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Chemical defenses, nutritional quality, and structural components in three sponge species: *Ircinia felix*, *I. campana*, and *Aplysina fulva*

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Abstract Allocating chemical defenses to regions or tissues most at risk for predatory attack may provide protection while simultaneously minimizing associated metabolic costs. Chemical defense allocation patterns were investigated in the aspiculate sponges *Ircinia felix*, *I. campana*, and *Aplysina fulva* collected between July 2005 and April 2006 from J Reef off the coast of Georgia, U.S.A. It was predicted that chemical defenses would be (1) higher in the outermost 2 mm layer of the sponge; (2) positively correlated with tissue nutritional quality; and (3) correlated with structural components such as spongin fibers. Whereas defensive chemicals were concentrated in the outer 2 mm of *A. fulva*, the *Ircinia* species had higher concentrations in deeper tissue layers. Furthermore, no significant positive or negative correlation between chemical defenses and nutritional quality or levels of structural components was observed in these sponges. Overall, these results do not support the prediction that predation pressure by fish and large mobile invertebrates significantly impacts chemical defense allocation in these sponges.

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Introduction

Secondary metabolites are thought to be the most common mode of defense against consumers in sessile marine and terrestrial organisms (Lincoln and Langenheim 1979; Rhoades 1979; Paul 1992; Hay 1996). These compounds may be especially common in areas of high predation intensity in marine ecosystems, and organisms lacking such defenses often dwell in cryptic locations to avoid consumers (Bakus 1964, 1981; Dunlap and Pawlik 1996; Pawlik 1997, 1998). While secondary compounds may be an effective defensive mechanism, they may be costly because their production requires use of limited resources that might otherwise be expended on growth or reproduction (McKey 1974; Rhoades 1979; McClintock and Baker 2001; Stamp 2003). This cost can be reduced by concentrating chemical defenses in tissues or regions of the body that are at greater risk of being consumed because of their proximity to predators or that lack effective structural defenses (McKey 1974; Tugwell and Branch 1989; Meyer and Paul 1992; Schupp et al. 1999). Furthermore, if food preferences of predators are dictated by nutrient intake, a positive correlation between nutrient content of prey tissues (particularly protein) and concentrations of chemical defenses might be expected (Bowers and Stamp 1992). Such differential allocation of chemical defenses within individuals was initially described in terrestrial plants (McKey 1974; Coley 1983; Liu et al. 1998) and has been substantiated in benthic marine organisms (see Hay 1996).

Secondary metabolites are not the only deterrent against predation. Structural components like silica or calcium carbonate spicules and fibers, while primarily providing support, may also serve a defensive role (Coley 1983; Pennings and Paul 1992; Burns and Ilan 2003; Ruzicka and Gleason 2009). Physical defenses are often less costly to

produce than chemical deterrents and so may be favored if highly effective against potential predators (Harvell and Fenical 1989; Meyer and Paul 1995). There is some evidence that structural and chemical defenses may act synergistically to enhance deterrence (Schupp and Paul 1994; Burns and Ilan 2003; Hill et al. 2005).

Sponges are sessile, soft-bodied organisms that are a major component of benthic communities from polar to tropical seas (Targett and Schmahl 1984; Alcolado 1991; Zea 1993; McClintock and Baker 2001). Sponges generally have high nutrient content, with mean protein values exceeding 20% of their dry mass compared to 0.8% to 13.7% in seaweeds (Montgomery and Gerking 1980; Chanas and Pawlik 1995). In addition, structural defenses like spicules and spongin fibers may be ineffective as feeding deterrents when tested alone or together (Chanas and Pawlik 1995, 1996). Thus, although all indications are that sponges should be highly palatable prey, their great abundance on reefs suggests that these organisms are adequately defended against predators (Paul 1992).

