ARE CHEMICAL DEFENSES ALLOCATED WITHIN SPONGES TO DEFEND

REGIONS MOST AT RISK TO PREDATOR ATTACK?

by

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(Under the Direction of Daniel F. Gleason)

ABSTRACT

The allocation of chemical defenses to regions most at risk to predator attack may provide adequate protection at minimal metabolic cost. This study examined chemical defense allocation within three sponge species from a temperate reef by investigating the predictions that: concentrations of chemical defenses are 1) higher in the outer 2 mm of the sponge tissue, 2) positively correlated with tissue nutritional quality, 3) negatively correlated with sponge structural components, and 4) varied enough to have differential effects on predator deterrence. The concentrations of chemical defenses varied within *Ircinia felix* and *Aplysina fulva*, but were equal throughout *I. campana*. There were, however, no consistent positive or negative correlations between chemical defenses and nutritional quality or structural components and no clear correlation between chemical defense concentration and predator deterrence. Together, these results suggest the need for a reevaluation of currently accepted ideas regarding chemical defense allocation within sessile prey.

INDEX WORDS: Temperate reefs, Sponge, Chemical defenses, Structural defenses, Nutritional quality, South Atlantic Bight, Secondary metabolites, Allocation of chemical defenses

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DEDICATION

This work is dedicated to my parents, John and Anne Freeman. They have supported my fascination with marine biology since I first became interested in it in 7th grade. They immediately ensured that I had numerous field guides and underwater cameras to document and identify all the marine creatures I found while on vacation and spent countless hours on the beach watching me snorkel. More recently, they helped me follow and broaden this interest by sending me to marine biology based summer camps and ultimately encouraging me to continue to follow my interest through my undergraduate and graduate degrees. I will always be extremely grateful to them for recognizing my strong interest and encouraging me to excel in what I have set out to do since then. Their love and constant support have been instrumental in my success all the way from that first day in the ocean to the completion of this project.

ACKNOWLEDGMENTS

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CHAPTER 1

ISOLATION OF CHEMICAL DEFENSES IN THREE SPONGE SPECIES FROM A

TEMPERATE REEF

Introduction

The production of secondary metabolites by sessile marine organisms is thought

to be the most common method for defense against biotic factors like predation, infection

by pathogens, or overgrowth by fouling organisms (Bakus et al. 1986, Paul 1992). The

role of these metabolites in deterring potential predators has received increasing attention

and has been substantiated in numerous marine phyla, including gorgonians (O'Neal &

Pawlik 2002), tunicates (Paul 1992), and sponges (Pawlik et al. 1995). These metabolites

are especially common in areas of high predator abundance and relegate organisms

lacking such defenses to cryptic locations to avoid consumers (Bakus 1964, Dunlap &

Pawlik 1996).

Sponges are a major component of benthic communities from tropical to polar

regions (Zea 1993, McClintock & Baker 1997). Because sponges are soft-bodied,

nutritious (Chanas & Pawlik 1995) organisms that typically lack effective physical

defenses, chemical defense, through the production of secondary metabolites, is thought

to be the most common means of predator deterrence (Paul 1992, Pawlik 1993, Wilson et

al. 1999). In the first large-scale survey of these chemical defenses, Pawlik et al. (1995)

reported that 49 of 71 species of Caribbean sponges have extracts deterrent to fish. Some

of the most deterrent sponges were from the orders Verongida and Dictyoceratida and

included two common Caribbean genera, *Aplysina* and *Ircinia*, that have well-

documented, effective secondary metabolites (Pawlik et al. 1995).

Sponges of the genus *Ircinia*, including species studied from Brazil (Epifanio et al. 1999), the southern Caribbean (Martinez et al. 1997), and the Bahamas and Florida Keys (Pawlik et al. 2002), have defensive chemicals known as furanosesterterpene tetronic acids (FTAs). Crude extracts containing FTAs are deterrent to both invertebrate (Waddell & Pawlik 2000a,b, Burns et al. 2003) and fish predators (Pawlik et al. 1995, Epifanio et al. 1999, Pawlik et al. 2002) even at levels well-below natural concentrations (Pawlik et al. 2002).

Species of *Aplysina* investigated in the Caribbean and Mediterranean have secondary metabolites known as brominated tyrosine derivatives that undergo biotransformation following mechanical damage (Teeyapant & Proksch 1993, Ebel et al. 1997, Puyana et al. 2003, Thoms et al. 2006). This "activation" is characterized by a conversion of high molecular weight brominated isoxazoline alkaloids to the low molecular weight brominated compounds aeroplysinin-1 and dibromocyclohexadienone (Teeyapant & Proksch 1993). Whether the high molecular weight precursors or activated defenses are more effective at deterring predators is unclear currently (Ebel et al. 1997, Thoms et al. 2004). Regardless, the crude extract and isolated metabolites of *Aplysina* sponges are deterrent fish predators (Pawlik et al. 1995, Ebel et al. 1997, Thoms et al. 2006).

Although FTAs and brominated tyrosine derivatives have been reported from species of *Ircinia* and *Aplysina* in both the Caribbean and Mediterranean, an evaluation of antipredator compounds in sponges of these genera in the temperate northwestern Atlantic has yet to be completed. Moreover, the identification and isolation of these defensive compounds is a required first step in the process of quantifying these

metabolites within sponges from this geographic region (Chapter 2), as well as for assessing their ability to deter predators common to this region (Chapter 3). The objectives of this investigation were to determine if FTAs and brominated tyrosine derivatives occur in *Ircinia* and *Aplysina* sponges from a temperate reef in the South Atlantic Bight (SAB) and, if so, isolate these compounds so they can be used as standards for compound quantification via high performance liquid chromatography (HPLC).

Methods

Sponge collection and identification

Sponges were collected at J Reef (31˚ 36.056 N, 80˚ 47.431 W), a hard bottom area in the SAB located about 32 km off the coast of Georgia, USA. This reef is characterized by sandstone and relic scallop shell ridges that provide areas of moderate relief projecting 2-3m above the bottom. These ridges serve as habitat for a diverse assemblage of invertebrates and fishes and are bordered by sand substrate in waters ranging from 18 to 20 m deep (Hunt 1974, Fioravanti-Score 1998).

The three species of sponges used in this study were chosen due to their high abundance at J Reef, their relatively large size, and because their antipredator secondary metabolites have been identified. *Ircinia felix* and *Ircinia campana*, the amorphous and vase-shaped stinker sponges are both aspiculate sponges characterized by a tough network of fibers. The sponge *Aplysina fulva* is an aspiculate sponge that exhibits a repent rope-like growth form along the substrata (Figure 1).

Sponges were collected from J Reef in June of 2005 by taking 100 to 300 g (wet weight) subsamples from large individuals. Samples were bagged and brought to the surface where they were placed on ice. Approximately 5 hours later these samples were

placed in a -70 ˚C freezer for storage. Sponges were identified based on morphological

characteristics and thin tissue sections. All identifications were substantiated by Dr. Rob

Van Soest of the University of Amsterdam.

<u>Identification and isolation of FTAs</u>

The isolation and identification of FTAs from *I. felix* and *I. campana* followed

methods used by Martinez et al. (1997). A sample (281.7 g wet weight) of *I. felix* or *I.*

campana collected from J Reef was extracted overnight twice in 400 ml Methanol

(MeOH) and once in 400 ml Ethyl Acetate (EtOAc). The extracts were dried by vacuum

evaporation at 30˚ C and the MeOH extract was re-extracted with 400 ml EtOAc. Both

EtOAc soluble fractions were combined and dried by rotary evaporation to produce a

brownish-yellow residue that was freeze dried to ensure complete removal of solvents

and water before derivatization. In order to make the extract volatile for analysis by gas

chromatography and mass spectrometry (GC-MS), the freeze dried sample (3.2 g) was

acetylated by adding 20 ml of 1:1 acetic anhydride:pyridine and the mixture was stirred

for 27 hours at room temperature in the dark. The acetic anhydride and pyridine mixture

was removed by rotary evaporation at 60-70˚ C and the remaining dark brown residue

was extracted with a 17 ml mixture of 4:1 hexane:EtOAc. For preliminary cleanup of

acetylated FTAs, 6 ml of this extract was run through a 6 ml Phenomenex Strata-X

polymeric sorbent (500 g) column using a syringe plunger. The first two fractions (6 ml

each) were found, initially by thin layer chromatography (TLC) and later by GC-MS

comparison, to contain all the FTAs. These fractions were collected, dried, and dissolved

in 100% Methylene Chloride (DCM) and filtered through a 45 μm syringe filter for GC-

MS analysis.

GC-MS analysis of extracts from both *Ircinia* species was conducted using a Hewlett Packard 5890 GC-MS equipped with a 30 m long 0.250 mm diameter DB-5 fused silica column and He gas mobile phase. The inlet and detector were kept at 300° and 280° C, respectively, while the oven temperature increased from 200° to 300° C during the run. All samples were injected manually in 2 µl volumes.

FTA standards for quantification via HPLC were prepared by evaporation of the 4:1 mixture of hexane:EtOAc from the extract of *I. felix* and re-dissolving this extract in MeOH at a concentration of 69 mg/ml for preparative chromatography. Preparative chromatography was performed on a Shimadzu HPLC system equipped with a SPD-10A-VP UV/VIS detector and SIL-10AF autoinjector using a Vydac 10 x 250 mm C-18 preparatory column (Grace Vydac, Hesperia, California). Concentrated FTAs were injected at a volume of 200 µl and separation was achieved using a solvent of 85:15 MeOH:H_2O for 28 minutes with a flow rate of 4 ml/min. Peak detection was monitored at 270 nm, the wavelength of maximum absorbance for FTAs (Martinez et al. 1997). Peaks corresponding to those produced by FTAs were collected, concentrated, verified by GC-MS, and diluted for use as standards for HPLC quantification.

Identification and isolation of brominated tyrosine derivatives

For *A. fulva*, a small sample (~50 g wet weight) from J Reef was extracted twice in 75 ml of a 1:1 mixture of DCM:MeOH, followed by a third extraction in 75 ml of acetonitrile ($CNCH_3$). The extracts were combined, filtered, and evaporated to dryness. The crude extract was dissolved in a 6 ml mixture of 1:1 $CNCH_3$:H_2O and passed through a 6 ml Phenomenex Strata-X polymeric sorbent (500 g) column with a plunger to remove contaminants. Fractions collected from this column were evaporated to dryness and

dissolved in MeOH at 45 mg/ml for analysis by liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS was carried out using a Phenomenex Gemini C-18 analytical column (4.6 x 250 mm) with a solvent gradient consisting of $CNCH_3$ and water buffered with 0.1% formic acid. The gradient was 90 % water for the first 3 minutes followed by an increase in the concentration of $CNCH_3$ to 100% over 28 minutes with a flow rate of 0.7 ml min^{-1}. Peaks were viewed using an Agilent 1100 diode array detector at 254 nm and identified based on their fragmentation patterns and molecular weight in a Micromass quadrapole time-of-flight mass spectrometer using positive electrospray ionization.

Once I confirmed the presence of 10 previously described brominated compounds from *A. fulva*, I isolated groups of these compounds using a Vydac C-18 preparatory column (10 x 250 mm). Concentrated samples of dissolved crude extract were injected at a volume of 200 μl and separation was achieved using non-buffered $CNCH_3$:H_20 with the same gradient as above at 4 ml min^{-1} with monitoring at 254 and 280 nm. This method did not allow for the purification of individual compounds, so standards to be used in HPLC analysis were mixtures of known chemical defense compounds. In *A. fulva*, there were two mixtures of compounds, one consisting of a group of three compounds with relatively short retention times and one composed of five compounds with longer retention times. Groups of peaks with these retention times were collected and concentrated until enough of the mixture was present for use as standards. A purified sample of Aeroplysinin-1 was also purchased from Axxora for use as a standard.

Results

GC-MS analysis identified five compounds from *I. felix* and three compounds from *I. campana* that show an ion peak at 440 m/z (Figure 2). The presence of the ion peak at 440 and the fragmentation patterns of these compounds suggest that FTAs are present in these *Ircinia* species from J Reef. The spectra from three of the *I. felix* peaks (#1, 2, and 4) and two of the *I. campana* peaks (#1 and 3) correspond to published values for the fragmentation of variabilin and multiple combinations of the cis/trans isomers felixinin and strobilinin (Figure 3). The spectra for the remaining two peaks (# 3 and # 5) of *I. felix* and one peak (# 2) of *I. campana* do not correspond to previously published FTA values and are currently unidentified (A. Martinez, pers. comm.) (Figure 2 and Appendix A). These peaks were also visible in HPLC analysis when a mobile phase of 85:15 MeOH:H_2O was used (Figure 4).

LC-MS analysis of *A. fulva* samples showed 9 peaks with spectra corresponding to published values for brominated tyrosine derivatives with anti-predator properties (Figures 5 and 6, Appendix B) (Ebel et al. 1997, Thoms et al. 2006). Compounds were identified by their spectra and molecular weights and retention times provided by other investigators (Appendix B) (R. Berlinck and C. Thoms, pers. comm.). Of these 9 compounds, 8 have been previously classified as high molecular weight precursor or starting molecules and one as a low molecular weight compound that is "activated" following mechanical damage to the sponge (Teeyapant & Proksch 1993, Ebel et al. 1997, Puyana et al. 2003, Thoms 2004, Thoms 2006) (Figure 6). In addition to the 9 compounds consistently observed by LC-MS, dibromocyclohexadienone, another "activated" compound (Figure 6), was observed in trace amounts in a couple of the LC-

MS analyses, but was not detectable in experimental samples run on the HPLC. These nine compounds were placed into three groups according to retention time and the ability to collect them using preparatory HPLC for use as standards (Figure 6).

Discussion

Secondary metabolites are thought to be the most effective defensive mechanism employed by many benthic marine invertebrates (Bakus et al. 1986, Pawlik et al. 1995). The deterrent ability of extracts containing these metabolites is well documented in many species of marine sponges (Pawlik et al.1995, Ruzicka 2005), and, in some species, the ability of specific compounds to deter predators has been reported (Wilson et al. 1999, Pawlik et al. 2002). In this study I identified and isolated previously reported defensive metabolites from two species of *Ircinia* (*I. felix* and *I. campana*) and one species of *Aplysina* (*A. fulva*) collected from the SAB region of the Atlantic coast of North America. In total, I have identified 5 FTAs from *I. felix* and *I. campana* and 10 brominated tyrosine derivatives in *A. fulva*. Documentation that these defensive metabolites occur in sponges from the SAB provides further evidence that their presence is independent of geographic location. These compounds have been observed in the same genera inhabiting the Caribbean and Mediterranean seas, Florida Keys, and Brazil (Martinez et al. 1997, Epifanio et al. 1999, Pawlik et al. 2002, Puyana et al. 2003, Thoms et al. 2006).

The FTAs identified in *I. felix* and *I. campana* in this study correspond to the most commonly found FTAs, including variabilin and at least two isomers of this compound (Figures 2 and 3) (A. Martinez, pers. comm.). These compounds have been shown to deter feeding by fish predators and hermit crabs (Waddell and Pawlik 2000a, Pawlik et al. 2002), even at concentrations below that naturally occurring within the sponge (Pawlik et

al. 2002), but may be less effective against some invertebrate or specialist sponge predators (Randall and Hartman 1968, Waddell and Pawlik 2000b).

