Intraspecific variation in seagrass anti-herbivore defenses along a natural gradient in phosphorus availability impacts herbivore feeding decisions

Jamila S. Roth[1,2,*], P. Christopher Wilson[3], LaTina Steele[4], Laura K. Reynolds[3]

[1]School of Natural Resources and Environment, University of Florida, Gainesville, FL 32611, USA
[2]Mote Marine Laboratory, Sarasota, FL 34236, USA
[3]Department of Soil, Water, and Ecosystem Sciences, University of Florida, Gainesville, FL 32611, USA
[4]Department of Biology, Sacred Heart University, Fairfield, CT 06825, USA

ABSTRACT: Plant nutritional properties, physical defenses, and chemical defenses impact herbivore feeding decisions. While herbivores often prefer plants enriched with nitrogen, less is known about how phosphorus impacts plant—herbivore interactions. In this study, we investigated how seagrass—herbivore interactions vary along a natural gradient in phosphorus availability, using turtle grass *Thalassia testudinum* collected from 3 sites in Florida (USA) along the gradient (Weeki Wachee: low phosphorus and nitrogen; Homosassa: high phosphorus and nitrogen; Crystal River: high phosphorus and low nitrogen) and 2 common herbivores, variegated sea urchin *Lytechinus variegatus* and emerald parrotfish *Nicholsina usta*. *T. testudinum* plants from the phosphorus-poor site (Weeki Wachee) exhibited increased physical and chemical anti-herbivore defenses. Plants in Weeki Wachee had higher leaf fiber content, lower specific leaf area, and up to 2 times higher concentrations of phenolic acids and condensed tannins. Both *L. variegatus* and *N. usta* avoided consuming the plants from Weeki Wachee, preferring plants from sites with higher nutrient availability. However, feeding preferences varied between the 2 herbivore species, with *L. variegatus* often consuming similar amounts of the plants from the 2 phosphorus-rich sites (Homosassa and Crystal River) and *N. usta* preferring the plants from Homosassa, which had the highest concentrations of both phosphorus and nitrogen. These findings suggest that plants at the phosphorus-poor site invest more resources in anti-herbivore defenses, and this higher investment in defenses, along with lower leaf tissue phosphorus concentrations, deterred multiple herbivore species.

KEY WORDS: Herbivory · *Thalassia testudinum* · Sea urchins · Parrotfish · Phosphorus · Condensed tannins · Phenolics · *Lytechinus variegatus* · *Nicholsina usta*

1. INTRODUCTION

Herbivory influences plant fitness, driving patterns in plant abundance and distribution (Lubchenco & Gaines 1981, Huntly 1991, Poore et al. 2012, Wood et al. 2017). To reduce grazing pressure, plants evolved chemical and physical anti-herbivore defenses that reduce palatability, which can include changes in secondary metabolite content, fiber content, leaf toughness, and nutrient content (Fritz & Simms 1992). However, allocating resources to anti-herbivore defenses reduces the availability of resources for growth and reproduction, and therefore, resource availability can impact plant defense strategies (Hernán et al. 2019).

Various hypotheses can explain the effects of resource availability on intra- and interspecific differ-

ences in plant anti-herbivore defenses (Stamp 2003). The resource availability hypothesis (RAH) predicts that species adapted to high-resource environments will have higher growth rates, lower constitutive defenses, and higher inducible defenses, while species adapted to resource-poor environments will have lower growth rates and higher constitutive defenses (Coley et al. 1985, Endara & Coley 2011). Similarly, the growth-differentiation hypothesis (GDH) predicts that plants in low-nutrient environments will have higher defenses due to higher re-growth costs following herbivory (Stamp 2003). In contrast, when examining intraspecific variation in plant defenses, recent studies predict the opposite trend, with higher constitutive defenses in resource-rich environments due to higher herbivore pressure (referred to as the intraspecific RAH; Hahn & Maron 2016). However, if herbivore pressure is not higher in resource-rich areas compared to resource-poor areas, patterns may not fit predictions from the intraspecific RAH (Lamarre et al. 2012).

Seagrass herbivores include mesograzers (e.g. amphipods), sea urchins, fish, turtles, sirenians (e.g. manatees), and waterfowl. Feeding decisions vary by taxa due to species-specific differences in sensory abilities and species-specific sensitivities to anti-herbivore defenses (Heck & Valentine 2006, Prado & Heck 2011). Additionally, the impact of herbivory on seagrass meadows varies based on herbivore identity (Heck et al. 2015, Roth et al. 2023). While meso-grazers often remove epiphytes from seagrass leaves, facilitating seagrass growth (Baden et al. 2010), sea urchins, parrotfish, and green turtles consume large amounts of seagrass tissue, potentially overgrazing seagrass meadows when present at high densities (Rose et al. 1999, Fourqurean et al. 2010, Heck et al. 2015, Lefebvre et al. 2017). Furthermore, populations of herbivorous green turtles, manatees, and emerald parrotfish are all increasing in the northern Gulf of Mexico due to tropicalization (i.e. range expansion of tropical species into subtropical and temperate regions) and successful conservation efforts (Fertl et al. 2005, Foley et al. 2005, Pabody et al. 2009, Fodrie et al. 2010, Avens et al. 2012). Overgrazing of seagrasses also leads to losses of valuable ecosystem services, including reduced blue carbon stores and habitat provisioning (Carnell et al. 2020, Orth et al. 2020). Therefore, understanding how seagrass nutrients, chemical defenses, and physical defenses impact feeding choices in multiple herbivore species can improve predictions of how changing abiotic and biotic conditions will impact seagrass meadows and their associated ecosystem services (Barbier et al. 2011).

