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Chemical defenses against herbivores and fungi limit establishment of fungal farms on salt marsh angiosperms

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ABSTRACT

Within coastal salt marshes of eastern North America, the snail *Littoraria irrorata* facilitates fungal growth on live plant tissues to gain access to a palatable and nutritious fungal food source. This snail–fungal mutualism increases exposure of the foundation species *Spartina alterniflora* to infection, whereas fungal farming on other local marsh plants is rarely observed. We sought to identify traits from five salt marsh angiosperm species, such as chemical defenses against snails or fungi, which restrict *L. irrorata* habitat choice, feeding patterns, and ability to establish fungal farms. In the field and in mesocosm experiments, *L. irrorata* densities were significantly higher on *S. alterniflora* than on other available plants, indicating that *S. alterniflora* is a favored habitat for *L. irrorata*. Highly avoided plants were rich in chemical defenses that rendered these plants unpalatable to *L. irrorata* in feeding trials, whereas *S. alterniflora* extracts deterred *L. irrorata* feeding only slightly. Removal of plant structure did not alter *L. irrorata* preferences indicating a negligible role of tissue toughness as a defense. All plants in our study produced compounds that inhibited growth of fungi typically farmed by *L. irrorata*, although *S. alterniflora* antifungal compounds were weaker than those of other plant species, consistent with the observation of fungi only on wounded *S. alterniflora* tissues. We propose that the weak chemical defenses produced by *S. alterniflora* make it a preferred plant for fungal farming, whereas less abundant species that invest in potent chemical defenses against grazers and fungi are not as frequently colonized, consumed, or subjected to fungal farming by *L. irrorata*. The inability of *S. alterniflora* to adequately deter herbivores and pathogens may lead to increasing losses in plant biomass and reduce the ecosystem services provided by this foundational species.

1. Introduction

The coastal Atlantic salt marshes of North America have historically been presented as an archetypical example of bottom-up forces controlling primary production (Teal, 1962), a system where most grazers were considered detritivorous intermediaries between highly productive cordgrass, *Spartina alterniflora*, and microbial decomposers (Barlocher and Newell, 1994a). However, studies involving geese (Buckeridge and Jefferies, 2007), insects (Bertness and Shumway, 1992), crabs (Bortolus and Iribarne, 1999) and snails (Silliman and Zieman, 2001) have shown that herbivores exert top-down control of salt marsh primary production and alter marsh landscapes (Silliman et al., 2005). Since healthy salt marshes provide a variety of ecosystem services, including buffering coastlines and forming nurseries for fish and shellfish (Gedan et al., 2009), understanding the mechanisms by which plant community members respond to these biotic threats has become increasingly important.

The periwinkle snail *Littoraria irrorata* is an abundant grazer in southeastern Atlantic marshes (Pennings and Silliman, 2005) that poses a unique double threat to *S. alterniflora*, which composes the bulk of plant biomass in these marshes (Pennings and Bertness, 2001). Periwinkles create grazing scars along the blades of *S. alterniflora*, selectively depositing hyphae-laden feces within wounded plant tissues to facilitate fungal establishment and growth (Silliman and Newell, 2003). This behavior represents a rare example of fungiculture outside the class Insecta (Mueller and Gerardo, 2002), although marsh crabs can also facilitate fungal infection of *S. alterniflora* (Daleo et al., 2009). *L. irrorata* later consume fungal hyphae during garden maintenance, obtaining a preferred food item that is more nutritious than uninfected *S. alterniflora* tissue (Barlocher and Newell, 1994a, 1994b; Barlocher et al., 1989). Through a facultative mutualism, fungi and snails gain access to more nutritious resources than would be expected in the absence of farming, but *S. alterniflora* becomes simultaneously exposed to tightly associated grazers and pathogens. At high *L. irrorata* densities and in the presence of compounding abiotic stressors, fungal infections mediated by *L. irrorata* can rapidly transform healthy stands of *S. alterniflora* into mudflats (Silliman and Zieman, 2001; Silliman et al., 2005). Periwinkles are thus not mere detritivores or mesograzers, but high impact fungal farmers exerting top-down control on marsh ecosystems.

