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Resource availability and plant age drive defense against herbivory in salt marshes

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The resource-availability hypothesis (RAH) and the intraspecific RAH (RAH_{intra}), posit that resources, (i.e. nutrients) control plant antiherbivore defenses. Both hypotheses predict that in low-resource environments, plant growth is slow, and constitutive defense is high. In high-resource environments, however, the RAH predicts that plant growth is fast, and constitutive defense is low, whereas the RAH_{intra} predicts that increased resources attract more herbivores, and this intensified grazing pressure leads to high constitutive defense. Salt marshes are nutrient-limited ecosystems threatened by eutrophication and chronic herbivory, yet we know little about how these stressors shape saltmarsh plant antiherbivore defenses, which influence trophic interactions and ecosystem resilience. We manipulated resource availability via nutrient addition and herbivory via the marsh periwinkle *Littoraria irrorata*, on the saltmarsh foundation species *Spartina alterniflora*, in mesocosms. Because plant age can also influence trait variation, we measured traits in both original and clonally-grown new stems. Feeding assays then evaluated how treatments and plant age affected subsequent *Littoraria* consumption of *Spartina*. Nutrient addition stimulated growth, while decreasing defensive traits (e.g. fiber and silica content), following the RAH. Herbivory enhanced belowground production and increased stem diameter, yet did not induce defensive traits, contrary to our expectations. Herbivory plus nutrients increased *Spartina* biomass and reduced phenolics, a defensive trait, further supporting the RAH. Regardless of treatment, clonally-grown new stems had greater variation in measured traits. Despite altered traits, however, treatments and plant age did not affect *Littoraria* consumption. Our results support the RAH and part of the RAH_{intra} and suggest: 1) nutrient availability is a primary driver of plant trait change and 2) plant age controls the magnitude of trait variation in *Spartina*. Further, our findings indicate that eutrophic conditions may not always increase top–down control by herbivores, and in some instances can enhance saltmarsh resilience against sea-level rise via stimulated *Spartina* biomass production.

Keywords: compensatory growth, growth-rate hypothesis, plant functional traits, resistance, *Sporobolus alterniflorus*, tolerance

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Introduction

Herbivores shape plant biomass, abundance, and ecosystem service provision (Silliman and Zieman 2001, Daleo et al. 2015, Freitas et al. 2016). In response to grazing from herbivores, plants can alter their morphological, chemical, and structural traits to mitigate or deter future attack. Plants mitigate grazing by overproducing or reallocating biomass, altering morphology, and/or increasing photosynthetic capacity to compensate for mass lost to herbivores (Strauss and Agrawal 1999, Stowe et al. 2000, Tiffin 2000). In contrast, plants deter grazing by altering chemical or structural defenses which decrease plant palatability, thus shaping herbivore preference and preventing or limiting further grazing (Painter 1951, Strauss et al. 2002). In addition, these plant traits can be either constitutive (i.e. inherent to the plant without herbivores present) or induced (i.e. direct result of herbivory) (Karban and Baldwin 1997, Garcia et al. 2021).

Defense against herbivory comes at a cost, as resources allocated to defense cannot be used for reproduction or new growth (Herms and Mattson 1992, Strauss et al. 2002, Leimu et al. 2006). This tradeoff between anti-herbivore defense and plant growth forms the foundation of the resource availability hypothesis (hereafter 'RAH'), which offers a potential driver of this tradeoff (Coley et al. 1985, Bryant et al. 1989, Endara and Coley 2011). The RAH posits that in low-resource environments, plants have slow growth due to nutrient limitation and high constitutive defense to protect existing tissues from herbivory. In contrast, in high-resource environments, plants have low constitutive defense and fast growth to compensate for herbivore damage. The RAH has been primarily tested in terrestrial ecosystems between plant species (interspecific; Bryant et al. 1989, Massey et al. 2007, Endara and Coley 2011). Recent studies have suggested that the RAH may not apply within a species (intraspecific), leading to the development of the intraspecific RAH (RAH_{intra}), which asserts that the effects of resource availability on plant defenses is mediated through two distinct pathways: physiological traits and herbivory pressure (Hahn and Maron 2016, López-Goldar et al. 2020). The RAH_{intra} predicts that in physiologically stressful environments with low resource availability, plants have high constitutive defense, a prediction similar to the RAH. In contrast, low stress environments with high resource availability facilitate and support higher herbivore abundances and thus grazing pressure, causing plants in these areas to have greater constitutive defense, a pattern opposite of the RAH (Hahn and Maron 2016, López-Goldar et al. 2020). Since the development of the RAH_{intra}, both hypotheses have been applied in various ecosystems (seagrasses: Hernán et al. 2019, Hernán et al. 2021; deciduous forests: Lynn and Fridley 2019; pine forests: López-Goldar et al. 2020; grasslands: Hahn et al. 2021), with mixed support for each hypothesis, suggesting that whether the RAH or the RAH_{intra} predicts plant response to herbivory may be ecosystem-dependent.

In addition to resource availability, plant age can also influence the magnitude of trait expression and defense (Henn and Damschen 2021). For example, in terrestrial plants, newer leaves tend to have traits associated with fast growth (e.g. higher chlorophyll), and reduced defensive traits (e.g. higher nitrogen and protein), than older leaves, and these traits tend to be more plastic and have greater variation in younger plants (Chen and Poland 2009, Cope et al. 2020, Sola et al. 2020, Funk et al. 2021). Thus, increased presence of newer leaves and stems, which grow quicker but are more nutritionally dense, may further shape herbivore preference.

We used saltmarshes to examine the effects of resource availability, herbivory, and plant age on plant traits associated with growth and defense against herbivores. Salt marshes are intertidal grasslands found at the land-sea interface and provide shoreline protection from storms, nutrient cycling, and habitat for valuable species (Friess et al. 2020, Whitfield 2020). Similar to other vegetated coastal ecosystems, salt marshes are threatened by chronic herbivory (Gedan et al. 2009) and nutrient runoff from land (Deegan et al. 2012). In US Atlantic coast salt marshes, insect herbivory can suppress plant production by approximately 60% (Gedan et al. 2009), crab herbivory can clear up to 90% of vegetation from creekbanks (Holdredge et al. 2009), and snail herbivores can remove around 38% of all vegetation (Silliman and Zieman 2001), at times converting these ecosystems into mudflats (He and Silliman 2016, Angelini et al. 2018, Williams and Johnson 2021). Coastal eutrophication can facilitate saltmarsh loss by stimulating microbial decomposition and causing biomass reallocation from below to aboveground in plant foundation species, ultimately destabilizing creekbanks and promoting erosion (Deegan et al. 2012). Salt marshes along the Gulf and Atlantic coasts of North America are dominated by the smooth cordgrass, *Spartina alterniflora* (hereafter *Spartina*). *Spartina* is a perennial, clonal graminoid in the family Poaceae native to the Atlantic and Gulf Coasts of North America. In many other areas, including the US Pacific coast and parts of China, *Spartina* has become invasive (Levin et al. 2006, Li et al. 2009). As a foundation species in North American salt marshes, the above- and belowground biomass of *Spartina* is critical for vertical accretion, the process by which salt marshes keep pace with sea-level rise. Thus, understanding the drivers of trait change and *Spartina* defense against herbivores can provide direct insight into ecosystem resilience to rising seas and eutrophication (Lavorel 2013, Wright et al. 2016).

