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LaTina Steele
Sacred Heart University

John F. Valentine
University of South Alabama

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Seagrass deterrence to mesograzer herbivory: evidence from mesocosm experiments and feeding preference trials

LaTina Steele[*1,2,3] and John F. Valentine[1,2]

[1]Dauphin Island Sea Lab, 101 Bienville Blvd., Dauphin Island, AL 36528, USA
[2]Department of Marine Sciences, University of South Alabama, Mobile, AL 36688, USA
[3]Current address: Department of Biology, Sacred Heart University, 5151 Park Ave. Fairfield, CT 06825, USA

*__Corresponding author__: Email: steelel@sacredheart.edu; Telephone: 203-396-8485

Running head: Effects of mesograzer feeding on seagrass phenolics

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Abstract

Two laboratory experiments documented the effects of mesograzer feeding (i.e., the gastropod *Crepidula ustulatulina* and the isopod *Paracerceis caudata*) on phenolic acid and condensed tannin production in two regionally abundant seagrasses (*Thalassia testudinum* (turtlegrass) and *Halodule wrightii* (shoalgrass)). Subsequent paired choice experiments tested the hypothesis that phenolic acids and condensed tannins produced by these seagrasses deter mesograzer feeding. At the scale of the shoot, grazing by gastropods and isopods led to ~40-50% decreases in concentrations of some phenolic acids and ~20% decreases in condensed tannins in turtlegrass leaves. At a more refined spatial scale, concentrations of two of these compounds increased by 25-85% in areas near tissues damaged by *C. ustulatulina* and *P. caudata* in turtlegrass. In contrast, isopod feeding increased concentrations of some shoalgrass phenolic acids by about 30-50%, while gastropod grazing led to approximately 25-50% higher concentrations of condensed tannins in shoalgrass leaves, suggesting that grazer identity and seagrass species play important roles in seagrass deterrent production. Amphipods (*Batea catharinensis*) consistently preferred agar food made from seagrass leaves with low phenolic concentrations in choice feeding experiments, indicating that phenolics can act as feeding deterrents to these mesograzers.

Keywords: *Thalassia testudinum, Halodule wrightii, condensed tannins, phenolics, grazer*

Introduction

While the ability of inducibly produced chemical compounds to deter attacks by herbivores is well known for terrestrial food webs (Agrawal 1999), the efficacy of such episodically produced compounds in deterring attacks by marine herbivores remains inadequately understood. Historically, marine studies have focused on documenting the ability of these compounds to alter herbivore feeding preferences and determine the persistence of macroalgae in areas of intense grazing (e.g., Hay 1996, Targett and Arnold 1998). Notably, most studies detected the presence of induced feeding deterrents, suggesting that this defense strategy is widespread among marine macroalgae (but see Steinberg 1994 and Long and Trussell 2007). In some cases, the presence of chemical deterrents in macroalgae is highly localized, concentrated in tissues perceived to be valued by the plant (Steinberg 1984, Macaya et al. 2005, Lima et al. 2008, Pansch et al. 2008, Rhode and Wahl 2008). In other cases, the production of these deterrents is spatially limited to areas near grazing injuries (Järemo et al. 1999, Hemmi et al. 2004), which is hypothesized to lead to the dispersion of damage caused by mesograzers to other parts of the plant, indirectly reducing breakage that would occur if these grazers continued to feed in the damaged area (Hemmi et al. 2004).

A growing body of literature supports the hypothesis that chemical feeding deterrents are similarly inducibly produced by multiple seagrass species from several locations (Aragones et al. 2006, Moran and Bjorndal 2007, Arnold et al. 2008). In most cases, though, seagrass responses resulted from mechanical simulations of vertebrate grazing, with few studies utilizing either live animals or invertebrate herbivores. It is unclear if observed differences in compound concentrations in these studies were due to experimental artifacts attributable to the mechanical damage of the leaves (Baldwin 1990) or due to seagrasses using different defense strategies to deter future grazer damage.

All of the studies testing herbivore-induced production of chemical feeding deterrents in seagrasses to date have used macrograzers. The ability of smaller mesograzers to induce the production of these feeding deterrents remains unknown, despite an accumulating body of evidence showing that mesograzers can heavily damage marine angiosperms (Unabia 1980, Zimmerman et al. 1996, 2001, Boström and Matila 2005, Rueda et al. 2009). As such, it stands to reason, when coupled with our previous study using an urchin macrograzer, that seagrasses might use inducibly produced chemical compounds to deter grazing by these small herbivores (cf., Hay 1996, Steele and Valentine 2012).

While there have been several evaluations of the ability of grazers, or simulated grazing, to induce chemical responses in seagrasses (Aragones et al. 2006, Moran and Bjorndal 2007, Arnold et al. 2008), to date, few studies have assessed of the efficacy of these compounds as feeding deterrents. Extracts from *Zostera marina* leaves thought to contain phenolic acids have been shown to deter amphipod feeding (Harrison 1982). Verges et al. (2007) documented the deterrent properties of *Posidonia oceanica* extracts, which contained both lipophilic metabolites and hydrophilic metabolites (e.g., phenolics), against attacks by herbivorous fish, sea urchins, and a gastropod. In the latter study *P. oceanica* extracts were found to deter feeding by some consumers but not others. These studies show that chemical compounds can act as deterrents to feeding by some seagrass herbivores.

Although it seems that chemical deterrents can reduce seagrass losses to herbivory, it should be noted that elevated nitrogen, which co-varies with some phenolics (Cronin 2001), can trigger grazing by vertebrates. McGlathery (1995) and Goecker et al. (2005) independently found that bucktooth parrotfish preferentially consumed nitrogen-rich turtlegrass leaves. Goecker et al. (2005) also reported low phenolic concentrations in turtlegrass leaves high in nitrogen,

alternatively suggesting that phenolics may have played a contributory role in determining parrotfish feeding preferences. In addition, turtles have been found to preferentially feed on nitrogen-rich turtlegrass leaves (Bjorndal 1985). Notably, no studies have assessed the effects of nitrogen on mesograzer feeding preferences in seagrasses.