Secondary metabolites are the most commonly demonstrated antipredator mechanism in sponges (Paul 1992; Pawlik 1993; Pennings et al. 1994; Chanas and Pawlik 1996; Becerro et al. 1998), but our understanding of how these 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 chemicals in the exposed, outer region of the sponge. In contrast, extracts from the outer 2 mm of *Chondrilla nucula* (Swearingen and 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 generalization about the relationship between the distribution of chemical defenses within individuals and the susceptibility of sponge tissues to predatory attack difficult. Nevertheless, differential allocation of defenses within individuals may be ecologically and evolutionarily relevant, as suggested by reports of fish biting or mouthing sponges without removing tissue (Schulte and Bakus 1992; Dunlap and Pawlik 1998) or rapidly consuming a sponge once the outer region has been removed (Wulff 1997).

To better understand chemical defense allocation patterns within sponges, we investigated the relationships among the concentration of chemical defenses, nutritional quality, and structural components in three species of sponges from a temperate reef. *Ircinia felix* and *I. campana*, the amorphous and vase-shaped stinker sponges, are both asipculate sponges characterized by a tough network of fibers. The sponge *Aplysina fulva* is an asipculate sponge that exhibits a rope-like growth form along the substrata. All three of these species are chemically defended against the generalist predatory fishes present at this site (Ruzicka and Gleason 2009). These sponges were selected due to: (1) their high abundance on these reefs and previously described secondary metabolites (Martinez et al. 1997; Freeman 2007; Nuñez et al. 2008) and (2) their large size, which ensured that there would be enough tissue from the outer 2 mm to carry out all laboratory analyses.

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) correlated with structural components in a manner that supports either the role of sponge structural materials in deterring predators or the presence of a synergism between these two defense mechanisms.

Methods

Collection and identification

The sponges *Ircinia felix*, *I. campana*, and *Aplysina fulva* were collected on two separate days per species (Table 1) at J Reef (31° 36.056N, 80° 47.431W), a hard bottom area in the South Atlantic Bight (SAB) about 32 km off the coast of Georgia, USA. This reef is characterized by sandstone and relic scallop shell ridges that provide moderate relief and support diverse assemblages of tropical and temperate sponge species (Freeman et al. 2007; Ruzicka and Gleason 2008).

During collection, a large portion (>1 kg wet weight) of an individual sponge was removed from the substrate with a dive knife and placed in a plastic bag. To avoid rapid changes in the chemical makeup of the sponge, while on the boat, the outer 2 mm (hereafter referred to as outer region) was removed from a portion of each sponge with a razor blade. A depth of 2 mm was chosen as the division

Table 1 Collection dates and numbers of samples collected for each sponge species at J Reef off the coast of Georgia, USA

Species	Date	No. of samples
Ircinia felix	Jul 25, 2005	11
	Sep 2, 2005	9
Ircinia campana	Oct 20, 2005	10
	Jan 19, 2006	12
Aplysina fulva	Mar 3, 2006	14
	Apr 21, 2006	10

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 color differences resulting from the frequent presence of cyanobacterial symbionts in the outer layer (Turon et al. 2000; Becerro et al. 2003; Becerro and Paul 2004) (S1). 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 that was neither dissected nor 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. Once removed from liquid nitrogen, samples for chemical analysis were also stored at −70°C until they were processed. There was no evidence of reproduction in the form of sponge larvae in either the *Ircinia* spp. (in a family exhibiting internal development) or *Aplysina fulva* (in a sponge family exhibiting external development) during collection or dissection of these sponges (Maldonado 2006).

Identification and isolation of defensive compounds

Furanosesterterpene tetronic acids (FTAs) from the *Ircinia* species were extracted following methods adapted from Martinez et al. (1997). A sample (250–280 g wet weight) of *I. felix* and *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 h 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 mg) 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^{-1} 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 × 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 min with a flow rate of 4 ml min^{-1}. 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.

To isolate and identify brominated tyrosine derivatives from *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_20 and passed through a 6-ml Phenomenex Strata-X polymeric sorbent (500 mg) column with a plunger to remove contaminants. Fractions collected from this column were evaporated to dryness and dissolved in MeOH at 45 mg ml^{-1} 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 × 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 min followed by an increase in the concentration of $CNCH_3$ to 100% over 28 min 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 quadrupole time-of-flight mass spectrometer using positive electrospray ionization.