Under ambient conditions, salt marshes are historically nutrient limited ('low-resource environment'; Deegan et al. 2007, Lu et al. 2019, Bowen et al. 2020) and create physiologically stressful conditions for plants (e.g. frequent inundation, waterlogged sediments, high salinities, Pennings and Bertness 2001). Thus, according to the RAH and the RAH_{intra}, we would expect saltmarsh plants to have relatively high constitutive defense. However, under eutrophic conditions driven by anthropogenic nutrient loading ('high-resource environment'), the RAH predicts saltmarsh plants would have increased growth and decreased defense, while the RAH_{intra} predicts that an increase in resources may elevate grazing pressure and thus constitutive defense. Overall, this

suggests that, in some cases, higher nutrient availability via runoff could alter plant defense against herbivores.

The overarching goal of this study was to use predictions from both the RAH and the RAH_{intra} to test how increased resource availability influences *Spartina* growth and defense and to understand how an understudied driver, plant age, may mediate trait variation. We expected that relative to ambient conditions: 1) nutrients alone would decrease constitutive defense and stimulate growth, 2) herbivory alone would induce defensive traits and 3) herbivory plus nutrients would stimulate growth. Further, we expected newer, clonally grown stems to have higher growth traits and lower defensive traits than older stems and that traits altered by our treatments would influence *Spartina* palatability, affecting subsequent feeding by herbivores. To test these hypotheses, we first ran a factorial mesocosm experiment manipulating nutrient availability via fertilizer addition and herbivory from the marsh periwinkle, *Littoraria irrorata* (hereafter *Littoraria*), a marine gastropod in the family Littorinidae native to the salt marshes of the Atlantic and Gulf coasts of North America. *Littoraria* is an herbivore and most often associated with emergent vegetation, especially *Spartina*, which it climbs at high tide to avoid intertidal predators and to forage on *Spartina* leaves and its associated fungal colonies (Silliman and Newell 2003, Rietl et al. 2018, Failon et al. 2020). Following the mesocosm experiment, w measured several traits related to the growth and defensive ability of clonally-grown new stems and original *Spartina* stems. Lastly, we conducted a feeding assay with *Littoraria* and experimentally manipulated *Spartina* to assess how altered traits and plant age affected herbivore preference and consumption.

Material and methods

Collection site

In June of 2018, 300 roots and shoots of *Spartina* were collected from Cushman's Landing marsh in Cape Charles, Virginia, USA (37°10'31.422"N, 75°56'33.4968"W). Cushman's Landing marsh has an average salinity of 28 ppt (McGlathery and Christian 2022) and experiences semi-diurnal tides with an average height of 1.35 ± 0.27 m (standard deviation; Porter et al. 2022). Individual *Spartina* stems were collected at least 1 m apart to minimize collecting ramets from the same genetic clone. Following collection, plants were immediately transported to the Virginia Inst. of Marine Science (VIMS) in Gloucester Point, VI, USA.

Mesocosm set-up and maintenance

Within 24 h of collection, 10 individual *Spartina* stems were planted in 11-l nursery pots containing a 90:10 potting mix to sand ratio, with a total of 28 pots planted (Fig. 1A). This stem density (~ 144 stems per m^2) was slightly less than average stem densities at this site (~ 208 per m^2). At the time of planting, *Spartina* plants had an average height of 73.8 ± 14.2 cm, stem diameter of 9.9 ± 2.1 mm, and 9 ± 1.5 leaves (Wittyngham 2022) and there were no initial differences in morphometrics by pot or by treatment. A waterproof paint line was added to each stem at the sediment surface so that stem height could be tracked throughout the course of the experiment. Of the ten shoots planted, five were randomly selected and marked with a colored zip tie (Fig. 1A). These

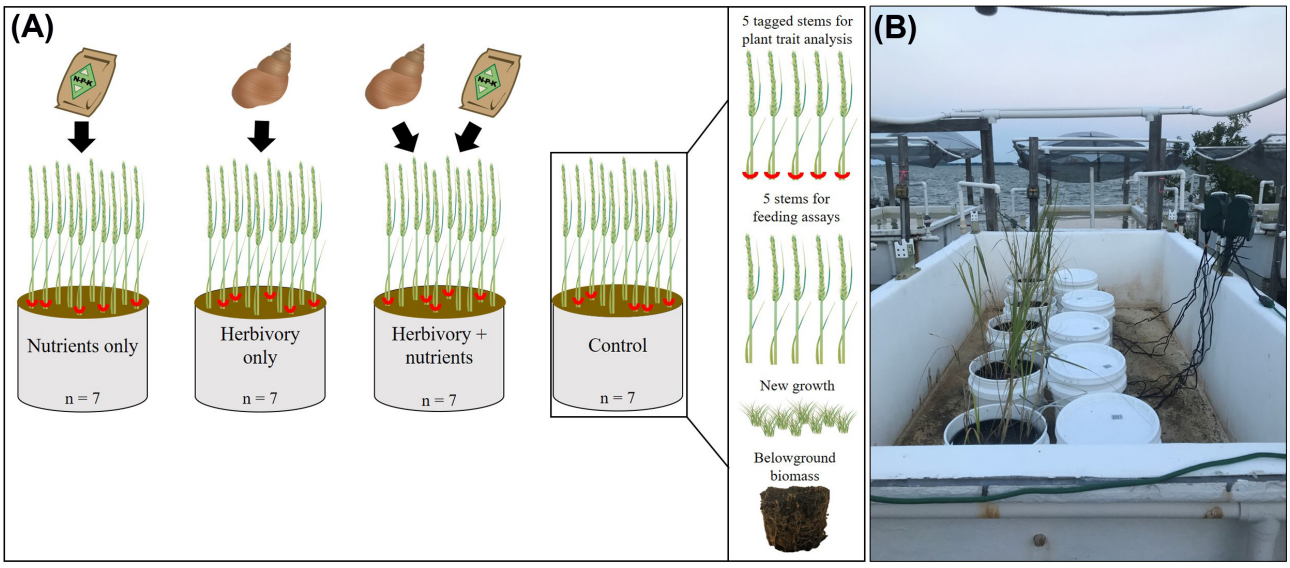


Figure 1. (A) Schematic of experimental design including mesocosms per treatment (nutrients only, herbivory only, herbivory plus nutrients and control; n = 7 per treatment). In each mesocosm, ten *Spartina* stems were planted, with five plants tagged at the sediment surface (shown by red semi-circle). At the end of the experiment, all plant material from mesocosms was harvested and separated into four categories: 1) the five tagged stems for plant trait analysis, 2) the five non-tagged stems for feeding assays, 3) new growth and 4) belowground biomass. Images were open-sourced from the Univ. of Maryland center for environmental science (UMCES) integration and application network (IAN) library. (B) Photo showing mechanically-tidal mesocosm system used for this experiment.