The high molecular weight brominated tyrosine derivatives identified in *A. fulva* can be further divided into two groups: those with one (Aerophobin-1) and two (Aerothionin) isoxazoline moieties (Figures 5 and 6). The occurrence of both types of isoxazoline alkaloids in a single species of sponge has not been reported previously. The identification of both types of alkaloids in this study may represent the fact that secondary metabolites in *A. fulva* have not been well-investigated previously, or that the presence of both moieties varies geographically. In a study on the activated defenses of *Aplysina insularis* and *A. archeri* from the Caribbean, Puyana et al. (2003) reported compounds with only two isoxazoline moieties. In a follow-up study by Thoms et al. (2006), using *A. fistularis* and *A. archeri* from the Caribbean and *A. aerophoba* and *A. cavernicola* from the Mediterranean, only compounds with one moiety were reported. Having both types of alkaloids, such as seen here in *A. fulva*, may confer additional protection from predators if activated compounds are, in fact, more deterrent to predators (Ebel et al. 1997). This added protection may stem from the fact that alkaloid precursors with two isoxazoline moieties produce two molecules of the activated aeroplysinin-1 upon enzymatic cleavage (Ebel et al. 1997). The high number of aeroplysinin-1 molecules produced from the conversion of these, as well as from the co-occurring alkaloids with one moiety in *A. fulva*, may provide enhanced protection from predators following mechanical damage.

In summary, the documentation of defensive compounds in these sponge species from the SAB suggests that these sponges, like others in these genera from the

Mediterranean and Caribbean, should be chemically defended from invertebrate and fish predators. In order to adequately defend themselves against predators, these sponges may allocate chemical defenses to regions of their body most susceptible to predation. Based on observations during the collection of these species, I predict that *I. felix* and *I. campana* may have higher concentrations of chemical defenses towards the interior of their bodies to defend against the large number of invertebrates I commonly observed living inside canals and large, hollowed out regions of these sponges. The smaller rope sponge, *A. fulva*, on the other hand, may have higher concentrations of chemical defenses toward the exterior of its body, to defend against feeding by fish and mobile invertebrate predators in the water column. In order to assess whether or not such chemical variation occurs within these sponges and the significance of such variation as a predator deterrent, I quantified FTAs and brominated tyrosine derivatives in the inner and outer regions of these three sponges (Chapter 2) and conducted feeding assays against urchin and fish predators (Chapter 3).
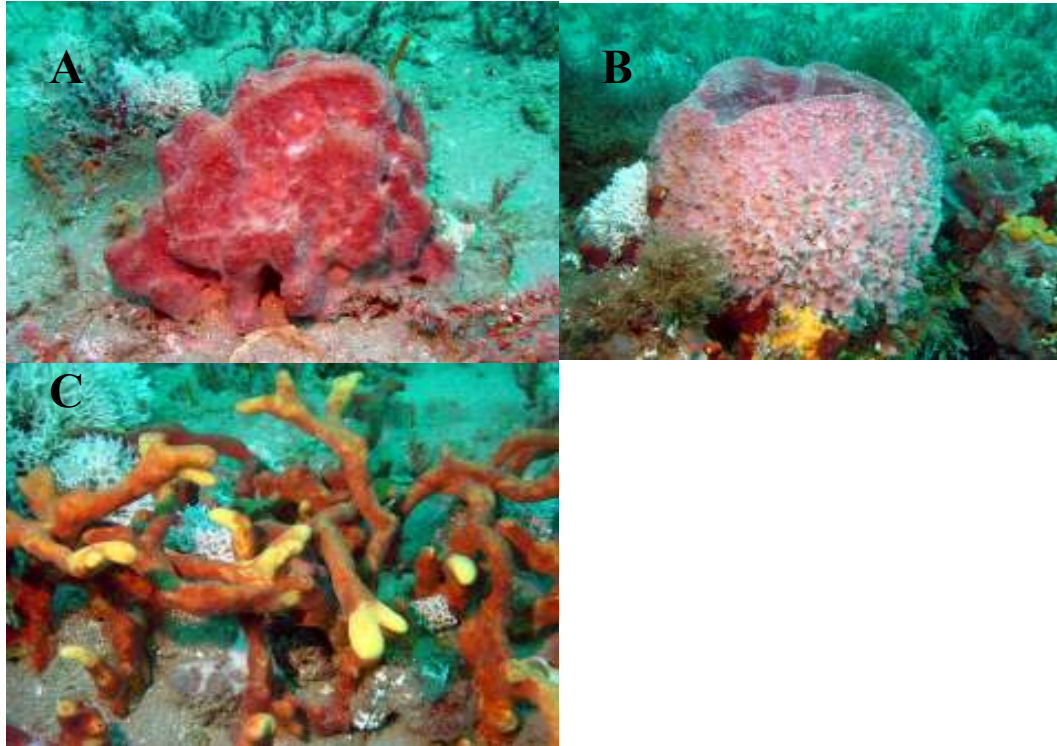
Figure 1: *In situ* photographs of (A) *I. felix*, (B) *I. campana*, and (C) *A. fulva* from J Reef. At J reef, *I. felix* is generally about 30 cm in width and can be up to 30 cm in height, *I. campana* can reach 45-50 cm in width and 60 cm in height, and the branches of *A. fulva* can be 60-90 cm long and 3-6 cm wide.
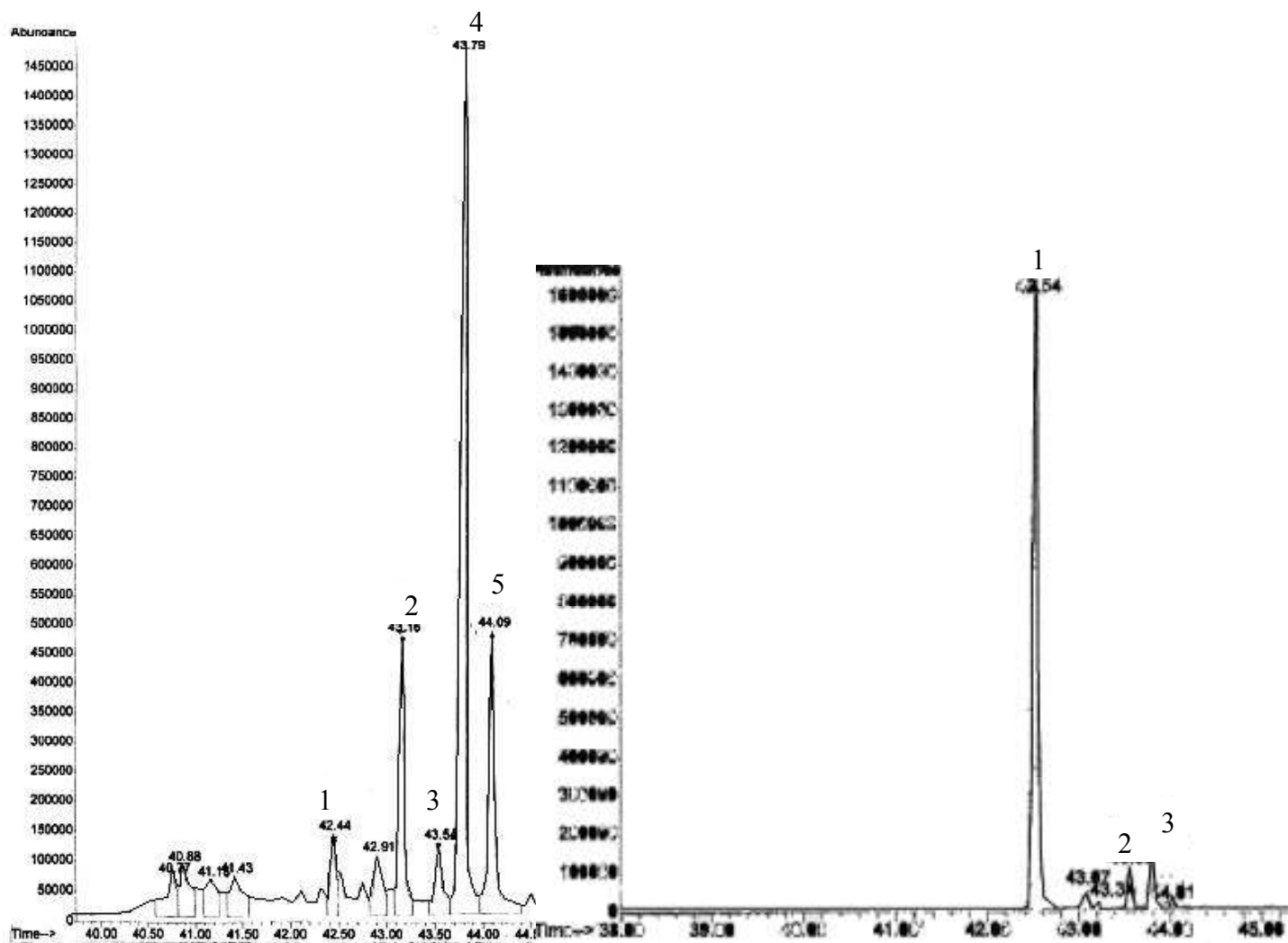
Figure 2: GC-MS chromatogram for *I. felix* and *I. campana*. Peaks labeled 1-5 (*I. felix*) and 1-3 (*I. campana*) represent peaks with spectra corresponding to FTAs

Strobilinin Acetate

Felixinin Acetate

Strobilinin Acetate

Felixinin Acetate

Variabilin Acetate
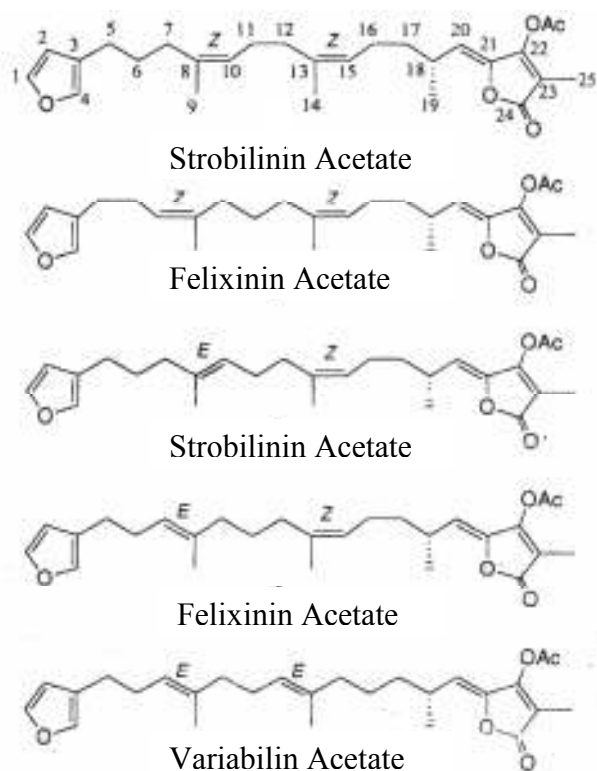
Figure 3: Structures of Furanosesterterpene Tetronic Acids (FTAs) commonly observed in species of *Ircinia* in their acetate forms. Variabilin was the major FTA encountered in the samples in this study. GC-MS analyses revealed 5 peaks with the same molecular weight as variabilin (440 m/z) that could be variations of the individual isomers felixinin or strobilinin or combinations of the possible isomers seen above. The above structures have been adapted from (Martinez et al. 1997).
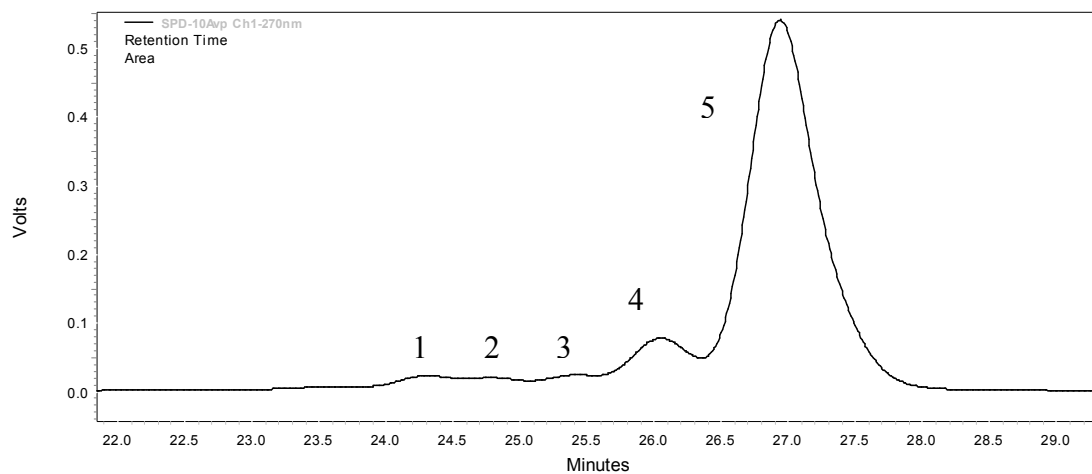
Figure 4: HPLC chromatogram of FTAs in *I. felix*. Sample was run at 85:15 MeOH:H20 to verify the presence of the 5 FTA peaks. Extracts from *I. campana* were similar to this, but with only 3 detectable peaks.
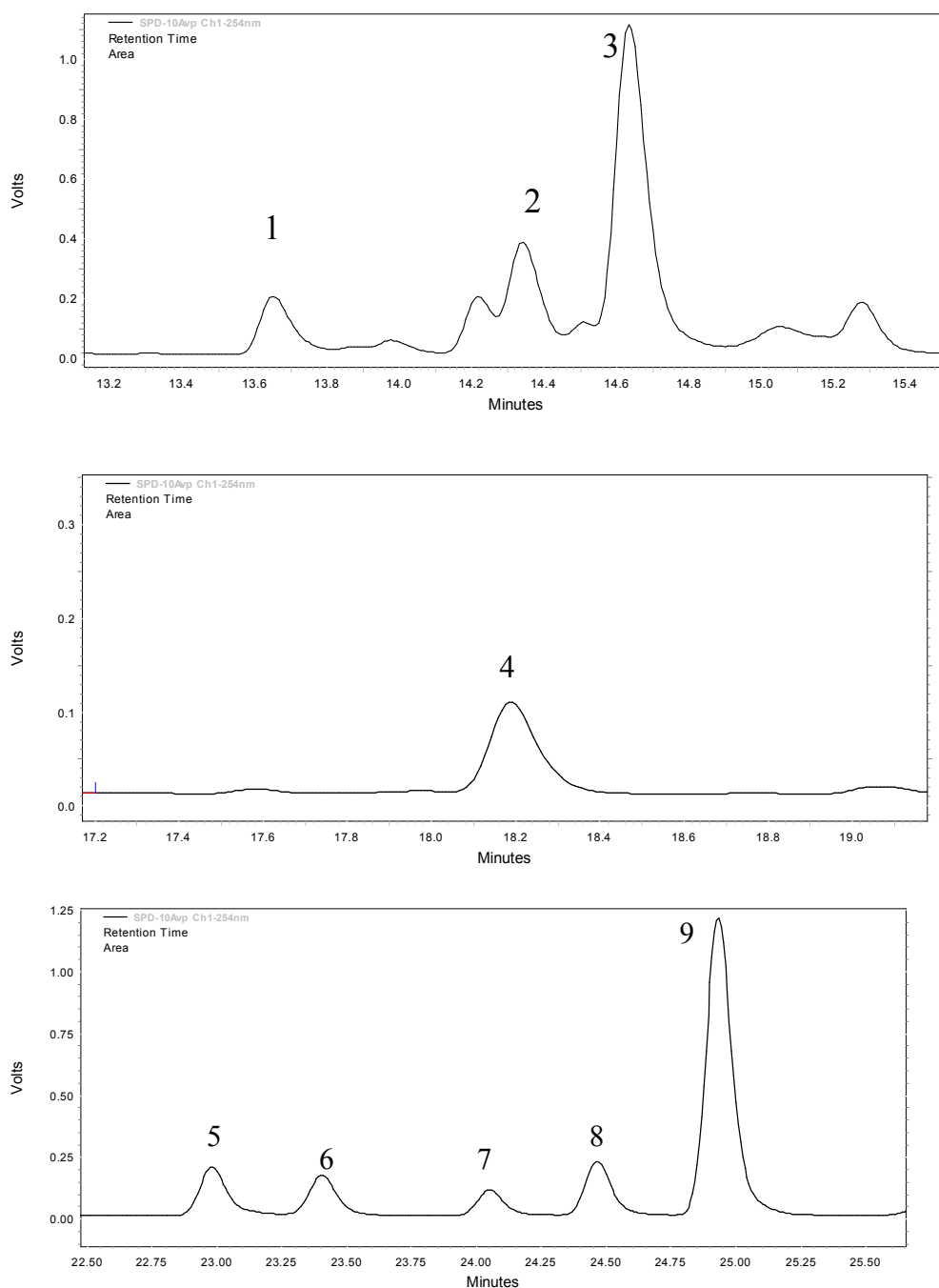
Figure 5: High performance liquid chromatography (HPLC) chromatograms displaying the composition of brominated defensive metabolites in the crude extract of *A. fulva*. Peak numbers correspond to compounds shown in Figure 6. Each of these chromatograms represents a group of peaks (#s 1-3), (# 4), and (#s 5-9) that were pooled for quantification of these metabolites (see Chapter 2). Dibromocyclohexadienone was only present in trace amounts in most samples and is therefore not included in these chromatograms.