In some cases, grazer identity, in addition to grazer density, can also determine plant production of feeding deterrents. Grazer-specific changes in the production of deterrent compounds have been documented in a number of terrestrial plants (Valkama et al. 2005, Van Zandt and Agrawal 2004, Stout et al. 1998, Traw and Dawson 2002). Gastropod (*Littorina obtusata*) grazing can similarly induce the production of chemical feeding deterrents in brown algae (*Ascophyllun nodosum*), while isopod (*Idotea granulosa*) grazing cannot (Pavia and Toth 2000). In the only seagrass example that we know of, Arnold et al. (2008) found that turtlegrass responded differently to simulated parrotfish grazing and live sea urchin grazing. Given the prevalence of grazer-specific responses in other plant species, it seems likely that many seagrasses would also exhibit such responses to herbivory.

This study examined the extent to which mesograzer identity, using two mesograzers (the gastropod *Crepidula ustulatulina* and the isopod *Paracerceis caudata*), can affect seagrass production of phenolic acids and condensed tannins. Although gastropods of the genus *Crepidula* are assumed to be filter feeders (Hoagland 1979), *C. convexa* individuals, only recently distinguished from *C. ustulatulina* (Collin 2002), are known to feed on algae using the radula as juveniles (Hoagland 1979). A well-developed radula is retained by *C. convexa* into adulthood (Hoagland 1979), suggesting that this gastropod is capable of feeding directly on plant tissue even as an adult. Radula scars were apparent on seagrass leaves occupied by *C. ustulatulina* in the field, as well as on leaves harvested during laboratory experiments where no

other grazers were present. Coupled with the fact that these animals were found on the inner leaves of turtlegrass, where there are few epiphytes, this evidence indicates *C. ustulatulina* does consume seagrass tissue.

We also sought to determine the generality of any responses to grazing using two seagrass species (turtlegrass *Thalassia testudinum* and shoalgrass *Halodule wrightii*) common to the northern Gulf of Mexico. Additionally, this study aimed to determine if differences in leaf damage (by *C. ustulatulina* and *P. caudata*) also triggers differences in the production of these compounds (i.e., systemically (across all leaves on a grazed shoot) or locally (higher concentrations near mesograzer damage)). Leaves of seagrasses at our study site were previously documented to contain low levels of phenolic acids and condensed tannins (Steele et al. unpublished data), so an induced response should have been detectable, if in fact, seagrasses produce these compounds to deter attacks by mesograzers. Lastly, we tested the hypothesis that co-varying concentrations of phenolic acids and leaf nitrogen in the leaves of these two seagrasses can, cumulatively or interactively, determine feeding preferences of two locally abundant crustacean mesograzers (*P. caudata* and the amphipod *Batea catharinensis*).

Materials & Methods

Study Species Collection

Because it was nearly impossible to create a true mesograzer-free control treatment in the field (c.f. Carpenter 1986), two separate laboratory experiments were conducted to answer the questions posed above. Mesograzers and plants were collected from the Gulf Islands National Seashore in Perdido Key, FL. Isopods and amphipods were collected by anchoring pre-rinsed, cut-to-fit, air conditioner filters within mixed turtlegrass and shoalgrass meadows, using rebar, (Rabalais et al. 1995) for three to five days. Upon retrieval, filters were immediately rinsed over

large plastic containers filled with ambient seawater. After rinsing, the retained organisms and seawater were poured over a 500 µm sieve, and a pipette was used to transfer isopods and amphipods captured on the sieve to aerated buckets filled with seawater.

Gastropods, *Crepidula ustulatulina*, were collected from the surfaces of turtlegrass leaves by hand. Gastropods were placed in an aerated bucket containing seawater as well, then transported to the laboratory, where animals were acclimated to laboratory conditions and starved for 24 hours prior to the start of each experiment.

Approximately 250 turtlegrass shoots with intact roots/rhizomes were collected from the same site as the mesograzers and transported to the lab in aerated buckets, and animals were removed from each shoot by hand. Shoots were then rinsed with fresh water for five minutes to remove remaining undetected animals, then returned to aerated containers holding clean seawater (free of the detritus from previously held shoots). First and second rank leaves (youngest and next youngest leaves) were chosen for chemical analysis because they are considered to be of greater photosynthetic value to the plant than older outer leaves (Durako and Kunzelman 2002), and we predicted that, if seagrasses produce chemical deterrents in response to grazing, the responses were more likely to be detected in these leaves. For this reason, older leaves were removed prior to placement of each shoot in laboratory mesocosms. Approximately 800 *Halodule wrightii* shoots, also with intact roots/rhizomes, were collected for the second experiment. Animals and leaves older than first and second rank were removed from shoots, as described above, prior to placement of each shoot in laboratory mesocosms. All plants were acclimated to laboratory conditions for 18-24 hours prior to use in experiments.

Laboratory Induction Experiments

Two separate laboratory induction experiments were designed for each of the seagrass species to test three competing hypotheses: 1) grazing induces production of phenolic acids and condensed tannins on both leaves of a seagrass shoot, 2) grazers induce production of phenolic acids and condensed tannins only in damaged leaves and not in ungrazed leaves on grazed shoots, and 3) grazing induces production of phenolic acids and condensed tannins only in areas in close proximity to the wounded area of a grazed leaf.

Ten of the harvested turtlegrass shoots were haphazardly selected for placement into each of 18 cylindrical 38 L tanks containing filtered seawater obtained from the U.S. Environmental Protection Agency Laboratory in Gulf Breeze, Florida (USA). To ensure that the selected plants were healthy, and to evaluate the possibility that production of chemical deterrents is costly for seagrasses, the leaves of an additional two shoots/tank (n = 36 shoots) were marked by puncturing the base of the leaves with a hypodermic needle and placed in each tank to document treatment effects on growth during each experiment (Valentine and Heck, 2001).