plants served as a composite sample for each replicate pot to be analyzed for plant traits at the end of the experiment. The remaining five plants were designated as composite samples for feeding assays. Each nursery pot was suspended in its own 19-l bucket, and all buckets were made tidal mechanically (MacTavish and Cohen 2014, Wittyngham 2021) using programmable timers and dosing pumps to simulate the natural diurnal tidal cycle of the Chesapeake Bay region (Fig. 1B). Each nursery pot had its own reservoir bucket, which was filled with seawater directly from the York River (average salinity of 17–20 ppt) via a flow-through system. This water was replaced every three days to avoid excess algal growth and nutrient depletion.

Mesocosms were then randomly assigned one of the four treatments manipulating *Littoraria* herbivory and nutrient availability: 1) herbivory only, 2) nutrients only, 3) herbivory plus nutrients and 4) an unmanipulated control, with seven replicates per treatment (Fig. 1A). After approximately two weeks of growth, *Littoraria* with an average height of 23 ± 1.7 mm and an average width of 17.5 ± 1.2 mm (measured with digital calipers, Failon et al. 2020) were collected from Cushman's Landing marsh. Twelve adult snails were added to each replicate mesocosm for herbivory only and herbivory plus nutrient treatments (n = 7 replicates per treatment) for a density of 192 snails per m^2. Although this treatment density was ~ three times the average natural density in Cushman's Landing marsh (64 snails per m^2; unpubl.), it was still within the range of natural densities observed in Virginia (Failon et al. 2020). This treatment density was selected to ensure herbivory would elicit a response in *Spartina*. Each week, snails were counted and replaced as necessary to maintain these densities. In nutrient addition treatments (nutrients only, herbivory plus nutrients), two 15 ml centrifuge tubes drilled with holes and filled with ~ 14 g of ammonium-nitrate fertilizer (Plantacote slow-release fertilizer, N:P:K = 14:14:14) were pushed into the sediment of their corresponding mesocosm.

At the end of the experiment (June–August, 10 weeks total), the stem height, diameter, and the number of leaves were measured for each of the ten originally planted *Spartina* stems. The number of new stems produced was also counted and recorded on a per mesocosm basis. Any remaining fertilizer was dried and weighed, and the average nitrogen loading rate was calculated as 0.65 ± 0.012 g N m^{-2} day^{-1}. This loading rate falls between those of other enrichment studies (lower than: Langley et al. 2013, 1.79 g N m^{-2} day^{-1}; similar to: Hill et al. 2020, 0.63 g N m^{-2} day^{-1}; higher than: Johnson et al. 2016, 0.47 g N m^{-2} day^{-1}), and is almost two orders of magnitude higher than ambient loading rates in this region (0.0038 g N m^{-2} day^{-1}; Stanhope et al. 2009, Giordano et al. 2011). All plant material was rinsed in an outdoor sieve (1 mm^2 mesh) to remove sediments, and belowground biomass was separated from aboveground biomass. Because it formed thick root mats that could not be disentangled, belowground biomass was compiled on a per pot basis, not per stem. Belowground biomass was placed in a 60°C drying oven for 12 days and dry masses were recorded. The remaining aboveground biomass was divided into three groups: 1) the five stems marked with zipties for plant trait analysis, 2) the five stems selected for feeding assays and 3) new stems (i.e. not originally planted stems) (Fig. 1A). Dead stems were recorded and removed when applicable. All aboveground biomass (for plant traits, feeding assays and new stems) was rinsed to remove sediments and then was stored in a −80°C freezer.

Plant-trait analysis

Above- and belowground biomass, root:shoot ratio, carbon content, and chlorophyll content (a, b, total, as a proxy for photosynthetic capacity (Croft et al. 2017)) were considered growth traits, as these variables influence a plants' ability to mitigate damage from herbivory (Hernán et al. 2019). In comparison, leaf nitrogen content, total phenolics, total soluble protein, neutral detergent fiber (hereafter 'fiber'), and biogenic silica (hereafter 'silica') content were considered defensive traits, as they can affect the palatability and forage quality of plant tissues, which may influence herbivore preference (Pennings et al. 1998, Massey et al. 2007, Hernán et al. 2019). Although nitrogen is critical for plant growth and can be positively correlated with chlorophyll, we considered leaf nitrogen as a defensive traitbecause it specifically relates to plant nutritional quality and herbivore metabolism (Mazid et al. 2011, Hernán et al. 2019). Thus, elevated nitrogen, along with protein content, represented a decline in defense, as these traits can enhance palatability and herbivore consumption (Nordhaus et al. 2011, Richman et al. 2015, Hernán et al. 2019), whereas increased phenolics, fiber, and silica content signified increased defense, as these structural variables can directly impede herbivore access or ease of grazing (Dudt and Shure 1994, Pennings et al. 1998, Martínez-Crego et al. 2016).

All *Spartina* aboveground biomass (for plant traits, feeding assays and new stems) was freeze-dried and then ground to a fine powder (40 mesh sieve). All three groups (plant traits, feeding assays and new stems) were analyzed for carbon, nitrogen, chlorophyll, phenolic, protein, silica and fiber content. We measured each of these traits in both original *Spartina* stems and clonally grown new stems. Samples were run on an elemental analyzer for carbon and nitrogen analysis and compared to a standard curve for acetanilide. Chlorophyll content (a, b, total) was measured following methods from Warren (2008), Tran et al. (2018) and Nguyen et al. (2020). Absorbance was read on a molecular devices plate reader at 652 and 665 nm and then corrected to a 1 cm pathlength using equations from Warren (2008) and Tran et al. (2018). Chlorophyll a and b concentrations were calculated using equations from Wellburn (1994) and Tran et al. (2018). Total chlorophyll concentrations are the sum of both chlorophyll a and b (Tang et al. 2018). Total phenolic concentrations were measured using a modified Folin–Ciocalteu protocol (Ainsworth and Gillespie 2007, Wittyngham et al. 2019, Wittyngham 2021) and absorbance was measured on a plate reader at 765 nm and compared to a gallic acid standard curve.

