followed by Tukey's HSD multiple comparisons (Zar 2010). For Expt 2, ergosterol content and the percentage of leaf area damaged were compared among the same 3 treatments and the 2 clump types using 2-way ANOVAs followed by Tukey's HSD multiple comparisons. In all analyses, ergosterol content data were $\log_{10}(x+1)$ transformed.

RESULTS

Crab density and abiotic conditions

At the study site, the average density of crab burrows was 15.0 ± 1.0 m^{-2} (standard error; n = 60). Air temperature ranged from 12 to 33°C and 10 to 24°C during the first and second experiment, respectively. The study site was flooded 71% of the days over which the 2 experiments occurred. Average salinities of the estuarine surface water at the fixed station and pore water at the study site were 21.7 ± 6.5 and 25.2 ± 8.3 g l^{-1}, respectively.

Effects of crab grazing and fungal infection on *Spartina alterniflora*

In Expt 1, 100% of *Spartina alterniflora* clumps protected with exclusion cages survived, while only 65% of the clumps in partially enclosed cages survived (70% in crab grazing + with fungi and 60% in crab grazing + without fungi treatments). The height and leaf number of *S. alterniflora* tillers were significantly affected by natural crab grazing but were not affected by fungicide application (Table 1). Tillers growing in exclusion cages (pooled across no-grazing and simulated-grazing treatments) were on average 12 cm taller (+36%) and had 2 more live leaves (+125%) than those in partially enclosed cages (Fig. 2A,B). Natural crab grazing also had significant effects on the aboveground biomass and number of tillers of *S. alterniflora* transplants (Table 1). Crab grazing (in partially enclosed cages) caused a 31 to 40% reduction in aboveground biomass relative to that protected against grazing (Fig. 2C), whereas tiller number per clump was significantly higher in simulated-grazing treatments than in no-grazing or crab-grazing treatments (Fig. 2D). Grazing by *Neohelice granulata* explained more than 55% of the variability in the number of leaves and tillers, but it accounted for only 15

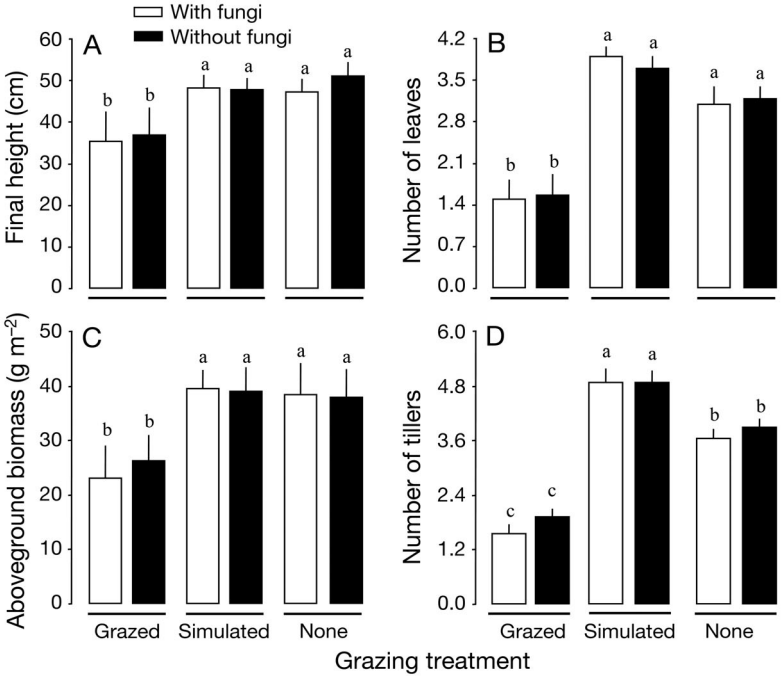

Fig. 2. Mean values (+SE) of (A) height (cm) and (B) leaf number of marked tillers and (C) aboveground biomass (g m^{-2}) and (D) number of tillers of *Spartina alterniflora* clumps in combinations of crab grazing treatments with and without fungi in the mudflat. Different lowercase letters indicate significant differences (p < 0.05)