1) Aerophobin-1   2) Aerophobin-2

3) Aplysinamisin-1   4) Aeroplysinin-1

5) Hydroxyaerothionin   6) Hydroxy-oxo-aerothionin

7) Homoaerothionin   8) Aerothionin

9) Fistularin 3   Dibromocyclohexadienone
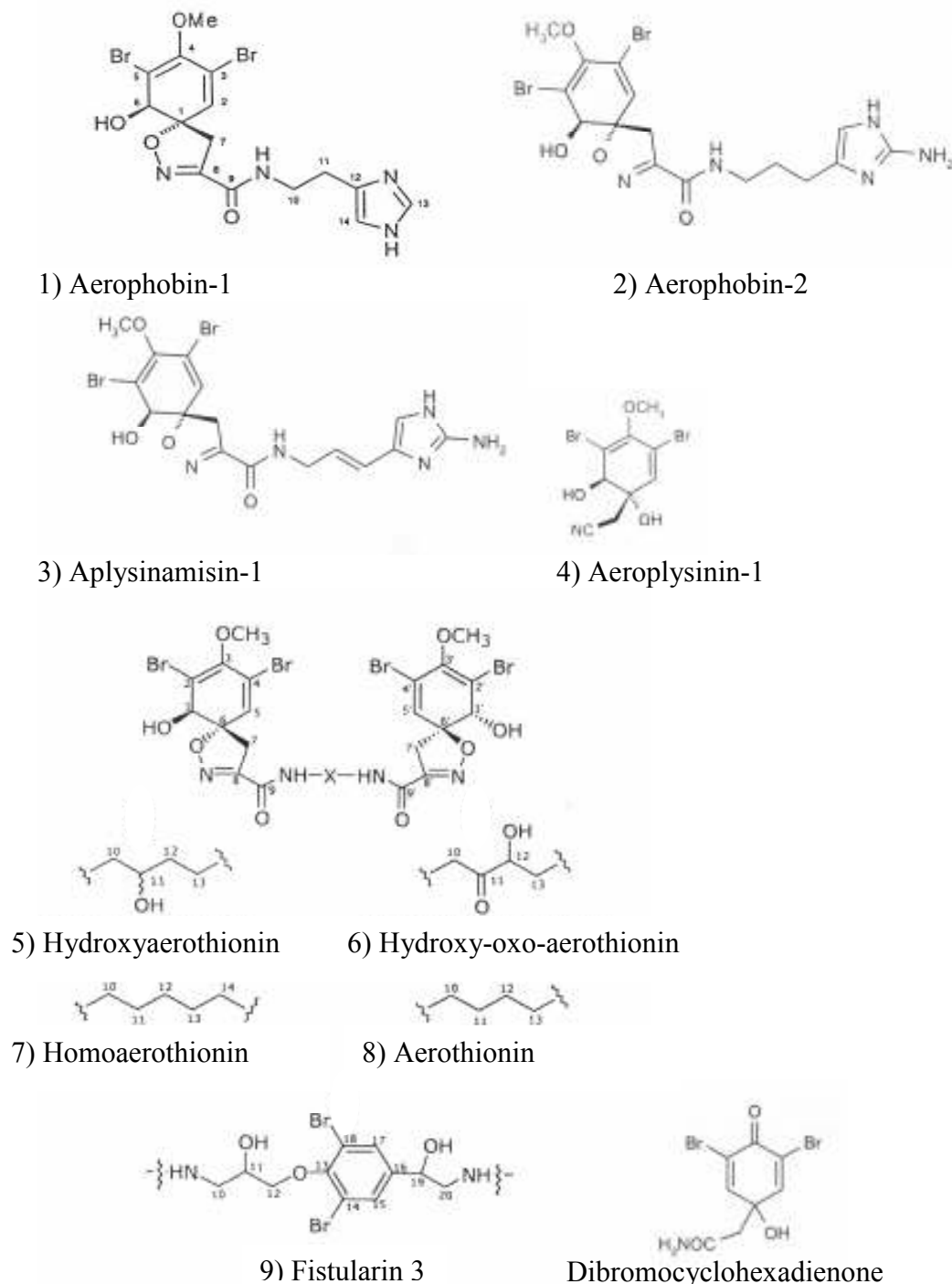
Figure 6: Brominated compounds identified in *A. fulva* from J Reef. Compound #s 1-3 and #s 5-9 are high molecular weight precursors of the "activated" compounds # 4 and dibromocyclohexadienone. The concentration of these compounds (1-9) (as purified mixtures) was also quantified using HPLC in the crude extracts of experimental samples. Structures were adapted from (Teeyapant & Proksch 1993, Puyana et al. 2003, Thoms 2006). Dibromocyclohexadienone was initially identified in trace amounts by LC-MS, but was not detected in experimental samples.

CHAPTER 2

VARIATION IN CHEMICAL DEFENSES, NUTRITIONAL QUALITY, AND

STRUCTURAL COMPONENTS WITHIN INDIVIDUALS OF THREE SPONGE

SPECIES

Introduction

Predation is a prominent factor influencing the standing crop, productivity, and community structure of marine (Hixon 1997) and terrestrial (Coley 1983) ecosystems. Intense predation selects for traits that allow prey to avoid, deter, or possibly tolerate predators (see Coley 1983, Hay 1991). Such protective and defensive traits include burrowing, growth in cryptic locations, physical defense, variability in shape or form, and chemical defense (Bakus 1968, Pennings & Paul 1992).

Secondary metabolites are thought to be the most common method for defense against consumers in sessile marine and terrestrial organisms (Rhoades 1979, Paul 1992, Hay 1996). These compounds may be especially common in areas of high predation intensity in marine ecosystems, resulting in organisms lacking such defenses being relegated to cryptic locations to avoid consumers (Bakus 1964, Bakus 1981, Dunlap & Pawlik 1996, Pawlik 1997, 1998). While secondary compounds may be an effective defensive mechanism, they may exert a biological cost because their production requires use of limited resources that might otherwise be expended on growth or reproduction (McKey 1974, 1979, Rhoades 1979). This cost can be minimized by concentrating chemical defenses in tissues or regions of the body that have higher fitness value to the organism or that have greater risk of being consumed because of their proximity to

predators, high nutritional quality, or low levels of structural defense (McKey 1974b,

Tugwell & Branch 1989, Meyer & Paul 1992, Schupp et al. 1999). Such differential

allocation of chemical defenses within individuals was initially described in terrestrial

plants (McKey 1974, Coley 1983, Liu et al. 1998), but has since been substantiated in

corals (Harvell & Fenical 1989) and algae (Paul & Van Alstyne 1988, Hay et al. 1988a,

Tugwell & Branch 1989, Meyer & Paul 1995).

If food preferences by predators are dictated by nutrient intake, a positive

correlation between nutrient content (particularly protein) and concentrations of chemical

defenses may enhance survival and protect nutritious regions of prey (McKey 1974,

Feeny 1976, Bowers & Stamp 1992, Toft 1999, Mayntz et al. 2005). Furthermore, such a

correlation might be selected for in order to protect regions involved in production or

storage of nutrients (Rhoades 1979). Allocation patterns such as these may be especially

common in marine systems, where sessile invertebrates survive in nitrogen limited

environments surrounded by potential predators that may be selecting nutrient-rich foods

(Grigg et al. 1984, Duffy & Paul 1992, Pennings et al. 1994).

Secondary metabolites are not the only weapon available for deterring predation.

While structural components primarily provide support, they may also serve a secondary

role as defensive mechanisms (Coley 1983, Pennings & Paul 1992). Minerals such as

silica or calcium carbonate, fiber content and overall toughness (Coley 1983, Pennings &

Paul 1992) may deter feeding by providing a stiff, protective outer layer (Hill & Hill

2002), lowering the palatability or nutritional value (Duffy & Paul 1992), irritating the

mouth, gut, and stomach of predators (Meylan 1988, Randall & Hartman 1968), or

having a buffering effect on the gut pH of herbivores (Hay et al. 1994). The lower

susceptibility of heavily calcified algae to herbivores (Paul and Hay 1986, Hay et al.

1994), as well as reduced feeding on artificial diets after the addition of calcite or

aragonite (Pennings & Paul 1992, Schupp & Paul 1994), supports the feeding deterrent

role of these structural components. If structural defenses reduce the overall palatability

of prey, it might be expected that individuals or regions of an individual that are

structurally well-defended will exhibit lower levels of chemical defenses, suggesting an

energetic tradeoff between the two (Harvell & Fenical 1989, Meyer & Paul 1995).

However, because a single defensive trait is unlikely to be effective against a wide range

of predators, both chemical and structural defenses are more likely to be present

simultaneously and may even act synergistically to deter predators (Hay et al. 1994,

Meyer & Paul 1995).

Sponges are sessile, soft-bodied organisms found in benthic communities ranging

from polar to tropical seas (Targett & Schmahl 1984, Alcolado 1991, Zea 1993, Chanas

& Pawlik 1995, McClintock & Baker 1997). They are a major part of the benthic

community on both hard and soft substrates (Reiswig 1973, Sara & Vacelet 1973,

Wenner et al. 1983, Targett & Schmahl 1984). Marine sponges generally have high

nutrient content, with a mean protein value exceeding 20% of their dry mass compared to

0.8% to 13.7% in seaweeds (Montgomery 1980, Chanas & Pawlik 1995). In addition, the

physical defenses of sponges may be ineffective as feeding deterrents when tested alone

(Chanas & Pawlik 1995, Ruzicka 2005). Thus, as a group, sponges should be highly

palatable prey, quickly consumed by predators seeking out protein-rich organisms or

tissues, as has been reported in terrestrial plant-herbivore systems (reviewed by Mattson

1980, Fagan et al. 2002, Mayntz et al. 2005). The high abundance of sponges on reefs,

however, suggests that these organisms are adequately defended from predators (Paul 1992).

Deterrence via production of secondary metabolites is thought to be the most common antipredator mechanism used by sponges (Paul 1992, Pawlik 1993, Pennings et al. 1994, Chanas & Pawlik 1996, Becerro et al. 1998, Wilson et al. 1999). Despite the well documented presence of these chemical defenses across sponge species, our understanding of how these secondary compounds are distributed within individuals is scant. Investigations using the sponges *Cacospongia* sp. (Becerro et al. 1998), *Ectyoplasia ferox* (Kubanek et al. 2002), *Latrunculia apicalis* (Furrow et al. 2003) and *Rhopaloeides odorabile* (Thompson et al. 1987) indicate localization of chemical defenses in exposed regions like the outer 2 mm of the sponge tissue layer. In contrast, extracts from the outer 2 mm of *Chondrilla nucula* (Swearingen & Pawlik 1998) and the ectosome of six species of Red Sea sponges (Burns et al. 2003) are no more deterrent than extracts from inner regions. These contrasting results make it difficult to generalize about the relationship between the distribution of chemical defenses within individuals and the susceptibility of sponge tissues to predatory attack. Nevertheless, such allocation may be ecologically and evolutionarily important, as suggested by reports of fish biting or mouthing outer regions of sponges without removing tissue (Schulte & Bakus 1992, Dunlap & Pawlik 1998) or rapidly consuming a sponge once the outer region has been removed (Wulff 1997).

To better understand if chemical defense allocation patterns within sponges are consistent with predictions originally proposed in terrestrial systems, I investigated the relationship between the concentration of chemical defenses, nutritional quality, and

structural components within three species of sponges from a temperate reef. The specific

goals of this study were to determine if chemical defenses in sponges are 1) higher in the

outermost 2 mm of the sponge body, thus protecting the tissues most exposed to

predation by fish and large mobile invertebrate predators, 2) positively correlated with

tissue nutritional quality, thus guarding the most nutritious and expensive tissues from

predatory damage, and 3) negatively correlated with structural components, thus

providing support for the role of sponge structural materials in deterring predators.

<div align="center">Methods</div>

<u>Collection, identification, and extraction</u>

The sponges *Ircinia felix*, *Ircinia campana*, and *Aplysina fulva* (see Fig. 1,

Chapter 1) were collected at J Reef (31˚ 36.056 N, 80˚ 47.431 W) with two separate

collection days per species (Table 1). In addition to their high abundance and previously

described secondary metabolites (Chapter 1), these sponges were chosen for this study

because their large size ensured that there would be enough tissue from the outer 2 mm to

carry out all my laboratory analyses. During collection, a large portion (>1kg wet weight)

of an individual sponge was removed from the substrate with a dive knife and placed in a

plastic bag. In order to prevent changes in the chemical makeup of the sponge during

travel to the lab, while on the boat I removed the outer 2 mm (here after referred to as

outer region) from a portion of each sponge with a razor blade. A depth of 2 mm was

chosen as the point of division between the outer and inner regions because: 1) previous

studies using sponges have found the outer 2 mm to be the region of highest secondary

metabolite concentration (Kubanek et al. 2002, Furrow et al. 2003), and 2) the outer 2

mm could be teased out with a high degree of reproducibility because of the color

differentiation resulting from the presence of cyanobacterial symbionts in the outer layer (Fig 7). The outer 2 mm and the inner region directly beneath were packed separately in aluminum foil and immediately flash frozen in liquid nitrogen. Sponge tissue not dissected and flash frozen on the boat was stored on ice and later placed in a -70 ˚C freezer for future analysis of nutritional quality and structural components, and for feeding assays. Once removed from liquid nitrogen, samples for chemical analysis were also stored at -70 ˚C until they were processed.

Sponge tissue from all three species was prepared for extraction by lyophilizing it overnight, weighing the dried tissue, and chopping it into small pieces using a razor blade. Tissue was homogenized using a mortar and pestle and extracted 3 times for 24 hours at 4˚ C with 10 ml of 1:1 Dichloromethane:Methanol (DCM:MeOH). During freeze drying and extraction, sponges and their extracts remained completely covered in aluminum foil to prevent degradation of compounds from light exposure. Samples were inverted every 12 hours during the extraction to ensure that solvents were thoroughly mixed. The efficiency of this extraction technique for all three species was confirmed by thin layer chromatography (TLC) with H_2SO_4 charring. The first two extractions contained similar numbers and colors of dots along the plate, but the absence of these dots in the third extract suggested that compounds were adequately removed from the sponge tissue after the second extraction. Crude extracts obtained from all three extractions were combined and filtered into pre-weighed vials using coarse porosity (20-25 μm particle retention) filter paper. The solvent was removed by vacuum evaporation and the resulting crude extract was weighed to the nearest 0.0001g using an electronic balance (APX-60, Denver Instruments, Denver, CO).

Quantification of defensive metabolites

To determine whether the concentration of chemical defenses was higher in the outer or inner regions of these sponges, I quantified the secondary metabolites from these two regions in 20 individuals of *A. fulva* and 15 individuals each of *I. felix* and *I. campana*. Although these sample sizes do not represent all the samples available, this replication was adequate to account for the variation that was observed in the population. The samples used were chosen by lottery from the total number of samples available for each species: 20 for *I. felix*, 22 for *I. campana*, and 24 for *A. fulva*.

