PLANT-MICROBE-ANIMAL INTERACTIONS – ORIGINAL RESEARCH



Herbivory and resource availability shift plant defense and herbivore feeding choice in a seagrass system

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Abstract

Numerous hypotheses have been posited to explain the observed variation in plant defense strategies against herbivory. Under resource-rich environments, plants are predicted to increase their tolerance (limiting resource model; LRM) and, while the resource availability hypothesis (RAH) predicts a decrease in constitutive resistance in plant species growing in resource-rich environments, at the intraspecific level, plants are predicted to follow an opposite pattern (intraspecific RAH). Furthermore, the effect of multiple factors in modulating plant defense strategies has been scarcely explored and is more difficult to predict. Our aim was to understand how plant defense traits respond to herbivory, resource availability and their interactions, and to assess the effects on plant palatability. To this end, we performed an in situ factorial experiment at two sites simulating three herbivory levels and two nutrient availability conditions with the seagrass *Posidonia oceanica*. Additionally, we performed a series of feeding experiments with its two main herbivores. While plants decreased their constitutive resistance under nutrient fertilization (contrary to intraspecific RAH but in accordance to the RAH), and did not increase allocation to tolerance (likely due to resource limitation, LRM), simulated herbivory induced resistance traits. However, we found no interactive effects of nutrient fertilization and herbivory simulation on plant defense. Both herbivores responded similarly to changes in plant palatability, strongly preferring nutrient-enriched plants and non-clipped plants. This work highlights the need to better understand the drivers of plant defense intraspecific variability in response to resources, particularly in habitat-forming species where changes in plant traits and abundance will cascade onto associated species.

Keywords Plant–herbivore interactions · Limited resource model · Resource availability hypothesis · Nutrients · *Posidonia* oceanica

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Introduction

Herbivory is a key ecological process that regulates the flow of energy to upper trophic levels and the composition, abundance and distribution of plant communities (Huntly 1991; Wood et al. 2016). Herbivory has strong effects on plant fitness and can be an important pathway of biomass and nutrient loss in plants (Cebrian 1999). Therefore, plants have evolved diverse defense strategies against herbivory; tolerance strategies that reduce the impact of herbivory on plant fitness (e.g., increased growth to compensate for leaf loss), and resistance strategies that reduce the preference or performance of herbivores (e.g., production of phenolic compounds; Fritz and Simms 1992). Plant allocation to these different strategies is determined by a trade-off between benefits (i.e., better defense against herbivores) and costs (i.e., less resources available for growth and/or reproduction; Bazzaz et al. 1987). Although initially thought otherwise

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(Van Der Meijden et al. 1988), recent studies suggests that plants present mixed tolerance-resistance defense mechanisms (Agrawal 2011; Carmona and Fornoni 2013), but that a trade-off exists between both strategies whereby selection for increased resistance can lead to decreased tolerance (Fineblum and Rausher 2002). In addition, these defenses can be either constitutive (i.e., expressed regardless of the herbivory pressure suffered by the plant) or induced (i.e., produced in response to herbivory damage; Agrawal and Karban 1999; Vergés et al. 2008). Recent studies suggest a trade-off between both strategies in which plants with higher induced resistance invest less in constitutive resistance (e.g.; Kempel et al. 2011; Rasmann et al. 2015).

Since defense has a cost, availability of resources is an important determinant of defense strategies in plants. As an explanation of how resources influence plant allocation to defense, the Resource Availability Hypothesis (RAH) postulates that species adapted to resource-rich environments have higher growth rates and have less constitutive defenses but higher inducible defenses. On the contrary, species adapted to environments with low resource availability will have lower growth rates and invest more in constitutive defense strategies (reviewed in Endara and Coley 2011). Although the RAH has been refuted by some studies (see Endara and Coley 2011 and references therein) it is considered as an adequate framework to explain the variation observed in defense strategies under different resource availability conditions. Recent studies have explored this hypothesis in the context of intraspecific variations in plant defense (hereafter intraspecific RAH) suggesting the opposite pattern. In this case, individuals of the same species have higher constitutive resistance in high resource environments mediated by the higher herbivory pressure found in these environments. Accordingly, in low-resource environments, inducibility should be more effective and would trade-off with constitutive resistance (Hahn and Maron 2016). However, some plant species inhabiting high resource environments, such as tropical forests, do not bear high herbivory rates and thus, the predictions of the intraspecific RAH may not be applicable (Lamarre et al. 2012). Similarly, high resource availability should also allow plants to better tolerate and/or compensate herbivory. In this regard, the Limiting Resource Model (LRM; Wise and Abrahamson 2005) states that a plant will become more tolerant when the availability of resources, including limiting resources, increases. However, when herbivory damage affects the use or acquisition of an alternate resource, plants under high resource availability will not increase their investment in tolerance (Hay et al. 2011; Hattas et al. 2017). Regarding marine macrophytes (i.e., seagrasses and algae), leaves and blades or thalli do not only perform photosynthesis but also can be a major source of nutrients via absorption from the water column (Romero et al. 2006). Thus, higher rates of herbivory may

result in higher limitation not only of carbon fixation but also of nutrient acquisition.

In addition to resource availability, plant defense strategies against herbivores can respond to different consumption levels. Indeed, herbivore intensity and duration modify plant defense responses, with some traits only being induced under high herbivory (e.g., secondary metabolites, mineral crystals; Vergés et al. 2008; Dostálek et al. 2016; Hartley et al. 2016), while others tend to be induced under moderate herbivory (e.g., compensatory growth; Ruiz-R et al. 2008; Vergés et al. 2008; Sanmartí et al. 2014). Therefore, increased resource availability (when limiting resources are considered) could minimize the costs of defense under different levels of herbivory pressure. Yet, few studies have assessed the interactive effects of resource availability and varying herbivory pressure on plant defense strategies (i.e., tolerance and resistance).

