CHEMICALLY-MEDIATED INTERACTIONS IN SALT MARSHES: MECHANISMS THAT PLANT COMMUNITIES USE TO DETER CLOSELY ASSOCIATED HERBIVORES AND PATHOGENS

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CHEMICALLY-MEDIATED INTERACTIONS IN SALT MARSHES: MECHANISMS THAT PLANT COMMUNITIES USE TO DETER CLOSELY ASSOCIATED HERBIVORES AND PATHOGENS

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To my parents, for being a source of encouragement,
to my wife, for reminding me to eat,
and to Harold, it's been a blast.

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SUMMARY

Herbivores and pathogens pose a consistent threat to plant productivity. In response, plants invest in structural and/or chemical defenses that minimize damage caused by these biotic stressors. In salt marshes along the Atlantic coast of the United States, a facultative mutualism between snails (*Littoraria irrorata*) and multiple species of fungi exert intense top-down control of the foundation grass species *Spartina alterniflora*. Snails facilitate fungal infection by depositing fungal spores into freshly grazed plant tissues, and selectively consume the fungus as it progressively infects the plant. Although these multitrophic interactions have existed in salt marshes for long enough to exert selection pressure on all parties involved, losses of top marsh predators such as blue crabs has threatened the trophic cascade that historically kept snail populations at least partly in check. As a result, the negative repercussions of the facultative mutualism between snails and pathogens on salt marsh communities have increased in severity.

Since exposure to herbivores and pathogens are tightly coupled in this system, I investigated whether *S. alterniflora* utilizes chemical and/or structural defenses to deter both groups within this farming mutualism (snails and fungi), and examined how plant defenses varied among *S. alterniflora* individuals and populations. I also assessed how other marsh plants prevent snails from establishing farms, and considered whether interspecific variation in plant chemical defenses influences marsh community structure.

Initial experiments revealed that *S. alterniflora* chemical defenses inhibited *L. irrorata* and two fungi that snails commonly farm. However, the magnitude of these

interactions varied between tall and short growth forms of *S. alterniflora,* such that short form plants regularly exposed to higher snail densities were constitutively defended against grazers and pathogens, while tall form *S. alterniflora* that experience lower herbivore pressure were not chemically defended. A caging experiment determined that production of short form *S. alterniflora* chemical defenses could not be induced in the presence of snails and fungi, nor relaxed in their absence. Through separations chemistry guided by ecological assays, we isolated two distinct classes of chemical defenses from short form *S. alterniflora*, one of which inhibited fungal growth and the other decreased plant palatability. Spectroscopic analysis of these molecules determined that a suite of phenolic compounds, including the flavonoid glycoside orientin reduced *S. alterniflora* palatability to snails, whereas a fatty acid (α-dimorphecolic acid) inhibited growth of a fungal pathogen (*Mycosphaerella* sp.). In lab assays, natural concentrations of α-dimorphecolic acid from whole plant extracts were capable of preventing *Mycosphaerella* sp. growth, but concentrations of the fatty acid on plant surfaces were not. Another fungus (*Phaeosphaeria spartinicola*) was inhibited by *S. alterniflora* crude extracts, but not α-dimorphecolic acid at natural concentrations, suggesting that additional molecules may defend *S. alterniflora* against other fungal pathogens.

In a community context, the chemical defenses produced by *S. alterniflora* were relatively weak compared to those of four other salt marsh plant species, which produced compounds that completely inhibited *L. irrorata* grazing and strongly hindered fungal growth in lab assays. Nutritional and structural differences among marsh plants did not influence feeding preferences, suggesting that differences in plant chemistry were the primary driver for food selection by snails. In field surveys and mesocosms *L. irrorata*

was overwhelmingly found residing on *S. alterniflora*, and evidence of fungal farming was only detected on *S. alterniflora* tissues. Despite being a susceptible target to snails and fungi, *S. alterniflora* was the most abundant plant in surveyed marshes. It appears that *S. alterniflora* produces weak chemical defenses that slow down or limit fungal growth and snail herbivory, and may compensate for tissue losses by producing new growth. In contrast, less abundant marsh plants express chemical defenses that completely inhibit fungal farming and deter snail grazing, but doing so may come at a cost to growth or competitive ability against *S. alterniflora*.

Our results apply to southern salt marsh plant populations that are exposed to exceptionally high rates of herbivory, and it remains to be seen how abiotic stressors and genetic differences among marsh plant populations also alter expression of chemical defenses in conjunction with biotic threats. Since our studies were conducted in the summer season, we cannot say whether plant investment in chemical defenses would have been greater earlier in plant ontogeny in order to defend developing plant tissues. However, as marsh dieback continues with rising herbivore densities and compounding abiotic stressors, the ecosystem services that these environments provide may be lost. Therefore, understanding how and under what conditions salt marsh plants resist losses to herbivores and pathogens will help predict which marsh communities are most likely to be threatened in the future.

CHAPTER 1

INTRODUCTION

Top-down control of plant productivity by herbivores can fundamentally structure communities in marine, terrestrial, and freshwater systems (Hairston et al. 1960; Carpenter et al. 1985). To prevent or resist herbivory, plants can allocate finite resources towards growth to compensate for consumed tissue, or invest in chemical or structural defenses that prevent damage from occurring (Herms and Mattson 1992). Many environmental factors, including nutrient availability, herbivore identity and density, and the presence of predators that control herbivore populations influence the type and quantity of defenses that are used to protect plant biomass. Producing defenses is assumed to be costly to plants, because they divert resources that could have otherwise been used for primary plant functions (Koricheva 2002). A variety of hypotheses have been postulated to clarify when plants are most frequently defended (Feeny 1976; Coley et al. 1985; Herms and Mattson 1992), and these theories continue to be debated (Stamp 2003). In general, plant traits that deter herbivores should be favored if the threat of herbivory is high, while employing such defense strategies when plant growth is not controlled by herbivores can severely reduce plant fitness (van Dam and Baldwin 1998; van Dam et al. 2000).

In some instances, facilitation between organisms can significantly increase top-down pressure exerted on plants, which can in turn lower species richness or decrease overall community productivity (O'Dowd et al. 2003; Silliman et al. 2005). For instance, a facultative mutualism between invasive yellow crazy ants (*Anoplolepis gracilipes*) and scale insects has led to an "invasional meltdown" of Pacific islands (O'Dowd et al. 2003).

The ants protect scale insects from predation in exchange for honeydew, and higher densities of both insects led to reductions of omnivorous crab populations by ant aggression and canopy tree die off due to scale insect feeding (O'Dowd et al. 2003). The negative impacts of these facultative mutualists have reduced the abundance and richness of native flora and fauna, and paved the way for subsequent invasion by other exotic species (O'Dowd et al. 2003).

In salt marshes, grazing damage from the periwinkle snail *Littoraria irrorata* creates wound sites on the dominant marsh grass, *Spartina alterniflora* (Silliman and Newell 2003). Snails then facilitate subsequent fungal infection of the plant by defecating feces rich in fungal spores into damaged plant tissues, and then tend and consume fungal "gardens" as they develop (Silliman and Newell 2003). The loss of snail predators enhances the top-down control exerted by fungal farms on marsh communities (Silliman and Bertness 2002), which is compounded by abiotic stressors such as drought (Silliman et al. 2005). These interactions highlight another instance in which a facultative mutualism leads to the collapse of a productive ecosystem.

Salt marsh plants are capable of using chemical defenses to deter a variety of vertebrate and invertebrate grazers, including birds (Buchsbaum et al. 1984), crabs (Siska et al. 2002), snails (Hendricks et al. 2011; Long et al. 2011), and insects (Siska et al. 2002). The defenses produced by these plants tend to be more potent in marshes that have an intense, constant threat of herbivory (Pennings et al. 2001; Siska et al. 2002). In some cases these defenses can be induced by herbivores (Long et al. 2011), suggesting that plants cue into when future damage is likely to occur, and adjust investment in defenses accordingly. However, we know very little about the mechanisms plants use to

inhibit fungal pathogens in these systems. Furthermore, the compounds used in defense are currently unknown, but are frequently attributed to broad classes of molecules such as phenolics (Buchsbaum et al. 1984). To date, no pure compounds responsible for antiherbivore or antifungal activity have been identified from salt marsh plants, although a subset of common phenolic compounds found in *S. alterniflora* detritus can reduce grazing by marsh herbivores (Valiela et al. 1979; Valiela and Rietsma 1984).

Salt marsh plants frequently exposed to fungal farming may simultaneously defend against *L. irrorata* farmers and their fungal crop, which could reduce the resources allocated towards primary plant functions. Since the threat posed by herbivores and pathogens are tightly coupled in this system, one strategy would be to produce a broad chemical defense capable of deterring both threats simultaneously, similar to how chemical defenses employed by some diatoms (Ianora and Miralto 2010), terrestrial plants (Biere et al. 2004) and algae (Schmitt et al. 1995) are effective against microbes, herbivores, and competitors. A generalized defense should reduce mechanistic or energetic costs associated with defense, but could prove ineffective if either partner in the mutualism is able to resist the defense. Alternatively, plants could produce multiple classes of chemical defenses to target either herbivores or pathogens, although this strategy has the potential to be energetically costlier to the plant.

The plant most frequently farmed by *L. irrorata* is the dominant marsh grass *S. alterniflora* (Silliman and Newell 2003). Previous studies have shown that *S. alterniflora* is chemically defended against herbivores (Siska et al. 2002, Long et al. 2011), but to date no studies have determined whether *S. alterniflora* chemical defenses can also inhibit fungal growth. Since top-down control from fungal farms can lead to massive

losses in *S. alterniflora* biomass (Silliman et al. 2005), I hypothesized that *S. alterniflora* should produce chemical defenses against both snails and fungi if the threat of farming is severe. Furthermore, the allocation of these defenses among plant tissues or populations can be addressed by identifying the specific molecules involved in *S. alterniflora* defense. However, since snails commonly reside on *S. alterniflora* and farms are rarely observed on other plant species, I examined how other members of the salt marsh plant community respond to the threats imposed by herbivores and fungi. Examining the different strategies plants use to defend against multiple threats could in turn enhance our understanding of the factors that influence when, where and how species are chemically defended.

The following chapters of my dissertation examine how plant chemical defenses against herbivores and pathogens influence salt marsh community structure. I first review the state of the field for chemically mediated interactions among marine angiosperms. Mangroves, seagrasses, and salt marsh plants at the interface of marine and terrestrial systems experience a wide variety of biotic and abiotic challenges, and provide unique opportunities to test new hypotheses in chemical ecology. In the chapters that follow this review, I discuss the results of my research investigating mechanisms of plant defense at the species and community level against tightly coupled herbivores and pathogens. I conclude with perspectives for future research regarding allocation of plant chemical defenses at individual, population, and community scales.

CHAPTER 2

CHEMICAL ECOLOGY OF MARINE ANGIOSPERMS: STATE OF THE FIELD AND OPPORTUNITES AT THE INTERFACE OF MARINE AND TERRESTRIAL SYSTEMS

Abstract

This review examines the state of the field for chemically mediated interactions between marine angiosperms (seagrasses, mangroves, and salt marsh angiosperms) and their environments. Small scale interactions among marine angiosperms and herbivores, pathogens, fouling organisms, and competitors are explored, as are the roles of exudates from these plants in settlement and development of larval fishes and invertebrates. Ecosystem level effects are also examined including how antifeedant compounds alter herbivore behavior following plant senescence, as well as how plant secondary metabolites influence interactions across multiple trophic levels. While our understanding of chemical ecology of marine angiosperms lags behind that of terrestrial and other marine organisms, it is evident that ecological interactions among individual plants, populations, and communities are shaped by chemical cues and signals. Throughout this review, we point out areas of need for future study, and highlight opportunities for creative new directions in chemical ecology.

Introduction

Plants utilize chemical compounds to attract pollinators, deter herbivores, signal parasitoids of herbivores, inhibit competitors, and prevent establishment of microbial pathogens. Such interactions are well documented in terrestrial and marine systems (see comparison by Hay and Steinberg 1992), yet there are surprisingly few studies investigating chemically mediated interactions at the interfaces of these environments. Mangroves, seagrasses, and salt marsh plants are more similar taxonomically and physiologically to terrestrial plants than to other marine autotrophs (Soltis et al. 2008; Wissler et al. 2011) and face a unique series of challenges in marine habitats. For instance, marine angiosperms interact with diverse herbivores, including fish (Goecker et al. 2005; Prado and Heck 2011), crustaceans (Camilleri 1989; Siska et al. 2002), echinoderms (Verges et al. 2007a), gastropods (Silliman and Zieman 2001; Hendricks et al. 2011), arthropods (Denno et al. 2000; Pennings et al. 2009), reptiles (Moran and Bjorndal 2007), mammals (Preen 1995; Lefebvre et al. 2000) and waterfowl (Buchsbaum and Valiela 1987), representing a wider variety of grazers than terrestrial counterparts are likely to encounter. There is mounting evidence supporting the importance of chemical cues in mangroves, seagrasses, and salt marsh habitats, although the compounds responsible for such interactions have only been characterized in a handful of studies (Jensen et al. 1998; Qi et al. 2008; Sieg Chapter 3).

Phenolic compounds fulfill a range of primary and secondary roles for aquatic angiosperms, including cell wall support, osmoregulation, and defense against herbivores, pathogens, and fouling organisms (see review by Arnold and Targett (2002)). Understandably, marine angiosperm chemical defenses are frequently attributed to

complex mixtures of tannins or other phenolics, as it can be difficult to resolve which specific molecule(s), among a complex mixture of similar compounds, are responsible for a particular ecological effect. However, since minor differences in the chemical structure of phenolics can lead to dramatic changes in ecological function (Appel 1993; Salminen and Karonen 2011), researchers should transition from using this class of compounds as a catch-all metric for chemical defense. For instance, within herbivore digestive tracts, condensed tannins and gallotannins reduce plant nutritive quality by precipitating proteins (Feeny 1970; Appel 1993) whereas ellagitannins can cause oxidative stress (Feeny 1970; Appel 1993; Salminen et al. 2011). Furthermore, the protein-precipitating efficacy of tannins largely depends on pH conditions within the herbivore, meaning that some tannins may be rendered useless in the more alkaline guts of herbivorous insects such as caterpillars (Appel 1993; Harrison 2001). Therefore, a more specific classification of defensive molecule identity and subsequent determination of when and why they are produced will allow researchers to accurately measure plant investment in chemical defenses against a variety of herbivores, pathogens, and other threats.

The purpose of this review is to present the state of the field for marine angiosperm chemical ecology, and by doing so, clarify opportunities and new directions for future research. The review is organized into sections examining plant-herbivore and plant-pathogen interactions, chemically mediated competition (also known as allelopathy), chemical cues affecting larval settlement, and community or ecosystem level effects of chemically mediated interactions. Within each section, we discuss the areas of most critical need, highlight new avenues in chemical ecology that are amenable for study with marine angiosperms, and explore how chemical cues are linked among

different ecological interactions. Our review focuses on chemical ecology within salt marsh, seagrass, and mangrove systems, but we also wish to point out a few previous reviews of note for interested readers. Arnold and Targett (2002) provide an excellent review comparing the ecological roles of phenolics in marine vascular and non-vascular plants. Heck and Valentine (2006) discuss interactions between seagrasses and herbivores. Gopal and Goel (1993) as well as Gross (2003) touch upon seagrass chemical defenses in the broader context of macrophyte competition in marine and freshwater systems. Reviews on advances in marine benthic chemical ecology, including interactions within seagrass and mangrove habitats are published roughly every two years (Paul et al. 2011). Wu et al. (2008a) provide an extensive review on natural products of mangroves, including their biomedically and agriculturally relevant activities.

Interactions Between Marine Angiosperms and Herbivores

Plant-Herbivore Interactions in Salt Marshes

For decades, the prevailing view was that bottom-up forces controlled salt marsh production (Odum and De La Cruz 1967), and the effects of grazers on plant productivity were considered negligible (Smalley 1960; Teal 1962). However, several studies have shown that salt marshes can be top-down controlled, with grazing by a relatively small number of herbivore species having drastic impacts on marsh productivity (Silliman et al. 2005; Sala et al. 2008). Increasing evidence supporting top-down regulation of salt marshes has created renewed interest in plant defenses against herbivores in these systems (Long et al. 2011; Sieg Chapter 4). Given that salt marshes are declining worldwide (Gedan et al. 2009), it is important to integrate how bottom-up factors such as eutrophication (Deegan et al. 2012) and drought (Silliman et al. 2005) interact with top-down regulators such as trophic cascades (Silliman and Bertness 2002; Marczak et al. 2011) to determine the overall productivity of these ecosystems (Denno et al. 2002; Silliman and Bertness 2002; Silliman et al. 2005; Marczak et al. 2011).

The factors that influence gastropod consumption of salt marsh plant detritus have received a fair amount of attention, given that gastropods have traditionally been viewed as important detritivores in salt marsh systems (Rietsma et al. 1982; Zimmer et al. 2004). Consumption of *Spartina alterniflora* detritus by the snail *Littoraria irrorata* increased as cinnamic acids such as ferulic acid (**1**) and *p*-coumaric acid (**2**) leached from plant tissues (Valiela et al. 1979), and these compounds influenced snail preferences for aged detritus over senescing blades (Barlocher and Newell 1994b). Specifically, polar detritus extracts embedded into artificial diets inhibited *L. irrorata* grazing to a similar degree as diets

containing **1** (Barlocher and Newell 1994b). Increasing concentrations of **1** and **2** in artificial diets decreased grazing by the snail *Melampus bidentatus* and two isopod species in a dose-dependent manner (Valiela et al. 1979, Valiela and Rietsma 1984). Increasing the nitrogen content and pH of artificial *S. alterniflora* diets or decreasing diet salinity also made diets more palatable to snails (Valiela and Rietsma 1984), suggesting that *M. bidentatus* is capable of selecting diets based on nutritional as well as defensive traits (Valiela and Rietsma 1984; Valiela et al. 1984; Rietsma et al. 1988). Snails readily consumed diets containing **1** when nitrogen content was high, but not when nitrogen content was low (Valiela and Rietsma 1984). Thus, the nutritional quality of the plant was a more important factor for snails than overall plant palatability. These studies chose to examine **1** and **2** because they were the most abundant phenolic compounds in decaying tissues. Chemical defenses of living tissues were not considered in these studies, which is unfortunate considering that defending detritus provides no fitness advantage to the plant, whereas chemical traits that limit herbivory on live tissues should be positively selected over evolutionary time.

A variety of traits limit damage to salt marsh plants caused by invertebrate mesograzers, although the relative impacts of chemical, structural, and nutritional plant traits appears to vary among herbivore species. In most studies, polar plant chemical defenses along with tougher tissues reduced feeding by invertebrates, while increased nitrogen content often increased plant palatability (Valiela and Reitsma 1984; Pennings et al. 1998; Siska et al. 2002; Sieg Chapter 4). In choice assays, the crab *Armases cinereum* consistently preferred marsh plants with softer tissues but were less discriminatory when plant structure was removed or plant extracts were embedded in artificial diets (Pennings

et al. 1998). These data suggest that tissue toughness deters generalist omnivores like *A. cinereum* that have a wide diet breadth, possibly because they lack the specialized appendages that some specialists use against tougher plants (Pennings et al. 1998). In contrast, moderately polar chemical defenses reduced grazing by the snail *L. irrorata* on the invasive reed *Phragmites australis* (Hendricks et al. 2011) and *S. alterniflora* (Long et al. 2011; Sieg Chapter 3). Polar extracts from a panel of other marsh plants completely inhibited *L. irrorata* herbivory, while snails did not discriminate among diets based on nutritional or structural characteristics (Sieg Chapter 4). A mixture of phenolic compounds isolated from *S. alterniflora* including the flavonoid glycoside orientin (**3**) reduced snail herbivory (Sieg Chapter 3), although these chemical defenses were weaker than unknown deterrent compounds of plants growing in proximity to *S. alterniflora* (Sieg Chapter 4). Because *L. irrorata* grazing frequently facilitates fungal infection (see further discussion in community and ecosystem effects section), it should be highly advantageous for plants to limit damage caused by these snails.

It is unclear whether investment in chemical defenses comes at a cost to other plant functions, such as growth or competitive ability. For instance, *S. alterniflora* is a dominant foundation species within coastal Atlantic marshes in the United States despite being more susceptible to snail herbivory and fungal infection than other co-occurring plants (Sieg Chapter 4). *Spartina alterniflora* is found at lower marsh elevations due to competitive exclusion from higher elevations by other marsh plants (Bertness 1991; Emery et al. 2001), but is also far more tolerant of the anoxic sediments associated with lower marsh zones than other species (Howes et al. 1986; Bertness 1991). To thrive in these habitats, *S. alterniflora* utilizes physiological mechanisms to cope with a stressful

habitat (such as salt excretion and soil oxidation), which is expected to reduce the resources available for chemical defenses. In contrast, plants residing in less physically stressful marsh zones may have proportionally more resources to be allocated to secondary plant functions including chemical defense. Thus, inherent differences in salinity tolerance or nutrient acquisition may limit plant investment in defense, which could increase how susceptible plants are to grazers and pathogens. However, it is also possible that rapid growth (Schubauer and Hopkinson 1984; Houghton 1985) allows *S. alterniflora* to withstand herbivory and invest less in chemical defenses than other marsh species that are restricted to higher marsh elevations. Given that invertebrates select among plants from different soil salinities or marsh elevations (Bowdish and Stiling 1998; Goranson et al. 2004), plant trade-offs between primary production, stress tolerance, and defense could determine to what extent marsh plants are susceptible to herbivory.

Geese are deterred by salt marsh plant chemical defenses, although there are relatively few studies investigating these defenses in relation to other plant traits. Based upon field observations and feeding assays using 15 plant species, captive Canada geese typically consumed plant species with low phenolic content (< 2% by dry mass), whereas nutritional differences among species did not influence which plants were preferred (Buchsbaum et al. 1984). In other studies, seasonal variation in nutrient content and digestibility of plant tissues also affected which plants were consumed by geese (Buchsbaum et al. 1986; Buchsbaum and Valiela 1987). When coated on an artificial diet, polar extracts containing phenolics from three non-preferred marsh plants (*Limonium carolinianum, Salicornia europaea,* and *Solidago sempervirens*) were less

palatable to geese than controls, while lipophilic compounds from a fourth species (*Salicornia bigelovii*) were also deterrent (Buchsbaum et al. 1984). Geese also avoided artificial foods coated with tannins or mixtures of phenolic acids including **1** and **2** (Buchsbaum et al. 1984). Phenolic compounds deter grazing by geese, as do other less polar compounds, which could explain why phenolic content did not positively correlate with antifeedant properties for all surveyed species (Buchsbaum et al. 1984). In a separate study in the field and in aviaries, geese overwhelmingly preferred native *Spartina foliosa* to an exotic hybrid *Spartina* when presented with whole plants or plants embedded in turf, but could not distinguish between clippings of the two species (Grosholz 2010). Geese did not discriminate based upon chemical and nutritional differences among species; instead birds selected diets based on how easily they could be pulled from marsh sediments (Grosholz 2010). The invasion of the hybrid *Spartina* therefore is facilitated by its ability to withstand tugging strain by geese, highlighting a mechanism of structural defense that is easily overlooked when examining plant defenses using small mesograzers.

Due to the remarkably similar plant species pool from northern to southern latitudes (Pennings and Bertness 2001), salt marshes form an attractive system to compare production of chemical defenses across a range of environmental conditions. In a recent meta-analysis, salt marshes were one of the only systems in which the trend for greater herbivory and higher investment in defenses in lower latitudes was consistently supported (Moles et al. 2011). Many marshes from northern sites experience lower herbivore pressure, shorter growing seasons, and reduced plant investment in chemical defenses relative to southern counterparts (Pennings et al. 2001; Siska et al. 2002;

Salgado and Pennings 2005; Pennings et al. 2009). Herbivore abundance, densities, and per capita grazing rates are far greater in low latitude marshes (Silliman and Bortolus 2003; Pennings and Silliman 2005; Pennings et al. 2009), which may have selected for resistance mechanisms produced by low latitude plant populations. High latitude plants transplanted into southern marshes experienced up to 100 times greater grazing damage than did native low latitude plant populations (Pennings et al. 2009), which was likely caused the inability of weakly defended northern plants to resist or repel herbivores. When offered a choice of plants collected from high or low latitude marshes in feeding assays, herbivores typically preferred plants from high latitudes, which can be caused by greater investment in structural and chemical defenses by low latitude plants (Pennings et al. 2001; Siska et al. 2002). For instance, polar plant chemical defenses, as well as tougher tissues and lower nitrogen content reduced grazer preferences for ten low latitude marsh plant species over high latitude conspecifics (Siska et al. 2002). In common garden experiments, the reduced palatability of low relative to high latitude plants can persist for up to four generations, suggesting that traits that limit herbivory are controlled by genetic differences among plant populations (Saldago and Pennings 2005). While the above studies focused on salt marshes along the Atlantic coast of the United States, crabs in western European marshes also selectively consumed high latitude plants more frequently than low latitude plants (Pennings et al. 2007). Collectively, these data support the hypothesis that plants from lower latitudes are less palatable to herbivores than their high latitude counterparts. The consistency of flora and fauna within a wide latitudinal range of marsh habitat, coupled with evidence for population level variation in

plant chemical defenses make salt marshes an excellent system for testing the influence of environmental factors in altering production of defenses.

The dominant marsh grass *S. alterniflora* can alter expression of both primary and secondary metabolites in response to increasing herbivory pressure, changing how future interactions between plants and herbivores will occur. For instance, the concentration of essential amino acids in *S. alterniflora* decreased in response to grazing by the planthopper *Prokelisia dolus*, which reduced overall nutritional quality of *S. alterniflora* tissues and changed distribution patterns and development of other planthoppers that compete with *P. dolus* (Olmstaed et al. 1997; Denno et al. 2000). In mid latitude marshes where grazing intensity is moderately high, exposure to a natural suite of herbivores resulted in *S. alterniflora* chemical defenses that were 50 % more deterrent to *L. irrorata* relative to caged control plants, whereas no evidence of chemical defenses were observed in high latitude sites where herbivory pressure is less intense (Long et al. 2011). Total phenolic content did not predict differences in palatability, suggesting that specific compounds (individual phenolic compounds or members of other molecular classes) were responsible for feeding behavior (Long et al. 2011). In a similar study conducted in low latitude marshes where snail grazing is up to three times higher than in mid latitude marshes (Silliman and Newell 2003), *S. alterniflora* was constitutively defended against both grazers and fungi even when herbivores were removed for four weeks from the system (Sieg Chapter 3). Collectively, these data highlight that there is a gradient in plant responses to herbivory, whereby populations of *S. alterniflora* are relatively undefended in high latitudes (Siska et al. 2002; Long et al. 2011), exhibit induced chemical defenses in mid latitudes (Long et al., 2011), and are constitutively chemically

defended in low latitudes (Siska et al. 2002; Sieg Chapter 3). These studies suggest that *S. alterniflora* populations are capable of responding to changing herbivore pressure over relatively short periods of time, but it is unclear how other environmental factors along a latitudinal gradient also cause plants to upregulate production of chemical defenses. It is also possible that *S. alterniflora* responds to these threats differently during plant ontogeny, which would not have been easily detected during shorter induction studies.

Plant-Herbivore Interactions Involving Seagrasses

There is growing evidence that chemical defenses play an important role in defending seagrasses against marine herbivores, although few examples of specific antiherbivore compounds exist. In surveys of Pacific Ocean macrophyte chemical defenses, crude extracts of the seagrass *Enhalus aeoroides* deterred feeding by both orangespine surgeonfish (Meyer et al. 1994) and adult, but not juvenile rabbitfish (Paul et al. 1990). Crude extracts of the Mediterranean seagrass *Posidonia oceanica* reduced grazing by multiple sea urchin species and a natural fish assemblage, but had no effect on gastropod feeding behavior (Verges et al. 2007a). Many studies have described structures of secondary metabolites or measured concentrations of groups of compounds that are hypothesized to act as plant defenses in seagrasses, without examining the ecological role(s) of these compounds. For instance, the presence or absence of eight phenolic acids common to terrestrial plants were reported among blades and rhizomes of 25 Caribbean and Pacific seagrasses (Zapata and McMillan 1979). In this broad survey, six phenolic acids (including **1** and **2**) were found in over 50% of seagrass species, while *p*-hydroxybenzoic acid (**4**) was found in all 25 species (Zapata and McMillan 1979).

Therefore, the pool of phenolic acids that could play a role in defense is relatively similar among seagrasses and closely aligns with common compounds found in terrestrial plants. Similar studies have also conducted surveys of which seagrasses produce condensed tannins (McMillan 1984) and sulfated phenolics (McMillan et al. 1980; McMillan 1986). Other studies have identified and quantified specific phenolic compounds in individual seagrass species including *P. oceanica* (Agostini et al. 1998)*, Zostera marina* (Quackenbush et al. 1986), *Thalassia hemprichii* (Qi et al. 2012), and *Halophila johnsonii* (Meng et al. 2008). While a pool of common phenolic acids is associated with many seagrass species, these common compounds appear to be produced as part of blends unique to individual species; in addition, some species produce unusual compounds. However, these studies have typically not explored the ecological function(s) of these compounds, a limitation that has been raised in recent reviews on seagrass ecology (Heck and Valentine 2006). Given that certain phenolics are conserved across seagrass taxa while others are more species-specific, seagrasses could provide a tractable system to examine how expression of chemical defenses has diverged across evolutionary time in response to changing environmental conditions and emerging threats.

In a few instances the ecological roles for seagrass phenolics have been described, including reducing plant palatability to herbivores (e.g. Harrison 1982) and inhibiting microbial settlement or growth (e.g. Todd et al. 1993; Jensen et al. 1998). Concentrations of total phenolic compounds often negatively correlate with seagrass tissue losses due to herbivory, although other traits such as tissue toughness or lower nutritional quality are also associated with lower rates of herbivory (Goecker et al. 2005; Prado et al. 2010;

Prado and Heck 2011; Tomas et al. 2011). Ultimately, herbivores select a preferred food item based on more than a single trait. For instance, fertilized turtlegrass (*Thalassia testudinum*) blades with higher nitrogen and lower phenolic content were consumed by bucktooth parrotfish by a five to 20 fold margin over unfertilized controls (Goecker et al. 2005). Since nitrogen content and total phenolic concentration co-varied in this experiment, it was unclear whether turtlegrass nutritive value, defensive traits, or both influenced parrotfish feeding preferences. The authors speculated that phenolics could act as an olfactory and/or gustatory cue, in which case higher concentrations of phenolics in less nutritious tissues may convey low relative quality of food items to herbivores (Goecker et al. 2005). Nutrient enrichment and exclusion of larger herbivores also stimulated feeding by a natural fish assemblage on *T. testudinum* (Olsen and Valiela 2010). Phenolics were not quantified among treatments and no explicit tests of chemical defenses were conducted in this study (Olsen and Valiela 2010), therefore it is unclear whether increased grazing rates were a result of reductions in chemical defenses or if the fish assemblage simply preferred a more nitrogenous diet with a reduced risk of predation.

Mesograzers are able to discriminate among individual blades of the same species to consume the most nutritious or palatable seagrass genotypes. In choice assays, isopods preferred *Z. marina* individuals with a lower C:N ratio and low phenolic content, while removal of plant structure did not change which *Z. marina* genotypes were preferred by isopods (Tomas et al. 2011). Surprisingly, nitrogen enrichment reduced plant palatability relative to unfertilized controls from the same genotype but did not alter isopod preferences among genotypes (Tomas et al. 2011). Because isopods were unable to

distinguish among fertilized or unfertilized tissues once plant structure was removed, and since nutrient enriched blades were tougher than non-fertilized plants, the authors speculated that enrichment made blades less palatable due to changes in structural, but not chemical traits (Tomas et al. 2011). Therefore, the combination of higher nutritional quality and lower quantities of chemical defenses predicted which *Z. marina* genotypes were most favored by isopods, while nutrient enrichment reduced overall plant palatability due to increases in plant toughness (Tomas et al. 2011).