Once we confirmed the presence of 9 previously described brominated compounds from *A. fulva*, we isolated groups of these compounds using a Vydac C-18 preparatory column (10 × 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_2O with the same gradient as above at 4 ml min^{-1} with monitoring at 254 and 280 nm. This method did not allow purification of individual compounds, so standards used in HPLC analysis were mixtures of known chemical defense compounds. In *A. fulva*, there were two groups of compounds, one consisting of a mixture 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 purchased from Axxora for use as a standard.

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 h 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 h 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 0.0001 g using an electronic balance (APX-60, Denver Instruments, Denver, Colorado).

Quantification of defensive metabolites

To determine whether the concentration of chemical defenses was higher in the outer or inner regions of these sponges, we quantified the secondary metabolites from these two regions in 15 individuals each of *I. felix* and *I. campana* and 20 individuals of *A. fulva*. Although these sample sizes do not represent all the samples available, this replication was adequate to account for the variation in chemical concentrations observed in the J Reef populations. 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 h at room temperature in the dark. After 9 h, this acetylated mixture was rapidly added to 30 ml of cold (9°C) filtered and deionized water and stirred 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 (4.6 × 250 mm) and a solvent of 93:7 MeOH:H_20 with UV detection at 270 nm (Freeman 2007). Quantification of secondary metabolites in *I. campana* and *I. felix* was completed by comparing peak areas of experimental samples to those of 9-µl volumes of a group of purified FTA standards ranging in concentration from 12.5 to 2,000 µg ml^{-1} (Freeman 2007).

In *A. fulva*, 100% MeOH was added to the dried crude extract to bring the concentration to 20 mg ml^{-1}, and this mixture was sonicated to ensure complete dissolution. In order to remove contaminants, we 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 the HPLC system described earlier. UV detection was at 254 and 280 nm using a solvent gradient consisting of $CNCH_3$ and water buffered with 0.1% formic acid. The gradient was 90% water for the first 3 min followed by increasing concentrations of $CNCH_3$ to 100% over 28 min with a flow rate of 0.7 ml min^{-1} (Freeman 2007; Nuñez et al. 2008). 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 1,000 µg ml^{-1} (Freeman 2007). We used the Wilcoxon's signed ranks test in SPSS 15.0 to determine if there were differences in the concentration of chemical defenses between the inner and outer regions of these three sponge species, because some data sets did not meet parametric assumptions even after transformation.

Nutritional analysis of sponge tissue

To determine if the concentration of defensive compounds was positively correlated with nutritional quality, we quantified soluble protein and carbohydrate content in the inner and outer regions of these three sponge species. The sponge samples that were initially placed on ice in the field were thawed, the outer 2 mm layer was removed, and the inner and outer regions were frozen and lyophilized. The dried sponge was cut into small pieces, homogenized using a mortar and pestle, and stored at −20°C. Soluble 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 h at room temperature. Protein concentration was determined by comparison to a calibration curve produced from bovine serum albumin (BSA). In all cases, protein concentrations were expressed as percent dry weight of sponge tissue. Duplicate protein assays conducted on each sample 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 and Pawlik 1995). We dissolved 10–15 mg of dried tissue in 1 ml filtered, deionized water for 1.5 h. A 250-μl subsample of this mixture was then transferred to a 16 × 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 min and allowed to cool to room temperature by immersion in tap water. We transferred 2 ml of each sample to a 16 × 150-mm test tube and added 1 ml of 5% phenol followed by rapid addition of 5 ml of H_2SO_4. This solution was allowed to stand at room temperature for 10 min, after which it was mixed and placed in a 25°C water bath for 15 min. Absorbance of each sample was read at 490 nm, and concentrations were calculated from a standard curve of known D-glucose 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.

We used the Wilcoxon's signed ranks test in SPSS 15.0 to identify significant differences in the protein and carbohydrate content between the inner and outer regions of the sponge. In order to determine whether there was a positive correlation between the concentration of chemical defenses and nutritional quality, we used a combination of a univariate partial correlation analysis in SPSS 15.0 and a multivariate Principal Components Analysis (PCA) using SYSTAT 10.0 as described in the next section.