Three grazer treatments, consisting of a control (no grazers), gastropods, or isopods, each stocked at field densities, were randomly assigned to each tank. Each treatment was replicated six times (n=18 tanks). No grazers were added to the six control tanks, which were examined periodically to ensure that controls were grazer free. In the gastropod treatment, replicate tanks were stocked with 12 individuals (one/shoot), similar to densities observed in the field. Each isopod replicate was stocked with four individuals, similar to densities reported by Valentine and Heck (1993). To avoid pseudo-replication (*sensu* Hurlbert 1984), tanks were aerated individually rather than using a recirculating seawater system. Salinity and temperature were held constant at 30 psu and 28-30 °C, respectively, to mimic field conditions at the time of collection, and lights operated on a 12 hour light: 12 hour dark cycle. Grazers were allowed to feed for 15 days.

Seagrass shoots were held in bare-root culture (no sediment in the tanks) for the experiment's duration. This method has been successfully used in short-term turtlegrass culture experiments to assess phenolic induction (Steele et al. 2005, Arnold et al. 2008). Two 2.0 ml microcentrifuge tubes filled with sterilized playground sand were attached to the base of each shoot with plastic cable ties to weight the shoots. Again each tank was considered to be a replicate.

Turtlegrass leaves were harvested after 15 days. Leaves within each replicate tank were pooled according to rank to ensure sufficient tissue was available for chemical analysis. To test the hypothesis that production of deterrents is limited to grazed areas of leaves, rather than being induced throughout all of the leaves on a shoot, grazed turtlegrass leaves taken from replicate grazing treatments were further subdivided and assigned to one of four categories according to distance from the wound (cf. Ralph and Short 2002, Steele et al. 2005): the damaged (grazed) area of leaf tissue, leaf tissue 2 cm above the damaged tissue, 2 cm below grazer damage, and remaining tissue (>2 cm above and below grazing scars). Tissues > 2 cm above grazer damage and > 2 cm below grazer damage were pooled to ensure sufficient tissue for analysis. These tissue categories were chosen based on previous studies which showed that seagrass physiological responses can vary with proximity to damage (Ralph and Short 2002, Steele et al. 2005). Ungrazed leaves from control treatments and ungrazed leaves from shoots in which at least one leaf was grazed were harvested separately and formed two additional turtlegrass tissue categories.

Ungrazed leaves from shoots on which at least one leaf was grazed were separated into another category to test the hypothesis that production of chemical deterrents by seagrasses is systemic. If true, grazing on older leaves should lead to an increase in production of phenolic

acids and condensed tannins in ungrazed younger leaves on the same shoot, which would not be expected if chemical responses to grazing are highly localized. Tissues from damaged leaves were pooled to address the hypothesis that grazing induces production of phenolic acids and condensed tannins on all leaves of a grazed seagrass shoot.

Because *H. wrightii* shoots are much smaller and thinner than *T. testudinum* shoots, ten haphazardly selected groups of five shoots were placed in each replicate tank and anchored using sand-filled microcentrifuge tubes in the second experiment as previously described. Otherwise, this experiment was identical to the *T. testudinum* experiment. Two additional groups of five shoots were marked for growth measurements and placed in each tank as well. Because damage was observed along much of the length of all shoalgrass leaves from tanks with grazers, shoalgrass leaves could not subdivided according to distance from a wound.

In both experiments, leaf samples were flash frozen in liquid nitrogen immediately after harvesting and stored in a -80 °C freezer to prevent oxidation of phenolics in the leaves. In preparation for chemical analysis, samples were freeze-dried then ground in liquid nitrogen. Ground samples were returned to the -80 °C freezer until chemical analysis.

Chemical Analyses

High performance liquid chromatography (HPLC) was used to quantify the concentrations of each of the phenolic acids identified in turtlegrass and shoalgrass leaf tissues following Ravn et al. (1994). HPLC was performed using a C-18 column and an isocratic solvent system (1:1:7 methanol: 2-propanol: 2% acetic acid) with a flow rate of 0.8 ml/min and a wavelength of 254 nm. Tissue samples were co-injected with nine of the most common of the phenolic acids reported in seagrasses (Zapata and McMillan 1979), one phenolic acid at a time, to identify peaks. Six of the nine targeted phenolic acids were detected in turtlegrass leaves:

gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid, and p-coumaric acid. Concentrations of these six phenolic acids were summed to calculate combined phenolic acids in turtlegrass. Seven phenolic acids were identified in shoalgrass: gallic acid, 3,4-dihydroxybenzoic acid, syringic acid, gentisic acid, p-hydroxybenzoic acid, ferulic acid, and p-coumaric acid. Since gentisic acid was present in only a few samples, and these few samples encompassed all three treatments, it was excluded from statistical analyses and calculations of combined phenolic acids. Thus, six phenolic acids, excluding gentisic acid, were summed to calculate combined phenolic acids in shoalgrass. A number of peaks present in the HPLC chromatographs remained unidentified; therefore, it is likely that additional phenolic acids were present in both turtlegrass and shoalgrass.

The colorimetric assay described by Arnold and Schultz (2002) was used to quantify condensed tannin concentrations in the samples. Because a commercially available quebracho tannin standard was used for this analysis rather than a standard made from seagrass tannins, values generated should not be considered absolute concentrations. However, these values did provide the basis to make comparisons of treatment effects on the production of feeding deterrents in the leaves of *Thalassia testudinum* and *Halodule wrightii*.

Feeding Preference Experiments

Because assessments of plants' ability to inducibly produce feeding deterrents cannot be done via simple comparisons of compound concentrations in damaged and undamaged tissues (Hay 1996), we also assessed the impacts of phenolic acid concentrations on mesograzer feeding preferences. Eight paired choice feeding experiments were conducted using two common mesograzers (four experiments with each grazer, see Table 1): the isopod *Paracerceis caudata* and the amphipod *Batea catharinensis*. The gastropod *Crepidula convexa* was not used in the

agar-based feeding experiments due to the nature of its grazing in the laboratory induction experiments. In this treatment, *Crepidula* removed only thin layers of epidermal tissue, suggesting that it would not consume detectable quantities of the agar food during the feeding trials.