Variation in seagrass leaf tissue nutrients, specifically leaf tissue nitrogen, can impact herbivore feeding decisions. In subtropical and tropical Atlantic seagrasses, nutrient addition (fertilizer containing nitrogen and phosphorus) enhanced consumption of turtle grass *Thalassia testudinum* by macrograzers (McGlathery 1995, Campbell et al. 2018), and bucktooth parrotfish *Sparisoma radians* consistently preferred nitrogen-enriched *T. testudinum* (Goecker et al. 2005). However, sea urchins do not always follow this pattern of increased grazing rates with higher nitrogen concentrations (Valentine & Heck 2001, Vergés et al. 2011, Steele & Valentine 2012). In some cases, urchin feeding decisions are primarily driven by morphology and structural defenses (Vergés et al. 2011), and in other cases, urchins compensate for low nutritional quality by consuming more of the low-nitrogen option than the nitrogen-enriched option (Valentine & Heck 2001). The impact of seagrass leaf tissue phosphorus on herbivore feeding decisions is also less studied than the impact of leaf tissue nitrogen, possibly because nitrogen is a more common aquatic pollutant. However, we expect phosphorus availability to also influence feeding decisions since consumers are often phosphorus-limited (Hood et al. 2005, Prado & Heck 2011, Benstead et al. 2014).

Along the north-central region of the Gulf Coast of Florida (Weeki Wachee to Waccasassa, FL), extensive water quality and seagrass monitoring data have revealed the existence of a natural gradient in water column total phosphorus (TP) concentrations, *T. testudinum* leaf tissue %P, and water column chlorophyll *a* concentrations, with concentrations increasing from south to north (Frazer et al. 1998, Jacoby & Frazer 2013, Jacoby et al. 2015, Barry et al. 2017; Fig. 1). Since there are correlations between TP, chlorophyll *a* concentrations, and latitude (Frazer et al. 1998, Jacoby et al. 2012, 2015), this creates a natural laboratory to study the impact of TP on seagrass characteristics. Bottom irradiance does not vary with TP, and the percent surface irradiance is above the reported thresholds for *T. testudinum,* indicating that the plants are not light limited in this region (Barry et al. 2017). While limited data are available on herbivore density and grazing pressure, seagrass monitoring in this region documents low grazing pressure with minimal grazing scars across the entire study region (Barry et al. 2023). Previous studies found positive correlations between TP and *T. testudinum* leaf surface area, shoot height, aboveground:belowground biomass ratio, and productivity, and a negative relationship between TP and *T. testudinum* shoot density along the latitudinal gradient in TP availability, with

the decline in shoot density possibly due to self-thinning that reduces shading from taller, wider leaves (Jacoby & Frazer 2013, 2015, Barry et al. 2017, 2018). Leaf nutrient ratios also indicate potential phosphorus limitation across this entire region (Barry et al. 2017). Variation in TP concentrations is expected to also influence seagrass anti-herbivore defenses and herbivore feeding decisions, thereby modifying the impact of increased grazing pressure on this region.

In this study, we analyzed constitutive *T. testudinum* anti-herbivore defenses and herbivore feeding decisions using *T. testudinum* from 3 sites along the gradient in TP (northern sites with higher TP: Crystal River and Homosassa; southern site with lowest TP: Weeki Wachee; Fig. 1). We expected patterns in anti-herbivore defenses to follow predictions from the RAH and GDH rather than the intraspecific RAH, with higher constitutive defenses in resource-poor areas. Seagrass monitoring data reveal low and similar grazing pressure across the study region, which indicates that the intraspecific RAH may not be applicable (Barry et al. 2023). We also explored the impact of variation in plant traits on feeding decisions of variegated sea urchins *Lytechinus variegatus* and emerald parrotfish *Nicholsina usta*, predicting that herbivores will prefer nutrient-enriched seagrass with lower defenses and that leaf nutrient content may have a greater impact on *N. usta* feeding behavior than on *L. variegatus* feeding behavior. Understanding *N. usta* feeding decisions is especially important, as a 22-fold increase in the abundance of *N. usta* from the 1970s to 2006−2007 has been observed in the northern Gulf of Mexico (approximately 149 to 188 km north of our study region) due to tropicalization (Fodrie et al. 2010). With increasing herbivore populations in the northern Gulf of Mexico (Fodrie et al. 2010, Avens et al. 2012), we hope to advance understanding of the potential impacts of tropicalization in temperate and subtropical regions as well as the impact of nutrient availability on plant—herbivore interactions.

Fig. 1. Sampling sites along the Gulf Coast of Florida, USA

2. MATERIALS AND METHODS

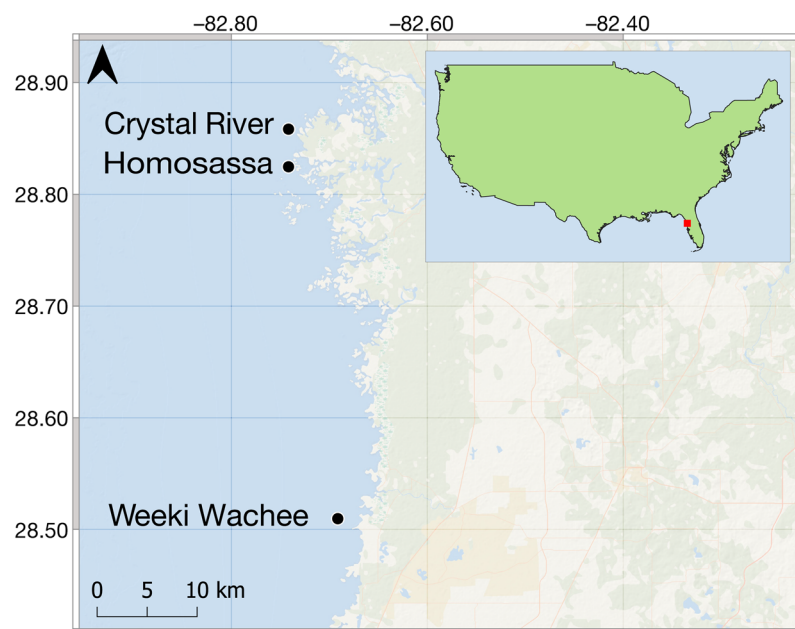To investigate how seagrass—herbivore interactions vary along a gradient in phosphorus availability, we analyzed plant physical and chemical characteristics and conducted feeding trials using multiple seagrass herbivore species and seagrass from multiple sites along the gradient (Jacoby & Frazer 2013, Jacoby et al. 2015, Barry et al. 2017, 2018). To capture variability in TP availability, we collected *Thalassia testudinum* from 3 sites in Florida: Crystal River (northern site with high TP), Homosassa (northern site with high TP), and Weeki Wachee (southern site with low TP; Figs. 1 & 2, Table 1). Sites were selected to minimize confounding variations (e.g. depth and salinity; Barry et al. 2017). While there is no systematic variation in water column total nitrogen concentrations along the phosphorus gradient (Frazer et al. 1998, Jacoby et al. 2012, 2015), we found that *T. testudinum* plants from Homosassa were enriched in nitrogen compared to those from the other 2 sites (Fig. 2). We measured changes in plant physical and chemical characteristics between sites, speculating that nutrient conditions may be driving changes in plant anti-herbivore defenses.