Furthermore, the consequences of resource availability and herbivory pressure on plants may extend beyond the individual species of herbivore, as we often find several herbivore species feeding on the same plant species. Indeed, herbivory damage exerted by one guild or group of herbivores often has strong effects on other herbivores within the same community due to changes in plant community structure or abundance (Foster et al. 2014) or plant defenses (Delphia et al. 2007; Ramirez and Eubanks 2016). This is even more relevant when herbivory is exerted upon foundation species that are the base of ecosystems such as terrestrial forests, coral reefs, or kelp beds, since in these ecosystems, grazing-mediated changes cascade through the food web and can have profound ecological impacts such as loss of feeding resources or refuge (Steneck et al. 2002; Ellison et al. 2005; Pagès et al. 2012).

Seagrasses are important foundation species, forming extensive underwater meadows that perform important functions and provide crucial ecosystem services. Due to their high primary productivity and structural complexity, they provide food and shelter for many marine species (Jackson et al. 2015) and they protect the shore against coastal erosion, stabilize the substrate, and increase water clarity (Nordlund et al. 2016). These marine systems in temperate regions are undergoing an increase in herbivore pressure due to the expansion of tropical species (Vergés et al. 2014; Hyndes et al. 2016) that, together with the predicted increase in herbivore feeding rates resulting from higher seawater temperature (Carr and Bruno 2013), may increase the grazing pressure in seagrass meadows. In addition, the commonly observed increases in nutrient concentrations in coastal waters (Smith 2003) have important detrimental effects in seagrass performance (Ralph et al. 2006). Thus, understanding the independent and interactive effects of herbivory and higher nutrient availability on trophic interactions has important implications for management and conservation of seagrass ecosystems. To our knowledge, only one other study has previously addressed this question (Tomas et al. 2015), and the work was performed with a fastgrowing species (*C. nodosa*), during a shorter experimental period (see methods) and in an already relatively eutrophic system (eastern Atlantic), and therefore, their results may not be applicable to other seagrass species or much more oligotrophic conditions.

In this study, we aim to assess the predictions posed by the resource availability hypotheses in relation to tolerance and resistance strategies against herbivory considering the effect of different levels of herbivory damage. In addition, we assess how simulated fish herbivory may affect another herbivore species within the community. Specifically, we tested the hypothesis that in oligotrophic environments, plant productivity will be strongly limited by nutrient availability. Thus, under simulated herbivory, plants would induce the production of defenses related to resistance. In addition, by increasing the resources available (i.e., nutrients), plants would invest more towards tolerance and compensatory strategies. Finally, we hypothesized that the induction of resistance defenses and the capacity of plants to tolerate herbivory may differ with herbivory pressure and may affect differently the feeding behavior of other herbivores within the same community. To examine these hypotheses, we performed an in situ manipulative experiment in which we simulated different levels of fish herbivory and two levels of resource (i.e., nutrient) availability in a factorial experiment with Posidonia oceanica, a dominant seagrass species inhabiting the oligotrophic coastal waters of the Mediterranean Sea. We assessed the main and interacting effects of nutrient addition and simulated herbivory on size and plant traits related to tolerance and resistance against herbivory. We also explored what the species-specific effects of simulated herbivory and nutrient-driven changes were on leaf palatability by a series of feeding experiments with the two main herbivores in P. oceanica meadows; the sea urchin Paracentrotus lividus and the fish Sarpa salpa.

Materials and methods

Study sites and experimental design

To test the combined and independent effects of nutrient availability and herbivore pressure on defense strategies against herbivores and the induced changes in seagrass palatability, 24 plots of 1×1 m were installed in two continuous *P. oceanica* meadows North ($A = 39^{\circ}29'52''N$, $2^{\circ}32'15''E$) and South ($B = 39^{\circ}27'53''N$, $2^{\circ}43'56''E$) of Palma Bay at depths of 6.6 ± 0.8 m at both sites. Mean initial density of the plots was 416.5 ± 7 shoots/m² and there were no differences in density between sites (Three-way ANOVA: $F_{(1/72)} = 0.146; P = 0.7$), treatments (Three-way ANOVA: $F_{(11/72)} = 0.67; P = 0.75$), or replicates (Three-way ANOVA: $F_{(1/72)} = 0.38; P = 0.54$).

The effects of herbivore pressure (C = ambient, H = moderate and HH = high) and nutrient enrichment (A = ambient and N = enriched) were tested in a factorial design resulting in six experimental treatments randomly assigned along the 24 plots, having, thus, four replicates per treatment. To avoid nutrient contamination and minimize nutrient transport among rhizomes, plots were set 3.5-4 m apart from each other. The experiment started in May 2014 and lasted for 4 months coinciding with the growing season of P. oceanica (Alcoverro et al. 1995). To have samples comparable in time and confirm the effectiveness of the fertilizing treatment, reference plant samples were collected in June (T1; i.e., ca. 1 month and a half after the beginning of the experiment) and at the end of the experiment in September (T2) at both sites. In addition, the induced changes in seagrass palatability were tested by a series of feeding choice experiments with different herbivores (fish and sea urchins; see below). These experiments were conducted during August and September.

Experimental treatments

Nutrients were added in the sediment by introducing 1-g fertilizing bars (COMPO®) homogeneously distributed across the plot, at a loading rate of 100 g/m²/month (N:P:K molar ratio = 13:6:10). Plots were fertilized once a month and, as a perturbation control, non-fertilized plots were disturbed by perforating the sediment using plastic bars with a similar size to that of the fertilizing bars. The nutrient loading rate selected was above the plant requirements (Alcoverro et al. 2000) and below the concentrations in which negative effects from toxicity have been previously reported (Invers et al. 2004). Two replicate porewater samples were collected at the end of the experiment in each plot. Upon collection, samples were filtered (0.22 µm), put on ice, and brought to the laboratory for analyses. Porewater nutrients were analyzed in a continuous flow autoanalyzer (trAAcs-800, Bran+Luebbe, Inc., IL USA) as described by Arjonilla et al. (1991). The fertilization method significantly increased the concentrations of nitrite, nitrate, ammonium and phosphate in the nutrient-added porewater plots (Supplementary material, Table S1, S2).