Because consistent treatment effects on the production of feeding deterrents were not detected (see Results), we collected seagrass leaves from areas with naturally varying nitrogen and phenolic content to test their relative effects on mesograzer feeding preferences. Variation in leaf phenolic and nitrogen concentrations in turtlegrass and shoalgrass leaves was documented at these two sites in the Florida panhandle (St. Joseph's Bay and Perdido Bay) during a separate survey (Steele et al. unpublished data), allowing us to use seagrass leaves from these two sites as high and low phenolic and nitrogen treatments in our feeding experiments (Table 1). The paired choices presented in Table 1 were offered to mesograzers in eight separate laboratory feeding experiments (four experiments per mesograzer). Leaf pairs with comparable nitrogen concentrations (Experiment 2, see Table 1) were included to control for preferences based on nitrogen, rather than phenolics. Leaf pairs with similar phenolic and nitrogen concentrations (Experiment 3) were used to consider the possibility that other factors determined mesograzer feeding preferences, which would be indicated if a preference for one treatment or the other were detected in Experiment 3 (see Table 1). Each of these four experiments was conducted first using isopods then repeated with amphipods, for a total of eight choice feeding experiments.

In order to quantify leaf consumption, while controlling for possible structural differences within leaves between sites and species on mesograzer feeding preferences, seagrass tissue was ground into a powder as previously described, then embedded in an agar matrix following Hay (1984) and Goecker et al. (2005). A mixture of 20 ml distilled water and 0.3 g agar was heated

then allowed to cool for approximately five minutes before adding 0.4 g (shoalgrass) or 0.6 g (turtlegrass) of ground seagrass tissue (amount of tissue in a seagrass leaf of equivalent length and width) to the mixture. This mixture was poured over pre-cut window screen (20 strips cut to 5 mm x 17 cm), with the bottom 2 cm of screen covered with tape to allow for the attachment of two vinyl-covered paperclips to anchor the "leaves" in experimental tanks. An acrylic sheet covered the strips until the agar solidified to ensure a uniform "leaf" thickness.

Mesograzer consumption was quantified by counting the number of empty squares present on the window screen at the end of each trial (after 24 hours). Trials in which no evidence of grazing was detected were omitted from subsequent statistical analyses, as they provided no information on mesograzer feeding preferences. Autogenic controls (leaf pairs without mesograzers) were included in each experiment to account for agar loss due to handling. It should be noted that agar loss was not observed in any of the autogenic control trials. Since the isopods failed to consume quantifiable amounts of agar food after 24 hours, the experimental duration was lengthened to 5 days for this comparison.

Chemical extraction and analysis were performed on agar leaves after soaking in seawater for 24 hours using methods described above to ensure that the process of making the agar food did not degrade phenolic acids in seagrass tissue embedded in agar and that phenolics were not leached completely from the agar during the experiments. Phenolic acid concentrations in agar leaves were approximately 50-60% of concentrations observed in a previous survey (Steele et al. unpublished data), so the likelihood of our making a type one error seems small.

Paracerceis caudata (used in the initial choice experiments) and *Batea catharinensis* (used in the subsequent choice experiments) were collected and processed as described above. Identical methods were used for choice tests using *P. caudata* and *B. catharinensis*. Individual

grazers were not re-used in these experiments. Prior to an experiment's start, 3 individuals of approximately the same size were placed in 15 replicate 1 L glass jars, each equipped with an air stone (n=3 mesograzers/jar, n=15 replicate jars/experiment) and held for 24 hours without food. After 24 hours, one of the agar leaf pairs described above (e.g., one leaf made with turtlegrass tissue from St. Joseph's Bay and one leaf made with turtlegrass tissue from Perdido Bay) was placed in each of the 15 treatment jars. Additional leaf pairs were placed in three other jars without mesograzers to serve as autogenic controls. High- and low-phenolic agar treatments were differentiated from each other using different colored paperclips, which also anchored the base of the strips to the bottom of experimental jars. After 24 hours, agar leaves were removed from the jars, and the number of empty squares was recorded (evidence of feeding).

Statistical Analysis

To test the hypothesis that grazing by gastropods and isopods triggers increased production of phenolic compounds in turtlegrass and shoalgrass leaves, leaf chemical concentrations were compared among treatments using a 2-way Analysis of Variance (ANOVA) with leaf rank and grazing treatment (control, gastropod, and isopod) as factors. Leaf rank was included to determine if the plants limited production of these potential feeding deterrents to younger, more valuable leaves. When significant treatment effects were detected ($p < 0.05$), posthoc Tukey multiple comparison tests were used to identify where significant treatment impacts existed. To ensure that the data satisfied the assumptions of ANOVA, a normality test was conducted prior to all statistical tests. Levene's homogeneity of variance tests were also conducted to test for equal variance among treatments in all tests (Zar 1999). Data were arcsine transformed prior to statistical analysis (Sokal and Rohlf 1995). In all cases, the results of statistical comparisons were considered significant when $p < 0.05$. Separate statistical

comparisons were conducted for each of the phenolic acids detected in turtlegrass (n=6) and shoalgrass leaves (n=6), for combined phenolic acids, and for condensed tannins. Although multivariate analysis of variance (MANOVA) would be the most appropriate analysis if our phenolic acid concentrations co-varied (Quinn and Keough 2002), initial correlation matrices detected little covariation among phenolic acids in our dataset; thus, univariate ANOVAs on each compound were used.

To test the competing hypothesis that grazing triggered localized production of potential feeding deterrents in turtlegrass, separate one-way ANOVA's were also conducted on each of the phenolic acids and the condensed tannin concentrations with tissue location (ungrazed leaves, grazed area, 2 cm above grazer damage, 2 cm below grazer damage, and remaining tissue from grazed leaves) serving as a treatment in the turtlegrass experiment. Separate ANOVA's were conducted for each grazer, compound, and leaf rank in the turtlegrass experiment. One-way ANOVA's were used rather than a single 3-way ANOVA due to insufficient degrees of freedom when leaf rank and grazer were included as factors in the test.

Paired t-tests were used to compare agar consumption in the feeding experiments. Because isopods did not visibly consume agar food in any experiment, statistical analyses were only performed on data collected during the amphipod feeding experiments. Since there were no changes in autogenic controls, these data were not included in subsequent analyses. Agar leaf consumption was log transformed ($y + 1$) to normalize the data. Results were considered significant at $p < 0.05$. Four separate t-tests were performed, one for each of the amphipod feeding experiments.