In some cases, the astringent tastes or reduced digestibility of seagrasses putatively caused by phenolic compounds is outweighed by the benefit gained after consuming tissues that are highly nutritious. For instance, the fish *Sarpa salpa* preferred young *P. oceanica* blades that were nitrogen rich despite nearly two-fold higher phenolic concentrations in young tissues compared to older tissues (Verges et al. 2011). Additionally, the sea urchin *Paracentrotus lividus* preferred softer, less nutritious inflorescences when offered a choice between reproductive or vegetative *P. oceanica* tissues, even though phenolic concentrations were higher in inflorescences than leaves (Verges et al. 2007b). Removal of plant structure reversed this trend (Verges et al. 2007b), further suggesting that plant chemical defenses are less important to *P. lividus* than seagrass nutritional or physical structural characteristics. In a different study, nutrient enrichment coupled with high algal epiphyte loads stimulated *S. salpa* grazing on *P. oceanica* relative to non-enriched plants, although chemical defenses were not assessed in this study (Prado et al. 2010). In contrast, older *P. oceanica* tissues with lower phenolic and nutritional content were preferred by *P. lividus* over young tissues, possibly because epibionts coating older tissues functioned as an additional nutritional

source (Verges et al. 2011). For sea urchins, the degree of epibiont fouling on seagrass blades may be a more reliable indicator of preferred food items than differences in seagrass phenolic content (Verges et al. 2011). However, epibionts may also deter herbivores if the epibionts themselves are chemically defended (Vervoort et al. 1998). Thus, it appears that chemical and nutritional characteristics of both seagrasses and their surrounding epibiont community determine how susceptible seagrasses are to herbivory, similar to the "shared doom" effect seen in marine algae (Wahl et al. 1995). While several mechanisms that seagrasses employ to limit fouling are documented (see further discussion in the interactions of marine angiosperms with pathogens and fouling organisms section), it is currently unclear whether seagrasses are capable of recruiting chemically defended epibionts as a means to reduce herbivory on plant surfaces.

Many herbivores discriminate among seagrasses based on structural characteristics such as blade morphology or toughness, but the relative importance of these traits varies among herbivore guilds. When offered as whole tissues, omnivorous pinfish and filefish preferred seagrass species with thinner, narrower blades, but could not distinguish among three seagrasses if plant structure was removed (Prado and Heck 2011). In contrast, herbivorous parrotfish and sea urchins consistently consumed the most nutrient rich seagrasses when offered in either form (Prado and Heck 2011). Thus, structural features influenced food selection by omnivorous fish, whereas strict herbivores were more sensitive to nutritional differences among seagrasses (Prado and Heck 2011). Since seagrasses are exposed to a wide variety of herbivores (Lefebvre et al. 2000; Moran and Bjorndal 2007; Verges et al. 2011), caution is recommended when

considering when, where, and how these plants utilize chemical defenses in relation to other defensive traits.

In some cases, repeated exposure to herbivores can lead to induction of seagrass phenolic compounds. Mechanical wounds that simulated parrotfish grazing induced higher total concentrations of condensed tannins in older, second rank *T. testudinum* blades, although tannin concentrations were not elevated in younger first rank blades (Arnold et al. 2008). Neither exposure to jasmonic acid (a common signal used by terrestrial angiosperms to elicit a systemic defense response), cues from grazed conspecifics, nor sea urchin grazing resulted in a similar induction of tannins in leaves (Arnold et al. 2008). However, sea urchin grazing did stimulate a four-fold increase in condensed tannin concentrations within rhizome tissues (Arnold et al. 2008). Urchin grazing also led to elevated tannin content in *Halodule wrightii* and *T. testudinum*, as well as increased local concentrations of **4** near grazing wound sites in *T. testudinum* (Steele and Valentine 2012). Higher phenolic concentrations in seagrass diets did not deter urchin herbivory, but could have had an effect on smaller mesograzers (Steele and Valentine 2012). In a related study, concentrations of condensed tannins, nitrogen, and phosphorus increased in *T. testudinum* blades that were clipped to simulate green turtle grazing (Moran and Bjorndal 2007). It is unclear if induced tannin concentrations would deter future grazing events, since the benefit of more nutritious tissues could outweigh any decreases in plant palatability resulting from enhanced chemical defenses. Rhizome nutrient stores also decreased with repeated exposure to mechanical damage (Moran and Bjorndal 2007), and it is possible that prolonged grazing would eventually deplete remaining *T. testudinum* nutrient reserves (Moran and Bjorndal 2007). In contrast,

moderate simulated fish grazing decreased phenolic content in *P. oceanica* blades, suggesting that herbivore-induced stress reduced concentrations of phenolic compounds in vegetative tissues (Verges et al. 2008). Damaged and undamaged blades were equally palatable to fish and urchins, so differences in phenolic content did not alter herbivore preferences in this study (Verges et al. 2008). Instead, the authors suggested that grazing resulted in reallocation of plant resources away from phenolics and towards other primary plant functions, namely compensatory growth to account for tissue losses from herbivory (Verges et al. 2008). Unlike similar studies conducted using salt marsh plants (Long et al. 2011), it is unclear whether induced changes in seagrass chemistry impart a defensive function to seagrasses. Therefore, there is potential for induced chemical defenses in seagrasses, but more rigorous studies are required to ascertain whether previous exposure to herbivores reduces the likelihood of future grazing damage.

Plant-Herbivore Interactions in Mangrove Systems

There is currently little concrete evidence that mangroves are chemically defended against herbivory, and the evidence in support of mangrove chemical defenses comes primarily from observational instead of manipulative studies. In some cases, higher concentrations of phenolic compounds corresponded to lessened leaf damage by invertebrates (Kathiresan 2003; Erickson et al. 2004), but these types of observations are often confounded by structural or nutritional differences among species that co-vary with herbivore damage. The lack of manipulative tests of mangrove chemical defenses is unfortunate, considering that chemists have discovered at least 350 novel natural products from mangroves that have no known ecological function, including paracasolide A (**5**)

(Chen et al. 2011), gymnorrhizol (**6**) (Sun and Guo 2004), a variety of limonoids (Yin et al. 2006; Luo et al. 2009; Li et al. 2010; Pan et al. 2010; Ravangpai et al. 2011), as well as flavonoid glycosides (Kandil et al. 2004) and iridoid glucosides (Fauvel et al. 1995; Fauvel et al. 1997; Fauvel et al. 1999). For a detailed compilation of mangrove natural products and their biomedical activities, see the comprehensive review by Wu et al. (2008a). Knowledge of the ecological roles of these compounds is limited by the few experiments that have been conducted with herbivores that present natural threats to mangroves. Limonoids isolated from the bark, seeds, and fruit of Indo-Pacific mangroves of the genus *Xylocarpus* (Li et al. 2010; Pan et al. 2010; Tan and Luo 2011) deterred insect feeding (Wu et al. 2008b; Li et al. 2011), although these compounds were tested against agricultural pests instead of ecologically relevant herbivores. Xylogranatins isolated from seeds of *Xylocarpus granatum* (Yin et al. 2006; Wu et al. 2008b) reduced grazing by the wheat pest armyworm *Mythimna separata* (Wu et al. 2008b). Xylogranatin G (**7**), the most inhibitory xylogranatin tested, reduced *M. separata* feeding by up to 80% (Wu et al. 2008b). In another study, the limonoids khayasin (**8**) and 2'*S*-methylbutanoylproceranolide (**9**) produced by *Xylocarpus moluccensis* killed coconut palm pest *Brontispa longissima* larvae when coated onto coconut palm leaves (Li et al. 2011). Known ecological functions for the majority of mangrove natural products such as these remain ambiguous and warrant further investigation.

At present, it is difficult to assess whether mangrove phenolics reduce herbivory, largely due to the contradictory data currently available. A significant negative correlation between total tannin content and number of grazing scars was found when comparing ten Indian mangrove species (Kathiresan 2003), and concentrations of

condensed tannins negatively correlated with consumption of leaves from red (*Rhizophora mangle*), white (*Laguncularia racemosa*), and black (*Avicennia germinans*) mangroves by the mangrove tree crab *Aratus pisonii* (Erickson et al. 2004). While these data suggest that tannins reduce herbivory on mangroves, in other studies phenolics had a neutral to positive effect on invertebrate grazing (de Lacerda et al. 1986; Nordhaus and Wolff 2007). Since many of these studies rely on observations of leaf damage instead of explicitly testing herbivore preferences using choice assays, it is difficult to determine what role phenolics play in mangrove defense, especially when one considers that phenolic concentrations tend to decline as mangrove tissues age (Camilleri 1989; Turner 1995). However, in a few cases ecological roles for mangrove chemical defenses have been described, advancing our understanding of how specific herbivores interact with mangrove defenses. Flavologlycans, a class of condensed tannins produced by the mangrove *Ceriops tagal* (Neilson et al. 1986b) deterred feeding by the crab *Neosarmatium smithi* (Neilson et al. 1986a). When flavologlycans were coated onto dried *C. tagal* leaves and offered to crabs in a choice assay, *N. smithi* exhibited a strong, concentration-dependent avoidance of foods containing these compounds (Neilson et al. 1986a). The deterrent properties of flavologlycans may explain why *N. smithi* stores freshly fallen mangrove leaves in burrows for weeks to months prior to consumption, perhaps to allow deterrent leaf compounds to decompose or leach from tissues, increasing leaf palatability (see further discussion in community and ecosystem effects section). Given the current state of the field, it is unclear how other herbivores overcome mangrove chemical defenses, but it would be interesting to examine how unique digestive enzymes or gut microflora could increase the range of diets that these

herbivores can consume as seen in other systems (Barbehenn et al. 2001; Zimmer et al. 2002).

The majority of evidence suggests that mangrove herbivores do not discriminate among diets based on nitrogen or carbohydrate content of leaves, suggesting that nutritional traits rare less important than structural or chemical characteristics. For instance, tree crab herbivory was inversely correlated with nitrogen and carbohydrate content in red, white, and black mangroves (Erickson et al. 2004), and senescent leaves from *R. mangle* were significantly preferred over *A. germinans* by the mangrove crab *Ucides cordatus* even though *R. mangle* contained higher tannin and lower nitrogen content than *A. germinans* (Nordhaus and Wolff 2007). Furthermore, in choice assays mangrove crabs did not discriminate among freshly fallen leaves from four mangrove species based on nitrogen, organic, or tannin content (Micheli 1993), and damage caused by a suite of herbivores did not correspond to leaf nitrogen content among three mangroves (de Lacerda et al. 1986). Conversely, nutrient enrichment could enhance mangrove palatability by lowering leaf C:N or lowering phenolic concentrations (Onuf et al. 1977; McKee 1995). In many of the studies described above, leaves from less consumed mangroves were tougher and more fibrous than those of preferred species (de Lacerda et al. 1986, Nordhaus and Wolff 2007), although in other studies toughness did not influence herbivory (Micheli 1993). Given that many of the grazers in these studies are omnivorous and ingest a wide variety of foods, it is possible that plant toughness limits what herbivores can physically consume regardless of their nutritional or chemical status.

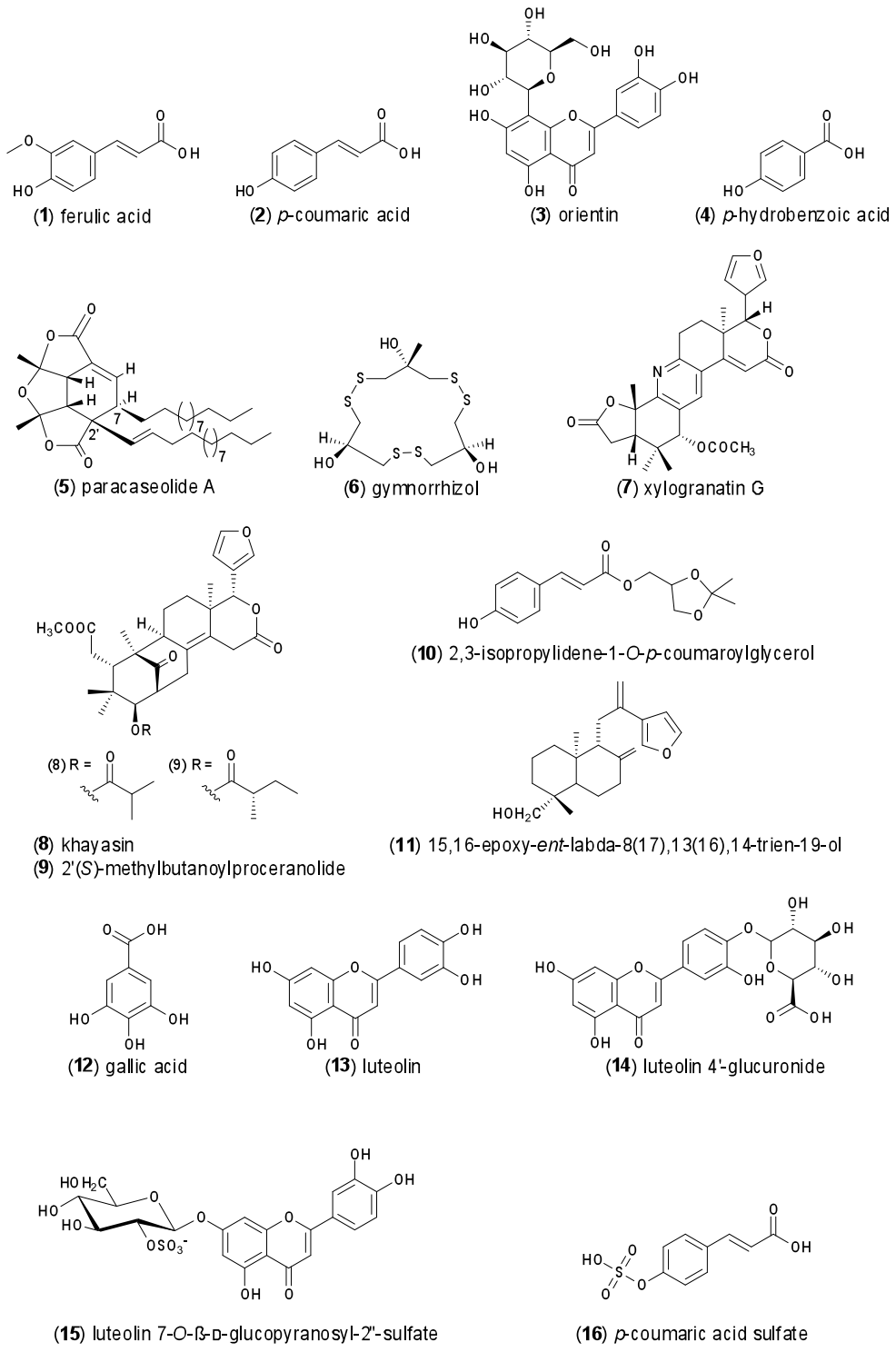

Figure 2.1. Secondary metabolites involved in chemically mediated interactions between marine angiosperms and organisms in their surrounding environment.

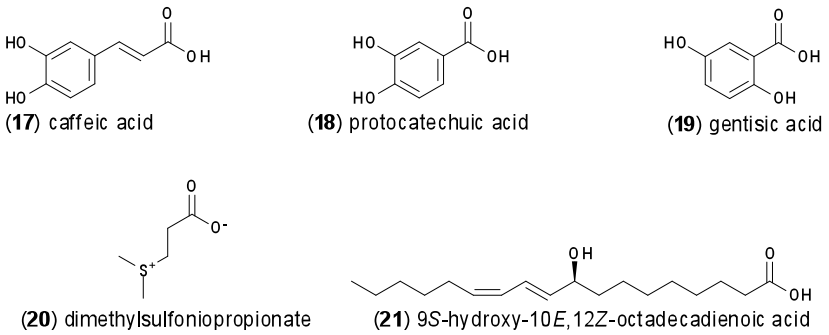

Figure 2.1. (continued) Secondary metabolites involved in chemically mediated interactions between marine angiosperms and organisms in their surrounding environment.

Allelopathic Interactions Between Marine Angiosperms

In the scope of this review, allelopathy is defined as the release of secondary metabolites by one species to inhibit the growth or success of a competitor. Such interactions have been documented in marine benthic (Rasher et al. 2011) and marine pelagic systems (Poulson et al. 2010), freshwater lakes (Mulderij et al. 2006), and terrestrial systems (Nishida et al. 2005). Despite growing evidence for such interactions among terrestrial and marine plants, there are currently very few examples of allelopathic interactions involving marine angiosperms.

Laboratory studies have shown that marine angiosperm natural products reduce the growth of common plants, suggesting that marine angiosperms may be allelopathic. For example, micromolar concentrations of several phenanthrenoid compounds from the spiny rush plant *Juncus acutus* and dihydrophenanthrenes, tetrahydropyrenes, and aromatic monoglycerides such as 2,3-isopropylidene-1-*O*-*p*-coumaroylglycerol (**10**) from the brackish soft rush (*Juncus effusus*) reduced growth of the aquatic microalga *Selenastrum capricornutum* by over 50% relative to controls, although a subset of the phenanthrenoids tested significantly stimulated algal growth (Della Greca et al. 1996; Della Greca et al. 1998; Della Greca et al. 2002a; Della Greca et al. 2002b). Multiple diterpenes from the salt-tolerant brackish marsh grass *Ruppia maritima* including 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-ol (**11**) were even more active, inhibiting algal growth at concentrations less than 0.8 μM (Della Greca et al. 2000). *Selenastrum capricorunutum* is a freshwater alga that is sensitive to changes in water quality and waterborne toxins (Baun et al. 2000; Regel et al. 2002) and is commonly used in natural product screening based on established toxicity protocols (Lytle and Lytle 2001), but

would be unlikely to compete or co-occur with angiosperms in estuarine environments. If algal growth is reduced by marine angiosperm exudates via inhibition of a conserved physiological plant function such as photosynthetic efficiency, then these natural products may indeed be allelopathic towards true competitors of marine angiosperms. Better insight into the ecological functions of these diverse natural products could highlight to what extent allelopathic interactions shape marsh community structure.

Currently, there exists one documented example of how allelopathy facilitates establishment of invasive species in marshes by releasing into sediments compounds that inhibit competitor growth (Rudrappa et al. 2007). This represents an example of root allelopathy similar to that observed in terrestrial systems (Ridenour and Callaway 2001; Rashid et al. 2010). Roots of the invasive marsh grass *Phragmites australis* were found to exude into the soil gallic acid (**12**), an allelopathic compound that inhibited *Spartina alterniflora* seedling growth as well as seedlings of several commercially important crops by inhibiting microtubule assembly within competitor roots (Rudrappa et al. 2007). Furthermore, the model plant *Arabidopsis thaliana* was more susceptible to exudates from exotic than from native *P. australis* genotypes, and concentrations of **12** in soil samples surrounding exotic *P. australis* stands matched those required to inhibit *S. alterniflora* seedling growth (Rudrappa et al. 2007). Allelopathy, coupled with potent chemical defenses against herbivores (Hendricks et al. 2011), could explain how *P. australis* has established a foothold in *S. alterniflora* dominated marshes. Since *S. alterniflora* rhizomes provide support and aerate anoxic marsh sediments in order to acquire nutrients in lower elevation marshes (Bertness 1991), compromising root integrity could deal a severe blow to the competitive ability of this species. The

distribution of plants within salt marshes is dependent on the stress tolerance and competitive ability of each species (Guo and Pennings 2012). It would be exciting to determine how mechanisms of interference competition such as allelopathy also contribute to the distinct patchiness of plants in higher elevation marsh zones, particularly when one considers the rapid spread (Saltonstall 2002) and increased competitive ability of exotic *P. australis* relative to native strains in mesocosm studies that simulate the effects of global warming (Mozdzer and Megonigal 2012).

Competitor identity may influence when and to what extent marine angiosperms produce allelopathic compounds. Production of a mixture of phenolic compounds including **1** was induced in the seagrass *Posidonia oceanica* in response to growing near the green alga *Caulerpa taxifolia,* but growing near *Caulerpa racemosa* did not cause a similar induction effect (Dumay et al. 2004). Greater quantities of specialized "tannin cells" were found in *P. oceanica* growing near *C. taxifolia* (Dumay et al. 2004), suggesting that *P. oceanica* physiologically responded to stressors associated with competition by producing cells that make and store phenolic compounds. However, the authors provided no evidence that induced phenolics increased the defensive or competitive success of *P. oceanica*. In another study, *C. taxifolia* responded to growing near *P. oceanica* with increased frond length and lowered caulerpenyne content, while *P. oceanica* augmented production of several phenolic compounds (including **1**) and increased leaf turnover rates (Pergent et al. 2008). The authors proposed that competitive interactions between *P. oceanica* and *C. taxifolia* led to different physiological responses (Pergent et al. 2008), whereby the alga relaxed investment in chemical defense (suggested by reductions in caulerpenyne) but increased growth, possibly to outgrow or

shade its competitor. In contrast, the seagrass produced more tannin cells and specific phenolic compounds, potentially investing in a chemical arsenal capable of inhibiting algal competitors (Pergent et al. 2008). However, in both of these studies, the allelopathic function of phenolic compounds was inferred but not directly tested. Therefore, changes in the quantity and identity of these compounds may have been a byproduct of physiological stress without direct effects on *P. oceanica* competitive ability.

Interactions of Marine Angiosperms with Pathogens and Fouling Organisms

A single drop of seawater contains thousands to millions of viruses, bacteria, fungi, and microalgae, inevitably including those that are pathogenic towards benthic plants and animals (Reinheimer 1992). Given this intense exposure, benthic organisms should be selected to defend themselves against diverse pathogens and fouling microalgae. Such chemical defenses of macroalgae and sponges have received increasing attention (see reviews by Engel et al. (2002); Goecke et al. (2010); Paul et al. (2011)), but the same cannot be said to date for marine angiosperms. Yet, seagrasses are sometimes included in broad screenings for new marine natural products with biomedical (Ballesteros et al. 1992) or ecological (Engel et al. 2006; Puglisi et al. 2007) antimicrobial functions. For the purposes of this review, we will only discuss studies that examine the antimicrobial potential of marine angiosperm compounds against ecologically relevant microbial targets. The prevalence of antifungal compounds in salt marshes has recently been investigated in conjunction with farming behavior by the marsh periwinkle *Littoraria irrorata* (Sieg Chapter 3; Chapter 4), but these multi-trophic interactions are discussed in the community and ecosystem effects section.

Both Caribbean and Pacific seagrass species appear to be well defended against marine microorganisms. Extracts of the Caribbean seagrasses *Thalassia testudinum* and *Halodule beaudettei* inhibited the growth of the saprophytic stramenopile *Halophytophthora spinosa* (Engel et al. 2006). Extracts of *H. beaudettei* and *Syringodium filiforme* also inhibited the stramenopile *Schizochytrium aggregatum* and the marine bacterium *Pseudoaltermonas bacteriolytica* (Engel et al. 2006), which causes kelp red-spot disease (Sawabe et al. 1998). In the Pacific, extracts of the seagrasses

Enhalus acorioides and *Halophila minor* inhibited *S. aggregatum* growth; *E. acoriodes* also demonstrated antifungal and antibacterial activity while *H. minor* antimicrobial activity was limited to effects on stramenopiles (Puglisi et al. 2007). Extracts from four Caribbean seagrasses (*T. testudinum, Halodule wrightii, Ruppia maritima* and *Halophila decipiens*) inhibited growth of the pathogenic fungus *Fusarium* spp. and three fungal species were also unable to grow on whole tissues from three of these seagrasses (Ross et al. 2008), suggesting that the localization of antifungal compounds in plant tissues prevents establishment of marine fungi *in situ*. Fungal contact also caused some seagrasses to release reactive oxygen species such as hydrogen peroxide (Ross et al. 2008), similar to how many terrestrial plants initially respond to microbial infection (Hueckelhoven et al. 2001). The specific compounds responsible for antimicrobial activity remain unknown for most seagrasses. However, the chemical characteristics of antimicrobial fractions differ among seagrass species even when targeting similar pathogens (Engel et al. 2006; Puglisi et al. 2007). Thus, seagrasses may have evolved a diverse chemical arsenal to inhibit marine pathogens.

In several instances compounds responsible for seagrass antimicrobial activity have been identified. Flavonoids isolated from *E. acoriodes* including luteolin (**13**) and luteolin 4'-glucuronide (**14**) inhibited the growth of fouling and pathogenic marine bacteria, and **14** also prevented settlement of the larval bryozoan *Bugula neritina* (Qi et al. 2008). In another study, the flavone glycoside luteolin 7-*O*-β-D-glucopyranosyl-2" sulfate (**15**) produced by *T. testudinum* inhibited growth of the zoosporic fungus *S. aggregatum* and prevented zoospores from attaching to artificial substrates (Jensen et al. 1998). Given that whole tissue concentrations of **15** within *T. testudinum* were up to 15

times that required to inhibit growth of *S. aggregatum,* it is likely that this compound was responsible for the majority of antifungal activity (Jensen et al. 1998). In contrast, crude extracts from a different collection of *T. testudinum* had no inhibitory effect on *S. aggregatum* growth (Engel et al. 2006), suggesting that these compounds may be genotype specific or only produced under certain environmental conditions. A sulfated phenolic compound isolated from the seagrass *Zostera marina, p*-coumaric acid sulfate (**16**)*,* inhibited barnacle attachment and biofilm formation of the marine fouling bacteria *Acinetobacter* sp., as did synthetic sulfated derivates of the compound (Todd et al. 1993).

Several other sulfated compounds, including flavonoids, flavones, galactans, and others, have been isolated from marine angiosperms but their ecological role(s) remain unknown (McMillan et al. 1980; McMillan 1986; Sun and Guo 2004; Aquino et al. 2005). Since there is strong evidence that sulfated phenolics protect seagrasses against microbes (Todd et al. 1993; Jensen et al. 1998; Qi et al. 2008), these compounds could prevent microbial settlement or growth. The antifouling compounds described above were isolated from whole plant tissue extracts instead of plant surfaces, so it is unclear if seagrasses relegate these compounds to plant surfaces that are vulnerable to pathogenic or fouling organisms. Compounds expressed on plant surfaces likely function as a preventative antifouling defense, while compounds isolated from tissue extracts may limit spread of microbial infection. In the absence of knowledge about the localized distribution and surface expression of secondary metabolites within plant tissues, most studies have quantified bulk tissue concentrations of compounds and tested these compounds at natural whole tissue concentrations which has limited our ability to predict the effects of antimicrobial and antifouling compounds in live plants. However, new

analytical techniques have been developed to map concentrations of antimicrobial compounds on biological surfaces (Lane et al. 2009; Abbas et al. 2012; Andras et al. 2012). These spatially resolved techniques can highlight which tissue surfaces are most heavily defended, and allow researchers to quantify allocation of chemical defenses on a much finer scale than was previously available. While these techniques have not yet been used for ecological studies involving marine angiosperms, they provide an exciting new way for researchers to assess how higher plants allocate defenses in response to grazing, infection, or fouling.

Fouling by epiphytic algae or bacteria can be detrimental to marine angiosperms, causing reductions in photosynthetic activity, increased risk of predation, or exposure to allelopathic agents (Sand-Jensen 1977; Williams and Ruckelshaus 1993; Heck and Valentine 2006). Marine angiosperms can prevent attachment or inhibit the growth of fouling organisms using chemical defenses (Harrison 1982; Todd et al. 1993). For instance, carbon uptake by epiphytic diatoms cultured from *Z. marina* was reduced up to 80% relative to controls when grown in media containing polar *Z. marina* extracts (Harrison and Durance 1985), and polar extracts from a panel of seagrass and mangrove species inhibited the growth of several fouling marine bacteria (Devi et al. 1997). In a lab study, polar extracts of young *Z. marina* blades inhibited establishment of the green alga *Platymonas* sp. and several marine bacteria isolates (Harrison 1982), while extracts from older tissues were less effective at preventing fouling by these organisms (Harrison 1982). Individual phenolic acids isolated from *Z. marina* (Zapata and McMillan 1979) including caffeic (**17**), protocatechuic (**18**) and gentisic acid (**19**) inhibited fouling to a similar degree as crude extracts (Harrison 1982), providing support for the function of

these phenolic acids as antimicrobial agents. Furthermore, shredding of seagrass blades by amphipod grazers was stimulated when exposed to extracts from detrital *Z. marina*, but was inhibited by young *Z. marina* extracts (Harrison 1982). If mesograzers cannot fragment seagrass detritus until defenses have leached or degraded from tissues, then the time until plant biomass enters the microbial loop is likely increased due to residual chemical defenses (see further discussion in community and ecosystem effects section).

Since antifouling properties of some seagrass extracts persist at dilute concentrations (down to ten parts per billion, Harrison and Durance (1985)), even minute quantities of seagrass defenses could delay microbial settlement on aged tissues. As with earlier studies, these experiments tested extracts from whole tissues instead of plant surfaces. Therefore, reports of such high activity at low concentrations in the lab may overestimate microbial exposure rates to these compounds in the field. Without quantifying the natural exposure rate of these compounds to fouling organisms and the grazers that may facilitate their establishment, it will remain difficult to tease apart how chemical defenses influence when grazing, fouling, and subsequent tissue degradation is likely to occur.

Protists of the genus *Labyrinthula* infect multiple seagrass species, causing necrotic lesions and mass mortality of seagrass meadows (Short et al. 1988; Robblee et al. 1991; also see review on labyrinthulomycetes by Raghukumar (2002)). It has been hypothesized that phenolic compounds limit the spread of *Labyrinthula* wasting disease (Buchsbaum et al. 1990; Vergeer et al. 1995; Vergeer and Develi 1997). If phenolic acids inhibit *Labyrinthula* growth, then seagrasses would benefit from upregulating production of these compounds upon *Labyrinthula* infection. However, environmental

(Buchsbaum et al. 1990; Vergeer et al. 1995) and seasonal (Harrison and Durance 1989) triggers can also increase the quantity or distribution of phenolic compounds. Blades of the seagrass *Zostera marina* infected with *Labyrinthula zosterae* contained up to five-fold higher concentrations of total phenolics compared to uninfected tissues (Vergeer and Develi 1997), and phenolic concentrations tended to be highest near sites of *L. zosterae* infection (Vergeer et al. 1995; Steele et al. 2005). Culturing experiments showed that increased light intensity amplified production of phenolic compounds in *Z. marina* (Vergeer et al. 1995), coinciding with a slight decrease in infection by *L. zosterae*. Other abiotic factors, such as increased temperature or salinity, did not increase production of *Z. marina* phenolic compounds (Vergeer et al. 1995; McKone and Tanner 2009), although increases in salinity coupled with *L. zosterae* infection resulted in higher concentrations of three phenolic acids, including **1** and **12** (McKone and Tanner 2009). Increased concentrations of these compounds did not, however, improve resistance to infection, suggesting that they could be produced as a general stress response rather than as a chemical defense against *L. zosterae* (McKone and Tanner 2009). Among the phenolic acids induced, only **17** (which made up the majority of phenolics acids in *Z. marina*) inhibited *L. zosterae* growth (Vergeer and Develi 1997). Other phenolic acids including **1**, **2**, and **12** had no effect on *L. zosterae* (Vergeer and Develi 1997), suggesting that these compounds may not play a strong role in preventing wasting disease. Oddly, **17** completely prevented establishment of *L. zosterae* in 60% of trials but had no effect at all in the remaining 40% despite several repetitions of the assay and the testing of compounds at concentrations representing infected tissues (Vergeer and Develi 1997). Thus, the role of **17** in defense against wasting disease is ambiguous but could be

clarified by testing the inhibitory role of this compound at concentrations spanning the range seen in healthy and infected blades.