At each site, we collected plants for fresh plant feeding trials, agar-based feeding trials, chemical analyses (carbon:nitrogen:phosphorus, fiber, condensed tannins, and phenolic acids), and specific leaf area (SLA) analysis. We selected plants without any visible grazing scars in order to measure constitutive anti-herbivore defenses rather than induced defenses. Plants collected for SLA analysis were stored in plastic bags on ice. For chemical analyses and agar-based feeding trials, we collected 10 samples from
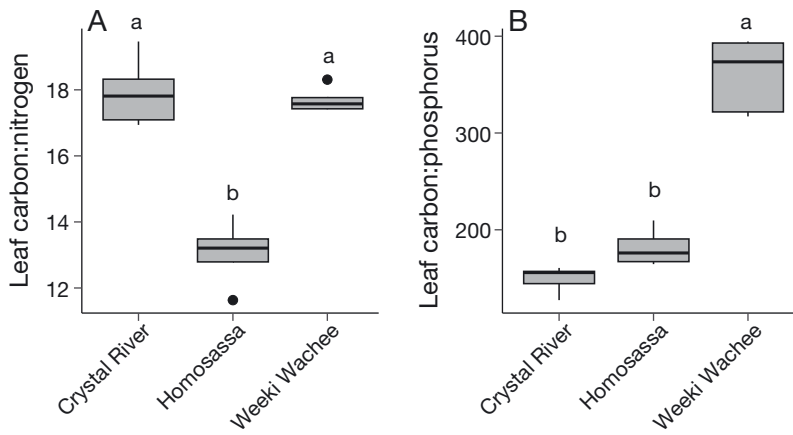
Fig. 2. Molar ratios of *Thalassia testudinum* leaf tissue nutrients by site. (A) Leaf carbon:nitrogen ratio was lowest in Homosassa, FL. (B) Leaf tissue carbon:phosphorus ratio was lowest in Crystal River and Homosassa, FL. Lowercase letters denote significant differences by site as determined by Tukey's HSD post hoc test using $\alpha = 0.05$. Statistics are presented in Table 1. In the plots, boxes represent the interquartile ranges; lines within the boxes represent the median values; error bars extend to the lowest and highest values within 1.5x the interquartile range; and points represent outliers

toughened leaves), which is a physical anti-herbivore defense (Hanley et al. 2007). We scanned the leaves using a flatbed scanner, calculated leaf surface area from scanned images using ImageJ 1.53i, and then dried the plants at 60°C for 48 h to obtain the dry weight (n = 10).

Using the ground leaf tissue, we analyzed leaf carbon (C), nitrogen (N), phosphorus (P), neutral detergent fiber (i.e. lignin, cellulose, hemicellulose), condensed tannin (i.e. proanthocyanin), and phenolic acid content. Neutral detergent fiber, condensed tannins, and phenolic acids are all expected to reduce leaf palatability, potentially deterring herbivory, while increased nutritional quality (higher N and P) is expected to increase leaf palatability (Thayer et al. 1984, Heck & Valentine 2006, Steele & Valentine 2015). Sample

each site, with each sample consisting of 40 second-rank (i.e. second-youngest) leaves pooled. We immediately placed samples on dry ice and subsequently stored samples in a −80°C freezer. Samples were freeze-dried and ground in liquid nitrogen. For fresh feeding trials, second-rank leaves were maintained in site water with aerators.

We calculated SLA (surface area in $cm^2 \ g^{-1}$ dry weight) for 10 plants from each site. SLA is negatively correlated with sclerophylly (hardened or

sizes varied because leaf tissue was limited and were based on the expected variability of the different compounds within and among sites. Phosphorus was analyzed on a Shimadzu spectrophotometer following a modified colorimetric method from Solórzano & Sharp (1980) at Florida International University's Blue Carbon Analysis Laboratory (FIU BCAL), and C and N were analyzed using a CHN analyzer (Fisons NA1500 or Thermo Flash EA 1112) at FIU BCAL (n = 5 for C, N, and P). Neutral detergent fiber (n = 8), measured as

Table 1. Effect of collection site on *Thalassia testudinum* chemical and physical properties as well as feeding behavior of *Lytechinus variegatus* and *Nicholsina usta*. 3,4-di: 3,4-dihydroxybenzoic acid; C: Crystal River; CT: condensed tannin; denom: denominator; DW: dry weight; fer: ferulic acid; gal: gallic acid; H: Homosassa; num: numerator; p-cou: p-coumaric acid; SLA: specific leaf area; VA: vanillic acid; W: Weeki Wachee

Response variable	F	Num. df	Denom. df	p	Pairwise p-values C–H	C–W	H–W
Carbon:nitrogen ratio	43.26	2	11	<0.001	<0.001	0.91	<0.001
Carbon:phosphorus ratio	89.56	2	11	<0.001	0.21	<0.001	<0.001
SLA	100	2	26	<0.001	<0.001	<0.001	<0.001
Fiber (% DW)	3.61	2	20	0.046	0.58	0.28	0.038
Gal (% DW)	85.58	2	27	<0.001	0.99	<0.001	<0.001
3,4-di (% DW)	18.43	2	27	<0.001	0.82	<0.001	<0.001
VA (% DW)	65.17	2	27	<0.001	0.003	<0.001	<0.001
Fer (% DW)	32.36	2	27	<0.001	0.96	<0.001	<0.001
p-cou (% DW)	10.72	2	27	<0.001	<0.001	0.053	0.093
CT (% DW)	10.18	2	9	0.005	0.64	0.005	0.021
L. variegatus consumption (g)	17.14	2	27	<0.001	0.59	0.025	0.070
L. variegatus consumption (agar squares)	6.06	2	24	0.007	0.63	0.049	0.034
N. usta consumption (g)	6.56	2	24	0.005	0.01	0.68	0.008
N. usta consumption (agar squares)	5.56	2	24	0.010	0.025	0.84	0.077