Herbivory simulation was performed once a month by clipping the leaves mimicking the grazing performed by the main vertebrate consumer of *P. oceanica* in shallow meadows, the fish *S. salpa* (Tomas et al. 2005; Prado et al. 2007). The experiment was performed during the period of maximum fish herbivory (Tomas et al. 2005; Prado et al. 2007). Clipping was performed as a proxy of fish herbivory (e.g., Vergés et al. 2008; Sanmartí et al. 2014; Tomas et al. 2015) inside the experimental plots as well as around a 15-cmwide margin surrounding the plot. The imitation of damage has also proved to be helpful in reaching the same effect between simulations and natural damage in terrestrial plants (Lehtilä and Boalt 2008). The control treatment (C) corresponded to the ambient herbivory, which was naturally low in both sites and was previously evaluated through a tethering experiment (relative fresh biomass consumed in 3 days, Site $A = 0.08 \pm 0.066\%$; Site $B = 0.08 \pm 0.048\%$). In addition, no bite marks were observed on the control plots during the experiment, or on the shoots collected for analysis or for feeding experiments. The moderate herbivory treatment (H) consisted of trimming 40% of the maximum blade length (i.e., ca. to 60 cm in length), while the high herbivory treatment (HH) consisted of performing ca. 80% removal (i.e., leaves were cut to ca. 20 cm in length). These values are in accordance with natural herbivory rates observed in P. oceanica (Tomas et al. 2005; Prado et al. 2007, 2008).

Herbivore feeding behavior experiments

To examine how changes in plant defense traits due to treatment effects (i.e. nutrient enrichment, herbivory, or their interactions) modify plant palatability, we performed a series of two-choice feeding trials with herbivorous sea urchins and fish at the end of the field experiment. Similar-sized sea urchins $(4.98 \pm 0.66 \text{ cm}, \text{ one-way ANOVA})$: $F_{(1/42)} = 1.695$; P = 0.21) of the species Paracentrotus lividus, the main invertebrate macro-herbivore on P. oceanica meadows, were collected and individually kept in plastic cages $(15 \times 15 \times 15 \text{ cm}^3)$ covered with a 1-cm mesh. These cages were placed in the experimental sites (A and B), over P. oceanica dead matte (natural substrate formed by this seagrass species) protected from currents. Sea urchins were maintained in the cages for 48 h and fed with Ulva sp. ad libitum prior to starting the feeding experiments. Replicates of two tethered leaf fragments (from two different treatments) attached with a clothes pin were placed inside the cages. To avoid confounding factors such as within tissue differences in structural (Enríquez 2005) or chemical traits (McKey 1979; Cronin and Hay 1996), the fragments offered were clipped from the young leaves avoiding basal and apical parts. The first centimeters above the ligule were discarded and the fragments used were within the following 15-20 cm.

Considering the low fish herbivory pressure measured and observed in the experimental sites (see above), the feeding experiments with fish were performed at a third site, a shallow cove in the middle of Palma Bay where *Cymodocea nodosa* is present and which harbors large groups of the herbivorous fish *S. salpa*. Two-choice feeding trials were set up in a manner similar to that used for sea urchins. In sandy patches and at least 3 m away from the *C. nodosa* meadow, replicates of two pegs with the tethered leaves attached with a clothes pin and a cable tie to a peg ca. 10 cm above the sediment were offered to the fish. In each replicate, sea urchins or fish were offered similar amounts of young leaf tissue (ca. 15–20-cm long) clean of epiphytes.

The experiments consisted of 25–30 replicates and ended when approximately 50% of initial material was consumed. Following the procedures of previous feeding behavior experiments in seagrasses (e.g., Tomas et al. 2015), replicates in which all the offered samples were either totally consumed or fully intact were not considered in the statistical analysis as they do not provide information on feeding preference. Consumption was calculated as leaf area eaten of each tissue relative to the total amount of leaf area eaten in the assay. To measure any potential changes in leaf tissue not related to grazing, control cages, the same type as the experimental ones but without herbivores, were used to correct for autogenic changes in area prior to the statistical analyses. Since we found no changes in area in these controls, those data were not used.

Plant size traits and epiphyte load

Leaf growth was measured in three shoots per plot at the end of the experiment by punching the leaves following the method of Zieman (1974). Leaf width, maximum leaf length, number of leaves, total leaf area, leaf biomass, specific leaf area (SLA; calculated as fresh leaf area divided by leaf dry mass), and percentage of necrotic surface were measured at the end of the experiment (T2) on these same shoots. Epiphytes were removed from both sides of each leaf by gentle scraping with a microscope slide, the water with the epiphytes was filtered and salt was removed by rinsing with distilled water. Filters were dried (60 °C for 48 h) and weighed, along with the corresponding dried leaves, and epiphyte dry mass was standardized by shoot dry mass and shoot leaf area to estimate epiphyte load.

Plant chemical traits

Regarding plant traits related to defense against herbivory, total phenolic compounds, nitrogen, sucrose, phosphorous and fiber contents of leaves are typically considered resistance traits since those change the nutritional quality (e.g., nitrogen), palatability or digestibility (e.g., fiber and phenols) of the plant, and therefore herbivore preference (Lincoln 1993; Valentine and Heck 2001; De Bruyn et al. 2002; Prado et al. 2010; de los Santos et al. 2012). Carbon, sucrose, starch, nitrogen and phosphorous contents of rhizomes are generally considered as tolerance traits since the accumulation of these resources belowground will allow plants to real-locate those resources to reproduction or biomass regrowth

after herbivory (Tiffin 2000; van Staalduinen and Anten 2005; Sanmartí et al. 2014).