Quantification of secondary compounds began in the two *Ircinia* species by lyophilizing the crude extract and then acetylating by adding 3 ml of 1:2 acetic anyhydride:pyridine to the dried extract and stirring for 9 hours at room temperature in the dark. After 9 hours, this acetylated mixture was rapidly added to 30 ml of cold (9 ˚C) filtered and deionized water and allowed to stir for 5 min. This mixture was extracted twice with 20 ml of EtOAc and both organic layers were collected and evaporated using rotary evaporation at 50-55˚ C. The yellow-brown residue was re-suspended in a known volume of MeOH, filtered through a PTFE 45 μm syringe filter, and 9 μl volumes injected into a Shimadzu HPLC system with a Phenomenex Gemini C-18 analytical column (4.6x 250 mm) (see Methods, Chapter 1) and a solvent of 93:7 MeOH:H_2O with UV detection at 270 nm. Quantification was carried out by comparing peak areas of experimental samples to those of 9 μl volumes of a group of purified FTA standards ranging in concentrations from 12.5 to 2000 μg/ml (see Methods, Chapter 1).

In *A. fulva*, 100% MeOH was added to the dried crude extract to bring the concentration to 20 mg/ml and this mixture was sonicated to ensure complete dissolution.

In order to remove contaminants, I filtered 1.5 ml of this mixture from each sample through a PTFE 45 μm syringe filter into an HPLC vial. The concentration of these compounds was quantified in 7 μl injections using a Shimadzu system with the Phenomenex Gemini C-18 analytical column (4.6 x 250 mm) (see Methods, Chapter 1). UV detection was at 254 and 280 nm using the same buffered solvent gradient as in the LC-MS work (Chapter 1). The compounds were quantified based on their retention times and comparison to peak areas of 7 μl injections of purified groups of compounds in known concentrations from 12.5 to 1000 μg/ml (see Methods, Chapter 1). I used the Wilcoxon's signed ranks test to identify differences in the concentration of chemical defenses between the inner and outer regions of these three sponges because not all data met parametric assumptions or could be transformed to meet these.

Nutritional analysis of sponge tissue

To determine if the concentration of defensive compounds was positively correlated to nutritional quality, I quantified the soluble protein and carbohydrate content in the inner and outer regions of these three species of sponges. The sponge samples that were initially placed on ice in the field were thawed, the outer 2 mm was removed, and the two regions were frozen and lyophilized. The dried sponge was cut into small pieces, homogenized using a mortar and pestle, and stored at -20 ˚C. Protein content was quantified using the Bradford assay (Bradford, 1976) as described previously by Becerro et al. (1998). Tissues from the inner and outer regions (10-15 mg dry weight) of a replicate were digested separately in 5 ml of 1 N NaOH for 12 hours at room temperature. Protein concentration was determined by comparison to a calibration curve run with a standard of bovine serum albumin (BSA). In all cases protein concentrations

were expressed as percent dry weight of sponge tissue. Duplicate absorbance values were always within 0.05 absorbance units of each other, so the mean of these two values was used for statistical analysis.

Carbohydrate content was quantified using the TCA-soluble carbohydrate assay (DuBois et al. 1956) as previously adapted for sponges (McClintock 1987, Chanas & Pawlik 1995). I dissolved 10-15 mg of dried tissue in 1 ml filtered, deionized water for 1.5 hours. A 250 µl subsample of this mixture was then transferred to a 16 x 100 mm test tube and total volume brought up to 1.0 ml using deionized water. Standards were prepared by dissolving D-glucose in filtered, deionized water to concentrations ranging from 20 to 120 µg ml^{-1}. Each sample and standard, assayed in duplicate, was mixed in 1 ml deionized water followed by 2 ml of 10 % trichloroacetic acid. Tubes were heated in a 100 ˚C water bath for 20 minutes and allowed to cool to room temperature by immersion in tap water. I transferred 2 ml of each sample to a 16 x 150 mm test tube and added 1 ml of 5% phenol followed by the rapid addition of 5 ml of H_2SO_4. This solution was allowed to stand at room temperature for 10 minutes, after which it was mixed and placed in a 25 ˚C water bath for 15 minutes. Absorbance of each sample was read at 490 nm and concentrations were calculated from a standard curve of known concentrations. Duplicate values were always within 0.05 absorbance units of each other, so the mean of these two values was used for statistical analysis.

I used the Wilcoxon's signed ranks test to identify significant differences in the nutrient content between the inner and outer regions of the sponge. In order to determine if there was a positive correlation between the concentration of chemical defenses and nutritional quality, I used a partial correlation analysis in which I measured the

correlation between chemical defenses and protein content while holding total structural components constant.

Structural components in sponge tissue

To determine if there is a negative correlation between the concentration of chemical defenses and structural components, I quantified the total structural content, including both fiber and ash content in the inner and outer regions of these three species of sponges. Methods for this analysis were adapted from Becerro et al. (1998). Samples of freeze dried sponge were weighed and dissolved in a 75:25 mixture of 3% hydrogen peroxide and 30% ammonium hydroxide for one week to remove all non-structural materials. After one week, the mixture was filtered through coarse porosity (20-25 μm particle retention) filter paper and structural material not dissolved was dried and weighed. In order to quantify fiber and ash content, samples were combusted in a furnace at 450˚C for 48 hours. The ash remaining after combustion was weighed and expressed as a percentage per dry mass. Fiber content was calculated by subtracting ash mass from total structural mass and also expressed as a percentage of dry mass.

As in the above chemical and nutritional quality analyses, I used the Wilcoxon's signed ranks test to identify differences in the concentration of structural components between the inner and outer regions of the sponge. In order to identify potential correlations between the concentration of chemical defenses and structural components, I used a partial correlations analysis in which I kept protein content constant. Likewise, to identify possible correlations between structural components and nutritional quality, I used a partial correlation analysis holding the concentration of chemical defenses constant.

I tested for temporal differences in the concentration of defensive chemicals, nutritional quality, and structural components using a one way ANOVA because sponges were collected at two different dates (Tables 2-4). In most instances, there were either no significant differences between collection dates or significant differences were observed, but patterns were similar (i.e. one region higher at both dates). If similar patterns were not observed (i.e. FTA concentration within *I. campana* and carbohydrate content in *A. fulva*), I conducted two (one for each date) separate paired T- tests comparing data from the inner and outer region. In all cases, these paired tests yielded non-significant results, suggesting that this discrepancy would not impact the results significantly if data from both dates were pooled for future analyses. For this reason, data from the two collections were pooled in all cases.

<div align="center">Results</div>

Chemical variation in tissues

The concentration of FTAs was highest in the inner region of the two *Ircinia* species, but this difference was only significant in *I. felix* when concentration was expressed as percent of sponge dry weight (Wilcoxon Signed Ranks Test: $Z = -2.045$, $p < 0.05$ and $Z = -0.284$, $p > 0.05$ for *I. felix* and *I. campana*, respectively) and as percent of crude extract (Wilcoxon Signed Ranks Test: $Z = -2.215$, $p < 0.05$ and $Z = -0.282$, $p > 0.05$ for *I. felix* and *I. campana*, respectively) (Figure 8 and Table 5). In *A. fulva*, the concentration of the 8 precursor compounds as well as Aeroplysinin-1 was significantly higher in the outer region of the sponge (Wilcoxon Signed Ranks Test: $Z = -3.920$, $p < 0.001$ for sum of precursors; $Z = -2.987$, $p < 0.01$ for Aeroplysinin-1) (Figure 9). The compounds found in *A. fulva* made up a much larger percentage of the sponge dry weight

than either of the *Ircinia* species and up to 34% of the crude extract (Figure 9 and Table 5).

Nutritional quality of tissues

The concentrations of protein and carbohydrate were significantly different between the inner and outer regions of both *Ircinia* species, but not in *A. fulva*. In both *Ircinia* species, the inner region had significantly higher soluble protein content than the outer region (Wilcoxon Signed Ranks Test: Z= -3.587, p<0.001, and Z=-2.203, p<0.05 for *I. campana* and *I. felix*, respectively) (Figure 10A). In contrast, the concentration of soluble carbohydrate was significantly higher in the outer tissue region for *I. felix* and the inner tissue region for *I. campana* (Wilcoxon Signed Ranks Test: Z= -2.576, p<0.05 and Z= -3.490, p<0.001 for *I. felix* and *I. campana*, respectively) (Figure 10B).

Foods of higher nutritional value that are most preferred by predators are generally thought to be those with higher protein:carbohydrate ratios. I calculated the protein:carbohydrate ratio and the only sponge with a significant difference in the P:C between the inner and outer regions was *I. felix* (Figure 11). This species showed a significantly higher P:C in the inner region (Wilcoxon Signed Ranks Test: Z= -3.360, p<0.01).

There was a positive correlation between the concentration of FTAs and nutritional quality, measured as protein content (Millikan 1982, Duffy and Paul, 1992), in the outer region of *I. felix*, but not in the corresponding inner region (Figure 12 A). No significant correlations between nutritional quality and the concentrations of antipredator compounds were found in either tissue region in *I. campana* and *A. fulva* (Figure 12 A and B).

Distribution of structural components

The total structural content, including both fiber and ash, was significantly higher in the interior of *I. felix* and *A. fulva* (Wilcoxon Signed Ranks Test: Z= -3.360, p<0.01 and Z= -3.102, p<0.01 for *I. felix* and *A. fulva*, respectively) whereas no significant differences between regions were evident in *I. campana* (Wilcoxon Signed Ranks Test: Z= -1.542, p>0.05) (Figure 13). The ash content measured in these sponges consisted mainly of sand grains and inorganic salts. Significantly higher ash content was measured in the inner region of *A. fulva* and the outer region of *I. campana* (Wilcoxon Signed Ranks Test: Z= -2.768, p<0.01 and Z= -2.873, p<0.01 for *A. fulva* and *I. campana*, respectively), but no differences were detected within *I. felix* (Wilcoxon Signed Ranks Test: Z= -1.269, p>0.20) (Figure 13). The tough spongin fibers found in these sponges were significantly higher in concentration towards the interior of all three species (Wilcoxon Signed Ranks Test: Z= -4.074, p<0.001; Z= -2.159, p<0.05; and Z= -3.509, p<0.001 for *I. campana*, *A. fulva*, and *I. felix*, respectively) (Figure 13).

There were no significant positive or negative correlations between the concentrations of chemical and structural defenses within these three species of sponges (Figure 14 A and B). Negative correlations between the concentration of structural components and protein in the inner region of *I. campana* and the outer region of *I. felix*, however, were significant (Figure 15 A and B).

Discussion

The production of secondary metabolites is a key defensive mechanism allowing for the survival of benthic invertebrates in marine systems (Bakus et al. 1986, Paul 1992, Wilson et al. 1999, Kubanek et al. 2000). Resources used in the production of these metabolites are limited, however, so there may be strong selection for the allocation of these defenses to regions most likely to come into contact with predators, regions of high nutritional quality, or regions with low levels of structural defenses (McKey 1974, 1979, Rhoades 1979). When separating inner and outer sponge tissue layers, two of the three sponge species examined in this investigation demonstrated significant differences in the distribution of chemical defenses (Figures 8 and 9). The distribution of chemicals in these two species, however, was not always consistent with patterns predicted by allocation models originally proposed in terrestrial systems. The concentration of chemicals in the outer 2 mm of *A. fulva* is in agreement with these models, but the elevated levels of FTAs in the inner region of *I. felix* are in direct opposition to these models. Although there was substantial variation in the nutritional quality and structural components within these species (Figures 10, 11, and 13), there were no consistent correlations between the concentration of chemical defenses and nutritional quality or structural components (Figures 12, 14, and 15). These results suggest that, in these sponges, chemical defenses are not consistently concentrated in regions that might be initially considered to be at an increased risk of predatory attack by fish and large mobile invertebrates.

Surprisingly, FTA concentrations were significantly higher in the inner region of *I. felix* (Figure 8). It is unlikely that concentrating FTAs in this region is an effective deterrent against feeding by fish and large, mobile invertebrate predators because these

consumers would have to first bite through the outer region in order to encounter these higher levels of chemical defenses. Instead, I propose that allocation of these defenses to inner tissue regions may be an adaptation to deter small cryptic invertebrates that I commonly observed residing in large, hollowed out regions of this sponge. Some amphipods, brittle stars, mollusks, nematodes, and, in particular, annelids may occur in large numbers (>15,000 individuals per ~900 ml sample of sponge) and be deeply embedded within *I. felix* (A. Greene, pers. comm.). These invertebrates may act as parasites by feeding on sponge tissue while simultaneously having a refuge from predators in the water column. Higher levels of chemical defenses within these inner sponge tissues may have been selected for to decrease feeding by these invertebrates in this susceptible region. The need for higher levels of chemical defense within inner tissue regions of *I. felix* is further supported by the demonstration that the concentrations required to deter fish and large invertebrate predators (Pawlik et al. 1995, Epifanio et al. 1999, Waddell & Pawlik 2000, Pawlik et al. 2002, but see Randall and Hartman 1968) may be ineffective against these smaller, resident invertebrates (Hay et al. 1988b).

The distribution of brominated tyrosine derivatives within *A. fulva* is consistent with studies reporting higher levels of chemical defenses towards the outer regions of the prey body that have the greatest probability of being attacked by predators (Tugwell & Branch 1989; Becerro et al. 1998, Kubanek et al. 2002, Furrow et al. 2003). My results with *A. fulva* are consistent with those of Thompson et al. (1983), who found the brominated compounds aerothionin and homoaerothionin to be localized within spherulous cells that are concentrated directly under the exopinacoderm of *Aplysina fistularis*. The antipredatory role of metabolites and crude extract from sponges in the

genus *Aplysina* has been verified (Ebel et al. 1997, Pawlik et al. 1995, Ruzicka 2005, Thoms et al. 2006), so I am confident that significantly higher concentrations of these defensive compounds in the outer region of *A. fulva* confers protection from predators.

The clear difference in allocation patterns between *I. felix*, *I. campana*, and *A. fulva* may be a result of differences in predation pressure on each sponge species or variation in the type or concentration of secondary metabolites. Although I commonly observed invertebrates within *I. felix*, and occasionally within *I. campana*, I rarely found any invertebrates living within *A. fulva*. The increased predation pressure exerted by these invertebrates may select for higher concentrations of FTAs in the inner region of *I. felix*, while *A. fulva* may be most vulnerable to predators in the water column and thus concentrates chemicals towards its periphery. Although the concentration of FTAs was not significantly higher in the interior of *I. campana*, the overall concentration of these compounds in *I. campana* was almost double that found in *I. felix*. This higher concentration of FTAs in both regions of *I. campana* may be sufficient to inhibit the colonization and feeding of invertebrates that might attempt to take up residence within the sponge tissues.

It should be noted that secondary metabolites may serve multiple ecological functions in marine organisms and that these compounds may also reflect requirements of the sponge to defend against competitors, fouling organisms, or microbial infection (Uriz et al. 1992, Kubanek et al. 2002). In fact, the presence of higher concentrations of FTAs towards the interior of *I. felix* has been documented previously and has been reported to play an antibiotic role rather than mediating external ecological interactions (Zea et al. 1999). Likewise, in species of *Aplysina*, the presence of brominated compounds has been

associated with inhibiting the growth of bacteria (see Thompson et al. 1983) as well as algae (Weiss et al. 1996).

Allocating chemical defenses to tissues having high nutritional value to predators may enhance prey fitness, especially if nutritious tissues are selectively targeted by predators (Rhoades 1979, Bowers and Stamp 1992) or if predators are willing to tolerate some level of defense to feed on highly nutritious tissue (Duffy & Paul 1992). Greater concentrations of FTAs in the high-protein inner region of *I. felix* (Figures 8 and 10A) are in agreement with this tenet of chemical ecology, but the general lack of significant correlations between these two factors, in all but one instance, (Figure 12) suggest that chemical defenses are not allocated to regions of increased nutritional quality.