the percent fiber in dry biomass, was analyzed following methods from de los Santos et al. (2012). Hereafter, we refer to neutral detergent fiber as 'fiber content.' Condensed tannin concentrations (n = 4) were analyzed using a colorimetric assay (cf. Arnold & Schultz 2002). Since we used a commercially available quebracho tannin as a standard for the condensed tannin analysis rather than a standard made from seagrass tannins, differences between collection sites should be interpreted as relative differences rather than absolute values. Phenolic acid concentrations (gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid, ferulic acid, and p-coumaric acid; n = 10) were analyzed using a Waters 6495 high performance liquid chromatograph equipped with a Waters 2487 UV detector and a C18 Luna column (150 × 4.6 mm; Phenomenex), following the methods of Ravn et al. (1994) and Steele et al. (2005).

Feeding choice experiments were conducted to test herbivore feeding preferences among *T. testudinum* options from Weeki Wachee, Homosassa, and Crystal River, using *Lytechinus variegatus* and *Nicholsina usta* as herbivores. To correct for autogenic changes in the feeding options (e.g. due to variability in wet weights), we ran all feeding trials both with an herbivore present (i.e. treatment) and without an herbivore present (i.e. control; following Prado & Heck 2011, Steele & Valentine 2012, Hernán et al. 2016). All feeding trials were run at 28°C. For the trials with fresh leaves, we collected second-rank *T. testudinum* leaves from the 3 sites within 48 h of the feeding trials, removing epiphytes with a razor blade and maintaining leaves in 5 gal (~19 l) buckets with site water and aeration. The leaves were cut to be 8.5 cm in length. Wet weights of the leaves were recorded before and after the trials. Herbivore consumption of fresh seagrass was calculated as:

$$preweight \times \left(\left(\sum_{i=1}^{n} \frac{control_i : postweight}{control_i : preweight} \right) / n \right) \quad (1)$$
$$- postweight$$

using the controls from the given site to incorporate autogenic changes in leaf weight. Since the initial leaf weight varied between replicates, the proportional change in weight in the controls was used to correct for autogenic changes.

In addition to conducting feeding choice trials with fresh *T. testudinum* leaves, we also conducted feeding choice trials with freeze-dried and ground *T. testudinum* that was embedded in an agar matrix to control for differences in leaf structure and shape. For each collection site, we heated 10 ml of deionized water to boiling and added 0.15 g agar. After the agar

and water solution cooled for around 3 min, we mixed in 0.3 g *T. testudinum* (previously freeze-dried and ground) from the collection site and poured it over 20 fiberglass window screen strips (0.5 × 8.5 cm) (methods from Steele & Valentine 2012, who determined the average dry mass of *T. testudinum* leaves of equivalent surface area). We placed the strips between 2 pieces of plexiglass until the agar solidified to achieve uniform thickness. We counted the number of empty window screen squares before and after the trials. Herbivore consumption of agar-based seagrass leaves was calculated as:

$$squares_b - \frac{\left(\sum_{i=1}^{n} control_i : squares_b - control_i : squares_a \right)}{n} \quad (2)$$
$$- squares_a$$

using the controls from the given site to incorporate autogenic changes in artificial leaves ($squares_b$ = the number of empty window screen squares before the trial and $squares_a$ = the number of empty window screen squares after the feeding trial).

L. variegatus were collected from Steinhatchee, FL, and maintained in a 100 gal (~375.5 l) tank with flowing seawater at the UF/IFAS Nature Coast Biological Station. The mean ± SD diameter of *L. variegatus* used in feeding trials was 6.9 ± 0.5 cm. *L. variegatus* were fed *T. testudinum* leaves ad libitum collected from Steinhatchee and Cedar Key, FL, with all food removed from herbivore tanks 24 h prior to feeding trials. We set up 10 experimental units (20 × 35 × 12 cm plastic containers with a window screen lid) that included an *L. variegatus* individual and 10 experimental units that served as controls with no *L. variegatus* present. Feeding trials lasted 80 min. For the trials with fresh *T. testudinum* from the study sites, we attached an 8.5 cm leaf segment from each collection site to a colored binder clip to distinguish between collection sites, placing a single binder clip from each of the 3 collection sites in each experimental unit. Similarly, for the trials with agar-based leaves, we attached an 8.5 cm artificial leaf segment from each collection site to a colored binder clip, adding a single binder clip from each of the 3 collection sites to each experimental unit.

N. usta were collected by the Gulf Specimen Marine Laboratory in Panacea, FL, and maintained in 120 gal (~454 l) tanks (n = 9, with 2 fish per tank) and flowing seawater at the University of Florida Whitney Laboratory for Marine Bioscience (under IACUC Protocol #202111424). The mean ± SD total length of *N. usta* used in feeding trials was 9.5 ± 2.8 cm. *N. usta* were fed strips of nori seaweed sheets, removing all food from tanks 24 h prior to feeding trials. To minimize

stress for the fish, we used the holding tanks as the experimental units (n = 9 tanks with 2 fish in each experimental unit). These trials with *N. usta* were 30 min long. For the control replicates associated with the *N. usta* trials, we placed the fresh seagrass or agar leaves in a 0.027 m³ cube made of plastic hexagonal mesh covered with fiberglass window screen and located in the *N. usta* holding tanks. For the trials with fresh *T. testudinum,* we clipped 8.5 cm leaf segments (n =3) from each site together in a binder clip, and we placed 2 binder clips from each of the 3 collection sites in each experimental unit and control cage. For the agar-based trials, we clipped a single 8.5 cm artificial leaf segment to a colored binder clip, placing 2 binder clips from each of the 3 collection sites in each experimental unit and control cage.