Pooled plant material of 3 shoots per plot collected at T1, and 8 shoots per plot collected at the end of the experiment (T2) were cleaned of epiphytes, ultrafrozen (- 80 °C), freeze-dried, and ground to a fine powder for further analyses of chemical traits. The collected plant material consisted of both the youngest and the second youngest leaves of each shoot (hereafter young leaves) and rhizomes. Nitrogen, carbon and phosphorous content of young leaves and rhizomes, as well as sucrose and starch content of rhizomes were analyzed from samples collected at the midst of the experiment (T1) and at the end of the experiment (T2). Sucrose, fiber and phenol content in young leaves were analyzed only from samples collected at the end of the experiment (T2).

Carbon and nitrogen contents in young leaves and rhizomes were analyzed using a CNH elemental analyzer (EA1108, Carlo-Erba, Italy). Phosphorus content in young leaves and rhizomes was analyzed following the protocol described by Fourqurean et al. (1992). Total phenols were analyzed as described in Hernán et al. (2016). Non-structural carbohydrates in young leaves (sucrose), and rhizomes (sucrose and starch) were measured using methodology described by Invers et al. (2004). Neutral detergent fiber content (NDF: referred as 'fiber content' hereafter) was determined as described in Hernán et al. (2016).

Statistical analyses

The effect of nutrient availability and simulated herbivory were analyzed by linear mixed effects models in which site was considered as a random effect. The models contrasted different combinations of two fixed effects, nutrient availability (i.e., ambient and nutrient addition) and herbivory level (i.e., control, moderate and high) independently or interacting. For dependent variables analyzed in two different times (i.e., T1, T2) during the experimental period, time was added as a fixed effect since we may expect differences in those variables with time due to plant phenology or length of the experimental treatment. We reduced the full linear mixed model of each response variable (initially containing all random factors, and all fixed factors and their interactions) using its AIC value (Supplementary material, Table S3), calculated as the sum of the model deviance and twice the number of parameters (Akaike's information criterion; Burnham and Anderson 2002). The model with the lowest AIC was taken as the best compromise between model adequacy (i.e., residual deviance) and model complexity (i.e., number of estimated parameters). Model reduction may withdraw independent variables and interactions during model selection due to lack of significance. Thus, tables and figures only show interactions between factors when they are considered in the final model (see Results section). All dependent variables were checked for normality with the Shapiro–Wilk test and homoscedasticity with the Bartlett test and transformed when needed. Post hoc analyses were performed using Tukey's honest significant difference test.

The analyses of two-choice feeding experiments were performed using a Wilcoxon signed-ranks paired test since data were not normal even after transformation. The overall effect of herbivory and nutrient addition on herbivore feeding preference was analyzed through meta-analysis of all individual feeding trials (i.e., 18 per site). The effect size was measured as the standardized mean difference using Hedges' d (bias corrected; Hedges 1981) and confidence intervals were obtained iteratively via the Q-profile method (Viechtbauer 2007). The differences in effect size were assessed with a random-effects model since plants came from two different sites and sea urchins and fish were different in each experiment. Models were fitted by restricted maximum likelihood. Meta-analysis considered experiments performed with sea urchins or fish individually, as well as combining both herbivores.

Results

Responses of plant size traits and epiphyte load

Increased nutrient availability and herbivore damage did not have interactive effects in plant size traits. On the other hand, increased nutrient availability reduced leaf biomass (Mean \pm SE; 11 \pm 2%), although it had no significant effects on any other leaf size trait measured (Fig. 1, Table 1). Both levels of simulated herbivory reduced all the size traits measured except mean leaf width and leaf growth, for which there were no significant effects. Clipped shoots had significantly lower leaf biomass ($H = 18 \pm 3\%$; $HH = 51 \pm 2\%$), leaf area (H = $32 \pm 2\%$; HH = $61 \pm 3\%$), maximum leaf length (H = $42 \pm 2\%$; HH = $66 \pm 1\%$), and SLA (H = $18 \pm 1\%$; $HH = 22 \pm 3\%$) than controls (Fig. 2, Table 1). Additionally, plants under the moderate clipping treatment exhibited a higher number of leaves $(12 \pm 6\%$ higher) when compared to the control and the high herbivory treatments, which had a similar number of leaves (Fig. 2, Table 1). Necrosis was significantly lower both in the moderate $(59 \pm 1\% \text{ lower})$ and the high clipping treatment $(85 \pm 7\%)$ than in the control treatment, while epiphyte load increased with clipping. The biomass of epiphytes per leaf area almost doubled in both clipping treatments, and epiphyte load by leaf biomass (i.e., g epiphyte/g seagrass) also increased ca. 36% under higher nutrient availability, while epiphyte load by area (i.e., g epiphyte/seagrass cm²) was not significant (Fig. 1, Table 1).

Fig. 1 Significant effects of increased nutrient availability on plant traits. Mean contents of plant traits from plants grown at the ambient (A; white) and increased nutrient availability (N; grey). Since there are no interactive effects between nutrient and herbivory treatments (see tables) the means have been calculated from the pooled herbivore treatments. Error bars represent standard error (N = 24 for Leaf sucrose, fiber and biomass, and epiphyte biomass; N = 48 for Leaf N and rhizome N, C/N, and sucrose)



Responses of plant chemical traits

Both nutrient addition and simulated herbivory modified plant chemical traits and there were no interactive effects of those two factors. Clipped plants exhibited lower nutrient content than unclipped ones. Nitrogen content of moderate and high herbivory treatments was lower than controls for both rhizome $(H = 21 \pm 5\%; HH = 25 \pm 5\%)$ and leaves $(H = 23 \pm 3\%; HH = 31 \pm 3\%)$ and, consequently, both tissues had higher C/N (H = $27 \pm 9\%$; HH = $33 \pm 9\%$ and $H = 30 \pm 4\%$; $HH = 42 \pm 5\%$, respectively) than controls. Similarly, phosphorous content in leaves was lower in the simulated herbivory treatments relative to controls $(H=27\pm6\%; HH=30\pm3\%)$ and was also marginally lower in rhizomes compared to controls. In addition, simulated herbivory increased rhizome sucrose content (H = $14 \pm 2\%$; $HH = 40 \pm 6\%$ higher than controls) but did not affect starch content. Plant clipping also increased leaf sucrose under the moderate herbivory level $(19 \pm 4\%)$ higher than C and HH; Fig. 2, Tables 2 and 3) and the production of fibers $(H=9\pm2\%; HH=5\pm0.04\%)$ and phenols $(H=9\pm1\%; HH=20\pm1\%; Fig. 2, Table 3)$ in leaves.