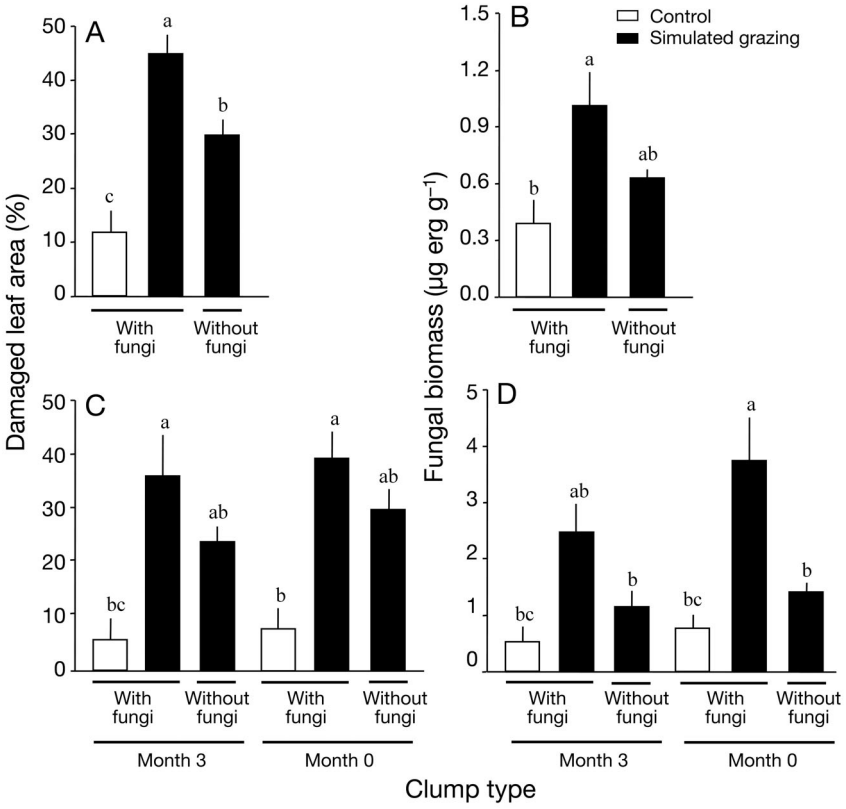

Fig. 3. Mean values (+SE) of the (A,C) percentage damaged area of leaves and (B,D) fungal biomass (µg ergosterol g^{-1} fresh weight) in leaves of *Spartina alterniflora* clumps transplanted to the tidal flat (for A,B Expt 1 and C,D Expt 2) in different treatments. Different lowercase letters indicate significant differences ($p < 0.05$, Tukey's HSD multiple comparisons)

to 17% of the variability in the height and aboveground biomass of *S. alterniflora* transplants (Table 1).

S. alterniflora transplants showed some degree of fungal damage in their leaf blades, and the intensity of the fungal damage differed significantly among crab-grazing treatments (df = 2, $F = 20.6$, $p < 0.001$), where simulated grazing had the highest damage and the control (not wounded ≈ undamaged) treatment had the lowest (Fig. 3A). Damaged areas in the control treatment were often on senescent tissues, located towards the tips of the leaves, the oldest part of the leaf and the part that experiences dieback first. The average fungal biomass (ergosterol content) of leaves in the control treatment (0.39 µg erg g^{-1}) was significantly lower than that of the simulated-grazing + with fungi treatment (1.0 µg erg g^{-1}; df = 2, $F = 5.4$; $p < 0.05$). In the simulated-grazing + without fungi treatment, the ergosterol content of *S. alterniflora* transplants was intermediate (Fig. 3B).