In many plants, including *Spartina*, phenolics can serve as an antiherbivore defense (Fraenkel 1959, Hartley and Jones 1997). However, we recognize that phenolics also assist in a variety of other functions, including the formation of structural compounds such as lignin (Jones and Hartley 1999) and UV protection (Close and McArthur 2002). Total soluble protein content was analyzed using a modified Bradford assay (Thermo Fisher Scientific Coomassie Plus Assay Kit, 2016; Wittyngham et al. 2019, Wittyngham 2021) and absorbance was measured on a microplate reader at 595 nm and compared to an albumin standard (BSA) curve. Silica was measured using a wet chemical alkaline extraction (DeMaster 1981, Conley and Schelske 2002) and then transferred to the VIMS analytical laboratory for measurement of dissolved silica concentrations in aliquots using the blue-molybdate colorimetric technique (Strickland and Parsons 1972). Fiber, comprised of lignin, cellulose, and hemicellulose, was analyzed by Waters Agricultural Laboratories, Inc. in Warsaw, NC, USA.

Feeding assays

We conducted a feeding assay to assess how: 1) treatment-induced changes in plant traits and 2) plant age (original versus new stems) influenced *Littoraria* consumption of *Spartina*. This full factorial, no-choice feeding assay measured *Littoraria* consumption of *Spartina* from each of the four mesocosm treatments (herbivory only, nutrients only, herbivory plus nutrients and unmanipulated control) across both plant ages (original stems and new stems), with 15 replicates per treatment combination (Supporting information for factorial design). Fifteen agar disks with no snails were included in assays to assess potential changes in food sources in the absence of *Littoraria*. To make each food source, 25 mg of plant powder was suspended in 25 ml of 2% agar solution and poured into petri dish lids (4 mm height × 50 mm diameter), covered in parafilm, and allowed to cool at 4°C overnight (Long et al. 2011, Hughes et al. 2015). On the morning of the feeding assay, *Littoraria* were collected from Cushman's Landing marsh and measured for height and width. Snails were introduced to their food sources within 5 h of collection. For each replicate, one petri dish lid containing an agar disk was placed in a 250 ml beaker with a single *Littoraria* snail and 1 ml of seawater collected from Cushman's Landing to prevent desiccation. Window screen was rubber-banded to each beaker to prevent snail escape. Snails were allowed to forage for 48 h. At this time, petri dishes were removed from their beakers, placed on top of window screen (1 mm^2 openings), and photographed. ImageJ software was used to count the number of squares consumed (Long et al. 2011, Hughes et al. 2015).

Statistics

All statistical analyses were conducted in R ver. 4.1.2 (www.r-project.org). To quantify differences in *Spartina* morphometrics, data was first averaged for all ten originally planted *Spartina* stems per mesocosm. We then ran a multivariate regression model with main factors of treatment (nutrients only, herbivory only, herbivory plus nutrients, control) and replicate number (i.e. replicate mesocosm), on the responses: stem height, stem diameter, number of leaves, and number of new stems. To assess differences in *Spartina* plant traits, data was first averaged for the five tagged original *Spartina* stems and for clonally-grown new stems for each mesocosm. We then conducted a multivariate regression model with main factors of treatment, plant age (original stem, new stem), and replicate number on the responses: aboveground biomass, belowground biomass, root:shoot ratio, carbon content, chlorophyll a, chlorophyll b, total chlorophyll, nitrogen content, phenolic concentrations, total soluble protein content, neutral detergent fiber, and biogenic silica. Due to high variation between values of each measured response, all plant-trait responses were scaled for standardization (function 'scale' in base R) prior to fitting the model. Lastly, to examine differences in *Littoraria* consumption, we conducted a multivariate regression model with main factors of treatment and plant age. For each regression model, variables met the assumptions of parametric statistics and included interaction terms.

Standardized effect sizes were then calculated for plant morphometrics, traits, and feeding assays using Glass's delta, rather than Cohen's D or Hedges' g, because there were unequal variances among our response variables (function *glass_delt*a in R package (www.r-project.org) 'effectsize', Ben-Schachar et al. 2020). For this calculation, the mean of the unmanipulated controls for each response variable was subtracted from the mean of each treatment group and divided by the standard deviation of the control group.

Results

The complete statistical reporting for all models can be found in the Supporting information. For each of the regression models conducted, there were no significant interactions between tested factors, thus model results are interpreted as main effects only.

Growth traits

Nutrient only treatments increased above- and belowground biomass ($p = 0.015$ and $p = 0.001$, respectively; Fig. 2A–B), the number of leaves per original stem ($p = 0.051$; Fig. 3C), and the number of new stems produced ($p < 0.001$; Fig. 3D). These treatments also decreased root:shoot ratio ($p = 0.012$; Fig. 2C). Nutrient only treatments had no effect on carbon content ($p = 0.070$; Fig. 2D), chlorophyll a ($p = 0.888$; Fig. 2E), chlorophyll b ($p = 0.682$; Fig. 2F), total chlorophyll content ($p = 0.9604$; Fig. 2G), original stem height ($p = 0.401$; Fig. 3A), or original stem diameter (Fig. 3B).

In contrast, herbivory only treatments increased belowground biomass ($p = 0.029$; Fig. 2B), root:shoot ratio ($p = 0.025$; Fig. 2C), and the diameter of originally planted stems ($p = 0.041$; Fig. 3B), while decreasing the number of