Results

Turtlegrass Experiment

Unexpectedly, the 2-way ANOVA's showed that grazer identity had significant negative impacts on the production of condensed tannins and several, but not all, of the identified phenolic acids in turtlegrass leaves (Figure 1). When significant differences were detected among grazer treatments, pairwise comparisons showed that concentrations of these potential feeding deterrents tended to be lower in leaves taken from tanks containing gastropods or isopods than in ungrazed control treatments (Figure 1), contrary to our hypothesis. These responses were comparable in first and second rank leaves, although fewer compounds in the older leaves differed among grazer treatments. Turtlegrass' response to isopod feeding was stronger than it was to gastropod feeding (Figure 1). Leaf rank affected concentrations of only two phenolic acids, p-hydroxybenzoic acid ($F_{1, 93} = 8.03$; $p = 0.006$) and vanillic acid ($F_{1, 81} = 54.77$; $p < 0.001$). In both cases, concentrations were higher in first rank leaves than in second rank leaves. No interaction between grazer and leaf rank was detected in any of the 2-way ANOVA's.

Because we found no evidence that mesograzer feeding induced production of phenolic compounds in turtlegrass at the shoot level, we considered the possibility that grazing triggered production of phenolic compounds only in damaged leaves and that, more specifically, induction occurred only in areas in close proximity to the grazing wound but was masked by declines in phenolic concentrations in areas away from the wound. This was true only for vanillic acid in first rank turtlegrass leaves in the gastropod treatment, which increased in tissue directly above gastropod damage than in any other tissue location on first rank leaves ($F_{5, 28} = 6.13$, $p = 0.001$; Figure 1), consistent with our hypothesis. In contrast, concentrations of ferulic acid and condensed tannin concentrations of damaged first rank turtlegrass leaves decreased significantly in areas near grazing wounds ($F_{5, 27} = 4.51$; $p = 0.006$ for ferulic acid; $F_{5, 26} = 4.04$; $F_{5, 24} = 8.45$, p

< 0.001 for condensed tannins; Figure 1), contrary to our predictions but consistent with results at the shoot level. No significant differences in the concentration of any phenolic acid, total phenolic acids, or condensed tannins were detected in the one-way ANOVA's conducted on damaged second rank turtlegrass leaves.

Grazing by isopods locally affected the concentrations of only one compound in second rank turtlegrass leaves (Figure 1). In contrast to the gastropod treatments, concentrations of individual phenolic acids and total phenolic acids did not vary with distance from damage in first rank turtlegrass leaves grazed by the isopod *P. caudata* (Figure 1). Localized impacts of isopod grazing led higher concentrations of p-coumaric acid in the grazed area than in all other tissues except tissue directly above grazer damage ($F_{4, 19} = 7.71$, $p = 0.001$; Figure 1).

Turtlegrass growth did not vary significantly among treatments ($F_{2, 17} = 0.09$, $p = 0.910$).

Shoalgrass Experiment

Unlike the turtlegrass experiment, results of the shoalgrass experiment were largely consistent with the hypothesis that mesograzer feeding induces production of some phenolic compounds in shoalgrass. Two-way ANOVA's showed that grazer treatment had significant effects on shoalgrass production of condensed tannins, p-hydroxybenzoic acid, and combined phenolic acids, but not the other five compounds measured (Figure 2). Leaf rank did not have a significant effect on the concentrations of any of the individual compounds measured. A significant interaction between grazer and leaf rank was detected, however, in the ANOVA conducted on combined phenolic acid concentrations ($F_{1, 34} = 3.32$, $p = 0.05$), but no significant interactions were detected in any of the other 2-way ANOVA's. Grazing by gastropods and isopods affected the production of different compounds. Both first and second rank shoalgrass leaves from the treatments stocked with gastropods contained more condensed tannins than did

leaves taken from the control treatments ($F_{2, 28} = 13.26$, $p < 0.001$, Figure 2). Concentrations of p-hydroxybenzoic acid ($F_{2, 16} = 5.88$, $p = 0.014$) and combined phenolic acids ($F_{2, 16} = 5.92$, $p = 0.014$) were higher in first rank leaves from isopod treatments than in first rank leaves from control treatments (Figure 2).

Shoalgrass growth did not vary significantly among treatments ($F_{2, 17} = 1.05$, $p = 0.375$).

Feeding Preference Experiments

In all choice feeding experiments, amphipods consumed significantly more of the low-phenolic seagrass treatment. When given a choice between agar containing *Thalassia testudinum* tissue collected from Perdido Bay (low phenolics, low N) and agar containing *T. testudinum* collected from St. Joseph's Bay (high phenolics, high N), amphipods consumed significantly more of the agar made with turtlegrass from Perdido Bay ($t = 2.76$, $p = 0.017$, Figure 3A). Similarly, when given a choice between agar containing *Halodule wrightii* tissue collected from Perdido Bay (low phenolics, no difference in N) and agar containing *H. wrightii* collected from St. Joseph's Bay (high phenolics, no difference in N), amphipods again consumed significantly more of the agar made from shoalgrass collected from Perdido Bay ($t = 1.95$, $p = 0.038$, Figure 3B). Importantly, amphipods consumed similar amounts of each treatment ($t = -0.30$, $p = 0.385$, Figure 3C) when offered agar made from turtlegrass collected from Perdido Bay and agar food made from shoalgrass collected from Perdido Bay (no difference in phenolics or N). When offered agar food made from turtlegrass collected from St. Joseph's Bay (high phenolics, high N) and agar food made from shoalgrass collected from St. Joseph's Bay (low phenolics, low N), amphipods consumed significantly more of the agar made from shoalgrass ($t = -3.15$, $p = 0.010$, Figure 3D).