Structural components may also act as effective predator deterrents in benthic marine invertebrates (Harvell et al. 1988, Pennings & Paul 1992, but see Chanas & Pawlik 1995, 1996), resulting in chemical defenses being allocated to areas of the body with low levels of structural defense (Hay et al. 1988a, Paul and Van Alstyne 1988, Harvell & Fenical 1989, Becerro et al. 1998). Such a tradeoff in the allocation of defenses was not evident in the three sponge species I investigated (Figure 14). The lack of such a relationship might be expected if structural components act primarily as skeletal elements and have little to do with predator deterrence (Chanas & Pawlik 1995, 1996). In contrast to a tradeoff between chemical and structural defenses, the co-occurrence of chemical defenses and total structural content in inner tissue regions of *I. felix* (Figures 8 and 13) may act synergistically and allow defense against a wider array of predators (Hay et al. 1994, Burns and Ilan 2003, Hill et al. 2005).

This study has documented variation in chemical defenses, nutritional quality, and structural components within individuals of three common reef sponges. In terms of antipredator chemical defenses, these species appear to show three distinct strategies: *I. felix* tends to allocate greater concentrations of chemicals to interior tissues, *A. fulva* to exterior tissues, and *I. campana* protects internal and external tissues equally. The true effectiveness of these different defensive strategies against predators is unclear, however, because the synergistic or additive effects of chemical and structural defenses against predators were not considered. Clearly, investigations into the combined effects of physical and chemical defenses on various predators are warranted (e.g., Hill et al. 2005). Furthermore, as demonstrated in my study, examining variation in the distribution of these defenses within individuals, not just within populations, may provide even greater insight into the antipredator strategies employed by sessile organisms (see Hay et al. 1996, Becerro et al. 1998). Finally, my results suggest that to truly understand the evolution of antipredator mechanisms in sessile marine animals we may want to reconsider what is meant by predation pressure. Specifically, large, biting fishes and mobile invertebrate predators may only be part of the story. Thus, we may want to expand our thinking of predation pressure to include small, cryptic, invertebrates that take up residence within sponge tissue and, by their combined effect, have the potential to cause substantial tissue loss.

Table 1: Collection dates and numbers of samples collected for each sponge species at J Reef.

Species	Date	# of Samples
Ircinia felix	25-Jul-2005	11
	2-Sep-2005	9
Ircinia campana	20-Oct-2005	10
	19-Jan-2006	12
Aplysina fulva	3-Mar-2006	14
	21-Apr-2006	10

Table 2: Mean concentrations (% of dry mass +/- SE) of chemical defenses, nutritional quality, and structural components in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of *A. fulva* collected on two different dates. The number of samples analyzed from the above collection date is indicated in parentheses after the SE. Samples were analyzed using a one way ANOVA and significant differences between the two collections are indicated in bold.

Sponge	Region	Test	Collection 1 (3-Mar-2006)	Collection 2 (21-Apr-2006)	F Ratio	P value
Aplysina fulva	Inner	Sum Precursors	5.12 +/- 0.29 (13)	4.27 +/- 0.39 (7)	3.0425	0.0982
		Aeroplysinin-1	**0.11 +/- 0.01 (13)**	**0.29 +/- 0.01 (7)**	**185.4468**	**<0.0001**
		Protein	17.69 +/- 1.15 (13)	16.38 +/- 1.31 (10)	0.5594	0.4628
		Carbohydrate	**3.98 +/- 0.31 (13)**	**2.97 +/- 0.35 (10)**	**4.5245**	**0.0454**
		Structural	43.37 +/- 2.94 (13)	35.68 +/- 3.35 (10)	2.968	0.0996
		Ash	9.93 +/- 2.44 (13)	9.76 +/- 2.78 (10)	0.0022	0.9628
		Fiber	**33.39 +/- 1.69 (13)**	**25.98 +/- 1.93 (10)**	**8.3201**	**0.0089**
Aplysina fulva	Outer	Sum Precursors	8.97 +/- 0.54 (13)	7.81 +/- 0.74 (7)	1.5982	0.2223
		Aeroplysinin-1	**0.19 +/- 0.02 (13)**	**0.29 +/- 0.02 (7)**	**13.6373**	**0.0017**
		Protein	18.93 +/- 1.2 (13)	17.80 +/- 1.37 (10)	0.3846	0.5418
		Carbohydrate	3.89 +/- 0.38 (13)	3.46 +/- 0.44 (10)	0.5797	0.4549
		Structural	33.24 +/- 1.75 (13)	30.42 +/- 1.20 (10)	1.1266	0.3006
		Ash	3.45 +/- 1.11 (13)	5.43 +/-1.27 (10)	1.3766	0.2538
		Fiber	29.70 +/- 1.51 (13)	25.00 +/- 1.72 (10)	4.2379	0.0522

Table 3: Mean concentrations (% of dry mass +/- SE) of chemical defenses, nutritional quality, and structural components in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of *I. felix* collected on two different dates. The number of samples analyzed from the above collection date is indicated in parentheses after the SE. Samples were analyzed using a one way ANOVA and significant differences between the two collections are indicated in bold.

Sponge	Region	Test	Collection 1 (25-Jul-2005)	Collection 2 (2-Sep-2005)	F Ratio	P value
Ircinia felix	Inner	FTAs	0.24 +/- 0.10 (7)	0.10 +/- 0.09 (8)	1.1552	0.302
		Protein	11.75 +/- 1.25 (11)	13.05 +/- 1.3847 (9)	0.4855	0.4948
		Carbohydrate	**2.74 +/- 0.24 (11)**	**1.71+/- 0.26 (9)**	**8.4622**	**0.0094**
		Structural	**56.04 +/- 1.71 (11)**	**61.75 +/- 1.89 (9)**	**5.0038**	**0.0382**
		Ash	20.15 +/- 2.88 (11)	26.45 +/- 3.19 (9)	2.1494	0.1599
		Fiber	35.86 +/- 1.71 (11)	35.42 +/- 1.89 (9)	0.0302	0.8639
Ircinia felix	Outer	FTAs	0.16 +/- 0.06 (7)	0.06 +/- 0.05 (8)	1.7362	0.2104
		Protein	10.15 +/- 1.07 (11)	11.27 +/- 1.18 (9)	0.4944	0.491
		Carbohydrate	3.03 +/- 0.25 (11)	3.47 +/- 0.28 (9)	1.3773	0.2559
		Structural	49.90 +/- 2.41 (11)	49.19 +/- 2.67 (9)	0.0389	0.8459
		Ash	20.15 +/- 2.54 (11)	21.16 +/- 2.81 (9)	0.0714	0.7923
		Fiber	29.58 +/- 2.13 (11)	27.88 +/- 2.36 (9)	0.2838	0.6007

Table 4: Mean concentrations (% of dry mass +/- SE) of chemical defenses, nutritional quality, and structural components in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of *I. campana* collected on two different dates. The number of samples analyzed from the above collection date is indicated in parentheses after the SE. Samples were analyzed using a one way ANOVA and significant differences between the two collections are indicated in bold.

Sponge	Region	Test	Collection 1 (20-Oct-2005)	Collection 2 (19-Jan-2006)	F Ratio	P value
Ircinia campana	Inner	FTAs	**0.15 +/- 0.04 (8)**	**0.50 +/- 0.04 (7)**	**37.8099**	**<0.0001**
		Protein	21.95 +/- 1.33 (10)	18.76 +/- 1.21 (12)	3.1664	0.0904
		Carbohydrate	1.62 +/- 0.22 (10)	1.58 +/- 0.20 (12)	0.0164	0.8994
		Structural	57.04 +/- 2.20 (10)	61.40 +/- 2.00 (12)	2.1496	0.1582
		Ash	24.75 +/- 3.62 (10)	28.61 +/- 3.31 (12)	0.6196	0.4404
		Fiber	32.29 +/- 2.19 (10)	32.79 +/- 1.20 (12)	0.0283	0.8682
Ircinia campana	Outer	FTAs	0.24 +/- 0.05 (8)	0.37 +/- 0.06 (7)	2.6236	0.1293
		Protein	**17.49 +/- 1.13 (10)**	**12.33 +/- 1.03 (12)**	**11.4581**	**0.0029**
		Carbohydrate	0.96 +/- 0.11 (10)	1.15 +/- 0.10 (12)	1.7281	0.2035
		Structural	58.72 +/- 2.44 (10)	53.87 +/- 2.23 (12)	2.1444	0.1586
		Ash	38.11 +/- 2.49 (10)	33.43 +/- 2.28 (12)	1.9246	0.1806
		Fiber	20.60 +/- 2.22 (10)	20.44 +/- 2.03	0.0028	0.9582

Table 5: Mean concentrations (+/- SE) of chemical defenses (as percent of crude extract) from the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of three species of sponges from J reef. N= 15 for the *Ircinia spp*. and N=20 for *A. fulva*.