An experiment by Steele et al. (2005) provided a novel explanation for the mixed data supporting the importance of phenolic compounds as defenses against *Labyrinthula* infection. Concentrations of common phenolic acids in tissues of *T. testudinum* were highest in proximal tissues just above, but not below, *Labyrinthula* lesion sites (Steele et al. 2005). The researchers speculated that phenolic compounds were not allocated to tissues infected with *Labyrinthula* as a defensive function. Instead, they hypothesized that phenolic compounds were produced in the distal blade tips and were unable to pass into carbohydrate sinks (such as rhizomes or new blades) because of damage to transport vessels caused by *Labyrinthula* lesions (Ralph and Short 2002; Steele et al. 2005), resulting in a backup of phenolic compounds around the lesion site (Steele et al. 2005). Treatment of blades with jasmonic or salicylic acid (terrestrial angiosperm hormones that trigger defenses) did not lead to increased phenolic concentrations in healthy *T. testudinum* blades (Steele et al. 2005). The authors referred to the accumulation of phenolic compounds as "pseudo-induction", because the elevated phenolic concentrations did not occur as a plant response to infection. Instead, damage to vascular tissues caused by *Labyrinthula* infection was postulated to result in a buildup of these compounds on one side of the wound site as the plant shuttled excess carbon (in the form of phenolics) from shoot to root (Steele et al. 2005). Further supporting this hypothesis, if these compounds were used in defense it would be counterintuitive to transport them away from younger, more valuable tissues near blade tips into older tissues unless doing so increased overall seagrass health. However, it is currently unclear whether distal portions

of *T. testudinum* are more biosynthetically active in producing phenolic acids than are basal tissues. Considering the mixed support concerning the role of phenolic compounds as defenses against *Labyrinthula* (Buchsbaum et al. 1990; Vergeer and Develi 1997; McKone and Tanner 2009), there is the possibility that pseudo-induction still benefits *T. testudinum* if the compounds that are "induced" also inhibit growth of *Labyrinthula*.

Settlement and Metamorphic Cues from Marine Angiosperms

The transition from pelagic to estuarine or reef habitats is a major event in the lifecycle in many benthic marine organisms, and there is a great deal of interest in what types of cues are utilized to assess the quality of a settlement site. There are excellent reviews covering the chemical ecology of larval settlement (Pawlik 1992) and colonization of benthic surfaces (Steinberg et al. 2002), examining cues mostly stemming from invertebrates, algae, and prokaryotes. There is little direct evidence for similar types of chemical cues from marine angiosperms, which is surprising given that these plants help to form estuarine nursery habitats. However, chemical cues must be considered in context along with the visual, auditory, and hydrodynamic characteristics of estuarine or reef habitats, as these cues can also provide useful information regarding habitat suitability.

There is considerable evidence suggesting that invertebrates use chemical cues from estuarine plants as a signal for larval settlement or molting. Several studies have investigated behavioral responses of larvae towards water conditioned with estuarine plant cues (Davis and Stoner 1994; Forward et al. 1996; Diaz et al. 2001), supporting the notion that larvae assess settlement site quality using chemical cues. Unfortunately, since conditioned water contains many different compounds at low concentrations it is difficult to identify the specific compound(s) used as settlement cues by these organisms. For instance, blue crab (*Calinectes sapidus*) megalopae rapidly molted to their first adult stage when exposed to either estuarine water or water conditioned with the seagrass *Zostera marina*, whereas offshore waters did not induce molting (Forward et al. 1994). Furthermore, molting times were shortened after exposure to water conditioned with the

seagrasses *Ruppia maritima, Halodule wrightii* or the salt marsh grass *Spartina alterniflora,* but not the open water alga *Sargassum* sp. (Forward et al. 1996). Compounds from estuarine water that accelerated molting were smaller than 10 kDa based on experiments using dialysis (Forward et al. 1996). In a separate study, blue crab megalopae preferred to settle in chambers containing *Z. marina* and *H. wrightii*, but not *S. alterniflora* tissues, although megalopae did not show positive chemotaxis towards plant cues in flume studies (Welch et al. 1997). When predator cues were included in chambers, settlement was reduced, suggesting that megalopae assessed both positive and negative cues when choosing appropriate settlement sites (Welch et al. 1997). In other studies, blue crab juveniles responded negatively (Diaz et al. 2003) or showed no response (Diaz et al. 1999) to estuarine water, and there is evidence that larval responses to visual and chemical stimuli change from one instar stage to another (Diaz et al. 2001).

Considering the multitude of abiotic and biotic differences among water sources, it is in many cases difficult to determine whether the cue(s) responsible for settlement are derived from vegetation or other associated features of the system. This is well illustrated by studies with queen conchs (*Strombus gigas*), whose larvae discriminate between "good" and "bad" nursery sites based on detrital composition (Davis and Stoner 1994). It was not turtlegrass, the major component of the detritus, that produced the strongest settlement cue for *S. gigas*, but red algae, including epibionts such as *Fosliella* sp. that were the source of the cue (Davis and Stoner 1994; Boettcher and Targett 1996). Given that red algae are favored foods for queen conch (Davis and Stoner 1994; Boettcher and Targett 1996), it seems appropriate for conch to cue to these minor components of the community. In this instance, chemical cues from the epibionts were

partially characterized, composing low molecular weight (<1 kDa), heat stable compounds (Boettcher and Targett 1996).

For a variety of species, it appears that the concentration of positive chemical cues from marsh vegetation relative to negative cues from predators reliably indicates the quality and proximity of nursery settlement sites. Marsh fiddler crab megalopae molting rates were highest in settlement cages placed adjacent to salt marsh vegetation, but dropped off significantly as distance between cage and marsh increased (O'Connor and Judge 2004). It is unclear whether the cues came from estuarine plants or another component of the marsh habitat, given that another fiddler crab species, *Uca pugnax*, was stimulated to molt when exposed to both estuarine water and marsh sediments (O'Connor and Judge 1999), but not *S. alterniflora* conditioned water (O'Connor and Gregg 1998). Pre-molt blue crab juveniles oriented themselves towards *S. alterniflora* or *Z. marina* water in a concentration dependent manner while simultaneously orienting away from predator cues (Forward et al. 2003). This behavior was consistent provided water flow rates were low, while settlement cues were less reliable in turbulent flow when waters were moving too quickly to sustain a reliable directional cue (Forward et al. 2003). In a separate study, hermit crabs (*Clibanarius antillensis)* found *Thalassia testudinum* nursery sites based on the concentration of predator or habitat chemical cues and different visual stimuli (Chiussi et al. 2001). Thus, a variety of crab species can assess the quality of nursery sites based on chemical cues, but the reliability of cues likely decrease under turbulent flow.

There is evidence that chemical cues from marine angiosperms also induce juvenile fish settlement into nurseries. Juvenile French grunts (*Haemulon flavolineatum*)

preferentially tracked to water collected from mangrove and seagrass habitats, but not to coral reef water associated with adult fish (Huijbers et al. 2008). Grunts also use a hierarchy of cues to determine proximity to suitable nursery habitats, namely auditory cues to hone to near-shore benthic habitats, and then a combination of visual or olfactory cues to find suitable nurseries (Huijbers et al. 2012). Chemical, but not visual cues from seagrass habitats also attracted juvenile spangled emperor (*Lethrinus nebulosus*) concurrent with a nasal olfactory organ in this age class developed enough to detect chemical cues (Arvedlund and Takemura 2006).

The strongest evidence for chemical settlement cues from marine angiosperms comes from studies of upside down jellyfish (*Cassiopea xamachana*) in mangrove stands. *Cassiopea xamachana* larvae overwhelmingly chose to settle on the underside of black (decayed) leaves of the red mangrove *Rhizophora mangle* compared to newly fallen or fresh leaves (Fleck and Fitt 1999). Extraction and fractionation of decaying *R. mangle* leaves led to a proline-rich peptide (~5.8 kDa) that induced larval settlement and metamorphosis (Fleck et al. 1999), as well as at least two other peptide-like molecules that could not be purified. These peptides likely leach from mangrove leaves into the water column when bacteria decompose leaf tissue (Fleck and Fitt 1999; Fleck et al. 1999), which is supported by the observation that heating or treatment of decaying mangrove leaves with antibiotics sharply reduced larval settlement (Fleck and Fitt 1999). Many of the larval settlement studies in salt marshes provide an excellent stepping off point from which new settlement cues could be isolated and identified using techniques such as those employed when studying jellyfish settlement in mangrove habitats.

Community and Ecosystem Effects

The fate of antifouling chemical defenses after plant death may have ecosystem-wide effects even after these compounds are of no further use to the organism that produced them. For instance, growth of phytoplankton and bacteria was inhibited by polar extracts from newly dead *Zostera marina* tissues (Harrison and Chan 1980), although antifouling potency of *Z. marina* extracts declined as detritus degraded (Harrison and Chan 1980). Given that tannins can take up to a month to leach from submerged *Rhizophora mangle* leaf litter (Cundell et al. 1979), it is not surprising that the load of fungal and bacterial decomposers on plant tissues remains low until most tannins have been removed (Cundell et al. 1979). This could explain the time lag between detachment of senescent tissues and their decomposition in the water column. Plant chemical defenses employed while the plant was alive can also prevent breakdown of dead tissue by mesograzers (Harrison 1982; Harrison and Chan 1980). Since shredding by detritivores facilitates subsequent microbial degradation of tissue, lingering chemical defenses delay the entrance of plant matter into the microbial loop. Given these implications for nutrient cycling, the mechanisms employed by mesograzers and microbial decomposers to overcome residual plant defenses warrant further study.

High phenolic concentrations in senescent leaves (up to 20% of dry leaf mass, Hernes et al. (2001)) can have a lasting effect on larger detritivores in the surrounding ecosystem, even if phenolics leach from decaying tissues or are eventually degraded by microbial decomposers (Robertson 1988). In several choice feeding assays, crustacean grazers consumed decaying mangrove leaves more frequently than freshly fallen leaves (Giddins et al. 1986; Camilleri 1989; Nordhaus and Wolff 2007), supporting the

hypothesis that crabs waited until phenolic content dropped before consuming leaves (Giddins et al. 1986). Alternatively, since C:N of leaves decreased and microbial growth increased as leaves degraded (Giddens et al. 1986; Camilleri 1989), crabs may have stored leaves to wait until nutritional quality increased. Additional studies are required to tease apart the relative importance of nutrition, structure, or chemical defenses as factors that influence detritivores. While mangrove crabs (*Neosarmatium smithi*) are hypothesized to store leaves from the yellow mangrove *Ceriops tagal* in burrows until condensed tannins degrade and overall palatability improves (Giddins et al. 1986), storage of white mangrove leaves by other closely related crab species did not improve leaf nutritional quality with age (Skov and Hartnoll 2002). Instead, crabs likely met their nitrogen requirement by consuming sediment detritus, which had a lower C:N than stored leaves (Skov and Hartnoll 2002). However, leaf storage could merely be a way for crabs to minimize predation risk by reducing the amount of time spent outside of burrows, while any increase in leaf nutritional quality comes as an added benefit (Micheli 1993). The species specificity of leaf-storing behavior, as well as its implications for leaf turnover rates and changes in tissue palatability to crabs remains open to debate.

When herbivores and pathogens are intimately associated, plants may develop multiple strategies to minimize tissue losses. The intensity of interactions between the foundation marsh species *Spartina alterniflora*, the periwinkle snail *Littoraria irrorata*, and a community of fungi cultured on grass blades by *L. irrorata* can change a salt marsh from a lush productive grassland to a barren mudflat in a matter of months (Silliman and Newell 2003; Silliman et al. 2005). Periwinkle snails facilitate growth of pathogenic fungi on blades of *S. alterniflora* by wounding plant tissue and then defecating hyphae-

rich fecal matter on wound sites (Silliman and Newell 2003). By seeding grazing scars with fungi, snails can selectively consume the fungus as it grows (Silliman and Newell 2003), but under drought conditions and increased snail densities brought on by the collapse of a trophic cascade, this can lead to massive marsh die-off (Silliman and Bertness 2002; Silliman et al. 2005). In lab studies, fungal establishment increased the palatability of *S. alterniflora* tissues to *L. irrorata*, possibly due to an up to 75% degradation of phenolic content in tissues (or powdered *S. alterniflora*) previously incubated with live fungi (Barlocher and Newell 1994a, 1994b). Artificial diets containing polar *S. alterniflora* extracts or **1** were deterrent to *L. irrorata*, but treatment of diets with fungal mycelium lessened the effect of *S. alterniflora* compounds (Barlocher and Newell 1994b). *Littoraria irrorata* digestive enzymes can degrade *S. alterniflora* and fungal material (Barlocher et al. 1989a), but *S. alterniflora* detritus colonized by fungi may be more nutritious to *L. irrorata* because fungi release amino acids from plant tissues (Barlocher et al. 1989b) and potentially break down *S. alterniflora* chemical defenses (Barlocher and Newell 1994b). In feeding trials, *L. irrorata* growth rates were highest on aged, colonized *S. alterniflora* litter as more available nitrogen became incorporated into fungal hyphae (Barlocher and Newell 1994a), and snails were also capable of discriminating between tissues infected with different fungal species (Barlocher and Newell 1994b), suggesting that the association between snails and fungi allows *L. irrorata* to overcome chemical defenses and other *S. alterniflora* traits to gain access to an abundant resource.

Littoraria irrorata grazing intensity on *S. alterniflora* can drastically change based on marsh elevation (Silliman and Bertness 2002) and latitude (Silliman and Newell

2003), and *L. irrorata* disproportionately resides on *S. alterniflora* relative to other available marsh plant species (Sieg Chapter 4). When given a choice of five different salt marsh plants in a mesocosm experiment, *L. irrorata* showed a five-fold preference for residing on *S. alterniflora* shoots over other available species (Sieg Chapter 4). *Littoraria irrorata* may prefer taller plants like *S. alterniflora* in part because they provide a refuge from predators during high tide (Hovel et al. 2001; Hughes 2012; Kimbro 2012), but chemical cues may also direct snails towards favored plants (Rahman et al. 2000; Kiehn and Morris 2010). *Littoraria irrorata* tracked towards *S. alterniflora* crude extracts in ring assays (Rahman et al. 2000) and was attracted to dimethylsulfoniopropionate (**20**) (Kiehn and Morris 2010), a compound produced by *S. alterniflora* as well as other vascular plants and marine algae (Iverson et al. 1989; Otte et al. 2004). In contrast, *L. irrorata* typically showed a negative response to predators, via cues from crushed conspecifics (Rahman et al. 2000; Kimbro 2012). These data support the idea that snails can respond to specific chemical cues and use them to track towards favored plant substrates while minimizing the risk of predation.

The prevalence of *L. irrorata* fungal farms on *S. alterniflora* but not on other marsh plant species begs the question of whether snails establish farms on *S. alterniflora* because it is often the most abundant and accessible plant, or because it is more weakly defended than other available plant species. A survey of tissue palatability and chemical defenses of five middle elevation salt marsh plants found that extracts of all five species were deterrent to both *L. irrorata* and two species of fungi (Sieg Chapter 4). Additionally, chemical defenses from *S. alterniflora* were not as deterrent as those of less abundant plant species including *Iva frutescens, Salicornia virginica, Batis maritima*, and

Borrichia frutescens (Sieg Chapter 4). Fungus was only detected on grazing sections of *S. alterniflora* but not other plants during field surveys, suggesting that fungal establishment is enhanced by snail grazing (Sieg Chapter 4). These data suggest that less abundant marsh plants heavily invest in chemical defenses to prevent tissue damage, while the foundation species *S. alterniflora* may compensate for tissue losses by producing new growth. Chemical defenses against grazers tended to be polar, relatively low molecular weight compounds, while antifungal compounds were frequently more lipophilic (Sieg Chapter 4), suggesting that marsh plants produce different types of chemical compounds to defend against herbivores or pathogens. Flavonoid glycosides including **3** isolated from *S. alterniflora* deterred *L. irrorata* grazing, while a fatty acid, 9*S*-hydroxy-10*E*,12*Z*-octadecadienoic acid (**21**) found in *S. alterniflora* tissues was responsible for inhibiting fungal growth (Sieg Chapter 3). However, the antifungal activity of **21** was weak relative to chemical defenses in other salt marsh plants (Sieg Chapter 3; Sieg Chapter 4).

At present, we do not know whether snails selectively farm local fungi that are most resistant to plant chemical defenses, nor do we know when the facultative mutualism evolved. It is possible that this association arose through mutual tolerance of *S. alterniflora* chemical defenses relative to those produced by other plants, and eventually led to cooperative behavior that allowed herbivores and pathogens to exploit a common resource (Sieg Chapter 4). We have only begun to understand the complex interspecific interactions that occur among marsh plants, snails and fungi, and may avenues for future research remain. For instance, we do not know whether farmed fungi compete for snail hosts within marsh communities, or whether local fungal populations

can adapt to the chemical defenses of salt marsh plants. It is possible that farmed fungi could also produce allelopathic compounds against other fungal species to maximize their chances of establishing on new wound sites as they open up, but this hypothesis remains untested at this point.

Toxins from pelagic phytoplankton, such as brevetoxins produced by the dinoflagellate *Karenia brevis* (Baden 1989; Baden et al. 2005), often cause mass mortalities of fish and marine mammals due to the accumulation of these compounds from lower trophic levels (Flewelling et al. 2005). For large herbivores such as manatees, the primary cause of this mortality comes from consumption of *Thalassia testudinum* containing a high quantity of epiphytes to which brevetoxins adsorb during red tides (Flewelling et al. 2005). High concentrations of brevetoxins on *T. testudinum* in bloom areas and in digestive systems of dead manatees suggested that consumption of brevetoxin-laden *T. testudinum* ultimately led to manatee deaths (Flewelling et al. 2005). Concentrations of brevetoxins on seagrass epiphytes can reach up to 1 $\mu g\ g^{-1}$ (Flewelling 2008), but usually drop to <50 $ng\ g^{-1}$ in the 6-8 months following a bloom (Flewelling 2008; Hitchcock et al. 2012). Brevetoxins persist on blades of *T. testudinum* for up to six months following a *K. brevis* bloom (Hitchcock et al. 2012), which is roughly three times longer than the average lifespan of a *T. testudinum* blade (Hitchcock et al. 2012), suggesting that motility of some mesograzers and epiphytes may keep these compounds in the seagrass community for extended periods of time. Feeding behavior and growth of amphipods were not inhibited by brevetoxin-laden foods at post-bloom concentrations (Sotka et al. 2009), suggesting that these mesograzers act as vectors leading to accumulation of toxins in planktivorous fish and subsequently piscivores such as

dolphins. Considering the large quantity of brevetoxin-laden seagrass tissues in herbivore guts (Flewelling et al. 2005), it is unlikely that brevetoxins reduced seagrass herbivory, but it remains to be seen if brevetoxins alter the way that seagrasses interact with other fouling organisms or pathogens.

Conclusions

Seagrasses, mangroves, and salt marsh angiosperms provide a host of services to their surrounding ecosystem by forming nursery habitats for juvenile fish and invertebrates, buffering coastlines against soil erosion and storm damage, and acting as nutrient sinks that limit eutrophication (Nagelkerken et al. 2008; Gedan et al. 2009; Waycott et al. 2009). The ways that these ecologically valuable plants interact with their environments remain a popular subject of study, but our understanding of marine angiosperm chemical ecology lags behind terrestrial or coral reef systems. Examples of chemically mediated interactions between marine plants and herbivores or microbes are well documented, but we know comparatively little about allelopathic interactions among marine angiosperms and other plant or algal competitors. There is also substantial evidence that fish and invertebrates can detect exudates from marine angiosperms and use them as a reliable cue for larval settlement, molting or habitat selection. Natural products produced by mangroves continue to be discovered, but the ecological function(s) of these compounds are rarely tested. We still have relatively few examples of specific marine angiosperm defense compounds or allelochemicals among seagrasses or salt marsh angiosperms. The hypothesis that phenolic compounds are responsible for the majority of antiherbivore and antimicrobial activity in marine angiosperms has mixed support, and the use of this broad class of compounds as a catch-all for chemical defenses among plant species may not be the most effective strategy. The fate of marine angiosperm chemical defenses after they are released into the environment, as well as how chemically mediated interactions can affect multiple trophic levels, remains an appealing topic that deserves further attention. Ample opportunities exist to investigate population, community, and

ecosystem level effects of chemically mediated interactions within salt marshes, seagrass beds, and mangrove stands. Communication and collaboration among natural products chemists, field ecologists, and evolutionary biologists will continue to expand our knowledge of these valuable ecosystems.

CHAPTER 3

MULTIPLE CHEMICAL DEFENSES PRODUCED BY *SPARTINA ALTERNIFLORA* DETER FARMING SNAILS AND THEIR FUNGAL CROP

Abstract

Plants are exposed to a variety of ecological threats, including herbivores, pathogens, and parasites, which can be tightly associated. In cases where chemical defenses play a role in resistance, plants may produce a single molecule (or group of molecules) that inhibits a diverse array of enemies, or they may invest in a suite of deterrent compounds that each protects against specific threats. The periwinkle snail *Littoraria irrorata* exerts substantial top-down control over smooth cordgrass *Spartina alterniflora* by culturing and grazing fungi on plant tissues as well as creating destructive wounding scars. To combat fungal farming, *S. alterniflora* produces chemical defenses that inhibit fungal growth and reduce *L. irrorata* grazing. Guided by ecological assays, we isolated a fatty acid (α-dimorphecolic acid) from *S. alterniflora* that inhibited growth of *Mycosphaerella* sp., a marsh fungus commonly farmed by *L. irrorata*. *Mycosphaerella* sp. was far more susceptible to the inhibitory effects of α-dimorphecolic acid than another farmed fungus, *Phaeosphaeria spartinicola*. In addition, several phenolic compounds isolated from *S. alterniflora* deterred grazing by *L. irrorata* when incorporated into artificial diets, of which one, the flavonoid glycoside orientin, was fully characterized. These defenses are not potent enough to completely deter fungi and snails, but may slow down or minimize the negative effects caused by fungal farming. In a marsh where grazing intensity was high, chemical defenses were constitutively expressed

in *S. alterniflora* even after a month long caging experiment in which exposure to fungi and herbivores was manipulated. Thus, *S. alterniflora* relies on multiple types of secondary metabolites instead of a single class of molecule to combat tightly associated snails and fungi. Although α-dimorphecolic acid was not expressed in sufficient concentration on plant surfaces to prevent fungal establishment, this defense may reduce fungal growth in plant tissues, increasing the resistance of *S. alterniflora* to fungal farming.

Introduction

Coastal wetlands rank among Earth's most productive ecosystems (Valiela 1995, Bertness et al. 2001) and are valuable ecological (Levin et al. 2001) and economic (Gedan et al. 2009) resources. Salt marsh primary productivity is controlled by a suite of bottom-up (Teal 1962; Deegan et al. 2012) and top-down (Srivastava and Jefferies 1996; Silliman and Zieman 2001) regulators that interact to affect overall ecosystem health (Hillebrand et al. 2007; Gruner et al. 2008). Recent evidence has shown that the periwinkle snail *Littoraria irrorata* controls productivity of the dominant North American Atlantic marsh grass *Spartina alterniflora* (Silliman and Zieman 2001; Silliman et al. 2005). Traditionally viewed primarily as a detritivore (Barlocher and Newell 1994b; Zimmer et al. 2004), *L. irrorata* establishes fungal farms on live *S. alterniflora* surfaces to gain access to a preferred fungal diet (Barlocher and Newell 1994a; Silliman and Newell 2003). Left unchecked in the presence of compounding abiotic stressors such as drought, *L. irrorata* and their associated fungal farms can cause drastic *S. alterniflora* die-offs in salt marsh communities (Silliman et al. 2005), particularly in the absence of secondary consumers such as blue crabs (Silliman and Bertness 2002).

Since fungal farming by snails can lead to massive losses in plant biomass, salt marsh grasses including *S. alterniflora* should be under selective pressure to limit damage or infection due to *L. irrorata* and their cultivated fungi. Marine and terrestrial autotrophs frequently employ structural and chemical defenses to deter herbivores (Hay and Steinberg 1992) and microbes (Pearce 1996; Lane and Kubanek 2008), as do salt marsh plants bordering these ecosystems (Siska et al. 2002; Hendricks et al. 2011; Sieg

Chapter 4). Recently, we demonstrated that *L. irrorata* prefers to establish fungal farms on *S. alterniflora* rather than on other available marsh plants, largely because *S. alterniflora* produces weaker chemical defenses against snails and fungi than do other marsh plant species (Sieg Chapter 4). However, *S. alterniflora* does possess chemical defenses: its extracts significantly inhibited fungal growth and *L. irrorata* grazing in lab assays relative to negative controls (Sieg Chapter 4), complementing other studies demonstrating that *S. alterniflora* produces chemical defenses against *L. irrorata* (Long et al. 2011) and other invertebrates (Siska et al. 2002).

Given that fungal establishment is often preceded by *L. irrorata* herbivory, *S. alterniflora* could minimize allocation costs by producing a common chemical defense to deter both grazers and pathogens, similar to the way some diatoms (Ianora and Miralto 2010), seaweeds (Schmitt et al. 1995) and terrestrial plants (Krischik et al. 1991; Marak et al. 2002; Biere et al. 2004) utilize a single class of secondary metabolite to defend against consumers, microbes, or competitors. Alternatively, *S. alterniflora* may utilize a varied chemical arsenal to defend against organisms as taxonomically distinct as gastropods and fungi. Although phenolic compounds contained in *S. alterniflora* detritus are known to act as antifeedants (Valiela et al. 1979; Barlocher and Newell 1994b), no specific chemical compounds from live salt marsh plants have previously been identified as defenses against herbivores or pathogens. Additionally, plant defenses should be localized to tissues most at risk. Even if a plant appears to be chemically defended based on a high concentration of defensive compounds contained within whole tissue extracts, the plant may be susceptible to damage if grazers and pathogens first encounter other plant parts (such as blade surfaces) that are weakly defended. Through the process of

bioassay-guided fractionation, we isolated multiple compounds responsible for *S. alterniflora* defense against *L. irrorata* and the closely associated fungi that snails cultivate on *S. alterniflora*. We also quantified *S. alterniflora* chemical defenses within plant tissues as well as on blade surfaces to determine whether these compounds were expressed at concentrations required to provide an adequate defense.

Ambient *L. irrorata* densities in Georgia salt marshes can exceed 600 snails m^{-2}, exerting greater top-down control of *S. alterniflora* production than in higher latitude marshes where snail densities are lower and grazing intensity is weak compared to Georgia marshes (Silliman and Bertness 2002; Silliman and Bortolus 2003). If herbivory is intense and constant, it may be beneficial to constitutively express defenses, whereas minimal investment in defenses against herbivores should be observed if the damage caused by herbivores is low. In cases of intermediate or cyclical herbivore pressure in which oncoming attack can be predicted by herbivore cues, many plants respond by inducing chemical or structural defenses (Karban and Baldwin 1997; Verschoor et al. 2004; Van Zandt 2007; Morrison and Hay 2011). For instance, grazing by a natural suite of herbivores in South Carolina salt marshes induced chemical defenses in *S. alterniflora* that then contributed to reduced herbivore damage (Long et al. 2011). However, chemical defenses were neither constitutively produced nor induced in *S. alterniflora* in New England marshes that contained lower ambient herbivore densities (Long et al. 2011), suggesting that *S. alterniflora* allocates resources towards defense only when herbivore grazing is substantial. Tissues and extracts from southern salt marsh plant populations also tended to be less palatable to herbivores than those from northern marshes (Pennings et al. 2001; Siska et al. 2002), and these differences in palatability

persisted for multiple plant generations even when grown in common gardens (Salgado and Pennings 2005).

Although it is known that herbivores induce chemical changes in some populations of *S. alterniflora*, it has been unclear whether other threats, such as exposure to fungal pathogens, also increase allocation of resources towards defense in this marsh plant. Using a caging experiment, we manipulated *S. alterniflora* exposure to herbivory and associated fungi in a Georgia salt marsh that experiences greater herbivory pressure than South Carolina sites where induction has been previously observed (Long et al. 2011). If induction of chemical defenses occurs in Georgia marshes, we hypothesized that the presence of herbivores and/or fungal pathogens would cause *S. alterniflora* to be more chemically defended than if *S. alterniflora* was protected from these biotic threats. Alternatively, if no change in chemical defenses was observed when herbivores or pathogens were excluded from *S. alterniflora* stands, then we would conclude that *S. alterniflora* invests constitutively in chemical defenses or that a four week timeframe is insufficient to observe significant changes in the defensive profile of the plant.

Overall, our study sought to a) establish whether *S. alterniflora* allocates resources towards producing a single class of chemical defenses or a diverse suite of molecules that deter closely coupled pathogens and grazers, b) measure expression of *S. alterniflora* chemical defenses on plant surfaces and inner tissues to assess if these defenses are likely to limit fungal farming in the field, and c) determine whether chemical defenses of *S. alterniflora* are induced or constitutively expressed.

Methods

Experimental Organisms

Short form smooth cordgrass (*Spartina alterniflora*) was collected from mid elevation marshes adjacent to the University of Georgia Marine Institute on Sapelo Island, GA (31 ° 23 ’ 47 ” N, 81 ° 17 ’ 00 ” W) in May-July 2011. Shoots were collected at least 10 m apart to increase likelihood of plant genetic diversity. All plant samples were rinsed and stored at -20 °C until extraction. A smaller suite of *S. alterniflora* samples were collected from Skidaway Island, GA (31 ° 57 ’ 37 ” N, 81 ° 01 ’ 39 ” W) in September 2012 to quantify surface and whole tissue concentrations of chemical defenses. *Littoraria irrorata* (average length 5.6 ± 1.9 mm) used for feeding assays were collected from Sapelo Island marshes, housed in plastic reptile cages, and fed a diet of powdered sea lettuce (*Ulva lactuca*) embedded in agar. Snails were checked periodically for the presence of fungal hyphae in their feces by rubbing snails along an agar plate and culturing resultant microbial colonies.

We obtained two species of fungi, *Mycosphaerella* sp. and *Phaeosphaeria spartinicola*, from the American Type Culture Collection. These fungi (SAP154 and SAP136, respectively) originally isolated from Sapelo Island, GA have been detected in *S. alterniflora* wound sites created by *L. irrorata* (Newell 2001, Silliman and Newell 2003). Fungi were cultured in sterile potato dextrose broth maintained at 29 °C.

Isolation of *S. alterniflora* Chemical Defenses

Prior to extraction, short form *S. alterniflora* leaves were cut into 2 cm segments to maximize exposed plant surface area. We generated a crude organic extract by

exhaustively extracting frozen *S. alterniflora* tissues with methanol and dichloromethane. Extracts were pooled and removed under rotary evaporation. We fractionated whole *S. alterniflora* extracts using a modified Kupchan liquid-liquid partitioning scheme (Kupchan et al. 1975), resulting in five fractions that differed in polarity. Deterrent fractions were further separated with silica gel (for antifungal compounds) or C_{18} silica gel (for antigrazer compounds), followed by size exclusion chromatography with Sephadex LH-20 resin. Antifungal and antigrazer compounds were purified after two rounds of semi-preparative reversed phase high performance liquid chromatography (HPLC, Waters 515 pump and Waters 2996 photodiode array detector, Grace Alltima C_{18} silica column) using a methanol/water gradient mobile phase. NMR spectroscopic data (^{1}H, ^{13}C, COSY, HSQC, and HMBC) were collected in $CDCl_3$ and 3:1 $MeOD/D_2O$ for pure compounds on a Bruker DRX-500 MHz Avance spectrometer. High resolution mass spectra of pure compounds were obtained using an Orbitrap mass analyzer in positive and negative electrospray ionization mode, and optical rotation of compounds in methanol was measured on a Jasco digital polarimeter. Structures of individual compounds were confirmed by comparing spectroscopic data with published reports of known compounds (Henry et al. 1987; Zhou et al. 2005).