We conducted all statistics using R v4.1.0, with α = 0.05 as a cutoff for statistical significance. For all statistical analyses, we examined plots of residuals versus predicted values and q-q plots when applicable to check for homogeneity of variance and normality of residuals. We used ANOVA with Tukey's HSD post hoc test to analyze the impact of collection site (categorical, 3 levels: Crystal River, Homosassa, Weeki Wachee) on plant traits (C:N, C:P, SLA, fiber content, gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid, ferulic acid, p-coumaric acid, and condensed tannins). An outlier from Crystal River was removed from the fiber data and an outlier from Homosassa was removed from the SLA data, as these data points were over 3 SD away from the mean and likely represented data entry errors. To account for the lack of independence among feeding trial options that were offered simultaneously, we analyzed the amount consumed in the feeding trials as a function of collection site using a generalized least squares linear model with an unstructured covariance matrix. We used the command 'gls' in the R package 'nlme' to conduct the generalized least squares model (Pinheiro et al. 2022) and the package 'emmeans' to analyze pairwise differences between the feeding trial options (Lenth 2021).

3. RESULTS

Thalassia testudinum leaves collected from 3 sites along a natural gradient in TP availability differed in nutrient concentrations, SLA, fiber content, phenolic acid content, and condensed tannin content. Plants from Weeki Wachee, which is located furthest south with the lowest phosphorus availability, were often chemically different from the plants collected from Homosassa or Crystal River, which are both located in the northern region of the gradient with higher phosphorus availability. Leaf tissue C:P ratio was on average 55% lower in Crystal River and Homosassa than in Weeki Wachee. Leaf tissue C:N ratio was on average 27% lower in Homosassa than in Crystal River and Weeki Wachee (Table 1, Fig. 2). SLA was lowest in Weeki Wachee, increasing by 33% on average in Crystal River and by 50% on average in Homosassa (Table 1, Fig. 3A), and fiber content was higher in Weeki Wachee than Homosassa (Table 1, Fig. 3B).

The concentrations of secondary metabolites also tended to be higher in plants from Weeki Wachee. Gallic acid concentrations increased by 93%, ferulic acid concentrations increased by 92%, and condensed tannin concentrations increased by 102% in plants from Weeki Wachee when compared to plants from both Homosassa and Crystal River (Table 1, Fig. 4A,D,F). Additionally, vanillic acid concentrations were 74% higher in Weeki Wachee than Crystal River and 40% higher in Weeki Wachee than Homosassa (Table 1, Fig. 4C). The concentration of 3,4-dihydroxybenzoic acid was on average 49% higher in Crystal River and Homosassa than in Weeki Wachee (Table 1, Fig. 4B), and the concentration of p-coumaric acid was on average 211% higher in Homosassa than Crystal River (Table 1, Fig. 4E).

Both *Lytechinus variegatus* and *Nicholsina usta* usually preferred the *T. testudinum* from Crystal River or Homosassa over that from Weeki Wachee.
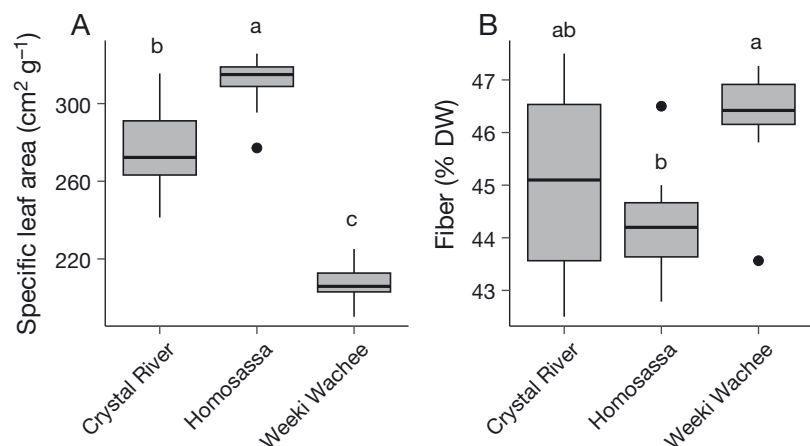