Sponge	Compound	% of Crude Mass Inner	% of Crude Mass Outer	p value
Ircinia felix	FTAs	1.36 (+/-0.52)	0.78 (+/- 0.26)	<0.05
I. campana	FTAs	2.85 (+/-0.55)	2.9 (+/- 0.45)	>0.05
Aplysina fulva	Aeroplysinin-1	0.89 (+/- 0.12)	0.90 (+/-0.07)	>0.05
A. fulva	Precursor Sum	24.1 (+/-1.05)	34.4(+/-1.29)	<0.001
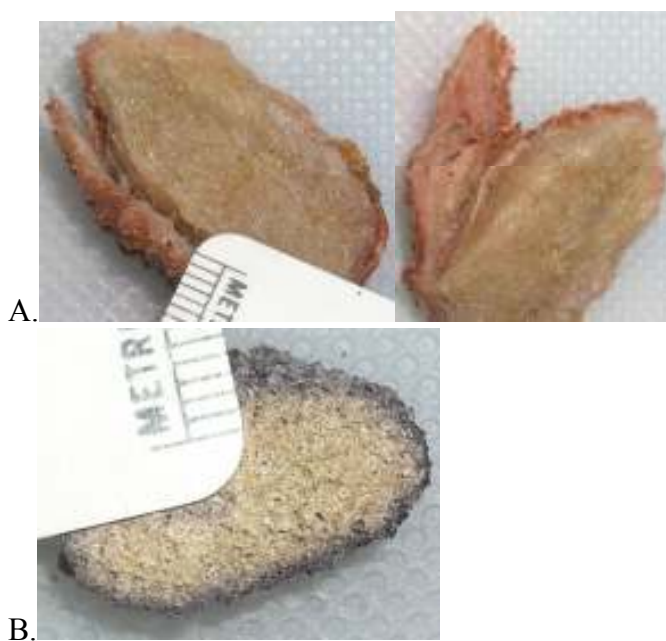
Figure 7: Photograph of *I. felix* (A) and *A. fulva* (B) displaying color differences between the outer 2 mm and the inner region. Both *Ircinia* species have similarly colored outer regions, so only *I. felix* is shown.
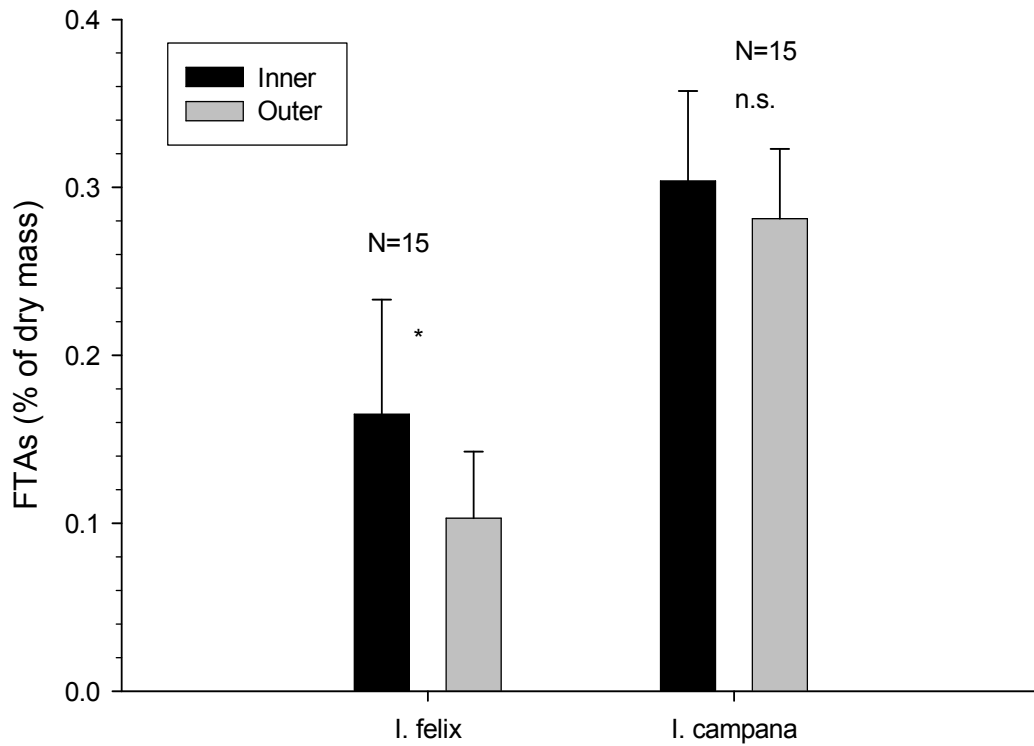
Figure 8: Concentration of FTAs (+/- SE) from outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of two species of *Ircinia* at J Reef. Means for the inner and outer regions of each species were compared with a Wilcoxon Signed Ranks test. *: $p<0.05$, n.s.: not significant.
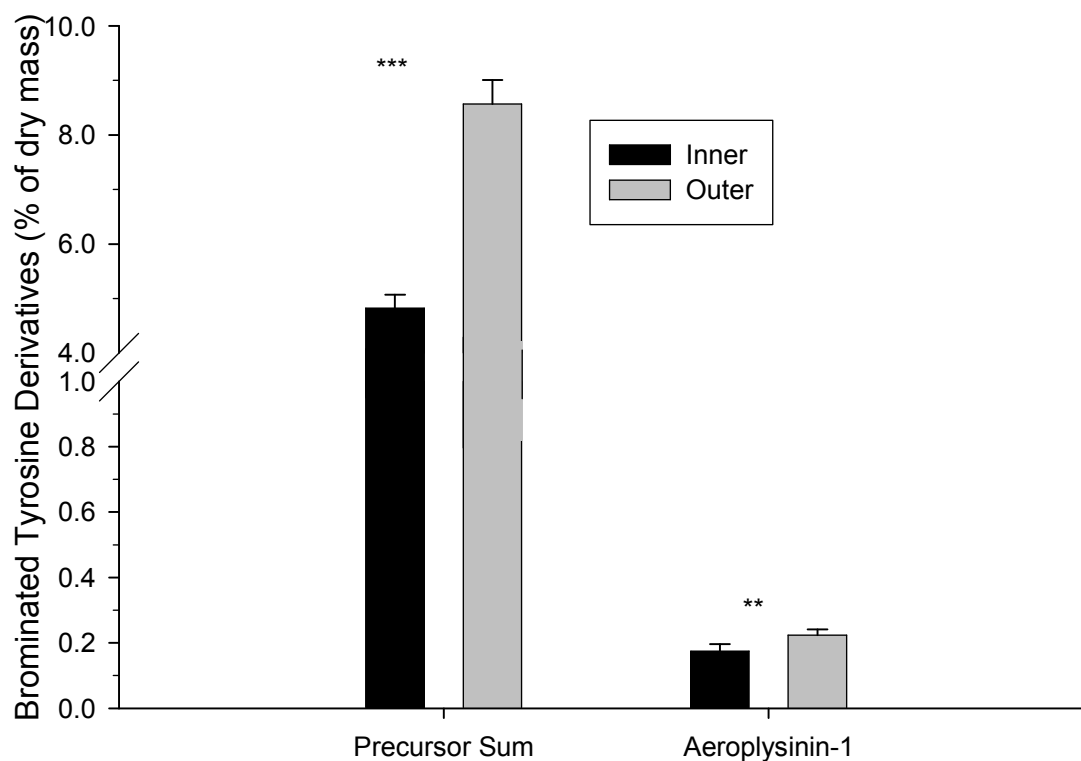
Figure 9: Concentration of secondary metabolites (+/- SE) from outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of *A. fulva* from J Reef. Precursor sum was calculated by pooling the data from the 8 high molecular weight peaks. Means for each group of compounds were compared with a Wilcoxon Signed Ranks test. ***: p<0.001, **:p<0.01. N=20.
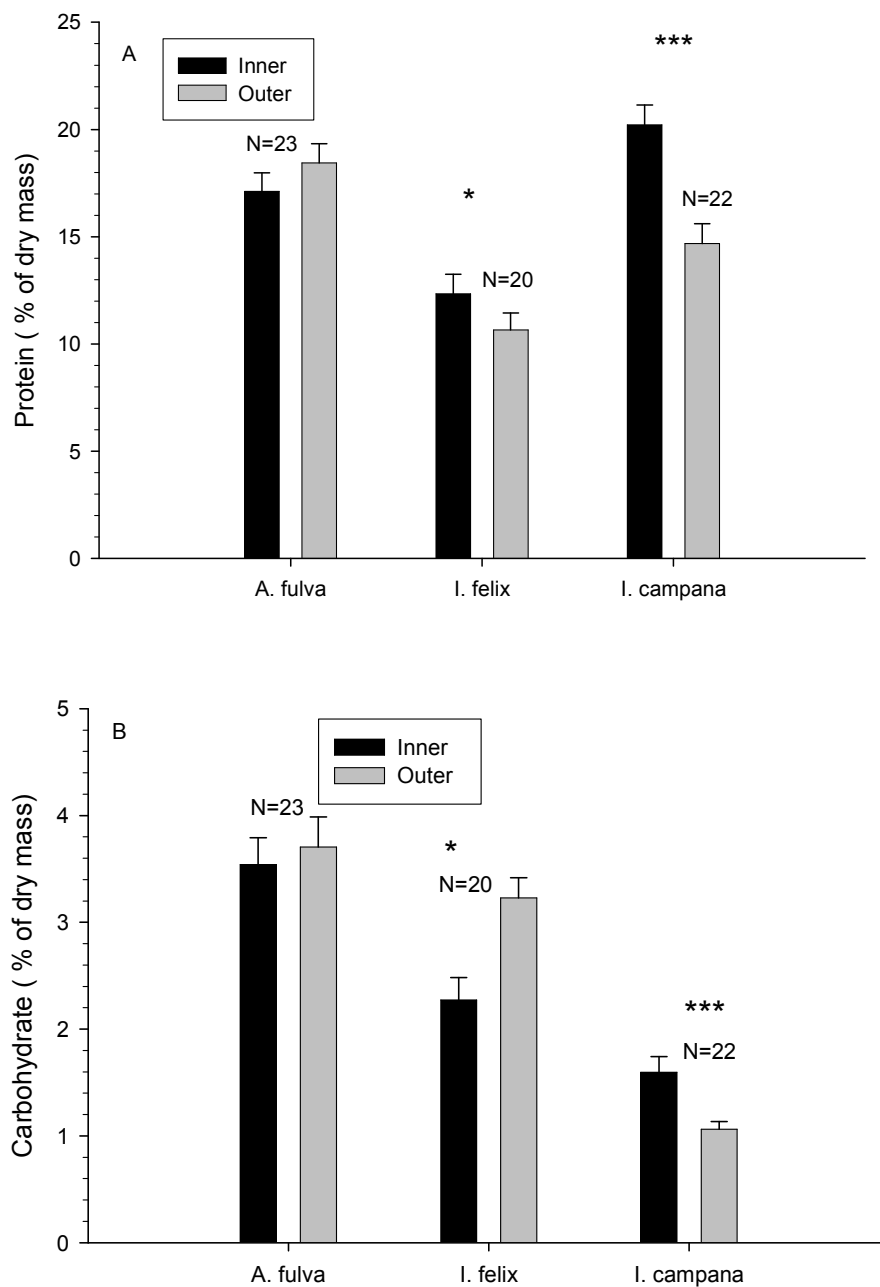
Figure 10: Concentrations of A) protein and B) carbohydrate (+/- SE) from outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of three species of sponges at J Reef. Means for each group of compounds were compared with a Wilcoxon Signed Ranks test. ***: $p<0.001$, *: $p<0.05$
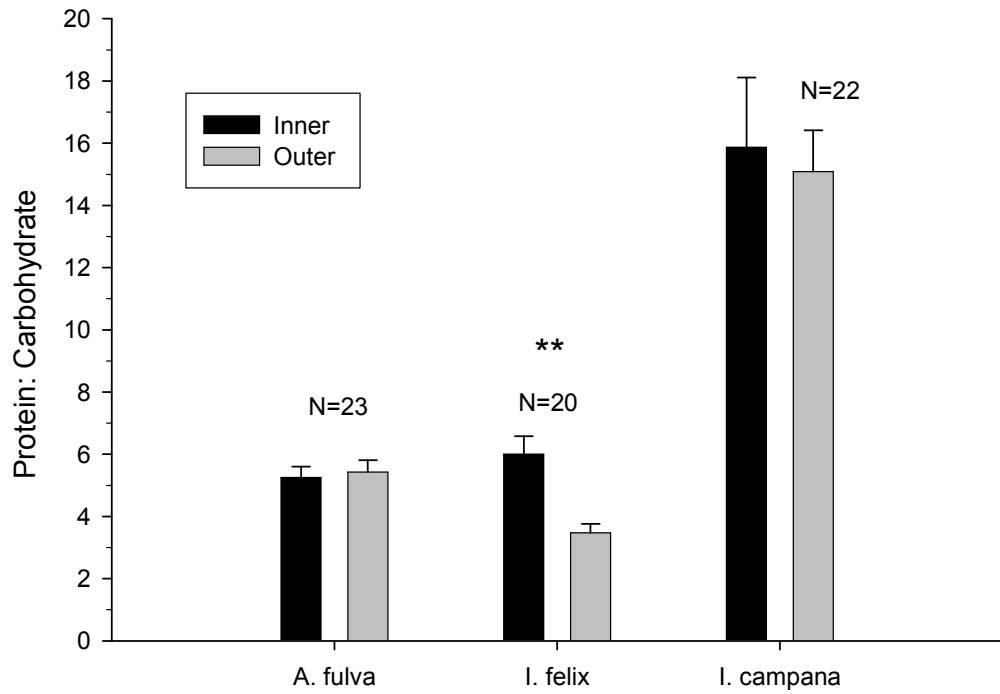
Figure 11: Distribution of the Protein: Carbohydrate ratio (+/- SE) from outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of three species of sponges at J Reef. **: $p < 0.01$.
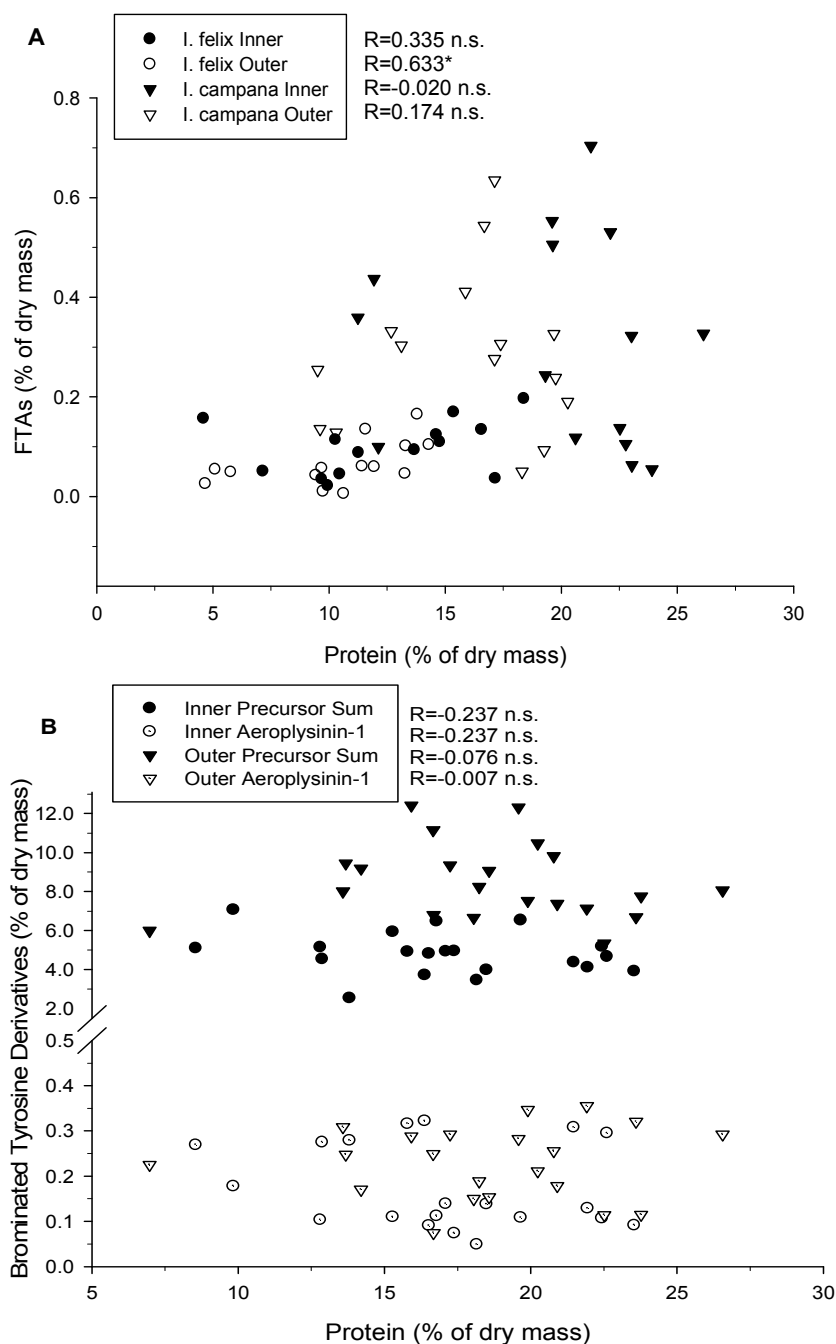
Figure 12: Correlations between the concentration of FTAs (A) or brominated tyrosine derivatives (B) and nutritional quality (protein content) in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of two *Ircinia* species (A) and *A. fulva* (B). Data were analyzed using partial correlation analysis in which the structural components were held constant. The correlation coefficient and significance of each measurement is indicated next to the legend. Sample size is 14, 15, and 20 for *I. felix*, *I. campana*, and *A. fulva*, respectively. *:$p<0.05$, n.s.: not significant.
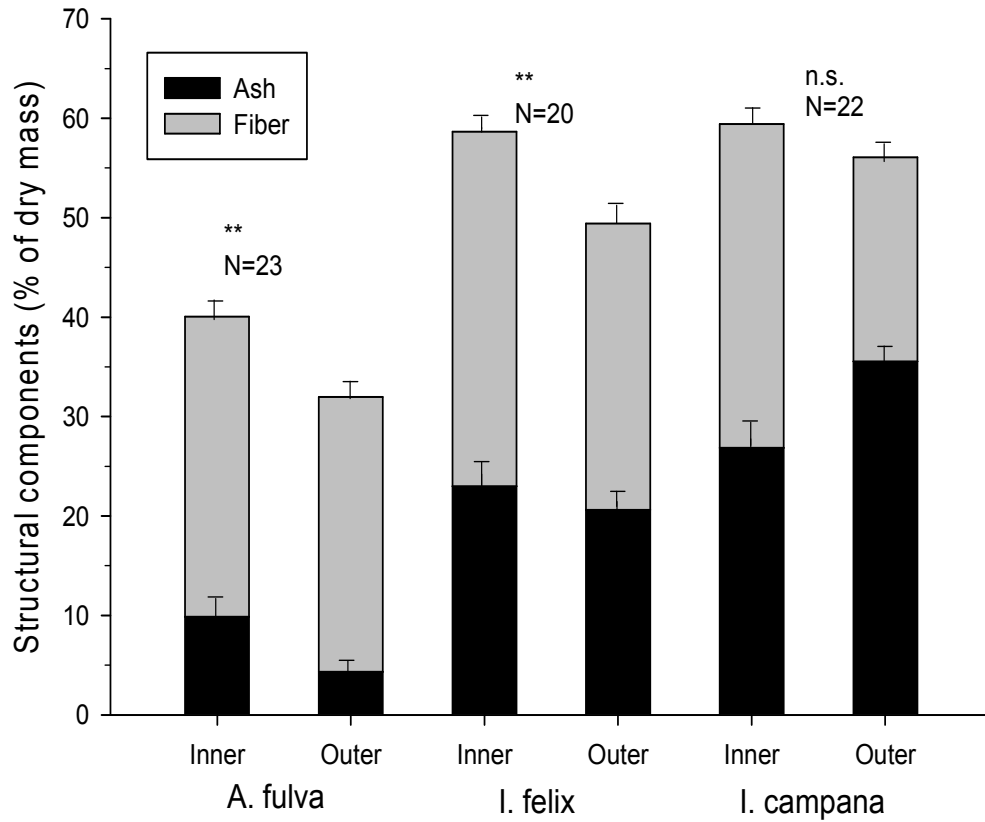
Figure 13: Distribution of total structural components (ash and fiber) from outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of three species of sponges at J Reef. Statistical data listed are for total structural component measurements: **:$p<0.01$, n.s.: not significant.
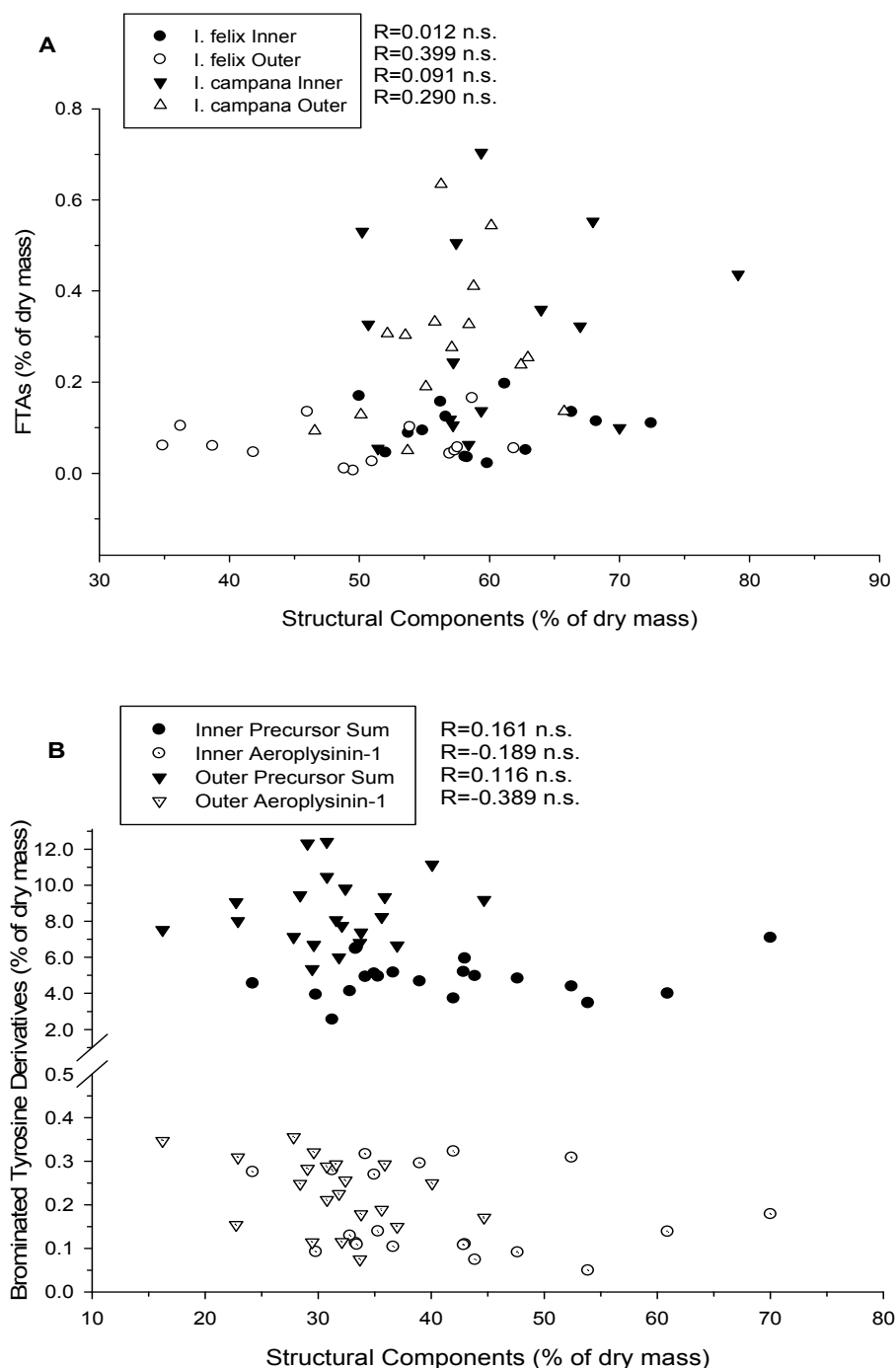
Figure 14: Correlations between the concentration of FTAs (A) or brominated defensive compounds (B) and structural components in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of two *Ircinia* species (A) and *A. fulva* (B). Data were analyzed using partial correlation analysis in which the protein content was held constant. The correlation coefficient and significance of each measurement is indicated next to the legend. Sample size is 14, 15, and 20 for *I. felix*, *I. campana,* and *A. fulva*, respectively. n.s.: not significant.
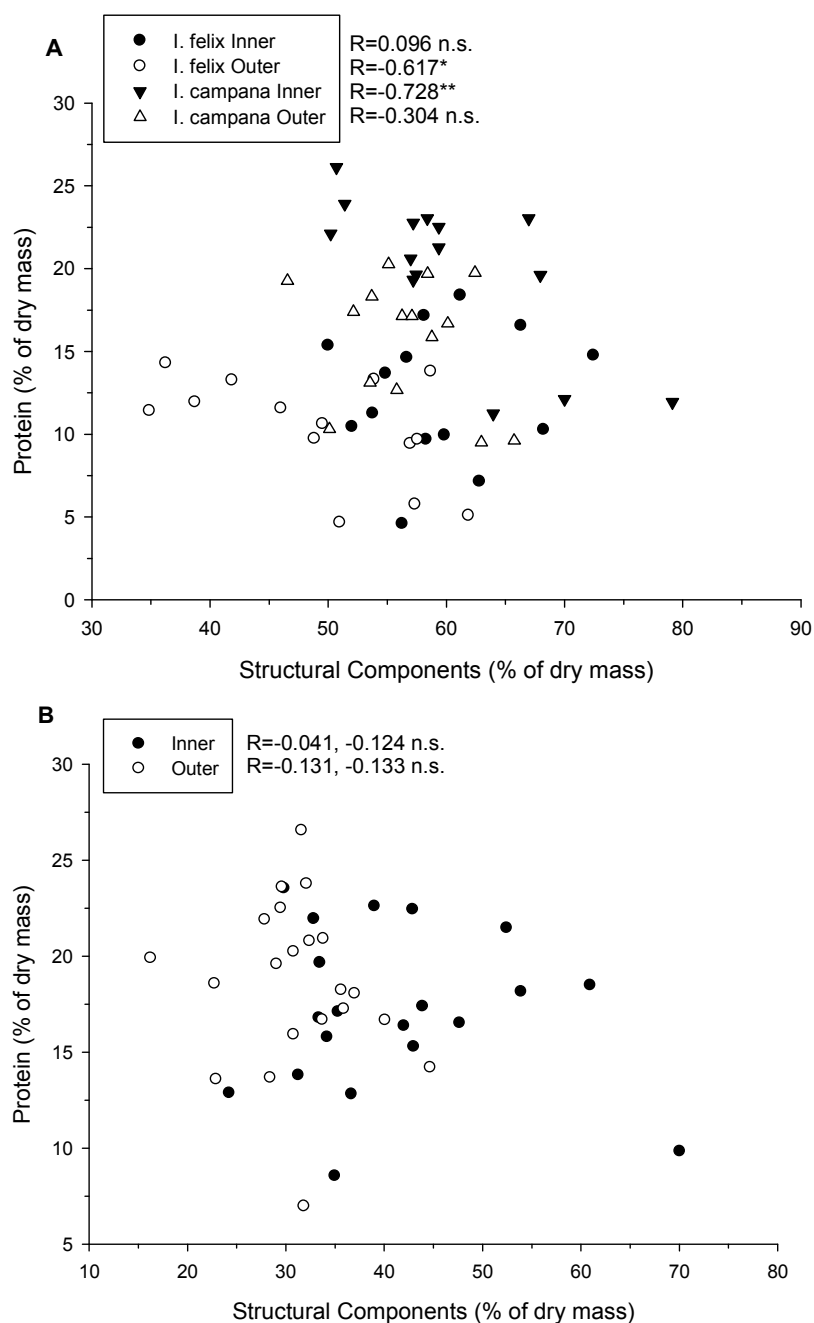
Figure 15: Correlations between the concentration of structural components and protein content in the outer (outer-most 2 mm of tissue) and inner (remaining tissue) regions of two *Ircinia* species (A) and *A. fulva* (B). Data were analyzed using partial correlation analysis in which the concentration of chemical defenses was held constant. The correlation coefficient and significance of each measurement is indicated next to the legend. For (B), correlation coefficients are shown for both the sum of precursors and Aeroplysinin-1. Sample size is 14, 15, and 20 for *I. felix*, *I. campana*, and *A. fulva*, respectively. *:p<0.05, **:p<0.005, n.s.: not significant.