Structural components in sponge tissue

To determine whether there was a negative correlation between the concentration of chemical defenses and structural components, we quantified the total structural content, including both fiber and ash content in the inner and outer regions of these three species of sponges. Because these three sponges are aspiculate, structural material consisted of fiber skeleton and sand grains and inorganic salts. The use of aspiculate sponges may underestimate the level of deterrence conferred by structural components.

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 1 week to remove all non-structural materials. After 1 week, the mixture was filtered through coarse (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 h. The ash remaining after combustion was weighed and expressed as percent per dry mass. Fiber content was calculated by subtracting ash mass from total structural mass and also expressed as percent of dry mass.

As in the earlier mentioned chemical and nutritional quality analyses, we used the Wilcoxon's signed ranks test in SPSS 15.0 to determine whether there were differences in the concentration of structural components between the inner and outer regions of each sponge species. We used univariate partial correlations to investigate the relationship between the concentration of chemical defenses and structural components, while keeping protein content constant. Using a partial correlation analysis, we also investigated the relationship between chemical defenses and protein content while holding total structural components constant. In addition, as mentioned earlier, we analyzed these data using a multivariate PCA analysis in SYSTAT 10. The PCA was run on 3 variables: protein, carbohydrate, and structural components. A PCA for each region (i.e., inner and outer) of each species was run separately. These analyses resulted in the formation of one or two principal component axes that had eigenvalues >1. The factor scores for each sample on these axes were obtained and run against the values for the concentration of chemical defenses using a bivariate correlation matrix with a Bonferroni correlation.

As a precautionary measure, we tested for differences in the concentration of defensive chemicals, protein, and structural components in each sponge species between the two sample dates (Table 1) using a one-way ANOVA. Significant differences between dates were observed only in *I. felix* for structural components in inner tissues, in *I. campana* for FTAs in inner tissues and protein in outer tissues, and in *A. fulva* for aeroplysinin-1 in inner and outer tissues and fiber in inner tissues (Freeman 2007). For instances where significant differences among dates occurred, we compared values in inner and outer tissues and found that in only one case (FTAs for *I. campana*) did the relationship between values found in inner and outer tissues reverse (i.e., FTA concentrations were higher in outer tissues on the first sample date and then in the inner on the second sample date). Paired *t*-tests (inner versus outer) conducted for this

sample on each date yielded non-significant results suggesting that this discrepancy was not great enough to impact the overall results if the data were combined. Given the earlier results, we felt justified in combining the data across sample dates for all three sponge species.

Results

Chemical variation in sponge tissues

Ircinia felix and *I. campana* from J Reef exhibited FTAs that have been found in sponges in this genus across a wide geographic range (Martinez et al. 1997; Epifanio et al. 1999; Pawlik et al. 2002; Freeman 2007). *Aplysina fulva* from J Reef had 9 brominated tyrosine derivatives that have previously been observed in sponges in this genus (Epifanio et al. 1999; Thoms et al. 2006; Freeman 2007). These compounds from *A. fulva* were categorized into two groups for further analyses: 8 high molecular weight compounds (designated as the Sum precursor) and the low molecular weight compound, aeroplysinin-1 (Freeman 2007).

Contrary to the prediction that antipredator compounds would be allocated to the outermost 2 mm of the sponge body, FTAs were in significantly higher abundance in the inner regions of *I. felix* relative to the dry mass of sponge tissue (Wilcoxon Signed Ranks Test: $Z = -2.045$, $P < 0.05$; Fig. 1). Also contrary to the prediction, no significant differences in FTA concentrations between inner and outer tissues were observed in *I. campana* (Wilcoxon Signed Ranks Test: $Z = -0.284$, $P > 0.05$; Fig. 1). In both species, the contribution of secondary compounds to the total sponge biomass was small (<0.5% of dry mass).