Total phenolics were quantified for a subset of chromatographic fractions using the Folin-Ciocalteu assay (Folin and Ciocalteu 1927), with tannic acid as standard. Sample absorbance (n = 3 per fraction) was recorded at 760 nm on a UV-visible spectrophotometer (Spectronic 21D) following two hours of development after addition of Folin-Ciocalteu reagent and sodium carbonate to sample aliquots.

Growth Inhibition Trials Using Marsh Fungi

Chemical defenses against fungi were assayed using a growth assay *sensu* Sieg (Chapter 4). In brief, *S. alterniflora* chromatographic fractions were dissolved in methanol, mixed with a sterile molten potato dextrose agar matrix, and transferred into a single well of a 24 well plate adjacent to a solvent control well (n = 3-5 wells fraction^{-1}). Compounds were embedded in agar at concentrations corresponding to the amount of extract generated from an equivalent volume of plant material, although pure compounds were tested at up to four times natural concentrations to account for progressive losses during the fractionation process. Plates were incubated at 29 °C after pipetting 100 μL of media containing macerated fungi (*Mycosphaerella* sp. or *P. spartinicola*) onto each well. The amount of agar surface covered by fungi was estimated to the nearest 5% under light microscopy after five days. A paired, 1-tail *t*-test was used to compare growth of marine fungi between treatments and controls. GraphPad Prism 6 was used for statistical analyses throughout this study, and all data were arcsine transformed prior to analysis to account for bound data sets.

Dose response curves were created by exposing both fungi to 9*S*-hydroxy-10*E*,12*Z*,-octadecadienoic acid (α-dimorphecolic acid) at eight concentrations ranging from 5.0 to 850 μM using the same bioassay methods described above (n = 4 for each concentration). The IC_{50} for each fungus was calculated by fitting a sigmoidal dose-response curve to a plot of fungal growth inhibition against the concentration of α-dimorphecolic acid. This design simulated exposure to antifungal compounds contained within plant tissues. To mimic surface exposure to plant chemical defenses, we coated agar blocks with α-dimorphecolic acid at three concentrations ranging from 0.5 to 5.0 μg

cm^{-2}. *Mycosphaerella* sp. and *P. spartinicola* inocula were added as described above to coated agar and growth was analyzed using the methods described for our previous fungal assays.

Concentrations of α-dimorphecolic acid were quantified in surface and whole tissue *S. alterniflora* extracts by separating plant extracts on a Grace Alltima C_{18} silica column attached to Waters 2695 separations module and Waters 2996 photodiode array detector coupled to a Micromass ZQ mass spectrometer run in negative electrospray ionization mode. Surface extracts were generated by dipping individual *S. alterniflora* blades (n = 15) in hexanes for 30 seconds to remove non-polar chemical constituents from plant surfaces (de Nys et al. 1998). Whole tissue extracts of single *S. alterniflora* blades (n = 15) were generated by exhaustive extraction in dichloromethane and methanol. After generating a standard curve of α-dimorphecolic acid from purified samples, we integrated mass spectrometric peaks for the $[M-H]^-$ ion at *m/z* 295 from crude extracts to determine α-dimorphecolic acid concentration relative to surface area or dry plant mass.

Feeding Trials Using *L. irrorata*

Spartina alterniflora compounds were screened for antigrazer activity by embedding extracts in artificial diets composed of powdered *U. lactuca* and agar, and offering treatment and control diets to *L. irrorata* simultaneously *sensu* Sieg (Chapter 4). Extracts were tested at concentrations corresponding to the amount of extract generated from a mass of plant equivalent to the mass of artificial diet, although semi-purified fractions were tested at higher concentrations to account for losses during separation.

Fractions were suspended in methanol as a carrier solvent to coat *U. lactuca*, while controls consisted of *U. lactuca* coated in methanol without plant extracts. Coated *U. lactuca* was mixed with molten agar medium, poured into 2.5 cm diameter Petri dishes, and allowed to cool (n = 20 dishes per fraction). Artificial foods were cut in half, after which control and treatment diets were offered to three snails on the same dish. After 48 hours, a paired, 1-tail t-test was used to determine statistical differences in consumption between diet types.

Induction of *S. alterniflora* Chemical Defenses

A field caging experiment was used to determine whether removal of *L. irrorata* and/or fungi relaxed expression of chemical defenses in defended populations of *S. alterniflora*. Five sites were selected within a mid-elevation marsh zone adjacent to the University of Georgia Marine Institute (31 ° 23 ’ 47 ” N, 81 ° 17 ’ 00 ” W) in June 2011. Within each site, 40 shoots of *S. alterniflora* were haphazardly selected, cleaned of mesograzers, and enclosed in a cage made from irrigation pipe and mesh (10 cm diameter x 60 cm height). Cages were placed 1.0 m apart, forming an 8 x 5 grid at each site. While placing cages, *S. alterniflora* rhizomes were severed using a spade to isolate caged shoots from the rest of population and minimize any effects caused by belowground signaling among *S. alterniflora* clones.

Four treatments (n = 10 per site) were designed to manipulate the presence or absence of *L. irrorata* and fungi within cages. We were unable to get permission to directly infect *S. alterniflora* with cultured fungi, so we estimated the effects of *L.*

irrorata or marsh fungi on production of *S. alterniflora* defenses using three levels of herbivory.

1) Herbivore exclusion (ambient fungus, no *L. irrorata*): Snails were excluded from cages, preventing establishment of fungal farms.

2) Artificial wounding (ambient fungus, *L. irrorata* mimic): Snails were excluded from cages, but a 5.0 cm scar was created on a single *S. alterniflora* blade to simulate snail grazing without chemical cues associated with *L. irrorata* herbivory or addition of fungi from snails.

3) Addition of snails lacking fungal hyphae (ambient fungus, *L. irrorata* present): Three lab-reared *L. irrorata* that had been flushed of fungal hyphae were added to each cage to measure effects of snail grazing while minimizing fungal exposure directly caused by *L. irrorata* herbivory.

4) Addition of snails with fungal hyphae (elevated fungus, *L. irrorata* present): Three *L. irrorata* collected from the experimental site were added to each cage, representing natural exposure to *L. irrorata* and associated fungi.

Cages were monitored twice weekly for four weeks, during which time cages were inspected to ensure treatments were maintained, and a pulse amplitude fluorometer was used to measure the photosynthetic efficiency of the second rank blade from each caged shoot. Photosynthetic efficiencies of 10 uncaged plants were also measured at each site to estimate caging effects on *S. alterniflora*.

After four weeks, each caged shoot was pulled from the sediment, rinsed and frozen at -20 °C until extracted. Differences in chemical defenses among treatments were determined using the bioassay methods described previously. Significant inhibition of

fungal growth or *L. irrorata* feeding by plant extracts was detected within a treatment by comparing growth or feeding inhibition of treatment extracts against paired solvent controls using a paired, 1-tail *t*-test. Since we were unable to directly manipulate the presence or absence of fungi across all levels of herbivory, significant effects due to fungi and snails could not be directly compared. However, a two-factor ANOVA was utilized to detect differences in chemical defenses among all treatments, sites, and the interaction of these two factors.

Results

Spartina alterniflora Produces a Fatty Acid Metabolite to Inhibit Fungal Growth

Crude *Spartina alterniflora* extracts significantly reduced growth of farmed fungi by over 50 % relative to controls ($P < 0.001$, Fig. 3.1A). With chromatographic fractionation of extracts guided by assays with *Mycosphaerella* sp., we determined that *S. alterniflora* contained a single lipophilic compound responsible for fungal growth inhibition (Fig. 3.1D). Spectroscopic analysis led to the identification of this compound as α-dimorphecolic acid (Fig 3.2; Henry et al. 1987), isolated with a yield of 0.0036% of dry plant mass, corresponding to 36 μg (g dry plant tissue)$^{-1}$ or 25 μM by plant volume. This isolated yield from bulk tissue was much lower than natural concentrations measured by LC-MS from crude extracts of individual *S. alterniflora* plants (220 ± 31 μM, which were 200 ± 40 μg (g dry plant tissue)$^{-1}$), suggesting that almost 90% of the compound was lost during purification steps. To account for such losses, α-dimorphecolic acid was initially tested at four times natural concentration, strongly inhibiting growth of *Mycosphaerella* sp. (Fig. 3.1D). This tested concentration (100 μM) was approximately half the average natural concentration of α-dimorphecolic acid detected in individual *S. alterniflora* extracts. Therefore, *S. alterniflora* appears to contain a high enough concentration of antifungal compound, as measured in whole tissue extracts, to significantly inhibit growth of *Mycosphaerella* sp.

Using a range of α-dimorphecolic acid concentrations of 5.0-850 μM, we measured the sensitivity of the fungi *P. spartinicola* and *Mycosphaerella* sp. to this antifungal agent. *Phaeosphaeria spartinicola* was more resistant to α-dimorphecolic acid (IC_{50} = 670 ± 4 μM, Fig. 3.3A) than *Mycosphaerella* sp. (IC_{50} = 57 ± 5 μM, Fig. 3.3A).

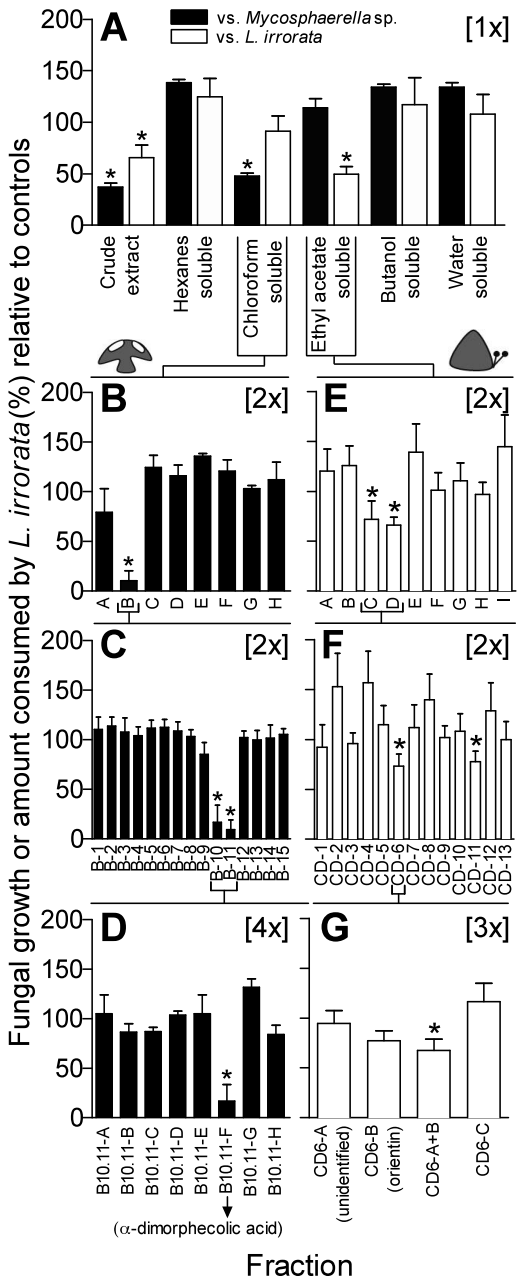

Figure 3.1. Isolation of antifungal and antigrazer compounds from short form *S. alterniflora*. Bars represent inhibition of the fungus *Mycosphaerella* sp. (A-D, black) or feeding by the snail *L. irrorata* (A, E-G, white) relative to paired controls. Separation methods include (A) liquid-liquid partitioning, (B) silica gel column chromatography or (E) C_{18} silica gel column chromatography, (C, F) Sephadex LH-20 size-exclusion column chromatography, and (D, G) C_{18} silica HPLC. Asterisks (*) represent significant differences between treatments and paired controls (1-tail *t*-test, n = 3-5 (vs. *Mycosphaerella* sp.), n = 16-20 (vs. *L. irrorata*)), while error bars represent 1 S.E. Tested concentrations relative to natural isolated yields are denoted in the upper right hand corner of each graph in square brackets.

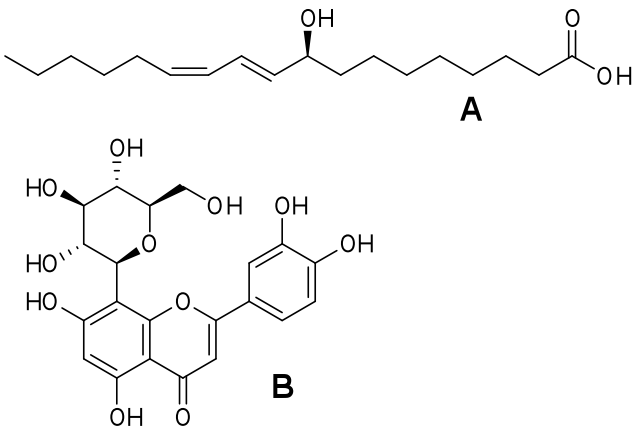

Figure 3.2. Chemical defenses produced by *S. alterniflora* to prevent fungal farming. (A) α-dimorphecolic acid, a fatty acid that inhibits growth of the fungus *Mycosphaerella* sp. and (B) orientin, which reduces grazing by the snail *L. irrorata* in conjunction with other phenolic compounds.

The minimum concentration of α-dimorphecolic acid that significantly inhibited *P. spartinicola* growth (340 μM, Fig. 3.3A) was equivalent to the bulk tissue concentration of α-dimorphecolic acid detected in four of 15 individual *S. alterniflora* samples, but was higher than the average concentration of this compound (220 μM) across all surveyed individuals. In contrast, *Mycosphaerella* sp. was significantly inhibited by α-dimorphecolic acid concentrations as low as 10 μM (Fig. 3.3A), which was well within the natural concentration of this fatty acid in *S. alterniflora* tissues.

In contrast to whole tissue extracts, natural concentrations of α-dimorphecolic acid on *S. alterniflora* surfaces appeared inadequate to inhibit growth of either fungus on our panel. Surface concentrations of α-dimorphecolic acid were below the limit of detection (1.1 μg cm^{-2}) of our LC-MS instrument. Neither *Mycosphaerella* sp. nor *Phaeosphaeria spartinicola* were inhibited by α-dimorphecolic acid when the compound was coated on agar surfaces at this threshold surface concentration (Fig. 3.3B), suggesting that even lower α-dimorphecolic acid concentrations present on plant surfaces would have little effect on fungal growth. However, growth of *Mycosphaerella* sp. was significantly reduced when α-dimorphecolic acid surface concentrations were elevated at least five-fold higher than concentrations detected in *S. alterniflora* tissue (5.0 μg cm^{-2}, 31% reduction in growth, $P = 0.044$, Fig. 3.3B).

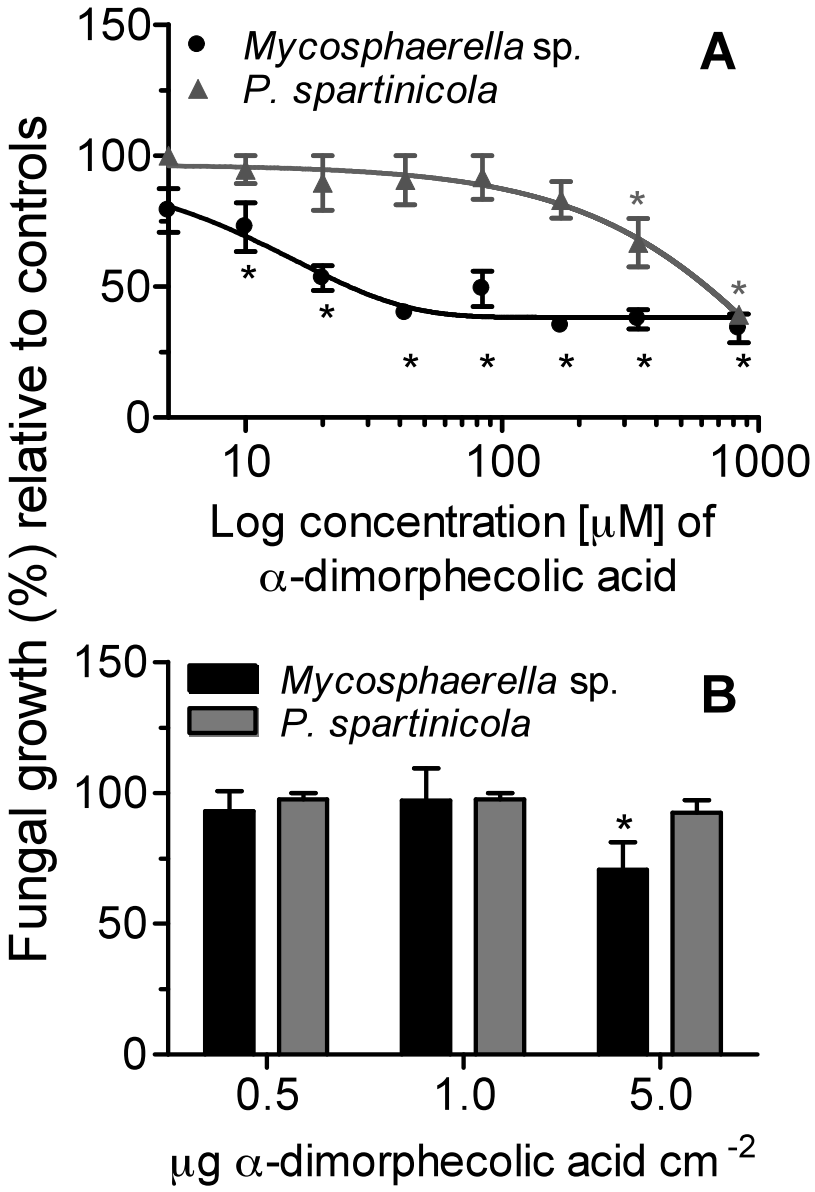

Figure 3.3. Growth inhibition of α-dimorphecolic acid against the fungi *Mycosphaerella* sp. (black) and *P. spartinicola* (grey) when embedded in agar representing whole tissue (A) or surface (B) concentrations. Significant differences in growth between paired treatments and controls determined by 1-tail *t*-test ($P < 0.05$, $n = 4$) and denoted by asterisks. Bars represent 1 S.E.

Polar Compounds Defend *S. alterniflora* from *L. irrorata* Herbivory

Crude extracts generated from short form *S. alterniflora* reduced *Littoraria irrorata* grazing on artificial diets by 35% relative to solvent controls ($P = 0.0016$, Fig. 3.1A), suggesting that *S. alterniflora* is chemically defended against herbivory. After liquid-liquid partitioning of crude extracts, chemical defenses from *S. alterniflora* that deterred *L. irrorata* were contained in the fraction collected from a moderately polar ethyl acetate layer (Fig. 3.1A). Bioassay-guided separation of this fraction using polarity-based and size-exclusion chromatography resulted in isolation of three compounds that contributed to deterrence of *L. irrorata* grazing (Fig. 3.1F, G). Two of these compounds (CD6-A and CD6-B) had a non-significant deterrent effect on snail feeding when each was embedded into artificial diets alone, but significantly reduced *L. irrorata* feeding when added to diets together at 3x the concentration that compounds were found in *S. alternifora*, suggesting that these compounds had an additive, deterrent effect on *L. irrorata* grazing (Fig. 3.1G). Other combinations using the pure compound CD6-C were not significant unless both CD6-A and CD6-B were both present (*data not shown*). Due to progressive losses during fractionation, the elevated concentrations at which compounds were tested (2-3X natural isolated yields) may actually be similar to those expected within live plant tissues. Both compounds were susceptible to decomposition as fractionation progressed. CD6-A was a moderately polar, small (<500 Da) molecular weight compound, but was not fully characterized despite attempts to limit compound oxidation by adding antioxidants. However, spectral analysis of compound CD6-B indicated that it was the known flavonoid orientin (Fig. 3.2; Zhou et al. 2005).

While orientin has been isolated from other grass species (van de Staaij et al. 2002), to our knowledge this is the first report of orientin from *S. alterniflora* tissues.

A third compound (CD-11) also significantly inhibited *L. irrorata* grazing (Fig. 3.1F), but rapidly decomposed after purification, preventing its identification. Subsequent attempts to isolate this compound were unsuccessful, but we can conclude that CD-11 is a polar, small molecular weight compound based on its retention on silica gel and size exclusion chromatography. These data suggest that other, as yet uncharacterized compounds limit *L. irrorata* consumption of *S. alterniflora*, in addition to orientin. The UV λ_{max} of CD6-A (225, 322 nm) and CD-11 (206 nm) are close to that of orientin (252, 345 nm), and analysis of samples with the Folin-Ciocalteu assay revealed that both unknown compounds contained phenolics. However, other non-active fractions separated by C_{18} silica gel column chromatography also contained phenolic compounds, suggesting that some, but not necessarily all phenolics produced by *S. alterniflora* functioned as feeding deterrents against *L. irrorata*.

Spartina alterniflora is Constitutively Defended Against *L. irrorata* and Fungi

Spartina alterniflora extracts generated after a month long field experiment manipulating grazing and fungal exposure were significantly deterrent to *L. irrorata* and fungal pathogens (Fig. 3.4). However, chemical defenses against snails and fungi remained similar whether snails and fungi were present or removed from *S. alterniflora*, rejecting the hypothesis that defenses are induced in this population of *S. alterniflora* (Fig. 3.4, Table 3.1). The potency of chemical defenses against either snails or fungi was not significantly affected by treatment, site, or interaction between factors (2-factor

ANOVA, Table 3.1), suggesting that chemical defenses were constitutively expressed by *S. alterniflora*. After 30 days, caging reduced the photosynthetic efficiency of *S. alterniflora* by an average of 11 % relative to uncaged plants, but this effect was consistent among treatments (*data not shown*). Thus, photosynthesis of *S. alterniflora* appeared unaffected by snail and fungal manipulations.

Crude extracts tested at natural and half natural concentrations reduced *L. irrorata* consumption of artificial diets by 25-40 % (n = 9-15, $P < 0.050$ for all treatments, Fig. 3.4). When tested at natural volumetric concentrations, *S. alterniflora* extracts inhibited growth of the fungi *Mycosphaerella* sp. and *P. spartinicola* by over 70% relative to controls ($P < 0.001$ for all treatments, Fig. 3.4A). However, when tested at half natural concentrations *S. alterniflora* extracts were still significantly deterrent to *Mycosphaerella* sp. (25-60% inhibition, P = 0.001-0.020, Fig. 3.4B) whereas growth of *P. spartinicola* was statistically similar to paired controls (P = 0.092-0.61, Fig. 3.4B). The inhibitory properties of *S. alterniflora* crude extracts against *Mycosphaerella* sp. are in accordance with assays testing pure α-dimorphecolic acid (Fig. 1D, Fig. 3.4). In contrast, *P. spartinicola* was much more susceptible to growth inhibition by *S. alterniflora* crude extracts (containing α-dimorphecolic acid) than pure α-dimorphecolic acid at similar concentrations to that found in crude extracts (Fig. 3.4). Given that the caging experiment was conducted at the same sites and in the same season as harvesting of *S. alterniflora* for isolation of chemical defenses, this suggests that other as yet unidentified compounds from within *S. alterniflora* tissues inhibit *P. spartinicola* growth.

Table 3.1. Two-factor ANOVA from month-long field caging experiment to test potency of *S. alterniflora* chemical defenses after altering exposure to herbivores or fungi.

Effect (*df*)		**Treatment (*3*)**	**Site (*4*)**	**Interaction (*12*)**
Antiherbivore				
vs. *L. irrorata* (1x)	*F*	1.51	1.51	0.45
	P	0.25	0.24	0.92
vs. *L. irrorata* (0.5x)	*F*	0.52	1.13	0.24
	P	0.67	0.36	0.99
Antifungal				
vs. *Mycosphaerella* sp. (1x)	*F*	0.45	1.39	0.45
	P	0.72	0.25	0.93
vs. *Mycosphaerella* sp. (0.5x)	*F*	1.05	0.75	0.92
	P	0.40	0.56	0.56
vs. *P. spartinicola* (1x)	*F*	0.93	1.57	0.91
	P	0.43	0.20	0.54
vs. *P. spartinicola* (0.5x)	*F*	1.15	0.94	1.06
	P	0.35	0.46	0.43

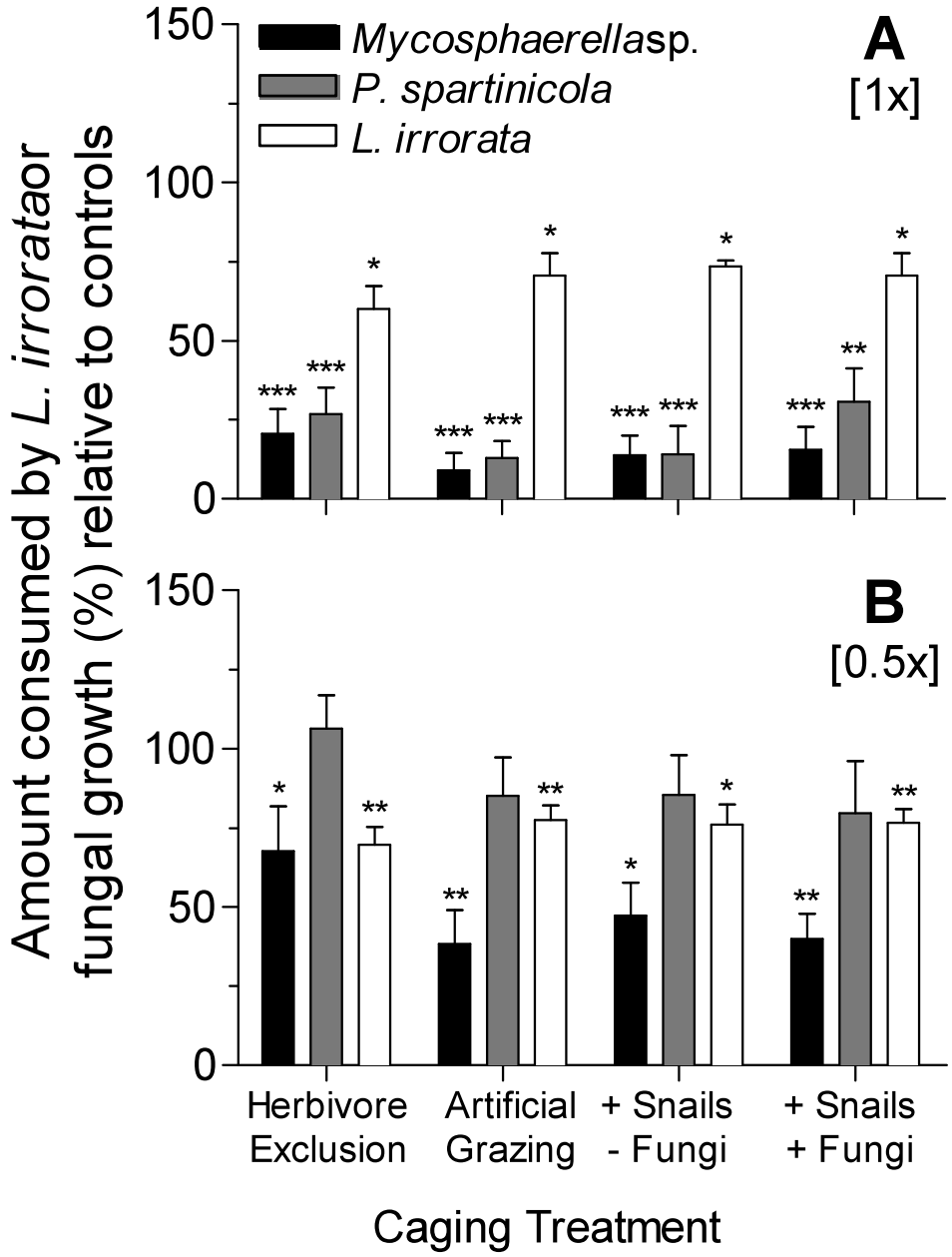

Figure 3.4. Chemical defenses in short form *S. alterniflora* after cage manipulations in the field against the fungi *Mycosphaerella* sp. (black bars) and *P. spartinicola* (grey bars) or the snail *L. irrorata* (white bars). Caging treatments denoted on the x-axis. Extracts were added to agar at (A) natural or (B) half natural isolated concentrations. Significant differences between paired treatments and controls determined by 1-tail *t*-test ($P < 0.05$, $n = 9$-15) and denoted by asterisks, (*, $P < 0.05$), (**, $P < 0.01$), (***, $P < 0.001$).

Discussion

Fungal farming by the periwinkle snail *Littoraria irrorata* has been detected in salt marshes along the southeastern United Sates (Silliman and Zieman 2001; Silliman and Newell 2003), but rarely occurs on marsh plants other than *Spartina alterniflora* (Silliman and Bertness 2002; Sieg Chapter 4). Selection of *S. alterniflora* as a farming substrate by *L. irrorata* may be influenced by the relative potency of chemical defenses among marsh plants. In a previous study, chemical defenses produced by *S. alterniflora* were less inhibitory towards *L. irrorata* and fungi than those expressed by four other co-occurring marsh plants (Sieg Chapter 4). Snails also showed a marked preference for *S. alterniflora* over other available plants, suggesting that *L. irrorata* assesses which plant substrates are most amenable to fungal farm establishment in selecting its habitat (Sieg Chapter 4). However, in the current study we show that *S. alterniflora* produces multiple polar compounds that collectively deter *L. irrorata* herbivory as well as the fatty acid α-dimorphecolic acid that inhibits the growth of the fungus *Mycosphaerella* sp. associated with *L. irrorata* farming (Fig. 3.1; Newell and Barlocher 1993; Newell 2001). These defenses are weaker than those produced by other marsh species, and the antifungal compound is not present on plant surfaces at concentrations adequate to prevent fungal establishment. Thus, while *S. alterniflora* is modestly chemically defended against both snail grazers and pathogenic fungi, it remains a preferred target relative to other, more defended marsh plants.

While several *S. alterniflora* compounds were isolated that additively deterred *L. irrorata* grazing, we were only able to fully characterize one of these, orientin. This flavonoid glycoside is produced by a variety of plants including grasses (van de Staaij et

al. 2002) and other species used as traditional medicines, such as rooibos (Bramati et al. 2002), holy basil (Devi et al. 2000) and lemongrass (Cheel et al. 2005). Orientin can function as an antioxidant (Cheel et al. 2005) and exhibits antiviral (Li et al. 2002), antibacterial (Cottiglia et al. 2001), and antifungal (De Campos et al. 2005) properties as well as a protective role against radiation injury (Devi et al. 2000). However, far less is known about the ecological role(s) of this compound, although it can act as a photoprotectant (van de Staaij et al. 2002) and defends against mildew infection in conjunction with other compounds produced in cucumber leaves (McNally et al. 2003). Although phenolic compounds similar to orientin are often hypothesized to act as antifeedant compounds (Haribal and Renwick 1998; Renwick et al. 2001; Haviola et al. 2007) and other natural products of phenylpropanoid metabolic origin have been shown to function as plant chemical defenses (Kubanek et al. 2001; Lane and Kubanek 2006; Haviola et al. 2007), to our knowledge ours is the first study to demonstrate that orientin functions as part of a chemical defense against herbivores.

Considering that orientin did not deter *L. irrorata* at the concentrations tested unless other similar compounds were also added into diets (Fig. 3.1), it could be that a subset of phenolic compounds produced by *S. alterniflora* collectively limit grazing by snails. This would support the practice of using total phenolic concentrations as a proxy for how defended a plant is against herbivory (Feeny 1976; Coley et al. 1985; Appel 1993). However, only a few of the chromatographic fractions isolated in the current study were deterrent to snail grazing (Fig. 3.1), whereas several non-deterrent fractions also contained phenolic compounds. Based on limited spectroscopic information, our unidentified antiherbivore molecules are also likely to be phenolic compounds, but these

deterrent molecules only make up a subset of the total phenolic pool in *S. alterniflora.* Therefore, all phenolics produced by *S. alterniflora* do not act as antiherbivore compounds; instead other phenolics might provide additional, unknown services to the plant. This explanation would be in accordance with previous studies that did not find a significant relationship between total phenolic concentrations in *S. alterniflora* and antiherbivore properties of plant extracts (Long et al. 2011; Sieg Chapter 4).