Nutrient fertilization triggered a reduction in rhizome and leaf sucrose content $(11 \pm 4\% \text{ and } 15 \pm 2\%, \text{ respectively})$ and in leaf fiber content $(6 \pm 1\%;$ Fig. 1, Table 3) relative to controls having no effect in rhizome starch. Additionally, plant rhizomes from fertilized plots had lower C/N content $(23 \pm 1\%)$; Fig. 1, Table 2) than control plots, and both rhizomes and leaves had higher N content $(29 \pm 4\%)$ and $13 \pm 6\%$, respectively; Fig. 1, Table 3). Furthermore, there was an interactive effect of nutrient availability and time. Leaf N content of plants growing under ambient conditions strongly decreased during the experimental period $(10 \pm 2\%$ decrease) in comparison to plants growing under nutrient-enriched conditions ($2\% \pm 0.3$ decrease). Finally, P in leaves and N in rhizome increased during the experiment $(12 \pm 4\%$ higher than initial samples), while C $(0.7 \pm 0.2\%)$, C/N $(17 \pm 1\%)$ and carbohydrate reserves of rhizomes

Table 1 Results of mixed-effects models on size traits

| Trait | Fixed | i | $\beta_{ m N}$ | $\beta_{ m H}$ | $\beta_{ m HH}$ | Random | i _{site} | $\beta_{\rm residual}$ |
|---|-------|---------|----------------|----------------|-----------------|--------|-------------------|------------------------|
| Leaf biomass (g DW) | Est. | 0.683 | - 0.057 | - 0.115 | - 0.336 | Var. | 0.000 | 0.008 |
| | SE | 0.025 | 0.025 | 0.031 | 0.031 | SD | 0.000 | 0.087 |
| | Р | < 0.001 | 0.028 | < 0.001 | < 0.001 | | | |
| Log (Leaf area (cm ²)) | Est. | 2.220 | - 0.044 | - 0.159 | - 0.410 | Var. | 0.000 | 0.006 |
| | SE | 0.024 | 0.023 | 0.028 | 0.028 | SD | 0.013 | 0.078 |
| | Р | < 0.001 | 0.061 | < 0.001 | < 0.001 | | | |
| Log (Maximum leaf length (cm)) | Est. | 1.835 | | - 0.231 | - 0.462 | Var. | 0.000 | 0.003 |
| | SE | 0.013 | | 0.019 | 0.019 | SD | 0.000 | 0.053 |
| | Р | < 0.001 | | < 0.001 | < 0.001 | | | |
| Mean leaf width (cm) | Est. | 0.956 | | 0.002 | - 0.023 | Var. | 0.000 | 0.001 |
| | SE | 0.009 | | 0.011 | 0.011 | SD | 0.005 | 0.032 |
| | Р | < 0.001 | | 0.870 | 0.053 | | | |
| Leaf number | Est. | 5.083 | | 0.490 | - 0.250 | Var. | 0.032 | 0.398 |
| | SE | 0.202 | | 0.223 | 0.223 | SD | 0.180 | 0.631 |
| | Р | < 0.001 | | 0.033 | 0.268 | | | |
| SLA (cm ² /g) | Est. | 255.494 | | - 45.279 | - 57.060 | Var. | 181.600 | 630.900 |
| | SE | 11.412 | | 8.881 | 8.881 | SD | 13.480 | 25.150 |
| | Р | 0.006 | | < 0.001 | < 0.001 | | | |
| Log (leaf growth (cm ² /day)) | Est. | - 0.157 | | 0.072 | - 0.044 | Var. | 0.002 | 0.012 |
| | SE | 0.044 | | 0.039 | 0.039 | SD | 0.048 | 0.111 |
| | Р | 0.077 | | 0.074 | 0.264 | | | |
| Leaf necrosis (% cm ²) | Est. | 16.436 | | - 9.664 | - 13.937 | Var. | 0.000 | 37.350 |
| | SE | 1.528 | | 2.161 | 2.161 | SD | 0.000 | 6.110 |
| | Р | < 0.001 | | <0.001 | < 0.001 | | | |
| Epiphyte biomass (g. epi./g. Leaf) | Est. | 0.132 | 0.077 | 0.129 | 0.144 | Var. | 0.002 | 0.012 |
| | SE | 0.044 | 0.032 | 0.039 | 0.039 | SD | 0.042 | 0.110 |
| | Р | 0.065 | 0.020 | 0.002 | 0.001 | | | |
| Epiphyte biomass (g. epi./cm ² leaf) | Est. | 0.001 | | 0.001 | 0.001 | Var. | 0.000 | 0.000 |
| | SE | 0.000 | | 0.000 | 0.000 | SD | 0.000 | 0.001 |
| | Р | 0.006 | | <0.001 | <0.001 | | | |

Intercept (i) and slopes (β) for the fixed nutrient addition (β_N), herbivory simulation moderate (β_H) and high level (β_{HH}) and the random site ($\beta_{residual}$) effects on size traits. Blank spaces indicate that these variables were dropped during model selection. Significant effects (P < 0.05) in bold

Est. indicates estimate, SE standard error, Var variance, SD standard deviation and P p value

(sucrose = $11 \pm 4\%$; starch = $37 \pm 2\%$) decreased through time (Supplementary material Fig. S1; Tables 2 and 3).