In *A. fulva*, concentrations of 8 antipredator precursor compounds as well as aeroplysinin-1 were, as predicted, significantly higher in the outer sponge tissue regions (Wilcoxon Signed Ranks Test: $Z = -3.920$, $P < 0.001$ for sum of precursors; $Z = -2.987$, $P < 0.01$ for aeroplysinin-1; Fig. 2). On average, the compounds found in *A. fulva* were more than an order of magnitude more concentrated on a sponge dry weight basis than in either of the *Ircinia* species (compare Figs. 1 and 2).

Nutritional quality of sponge tissues

Protein concentrations were significantly different between the inner and outer regions in the two *Ircinia* species, but not in *A. fulva*. In both *Ircinia* species, the inner region had higher soluble protein content than the outer region (Wilcoxon Signed Ranks Test: $Z = -3.587$, $P < 0.001$, and $Z = -2.203$, $P = 0.028$ for *I. campana* and *I. felix*, respectively) (Fig. 3a). 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: $Z = -2.576$, $P = 0.010$ and $Z = -3.490$, $P < 0.001$ for *I. felix* and *I. campana*, respectively) (Fig. 3b).

While there was a positive correlation between the concentration of FTAs and nutritional quality in the outer region of *I. felix*, no such pattern was found in the corresponding inner region (S2). Furthermore, significant correlations between nutritional quality and the concentrations of antipredator compounds were absent in both tissue regions in *I. campana* and *A. fulva* (S2).

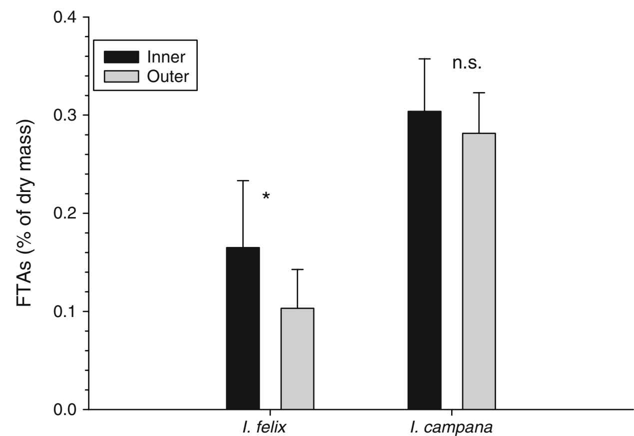

Fig. 1 Concentration of FTAs (±SE) from outer (outer 2 mm) and inner (remaining tissue) regions of two species of *Ircinia* at J Reef as % sponge dry mass. Means for inner and outer regions of each species were compared with a Wilcoxon Signed Ranks test. * $P < 0.05$, *NS* not significant. $N = 15$

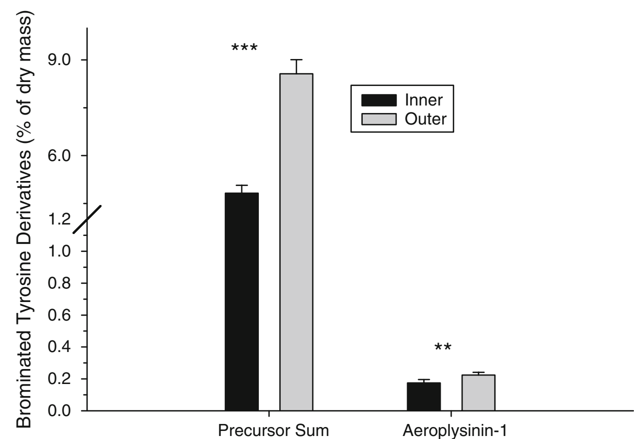

Fig. 2 Concentration of secondary metabolites (±SE) from outer (outer 2 mm) and inner (remaining tissue) regions of *A. fulva* from J Reef as % sponge dry mass. Precursor sum was calculated by pooling data from 8 high molecular weight peaks. Means for each group of compounds were compared with a Wilcoxon Signed Ranks test. *** $P < 0.001$, ** $P < 0.01$. $N = 20$. Notice *y*-axis break between 1.2 and 4