CHAPTER 3

DOES CONCENTRATING CHEMICAL DEFENSES WITHIN SPECIFIC REGIONS

OF MARINE SPONGES RESULT IN ENHANCED PROTECTION FROM

PREDATORS?

Introduction

Secondary metabolite production is thought to be the most common method of

defense against consumers in many benthic marine organisms (Paul 1992, Pawlik et al.

1995). Although the effectiveness of these chemicals against predators is well-

documented, their production may come with a metabolic cost (McKey 1974, 1979,

Rhoades 1979). Allocating more defensive chemicals to regions of the body most at risk

to predators may alleviate some of this cost (Rhoades 1979). This strategy of

simultaneously deterring predators while minimizing costs, will be effective, however,

only if predator deterrence is positively correlated with the concentration of defensive

chemicals.

In marine sponges, studies documenting preferential allocation of secondary

metabolites to regions at high risk of predator attack do not always examine the

relationship between chemical concentrations and predator deterrence. For instance,

brominated compounds have been found in spherulous cells common to the ectosomal

region of sponges in the genus *Aplysina* (Thompson et al. 1983, Turon et al. 2000).

Likewise, *Rhopaloeides odorabile* has higher levels of diterpenes in the outer 2 cm

(Thompson et al. 1987) and *Negombata magnifica* concentrates the metabolite latrunculin

B toward its periphery (Gillor et al. 2000). Although these studies documented higher

concentrations of these metabolites in specific tissue regions, none of them examined the significance of this allocation to predator deterrence. Furthermore, results from investigations that tested the effectiveness of differing chemical concentrations against predators are contradictory. For example, Furrow et al. (2003) reported that the Antarctic sponge *Latrunculia apicalis* sequesters defensive compounds in the outer 2 mm of its tissues and that chemical extracts from this region are significantly more deterrent against sea stars than extracts from the inner region. In contrast, chemical extracts from the outer regions of *Chondrilla nucula* and 6 species of Red Sea sponges do not display an enhanced ability to deter predators (Swearingen & Pawlik 1998, Burns et al. 2003).

Combining quantification of chemical defenses with tests of their ability to deter relevant consumers (as in Furrow et al. 2003, but not in Swearingen & Pawlik 1998, Burns et al. 2003) may provide much insight into the ecological significance of differentially allocating these compounds to specific regions of the sponge body. Using this methodology, Schupp et al. (1999), confirmed that increased concentrations of defensive chemicals in regions of a sponge most vulnerable to consumers made them deterrent to fish predators. In contrast, a study by Becerro et al. (1998) found that although major metabolites were higher in the tips than the base of the sponge *Cacospongia* sp., extracts from the tips were consumed by fish just as readily as extracts from the base. These results suggest that this pattern of allocation does not confer additional antipredator protection to the sponge tips. Contrasting results from studies such as those described above indicates that further work is needed to fully understand the relationship between allocation of chemical defenses within sponges and predator deterrence.

Concentrating chemical defenses in regions of the body most susceptible to predation or most likely to come into contact with predators (see Burns et al. 2003) would seem to be the most efficient means of enhancing fitness in sponges because these organisms typically lack specialized reproductive structures (but see Schupp et al. 1999) that must be protected from consumers. Using sponges with three different chemical defense allocation patterns [i.e., higher concentrations of chemical defenses in the inner region (*I. felix*), higher concentrations in the outer region (*A. fulva*), and no difference in concentrations (*I. campana*)], the goal of this study was to determine if allocating higher concentrations of chemical defenses to particular regions of the sponge body makes those portions more deterrent to predators.

Methods

Artificial food preparation

To determine if higher concentrations of defensive compounds within certain regions of sponges correspond with increased protection of those regions from predators, I compared the palatability of extracts from the inner and outer region of each of three sponge species to fish and urchin predators. Methods for the extraction of crude organic extracts from *I. felix*, *I. campana* and *A. fulva* and artificial food preparation followed those described by Becerro et al. (2003) and Ruzicka (2005). I did not have enough tissue to test the palatability of *I. felix* extracts against fish, so only *A. fulva* and *I. campana* were used in this assay. All three species were used in urchin feeding assays.

For fish feeding assays, frozen sponge samples were thawed and 5 ml of the outer and inner regions of each replicate were measured by displacement of water. These samples were freeze dried and extracted three times in DCM:MeOH (see Methods,

Chapter 2). The three extracts were combined, filtered, and evaporated to dryness. The dried crude extract was weighed and then reconstituted in 0.50 ml of MeOH and sonicated to ensure complete dissolution. Artificial food was made in 150 ml batches from a mixture of 7.5 g powdered squid mantle, 2.975 g Type I Carrageenan, 0.525 g agar, and 150 ml distilled water. This food mixture resulted in artificial food approximating the average nutritional quality present in sponges (Chanas & Pawlik 1995). Once heated, approximately 4.5 ml of food mixture was added to each 27 mm diameter x 58 mm height vial containing 0.50 ml of MeOH and dissolved sponge extract. The mixture was homogenized by stirring rapidly and then was allowed to cool. Once the artificial food cube had hardened, it was cut into 1 ml cubes for feeding assays. Control food cubes were prepared the same way, but with crude extract omitted. To ensure that selection of food cubes by predators was not influenced by color, food coloring was added to both control and treatment mixtures before heating until both appeared similar in color by visual comparison. For the urchin feeding assays, frozen sponge samples from all three species were thawed and processed in the same manner as in the fish trials, but with only 3 ml of tissue from the outer and inner regions of each replicate and 0.30 ml of MeOH.

Feeding assays

Fish feeding assays carried out with extracts from *A. fulva* and *I. campana* were conducted in the field at J Reef in one dive per species. The assays began by releasing several control food cubes to stimulate feeding activity of natural assemblages of reef fish. Inner, outer, and control food cubes were then offered one or two at a time. In total, 21 food cubes of each type (inner, outer, and control) were released for *I. campana* and

14 of each type were released for *A. fulva*. Foods cubes were released haphazardly so that no more than 4 of the same type of cube were released in a row and the fish could not habituate to a pattern of cube release or become accustomed to one type of food. A food cube was considered unpalatable if it was rejected by fishes two or more times or if it sank to the bottom uneaten.

Feeding assays were also conducted for *A. fulva*, *I. felix*, and *I. campana* in the wet lab at Georgia Southern University using the urchin *Arbacia punctulata* as the predator. This urchin is a common invertebrate grazer on hard bottom reefs off the coast of Georgia. While this species is not commonly observed feeding on sponges at J Reef, other investigators have reported that this species, as well as others in this genus, prey on sponges (reviewed in Ridder & Lawrence 1982).

The experimental design for the urchin assays followed a method similar to that employed by Hay et al. (1994) using fiberglass window screening to hold the artificial food in place. Before adding artificial food to the sponge crude extract, a small (~7 x 7 mm) piece of fiberglass window screening (with 1 x 2 mm openings) was added to the vial containing the extract to act as a point of attachment for the food cube. Approximately 2.7 ml of artificial food was then added to the vial and stirred vigorously until the extract was thoroughly mixed within the food. Before allowing the artificial food to harden, the small piece of window screening was pushed to the bottom of the vial in the middle of the food cube. Once the food had hardened, the food cube was removed from the vial, blotted dry, and weighed.

For each replicate, urchins were given a choice of cubes containing compounds from inner and outer sponge tissues or a control cube containing no sponge extract. To do

this, I attached pre-weighed food cubes to a large (~140 x 130 mm) piece of window screening by sewing the small piece of screen embedded in the food cube to the larger screen base with monofilament line. To ensure that food cubes were equidistant from each other, I used an acrylic template containing three holes oriented in a triangle to position the cubes on the large screen base. This triangular arrangement ensured that when an urchin was placed in the center, its tube feet would be in contact with all three food types.

In preparation for the feeding assay, the large screen base containing all three food cubes was attached to the bottom of a feeding arena using monofilament line. These arenas were 3.1 L Glad© plastic containers that had holes drilled in the sides, bottom, and lid to allow water flow. Stainless steel hex nuts were cable tied to the sides for negative buoyancy. The lids prevented the urchins from escaping during the assay period and also allowed me to conduct 20 assays at once by stacking arenas within the aquarium. In order to prevent positional effects, the orientation of the window screen within the feeding arena was adjusted haphazardly among replicates.

Arbacia punctulata were held in 75.7 L aquaria at Georgia Southern University. These aquaria contained filtered, artificial seawater at 30 to 32 ppt. Urchins were maintained on a diet of algal disks until 48 hours before their assay. All urchins used in the assay had a test diameter between 4 and 5.5 cm. An assay began when an urchin was placed in the center of the three food types, with as little stress due to exposure to air and handling as possible, and the arena was covered and placed on the bottom of the aquarium. A complete set of assays for a sponge species was completed in one day because these arenas could be stacked. Preliminary assays found that urchins allowed to

feed for more than 8 hours could consume all of at least one type of food, so all assays were stopped at 8 hours. I found that urchins typically moved around on the window screen, trying at least two types of food, but usually fed extensively only on one. Urchins that did not feed at all (~ 10 % of runs) provided no information on the relative deterrence of extracts and were not included in the statistical analysis. Urchins were only used once in these assays. Tests assessing the weight loss and degradation of food cubes resulting from the 8 hour exposure to water were conducted simultaneously with the assay by placing food cubes in feeding arenas lacking *A. punctulata*. At the completion of the assay, urchins were removed from the feeding arenas. Individual food cubes were removed from the window screening, blotted dry, and weighed to determine how much of each cube had been consumed.