A single fatty acid compound, α-dimorphecolic acid, was responsible for antifungal activity of *S. alterniflora* extracts against *Mycosphaerella* sp. (Fig. 3.1). This compound was previously isolated from terrestrial plants (Powell et al. 1967; Henry et al. 1987; McRae et al. 2008) and cyanobacteria (Mundt et al. 2003), but has not been reported from *S. alterniflora*. Given the antimicrobial properties of α-dimorphecolic acid (Mundt et al. 2003; McRae et al. 2008), as well as the antifungal (Rao et al. 1991; Calvo et al. 1999; Cowley and Walters 2005) and antiherbivore (Mohri et al. 1990) properties of closely related fatty acids, it is not surprising that a compound such as α-dimorphecolic acid constitutes the modest defense of *S. alterniflora* against the pathogenic fungus *Mycosphaerella* sp.

The two fungi used in the current study responded differently to α-dimorphecolic acid in lab assays. The average natural bulk concentration of α-dimorphecolic acid (220 μM) expressed within *S. alterniflora* tissues was sufficient to protect the plant against *Mycosphaerella* sp. but not *P. spartinicola* (Fig. 3.3A), assuming that the compound was evenly distributed in all tissues including on plant surfaces. However, use of bulk tissue concentrations may overestimate ecologically relevant exposure of fungi to α-dimorphecolic acid. We found natural surface concentrations of α-dimorphecolic acid to

be lower than that required to significantly deter fungal growth of either pathogen (Fig. 3.3B), which may allow fungi to successfully establish on outer plant tissues. However, fungi often bypass *S. alterniflora* surface tissues by being transported directly to inner plant tissues exposed in wounding scars created by *L. irrorata*. Thus, it may benefit *S. alterniflora* to concentrate antifungal compounds not at the surface, but within plant tissues where concentrations of α-dimorphecolic acid can significantly inhibit fungal growth after a wounding event.

In a related study *P. spartinicola* was consistently more resistant to chemical defenses from four other salt marsh plant species than was *Mycosphaerella* sp., which could make *P. spartinicola* the better crop for snails to cultivate (Sieg Chapter 4). We used *Mycosphaerella* sp. for all bioassays leading to isolation of α-dimorphecolic acid so it is possible that we overlooked other compound(s) *S. alterniflora* produces to inhibit *P. spartinicola* growth, which could explain why these two fungi were equally susceptible to *S. alterniflora* crude extracts when tested at natural concentrations (Fig. 3.4A).

In the current study, we have found that products of multiple biosynthetic pathways are utilized to partially defend *S. alterniflora* tissues against both farming snails and their fungal crop. Mixed products of the phenylpropanoid and polyketide metabolic pathways (i.e., orientin and related flavonoids) act as chemical defenses against grazers, while a fatty acid functions as an antifungal agent. We did not detect any overlap in antifungal or antigrazer activity within the chromatographic fractions during bioassay-guided fractionation (Fig 3.1A), so it is clear that α-dimorphecolic acid and orientin do not have reciprocal roles in defense against grazers or fungi, respectively. Investing in the production and maintenance of chemical defenses can impose fitness costs (see

review by Koricheva (2002)) but plants often employ a mixed chemical arsenal to defend against multiple threats. For instance, *Arabidopsis* utilizes glucosinolate hydrolysis products including nitriles and isothiocyanates to prevent caterpillar grazing (Lambrix et al. 2001), but relies on defensive proteins like defensins and thionins to defend against fungi (Epple et al. 1997). Other species rely on a single class of molecules to deter herbivores, pathogens, and competitors. The seaweed *Dictyota menstrualis* uses a suite of diterpene alcohols to prevent both fish herbivory and fouling by bryozoan larvae (Schmitt et al. 1995), and diatoms produce a blend of polyunsaturated aldehydes to defend against zooplankton grazers and to undermine competitors (see review by Ianora and Miralto (2010)). Even structurally related molecules produced by the same organism can affect herbivores and pathogens differently. The iridoid glycoside catalpol is more deterrent to herbivores than its precursor molecule aucubin, which functions as an antifungal agent in plantains (Bowers and Puttick 1988; Marak et al. 2002). Furthermore, the glycosylation status of molecules can affect their ecological targets, such that aglycones (non-glycosylated iridoids) inhibit specialist fungi, whereas iridoid glycosides deter generalist herbivores (Marak et al. 2002; Biere et al. 2004). It may be more efficient and less costly to produce generalized defenses that inhibit a range of organisms, but doing so may be disadvantageous if the targets of these defenses can adapt to or overcome defenses faster than the targeted organism can modify them.

The results of our caging experiment indicate that Georgia populations of *S. alterniflora* produce constitutive chemical defenses, and do not relax production of these defenses even when the threat of herbivory is removed for four weeks (Fig. 3.4). These results differ from a previous study of similar length conducted in South Carolina

whereby *S. alterniflora* exposed to a natural suite of herbivores responded by inducing more deterrent chemical defenses than those produced by plants for which herbivore pressure was removed (Long et al. 2011). Unlike other *S. alterniflora* induction studies (Long et al. 2011), we excluded all members of the salt marsh herbivore community apart from *L. irrorata*. It is possible that induction of *S. alterniflora* chemical defenses was initiated from grazing by other herbivore feeding guilds, such as planthoppers or aphids. Furthermore, induction of chemical defenses typically occurs in a few days following initial grazing events, while relaxation of these defenses can take weeks to months (Karban 2011). We cannot exclude the possibility that plants were caged too late in the season to undergo a noticeable change in chemical defenses, since the plant population would have been exposed to intense herbivory for several months prior to initiation of our caging treatments. Further studies are required to determine how long chemical defenses in *S. alterniflora* persist after initial induction events occur. However, these studies suggest that *S. alterniflora* populations vary in their expression of chemical defenses, such that southern populations consistently exposed to intense grazing by snails (i.e., in Georgia) are constitutively defended; populations with moderate grazing pressure induce chemical defenses upon exposure to herbivore cues; and populations exposed to low herbivore densities do not invest in chemical defenses at all. Increased investment in chemical defenses due to a greater threat of herbivory may also explain why plants from southern marshes are generally less palatable to a range of herbivores than their northern counterparts (Pennings et al. 2001; Siska et al. 2002; Salgado and Pennings 2005; Long et al. 2011).

While the ambient threat of herbivory may explain when *S. alterniflora* populations produce chemical defenses, other factors may also influence to what extent *S. alterniflora* populations are defended. For instance, our study was conducted in a salt marsh during a period of severe to extreme drought, which could have caused *S. alterniflora* to express chemical defenses differently than the South Carolina population studied by Long et al. (2011) where drought conditions were moderate. Reductions in freshwater inflow caused by drought can stress estuarine plants by creating hypersaline conditions and reducing nutrient input into salt marsh sediments (Alber 2002; Wetz et al. 2011). Secondary consumers of snails such as blue crabs are also more susceptible to parasites in warmer, more saline waters indicative of drought conditions (Lee and Frischer 2004), which may relieve snails from predation pressure. We did not quantify nutritional quality of marsh sediments in our study, and at present cannot say to what degree nutrient availability predicts *S. alterniflora* investment in chemical defenses. However, it is important to consider how abiotic environmental conditions can also explain population-level variability in *S. alterniflora* expression of chemical defenses.

In conclusion, *S. alterniflora* utilizes at least two classes of chemical compounds to prevent establishment of fungal farms. A suite of phenolics including orientin deterred *L. irrorata* herbivory, which is expected to limit snail grazing that facilitates fungal infection. A single fatty acid (α-dimorphecolic acid) produced by *S. alterniflora* inhibited the growth of a fungus that is commonly cultured by *L. irrorata*, although other compounds may be employed to defend against other common marsh fungi. Since fungal infection is often preceded by *L. irrorata* herbivory, *S. alterniflora* must invest in chemical defenses that deter both snail farmers and their fungal crop. However, these

defenses are clearly not potent enough to completely prevent establishment of fungal farms, since *S. alterniflora* is still colonized by snails (and fungi) far more frequently than other mid-elevation marsh species (Sieg Chapter 4). In contrast to previous studies, chemical defenses in *S. alterniflora* were not induced by exposure to herbivores or pathogens. Instead, *S. alterniflora* was constitutively defended against both fungi and snails, potentially due to intense, consistent grazing pressure within these populations. At present, we only have a partial understanding of the abiotic and biotic factors that cause *S. alterniflora* to express chemical defenses. The chemical defenses isolated in the current study may have been sufficient to allow *S. alterniflora* to reduce the spread of fungal farms in the past. As snail densities continue to increase due to reduced top-down predator control (Silliman and Bertness 2002), *S. alterniflora* is likely to be exposed more frequently to snails and the fungi they farm. In this scenario, the weak defenses that marginally limited fungal growth and snail herbivory previously may no longer sufficiently protect *S. alterniflora*, which could potentially explain why *S. alterniflora* dominated marshes continue to decline.

CHAPTER 4

CHEMICAL DEFENSES AGAINST HERBIVORES AND FUNGI LIMIT ESTABLISHMENT OF FUNGAL FARMS ON SALT MARSH ANGIOSPERMS

Abstract

Facultative mutualisms allow species to benefit more by working together than they would without cooperation. Occasionally, these interactions negatively impact the surrounding community, pressuring some community members to evolve mechanisms to limit the success of the mutualistic partners. Within coastal salt marshes of eastern North America, fungal farming by the snail *Littoraria irrorata* facilitates fungal growth on live plant tissues and provides a palatable food source for snails, while drastically reducing plant biomass. This interaction increases exposure of the foundation species *Spartina alterniflora* to fungal infection, whereas evidence of farming on other plants is rarely observed. We sought to identify traits from five salt marsh plant species, such as chemical defenses against snails or fungi, which restrict *L. irrorata* feeding patterns, habitat choice, 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 rendering 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 snail 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* defenses against fungi were weaker than those of the other

species, consistent with the observation of fungi only on wounded *S. alterniflora* tissues. We propose that due to its weak chemical defenses, *S. alterniflora* must withstand fungal farming by investing in new growth, whereas less abundant species heavily invest in chemical defenses against snails and fungi such that they are not as frequently colonized, consumed, or subjected to fungal farming by *L. irrorata*.

Introduction

Ecological interactions such as facilitation and mutualism are often seen as positive forces that enhance community structure, diversity, and the success of cooperative partners within stressful habitats (Stachowicz 2001; Bruno et al. 2003). These positive interactions can also have negative repercussions on organisms, directly or indirectly (Silliman and Newell 2003; Hay et al. 2004). For instance, the facultative mutualism between invasive yellow crazy ants and scale insects, which provide ants with honeydew in exchange for protection, has led to substantial losses in the diversity and abundance of native flora and fauna on Christmas Island (O'Dowd et al. 2003). At elevated densities, the ants rapidly eliminate populations of native red crabs, which are dominant omnivores on the forest floor, while scale insects directly and indirectly cause canopy tree dieback and pave the way for subsequent invasional "meltdown" (O'Dowd et al. 2003). Since mutualisms can fundamentally alter community structure, community members that are negatively affected by these interactions are expected to evolve resistance mechanisms to defend themselves or prevent mutualist success.

Salt marshes 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 mash primary production and alter marsh landscapes (Silliman et al. 2005). In particular, periwinkle

snails (*Littoraria irrorata*) create grazing scars along the blades of *S. alterniflora* that facilitate establishment of fungi (Silliman and Newell 2003), in a rare example of fungiculture outside the class Insecta (Mueller and Gerardo 2002). Snails selectively deposit spore-laden feces within wound sites to enhance fungal establishment and growth (Silliman and Newell 2003). *Littoraria irrorata* later consume fungal hyphae during garden maintenance, obtaining a preferred food item that is more nutritious than uninfected *S. alterniflora* tissue (Barlocher et al. 1989; Barlocher and Newell 1994a, 1994b). Through a facultative mutualism, fungi and snails gain access to more nutritious resources than would be expected in the absence of farming. In the presence of compounding abiotic stressors, these snail-mediated fungal infections 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.

Since snails and fungi can control salt marsh vegetation, plants that are susceptible to consumption or infection should employ mechanisms to thwart this facultative mutualism. Terrestrial plants produce a variety of structural (Milewski et al. 1991) and chemical (Agrawal and Kurashige 2003) defenses to 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 (Buchsbaum et al. 1984; Barlocher and Newell 1994b; Pennings et al. 1998; Siska et al. 2002), yet the relative palatability of plants to *L. irrorata*, especially in the context of fungal farming, is currently unclear and requires further consideration.

Despite the evidence that fungi are dominant decomposers in salt marsh ecosystems (Newell 2001a) and play a vital role in fungiculture (Silliman and Zieman 2001; Silliman and Newell 2003), we know surprisingly little about the prevalence of antifungal compounds among salt marsh plants. However, seagrasses, another class of marine angiosperms, 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). Chemical defenses against fungi are also documented in terrestrial plants (Terras et al. 1995). Therefore, 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 utilize chemical defenses against fungi, 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 species (*S. alterniflora, Batis maritima, Borrichia frutescens, Salicornia virginica* and *Iva frutescens*) common to middle elevation salt marsh zones (Chapman 1974; Pennings and Bertness 2001), to snails 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). *Spartina alterniflora* extracts have also been shown to

reduce *L. irrorata* grazing (Long et al. 2011), but extracts of none of the other plants on our panel have been previously tested against *L. irrorata*. Furthermore, no previous studies have determined whether these plants also utilize chemical defenses to inhibit growth of co-occurring fungi, thus 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, nutritional, and/or chemical defenses explain feeding preferences by *L. irrorata*? (3) Do salt marsh plants produce molecules that limit fungal infection, and if so, how variable are these defenses among plant species? And (4) does *L. irrorata* farm fungi on plants that have comparatively weak defenses against both fungi and herbivores? Using a combination of field studies and controlled laboratory assays, we demonstrate whether salt marsh plants simultaneously deter herbivores and pathogens using chemical defenses, and consider how the relative strength of these defenses may predict which species are most susceptible to *L. irrorata* and the pathogenic fungi that it cultures.

Methods

Surveys

We conducted surveys within the National Estuarine Research Reserve on Sapelo Island, GA in July 2011 to measure distributions and abundances of *L. irrorata* and salt marsh vegetation. Surveys were taken in middle elevation marshes 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 50 m transect (n = 3 transects per site). 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.

Littoraria irrorata densities on plants were measured by visually inspecting 100 individuals of the five most abundant mid marsh plant species (*B. maritima, B. frutescens, I. frutescens, S. alterniflora,* and *S. virginica*) and counting the number of snails observed per plant (n = 100 plants $species^{-1}$ $site^{-1}$). Statistical differences in *L. irrorata* densities 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.

Quantification of Fungi

We extracted ergosterol, a cell membrane component found only in fungi that is commonly used to estimate fungal load on plant tissues (Seitz et al. 1979; Newell et al. 1988), from plant samples collected in July 2011 at the aforementioned survey site adjacent to the marine institute. Samples from each of the five surveyed plants were cleaned, cut into 2 cm segments, pooled, 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.

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 hours, snail distributions among plants were recorded three times and averaged to provide an estimate of snail habitat preference. After each measurement, snails were placed in the center of the mesocosm and plants were rearranged. New plants and snails were used to begin each new series of trials. The five mesocosms were used for four rounds of experiments, thus 20 trials were conducted in total. Since data were not normally distributed, we ran a non-parametric Friedman's test with multiple comparisons to measure differences in snail habitat choice among plant species.

Plant and Animal Collections

To investigate plant responses to herbivory and fungal infection, we examined structural and chemical defenses among the five focal marsh plant species in our panel. Plant and animal samples were collected in July 2011. *Littoraria irrorata* collected for grazing assays (average length 5.6 ± 1.9 mm) were housed in plastic reptile cages with a bed of small rocks. Cages were kept damp with seawater at 25 °C, and snails were fed a diet of powdered sea lettuce embedded in agar between trials. All plant species other

than *I. frutescens* were removed intact from the marsh to minimize plant structural damage, while leaf and stem cuttings were taken for *I. frutescens*. We collected plants at least 10 m apart to maximize our chances of collecting distinct individuals instead of genetic clones. Harvested samples were cleaned and stored at -20 °C until extraction (see below). We estimated plant nutritional quality by measuring the protein, organic and phenolic content of dry, ground tissues from all surveyed marsh plants (n = 5-11 species^{-1}). Protein content was quantified using a modified Bradford protein assay (Bradford 1976), organic content was calculated as organic matter lost after combusting dry tissues at 500 °C for 24 h, and phenolic content was measured with the Folin-Ciocalteu assay using tannic acid as a standard (Folin and Ciocalteu 1927). All nutritional traits were expressed relative to dry mass. Differences in nutritional value among plant species were measured using a one-way ANOVA and Tukey's HSD test.

Assays Testing Plant Structural Defenses Against Herbivores

Preliminary multi-choice feeding trials using fresh whole plant tissues were unsuccessful due to the slow feeding behavior of snails, as plant tissues rotted before quantifiable grazing occurred. However, snails readily consumed artificial diets containing ground tissues, so an assay methodology where *L. irrorata* were offered a choice between several diets consisting of ground marsh plants and agar was employed *sensu* Hay et al. (1994). This assay enabled us to standardize plant structures among species and assess how important plant structure was in *L. irrorata* food selection. Frozen plant tissues were lyophilized and ground to a fine powder. Powdered plant tissue was added to a molten agar matrix at ¼ natural concentrations (measured by plant

volume) and poured into an 8 cm diameter Petri dish. Attempts to make artificial foods using higher concentrations of powdered plant material in agar were unsuccessful, as the matrix became too thick to pour. Once foods had solidified, they were cut into fifths, and a single wedge of each artificial food was placed in each dish, resulting in five food options per plate.

Dishes containing artificial foods (n = 10) were placed on a damp paper towel in a lidded plastic container, and 10 snails were added above the food dish. Snails consumed diets for 72 h, after which we placed each plate on a gridded background and counted the number of visible squares remaining in each diet to assess feeding preferences. Since these data were not normally distributed, we compared diet preferences using a Friedman's test with multiple comparisons (Roa 1992).

Assays Testing Plant Chemical Defenses Against Herbivores

To examine chemically mediated deterrence of focal marsh plants, we extracted plant tissues and incorporated them into artificial diets for snail feeding assays. For each extract (n = 8 species^{-1}), a single shoot was rinsed, cut into 1 cm portions and extracted in methanol for 1 h, followed by subsequent extractions with 1:1 and 1:2 mixtures of methanol and dichloromethane. Solvents were dried under rotary evaporation, generating a crude organic plant extract.

Artificial foods containing plant extracts were created by coating 0.6 g of ground *Ulva lactuca* (a green alga palatable to *L. irrorata*; Long et al. (2011)) with an equivalent amount of crude extract (based on plant dry mass) suspended in methanol. Solvents were removed under vacuum after coating. Coated *U. lactuca* was added to 20 mL of a molten

agar medium, shaken vigorously, pipetted in 2 mL aliquots into 2.5 cm diameter Petri dishes, and allowed to harden *sensu* Long et al. (2011). After hardening, each treatment food was cut in half and paired with a control food. Control foods were coated in carrier solvent without plant extracts, but otherwise prepared identically to treatments.

For each feeding trial, three snails were placed on a food plate in a plastic container lined with a moist paper towel. Snails consumed foods for 48 h, after which the area of treatment and control diet consumed was measured by placing each plate on a gridded background. Diet choice was recorded as the total number of visible squares underneath respective artificial foods. Due to wide variation in snail feeding, we used the average amount of food eaten from 20 plates to estimate palatability of each plant extract. If snails consumed less than 20% of total diet available on a single plate, that plate was excluded from analysis. A paired two-tail *t*-test was used to compare consumption of treatments and paired controls within a plant species, while differences in relative consumption among plant species was analyzed with a one-way ANOVA and Tukey's HSD test. Consumption values were bound by food size and not normally distributed, thus data were arcsine transformed before analysis.

Fungal Assays

We conducted fungal growth assays to assess the inhibitory properties of marsh plant extracts against commonly encountered fungi *sensu* Lane et al. (2009). Cultures of two fungi (*Mycosphaerella* sp. and *Phaeosphaeria spartinicola,* SAP154 and SAP 136, respectively) representing common salt marsh fungi that have been detected in infected *S. alterniflora* tissues (Newell and Barlocher 1993, Newell 2001b) were obtained from the

American Type Culture Collection. These strains were originally isolated from Dean Creek marsh on Sapelo Island, close to our survey sites. Fungal cultures were maintained in sterile liquid potato dextrose broth at 29 °C until needed for growth assays.

Growth assays were conducted by embedding extracts in molten potato dextrose agar at natural or half natural volumetric concentrations, and transferred into wells of a 24-well plate (n = 3 wells extract^{-1}, n = 8 extracts species^{-1}). Treatment wells were paired with control wells that contained agar and carrier solvent. Each well was inoculated with a 100 μL suspension of macerated *Mycosphaerella* sp. or *P. spartinicola*, plates were incubated for five days at 29 °C, and the proportion of each well covered by fungus was estimated to the nearest 10%. Differences between treatments and paired controls were determined using a paired two-tail *t*-test after arcsine transformation, and differences among plant species were compared using a one-way ANOVA and Tukey's HSD test. The three subsamples were averaged prior to analysis to obtain a more accurate estimate of inhibition for each extract.

Results

Field Distributions of Salt Marsh Plants and *L. irrorata*

Spartina alterniflora was the most abundant plant species in the middle elevation marshes surveyed during the summer (230 ± 31 g m^{-2}, n = 3 sites, Fig. 4.1A), and also had the highest *L. irrorata* densities (0.48 ± 0.03 snails (g fresh plant)$^{-1}$, n = 300 shoots, Fig. 4.1B). In contrast, mean *L. irrorata* densities on all other species were lower than 0.15 snails (g fresh plant)$^{-1}$, although these plants were also far less abundant than *S. alterniflora* (Fig. 4.1B). In field surveys, *L. irrorata* was never observed on *I. frutescens*; therefore this plant was excluded from statistical analysis. Snail density varied significantly among plant species ($F_{3,8}$ = 8.34, P < 0.01, Fig. 4.1B). Post-hoc analysis revealed this was largely driven by differences in snail densities between *S. alterniflora* and the other three plant species (Fig. 4.1B), which may be due to snails preferring *S. alterniflora*, or *S. alterniflora* being the most abundant plant species in middle elevation marshes (Fig. 4.1A). Thus, we proceeded with a mesocosm choice experiment.

L. irrorata Habitat Choices

When presented with an approximately equal mass of vegetation of five mid elevation marsh plant species, *L. irrorata* were 2.6 to 5.5 times as likely to choose to reside on *S. alterniflora* within 72 h than on any of the other available plants (χ^2 = 44.3, df = 4, P < 0.001, Fig. 4.2). Snail densities on *B. maritima, B. frutescens, I. frutescens* and *S. virginica* were all similar to one another (P > 0.05, Fig. 4.2) and consistently lower than *L. irrorata* densities on *S. alterniflora* (P < 0.001 for all comparisons, Fig. 4.2). In

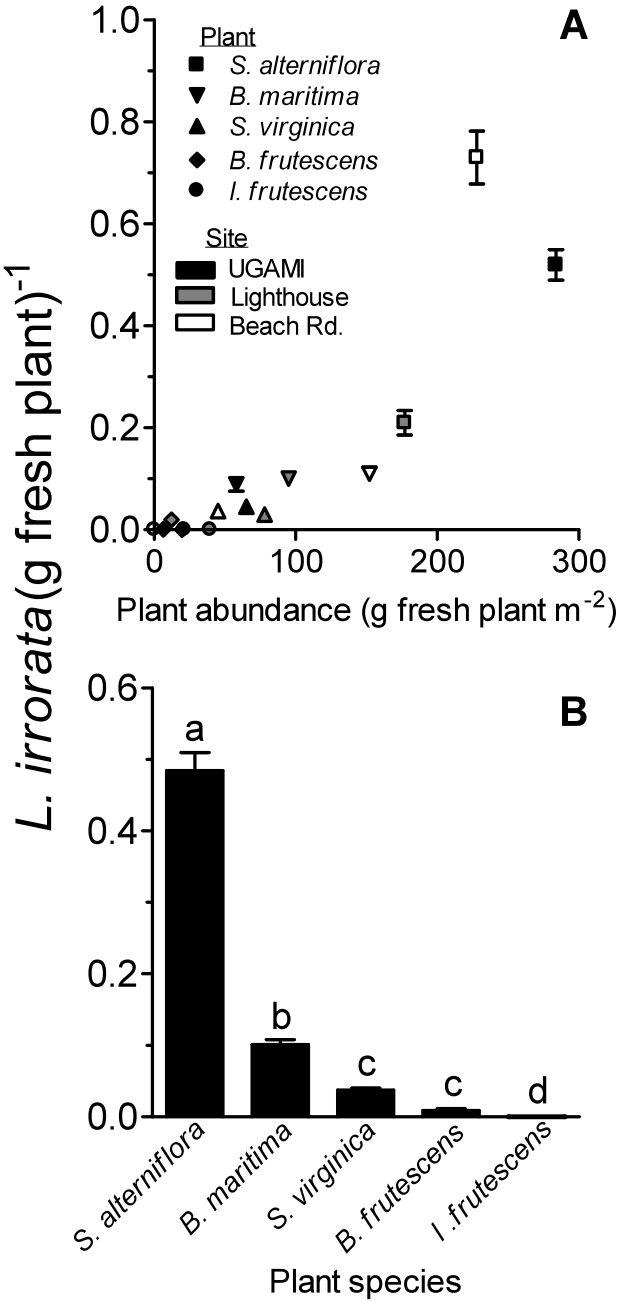

Figure 4.1. (A) *L. irrorata* densities relative to the abundance of five plant species among three middle elevation marshes on Sapelo Island, GA surveyed in July 2011; $n = 150$ quadrats site^{-1} to determine plant abundance, $n = 100$ plants site^{-1} surveyed to measure *L. irrorata* densities on each species. (B) Average *L. irrorata* densities across all sites; letters represent significant differences in snail densities among plant species (one-way ANOVA with Tukey's HSD test, $P < 0.01$, $n = 300$ plants species^{-1}). Error bars represent 1 S.E. in this and all later figures.

mesocosms, *L. irrorata* densities were 1.9 times higher on *S. alterniflora* than observed in the field (Figs. 4.1-2), even though snails had a choice of five plant species, four of which went mostly unoccupied. These data support the hypothesis that the high *L. irrorata* densities on *S. alterniflora* in the field are a result of snail preferences and not only due to the higher probability of encountering *S. alterniflora* in the field.

Feeding Preferences of *L. irrorata*

Littoraria irrorata preferred consuming artificial diets containing ground *S. alterniflora* and *B. maritima*, leaving the other three food options untouched ($\chi^2 = 38.8$, $df = 7$, $P < 0.001$, Fig. 4.3A): 61% of the *S. alterniflora* diet and 15% of the *B. maritima* diet was consumed in each trial. On non-preferred foods, there was occasional evidence of small rasping marks, suggesting that snails had tasted, but ultimately rejected these diets. Because these data reflect a similar hierarchy of preferences to snail colonization of live plants (Fig. 4.2), physical structure of plants does not appear to drive feeding preferences for *L. irrorata*. If snail diet preferences were driven by differences in plant structure, then we would expect snails to consume foods equally when offered in reconstituted diets whose structural differences had been removed. Nutritional analysis revealed that all five plants had equivalent protein content, while organic content of *S. alterniflora* was significantly higher than for all other species ($n = 5$, $P < 0.001$ for all comparisons, Table 4.1). The concentrations of phenolic compounds in tissues of all five species ranged from 1.1-2.3 % by dry mass. Phenolic contents of *S. alterniflora, S. virginica* and *B. maritima* were significantly lower than *B. frutescens*, which had the highest phenolic content of all species tested ($n = 11$, $P = 0.0012$-0.04, Table 4.1).

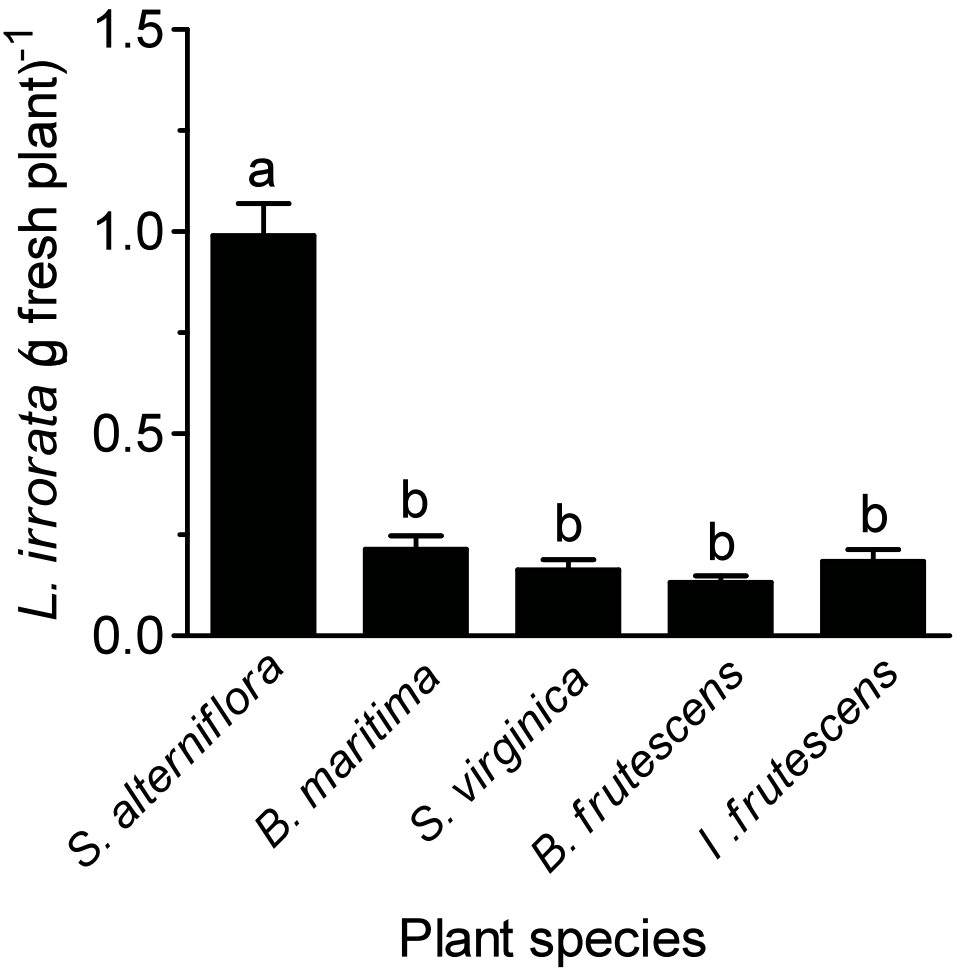

Figure 4.2. *L. irrorata* distributions in multi-choice mesocosm experiments measuring snail habitat preferences. Letters denote significant differences in snail distributions among plant species. Statistical differences among treatments determined by Friedman's test with multiple comparisons ($P < 0.001$, $n = 20$).

Table 4.1. Selected salt marsh plant traits. Expressed values represent mean percent content by dry mass ± 1 S.E for all traits. Superscript letters within a column represent significant differences among species for a given trait (one-way ANOVA with Tukey's HSD test, $P < 0.05$, $n = 5\text{-}11$).