Discussion

We predicted that mesograzer feeding would lead to elevated phenolic concentrations in the leaves of both *Thalassia* and *Halodule* and that phenolics may act as feeding deterrents against these small grazers. This was true to some extent in experiments using *Halodule* (Figure 2), but not *Thalassia* (Figure 1), and seagrass responses varied with grazer identity. In *Halodule*, feeding by gastropods led to increased levels of condensed tannins in grazed first and second rank leaves, while feeding by isopods led to increases in p-hydroxybenzoic acid and combined phenolic acids only in the youngest leaves (Figure 2). Concentrations of phenolic compounds were generally lower in turtlegrass leaves taken from the grazer treatments than in leaves taken from grazer-free controls (Figure 1). This suggests that the plant perceives production of phenolic compounds to be costly in some undetected way, as we found no evidence of an impact on shoot-specific growth. These results are consistent with those of Darnell and Heck (2013), who found that turtlegrass leaves grazed by parrotfish contained lower total phenolic concentrations than ungrazed leaves.

Grazer identity seems to play an important role in determining when a plant will produce potential chemical deterrents, with different grazers eliciting unique plant responses. Our results support the idea that mesograzer identity may be important in determining the effects of grazing on production of chemical deterrents by some seagrasses, since grazing by both *Paracerceis caudata* and *Crepidula ustulatulina* resulted in unique changes (either increases or decreases) in *Thalassia testudinum* and *Halodule wrightii* leaf phenolic concentrations, with different responses exhibited by the two seagrass species (Figures 1 & 2). The different seagrass responses to different grazers observed here are consistent with results from macroalgae. Pavia and Toth (2000) showed that gastropod grazing induced production of chemical feeding deterrents in brown algae, but isopod grazing did not. Other studies have also shown that variance in grazer

composition elicits different chemical responses in turtlegrass (Moran and Bjorndal 2007, Arnold et al. 2008).

Changes in plant chemical composition alone are not sufficient to infer that those compounds act as an inducible defense; impacts of differences in deterrent compound production on herbivore feeding preferences must also be assessed (Hay 1996). In addition, compounds that deter feeding by one herbivore may not deter feeding by another (Hay 1996), and plants can respond differently to feeding by different grazer species (Pavia and Toth 2000). Because of this, we tested the palatability of seagrasses with differing phenolic concentrations to two mesograzers (the isopod *Paracerceis caudata* and the amphipod *Batea catharinensis*). Although *P. caudata* feeding resulted in changes in phenolic concentrations in the leaves of both *Thalassia* and *Halodule* in the laboratory induction experiments, this isopod failed to consume quantifiable amounts of seagrass leaves embedded in the agar used in the feeding preference experiments. We cannot, therefore, make a statement as to how phenolics affect seagrass palatability for this isopod. When amphipods were presented with a choice between seagrasses containing either high or low phenolic concentrations, they always fed more heavily on the phenolic-poor food, even when it contained lower levels of nitrogen than the phenolic-rich food (Figure 3). Because phenolics deterred feeding by amphipods and grazing led to elevated concentrations of phenolics in *Halodule wrightii* leaves in the induction experiments, phenolics may act as a chemical defense against mesograzers in this seagrass species.

It should be noted that the use of agar rather than whole seagrass leaves may have introduced unidentified artifacts into the feeding preference experiments (cf. Peterson and Black 1994). Since agar was used in all treatments in the feeding experiments, artifacts should have been the same across all treatments and experiments. The amphipod *Batea catharinensis* fed well

on the agar offered in the feeding preference experiments, but the isopod *Paracerceis caudata* failed to feed on the agar, despite having fed on live seagrass tissue in the induction experiments. This indicates that agar was not suitable for assessing this isopod's feeding preferences.

Nitrogen content in seagrass leaves is thought to be a key determinant of which leaves will be grazed. McGlathery (1995) and Goecker et al. (2005) both found that, when presented with a choice, bucktooth parrotfish preferentially consumed turtlegrass leaves rich in nitrogen. Turtles also preferentially feed on nitrogen-rich turtlegrass leaves (Bjorndal, 1985). However, Goecker et al. (2005) also reported that turtlegrass leaves that were high in nitrogen were low in phenolics, suggesting that parrotfish might have preferred leaves with low levels of phenols, as well as high nitrogen. Sea urchins, on the other hand, are known to exhibit compensatory feeding when offered seagrass with low nitrogen content rather than preferentially consuming nitrogen-rich tissue (Valentine et al. 2001). The relationship between nitrogen in seagrass leaves and herbivore feeding rates has not been fully investigated, but the results of this study suggest that phenolics may be more important, and leaf nitrogen content less important, in determining seagrass grazing risk than previously thought.

Though it is clear from this study that differences in phenolic concentrations can influence herbivore feeding preferences, given the somewhat idiosyncratic changes in the concentrations of these compounds in turtlegrass when exposed to grazers, regardless of size, it is possible that these compounds have alternative roles in seagrasses. Studies have found that phenolic compounds (and crude extracts containing phenolics) from multiple seagrasses can reduce the incidence of infection by marine bacteria and fungi (Harrison 1982, Jensen et al. 1998, Ross et al. 2008). Caffeic acid, which is found in eelgrass, can inhibit the growth of the wasting disease pathogen *Labyrinthula* spp. in culture (Vergeer and Develi 1997). Phenolics are

also known to act as antioxidants, protecting against damage from ultraviolet radiation in terrestrial plants (Close and McArthur 2002). They may have a similar function in algae, since Pavia et al. (1997) found that increasing UV-B radiation increased phlorotannins levels in *Ascophyllum nodosum*. Considering that seagrasses often occur in shallow waters with high light exposure, we cannot discount the confounding role that ultraviolet radiation may have played in our study. Since our palatability experiments suggest that phenolics can act as feeding deterrents in seagrasses, rather than serving only one purpose, these compounds may act as a generalized defense against a host of attackers.

Although production of inducible chemical defenses is believed to be metabolically costly (Cipollini et al. 2003), we found no evidence of a cost, in terms of growth, to producing phenolics in either *Thalassia testudinum* or *Halodule wrightii*. Costs associated with producing phenolics may, however, have been evident in another way, such as shifting carbohydrate resources out of the rhizomes, where carbohydrates are stored in seagrasses. This should be addressed in future studies to determine if production of phenolics is costly to seagrasses. Similarly, since no differences in growth were detected among grazing treatments in shoalgrass (in which grazing led to increased phenolic concentrations), it seems that increasing phenolic concentrations does not improve seagrass fitness. Thus, it cannot be inferred from this study that phenolics act as an inducible defense in this seagrass, although these compounds did deter feeding by amphipods.