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Since *L. irrorata* and fungi can control salt marsh vegetation, plants that are negatively impacted are expected to evolve mechanisms to thwart this facultative mutualism. Terrestrial plants produce a variety of structural (Milewski et al., 1991) and chemical (Agrawal and Kurashige, 2003) defenses that deter herbivores, and plant defenses have also been documented in salt marsh ecosystems (Buchsbaum et al., 1984; Pennings et al., 1998). Chemical defenses of salt marsh plants limit consumption by a wide range of herbivores (Barlocher and Newell, 1994b; Pennings et al., 1998; Siska et al., 2002) but the relative palatability of plants to *L. irrorata*, especially in the context of fungal farming, requires consideration.

Despite the evidence that fungi are important decomposers in salt marsh ecosystems (Newell, 2001a) and can infect live *S. alterniflora* (Silliman and Newell, 2003; Silliman and Zieman, 2001), we know surprisingly little about the prevalence of antifungal compounds among salt marsh plants. However, seagrasses, another class of marine angiosperm, are known to produce antimicrobial compounds (Engel et al., 2006) including secondary metabolites such as flavone glycosides that inhibit zoosporic fungi (Jensen et al., 1998). Terrestrial plants also produce antifungal molecules (Terras et al., 1995), so salt marsh plants on the border of marine and terrestrial ecosystems may be similarly expected to possess chemical defenses that deter fungal colonization. However, no studies to date have determined whether salt marsh plants deter fungi via chemical defenses, even though fungal infection is a precursor to widespread marsh vegetative die-off, particularly when coupled with high densities of farming snails (Silliman et al., 2005).

Despite the small but consistent community of plant species found in Atlantic salt marshes (Chapman, 1974; Pennings and Bertness, 2001), there have been no reports of *L. irrorata* fungiculture on plants other than *S. alterniflora*. In the current study, we investigated resistance of five marsh angiosperm species (*S. alterniflora*, *Batis maritima*, *Borrichia frutescens*, *Sarcocornia* sp. and *Iva frutescens*) common in mid-to-high elevation salt marsh zones (Chapman, 1974; Pennings and Bertness, 2001), to *L. irrorata* and their fungal partners. In previous studies, extracts of *B. frutescens*, *I. frutescens* and *S. alterniflora* were unpalatable to crabs and grasshoppers (Pennings et al., 1998; Siska et al., 2002), although chemical defenses did not always explain herbivore preferences for live plant tissues (Pennings et al., 1998). *S. alterniflora* extracts have also been shown to reduce *L. irrorata* grazing (Long et al., 2011), but none of the other plants on our panel had been previously tested for chemical defenses against *L. irrorata*. Furthermore, no previous studies have determined whether these plants utilize chemical defenses to inhibit growth of co-occurring fungi, potentially combating both players of the fungal farming mutualism. Specifically, we addressed the following questions: (1) when given a choice of common marsh plants, do *L. irrorata* colonization patterns match observed snail distributions in the field? (2) Do structural defenses, nutritional differences, and/or chemical defenses explain feeding preferences by *L. irrorata*? (3) Do salt marsh plants produce chemical defenses against fungi, and if so, how variable are these defenses among plant species? Overall, we sought to test the hypothesis that *L. irrorata* farms fungi on plants that have comparatively weak defenses against both fungi and herbivores. Using a combination of field studies and controlled laboratory assays, we considered whether the relative strength of chemical defenses against herbivores and pathogens predicts which plants are most susceptible to fungal farming by *L. irrorata*.