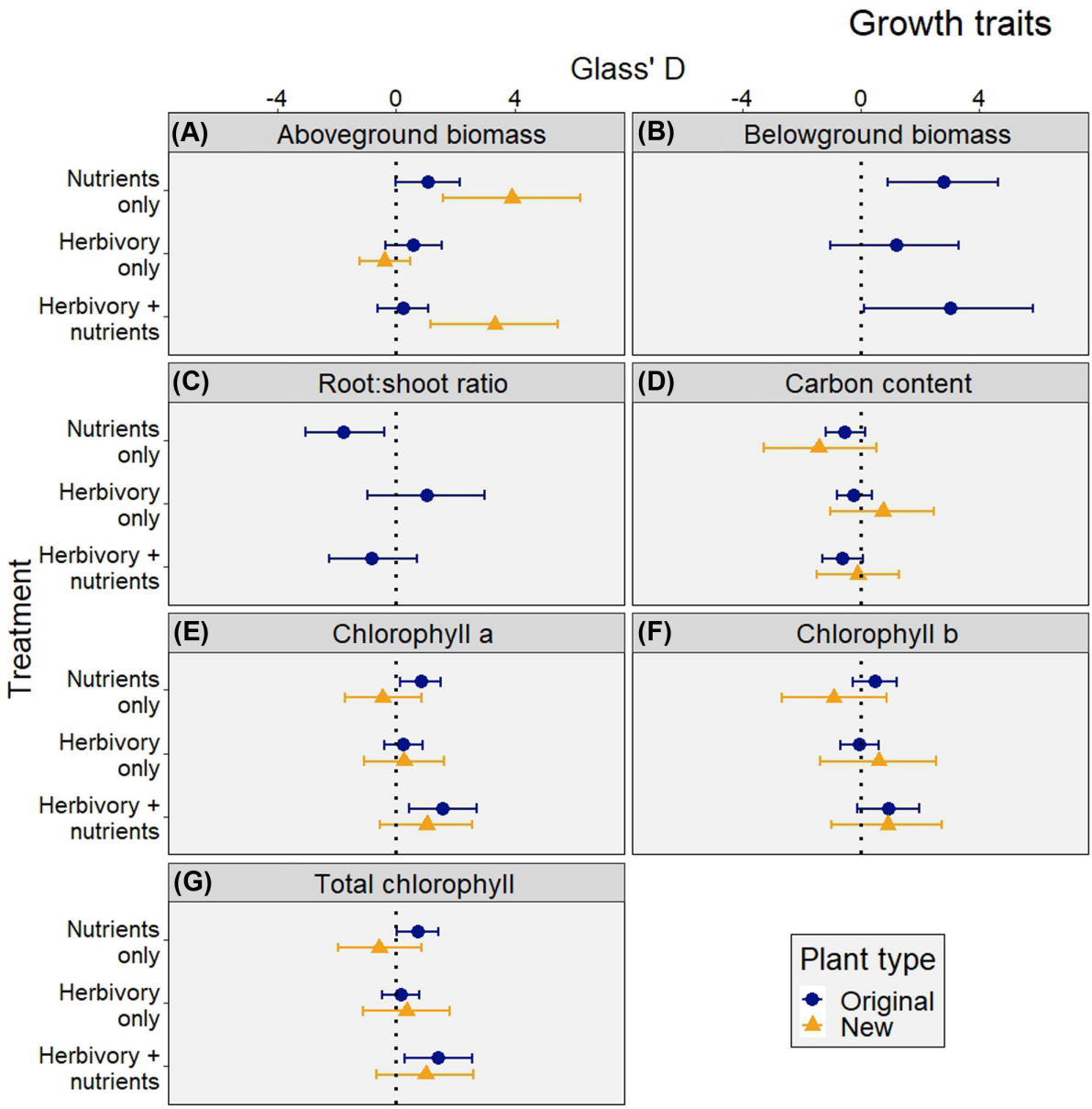


Figure 2. Standardized effect sizes (Glass's delta, 'Glass' D') for measured growth traits per treatment and plant age: (A) aboveground biomass, (B) belowground biomass, (C) root:shoot ratio, (D) carbon content, (E) chlorophyll a, (F) chlorophyll b and (G) total chlorophyll. Note that belowground biomass and root:shoot ratio were compiled per replicate, not per stem, thus they are displayed as 'original' stems only. Error bars represent 95% confidence intervals.

new stems produced ($p < 0.001$; Fig. 3D). Herbivory only treatments had no effect on aboveground biomass ($p = 0.901$; Fig. 2A), carbon content ($p = 0.831$; Fig. 2D), chlorophyll a ($p = 0.761$; Fig. 2E), chlorophyll b ($p = 0.587$; Fig. 2F), total chlorophyll ($p = 0.667$; Fig. 2G), height of originally planted stems ($p = 0.086$; Fig. 3A), and the number of leaves per original stem ($p = 0.508$; Fig. 3C).

Herbivory plus nutrients treatments increased belowground biomass ($p = 0.001$; Fig. 2B) and the number of new stems produced ($p < 0.001$; Fig. 3D). This treatment had no effect on aboveground biomass ($p = 0.087$; Fig. 2A), root:shoot ratio ($p = 0.214$; Fig. 2C), carbon content ($p = 0.179$; Fig. 2D), chlorophyll a ($p = 0.127$; Fig. 2E), chlorophyll b ($p = 0.518$; Fig. 2F), total chlorophyll ($p = 0.207$; Fig. 2G), original stem height ($p = 0.559$; Fig. 3A), original stem diameter ($p = 0.124$; Fig. 3B), or the number of leaves per original stem ($p = 0.320$; Fig. 3C).

On average, new stems had higher aboveground biomass ($p < 0.001$; Fig. 2A), chlorophyll a ($p < 0.001$; Fig. 2E), chlorophyll b ($p < 0.001$; Fig. 2F), and total chlorophyll ($p < 0.001$; Fig. 2G) than original stems. Plant age had no effect on carbon content ($p = 0.061$; Fig. 2D).