For both fish and urchin assays, the integrity of the chemical defenses in the artificial food cubes was confirmed by re-extraction of leftover cubes and analysis by TLC and HPLC. In all instances, the compounds extracted from the sponges were present in the artificial food and had not undergone degradation during the experimental procedure.

To test for significant differences in consumption of the different food cube types by fish (expressed as % of cubes consumed), I used 3 x 2 and 2 x 2 contingency tables with chi squared analysis. For the urchin feeding assays, I analyzed square root arcsine transformed data (expressed as % of weight loss of each food type) with a repeated measures ANOVA. A repeated measures analysis was used because all three foods cubes were exposed to one urchin simultaneously and were therefore not independent

.

Results

Fish that consumed experimental food cubes were primarily the black seabass (*Centropristus striata*) and spottail pinfish (*Diplodus holbrooki*), with occasional consumption by the grey triggerfish (*Balistes capriscus*) and pinfish (*Lagodon rhomboides*). In both *A. fulva* and *I. campana*, 100 % of the control food cubes were consumed while treatment cubes were consumed less frequently (*A. fulva*: Pearsons Test: $X^2 = 6.222$, $p<0.05$ and $X^2 = 10.435$, $p<0.01$ for inner vs. control and outer vs. control, respectively; *I. campana*: Pearsons Test: $X^2 = 4.909$, $p<0.05$ and $X^2 = 4.190$, $p<0.05$ for inner vs. control and outer vs. control, respectively) (Figure 16). The frequency at which the two treatment food cubes were consumed, however, was not significantly different in either sponge species (Pearsons Test: $X^2 = 0.735$, $p>0.30$ and $X^2 = 0.094$, $p>0.70$ for *A. fulva* and *I. campana*, respectively) (Figure 16).

In laboratory feeding assays with urchins, control food cubes lost more weight than both treatment cubes in all sponge species, but this trend was only significant in *A. fulva* ($F_{2,32} = 13.96$, $p<0.001$ for inner vs. control and $F_{2,32} = 8.246$, $p<0.01$ for outer vs. control). There was no significant difference in the percent change in weight between the two treatment cubes in any of the sponge species (Figure 17).

Discussion

There is a paucity of experimental evidence supporting the contention that allocating chemical defenses to regions of a sponge that are most vulnerable to consumption enhances protection from predators (Becerro et al. 1998, Schupp et al. 1999, Burns et al. 2003, Furrow et al. 2003). This lack of evidence exists because few studies have combined quantification of antipredator compounds within different regions of

sponges with bioassays to evaluate the ability of these differing chemical concentrations to deter consumers (Becerro et al. 1998, Schupp et al. 1999, Furrow et al. 2003). In this study, I investigated whether or not allocating higher concentrations of chemical defenses to particular regions of the sponge body makes those portions more deterrent to predators. Interestingly, data presented here do not support the hypothesis that increased concentrations of chemical defenses confer additional protection from predators. It is possible that the differential allocation of these compounds within these two species may be driven by other ecological factors such as deterring competitors or inhibiting infection or may be more effective against predators not tested in this investigation.

In an initial survey of the defensive compounds in the sponges *A. fulva*, *I. felix*, and *I. campana* (Chapter 1), I documented that they all have secondary metabolites that are known to deter predators (Chapter 1) (Pawlik et al. 1995, Ebel et al. 1997, Epifanio et al. 1999, Waddell & Pawlik 2000, Pawlik et al. 2002, Burns et al. 2003, Thoms et al. 2004). Further investigation showed that in at least two of these species, *A. fulva* and *I. felix*, concentrations of secondary metabolites in the inner and outer regions of these sponges differ (Chapter 2). Interestingly, however, the distribution of chemical defenses within all three species (except *A. fulva*) was not consistent with allocation models suggesting that defenses should be concentrated in regions most vulnerable to predation (McKey 1974, 1979, Rhoades 1979). In addition, the results of the feeding assays are not consistent with the antipredatory role that has been ascribed to differential allocation of chemical defenses within individuals. This is most evident in the inner region of *I. felix* and the outer region of *A. fulva*, where, although higher concentrations of chemical defenses were measured (Figures 8 and 9, Chapter 2), extracts from these regions did not

display enhanced deterrency (Figures 16 and 17). In addition, in *I. campana*, there was no significant difference in the levels of chemical defenses (Figure 8, Chapter 2) and there was no difference in predator deterrence by extracts from inner and outer tissue regions (Figures 16 and 17). Together, these results suggest the following: 1) chemical defense allocation patterns observed within *A. fulva* and *I. felix* may be selected for due to other ecological factors besides predation and 2) that the increased levels of chemical defenses observed in this study may be targeted to predators that were not tested in this investigation.

The feeding trials conducted here do corroborate previous findings that extracts from these sponge species deter predators because in all instances treatment food cubes were consumed less often than controls (Figures 16 and 17) (Pawlik et al. 1995, Ebel et al. 1997, Pawlik et al. 2002, Thoms 2004). The lack of evidence for a positive relationship between compound concentrations and predator deterrence may be attributable to at least two factors. First, defensive compounds may serve multiple functions and higher concentrations of these compounds may actually represent an adaptation to defend against competitors, reduce fouling, or inhibit microbial infection (Uriz et al. 1992, Kubanek et al. 2002). Second, because multiple factors typically play a role in prey deterrence, testing chemical defenses in isolation may not sufficiently address the suite of defenses, such as structural components, encountered by predators (Duffy & Paul 1992, Hay et al. 1994, Hay 1996).

It should also be pointed out that the different suites of chemical defenses are not necessarily equivalent when it comes to deterring predators (Hay et al. 1988b, Pennings & Paul 1992). The FTAs and brominated tyrosine derivatives identified here are known

to be deterrent compounds, but it is likely that they differ in their effectiveness against specific predators. This is supported in the current study, where FTA extracts from *I. campana* were only deterrent to fish predators whereas brominated tyrosine derivative extracts from *A. fulva* were deterrent to both fish and urchins (Figures 16 and 17). Extending these experimental methods to other predators may have yielded even a different set of results given that some small, cryptic invertebrates or mesograzers may be less affected by chemical defenses than other, larger predators (Hay et al. 1988b). To gain a better understanding of chemical defense allocation patterns in sponges, further assays should test the effects of these compounds at natural concentrations against large, biting predators in the water column; large, mobile invertebrates; and small cryptic invertebrates. Additionally, assays including, at the very least, the interactive effects of chemical and structural defenses may more accurately identify the effectiveness of variation in antipredatory defenses within sponges.

In conclusion, while I have documented significant within sponge variation in the distribution of defensive chemicals in two out of three sponge species from the SAB, this variation is not in consistent agreement with allocation models suggesting that at risk regions should be more heavily defended from predators (Chapter 2). Rather, results reported here suggest that relatively low levels of chemical defenses may adequately defend marine sponges from damage by large biting or mobile invertebrate predators. Whether concentrating chemical defenses within certain regions of these sponges is due to predation pressure from consumers not investigated here or an attempt at countering other ecological stresses are both topics in need of further investigation.
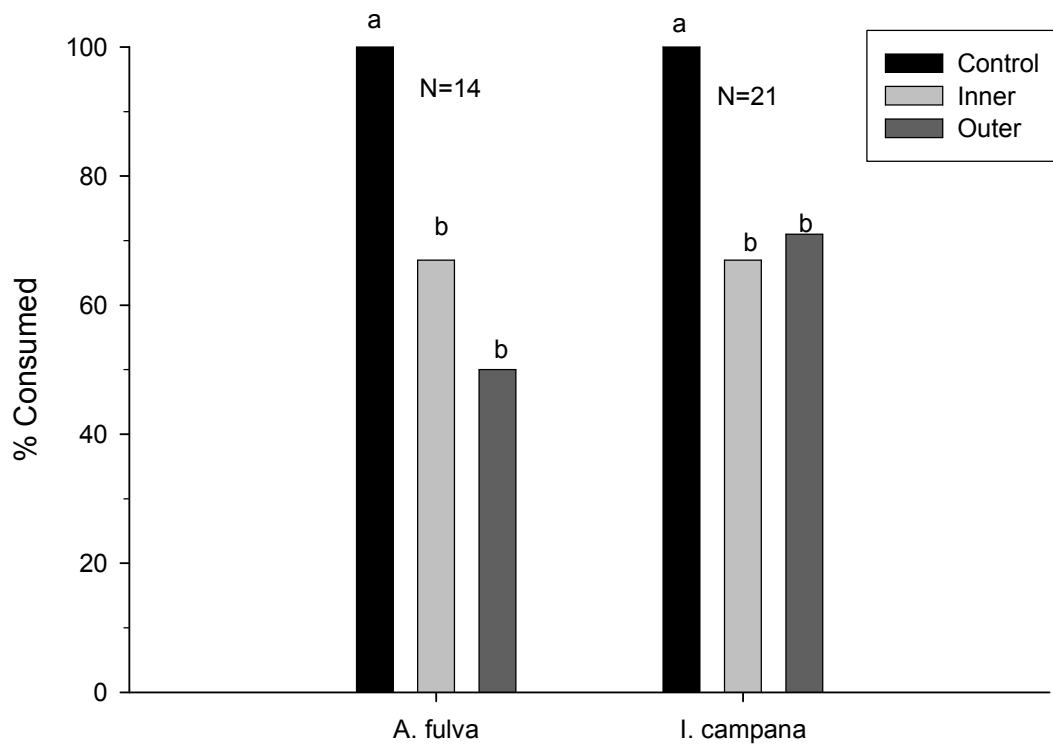
Figure 16: Consumption of food cubes by a natural assemblage of reef fish. Cubes containing crude extract from both the inner and outer regions of these sponges as well as control cubes were offered. Percentages are based on the number of cubes consumed out of the number indicated at the top of the bars. Data were analyzed using a combination of 3 x 2 and 2 x 2 contingency tables.
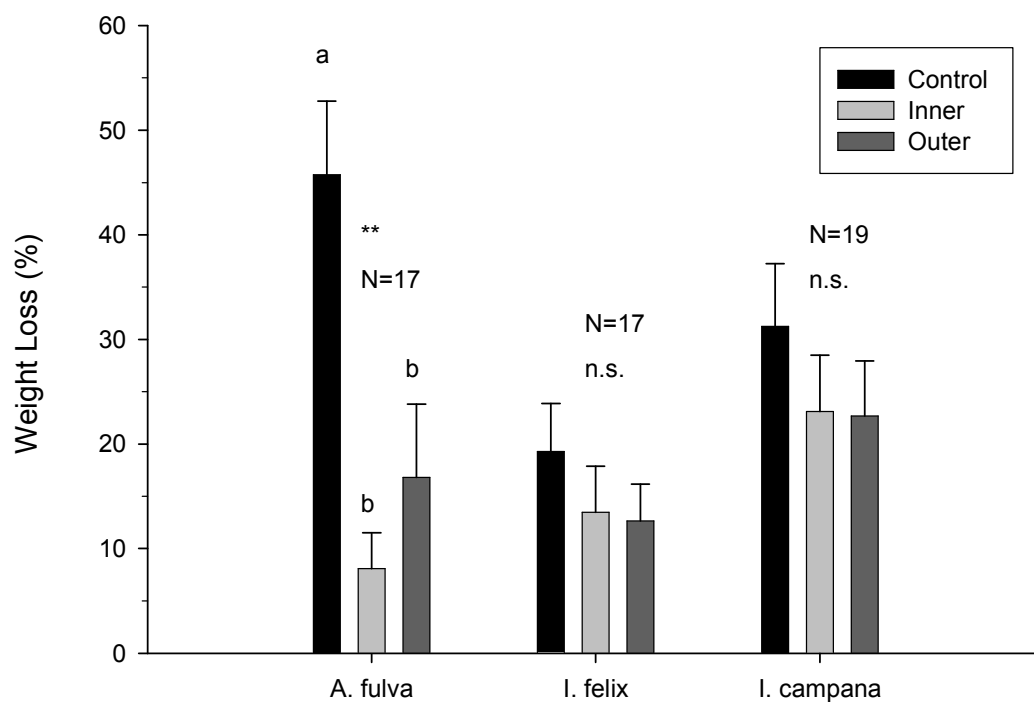
Figure 17: Percent weight loss (+/- SE) due to feeding by the urchin *Arbacia punctulata* on food cubes created from chemical extracts of 3 sponge species. Extracts were obtained from inner and outer tissue regions of each sponge. Controls represent food cubes containing no chemical extract. Data were analyzed using a repeated measures ANOVA followed by a least squares comparison. **:p<0.01 n.s.: not significant.

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APPENDICES

APPENDIX A: SPECTRAL DATA [M/Z (RELATIVE ABUNDANCE)] FROM GC-MS ANALYSIS OF FTA COMPOUNDS. PEAK NUMBERS CORRESPOND TO THOSE FROM THE GC CHROMATOGRAMS IN FIGURE 2 AND REPRESENT COMPOUNDS FROM FIGURE 3.

Peak 1: (Felixinin or Strobilinin Acetate (isomers of Variabilin), t_R: 42.44 min. from *I. felix* and 42.54 min. from *I. campana*): m/z (relative abundance): 55 (33), 69 (46), 81 (100), 95 (24), 109 (22), 123 (36), 135 (41), 153 (36), 175 (18), 189 (4), 203 (10), 215 (2), 231 (8), 249 (6), 425 (4), 440 (2).

Peak 2: (Felixinin or Strobilinin Acetate (isomers of Variabilin), t_R: 43.16 min from *I. felix*, not present in *I. campana*): m/z (relative abundance): 55 (33), 69 (45), 81 (100), 95 (21), 109 (23), 123 (36), 135 (40), 153 (34), 175 (20), 189 (3), 203 (9), 215 (2), 231 (8), 249 (4), 271 (1), 299 (1), 317 (1), 398 (1), 425 (1), 440 (2).

Peak 3: (Unidentified FTA, t_R: 43.54 min. from *I. felix* and 43.55 min. from *I. campana*): m/z (relative abundance): 55 (35), 69 (44), 81 (88), 95 (44), 107 (24), 121 (25), 135 (100), 149 (43), 161 (14), 175 (14), 189 (5), 203 (9), 215 (5), 229 (2), 243 (3), 440 (3).

Peak 4: (Variabilin Acetate, t_R: 43.79 min. from *I. felix* and 43.80 min. from *I. campana*): m/z (relative abundance): 55 (31), 69 (42), 81 (100), 95 (22), 109 (20), 123 (34), 135 (38), 153 (31), 175 (20), 189 (3), 203 (9), 215 (3), 231 (7), 249 (4), 271 (1), 425 (1), 440 (2).

Peak 5: (Unidentified FTA, t_R: 44.09 min. from *I. felix*, not present in *I. campana*): m/z (relative abundance): 55 (44), 69 (50), 81 (86), 95 (42), 107 (23), 121 (30), 135 (100), 149 (46), 161 (12), 175 (14), 189 (4), 203 (11), 215 (4), 229 (2), 243 (2), 257 (1), 271 (1), 289 (1), 335 (1), 440 (3).

APPENDIX B: MASS SPECTRA DISPLAYING THE MASS ION PEAK AND ISOTOPE PATTERNS FOR THE 10 BROMINATED TYROSINE DERIVATIVES IDENTIFIED FROM *A. FULVA* AT J REEF. NUMBERS DISPLAYED CORRESPOND TO THE NUMBER OF EACH COMPOUND IN FIGURES 5 AND 6.

1: Aerophobin-1

2: Aerophobin-2

3: Aplysinamisin-1

4: Aeroplysinin-1

5: Hydroxyaerothionin

6: Hydroxy-oxo-aerothionin

7: Homoaerothionin

8: Aerothionin

9: Fistularin-3

Dibromocyclohexadienone