Crab grazing and fungal infection dependence on *Spartina alterniflora* establishment duration

In Expt 2, clump type (establishment month 0 vs. month 3) significantly affected tiller production (Table 2) and height growth (Table 3). Height growth (24.4 ± 2.8 cm over the 35 d experimental period) and tiller production of establishment month 3 clumps (with and without fungi) (21.5 ± 3.1 tillers 35 d^{-1}) were 47 and 200% greater than those of Month 0 clumps (height growth 16.6 ± 1.7 cm 35 d^{-1}; production: 7.2 ± 0.9 tillers 35 d^{-1}) (Fig. 4A,B). However, tiller birth rate did not differ between clump types (Month 0: 3.60 ± 0.47 tillers tiller^{-1}; Month 3: 2.94 ± 0.37 tillers tiller^{-1}; Table 2). Clump type also did not affect the leaf production of the tillers with simulated grazing (Table 3). Analysis of paired tillers showed that simulated crab grazing significantly reduced the height growth and leaf production of tillers (Table 3, Fig. 4B,C), and these effects were not dependent on fungicide or clump type treatments (within-subject interactions not significant). Overall, damaged tillers grew 18.0% less and produced 14.4% fewer leaves than undamaged tillers.

As in Expt 1, fungicide application in Expt 2 significantly decreased the average percent damaged leaf area in simulated-grazing treatments, and this effect was independent of clump type (for with- and without-fungi treatments, respectively, establishment

Table 2. Summary of 2-way ANOVA results testing the effects of fungal presence and clump type (establishment duration = 0 mo or 3 mo) on tiller production and tiller instantaneous birth rate of *Spartina alterniflora* clumps. All clumps of both treatments were subjected to simulated crab grazing in the mudflat. Significant results are shown in **bold** ($p < 0.05$). Tiller production and tiller birth rate were $\log_{10}(x)$ transformed for the analysis

Factor	df	Production		Birth rate	
		F	p	*F*	p
Fungi (F)	1	0.10	0.72	1.17	0.28
Clump type (C)	1	17.7	**≪0.001**	1.21	0.29
C × F	1	0.01	0.90	0.01	0.92
Residual	36				

Table 3. Summary of 2-way repeated measures ANOVA for fungal presence, clump type (establishment duration = 0 mo or 3 mo), and simulated crab grazing effects on height growth and leaf production of *Spartina alterniflora* tillers in the mudflat. Significant results are shown in **bold** ($p < 0.05$). Height growth was $\log_{10}(x)$ transformed for the analysis

Factor	df	Height growth		Leaf production	
		F	p	*F*	p
Fungi (F)	1	0.9580	0.334	0.0166	0.898
Clump type (C)	1	**12.9120**	**0.001**	0.0166	0.898
C × F	1	2.4910	0.123	1.3435	0.254
Residual	36				
Within subjects					
Grazing (G)	1	**6.0640**	**0.019**	**10.3143**	**0.0028**
G × F	1	0.3920	0.535	0.2571	0.615
G × C	1	0.0390	0.844	0.0286	0.867
G × F × C	1	0.2530	0.618	0.2571	0.615
Residual	36				

month 3: 23.3 ± 3.3 and 36.7 ± 8.0%; establishment month 0: 30.0 ± 4.5 and 40.0 ± 5.1%; Table 4). Similarly, fungicide application significantly decreased the average leaf ergosterol content in simulated-grazing treatments (for with- and without-fungi treatments, respectively, establishment month 3: 1.17 ± 0.18 and 2.50 ± 0.51 µg erg g^{-1} fresh weight; establishment month 0: 1.43 ± 0.14 and 3.75 ± 0.80 µg erg g^{-1} fresh weight; Table 4). In both establishment types without fungicide, the average percentage of damaged leaf area in simulated-grazing treatments was significantly higher ($p < 0.05$) than that in control treatments (Fig. 3C). Simulated crab grazing significantly affected fungal biomass in *S. alterniflora* leaves, with a 375% increase in fungal biomass relative to control treatments (Fig. 3D).