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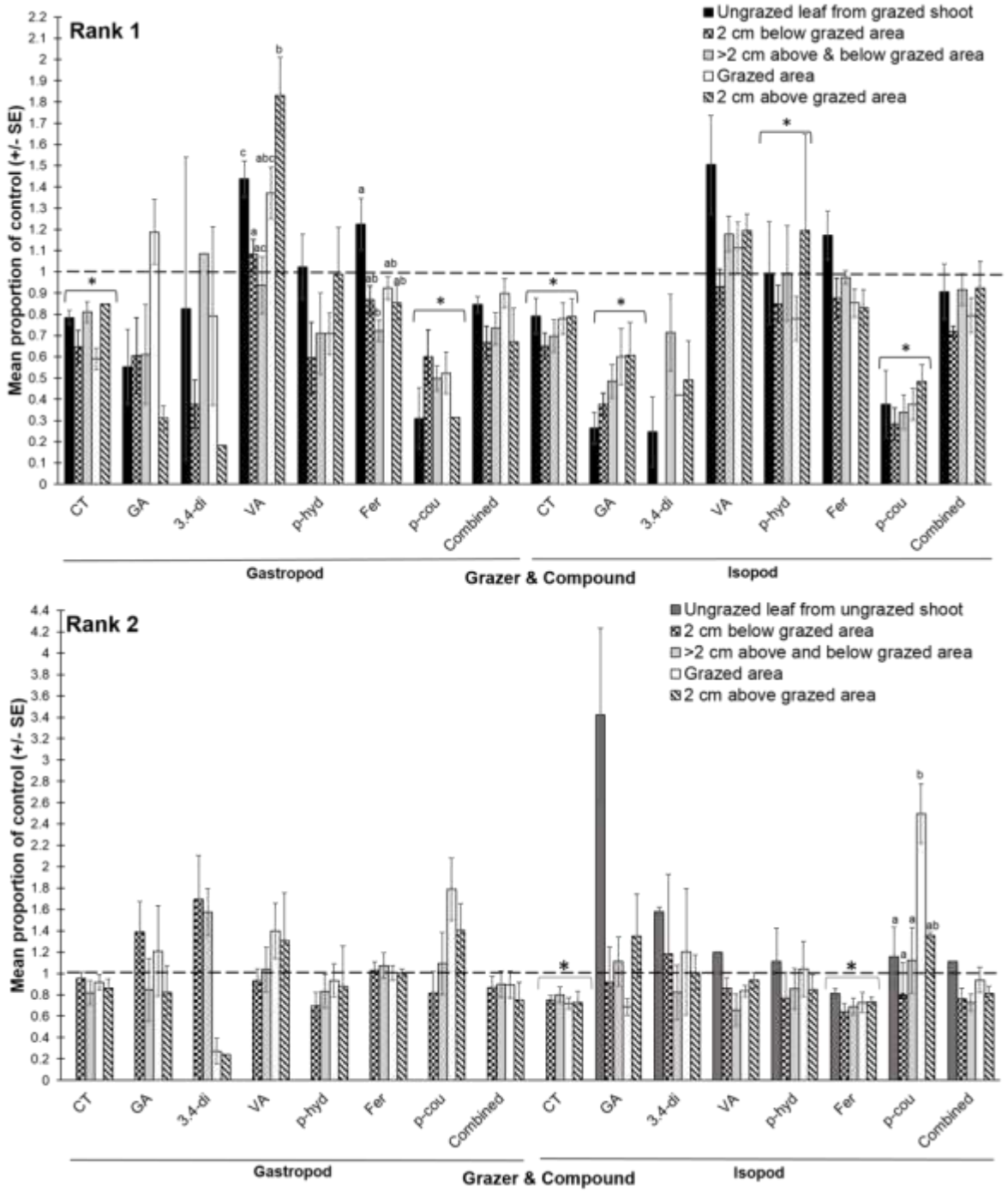


Figure 1. Condensed tannin (CT), gallic acid (GA), 3,4-dihydroxybenzoic acid (3,4-di), vanillic acid (VA), p-hydroxybenzoic acid (p-hyd), ferulic acid (Fer), p-coumaric acid (p-cou), and combined phenolic acid (Combined) content in first and second rank *Thalassia testudinum* leaves taken from either gastropod or isopod grazing treatments. Phenolic content is expressed relative to the concentration of each compound in control leaves (proportion of the amount in control leaves $\pm$ 1 standard error). Dashed lines denote the control value of 1. Values above the dashed line indicate higher concentrations of the compound in those tissues than in the control, and vice versa. Asterisks indicate a significant difference in levels of each compound between the grazer treatment (gastropod or isopod) and the control. Lowercase letters over the bars indicate significant differences among tissues ($p \leq 0.05$). Where ANOVA found no effect of tissue on compound concentration, no letters are included over the bars.