Herbivore feeding behavior experiments

Both nutrient- and herbivory-induced changes affected plant palatability. Sea urchins and fish exhibited similar feeding patterns with a clear preference for leaves grown under the control (i.e., no clipping) herbivory treatment, as well as for leaves from the nutrient-fertilized treatment (Fig. 3). The responses observed in the feeding experiments were consistent regardless of the site of origin of the leaf tissue. There was only one two-choice feeding experiment with contrasting responses between both herbivores. When offered leaves from moderate (HA) versus high herbivory (HHA) ambient nutrient treatments, sea urchins preferred the leaves from the high herbivory treatments, while fish preferred the opposite (Fig. 3c). When these plant tissues were fertilized, the prior preference disappeared and both herbivores preferred leaves from the moderate (HN) herbivory treatment (Supplementary material, Table S4 and Fig. S2).

Discussion

Nutrient addition reduced investment in constitutive resistance traits, contrary to the expectations from the intraspecific RAH (Hahn and Maron 2016), but in accordance with

Fig. 2 Significant effects of simulated herbivory on plant traits. Mean contents from plants grown at the control (C; white), moderate (H; grey) and high (HH; black) herbivory treatments. Since there are no interactive effects between herbivory and nutrient treatments (see tables), the means have been calculated from the pooled nutrient addition treatments. Error bars represent standard error (N = 16 for all traits except for: leaf and rhizome N and C/N, rhizome sucrose and leaf phosphorus, for which N=32). Different letters indicate statistically significant differences across treatments (Tukey)



the RAH (Endara and Coley 2011). In addition, simulated herbivory modified plant defense traits inducing resistance defenses and enhancing the accumulation of belowground reserves without compensatory growth. Interestingly, we found no interactive effects of nutrient availability and simulated herbivory in plant traits. In fact, higher nutrient availability did not enhance either inductive or constitutive resistance traits under herbivory, contrary to predictions of both RAHs (Endara and Coley 2011; Hahn and Maron 2016). Similarly, we did not observe an increased allocation to tolerance traits under nutrient fertilization (regardless of whether the plants were submitted to herbivory or not), which could be due to the existence of a limiting resource, as suggested by the LRM. Furthermore, some tolerance (e.g., number of leaves) and resistance (e.g., fiber) trait responses were strongest at the intermediate herbivory level. Both nutrient addition and clipping independently modified plant traits that altered plant palatability, with both herbivores responding similarly: sea urchins and fish preferred leaves from the nutrient-fertilized treatment (which were both more nutritious and contain less fiber), while they were deterred by clipped plants (which were more chemically and structurally defended).

Our results provide evidence supporting the notion of a decrease in constitutive resistance traits with higher resource (i.e., nutrient) availability whether without herbivory or under high herbivory, contrary to the prediction of the intraspecific RAH. Furthermore, our results show that the decrease in constitutive resistance enhances susceptibility to being consumed. Interestingly, even though a reduction in secondary compounds (e.g., phenols) is one of the most common responses to increased nutrient availability observed in terrestrial plants (Endara and Coley 2011) and seagrasses (e.g., Goecker et al. 2005; Tomas et al. 2011;

| | Log (Niti | (%) uogen | (| Sqrt (Cart | ((%) uoç | | LOG (UN | <u> </u> | | Log (Pho: | sphorus (| ((% | Sucrose (| (%) | | Log (St | arch (%)) | |
|---------------------------------------|----------------|-----------|---------|------------|----------------|---------|---------|----------|---------|-----------|----------------|-------|----------------|----------------|---------|---------|-----------|---------|
| Fixed | Est. | SE | Р | Est. | SE | Ρ | Est. | SE | Р | Est. | SE | Р | Est. | SE | Ρ | Est. | SE | Ρ |
| _ I | 0.441 | 0.090 | 0.105 | 6.479 | 0.022 | < 0.001 | 1.182 | 0.087 | 0.032 | - 0.816 | 0.085 | 0.022 | 14.561 | 0.732 | 0.003 | 0.574 | 0.022 | < 0.001 |
| $\beta_{\rm N}$ | 0.105 | 0.023 | < 0.001 | - 0.021 | 0.011 | 0.056 | -0.108 | 0.023 | < 0.001 | | | | - 1.903 | 0.397 | < 0.001 | | | |
| $\beta_{ m H}$ | -0.105 | 0.028 | < 0.001 | | | | 0.105 | 0.029 | < 0.001 | - 0.096 | 0.055 | 0.084 | 2.000 | 0.488 | < 0.001 | | | |
| $eta_{ m HH}$ | - 0.132 | 0.028 | < 0.001 | | | | 0.130 | 0.028 | < 0.001 | -0.101 | 0.054 | 0.067 | 5.743 | 0.485 | < 0.001 | | | |
| eta_{t} | - 0.058 | 0.023 | 0.014 | 0.025 | 0.011 | 0.024 | 0.061 | 0.023 | 0.010 | - 0.085 | 0.044 | 0.060 | 1.971 | 0.397 | < 0.001 | 0.189 | 0.031 | < 0.001 |
| Random | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | |
| i _{site} B | 0.015 | 0.112 | | 0.001 | 0.028 | | 0.013 | 0.112 | | 0.011 | 0.103 | | 0.678 3.694 | 0.824 | | 0.000 | 0.000 | |
| $l_{ m site}$ $eta_{ m rascidual}$ | 0.013 0.013 | 0.112 | | 0.003 | 0.053 0.053 | | 0.013 | 0.110 | | 0.047 | cut.u 0.216 | | 0.078 3.694 | 0.024 1.922 | | 0.022 | 0.1 | 8 8 |

Est. indicates estimate, SE standard error, Var variance, SD standard deviation and P, p value

Table 2 Results of mixed- effects models on rhizome chemical traits

Martínez-Crego et al. 2016; but see Tomas et al., 2015), we did not detect changes in phenolic compounds. On the other hand, our study species did follow one of the RAH predictions (i.e., decrease in resistance) when considering nitrogen and fiber content. Indeed decreases in fiber content have also been observed in terrestrial plants under nutrient fertilization (Johnson et al. 2001). Such decrease in fibers may enhance plant susceptibility to herbivory since fibers deter consumers by reducing tissue digestibility (Klumpp and Nichols 1983; Sanson 2006) and by increasing leaf toughness. In addition to modifying fiber content, the increase in external sources of nutrients enhanced nitrogen (but not phosphorous) content in plant tissues. Higher nutritional quality of plant tissue can increase herbivore performance (Minkenberg and Ottenheim 1990; De Bruyn et al. 2002; Hemmi and Jormalainen 2002) and can be an important determinant of susceptibility to herbivory due to an increased preference by consumers (e.g., Cebrian 1999; Ngai and Jefferies 2004) particularly in seagrass systems (Valentine and Heck 2001; Prado et al. 2010). Nonetheless, nutrient-driven changes in plant tissues can trigger opposite feeding patterns amongst seagrass consumers (Tomas et al. 2015). In our results, however, the addition of nutrients strongly modified plant palatability and consistently changed feeding behavior, as plants growing under the nutrient addition treatment were always preferred by both herbivores over non-fertilized plants. This suggests that the reduction of fiber content, as well as the higher nutritional quality (via increase in nitrogen), were the main drivers of feeding behavior.