Fig. 3. Specific leaf area and fiber content of *Thalassia testudinum* by site. (A) Specific leaf area was highest in Homosassa, intermediate in Crystal River, and lowest in Weeki Wachee. (B) Fiber (% dry weight, DW) was higher in Weeki Wachee than in Homosassa. Lowercase letters denote significant differences by site as determined by Tukey's HSD post hoc test using α = 0.05. Statistics are presented in Table 1. Refer to Fig. 2 for box plot description
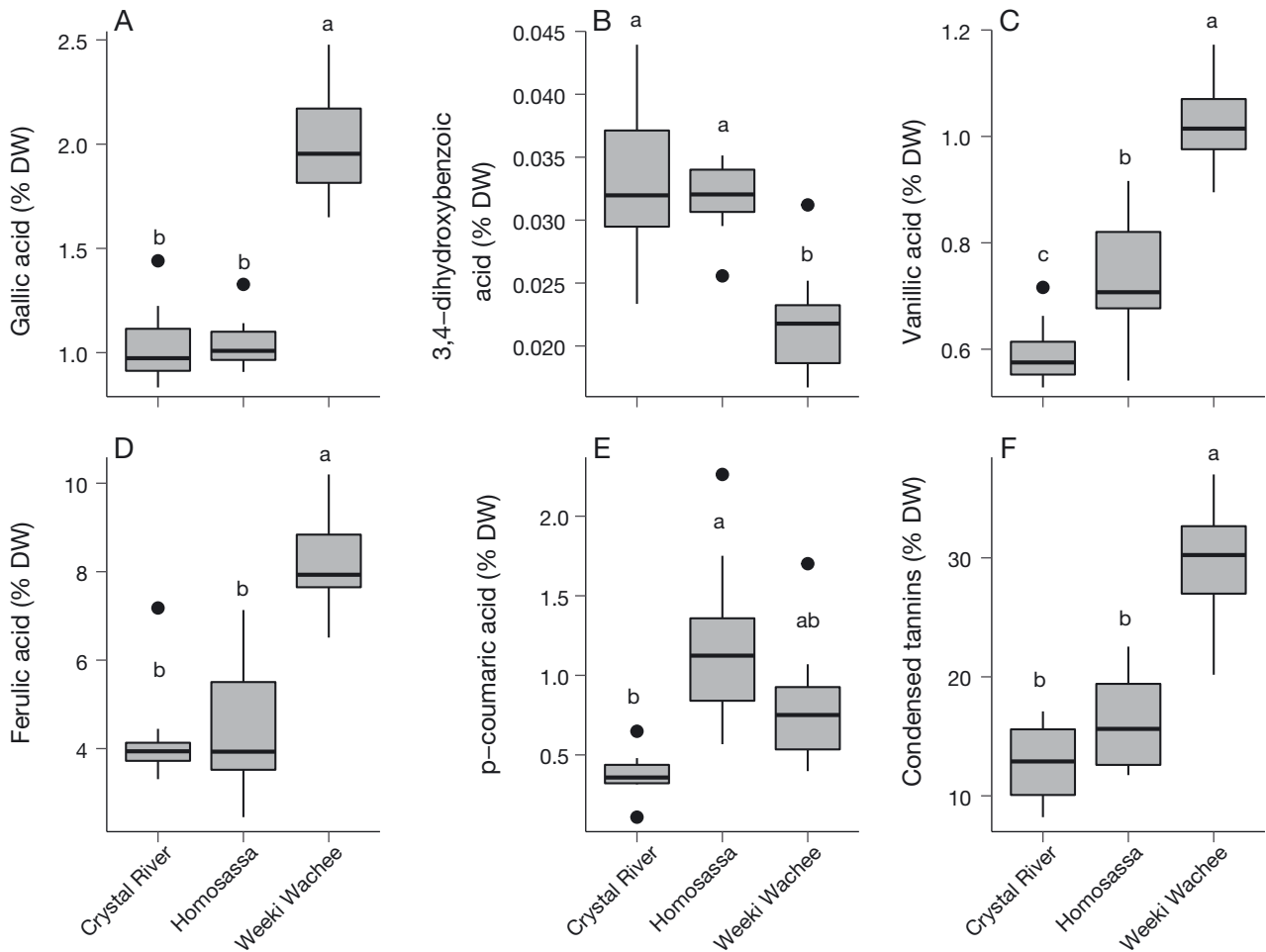
Fig. 4. Concentrations (% dry weight, DW) of phenolic acids and condensed tannins in *Thalassia testudinum* leaves by site: (A) gallic acid, (B) 3,4-dihydroxybenzoic acid, (C) vanillic acid, (D) ferulic acid, (E) p-coumaric acid, and (F) condensed tannins. Lowercase letters denote significant differences by site as determined by Tukey's HSD post hoc test using $\alpha = 0.05$. Statistics are presented in Table 1. Refer to Fig. 2 for box plot description

When offered fresh *T. testudinum*, *L. variegatus* individuals preferred the *T. testudinum* from Crystal River over the *T. testudinum* from Weeki Wachee, with no consumption of *T. testudinum* from Weeki Wachee observed (Table 1, Fig 5A). *L. variegatus* also preferred the agar-based leaves from both Crystal River and Homosassa over the agar-based leaves from Weeki Wachee, consuming on average 3.3× more of the Crystal River and Homosassa options than the Weeki Wachee option (Table 1, Fig. 5B). *N. usta* preferred the option from Homosassa, consuming on average 2.2× more of that option than the options from Crystal River or Weeki Wachee in the feeding trial with fresh *T. testudinum* (Table 1, Fig. 6A).
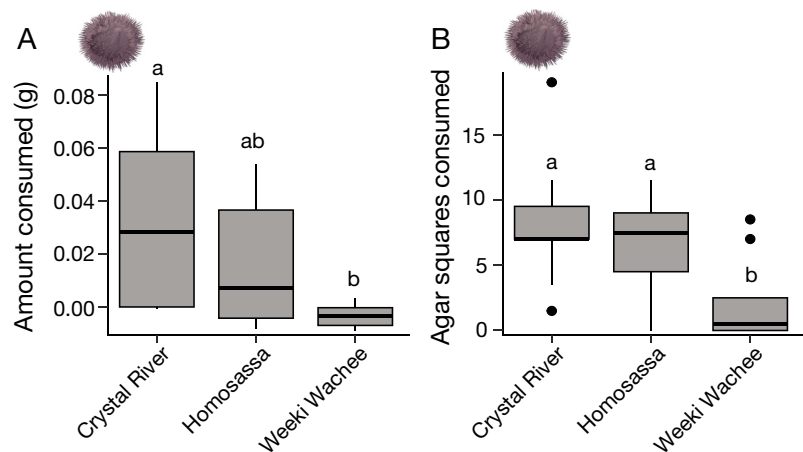

Fig. 5. *Lytechinus variegatus* feeding preference based on *Thalassia testudinum* collection site when using (A) fresh seagrass and (B) agar-based leaves. Lowercase letters denote significant pairwise differences using $\alpha = 0.05$. Statistics are presented in Table 1. Refer to Fig. 2 for box plot description. Images courtesy of Tracey Saxby, Integration and Application Network (ian.umces.edu/media-library)
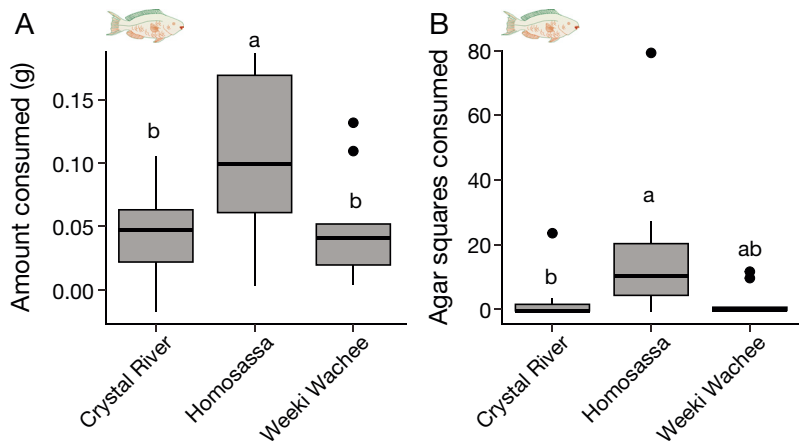
Fig. 6. *Nicholsina usta* feeding preference based on *Thalassia testudinum* collection site when using (A) fresh seagrass and (B) agar-based leaves. Lowercase letters denote significant pairwise differences using α = 0.05. Statistics are presented in Table 1. Refer to Fig. 2 for box plot description. Images courtesy of Diana Kleine, Marine Botany UQ (ian.umces.edu/media-library)

In the feeding trial with agar-based leaves, *N. usta* on average consumed 6.5× more of the option from Homosassa compared to the option from Crystal River (Table 1, Fig. 6B).