Plant	Protein content	Organic content	Phenolic content
Spartina alterniflora	3.1 ± 0.2^{a}	87.1 ± 0.6^{a}	1.4 ± 0.1^{a}
Batis maritima	2.9 ± 0.7^{a}	51.0 ± 0.9^{b}	1.1 ± 0.1^{a}
Salicornia virginica	3.3 ± 0.2^{a}	69.7 ± 0.8^{c}	1.1 ± 0.2^{a}
Borrichia frutescens	2.1 ± 0.2^{a}	77.8 ± 0.4^{d}	2.3 ± 0.3^{b}
Iva frutescens	2.4 ± 0.2^{a}	77.4 ± 0.4^{d}	1.7 ± 0.3^{ab}

Crude extracts of all five plants significantly depressed snail grazing relative to controls, although the magnitude of deterrence differed among plant species (Fig. 4.3B). Consumption of diets containing *S. alterniflora* extracts at natural concentrations reduced periwinkle grazing by ~10% relative to controls ($n = 8$, $P = 0.046$, Fig. 4.3B), compared to 67% reductions in feeding caused by *B. maritima* extracts and virtually 100% rejection for foods containing *S. virginica, B. frutescens,* and *I. frutescens* extracts ($n = 8$, $P < 0.001$ for all four species, Fig. 4.3B). Relative to their own controls, diets containing *S. alterniflora* extracts were consumed significantly more than diets with extracts from any other species ($n = 8$, $P < 0.001$, Fig. 4.3B). The effects of plant chemical defenses paralleled what was seen in the reconstituted plant tissue bioassays (Fig. 4.3A); diets containing either *S. alterniflora* extract or ground tissues were the most palatable food option to snails, whereas diets (other than *B. maritima*) were completely avoided. Thus, it appears that plant chemistry is a better predictor of snail preferences than plant structural characteristics or plant nutritional content.

Distribution of Fungi on Marsh Plants

We quantified ergosterol concentrations in *B. frutescens, B. maritima, I. frutescens, S. alterniflora,* and *S. virginica* extracts as a proxy for fungal growth in plant tissues because ergosterol is a cell membrane component exclusively found in fungi (Parks and Casey 1996, Seitz et al. 1979). Only wounded *S. alterniflora* tissues with visual evidence of grazing had detectable levels of ergosterol (30.7 ± 7.1 μg ergosterol (g dry plant)$^{-1}$, $n = 8$); ergosterol content in all other samples was below the limit of detection for our instrument (1.8 μg ergosterol (g dry plant)$^{-1}$). We interpret the lack of

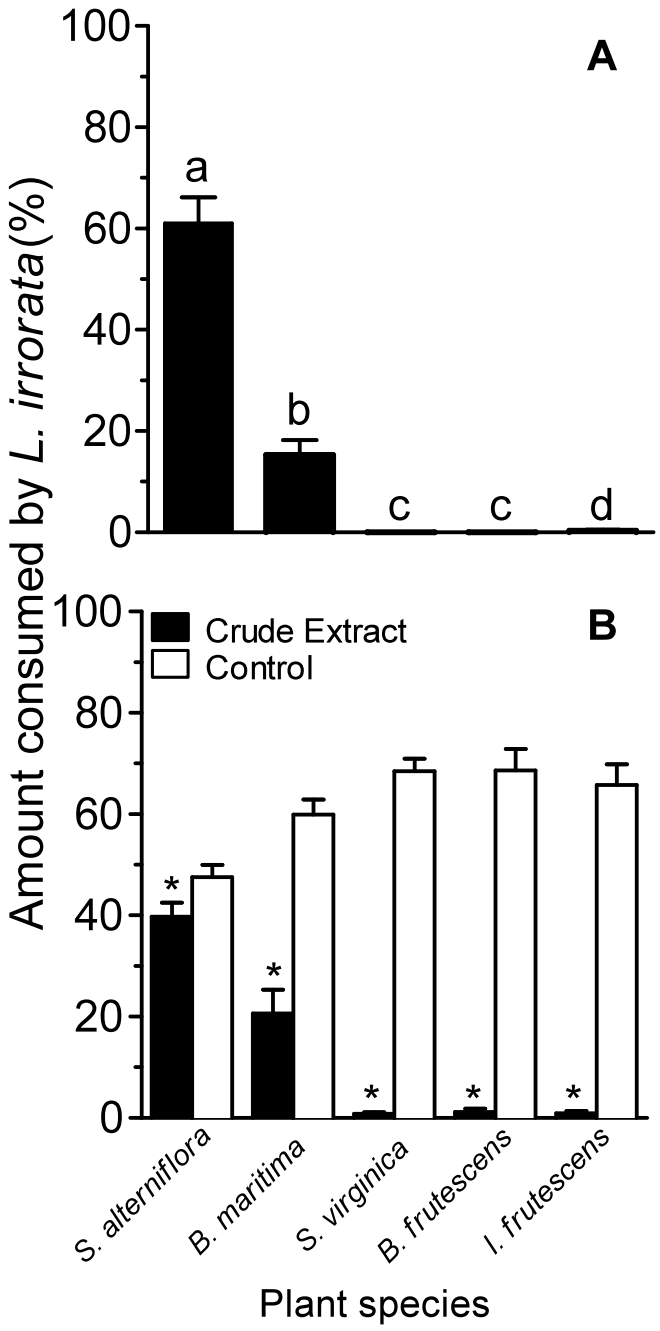

Figure 4.3. Feeding preferences of *L. irrorata* when offered plant constituents as either (a) ground, reconstituted tissue (to eliminate structural differences, all plants offered simultaneously) or (b) as crude extracts embedded in an artificial diet of palatable ground alga (to directly test chemical defenses, each extract offered with a paired control). Letters denote significant differences among snail food preferences (a, Friedman's test w/ multiple comparisons, $P < 0.001$, $n = 10$). Significant differences between extract-laden foods and paired solvent controls are denoted with an asterisk (b, paired *t*-test, $P < 0.05$, $n = 8$ extracts species^{-1}).

ergosterol in healthy plant tissues to mean that fungi had established only in wounded *S. alterniflora*.

Inhibitory Properties of Plant Extracts Against Salt Marsh Fungi

At natural concentrations, extracts of all plant species inhibited by over 80% the growth of marsh fungi *Mycosphaerella* sp. and *P. spartinicola* relative to controls ($n = 6$, $P < 0.001$, *data not shown*). Thus, we tested extracts at a lower concentration in order to better assess variation in antifungal defenses among species. At half natural concentrations, extracts of all species still significantly inhibited *Mycosphaerella* sp. growth relative to controls ($n = 7$, $P < 0.001$ for all species, Fig. 4.4). Extracts of *S. alterniflora* and *B. maritima* were similarly inhibitory to *Mycosphaerella* sp. ($P = 0.56$) but did not reduce fungal growth as much as the other three species ($P < 0.01$). *Iva frutescens* extracts were the most deterrent ($P < 0.01$ for all comparisons), reducing growth of *Mycosphaerella* sp. by over 90% compared to controls. Therefore, chemical defenses from all species in our panel inhibit *Mycosphaerella* sp., but these defenses vary in efficacy across species.

The fungus *P. spartinicola* was generally more resistant to plant defenses than *Mycosphaerella* sp. When tested at half natural volumetric concentrations, only *I. frutescens* extracts inhibited *P. spartinicola* growth by more than 50%, significantly more than any other species tested ($n = 7$, $P < 0.001$, Fig. 4.4). In contrast, *S. alterniflora* extracts did not significantly reduce the growth of *P. spartinicola* relative to controls at this low concentration ($n = 7$, $P = 0.095$, Fig. 4.4). Extracts of the other three species were significantly inhibitory to *P. spartinicola*, reducing growth by 12-27% relative to

controls ($n = 7$, $P < 0.025$-0.001, Fig. 4.4). *Phaeosphaeria spartinicola* thus appears to be more resistant to plant antifungal compounds than *Mycosphaerella* sp., although growth of both fungi was reduced by more than 50% by *I. frutescens* extracts even at half natural concentrations, suggesting that *I. frutescens* heavily invests in chemical defenses that inhibit fungal growth.

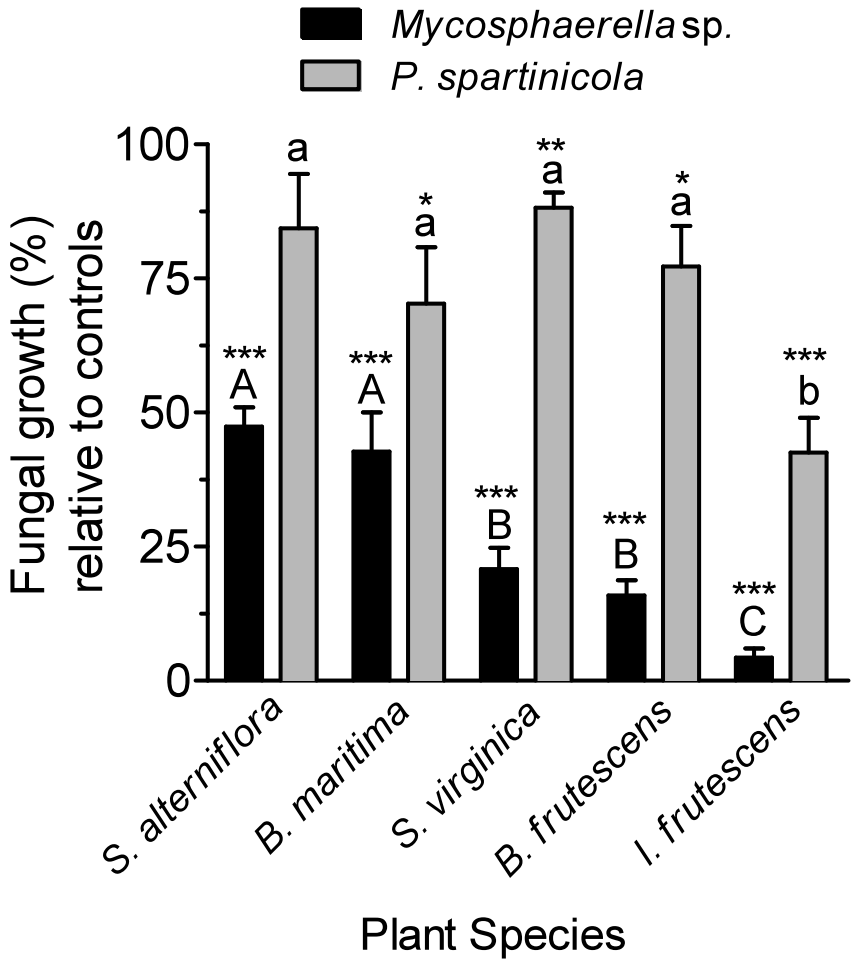

Figure 4.4. Effects of plant crude extracts on growth of marsh fungi *Mycosphaerella* sp. and *Phaeosphaeria spartinicola* when embedded in an agar matrix. Letters denote significant differences in fungal growth among plant species (one-way ANOVA with Tukey's HSD test, $P < 0.05$, $n = 7$ extracts species^{-1}). Significant differences in growth between extracts and their paired controls denoted by asterisks (*, $P < 0.05$), (**, $P < 0.001$), (***, $P < 0.0001$).

Discussion

Plant Chemistry Drives the Palatability of Salt Marsh Plants to *L. irrorata*

Based upon plant and herbivore surveys conducted in mid elevation marshes *L. irrorata* was most frequently encountered on *S. alterniflora,* which was also the most abundant plant in the surveyed areas of the marsh (Fig. 4.1). When plant biomass was standardized in mesocosm experiments, *L. irrorata* was more likely to reside on *S. alterniflora* over any other available plant species (Fig. 4.2). Collectively, these data suggest that *L. irrorata* chooses *S. alterniflora* over other available marsh plants. However, the mechanism(s) by which snails select resident plants was not addressed in our study, and could be influenced by plant height (Hughes 2012), tidal regime (Kimbro 2012), or detection of chemical cues released by plants and predators (Rahman et al. 2000).

Assessing palatability of fresh tissues to herbivores is a valuable first step in determining whether feeding preferences are influenced by plant structure, nutritional content, chemical defenses, or a combination of these traits. Due to the slow feeding behavior of *L. irrorata*, we were unable to address which plants were most palatable to snails when offered as whole tissues. However, for small herbivores like *L. irrorata*, habitat and food choice are intertwined. Therefore, we used a plant choice mesocosm experiment (Fig. 4.2) and feeding assays using ground plant tissues (Fig. 4.3A) to establish that *L. irrorata* prefers *S. alterniflora* to other mid elevation marsh plants. In addition, we saw frequent evidence of snail grazing scars on *S. alterniflora* when plants were visually inspected during field surveys, but rarely, if ever, saw markings characteristic of *L. irrorata* grazing on the other plants in our panel. These observations

support previous studies that have illustrated the intense grazing pressure these invertebrates impart onto *S. alterniflora* (Silliman and Bertness 2002). Furthermore, preferences for salt marsh plants can differ depending on herbivore identity. For instance, in the current study *I. frutescens* was infrequently colonized by and unpalatable to *L. irrorata*, while *S. alterniflora* was a preferred food item and habitat (Fig. 4.1-3). In contrast, the crab *Armases cinereum* has been shown to readily consume whole or ground *I. frutescens* and to avoid *S. alterniflora* (Pennings et al. 1998), suggesting that the plant traits responsible for diet choice varies among herbivore guilds (Pennings et al. 1998; Siska et al. 2002).

In contrast to structural defenses, chemical defenses were a consistent indicator of *L. irrorata* feeding preferences (Fig. 4.3). Crude extracts from all surveyed plants significantly inhibited *L. irrorata* feeding (Fig. 4.3B). However, the magnitude of deterrence varied among plant species: artificial diets embedded with extracts of *B. frutescens, I. frutescens,* and *S. virginica* were not consumed by snails whatsoever (Fig. 4.3B), while those from the dominant marsh species *S. alterniflora* were only marginally distasteful. We fractionated extracts of the plants on our panel, and for all species, chemical defenses against *L. irrorata* were exclusively located in polar organic fractions (*data not shown*), consistent with previous studies that showed polar plant compounds are more deterrent to salt marsh herbivores than lipid soluble chemicals (Siska et al. 2002; Long et al. 2011). The location of these defenses in hydrophilic fractions supports the hypothesis that polar compounds like phenolics deter herbivore grazing (Valiela et al. 1979; Buchsbaum et al., 1984; Barlocher and Newell 1994b). In our study, *B. frutescens*

was completely rejected by *L. irrorata* and had significantly higher phenolic concentrations than three of the other plants (Table 4.1, Fig. 4.3).

For any of these species, it is possible that phenolic compounds play a role in chemical defense. *Spartina alterniflora*, *B. maritima*, *I. frutescens* and *S. virginica* all contained similar phenolic concentrations that were statistically lower than those of *B. frutescens* (Table 4.1). Given that *S. virginica* and its extracts were completely rejected by *L. irrorata* yet had similar phenolic concentrations to the more palatable *S. alterniflora* and *B. maritima*, it appears that total phenolics are not a reliable metric for predicting the potency of chemical defenses in our study (Table 4.1, Fig. 4.3). However, it is possible that individual phenolics function to limit herbivory. Pure compounds responsible for feeding deterrence against herbivores have not yet been identified from most of the plants in our panel (but see Sieg Chapter 3), although the cinnamic acids ferulic and *p*-coumaric acid that are abundant in *S. alterniflora* detritus inhibit *L. irrorata* grazing when incorporated into artificial diets (Valiela et al. 1979; Barlocher and Newell 1994b). The palatability of *S. alterniflora* to marsh herbivores is known to vary seasonally (Siska et al. 2002) and by marsh latitude (Siska et al. 2002; Long et al. 2011), and grazing by a suite of herbivores can induce *S. alterniflora* to produce chemical defenses that are more deterrent to *L. irrorata* than those produced by plants removed from herbivory pressure (Long et al. 2011). We cannot differentiate between the possibilities that we have sampled an especially palatable population of *S. alterniflora*, or that *S. alterniflora* may invest less in chemical defenses relative to other plant species in mid elevation marshes. However, our data suggest that differences in plant structure or toughness are not as important as chemical defenses for *L. irrorata* when choosing a food

item. We are currently working towards identifying the plant compounds responsible for inhibiting *L. irrorata* herbivory (Sieg Chapter 3).

In addition to exhibiting weaker chemical defenses than the four other marsh plants, *S. alterniflora* had higher organic content (87% vs. 51-78% of dry mass), but equivalent protein content (Table 4.1), consistent with *S. alterniflora* estimates within previous studies (Barlocher and Newell 1994a, 1994b). Since *L. irrorata* preferred the most organically rich plant diet, nutritional content may have influenced feeding. However, when nutritional content was equalized in feeding assays in which plant extracts were incorporated into plant-based artificial diets (Fig. 4.3B), all five plant extracts were deterrent, not stimulatory, and the pattern of feeding preferences for *L. irrorata* was identical to the pattern observed on ground plants whose nutritional contents were different (Fig. 4.3A). Thus, differing organic content among these five marsh plants appears to play at most a marginal role in snail feeding preferences. In addition plant protein content, which did not differ among species, is expected to be the more important nutritional factor for herbivores that are often nitrogen-limited (Mattson 1980). Overall, weak chemical defenses appear to explain the preference of *L. irrorata* for *S. alterniflora* over other marsh plants, whose chemical defenses are highly effective (Fig. 4.3B).

The relative importance of chemical and structural defenses in determining grazing patterns of other marsh herbivores on *I. frutescens, S. alterniflora, S. virginica,* and other marsh plants not included in our panel have previously been reported for some herbivores (Buchsbaum et al. 1984; Pennings et al. 1998; Siska et al., 2002). Canada goose feeding preferences were driven more by differences in putative chemical defenses than by nutritional content or digestibility of plant tissues (Buchsbaum et al. 1984). In

contrast, feeding preferences of the generalist marsh crab *A. cinereum* can generally be explained more reliably by plant structure than by variations in plant chemistry (Pennings et al. 1998). Nutrient content, plant chemical defenses, and structural differences all explain consumption of marsh plant populations by grasshoppers and marsh crabs, but none of these traits act as a universal indicator of plant palatability (Siska et al. 2002). Intrinsic differences of northern and southern latitude marshes, including variation in grazing pressure and other environmental factors, also result in variable plant palatability among populations (Pennings et al. 2001; Siska et al. 2002), suggesting that marsh plants are capable of recognizing and responding to changes in their surrounding community (Long et al. 2011).

Chemical Defenses Against Fungi are Prevalent in Salt Marsh Plants

All five salt marsh plants examined in this study possessed chemical defenses that inhibited growth of common marsh fungi that are cultivated by *L. irrorata* (Fig. 4; Newell 2001b; Silliman and Newell 2003). As with chemical defenses against *L. irrorata*, deterrence varied among plant species. Against *Mycosphaerella* sp., *S. alterniflora* and *B. maritima* produced comparatively weak antifungal compounds; *S. virginica* and *B. frutescens* extracts were moderately inhibitory, whereas *I. frutescens* compounds almost completely inhibited growth of this fungus, even when tested at half natural concentrations (Fig. 4.4). In contrast, the fungus *P. spartinicola* was more resistant to crude extracts from all plants except those produced by *I. frutescens* (Fig. 4.4). At half natural concentrations, the remaining four plant species' defenses were only mildly inhibitory to *P. spartinicola* (Fig. 4.4). However, it is important to note that we

used extracts from whole plants, which may overestimate the concentration of antifungal molecules on plant surfaces where fungal infection may initiate. Therefore, our measurement of half natural concentrations may actually be closer to the natural dose of antifungal molecules than fungi would likely be exposed to *in situ*. We are currently investigating whether plants concentrate chemical defenses within or on outer tissues to estimate how marsh plants allocate and express antifungal compounds. Nevertheless, *I. frutescens* is clearly the plant least amenable to fungal farming, given that neither of the fungi farmed by *L. irrorata* can grow in the presence of *I. frutescens* compounds and extracts from *I. frutescens* completely deterred *L. irrorata* grazing.

The relative potency of antifungal compounds also explains why evidence of fungal infection was only detected in *S. alterniflora* tissues and not in the other four middle elevation marsh plants. Only wounded sections of live *S. alterniflora* tissues contained detectable levels of ergosterol, a fungal cell wall component that we used as a biomarker for the presence of fungi in plant tissues. However, concentrations of ergosterol from wounded *S. alterniflora* tissues in our study were approximately 90% lower than previously reported yields from standing dead or decaying *S. alterniflora* tissues (Newell 2001a). Despite being comparatively weak, *S. alterniflora* chemical defenses may slow the progression of fungal infection, given that we did not detect ergosterol in healthy *S. alterniflora* tissues. The fungi used in our study are dominant components of the microbial marsh community that can be found in wounded or decaying *S. alterniflora* tissues (Barlocher and Newell 1994a; Newell 2001b) and can account for at least 60% of the negative effects on *S. alterniflora* that are farmed by snails (Silliman and Newell 2003). Given these negative effects, plants that frequently

encounter these pathogens and develop defenses to prevent their growth or establishment are expected to be under positive selection pressure.

To our knowledge, this is the first study to document the prevalence of chemical in salt marsh plants that inhibit fungal growth. Since these fungi are among the species cultivated by snails (Silliman and Newell 2003), our data provide insights into some of the mechanisms by which salt marsh plants prevent farming behavior. *Littoraria irrorata* selectively consumes fungi (including *Mycosphaerella* sp. and *P. spartinicola*) instead of live or decaying *S. alterniflora* tissues (Newell and Barlocher 1993) and facilitates establishment of new fungal cultures on plants by defecating in wounded plant tissues (Silliman and Newell 2003). In return, farmed fungi may degrade plant substances that are reported to decrease snail enzymatic activity, increasing digestibility and nutritional quality of plant tissues (Barlocher et al. 1989). If *L. irrorata* relies on fungal degradation of deterrent plant chemistry to gain access to a food source, then the mutualism between snail and fungus may be more complex than originally suspected, and warrants further investigation into how fungi break down plant defenses. Additionally, if such chemical defenses are common, marsh fungi may depend on *L. irrorata* to gain access to inner tissues that would be inaccessible without snail herbivory. Furthermore, fungal farms should be immensely difficult to establish on plants such as *I. frutescens* that produce potent defenses against both snails and fungi. These plants are likely too distasteful for the snail to break through outer tissue layers; therefore no new wound sites are created.

Spartina alterniflora **is the Most Amenable Substrate for Fungiculture by** ***L. irrorata***

Given that fungal biomass on *S. alterniflora* can increase by over 20-fold in the presence of *L. irrorata* (Silliman and Newell 2003), traits that limit either the presence of snail farmers or their fungal crops should benefit plants that are regularly exposed to both fungi and *L. irrorata*. Based on our results, we propose that *L. irrorata* primarily establishes fungal farms on *S. alterniflora* because it produces the weakest chemical defenses against both herbivores and fungi among all accessible plants. At natural concentrations, *S. alterniflora* chemical defenses only reduced snail feeding by 10% relative to palatable controls, whereas other plants and their extracts were nearly inedible (Fig. 4.3). Chemical defenses against fungi were also found to be comparatively weak in *S. alterniflora* relative to other mid marsh species (Fig. 4.4). Given that *S. alterniflora* was the most palatable food option among live marsh plants, it is unsurprising that *L. irrorata* densities and evidence of snail grazing were highest on *S. alterniflora,* especially if consumption of *S. alterniflora* tissues results in a higher likelihood of subsequent fungal infection and establishment of a more palatable fungal crop. *Spartina alterniflora* is a rapid grower that is densely populated in middle elevation marshes, so the likelihood of snails encountering this favored species is high, making it a viable target for fungiculture.

The growth-differentiation balance hypothesis (Herms and Mattson 1992) posits that plants must make trade-offs when allocating resources towards growth to maintain a competitive edge, while simultaneously differentiating their tissues for secondary functions, such as production of structural and/or chemical defenses that deter herbivores. It is possible that *S. alterniflora* invests more in growth than defenses to produce new biomass faster than herbivores and pathogens can remove it, whereas less abundant plant

species allocate proportionally more resources towards differentiation (including producing chemical defenses) that prevent fungal growth or herbivore damage. Furthermore, competitive interactions relegate *S. alterniflora* to lower elevations of salt marsh habitat where other salt marsh angiosperms cannot grow (Bertness 1991; Emery et al. 2001). *Spartina alterniflora* must devote resources towards physiological mechanisms (such as aeration of anoxic soils) to tolerate the stressful lower elevations of the salt marsh (Howes et al. 1986; Bertness 1991). However, these zones also provide a competitive refuge, allowing *S. alterniflora* to thrive where other plants cannot (Emery et al. 2001). Due to this zonation pattern, it may be better strategically for *S. alterniflora* to devote resources towards growth and physiological stress responses while producing relatively weak chemical defenses that slow down, but do not prevent establishment of fungal farms. This strategy would allow *S. alterniflora* to resist herbivory and fungal infection, assuming that growth rates exceeded tissue losses due to farming.

Accurately assessing plant investment in growth relative to defense when considering the growth-differentiation balance hypothesis has proven exceptionally difficult (Stamp 2004). Thus, empirical evidence supporting this hypothesis is rare despite being one of the most advanced plant defense theories (Stamp 2002). In our study, we relied on plant abundance and previous reports of growth rates as indictors of plant investment in growth over differentiation. To further support the hypothesis that abundant plants like *S. alterniflora* allocate more resources towards growth over chemical defense, we require more explicit testing of the relative growth rates among mid elevation marsh species in relation to simultaneous production of chemical defenses.

Although the evolutionary timescale of the mutualism between fungi and *L. irrorata* remains unclear, mutualistic farming behavior between insects and fungi has occurred for over 50 million years (Mueller and Gerardo 2002), and has promoted a coevolutionary arms race of ants, cultivated fungi, and antibiotic-producing bacteria against parasitic fungal invaders (Currie et al. 2003). If the mutualism between farmable fungi and *L. irrorata* has developed on an evolutionary timescale, then salt marsh angiosperms would benefit from investing in traits (including chemical defenses) that minimize their risk of succumbing to infection and being consumed by gastropods. The potential for co-evolution of tripartite interactions between snails, fungi, and salt marsh angiosperms is intriguing. Yet, we only have the present snapshot of such interactions, and these are based upon a preliminary understanding of plant traits. As snail populations continue to grow in the wake of weakening marsh trophic cascades (Griffin et al. 2011; Silliman and Bertness 2002), marsh plants may be under greater pressure to deter both herbivores and fungi. Therefore, further investigation into the origin of symbiotic interactions between fungi and snails, the mechanisms of plant defense, as well as exploration into coevolutionary hotspots within the expanse of Atlantic salt marshes will prove fruitful for future studies.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The mechanisms by which primary productivity and community structure are controlled from the top-down and the bottom-up have been a central facet of ecological research for decades (Hairston et al. 1960, Polis and Strong 1996). While the relative importance of these forces is variable among ecosystems, most of the time plants must respond to the stresses caused by nutrient limitation and damage caused by herbivores and pathogens. When herbivores and pathogens are tightly associated, plants face an even greater need to evolve strategies to prevent or limit tissue losses, because damage caused by herbivory may facilitate subsequent losses due to infection (Silliman and Newell 2003). However, investment in chemical or structural defenses that protect plants is assumed to be costly and requires reallocation of resources away from primary plant functions (Herms and Mattson 1992). Plants must be able to cue into the ambient threat level, and utilize a strategy that maximizes the likelihood that genes will be passed down to the next generation. Unfortunately, as community landscapes shift due to global warming, anthropogenic interference, or invasive species, the strategies that were successful in the past may no longer impart a net positive effect for plants. This dissertation strives to explain the defense strategies utilized by a plant community to withstand top-down control by coupled herbivores and pathogens, and assesses how these strategies may change in the wake of a rapidly changing environment.

Within coastal salt marshes, a mixture of top-down and bottom-up forces controls plant community structure. While the bottom-up forces that limit plant productivity have been historically well represented (Teal 1962), our understanding of top-down control in

these systems is still being developed. Studies have shown that a seemingly innocuous grazer, the snail *Littoraria irrorata,* substantially reduces above ground biomass of the foundation species *Spartina alterniflora* due to a facultative mutualism between snails and pathogenic fungi (Silliman and Newell 2003). In Chapter 3, I investigated how *S. alterniflora* limits damage caused by herbivores and pathogens by producing multiple classes of chemical defenses. A fatty acid (α-dimorphecolic acid) reduced growth of a fungus (*Mycosphaerella* sp.) commonly farmed by *L. irrorata*, while a suite of phenolic compounds, including the flavonoid glycoside orientin, functioned as an antifeedant against snails. In salt marshes with intense, constant exposure to *L. irrorata*, production of these chemical defenses was constitutive. However, I found that the natural bulk concentration of these molecules were not adequate to prevent fungal growth on plant surfaces where infection is likely to commence. Thus, *S. alterniflora* can respond to the threat of fungal farming by constitutively expressing chemical defenses that can deter snails and fungi. However, since fungal farms are observed on *S. alterniflora* despite these defenses, it appears as though *S. alterniflora* chemical defenses slow down, but do not prevent damage caused by herbivores and pathogens.

One possibility that arose from my initial observations was that *S. alterniflora* was more palatable to *L. irrorata* compared to other salt marsh plant community members. In Chapter 4, I hypothesized that *S. alterniflora* was favored by *L. irrorata* because it was either more nutritious or invested less in structural or chemical defenses relative to other available plants. By giving *L. irrorata* an equal probability of encountering different marsh plants, we established that snails preferentially reside on *S. alterniflora*, despite plants having similar nutritional values. Second, I observed that

structural differences among plants did not account for which plants were most palatable to snails, but that chemical defenses from plants other than *S. alterniflora* made diets virtually inedible. All plants in our panel were chemically defended against two fungal pathogens, but of these plants, *S. alterniflora* chemical defenses were the weakest. In a community context, *S. alterniflora* is the most heavily farmed plant because it produces chemical defenses against grazers and fungi that are weak relative to other species, making it a tolerable plant on which to establish a fungal cultivar. This study highlights two different strategies employed by plants to combat top-down control. For plants like *S. alterniflora,* resources appear to be allocated towards functions that promote new growth and withstand a stressful abiotic environment, but a lower investment in chemical defenses makes plants more susceptible to herbivory or infection. In contrast, less abundant plants that thrive in higher marsh elevations allocate more resources towards chemical defense with the intent of limiting damage, but this may come at a cost to competitive ability in the more physically stressful low marsh (Bertness and Ellison 1987, Bertness 1991).

The findings presented in this dissertation have illustrated different mechanisms employed by primary producers to limit top-down control by tightly associated herbivores and pathogens. These studies explore how marsh community structure, as well as the prevalence of fungal farms, can be predicted by the relative investment by plants in chemical defense. Plants in these systems allocate a finite resource pool towards growth or defense (Herms and Mattson 1992), while simultaneously tolerating a stressful abiotic environment. Our studies primarily looked at production of chemical defenses caused by intense top-down control, but it is likely that bottom-up processes,

including nutrient availability, rates of tidal influx, and soil salinity also impact the resource pool available to salt marsh angiosperms. Furthermore, as herbivore densities continue to rise with the loss of secondary consumers (Silliman and Bertness 2002), the impact of herbivores and grazers on the salt marsh plant community will escalate. The defense strategy based on tolerance employed by *S. alterniflora* is unlikely to succeed if elevated herbivore densities remove new plant biomass before new growth can occur, which may contribute to the decline of these ecosystems (Silliman et al. 2005).

Our experiments addressed chemical defenses at the individual, population, and community scale. However, we have only scratched the surface of chemically mediated interactions among marsh plants, herbivores, and fungi. To conclude this dissertation, I have outlined three areas of interest that could provide fruitful directions for new projects stemming from my own research.