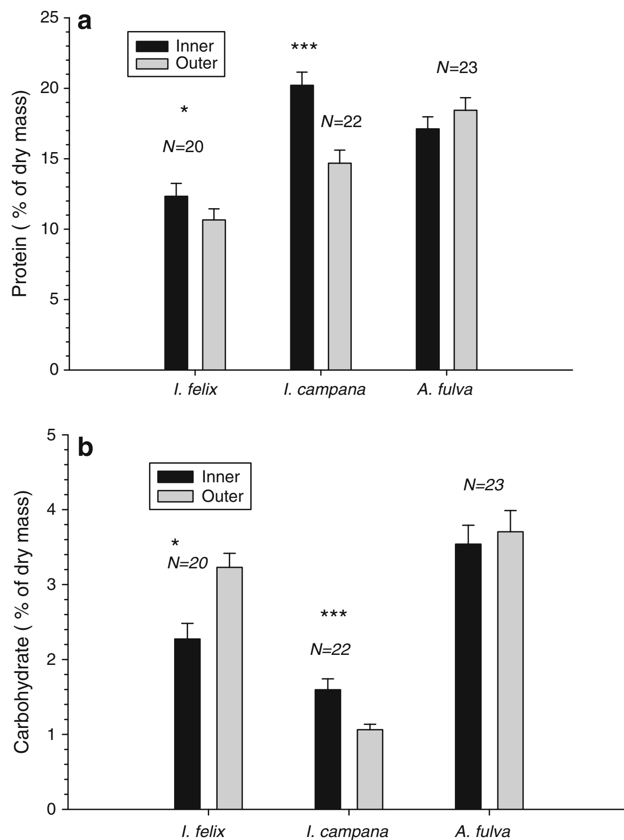

Fig. 3 Concentration of **a** protein (±SE) and **b** carbohydrate from outer (outer 2 mm) and inner (remaining tissue) regions of three species of sponges at J Reef. Means for each measurement were compared with a Wilcoxon Signed Ranks test. *** $P < 0.001$, * $P < 0.05$

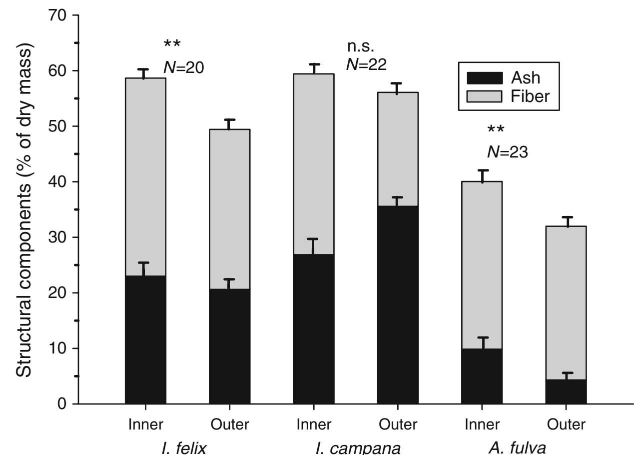

Fig. 4 Distribution of total structural components (ash and fiber) (±SE) from outer (outer 2 mm) and inner (remaining tissue) regions of three species of sponges at J Reef. Means for total structural components were compared using a Wilcoxon Signed Ranks test. ** $P < 0.01$, *NS* not significant

Distribution of structural components

To investigate the relationship between chemical defenses and structural components, we first quantified the structural content of inner and outer tissue layers of these three aspiculate sponges. 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: $Z = -3.360$, $P = 0.001$ and $Z = -3.102$, $P = 0.002$ for *I. felix* and *A. fulva*, respectively), whereas no significant differences between regions were evident in *I. campana* (Wilcoxon Signed Ranks Test: $Z = -1.542$, $P = 0.123$) (Fig. 4). The ash content of 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: $Z = -2.768$, $P = 0.006$ and $Z = -2.873$, $P = 0.004$ for *A. fulva* and *I. campana*, respectively), but no differences were detected within *I. felix* (Wilcoxon Signed Ranks Test: $Z = -1.269$, $P = 0.204$) (Fig. 4). The tough spongin fibers were in significantly higher concentration in the interior of all three species (Wilcoxon Signed Ranks Test: $Z = -3.509$, $P < 0.001$; $Z = -4.074$, $P < 0.001$; and $Z = -2.159$, $P = 0.031$; for *I. felix*, *I. campana*, and *A. fulva*, respectively) (Fig 4).