4. DISCUSSION

The chemical and physical traits of *Thalassia testudinum* varied based on collection site. The plants from Weeki Wachee, which were the most phosphorus-limited, exhibited the highest constitutive anti-herbivore defenses, with higher fiber content, lower SLA, higher condensed tannin concentrations, and higher gallic acid, vanillic acid, and ferulic acid concentrations. However, 2 out of the 5 phenolic acids measured (3,4-dihydroxybenzoic acid and p-coumaric acid) did not follow the same pattern, possibly because the various phenolic acids are driven by different limiting factors or different stressors. Additionally, the function of individual phenolic acids is unknown, and since they are all produced via the same biochemical pathway, increases in one acid may concomitantly decrease other acids that are intermediate steps in the pathway (Francenia Santos-Sánchez et al. 2019). Both *Lytechinus variegatus* and *Nicholsina usta* avoided consuming these heavily defended plants. Feeding preferences were likely driven by chemical rather than physical differences in the plants, as the trials with fresh leaves and the trials with agar-based leaves yielded similar results for both *L. variegatus* and *N. usta*, which is consistent with past findings for these herbivore species (Prado & Heck 2011).

We expect that there is genetic connectivity throughout the study region for *T. testudinum* plants, and therefore environmental variation and phenotypic plasticity rather than genetics are driving observed patterns. Phenotypic plasticity is well-documented in *T. testudinum* along with other seagrass species (Frankovich & Fourqurean 1997, Hackney & Durako 2004, Bricker et al. 2011, Maxwell et al. 2014, Barry et al. 2017, Darnell & Dunton 2017), and long-distance seed dispersal is also common for this species, with van Dijk et al. (2009) reporting *T. testudinum* dispersal distances of up to 360 km and a panmictic area of 350 km. In our study, the most northern site (Crystal River) was 39 km from the most southern site (Weeki Wachee). The high plasticity and genetic connectivity throughout the study region indicate that environmental differences, such as variation in nutrient availability, are likely driving differences in seagrass–herbivore interactions across the study sites.

Our findings that 6 out of the 8 anti-herbivore defenses studied were stronger in the phosphorus-poor environment, along with findings from Barry et al. (2017) of lower growth rates in phosphorus-poor environments, support the RAH and GDH hypotheses but not the intraspecific RAH (Endara & Coley 2011, Hahn & Maron 2016). These findings are consistent with patterns observed by Hernán et al. (2019) with *Posidonia oceanica.* However, the sites used in this study have low grazing pressure (Barry et al. 2023), and patterns in anti-herbivore defenses may change in regions with higher grazing pressure. Grazing intensity is a key factor that influences predictions under the intraspecific RAH, with this hypothesis proposing stronger constitutive anti-herbivore defenses due to higher grazing pressure at resource-rich sites (Hahn & Maron 2016). Plants from Weeki Wachee were better defended both chemically (i.e. higher condensed tannins, phenolic acids, and fiber) and physically (i.e. higher fiber and lower SLA) than plants from the other 2 sites. This site was uniquely phosphorus-limited and exhibited similar leaf tissue nitrogen content as the site at Crystal River, indicating that phosphorus limitation or co-limitation of phosphorus and nitrogen may be driving observed trends. Nitrogen alone likely did not drive these trends: plants from Crystal River and Weeki Wachee exhibited similar nitrogen content, but they exhibited differences in anti-herbivore defenses.

These findings indicate that phosphorus-limited *T. testudinum* plants may allocate more resources to anti-herbivore defenses. Both chemical and physical defenses were stronger at Weeki Wachee, suggesting that both components of the defense syndrome co-vary rather than exhibit trade-offs (Agrawal & Fishbein 2006, Hanley et al. 2007, da Silva & Batalha 2011). Next steps could include a manipulative experiment that tests the impact of phosphorus enrichment on seagrass anti-herbivore defenses, as we were not able to directly manipulate phosphorus availability in this study.

Feeding preferences often vary based on herbivore identity (Prado & Heck 2011, Darnell & Heck 2013). While plant nutrients, secondary metabolites (condensed tannins and phenolic acids), and physical structure can all influence feeding decisions (Goecker et al. 2005, Vergés et al. 2007a,b), the impact and relative importance of these factors may be different for different species. When examining the impact of prior grazing on seagrass palatability for *L. variegatus* and bucktooth parrotfish *Sparisoma radians*, Darnell & Heck (2013) found that parrotfish consistently preferred grazed leaves, while urchins demonstrated a preference for ungrazed leaves in some trials and no preference in other trials, indicating that parrotfish may exhibit higher discrimination than urchins. Similarly, our results indicate that urchins may be less selective than parrotfish in their feeding decisions, as *L. variegatus* preferred plants from both Homosassa (phosphorus and nitrogen-enriched) and Crystal River (phosphorus-enriched) over plants from Weeki Wachee (low phosphorus), while *N. usta* preferred only the plants from Homosassa (phosphorus and nitrogen-enriched). Herbivorous fish are often protein-limited (Horn 1988, Fris & Horn 1993, Bowen et al. 1995, Horn et al. 1995), and protein limitation may drive herbivores to target nitrogen-rich plants. Other studies have also found that parrotfish often prefer nitrogen-enriched seagrass (McGlathery 1995, Goecker et al. 2005, Campbell et al. 2018). Plants from Homosassa had lower fiber content and higher SLA, but since we found that *N. usta* feeding decisions were primarily driven by plant chemistry, these traits were likely not the primary drivers of their preference for plants from Homosassa. The avoidance of Weeki Wachee seagrass by both herbivore species could be directly due to lower C:P concentrations in these plants, or it could be due to increased anti-herbivore defenses, which may be an indirect effect of low nutrient availability.