Table 4. Summary of 2-way ANOVA results testing the effects of fungicide and clump type (establishment duration) on the percentage of leaf area damaged and fungal biomass of *Spartina alterniflora* clumps in the mudflat. All clumps of both treatments were subjected to simulated crab grazing in the mudflat. Significant results are shown in **bold** ($p < 0.05$). Fungal biomass was $\log_{10}(x + 1)$ transformed for the analysis

Factor	df	Fungal biomass		Damaged leaf area	
		F	p	*F*	p
Fungicide (F)	2	19.5	**≪0.001**	22.4	**≪0.001**
Clump type (C)	1	2.8	0.1	0.9	0.3
F × C	2	1.0	0.4	0.1	0.9
Residual	30				

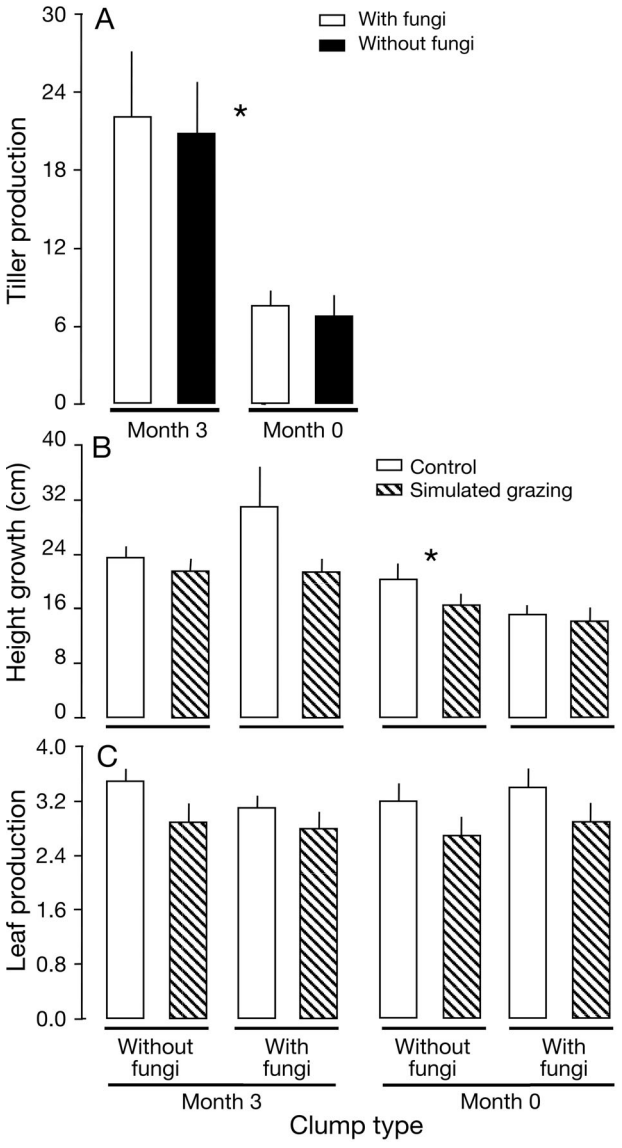

Fig. 4. Mean values (+SE) of (A) tiller production, (B) height growth, and (C) leaf production of marked tillers with and without simulated crab grazing of *Spartina alterniflora* in different clump type (establishment duration) and fungicide treatments in the mudflat. *Significant difference (Tukey's HSD test)

DISCUSSION

Effects of herbivory on *Spartina alterniflora* establishing in mudflats

The impact of *Neohelice granulata* herbivory on newly colonizing *Spartina alterniflora* plants agrees with previous studies in the SW Atlantic coast, where mortality of *S. alterniflora* tillers was found to be associated with *N. granulata* grazing (Marangoni & Costa 2012, Freitas et al. 2015). Alberti et al. (2007)

also demonstrated a high frequency of *N. granulata* grazing on *S. densiflora* and *S. alterniflora* on the edges of intertidal flats without vegetation over a range of more than 2000 km of the SW Atlantic. Results of these studies and our own reveal high vulnerability of newly colonizing or transplanted marsh plants to top-down control by herbivores.