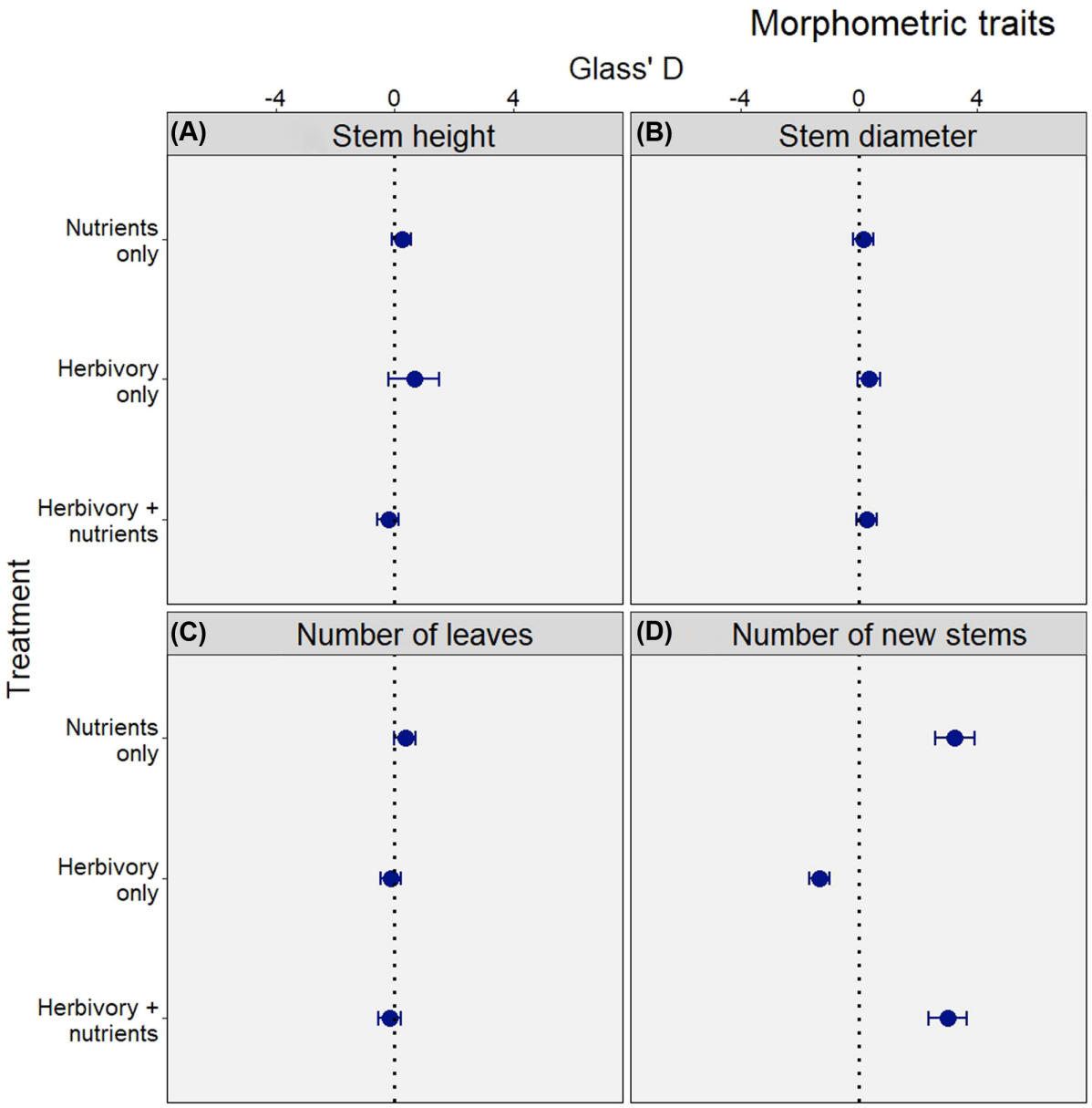


Figure 3. Standardized effect sizes (Glass's delta, 'Glass' D') for measured morphometric traits per treatment in original plants only: (A) stem height, (B) stem diameter, (C) number of leaves and (D) number of new stems. Error bars represent 95% confidence intervals.

Defensive traits

Nutrient only treatments decreased fiber content ($p=0.010$; Fig. 4D), and silica content ($p=0.018$; Fig. 4E), while having no effect on nitrogen content ($p=0.086$; Fig. 4A), phenolic concentrations ($p=0.193$; Fig. 4B) or protein content ($p=0.906$; Fig. 4C). Herbivory only treatments had no effect on defensive traits (nitrogen content: $p=0.835$, Fig. 4A; phenolics: $p=0.073$, Fig. 4B; protein: $p=0.400$, Fig. 4C; fiber: $p=0.080$, Fig. 4D; silica: $p=0.849$, Fig. 4E). Herbivory plus nutrients treatments increased nitrogen content ($p=0.044$; Fig. 4A), decreased phenolic concentrations ($p=0.013$; Fig. 4B) and had no effect on protein ($p=0.406$; Fig. 4C), fiber ($p=0.265$; Fig. 4D), or silica content ($p=0.836$; Fig. 4E).

On average, new stems had higher nitrogen ($p=0.022$; Fig. 4A) and protein content ($p < 0.001$; Fig. 4C) and lower silica content ($p < 0.001$; Fig. 4E) than original stems. Plant age had no effect on phenolic concentrations ($p=0.880$; Fig. 4B) or fiber content ($p=0.798$; Fig. 4D).

Feeding assays

Littoraria snails used in the feeding assays had an average height of 22.9 ± 1.1 mm and an average width of 17.8 ± 1.0 mm (Wittyngham 2022). *Littoraria* consumption of *Spartina* was not affected by treatment (herbivory only: $p=0.414$; nutrients only: $p=0.101$; herbivory plus nutrients: $p=0.691$; Fig. 5) nor plant age ($p=0.151$; Fig. 5).