In addition to reducing resistance to herbivory, nutrient addition also modified tolerance potential by reducing belowground carbohydrate reserves (i.e., sucrose), which would imply a lower capacity for plants to invest in regrowth after damage, potentially further exacerbating negative impacts of grazing that would result from higher palatability. Such carbohydrate reduction may have resulted from the plant's requirements for carbon skeletons needed for nitrogen assimilation (Touchette and Burkholder 2000; Invers et al. 2004), and it is not an uncommon response of plants to nutrient additions (Bloom et al. 1985). Interestingly, plant phosphorus content did not increase despite the increase of P in porewater, which suggests that P may have not been available for the plant, perhaps due to adsorption to sediment particles (Stumm and Morgan 1996) or to the carbonate matrix of the sediment (Short 1987). Hampered P absorption by seagrass roots likely resulted in P limitation, which may have hindered the allocation to tolerance traits under herbivory pressure, as suggested by the LRM (Wise and Abrahamson 2008).

Herbivory simulation induced plant resistance to herbivory through enhanced production of leaf phenolic compounds and fibers, especially at the moderate herbivory treatment, as well as the decrease in leaf nutrient (N and P)

| | Nitrogen | (%) | | Carbon | (%) | | C/N | | | Phosph | orus (%) | | Sucrose | (%) | | FIDTE (%) | _ | | Phenols | (%) | |
|----------------------|----------|---------|---------|--------|-------|-------|---------|-------|---------|---------|----------|---------|---------|---------|---------|-----------|-------|---------|---------|--------|-------|
| Fixed | Est. | SE 1 | | Est. | SE | Р | Est. | SE | Р | Est. | SE | Р | Est. | SE I | | Est. | SE | Ь | Est. S | E P | |
| | 0.104 | 0.032 | 0.103 | 36.252 | 0.246 | 0.001 | 28.674 | 3.152 | 0.042 | - 0.970 | 0.040 | 0.001 | 0.592 | 0.025 < | < 0.001 | 46.538 | 0.498 | < 0.001 | 7.215 0 | .289 < | 0.001 |
| $\beta_{\rm N}$ | 0.075 | 0.023 | 0.002 | | | | - 4.057 | 2.327 | 0.187 | | | | - 0.075 | 0.025 | 0.004 | - 2.893 | 0.488 | < 0.001 | | | |
| $\beta_{ m H}$ | - 0.131 | 0.023 < | < 0.001 | | | | 10.259 | 1.607 | < 0.001 | - 0.134 | - 4.245 | < 0.001 | 0.082 | 0.030 | 0.009 | 4.1556 | 0.598 | < 0.001 | 0.662 (| .304 | 0.035 |
| $\beta_{ m HH}$ | - 0.186 | 0.023 < | < 0.001 | | | | 14.707 | 1.607 | < 0.001 | -0.140 | - 4.434 | < 0.001 | 0.013 | 0.030 | 0.673 | 2.462 | 0.598 | < 0.001 | 1.414 0 | .304 < | 0.001 |
| $\beta_{\rm t}$ | 0.035 | 0.023 | 0.134 | 0.290 | 0.171 | 0.093 | - 1.504 | 1.607 | 0.352 | - 0.052 | - 2.003 | 0.048 | | | | | | | | | |
| $\beta_{ m N*H}$ | - 0.023 | 0.033 | 0.483 | | | | - 0.593 | 2.273 | 0.795 | | | | | | | | | | | | |
| $\beta_{ m N*HH}$ | 0.029 | 0.033 | 0.376 | | | | - 4.359 | 2.273 | 0.059 | | | | | | | | | | | | |
| $\beta_{ m N*t}$ | - 0.068 | 0.033 | 0.041 | | | | 3.248 | 2.273 | 0.157 | | | | | | | | | | | | |
| $\beta_{ m H^{*t}}$ | 0.029 | 0.033 | 0.372 | | | | - 3.464 | 2.273 | 0.131 | | | | | | | | | | | | |
| $eta_{ m HH*_t}$ | 0.006 | 0.033 | 0.854 | | | | - 2.078 | 2.273 | 0.363 | | | | | | | | | | | | |
| $\beta_{\rm N*H*t}$ | 0.051 | 0.046 | 0.268 | | | | -0.165 | 3.215 | 0.959 | | | | | | | | | | | | |
| $\beta_{\rm N*HH*t}$ | 0.043 | 0.046 | 0.352 | | | | - 0.952 | 3.215 | 0.768 | | | | | | | | | | | | |
| Ran- dom | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. S | D | |
| | | | | | | | | | | | | | | | | | | | | | |
| isite | 0.002 | 0.039 | | 0.091 | 0.302 | | 17.291 | 4.158 | | 0.002 | 0.043 | | 0.000 | 0.000 | | 0.020 | 0.140 | | 0.074 (| .273 | |
| $eta_{ m residual}$ | 0.002 | 0.046 | | 0.701 | 0.837 | | 10.334 | 3.215 | | 0.016 | 0.126 | | 0.007 | 0.085 | | 2.859 | 1.691 | | 0.741 0 | .861 | |