Grazing by consumers including *N. granulata* (Costa et al. 2003, Silliman & Bortolus 2003, Daleo et al. 2009, Alberti et al. 2010) and other herbivorous invertebrates severely reduces photosynthetic area and biomass of salt marsh plants (Silliman & Bertness 2002, Silliman et al. 2005, Bertness et al. 2014, He et al. 2015). Our study shows that the capacity of *S. alterniflora* to colonize new space by tillering was strongly affected by crab grazing as well. The average aboveground biomass of *S. alterniflora* protected from crab grazing was up to 56% higher and tiller production was up to 140% greater than that in partially enclosed cages. Our result that simulating grazing by removing a relatively small part of plant leaves did not significantly affect *S. alterniflora* biomass but increased tiller production is likely, because *S. alterniflora* plants are tolerant of wounding caused by herbivory, and damage often enhances tiller production in monocots (Hawkes & Sullivan 2001). With increasing grazing intensity, the impact of herbivory on plant production can shift from positive to negative (Atkins et al. 2015).

While simulated crab grazing reduced the height growth and leaf production of directly affected tillers by about 14 to 18%, neither establishment duration nor fungal presence affected these outcomes. Thus, we found no effect of establishment length on the strength of herbivory on *S. alterniflora*. Independently of establishment length, *S. alterniflora* appeared to respond to tiller damage by allocating energy more towards the formation of new tillers rather than regrowth of already affected tillers. Both establishment length treatments had twice as many tillers after 35 d than at the beginning of Expt 2. This high tiller production in response to partial leaf damage is consistent with the result of Expt 1. Clumps with a 3 mo difference in their establishment history showed similar tiller birth rates, suggesting neither restriction of soil resource supply for larger establishment nor different abilities of clump types to obtain local resources during the 35 d experiment. According to Brewer (2011), *S. alterniflora* is a species that exhibits higher tillering and clonal growth rates, which are strongly limited by nutrients. A plentiful resource supply in the tidal flat may have allowed both clump types to respond in a similar way to our experimental treatments. This result also suggests that 3 mo establishment clumps planted 1 m apart were not yet competing with each other for soil resources at the end of Expt 2. Significant reductions of birth rates of perennial plant modules (e.g. tiller or leaves) have been interpreted as strong evidence of intraspecific (Costa & Seeliger 1988) and interspecific competition (Brewer 2011) as net resource supply decreases. However, other studies (Bertness 1991, Marangoni & Costa 2012, Daleo et al. 2015) have found that small and newly establishing *S. alterniflora* plants are more sensitive to environmental stresses (e.g. high salinity, anoxia, and herbivory) than taller and/or densely rooted plants.

Effects of fungal infection on *Spartina alterniflora*

In general, the facilitation of fungal infection did not significantly affect the biometric parameters of *S. alterniflora* plants colonizing the intertidal flat in our experiments. These results contrast with other studies showing large reductions in *S. alterniflora* biomass due to fungal infection occurring in scars of invertebrate herbivores–detritivores in densely vegetated marsh stands (Silliman & Newell 2003, Silliman et al. 2005, Daleo et al. 2009). Simulated *N. granulata* grazing facilitated fungal infection in *S. alterniflora* leaves, and this result is consistent with other studies (Daleo et al. 2009, Freitas et al. 2015). The absence of an effect on plants in our experiments suggests a saprophytic rather than a pathogenic role of fungal species in this region or in this ecological context. In a recent study on diversity of fungi on *S. alterniflora* leaves at Pólvora Island, Silveira (2012) found that the most frequent fungi genera during summer and autumn were *Fusarium* and *Rhizoctonia*, 2 genera with little evidence for a pathogenic effect on *S. alterniflora* (Elmer et al. 2012).