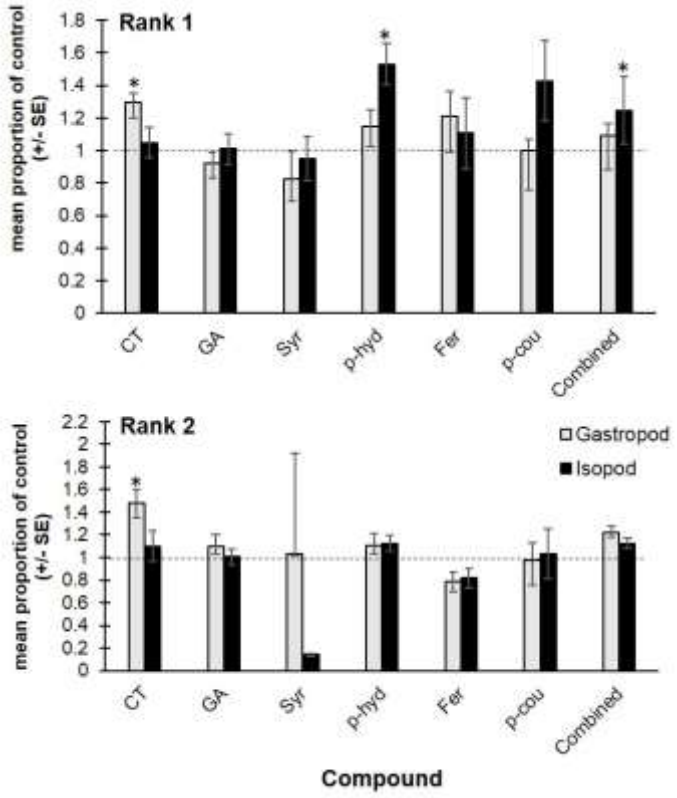


Figure 2. Condensed tannin (CT), gallic acid (GA), syringic acid (Syr), p-hydroxybenzoic acid (p-hyd), ferulic acid (Fer), p-coumaric acid (p-cou), and combined phenolic acid (Combined) content in first and second rank *Halodule wrightii* leaves taken from either gastropod or isopod grazing treatments. Phenolic content is expressed relative to the concentration of each compound in control leaves (proportion of the amount in control leaves $\pm$ 1 standard error). Dashed lines denote the control value of 1. Values above the dashed line indicate higher concentrations of the compound in those tissues than in the control, and vice versa. Asterisks indicate a significant difference in levels of each compound between the grazer treatment (gastropod or isopod) and the control.

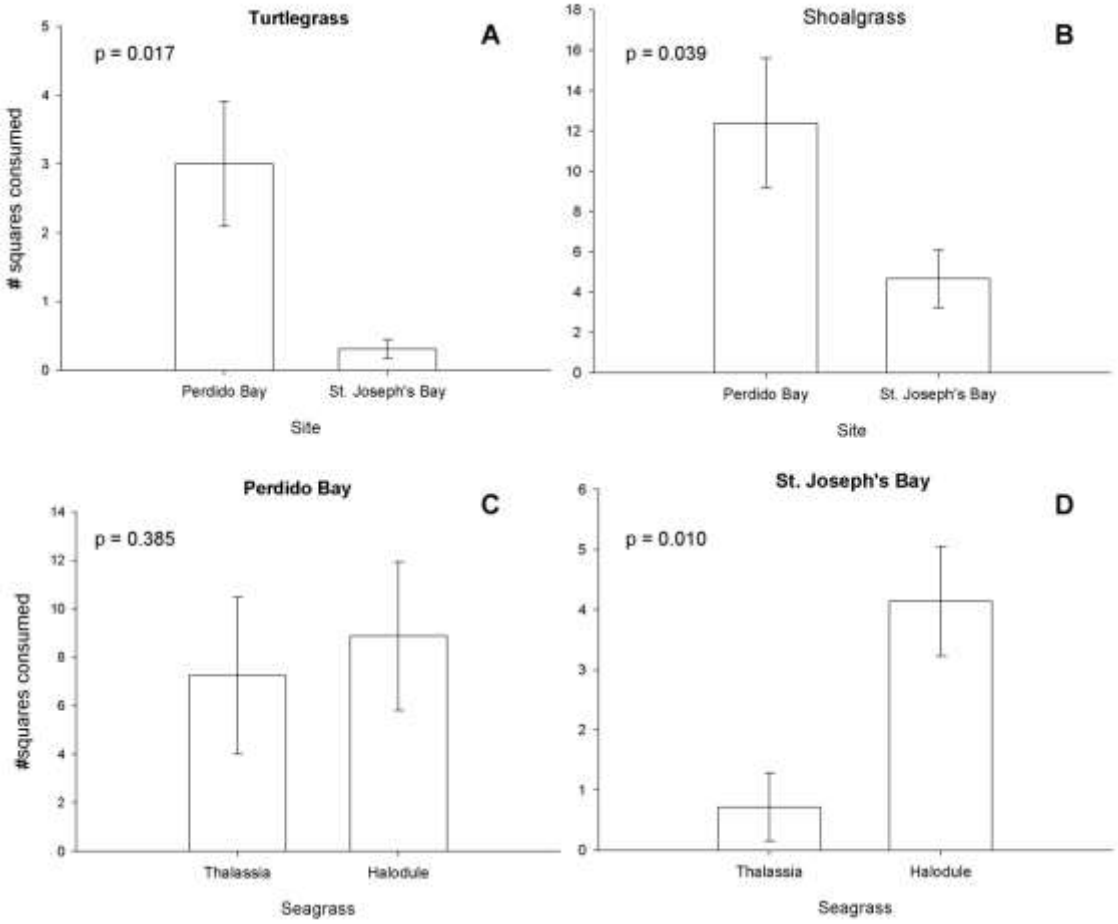


Figure 3. Amount of agar food made with seagrass tissue consumed by the amphipod *Batea catharinensis* when offered the following agar leaf pairs: A) *Thalassia* from Perdido Bay (low phenolics, low N) and St. Joseph's Bay (high phenolics, high N), B) *Halodule* from Perdido Bay (low phenolics) and St. Joseph's Bay (high phenolics; no difference in N), C) *Thalassia* and *Halodule* from Perdido Bay (no difference in phenolics or N), and D) *Thalassia* (high phenolics, high N) and *Halodule* (low phenolics, low N) from St. Joseph's Bay.

Table 1. Table of paired choices used in mesograzer feeding preference tests.

	Choice 1	Choice 2
Experiment 1	*Thalassia* from Perdido Bay (low phenolics, low N)	*Thalassia* from St. Joseph's Bay (high phenolics, high N)
Experiment 2	*Halodule* from Perdido Bay (low phenolics, similar N)	*Halodule* from St. Joseph's Bay (high phenolics, similar N)
Experiment 3	*Thalassia* from Perdido Bay (similar phenolics and N)	*Halodule* from Perdido Bay (similar phenolics and N)
Experiment 4	*Thalassia* from St. Joseph's Bay (high phenolics, high N)	*Halodule* from St. Joseph's Bay (low phenolics, low N)