Table 3Results of mixed-effects models on leaf chemical traits

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Fig. 3 a, b Hedges' d effect size and 95% confidence intervals of herbivory-driven (squares; a) and nutrient-driven (triangles; **b**) changes in feeding preference of fishes and sea urchins. Numbers above symbols indicate the number of experiments. Negative values of Hedges' d indicate higher consumption of clipped (a) or fertilized (b) leaf tissue. **c** The percentage of leaf area consumed in the two-choice experiments whose results differed between herbivore types (left: experiment performed with plants from site A, right: experiment with plants from site B). HA = non-fertilized leaf tissue of the moderate herbivory treatment, HHA = non-fertilized leaf tissue of the high herbivory treatment. Standard error (bars), number of replicates (n) and p value (P) shown in figures. Asterisk indicates statistically significant differences between treatments in the Wilcoxon signed-ranks paired test since data. See Supplementary material (Table S4 and Fig. S2)



content. However, the induction of resistance traits (such as fibers, nitrogen content) did not change with nutrient fertilization, contrary to the predictions of both RAHs (Endara and Coley 2011; Hahn and Maron 2016). While seaweeds can exhibit contrasting responses to the interaction between nutrient fertilization and herbivory in relation to the induction of chemical defenses (e.g., increasing or decreasing polyphenolic content; Yates and Peckol 1993; Mayakun et al. 2013), the only study to our knowledge to explore this interaction in seagrasses also found no effects of nutrient addition on defense induction (Tomas et al. 2015). On the other hand, in addition to inducing the production of chemical defenses (i.e., phenols) and fiber, which are common responses for many terrestrial and aquatic plants (e.g., Delphia et al. 2007; Toth and Pavia 2007; Hartley et al. 2016), herbivory also enhanced leaf structural defense by reducing SLA ($cm^2 g^{-1}$) which relates to tougher and thicker leaves (Enríquez 2005; Paul et al. 2012). This induced resistance from clipping produced a deterrent effect in both herbivores, although the specific mechanisms driving feeding behavior may have differed in several instances between fish and sea urchins. For example, we detected a difference in preference between fish and sea urchins when considering clipped plants (i.e., moderate vs. high herbivory) under ambient nutrient conditions. Sea urchins preferred the leaves from the high herbivory treatment, which were lower in fiber but higher in phenols, and lower both in sucrose and nitrogen contents. Fish, on the other hand, preferred the plants from the moderate herbivory treatment, which were higher in nitrogen and sucrose, lower in phenols, but higher in fiber content. The difference in feeding behavior suggests that under ambient nutrient conditions, phenolic compounds exert a higher deterrent effect on fish while sea urchins are more sensitive to fiber content (Vergés et al. 2010). When nutrients were added, however, preference in sea urchins shifted towards the moderate herbivory plants. Higher nitrogen content and generally lower fiber of fertilized tissues may have reduced or compensated for the effect of fiber originally deterring sea urchins.

In addition to modifying plant biomass and palatability, simulated herbivory increased epiphyte load likely due to a reduction in plant shelf-shading, and thus higher light exposure of the inner leaves. Consequently, algal epiphytes would have a higher surface with optimal light conditions available for settlement and growth (Borowitzka et al. 2006). The reduction of leaf surface by defoliation may also hinder N absorption through leaves, hampering allocation to tolerance (Hay et al. 2011). Indeed, in our study, simulated herbivory reduced N and P contents in the leaves as well as in the rhizomes. On the other hand, the reduction in leaf area did not seem to affect carbon fixation, since clipped plants accumulated sucrose in belowground tissues, and this phenomenon may be associated with a lower need for C to assimilate nitrogen. These results contradict previous works in which carbohydrate reserves and nutrients were reduced due to seagrass investment in regrowth following defoliation (Thayer et al. 1984; Vergés et al. 2008; Sanmartí et al. 2014). Yet, while plants in this study did not increase their growth rate under high herbivory (i.e. lack of compensatory growth), we did observe a high tolerance potential to herbivory, since plants sustained similar growth rates in all herbivory treatments. Moreover, shoots from the moderate treatment had a higher number of leaves and these exhibited higher sucrose content, which suggests a potential compensatory increase in photosynthetic activity under moderate levels of defoliation (Lunn 2008). In our study, plants appear to invest in trying to deter further loss of leaf area (through increase in resistance traits) and storing available carbon in belowground tissues for future tolerance rather than generating extra photosynthetic tissue, which suggests no trade-off between resistance and tolerance strategies.

Overall, in our system of study, the applicability of the intraspecific RAH seems to be limited. The seagrass P. oceanica responded to external sources of nutrients as predicted by the RAH, i.e., with a reduction on its constitutive resistance. Importantly, increased nutrient availability did not enhance resistance or tolerance in fertilized plants when undergoing simulated herbivory, perhaps due to the existence of limiting resources (potentially P), as predicted by the LRM. On the other hand, plants exhibited a mixed defense strategy under herbivory pressure with induction of tolerance (i.e., increased number of leaves and sucrose content) and resistance traits (i.e., phenolic compounds, fiber). Some traits, however, did not respond linearly to herbivory pressure, exhibiting stronger responses under moderate levels of clipping (e.g., number of leaves). Resource availability and herbivore damage can change plant traits that influence the feeding patterns of different herbivores within the same community. This is highly relevant in systems with one or few dominant primary producers, as they are the main food

source for all the associated herbivores in the community. Furthermore, understanding the general patterns between herbivory and plant defense under different environments will become increasingly relevant in future scenarios, as herbivore damage is already increasing in temperate systems (e.g., Heck et al. 2015; Hyndes et al. 2016; Vergés et al. 2016) with tropical consumers expanding polewards (Vergés et al. 2014).

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Author contribution statement GH, FT and JT conceived the experiment. GH, JT and IC performed the field experiment. IC performed the phosphorus analysis. GH performed plant size, plant chemical traits and statistical analysis. FT and JT funded field and laboratory material and analyses. GH and FT prepared the manuscript. All authors discussed and reviewed the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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