These results do not support our prediction that fungal infection facilitated by *N. granulata* grazing regulates newly establishing *S. alterniflora* plants in intertidal flats, suggesting the context-dependent nature of direct and indirect top-down control of marsh plants across regions and herbivores. The effects of fungal infection on plants may also vary with grazing intensity and the physiological conditions of marsh plants. Certain endophytic and surface saprophytic fungi can act as opportunistic pathogens when host plants are stressed by environmental conditions. For instance, high densities of invertebrate herbivores and extensive grazing (Silliman & Newell 2003), as well as prevalence of dry periods and

higher salinities (i.e. drought: Silliman et al. 2005, Elmer et al. 2012), have been associated with massive diebacks of *S. alterniflora* due to the combination of physiological stress and fungal infection.

Lower levels of fungal biomass may also be explained by a less efficient mechanism of fungal growth enhancement by *N. granulata* than by other intertidal herbivores. For instance, Sieg et al. (2013) reported up to 6 times higher (30.7 ± 7.1 µg erg g^{-1} fresh weight) fungal biomass in *S. alterniflora* leaves heavily damaged by the snail *Littoraria irrorata* in Georgia, USA, than that found at the end of our Expt 2. *Littoraria* snails are known to concentrate deposition of nitrogen- and hyphae-rich fecal pellets on their radulations, and this activity enhances fungal growth (Silliman & Newell 2003).

Implications for salt marsh restoration

While restoration is increasingly being used as a major conservation strategy aimed at curbing the loss of important coastal wetlands, protection of transplanted marsh propagules has not been widely considered to enhance restoration success. Results of our work reveal that newly establishing marsh transplants or those naturally colonizing damaged mudflats are often susceptible to grazing by herbivores. This finding suggests that marsh restoration projects in areas with high densities of herbivores should include protection strategies for new plantings. This is in line with Powers & Boyer (2014), who suggested that marsh restoration should consider the local context of consumer abundance and potential damages caused by grazers, especially during the establishment periods of new transplants or seedling recruits. Indeed, restoration projects of *S. foliosa* in San Francisco Bay have used cages to protect new plantings from goose grazing (Thornton 2013). However, the protection of transplants with cages is likely to increase monetary investment and time but is not the only solution. We suggest that clumping marsh grass plantings can be another effective method, where large transplant clumps protect inner grasses from grazing damage, allowing them to establish. For example, Alberti et al. (2008) found higher crab herbivory on the edge of patches of *S. densiflora*, and larger patches were better able to withstand herbivory and outcompete other neighboring plants. Clumping marsh grasses together will therefore actively harness a positive species interaction (e.g. group benefits: Bertness & Callaway 1994) often used by animals in nature, including schooling fishes (Brock & Riffenburgh 1960) and aggregations of hermit crabs (Powell & Nickerson 1965), where aggregations protect individuals from predation.

CONCLUSIONS

Herbivores such as *Neohelice granulata* are important regulators of the survival, growth, and tillering of habitat-forming *Spartina alterniflora* colonizing tidal flats in the SW Atlantic. This work importantly reveals that newly establishing marsh plants are vulnerable to grazing by herbivores. Thus, marsh plants used in coastal wetland restoration projects may need to be transplanted in large clumps in an effort to protect the majority of plants in the interior of the clump or with herbivore exclusion cages to protect transplants from grazing during early establishment periods. Although herbivore grazing facilitated fungal infection in *S. alterniflora* leaves, the presence of fungal infection had little to no effect on *S. alterniflora* growth, suggesting a saprophytic rather than a pathogenic role of fungi in this system. Thus, whether fungicide should be used as a tool to aid or enhance marsh restorations or mitigate salt marsh dieback may vary by ecosystem.

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