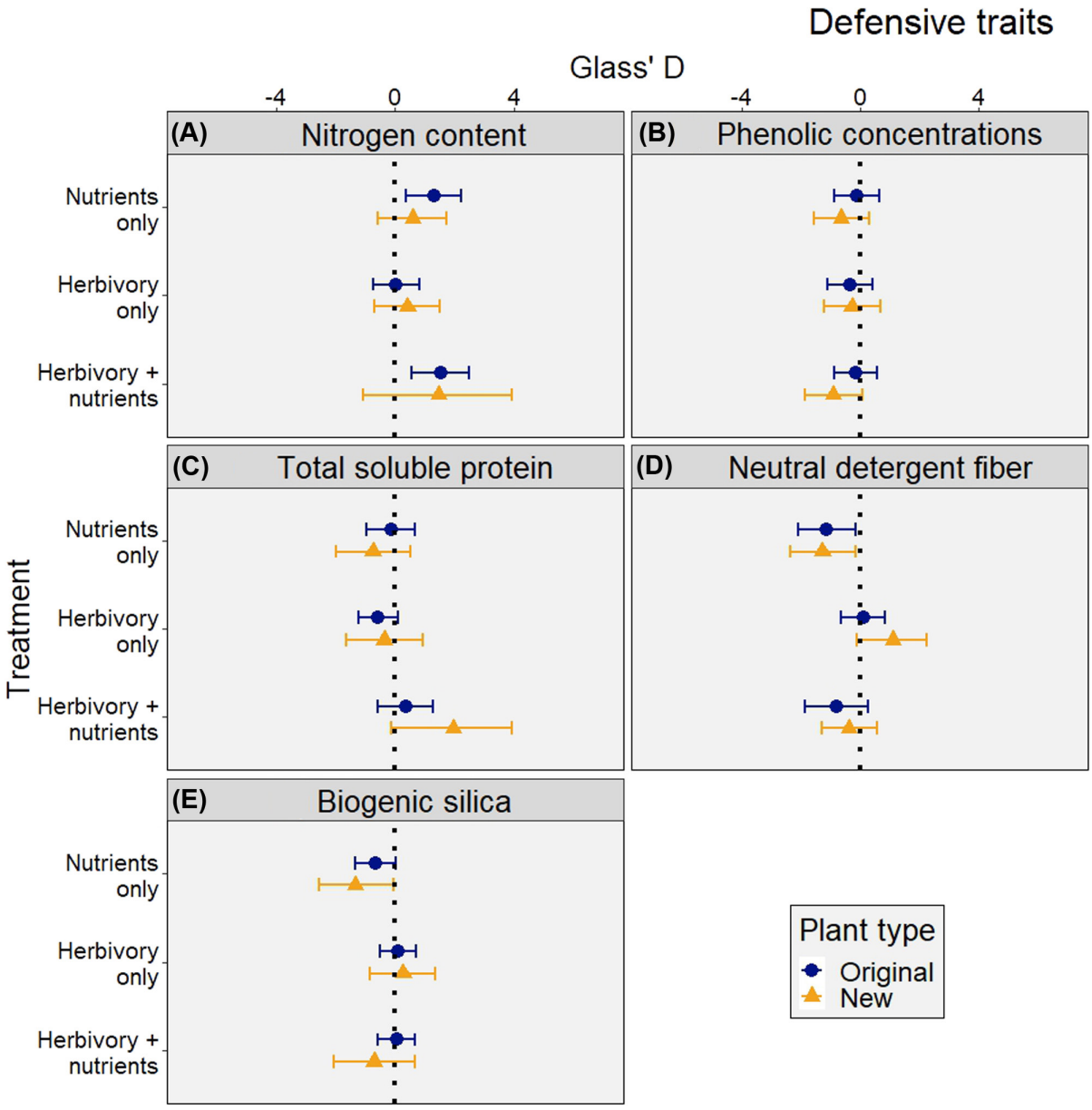


Figure 4. Standardized effect sizes (Glass's delta, 'Glass' D') for measured defensive traits per treatment and plant age: (A) nitrogen content, (B) phenolic concentrations, (C) total soluble protein, (D) neutral detergent fiber and (E) biogenic silica. Error bars represent 95% confidence intervals.

Discussion

Coastal ecosystems, such as salt marshes, are historically nutrient-limited (Deegan et al. 2007, Lu et al. 2019, Bowen et al. 2020), with nutrient availability directly tied to anthropogenic activities (e.g. runoff from land, Billen et al. 2013). This eutrophication of coastal waters can increase erosion and decomposition, facilitating saltmarsh loss (Deegan et al. 2012). Further, these ecosystems are threatened by chronic herbivory (Gedan et al. 2009) which, through the direct removal of plant foundation species, can alter their provision of critical services and saltmarsh resilience to sea-level rise. Thus, understanding the controls on trait change and plant defense against herbivores directly influences ecosystem resilience to sea-level rise and eutrophication (Lavorel 2013, Wright et al. 2016). Here, we found that nutrient addition stimulated traits associated with growth (Fig. 2) while reducing defensive traits, supporting predictions from the RAH. This indicates that elevated nutrient availability potentially switched *Spartina* strategy from defense to growth. Further, trait induction was mediated by plant age, with more pronounced trait variation in new growth. A decline in defensive traits often increases plant palatability, ultimately leading to higher grazing pressure (Hernán et al. 2019) and potentially

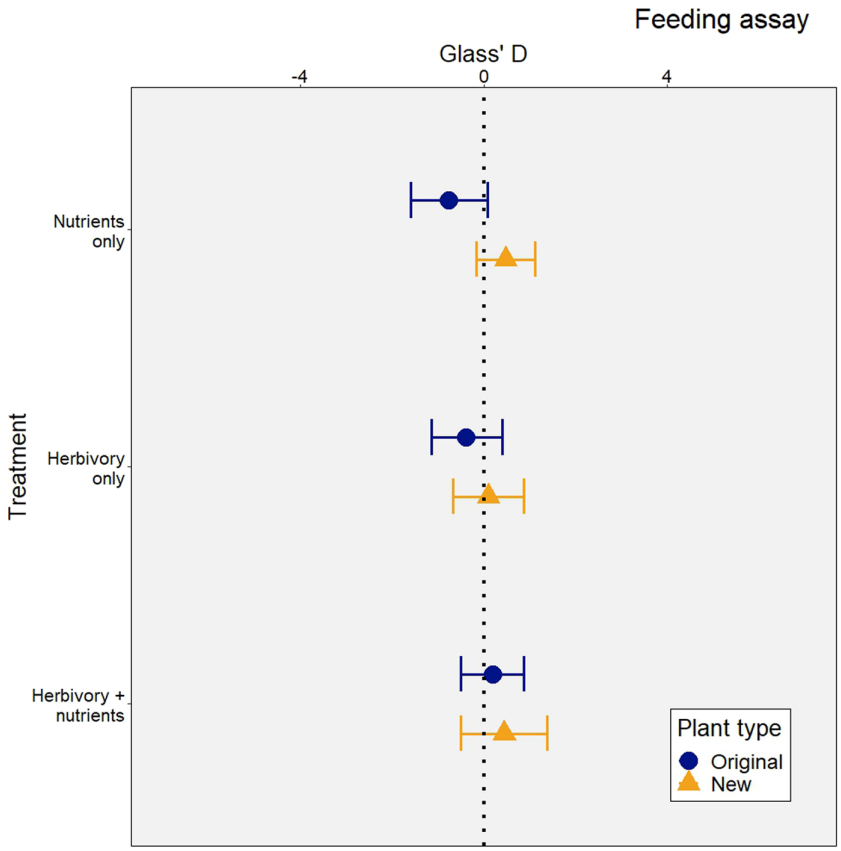


Figure 5. Standardized effect sizes (Glass's delta, 'Glass' D') for measured consumption of *Spartina* by *Littoraria* per treatment and plant age. Error bars represent 95% confidence intervals.

ecosystem loss. However, despite altered traits, feeding assays revealed *Littoraria* consumption was similar across treatments and plant age (Fig. 5), a finding in contrast to previous work showing that elevated nutrients can increase grazer damage (Bertness et al. 2008, Tomasula et al. 2023). Our study did find that nutrient only treatments decreased both fiber and silica content of *Spartina*, although these structural defenses were most likely lost in the process of creating agar-based foods (Long et al. 2011, Hughes et al. 2015), potentially influencing our feeding assay results. Overall, our results suggest that eutrophic conditions may not always increase top–down control (Silliman and Zieman 2001, Sala et al. 2008), and that a shift in *Spartina* to growth rather than defense could increase vertical accretion via stimulated biomass production (above-, belowground, new stem), potentially enhancing marsh resilience to sea-level rise (Graham and Mendelssohn 2014).

Based on the RAH, we expected nutrient addition alone to stimulate growth and reduce constitutive defensive traits in *Spartina*. Indeed, these treatments enhanced above- and belowground biomass, new stem production and the number of leaves on original stems (Fig. 2, 3), while reducing defensive traits (e.g. fiber and silica content; Fig. 4). This follows our hypothesis and aligns with previous nutrient enrichment studies in salt marshes (Johnson et al. 2016, Li et al. 2017, Crosby et al. 2021, Moore et al. 2021). Interestingly, in two other studies that evaluated the RAH in seagrasses, elevated nutrient availability decreased defensive traits yet had no effect on growth (Hernán et al. 2019, 2021). The authors attributed this result to a potential limiting resource other than nitrogen (e.g. phosphorus) that hindered plant growth. Our results, however, show an almost 40% increase in new stem production in response to nutrient enrichment relative to controls (Fig. 3D). This suggests that, in our study system, biomass production was nutrient limited, further supporting a well-established trend in the salt marsh literature (Valiela et al. 1985, Deegan et al. 2012).