Accurate Surface Analysis of *S. alterniflora* Expression of Chemical Defenses

A central component of plant defense theory is how plants allocate chemical or structural defenses to tissues in accordance with their value or risk of damage (Feeny 1976, Rhoades 1979, Herms and Mattson 1992). For *S. alterniflora*, localization of antifungal or antigrazer molecules may be best expressed on plant surfaces, where initial exposure to grazers or pathogens is most likely to occur. Until recently, the most efficient method to quantify chemical defenses on plant surfaces was using the "hexane dip" method (de Nys et al. 1998), in which lipophilic compounds are extracted from plant surfaces by dipping fresh tissues in hexanes. While this method is advantageous for quantification of non-polar metabolites that are likely to adsorb to plant surfaces, it is

inefficient at removing polar compounds. Furthermore, it is difficult to quantify chemical defenses at a fine spatial scale due to the methodological constraints involved in dipping an undamaged plant into solvent without extracting molecules from inner plant tissues. However, this method is straightforward and can be performed rapidly if fresh plants are accessible.

In Chapter 3, I utilized the hexane dip to quantify concentrations of α-dimorphecolic acid expressed on *S. alterniflora* blades, but was unable to extract a sufficient quantity of compound to generate an accurate estimate of surface concentrations of this defense. Luckily, advances in mass spectrometry are being incorporated into ecological research, and are being employed to examine surface chemical defenses at a much finer spatial scale than was previously available. One such advancement is the use of desorption electrospray ionization mass spectrometry (DESI-MS), which allows metabolites to be quantified at picomol mm^{-2} surface concentrations on algal surfaces (Lane et al. 2009, Andras et al. 2012). Additionally, this methodology can enable creation of a spatial map for the concentration of compound across intact biological surfaces, from live or preserved specimens, allowing researchers to compare allocation of defenses among wounded and healthy sections (Lane et al. 2009) or new and old tissues (Andras et al. 2012).

Given that fungal farms are easily detected on *S. alterniflora* due to localized necrosis of blade tissue, DESI-MS would be useful to answer how higher plants chemically respond to fungal pathogens as well as herbivores. By screening portions of *S. alterniflora* proximal and distal to wound sites created by *L. irrorata*, researchers could determine whether *S. alterniflora* allocates defenses to plant tissues that are currently

under attack. Additionally, the greater sensitivity of DESI-MS could provide an accurate estimate for constitutive levels of both non-polar antifungal molecules like α-dimorphecolic acid and moderately polar antigrazer phenolic compounds including orientin, which could not be quantified using a hexane dip due to solubility constraints. Using DESI-MS, researchers could quantify simultaneous expression of antifungal and antigrazer metabolites before, during, and after fungal farms had been established to observe induction of chemical defenses in real time. Considering the current access our research group has to DESI-MS equipment and collaborators adept at using this technique, there are ample opportunities to embrace this new technology and incorporate it into future studies of salt marsh chemical ecology, particularly in the context of fungal farming.

Variation of *S. alterniflora* Chemical Defenses at Local and Regional Scales

The intensity of predation, herbivory, and competitive interactions is hypothesized to increase at lower latitudes, partially due to a warmer, more stable climate allowing for greater speciation and niche differentiation (MacArthur 1972, Coley and Aide 1991). Since higher herbivore densities in lower latitudes exert greater top-down control of plant productivity (MacArthur 1972, Coley and Aide 1991, Pennings and Silliman 2005), plants in low latitudes are expected to invest more into production of chemical defenses than their high latitude counterparts (Cronin et al. 1997, Siska et al. 2002, Moles et al. 2011). While there is mixed support for the hypothesis that plants in lower latitudes are better defended against herbivory (Moles et al. 2011), studies in salt marshes consistently support this hypothesis (Pennings et al. 2001, Siska et al. 2002,

Pennings and Silliman 2005, Salgado and Pennings 2005, Long et al. 2011). Salt marshes provide a tractable system to study latitudinal variation in plant defense, because they maintain a similar pool of flora and fauna across a wide latitudinal gradient despite vast differences in abiotic and biotic stressors such as salinity stress, ice wrack, tidal regime, length of growing season, temperature, and herbivore density or grazing intensity (Pennings and Bertness 2001, Pennings and Silliman 2005). Plants from populations consistently exposed to high rates of herbivory are better defended (Siska et al. 2002) and express defensive traits for several generations even after the threat of herbivory is removed (Salgado and Pennings 2005), suggesting that the evolutionary history between herbivores and plants has led to genotypic variation in trait expression among plant populations.

In my dissertation, I found that *S. alterniflora* exposed to a constant, intense threat of herbivory were constitutively defended even after being removed from herbivory pressure for one month, in contrast to plant populations with lower ambient herbivore densities that are either not chemically defended or induce chemical defenses in response to previous herbivore grazing (Long et al. 2011). These studies support the hypothesis that the density and/or grazing intensity of herbivores varies by latitude, which affects the defense strategy employed by *S. alterniflora.* However, it is possible that other factors could explain the discrepancy between the findings of my dissertation and previous studies. For instance, severe to extreme drought conditions were in place during the course of my research on Sapelo Island, which were more intense than the moderate drought conditions experienced in South Carolina in 2006 during the Long et al. (2006) induction experiment. Drought induced rises in water temperature and salinity negatively

impacts blue crab populations by increasing their susceptibility to dinoflagellate parasites (Lee and Frischer 2004). The high densities of *L. irrorata* observed during my experiments may have been partly caused by drought-induced reductions in the number of blue crab predators, either through direct mortality or their retreat to less saline waters. Furthermore, drought may cause additional stress in *S. alterniflora* by altering soil chemistry and increasing plant susceptibility to pathogens (Silliman et al. 2005, Alber et al. 2008, Hughes et al. 2012). Therefore, while differences in grazing intensity may influence whether *S. alterniflora* is inductively or constitutively defended, other environmental and abiotic factors that are difficult to control could also change when and where defenses are most likely to be produced, as well as how long it takes for *S. alterniflora* to respond to its surrounding environment.

While differences in plant palatability across large regional scales are well documented in salt marshes, we know comparatively little about how plants adjust to environmental stressors on local scales, such as between elevations in a single marsh or among neighboring marshes. For instance, *S. alterniflora* produces two distinct growth forms depending on marsh elevation, a tall form near creek beds, and a short form at higher elevations. While genetically homologous (Mooring et al. 1971, Shea et al. 1975), these growth forms represent physiological responses to different environmental conditions (Linthurst and Seneca 1981, Howes et al. 1986), and could also entail plastic responses towards growth or defense depending on the threat of herbivory. *Littoraria irrorata* tends to avoid tall *S. alterniflora* because of increased predation risk in lower marsh elevations, but readily consume this growth form if predation pressure is removed (Silliman and Bertness 2002). In contrast, *L. irrorata* densities in short *S. alterniflora*

zones can exceed 600 snails m^{-2}, yet short plants can withstand *L. irrorata* herbivory (Silliman and Bertness 2002).

Since herbivory is a more intense threat in short *S. alterniflora* zones, short *S. alterniflora* should allocate proportionally more resources towards chemical defense than tall plants. During initial plant surveys, I observed that tall *S. alterniflora* were not chemically defended against snail herbivory, yet extracts from short *S. alterniflora* collected less than 50 meters from the tall *S. alterniflora* zone were unpalatable to snails (Fig. A.1). Snail densities were also much higher on short *S. alterniflora* than on tall plants, suggesting that herbivore exposure led to expression of chemical defenses (RDS*, unpublished data*). These observations were reminiscent of how low latitude marsh plants exposed to higher densities of herbivores were more defended than high latitude plants experiencing minimal herbivory (Pennings et al. 2001, Siska et al. 2002, Pennings et al. 2007).

Besides herbivore density, many other environmental factors co-vary with increased *L. irrorata* densities in short form marshes, such as greater salinity stress and lower concentrations of sediment nutrients (Valiela et al. 1978, Linthurst and Seneca 1981, Delaune et al. 1983). Given that allocation of resources towards growth or defense can be dependant on nutrient acquisition or environmental stress (Coley et al. 1985, Herms and Mattson 1992), future studies could examine how *S. alterniflora* clones, grown from seed in either high or low marsh elevations would differ in their allocation of resources towards primary or secondary plant functions. A comparison of resource acquisition, growth rates, and chemical defenses in the presence and absence of compounding biotic stressors (such as snails or fungi) between tall and short form *S.*

alterniflora over the course of a season could tease apart how abiotic and biotic stressors lead to production of chemical defenses. Since there is evidence for both phenotypic plasticity (Long et al. 2011) and genetic control (Salgado and Pennings 2005) of *S. alterniflora* defensive traits, a more in depth comparison of resource allocation between tall and short form *S. alterniflora* would provide an accessible system to test the environmental factors that force plants to make trade-offs between growth and defense.

While data on regional scales suggests that low latitude plants are more defended than plants from higher latitudes, I detected substantial variation in chemical defenses among local short *S. alterniflora* populations as well. During preliminary surveys, I generated *S. alterniflora* extracts from three Georgia salt marshes on Sapelo Island, Skidaway Island, and Tybee Island that were within 40 miles of one another. All three of these sites would classify as low latitude marshes, yet Sapelo Island *S. alterniflora* was the only population that contained chemical defenses capable of inhibiting fungal growth (Chapter 3 and Figure A.2). Since the plants used for preliminary screenings all came from equivalent marsh elevations that would experience similar abiotic stressors, the major difference between these sites was the density of *L. irrorata*. Densities of *L. irrorata* seen in Tybee and Skidaway *S. alterniflora* were approximately one-third of that observed in Sapelo Island marshes (RDS, *unpublished data*). Previous studies have also shown that Sapelo Island marshes contain greater *L. irrorata* densities than other low latitude marshes (Silliman and Bortolus 2003). Furthermore, *L. irrorata* densities can vary widely just among Sapelo Island sites, ranging from 350-3300 snails m^{-2} (Silliman and Bortolus 2003). Thus, plants in neighboring marshes experience vastly different

grazing intensity, which could reflect differences in top-down herbivore control by predators (Silliman and Zieman 2001).

As discussed in Chapter 3, Sapelo Island *S. alterniflora* was constitutively defended against both grazers and fungi, even when herbivory pressure was relieved, but the plants used in our study are exposed to a near constant threat of herbivory. Therefore, it was unsurprising that chemical defenses were constitutively expressed among these plants. However, had this study extended into nearby marshes either on Sapelo Island or adjacent salt marshes that contained lower *L. irrorata* densities, it is possible that our caging treatments could have either relaxed or induced chemical defenses produced by these plants. While the data for latitudinal variation in trait expression is evident in marshes, local variation in biotic stressors including herbivory may also have a pronounced effect on when *S. alterniflora* tissues will be chemically defended.

Implications of Invasive Species on Fungal Farming Dynamics

While the chemical arsenal employed by *S. alterniflora* is not sufficient to prevent *L. irrorata* from establishing fungal farms on plant tissues (RDS, Chapter 3), they may dampen the negative effects caused by herbivores and pathogens. However, relative to other members of the salt marsh plant community, the chemical defenses produced by *S. alterniflora* are comparatively weak, which could explain why fungal farms are not observed on less abundant salt marsh plants and snails prefer to reside on *S. alterniflora* (RDS, Chapter 4). As *L. irrorata* densities continue to increase as trophic cascades controlling marsh herbivore populations break down (Silliman and Bertness 2002, Griffin et al. 2011), the weak chemical defenses that allowed *S. alterniflora* to resist fungal farms

in the past may be insufficient to handle greater exposure to herbivores and pathogens, which could contribute to marsh degradation (Silliman et al. 2005).

Apart from weak chemical defenses, losses of *S. alterniflora* biomass are increasing due to the invasion of an exotic species that can withstand the harsh environmental conditions and intense herbivore pressure characteristic of coastal Atlantic salt marshes. In particular, an exotic Eurasian strain of the reed *Phragmites australis* has established a foothold in salt marsh habitats historically occupied by *S. alterniflora* (Chambers et al. 1999, Saltonstall 2002). Many factors contribute to *P. australis* success, including efficient nutrient acquisition and rapid growth rates (Mozdzer and Megonigal 2012), release of allelochemicals into the rhizosphere to inhibit competing marsh grasses (Rudrappa et al. 2007), and production of chemical defenses that are more deterrent to *L. irrorata* than defenses from native *S. alterniflora* (Hendricks et al. 2011). Furthermore, mesocosm studies have shown that exotic *P. australis* thrives in conditions that simulate the effects of global warming, including higher CO_2 concentrations, elevated sea levels, and nitrogen enrichment (Mozdzer and Zieman 2010, Mozdzer and Megonigal 2012).

The range expansion of *P. australis* threatens to fundamentally alter the community composition and ecological interactions that occur within coastal Atlantic salt marshes in the United States. Noticeable shifts and reductions in marsh faunal species composition have been observed as *P. australis* expands into *S. alterniflora* zones (Jivoff and Able 2003, Posey et al. 2003, Gratton and Denno 2006), possibly caused either by the decreased palatability of *P. australis* to local herbivores (Gratton and Denno 2006, Hendricks et al. 2011), or decreased flooding frequency due to increases in marsh elevation created by sediment trapping by dense *P. australis* stands (Osgood et al. 2003).

Exotic *P. australis* has also reduced plant diversity as it spreads through New England salt marshes by outgrowing or facilitating physical disturbance of competitors before they can establish in soils (Minchinton and Bertness 2003, Silliman and Bertness 2004, Minchinton et al. 2006).

As *P. australis* continues to spread south into lower latitude salt marshes (Saltonstall 2002), it will fundamentally alter the tripartite interaction between *S. alterniflora, L. irrorata*, and pathogenic fungi. As mentioned previously, *P. australis* can inhibit *S. alterniflora* growth by exuding gallic acid into the soil (Rudrappa et al. 2007) and *P. australis* (like other native marsh plants) is less palatable than *S. alterniflora* to snails due to polar antifeedant molecules (Hendricks et al. 2011). Thus, invasion by *P. australis* negatively impacts at least two of the organisms involved in fungal farming. If *P. australis* continues to disrupt marsh ecosystems, *S. alterniflora* will have to cope with biotic stressors from the top-down (fungal farms) as well as from highly competitive grasses, which could cause *S. alterniflora* to decline at even more rapid rates than would be expected from top-down control alone. As *S. alterniflora* dominated marshes transition into *P. australis* stands, *L. irrorata* may have to find a different species on which to establish fungal farms. At present, it is unknown whether *P. australis* is chemically defended against fungal pathogens, but chemical defenses against snails (Hendricks et al. 2011) should hinder or prevent establishment of fungal farms on *P. australis* tissues. Snails and fungi in *P. australis* dominated marshes will have to develop new mechanisms to overcome chemical defenses produced by other members of the marsh plant community, or run the risk of losing the associational benefit gained through facilitation. Studies investigating the capacity for snails to establish fungal farms on

other plants *in situ*, particularly with species of fungi that differ in resistance to plant chemical defenses, could assess whether fungal farming can be expected to persist in the future.

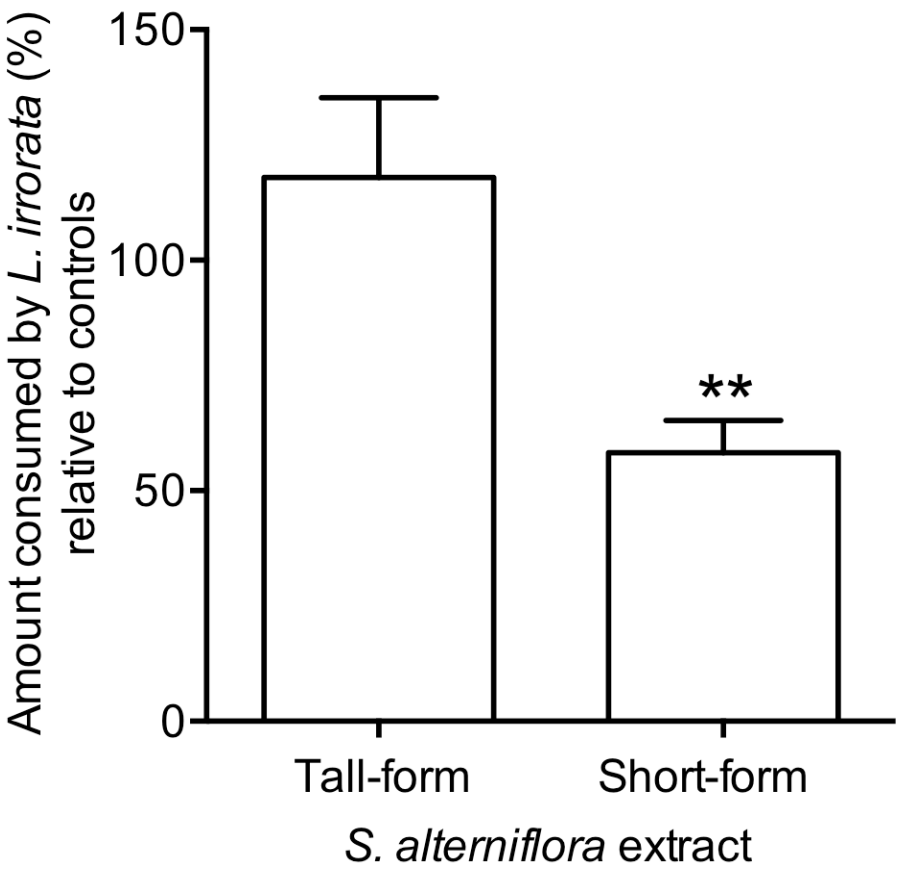

Figure A.1. *L. irrorata* grazing on artificial diets containing tall or short form *S. alterniflora* organic extracts. Asterisks (**) represent significant differences between treatments and paired controls (1-tail *t*-test, $P < 0.01$, n = 25), while error bars represent 1 S.E.

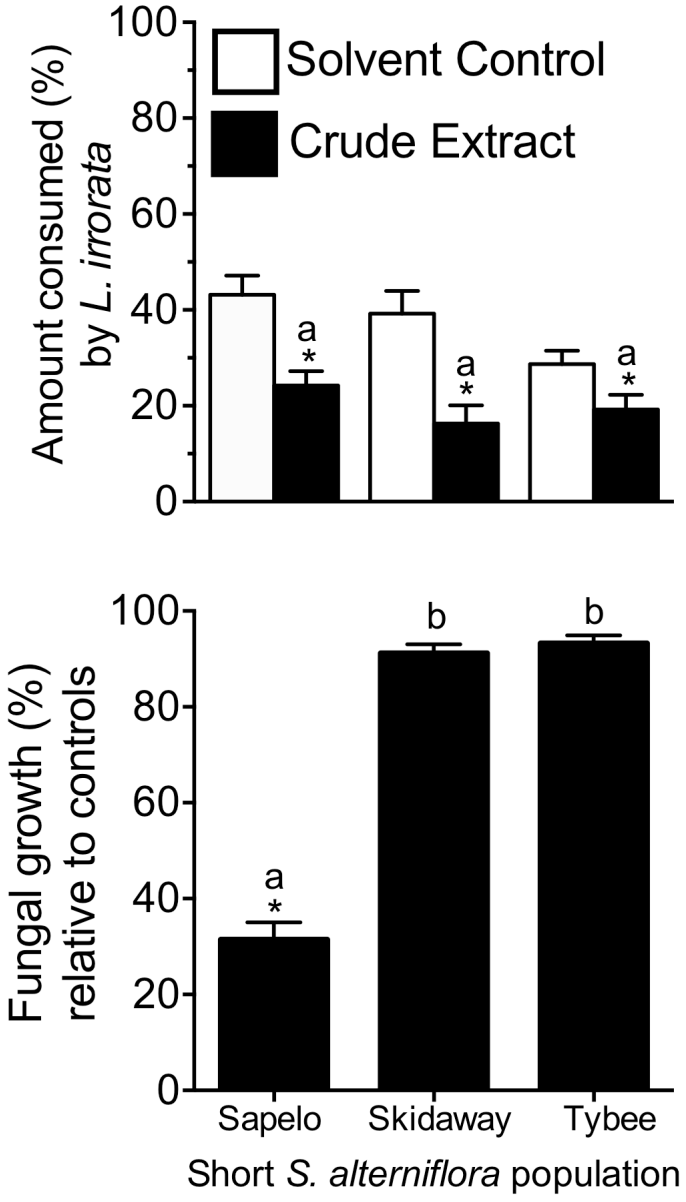

Figure A.2. Chemical defenses in short form *S. alterniflora* collected from three Georgia salt marshes against (A) the snail *L. irrorata,* and (B) the fungus *Mycosphaerella*. Salt marsh location is reported on the x-axis. Extracts were added to agar at natural isolated concentrations. Significant differences between paired treatments and controls determined by 1-tail *t*-test ($P < 0.05$, $n = 4$-25) and denoted by an asterisks (*). Differences among marsh populations determined by Kruskall-Wallis test with Dunn's multiple comparisons and denoted by lower-case letters.

Figure B.1. Representative cage set-up from induction experiment reported in Chapter 3. Each cage was made from irrigation pipe and window screen mesh (10 cm diameter x 60 cm height). This image was a test cage, which is why the total height is less than 60 cm.

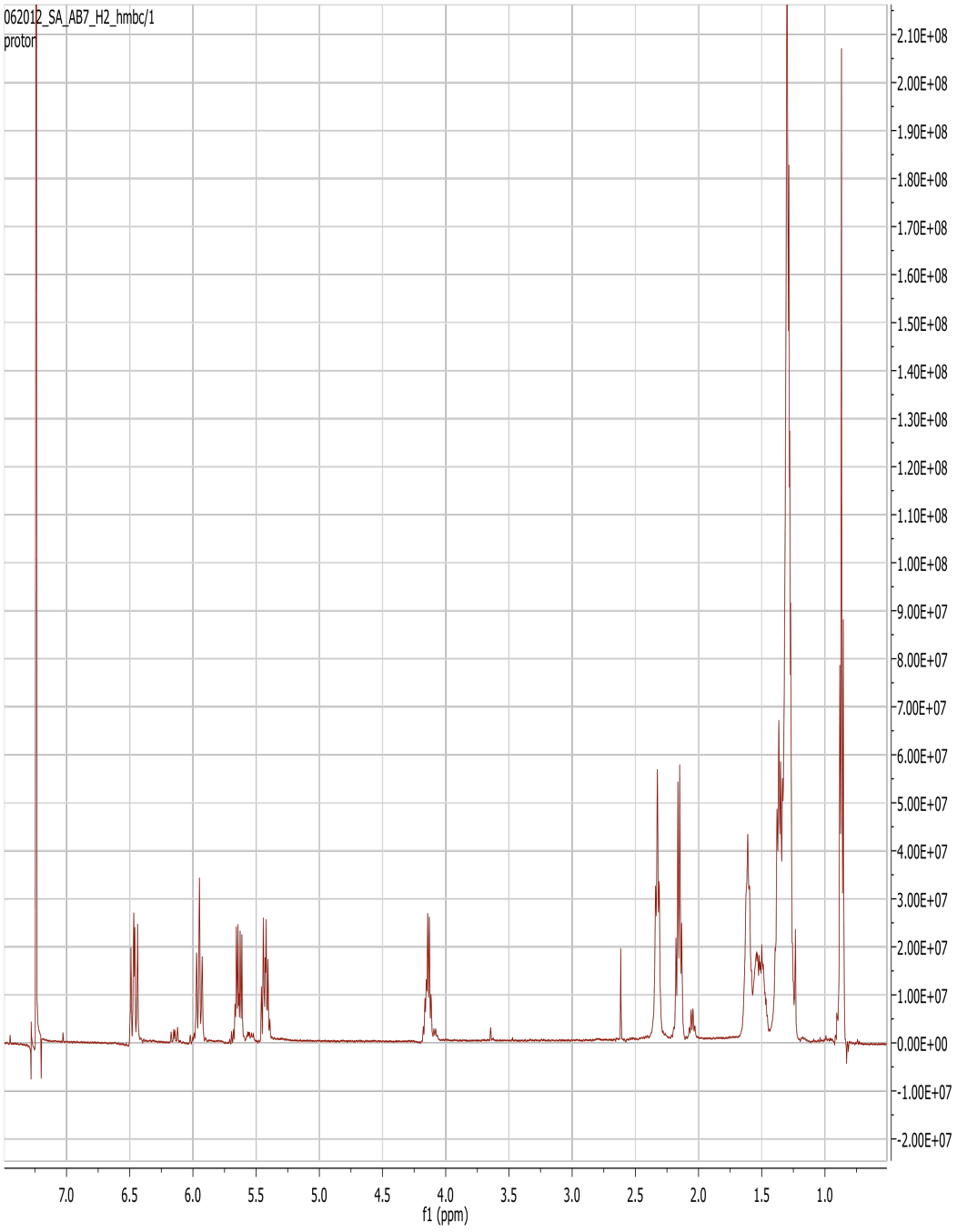

Figure C.1. ^{1}H NMR spectrum of α-dimorphecolic acid (500 MHz, $CDCl_3$).

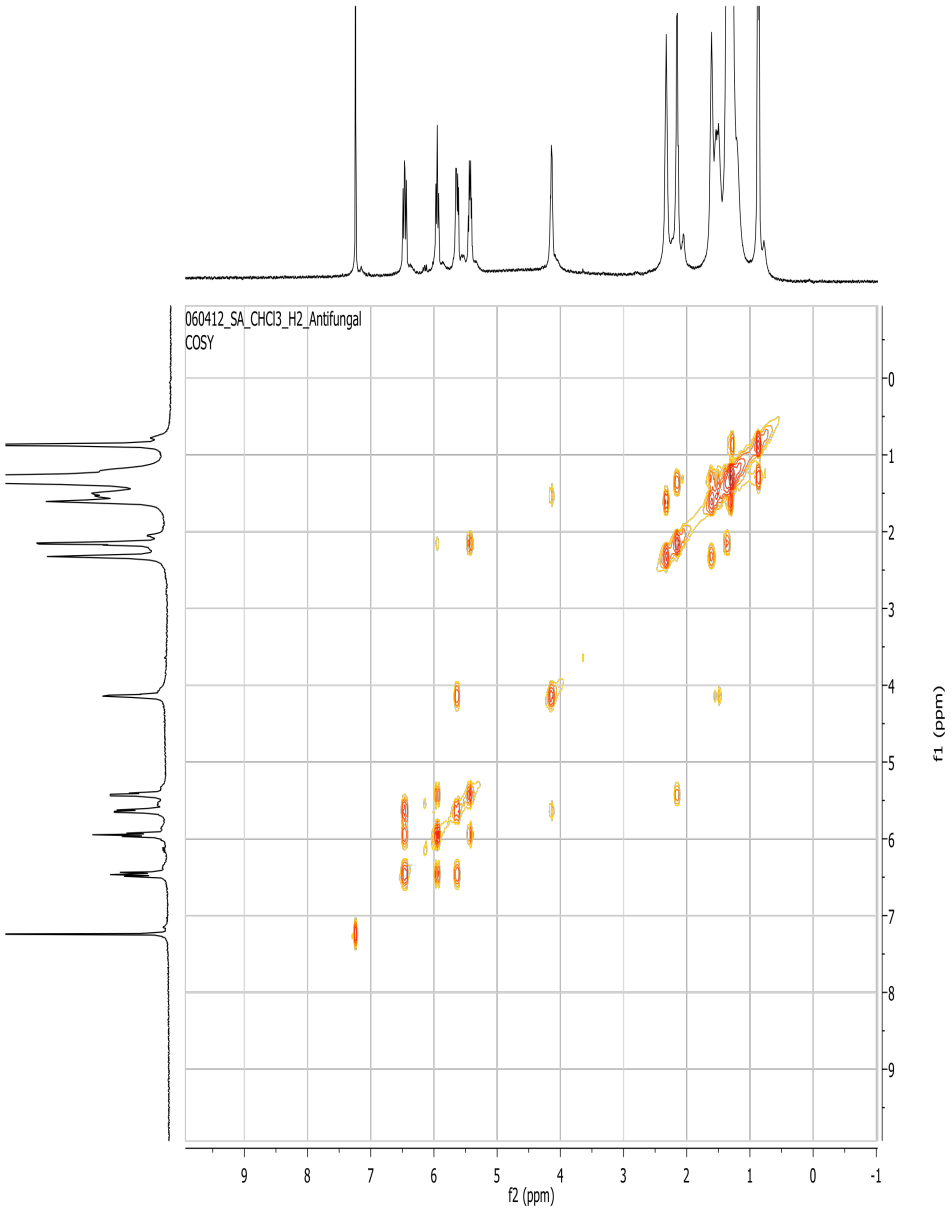

Figure C.2. COSY NMR spectrum of α-dimorphecolic acid (500 MHz, $CDCl_3$).

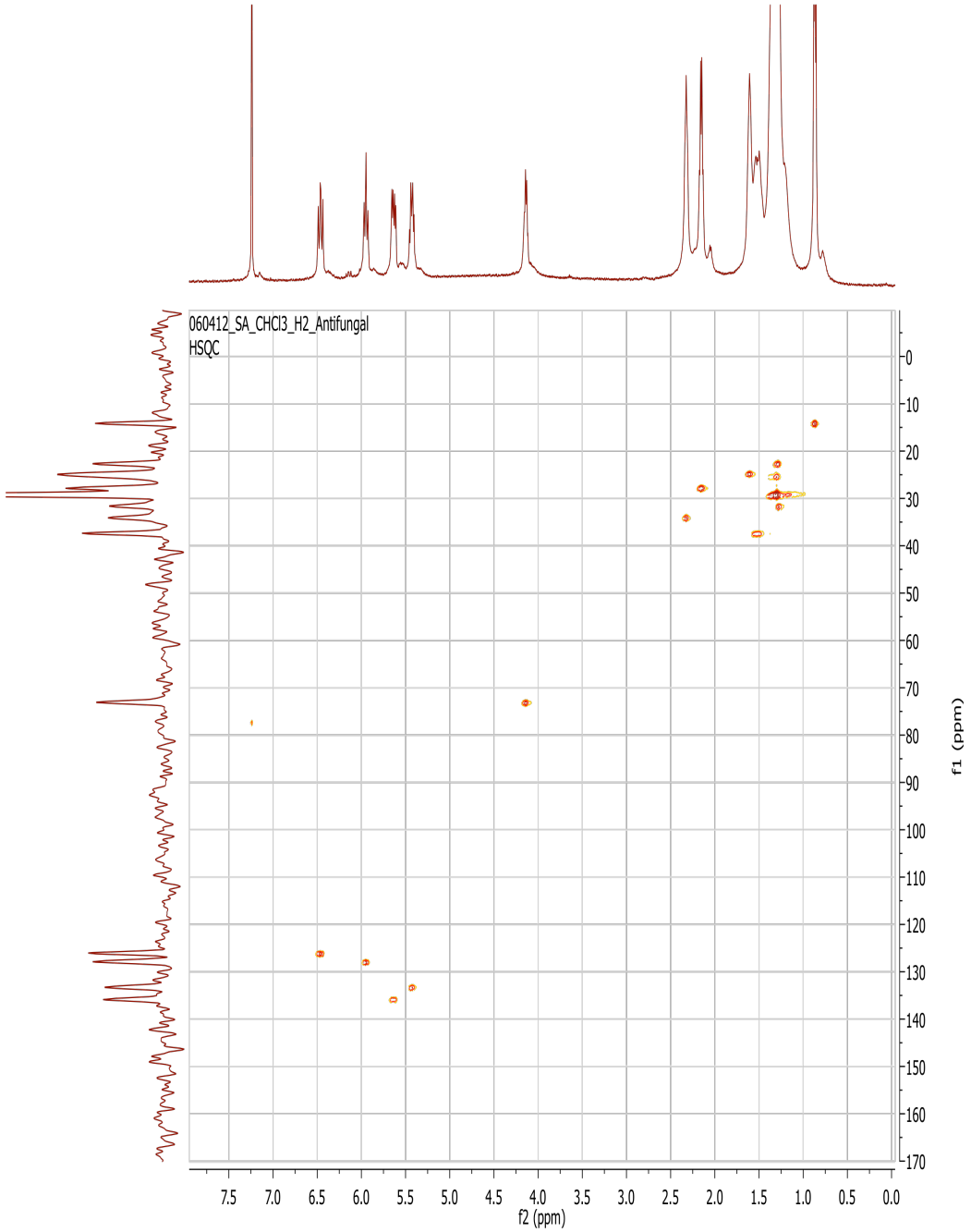

Figure C.3. HSQC NMR spectrum of α-dimorphecolic acid (500 MHz, $CDCl_3$).