A pattern indicative of a trade-off between chemical and structural defenses was not evident in these sponge species. No significant negative correlations between the concentrations of chemical and structural defenses were present within these three species when analyzed by univariate analyses (S3). Likewise, there were no significant positive correlations that might suggest a synergistic effect between these variables (S3). In the multivariate analyses, the PCA generated one or two (PC1 or PC2) principal components that together explained up to 87% of the variance in tissue nutritional and structural composition (S4). Factor scores from these axes, however, were not correlated with the concentrations of chemical defenses in any of the three species or regions of sponges.

Discussion

We found that while higher concentrations of chemicals in the outer tissue layers of *A. fulva* were congruent with predator deterrence allocation models proposed originally in terrestrial systems, the elevated levels of FTAs in the inner region of *I. felix* and the lack of significant differences between inner and outer regions in *I. campana* were not. Furthermore, chemical defenses did not appear to be concentrated in highly nutritious sponge tissues nor correlated with structural components, such as fibers, that may provide some level of physical defense against predators. These findings are counter to what would be expected under the assumption that resources used in the production

of these defensive metabolites are limited and so these metabolites should be found chiefly in areas more prone to predatory attack (McKey 1974, 1979; Rhoades 1979).

Evidence for spatial variation in antipredator chemicals within individual sponges is scant (but see Becerro et al. 1998; Kubanek et al. 2002; Furrow et al. 2003; Becerro and Paul 2004) relative to other benthic marine organisms (Hay 1996). Our demonstration that spatial variation in chemical defenses does exist within individuals of certain sponge species allows generation of testable hypotheses regarding the factors selecting for the observed patterns. For instance, we found that FTA concentrations in *I. felix* were significantly higher in inner as opposed to outer tissue regions. Such an allocation pattern may not be effective against predators that mouth the outer surface or remove small portions from the outer region of the sponge (Wulff 1994), but it may be an effective deterrent against large predators that bite through the first 2 mm into the inner tissues. Potential sponge predators on reefs in the South Atlantic Bight span this range of feeding types, e.g., generalist fishes (*Centropristus striata*, *Haemulon aurolineatum*, and *Diplodus holbrooki*) that lack adaptations for the consumption of sponges and may bite the outer layer without removing substantial tissue (Dunlap and Pawlik 1998), and sponge specialists like angelfish (*Holacanthus* sp. and *Pomacanthus* sp.), trunkfish (*Acanthostracion* sp.), and filefish (*Cantherhines macrocerus*) (Sedberry and Van Dolah 1984; Ruzicka and Gleason 2009). The latter bite repetitively and rapidly remove large areas of the sponge or entire sponges (Hourigan et al. 1989; Dunlap and Pawlik 1996). Given the higher concentration of antipredator defenses in inner tissues in *I. felix*, we hypothesize that the specialist group of predators exerts greater selective pressure on the distribution of secondary compounds. Thus, the higher concentrations of FTAs may act to protect the interior of the sponge, where basic sponge physiological processes such as reproduction and water pumping occur. In a recent study in Western Antarctica (Peters et al. 2009), the inner tissue of more than 50% of sponges assayed was deterrent to sea stars. These authors hypothesized that amphipod mesograzers may drive the production of defenses in the internal regions of these sponges (Peters et al. 2009). The evolution of chemical defenses in the interior of *Ircinia felix* may be an adaptation to deter feeding by the snapping shrimp, amphipods, and other mesograzers that we frequently observed within these sponges during dissection.