2. Methods

2.1. Surveys

We conducted surveys within salt marshes on Sapelo Island, GA in July 2011 to measure distributions and abundances of *L. irrorata* and salt marsh vegetation. Surveys were conducted adjacent to the University of Georgia Marine Institute (31° 23′ 47″ N, 81° 17′ 00″ W), near the Sapelo Island Lighthouse (31° 23′ 22″ N, 81° 17′ 02″ W) and Beach Road (31° 23′ 35″ N, 81° 16′ 17″ W). Plants were surveyed by randomly tossing a 30 cm^2 quadrat at 1 m intervals along a 100 m transect ($n = 3$ transects per site). Transects ran perpendicular to the shoreline starting in the mixed vegetation of the high elevation marsh zone and ended within stands of short-form *S. alterniflora*. The most abundant plant species, along with the total number of plant shoots were recorded for each quadrat. We estimated shoot biomass for each species by weighing 20 randomly selected shoots at each site. Plant species abundance was estimated at each site by multiplying average shoot mass by number of shoots observed, and dividing by survey area.

The quantity of *L. irrorata* residing on plants was measured by visually inspecting 100 individuals of the five plant species on our panel (*B. maritima*, *B. frutescens*, *I. frutescens*, *S. alterniflora*, and *Sarcocornia* sp.). The number of snails observed on each plant ($n = 100$ plants $species^{-1}$ $site^{-1}$) was counted during morning low tide at each site. Statistical differences in *L. irrorata* distributions among plant species were analyzed using a one-way ANOVA with Tukey's HSD test. We used GraphPad Prism 6 (Graph Pad Software, Inc.) and R (R Development Core Team) for all statistical tests throughout this study.

2.2. Quantification of fungi

We extracted ergosterol, a cell membrane component found only in fungi that are commonly used to estimate fungal load on plant tissues (Newell et al., 1988; Seitz et al., 1979), from plant samples collected in July 2011 at the aforementioned survey site adjacent to the marine institute. Ten to 15 individuals from each of the five surveyed plants were cleaned and cut into 2 cm segments to maximize extraction efficiency. Segments were pooled among individuals, divided into groups of five segments ($n = 8$ $species^{-1}$), and extracted sensu Newell et al. (1988). For *S. alterniflora*, segments were also categorized based on whether or not they displayed visual evidence of fungal infection such as yellowing or blackened tissue. After partitioning crude refluxed extracts, the lipophilic layer (containing ergosterol) was transferred to a scintillation vial, dried, and resuspended in Optima grade methanol for analysis by liquid chromatography-mass spectrometry (LC-MS). Separations were conducted on analytical Grace C_{18} silica columns with an aqueous methanol and 2% acetic acid mobile phase, using Waters HPLC and UV detection hardware and a Micromass ZQ mass spectrometer. Ergosterol was quantified based on characteristic UV absorption of peaks at 282 and 293 nm, as well as detection of the $[M + H]^+$ ion at m/z 397. Integrated mass peak areas at m/z 397 were compared to a standard curve generated from commercial 95% ergosterol (Sigma-Aldrich) to determine ergosterol content relative to dry plant tissue mass.

2.3. Herbivore habitat choice assays

In the field, *L. irrorata* was observed most frequently on blades of *S. alterniflora*, which was also the most abundant plant in marsh surveys. To determine whether snails were distributed according to plant abundance or preferentially resided on specific plant species, we conducted a mesocosm choice assay in May 2012. Mesocosms were filled with a layer of marsh sediment and estuarine water ($n = 5$), and covered with a mesh screen lid. Two shoots of each plant species on our panel were randomly placed in each mesocosm, keeping plant biomass constant among species. All plant samples were placed intact apart from *I. frutescens*, which was too large to fit into our mesocosms. Instead, two fresh clippings were used to represent *I. frutescens*. Fifty *L. irrorata* collected in marshes adjacent to the marine institute were added to the center of each mesocosm.

Over 36 h, snail distributions among plants were recorded three times and averaged to provide an estimate of snail habitat preference.