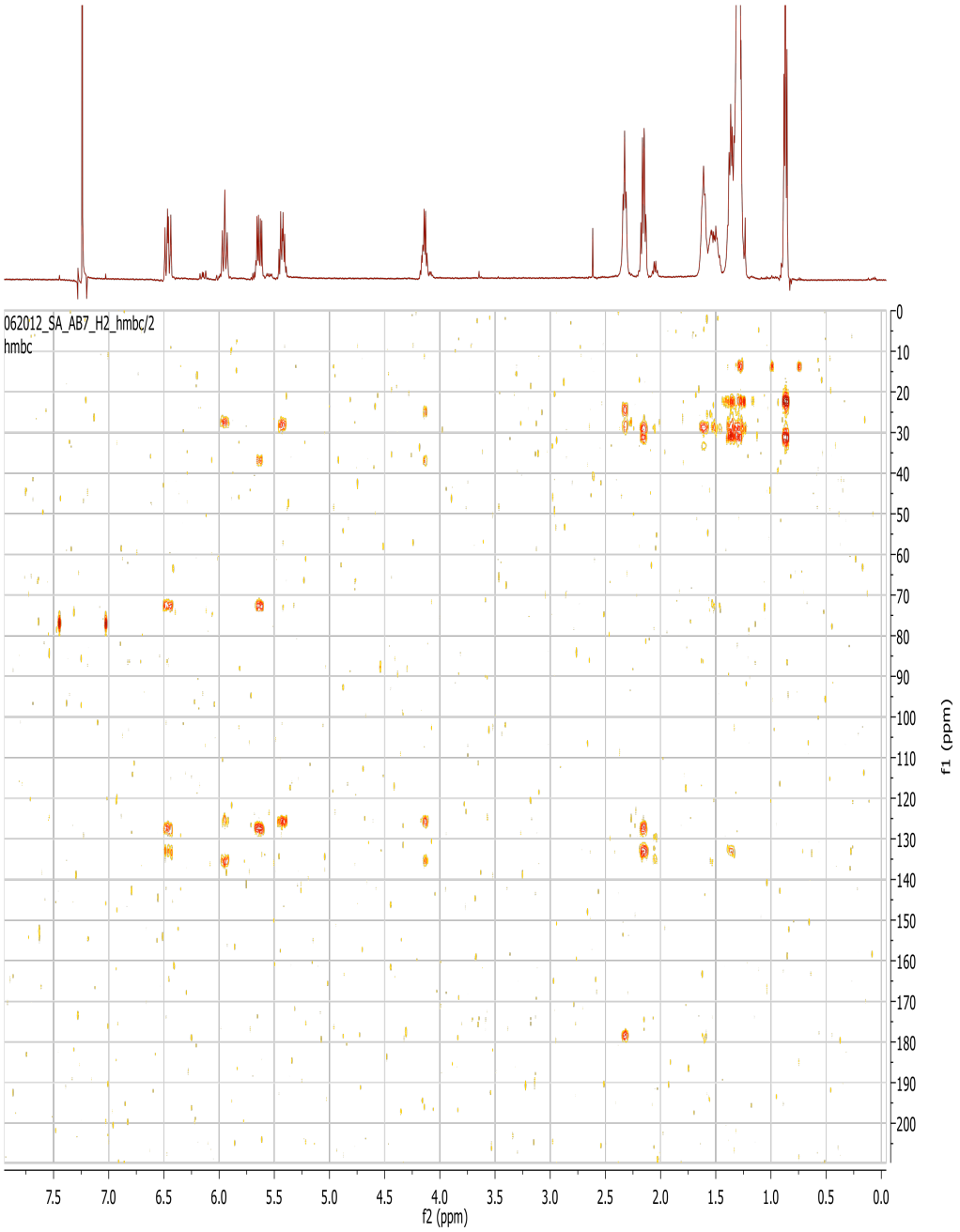

Figure C.4. HMBC NMR spectrum of α-dimorphecolic acid (500 MHz, $CDCl_3$).

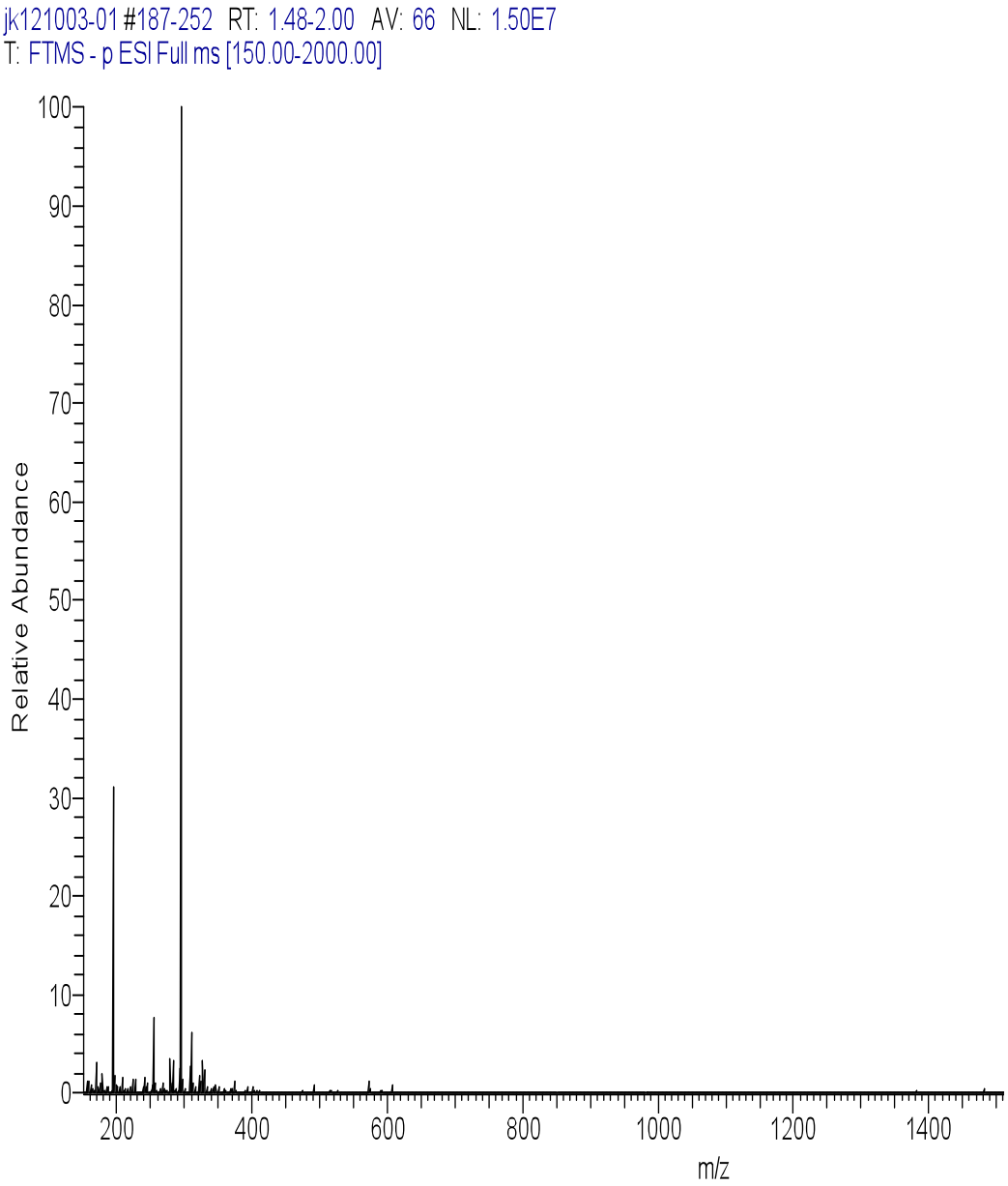

Figure C.5. High resolution mass spectrum (ESI – mode) of α-dimorphecolic acid generated on an Orbitrap Mass Analyzer (in MeOH).

Table C.1. Comparison of carbon and hydrogen chemical shifts for α-dimorphecolic acid collected by RDS to literature values.

α-dimorphecolic acid (from *S. alterniflora*)			α-dimorphecolic acid (literature values)		
no.	δ_c	δ_H (J in Hz)	no.	δ_c	δ_H (J in Hz)
1	178.9	-	1	178.02	-
2	34.1	2.34, 2H, t, (6)	2	35.26	2.36, 2H, t, (7.0)
3	24.8	1.63, 2H, t, (6.5)	3	26.29	1.20-1.70, 2H, m
4	29.2	1.30, 2H, m	4	30.68	1.20-1.70, 2H, m
5	29.2	1.30, 2H, m	5	30.55	1.20-1.70, 2H, m
6	29.4	1.30, 2H, m	6	30.36	1.20-1.70, 2H, m
7	25.4	1.30, 2H, m	7	26.68	1.20-1.70, 2H, m
8a	37.4	1.50, 1H, m	8	38.57	1.20-1.70, 2H, m
8b		1.54, 1H, m			
9	73.1	4.14, 1H, q, (6.5)	9	73.5	4.17, 1H, q, (7.0)
10	135.8	5.63, 1H, dd, (15.3, 6.8)	10	137.43	5.67, 1H, dd, (15.0, 7.0)
11	126.1	6.46, 1H, dd, (14.8, 11.0)	11	126.67	6.50, 1H, dd, (15.0, 11.0)
12	127.8	5.95, 1H, dd, (11.0, 10.5)	12	129.48	5.98, 1H, dd, (11.0,11.0)
13	133.3	5.42, 1H, dd, (10.5, 7.3)	13	133.12	5.47, 1H, dt, (11.0, 7.0)
14	27.8	2.17, 2H, ddd (7.0)?	14	28.75	2.20, 2H, m
15	29.4	1.36, 2H, m	15	30.64	1.20-1.70, 2H, m
16	31.7	1.28, 2H, m	16	32.71	1.20-1.70, 2H, m
17	22.7	1.28, 2H, m	17	23.74	1.20-1.70, 2H, m
18	14.1	0.87, 3H, t, (6.5)	18	14.56	0.90, 3H, t, (7.0)

Source data	Sieg et al., *in prep*	McRae et al 2008	Henry et al 1987
Solvent, NMR	$CDCl_3$, 500MHz	CD_3OD, 500MHz	$CDCl_3$, 400MHz

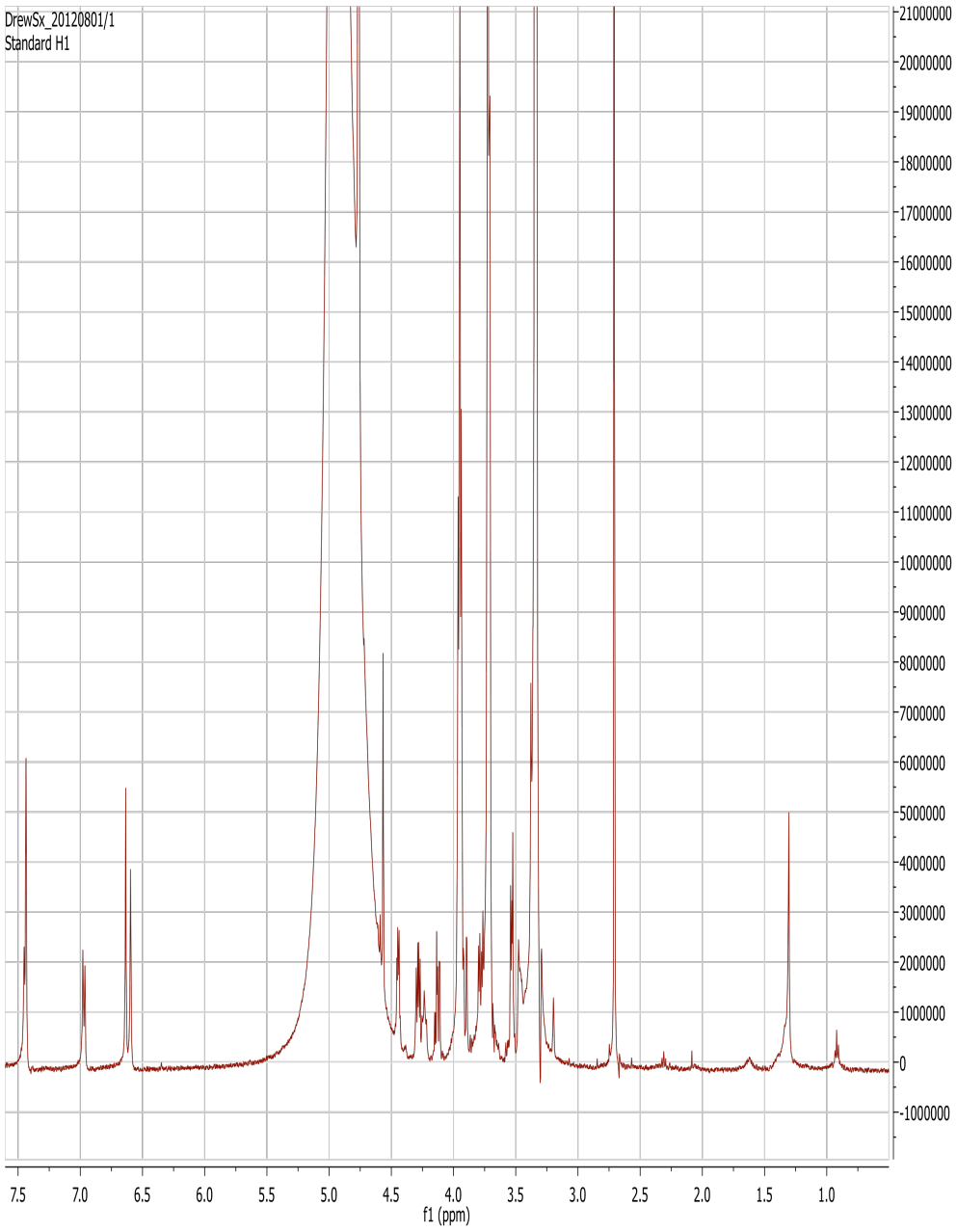

Figure C.6. ^{1}H NMR spectrum of orientin (500 MHz, 3:1 MeOD:D_2O).

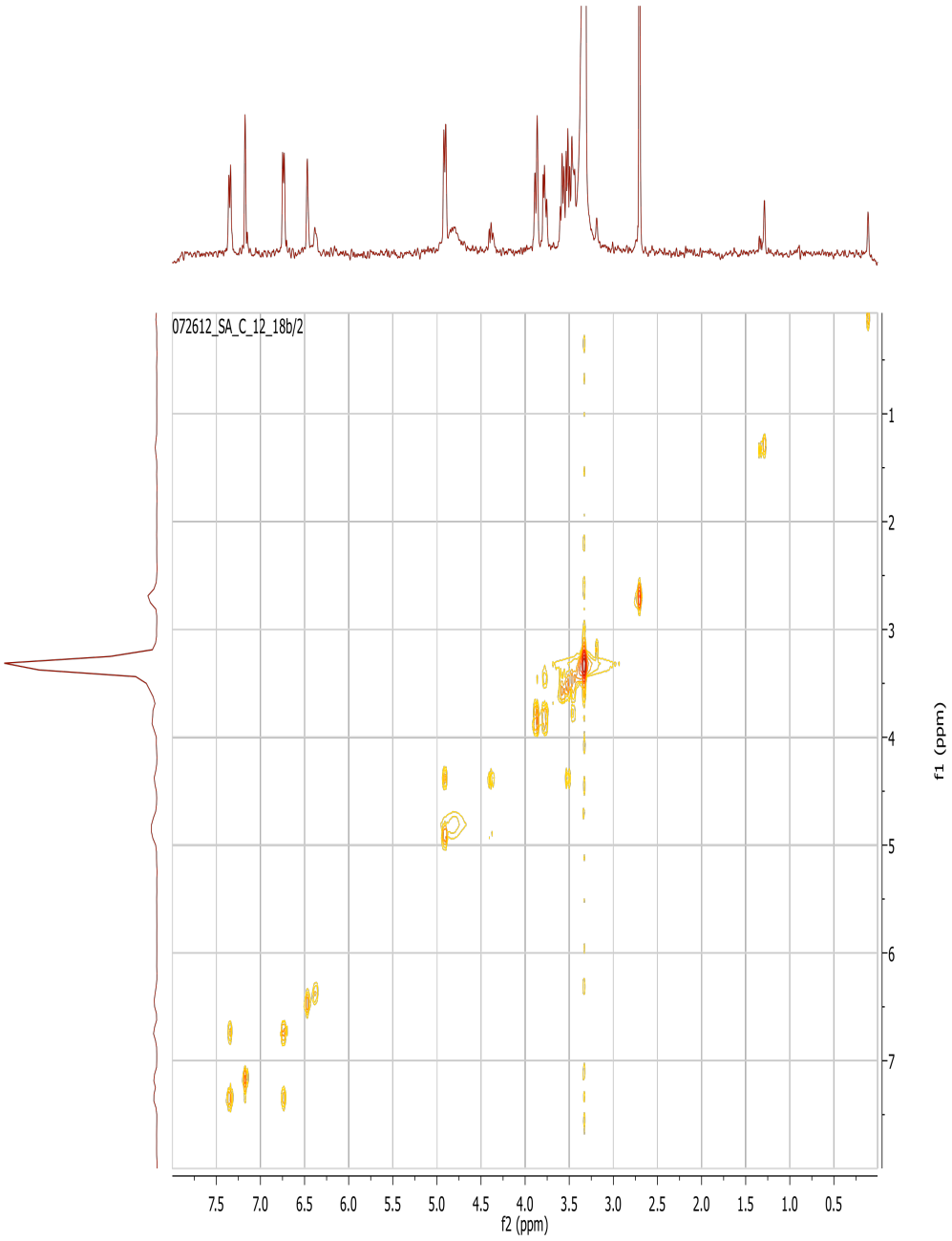

Figure C.7. COSY NMR spectrum of orientin (500 MHz, 3:1 MeOD:D_2O).

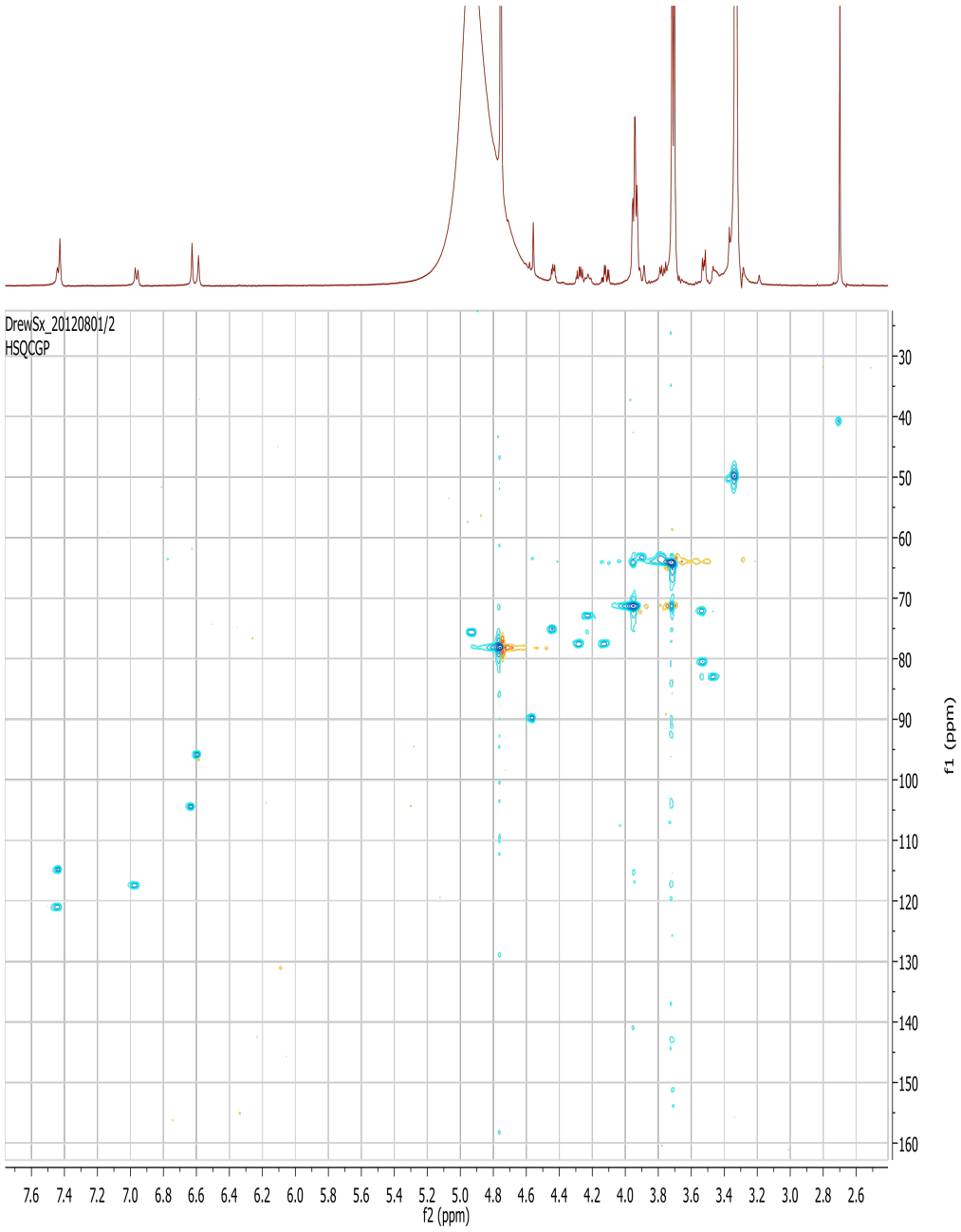

Figure C.8. HSQC NMR spectrum of orientin (500 MHz, 3:1 MeOD:D_2O).

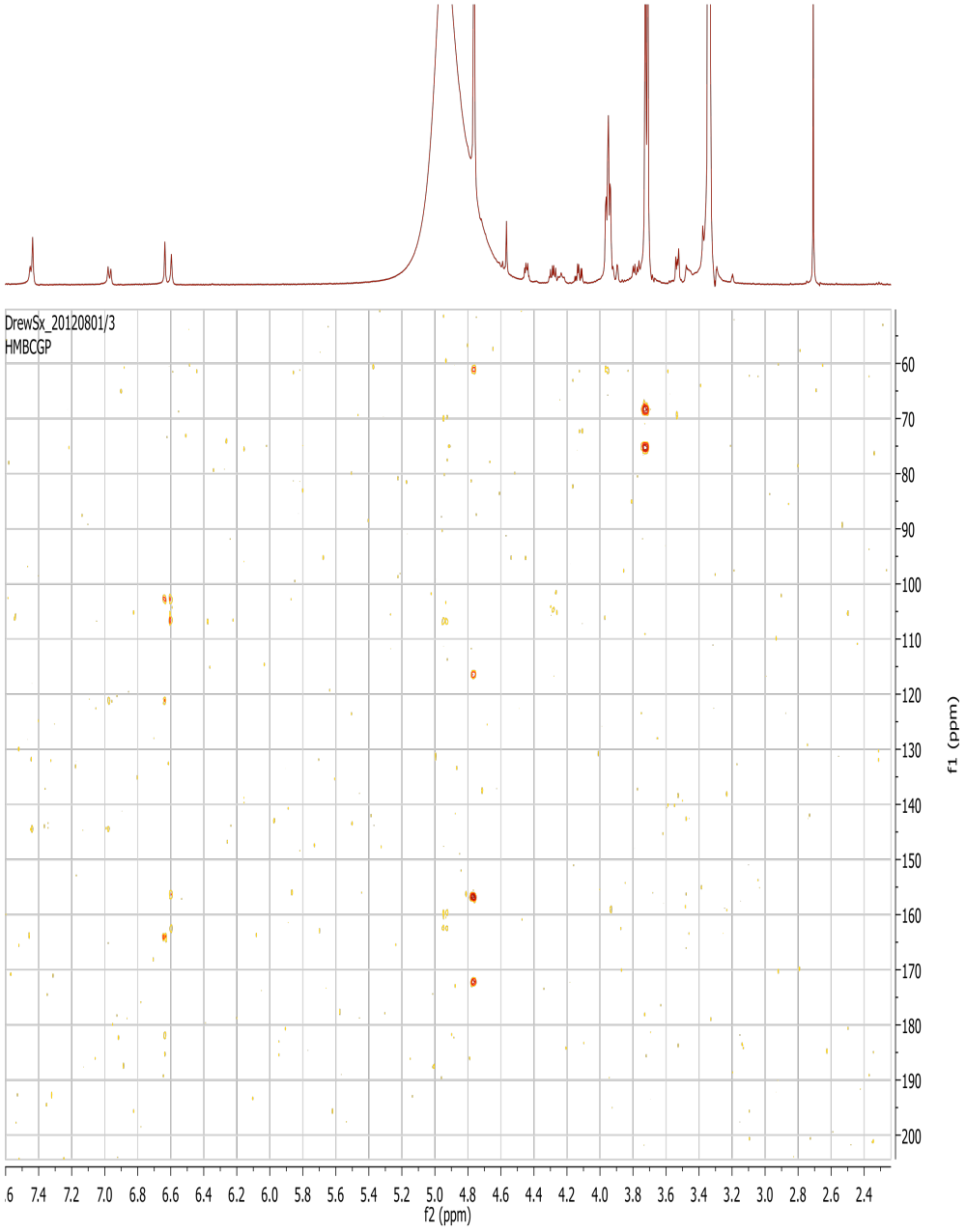

Figure C.9. HMBC NMR spectrum of orientin (500 MHz, 3:1 MeOD:D_2O).

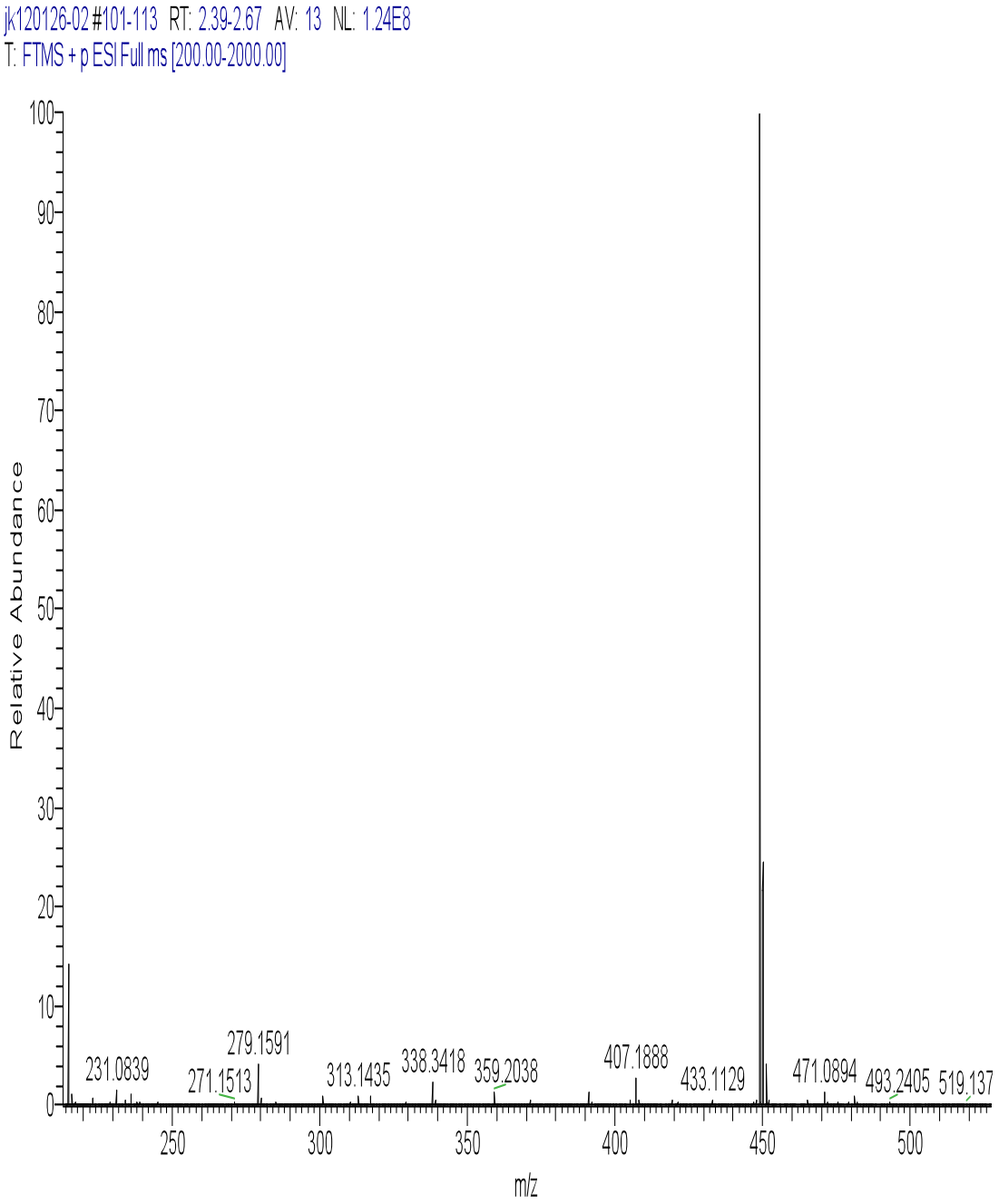

Figure C.10. High resolution mass spectrum (ESI + mode) of orientin generated on an Orbitrap Mass Analyzer (in MeOH).

Table C.2. Comparison of carbon and hydrogen chemical shifts for orientin collected by RDS to literature values.

Orientin (from *S. alterniflora*)			Orientin (literature values)		
no.	δ_c	δ_H (J in Hz)	no.	δ_c	δ_H (J in Hz)
1	-	-	1	-	-
2	163.8	-	2	164.2	-
3	104.4	6.64, 1H, s	3	102.4	6.65, 1H, s
4	181.9	-	4	182.0	-
5	156.3	-	5	160.5	-
6	95.9	6.60, 1H, s	6	98.3	6.25, 1H, s
7	162.5	-	7	162.8	-
8	106.5	-	8	104.7	-
9	159.6	-	9	156.0	-
10	102.7	-	10	104.0	-
1’	121.1	-	1’	122.0	-
2’	114.9	7.44, 1H, d, (1.5)	2’	114.1	7.44, 1H, d, (2.1)
3’	144.5	-	3’	146.0	-
4’	144.5	-	4’	149.9	-
5’	117.5	6.97, 1H, d, (8.5)	5’	115.6	6.90, 1H, d, (8.2)
6’	121.2	7.45, 1H, dd, (8.5. 1.5)	6’	119.5	7.50, 1H, dd, (8.0, 2.1)
1”	75.7	4.94, 1H, d, (9.5)	1”	73.5	4.72, 1H, d, (9.0)
2”	73.0	4.24, 1H, dd (8.8,8.8)	2”	70.9	3.22-3.88, 1H, m, glucosyl-H
3”	80.6	3.53, 1H, dd, (9.0,9.0)	3”	76.8	3.22-3.88, 1H, m, glucosyl-H
4”	72.0	3.54, 1H, dd, (9.3, 9.3)	4”	70.8	3.22-3.88, 1H, m, glucosyl-H
5”	83.0	3.47, 1H, m, glucosyl-H	5”	82.0	3.22-3.88, 1H, m, glucosyl-H
6”a	63.7	3.78, 1H, dd, (12.3, 4.8)	6”a	61.8	3.22-3.88, 1H, m, glucosyl-H
6”b		3.91, 1H, dd, (12.8, 1.8)	6”b		3.22-3.88, 1H, m, glucosyl-H

Source data	Sieg et al., *in prep*	Henry et al 1987
Solvent, NMR	3:1 MeOD:D_2O, 500MHz	DMSO, 500MHz

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