In contrast to the two *Ircinia* species, the distribution of brominated tyrosine derivatives within *A. fulva* was consistent with the hypothesis that levels of chemical defenses are higher in tissue regions with the greatest probability of being attacked initially by predators (Tugwell and Branch 1989; Becerro et al. 1998; Kubanek et al. 2002; Furrow et al. 2003). The antipredatory role of metabolites and crude extracts from sponges in the genus *Aplysina* has been clearly demonstrated (Pawlik et al. 1995; Ebel et al. 1997; Thoms et al. 2006; Ruzicka and Gleason 2009), but the ecological significance, in terms of predator deterrence, of differences on the order of 8.6% dry weight of sponge tissue toward the periphery of a sponge versus 4.8% in the inner region is largely untested. Chemical concentrations in the outer layer of *A. fulva* fell within the reported range of 7–12% found in natural populations of *Aplysina aerophoba* (Teeyapant et al. 1993), but the inner region concentrations were well below this level. Precursor molecules from various species of *Aplysina* have been shown to deter feeding at these natural concentrations (Thoms et al. 2004), and we hypothesize that the elevated concentrations in the outer regions of *A. fulva* are sufficient to defend this species from both generalist and specialist predators at reduced metabolic cost.

Allocating chemical defenses to tissues of high nutritional value to predators may also enhance prey fitness, especially if nutritious tissues are selectively targeted by predators (Rhoades 1979; Bowers and Stamp 1992) or if predators tolerate some level of defense to feed on highly nutritious tissue (Duffy and Paul 1992; Pennings et al. 1994). A positive correlation between concentrations of FTAs and protein in the outer tissue regions of *I. felix* agrees with this tenet of chemical ecology, but the absence of significant correlations between these two factors in the inner tissues of *I. felix* and inner and outer tissues of *I. campana* and *A. fulva* suggests that chemical defenses are not generally allocated to regions of high nutritional quality in sponges. Thus, either predators do not target regions with higher protein content intensely enough to select for increased levels of chemical defenses in these areas or the additional protection that would be obtained is not sufficient to warrant differential allocation. This lack of correspondence between antipredator compounds and protein concentrations is in agreement with Chanas and Pawlik (1995), who reported no overall differences in protein content between palatable and unpalatable sponge species.

Structural components might serve as effective predator deterrents in benthic marine invertebrates (Harvell et al. 1988; Pennings and Paul 1992; Ruzicka and Gleason 2009; but see Chanas and Pawlik 1995; 1996). If structural components are effective at deterring predators, then a tradeoff between chemical and structural defenses may exist where individuals or regions of individuals that are physically defended require lower investment in chemical defenses (Hay et al. 1988; Harvell and Fenical 1989; Becerro et al. 1998; Ruzicka and Gleason 2009). Alternatively, physical and chemical defenses may act synergistically to deter predators (Burns and Ilan 2003; Hill et al. 2005; Jones et al. 2005), resulting in high levels of chemical and physical defenses in regions of the sponge most likely to encounter

predators. In the current study, no tradeoffs (negative correlations) or evidence of synergy (positive relationships) were observed between these variables, suggesting that structural components in these three species are involved more in skeletal integrity of the sponge than in defense from predators (Chanas and Pawlik 1996).

We reiterate that the sponge species we investigated lacked spicules and this may have skewed our ability to detect correlations between structural and chemical defenses. Although spongin fibers may be largely indigestible for some predators, in general, they likely provide limited protection. Previous studies have shown that the ability of the intact spongin skeleton and associated structural materials in *I. felix*, *I. campana*, and *A. fulva* to deter generalist reef fish is significantly lower than that of their chemical defenses (Ruzicka and Gleason 2009). In contrast, spiculate sponges displayed higher levels of structural deterrence than aspiculate species (Ruzicka and Gleason 2009). Closer correlations between the concentration of structural components and antipredator compounds might have been observed had we included spiculate forms.

In conclusion, this study has documented variation in chemical defenses, nutritional quality, and structural components within individuals of three common temperate reef sponges of the South Atlantic Bight. In general, allocation patterns for secondary metabolites within individuals were not as we predicted, but in two of the three species, *I. felix* and *A. fulva*, there were significant differences in concentrations of chemical defenses in inner versus outer tissues. In light of this result, it may be informative to investigate the ecological significance of this chemical variation by testing the effectiveness of these differing concentrations in deterring predation by fishes and mobile macroinvertebrates.

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