While we found that *L. variegatus* and *N. usta* feeding preferences were primarily driven by chemical traits, the relative impacts of plant chemical and physical defenses on herbivore feeding preferences will vary based on herbivore species identity (Prado & Heck 2011, Martínez-Crego et al. 2016). Mesograzers, such as amphipods and isopods, need to scrape or bite through the entire leaf (Sanson 2006). Therefore, physical defenses, such as low SLA, may be a stronger deterrent for mesograzers with smaller mouthparts than for larger herbivores with larger and stronger mouthparts (Sanson 2006), as SLA is negatively correlated with sclerophylly (i.e. toughened or hardened leaves; Hanley et al. 2007, Martínez-Crego et al. 2016). We found that plants from Weeki Wachee had the lowest SLA of all sites and plants from Homosassa had the highest SLA, indicating that some mesograzers might prefer to consume plants from Homosassa and avoid consuming plants from Weeki Wachee. Furthermore, mesograzers can also be deterred by secondary metabolites, providing an additional mechanism that may deter consumption of *T. testudinum* in Weeki Wachee by mesograzers (Tomas et al. 2011, Steele & Valentine 2015, Martínez-Crego et al. 2016).

Minimal data are available on macrograzer abundance along the Nature Coast region of Florida. However, there are data on mesograzer abundance, with Barry et al. (2021) reporting that the macroinvertebrate community at all 3 sites is dominated by grazers that eat epiphytes, macroalgae, and potentially seagrass. The identity of the grazers varies by location: the macroinvertebrate community at Weeki Wachee is dominated by gastropod grazers, the community at Homosassa is dominated by crustacean grazers, and the community at Crystal River is dominated by a mix of gastropod and crustacean grazers (Barry et al. 2021). This variation in grazer identity could be due to changes in plant and algae palatability as well as changes in plant morphology and density (Barry et al. 2017). Mesograzer identity could also influence consumption of epiphytes, macroalgae, and seagrass as well as seagrass traits, including secondary metabolite production, across the study region (Heck & Valentine 2006, Steele & Valentine 2015). In fact, Steele & Valentine (2015) demonstrated that grazing by a gastropod and crustacean (isopod) induced changes in the concentrations of condensed tannins and phenolic acids in *T. testudinum* and *Halodule wrightii* leaves.

We observed both overlap and division among feeding decisions by *L. variegatus* and *N. usta.* We expect increased grazing pressure by *N. usta* on seagrasses in this region due to tropicalization (Fodrie et al. 2010, Heck et al. 2015), and our results suggest that *N. usta* may target *T. testudinum* in Homosassa. The

T. testudinum from this site was also preferred by *L. variegatus* over *T. testudinum* from Weeki Wachee and exhibited the highest SLA, potentially increasing susceptibility to mesograzer consumption and indicating that multiple herbivore species may all target similar plants. However, we found that only *L. variegatus* selectively consumed *T. testudinum* from Crystal River and neither herbivore species selectively consumed *T. testudinum* from Weeki Wachee. This diversity and specificity in herbivore feeding behavior may lead to asynchronous grazing pressure on *T. testudinum* in the Gulf of Mexico, allowing less impacted plants to compensate for losses in heavily grazed plants and increasing ecosystem stability (Mori et al. 2013).

Anthropogenic nutrient addition will likely have many indirect impacts on coastal ecosystems. For example, nitrogen enrichment reduces *T. testudinum* flowering output, indicating that nutrient loading may lead to reduced seagrass sexual reproduction and ultimately reduced genetic diversity (Darnell & Dunton 2017). Additionally, nutrient-enriched seagrass is often grazed at higher rates than unenriched seagrass, as demonstrated in this study and others (McGlathery 1995, Goecker et al. 2005, Campbell et al. 2018), indicating the potential for increased grazing pressure with nutrient loading. In this study, we also found a trend for reduced anti-herbivore defenses in phosphorus-enriched seagrass, which could again lead to stronger grazing pressure with nutrient loading. Changes in anti-herbivore defenses may also interact with tropicalization. Feeding decisions depend on herbivore identity (Prado & Heck 2011, Darnell & Heck 2013), and the abundance and identity of herbivores is changing with warming temperatures (Vergés et al. 2014). High nutrient concentrations often result in algal blooms and reduced light availability, and light limitation may increase seagrass regrowth cost following herbivory, causing seagrasses to invest more in anti-herbivore defenses. However, it is important to note that this study was conducted under relatively low nutrient conditions (averaging 7–17 µg l^{-1} total phosphorus and 304–442 µg l^{-1} total nitrogen; Barry et al. 2017), and so manipulative experiments with higher nutrient levels are needed to directly test the effect of increased phosphorus availability and anthropogenic nutrient loading on seagrass anti-herbivore defenses.

5. CONCLUSIONS

Overall, we found that seagrass physical and chemical anti-herbivore defenses vary along a natural gradient in TP availability, with the strongest defenses at the site with the lowest phosphorus availability (Weeki Wachee). Barry et al. (2017) reported lower growth rates in Weeki Wachee compared to the other sites, indicating that *Thalassia testudinum* in phosphorus-poor environments may exhibit stronger defenses due to high regrowth costs. Changes in *T. testudinum* defense syndrome differentially impacted *Nicholsina usta* and *Lytechinus variegatus* feeding decisions, although both herbivores avoided *T. testudinum* from Weeki Wachee, which had the lowest P content and the strongest anti-herbivore defenses. Since the different herbivore species responded differently to variation in plant defense syndromes, this could spread grazing pressure among multiple locations, plant age groups, and plant species, rather than concentrating grazing pressure on a specific phenotype. Understanding the impact of gradients in nutrient availability on plant–herbivore interactions is especially important given increases in anthropogenic nutrient loading and range expansion of tropical herbivores. Our findings highlight intraspecific variability in plant anti-herbivore defenses and variability in herbivore responses to plant defenses. Maintaining diversity of both producers and herbivores can spread grazing pressure, with some plants remaining less targeted by herbivores, potentially increasing ecosystem-level tolerance of grazing.

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