Contrary to our expectations, herbivory alone did not induce defensive traits (Fig. 4). In fact, *Littoraria* grazing only influenced growth traits, including greater belowground biomass production and thicker original stems (Fig. 2B, 3B). Surprisingly, grazing alone also decreased new stem production by more than 20% in comparison to controls (Fig. 3D). Herbivory only treatments also had no effect on aboveground biomass production (Fig. 2A), regardless of active removal of aboveground tissues via grazing, suggesting *Spartina* compensated for biomass lost to herbivores. Despite declines in new growth, enhanced belowground biomass and plant morphology combined with compensatory aboveground biomass production suggests *Spartina* prioritized growth in response

to *Littoraria* grazing, even in low resource conditions. A reallocation of biomass belowground is recognized as a consequence of herbivory in terrestrial ecosystems (Thomas et al. 2017) and may ultimately be the result of our experimental design using *Littoraria*, an herbivore which only attacks aboveground portions of *Spartina*. There are two possible explanations for a lack of induced defense by *Littoraria* grazing. First, *Littoraria* herbivory on *Spartina* can induce chemical defensive compounds other than phenolics (Long et al. 2011, Sieg et al. 2013, Kicklighter et al. 2018), so perhaps a defense response was elicited that was not captured by our measured traits. Alternatively, there is a tradeoff between constitutive and induced defenses, such that plants with high constitutive defense will not invest resources in induced defense (Kempel et al. 2011). Cushman's Landing marsh is relatively free from anthropogenic disturbance and thus simulates a low resource environment. Accordingly, *Spartina* collected from this site may already have high constitutive defenses, thus *Littoraria* herbivory would not have induced a further defensive response. In fact, *Spartina* collected from Cushman's Landing marsh (control treatment) had an average total phenolic content of 9.0 ± 0.46 mg g^{-1} gallic acid equivalent (GAE), which is 19% higher than *Spartina* collected from two other marshes along the eastern shore of Virginia (Wittyngham et al. unpubl.). This finding also provides support for the physiological trait prediction of the RAH_{intra} which states that low resource, high stress environments will produce plants with higher constitutive defenses, such as elevated phenolic concentrations.

If adhering to the RAH, we expected *Littoraria* herbivory plus nutrients to stimulate growth while decreasing defense (Coley et al. 1985). Indeed, these treatments stimulated belowground biomass and new stem production (Fig. 2, 3), while reducing defenses (e.g. increased nitrogen content, decreased phenolic concentrations; Fig. 4). Our results agree with previous studies of other aquatic macrophytes which showed that compensatory growth (e.g. increased biomass) led to decreased phenolics (seagrasses: Vergés et al. 2008; mesohaline marsh plants: Rejmánková 2016). Interestingly, herbivory plus nutrients treatments appeared to act as an intermediary, with some similar responses to both nutrients only (above- and belowground biomass, new stem production) and herbivory only (silica content) treatments.

In addition to mesocosm treatments, we expected plant age to affect *Spartina* traits. Similar to our predictions, we found new stems had higher chlorophyll content (a, b, total) (Fig. 2) and reduced defensive (e.g. higher nitrogen, protein; Fig. 4) traits when compared to original stems. This suggests that, regardless of herbivore presence or nutrient availability, there is higher resource investment in clonally-grown new growth than in original tissues. Plant new growth tends to have higher nitrogen content than older leaves and stems (Tomczak and Müller 2017, Funk et al. 2021), and herbivores often forage for nitrogen-rich food sources (Mattson 1980). Thus, resources may be devoted to fast new growth and high rates of photosynthesis to compensate for lower leaf surface area or to mitigate intense herbivory pressure. Although the influence of age on traits is understudied in saltmarsh plants, these results follow similar trends to those of a seagrass species and terrestrial studies (seagrasses: Sola et al. 2020; forests: Chen and Poland 2009; coastal sage scrub: Funk et al. 2021; tallgrass prairies: Henn and Damschen 2021). Further, we saw much greater variation in measured plant traits among new stems than in original stems, supporting others' recent findings that plant age strongly determines intraspecific variation in plant traits (Cope et al. 2020, Funk et al. 2021, Henn and Damschen 2021).

Contrary to our expectations, neither treatments nor plant age affected *Littoraria*'s consumption of *Spartina* (Fig. 5). Although altered nutritional quality and palatability can influence grazing intensity and herbivore abundance in salt marshes (Pennings et al. 1998, Long et al. 2011), our results support previous findings showing that *Littoraria* abundance and density were not impacted by *Spartina* traits or defense specifically in the U.S. mid-Atlantic region (Kicklighter et al. 2018, Failon et al. 2020). Thus, despite differences in growth and defensive traits driven by resource availability, herbivory, and plant age, our findings showed that potential changes in palatability did not increase top–down grazing pressure from *Littoraria*.

Our results suggest that, in some instances, salt marshes may be resilient against sea-level rise under moderate levels of herbivory and eutrophication. Under eutrophic conditions (high resource environment) *Spartina* had lower defensive traits and higher growth. Enhanced growth traits can directly influence salt marsh stability and resilience to sea-level rise, as *Spartina* is a foundation species responsible for regulating vertical accretion, the process by which salt marshes keep pace with sea-level rise (Kirwan and Megonigal 2013). Nutrient-induced above- and belowground biomass production, in particular, may enhance sediment deposition and organic matter accumulation (Graham and Mendelssohn 2014), both of which contribute to marsh elevation. Additionally, *Littoraria* grazing further stimulated belowground biomass production and produced thicker stems, which could aid wave attenuation and promote sediment capture (Lu et al. 2019). Interestingly, when both stressors (elevated nutrients and herbivory) were combined, a treatment most closely resembling field conditions, the most beneficial outcomes for vertical accretion were produced, with increases in both belowground biomass and new stem production. Thus, evaluating how resource availability influences plant trait variation and antiherbivore defenses provides direct insight into ecosystem stability and resilience under future climate change and anthropogenic disturbance scenarios.

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Author contributions

Serina S. Wittyngham: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Joanna Carey**: Data curation-Supporting, Formal analysis-Supporting, Methodology (equal); Resources (equal); Writing – review and editing (equal). **David Samuel Johnson**: Conceptualization (equal); Formal analysis-Supporting, Funding acquisition (lead); Investigation-Supporting, Methodology (equal); Project administration (lead); Resources (equal); Supervision (lead); Visualization-Supporting, Writing – review and editing (equal).

Data availability statement

Data were submitted through the Virginia Coast Reserve (VCR) Long-Term Ecological Research (LTER) repository and are publicly available from the Environmental Data Initiative: https://doi.org/10.6073/pasta/0b70b4ed9cd773475609eb1a97cec9bd (Wittyngham 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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