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Inducible Chemical Defenses in Temperate Reef Sponges of the South Atlantic Bight, U.S.A.

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INDUCIBLE CHEMICAL DEFENSES IN TEMPERATE REEF SPONGES OF THE SOUTH ATLANTIC BIGHT, U.S.A

by

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(Under the Direction of Daniel F. Gleason)

ABSTRACT

Sessile organisms employing inducible defenses may receive protection from consumers while simultaneously minimizing the metabolic costs of maintaining these defenses. To investigate if reef sponges in the South Atlantic Bight employ inducible chemical defenses, I tested two predictions with *Ircinia campana*, *I. felix*, and *Aplysina fulva*. First, concentrations of antipredator compounds should covary with the abundance of sponge predators. Second, higher compound concentrations should be more effective at deterring predators. Secondary metabolite concentrations in two (*I. campana* and *I. felix*) of the sponge species showed temporal variation, which is consistent with the inducible defense hypothesis. Interestingly, higher concentrations of these compounds did not deter feeding by fish or urchins. In contrast, *A. fulva*, showed no significant temporal variation in the concentration of secondary metabolites, but had deterrent effects on fish predators even at low concentrations. Combined, these results do not support an antipredator strategy based on inducible chemical defenses.

INDEX WORDS: Temperate Reefs, Sponge, Inducible Chemical Defenses, South Atlantic Bight, Secondary Metabolites

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DEDICATION

I dedicate this work to Maria, my mother. I thank you for inspiring me to develop strength of character and passion for life. Though we have been separated for long periods of time throughout our lives, I always remembered your cheering words and sensed your wholehearted support. I feel so fortunate to have grown under the guidance and love of such a devoted mother. I want to show you my gratitude by dedicating this work to you Mom, and hope that I have made you proud.

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Introduction

Anti-predator and anti-herbivore defenses that are maintained in a constant shape, size, or concentration are termed constitutive; whereas, defenses that are newly formed or synthesized, or change in size or concentration following attack by consumers are known as inducible (Karban and Myers 1989, Harvell 1990). Most theoretical models addressing constitutive and inducible defenses in terrestrial plants assume that defenses are metabolically costly, and that organisms evolve defensive strategies that maximize survival while minimizing energy expenditure (Feeny 1976, McKey 1979, Rhoades 1979, Coley et al. 1985, Fagerstrom et al.1987). Inducible defenses may be favored when attack by consumers varies over spatial or temporal scales, and when this variation in attack rates is a reliable predictor of future tissue damage (Harvell 1990, Hay 1996, Karban and Adler 1996). For example, some terrestrial plants exposed to temporally variable levels of herbivory increase production of herbivore-deterrent compounds following tissue damage by grazers in preparation for the predictable increase in attack incidence (reviewed in Harbone 1986 and Havel 1987).

While inducible defenses do exact some cost, manifested as a reduction in fitness components such as growth rate or reproductive output, this cost is lower than for constitutive defenses because they are produced only when needed (Feeny 1976, McKey 1979, Karban 1993b). In addition to lower fitness costs, benefits thought to be associated with induced defenses include the following: (1) diminished auto-toxicity, because maintaining an elevated and constant store of toxic compounds is not necessary (Baldwin

and Callahan 1993), (2) specificity, because it allows prey to modify defenses according to the nature of the stimulus (i.e. predator type) allowing the organism to respond to environmental variability (Harvell 1990, Iyengar and Harvell 2002), and (3) amplification, which allows defenses to increase as predation becomes more intense (Harvell 1992, Karban et al. 1997).

The ubiquity and diversity of herbivore-induced chemical defenses in terrestrial plants (reviewed in Rhoades 1983) has led researchers to investigate the relevance of these mechanisms in marine species (Tugwell and Branch 1989, Cronin and Hay 1996, Pavia and Toth 2000, Toth et al. 2005). Marine algae produce a diversity of secondary metabolites that deter feeding by herbivores (Norris and Fenical 1982, Faulkner 1984). Herbivore-induced secondary metabolites in marine algae have been demonstrated mainly in the phylum Phaeophyta (Van Alstyne 1988, Cronin and Hay 1996, Pavia and Toth 2000). Among marine animals, inducible defenses are predicted to be most common in colonial invertebrates because, like plants, they are modular, sessile as adults, and often subject to partial predation (Harvell 1999). In particular, the modular design of both plants and colonial marine invertebrates often allows one or more repeated units to be lost to consumers without mortality of the entire individual or colony (Harvell 1999). Thus, grazing can provide a reliable and non-lethal cue that enables mobilization of defenses that can effectively protect undamaged tissue (Harvell 1986).

Based on the above background we might expect to detect inducible defenses in colonial marine invertebrates exposed to distinct spatial and temporal variation in consumer activity (Harvell 1990, Hay 1996, Karban and Adler 1996). Accordingly, I conducted an initial investigation of the potential for inducible chemical defenses in

temperate reef sponges that occur in the South Atlantic Bight (SAB). The SAB encompasses a region from Cape Hatteras, NC to Cape Canaveral, FL with approximately 30% of this area consisting of temperate reefs composed of lithified limestone or sandstone embedded with fossilized scallop shells (Harding and Henry 1994, Erv Garrison pers. comm.). These reefs support a diversity of sessile and mobile invertebrates that include representatives from sponges, corals, bryozoans, tunicates, echinoderms, and crustaceans as well as a number of tropical and subtropical fishes (Struhsaker 1969, Miller and Richards 1980, Freeman et al. 2007).

A key feature of these SAB reefs that would favor inducible defenses in sessile benthic invertebrates is the observed seasonal variation in predatory fishes. Specifically, the distinct seasonal changes in water temperature (ranging from 13ºC in winter to 28ºC in summer) that occur on these reefs result in predator assemblages differing throughout the year with higher fish densities occurring during the warmer months, particularly August through September (Sedberry and Van Dolah 1984). Among colonial marine invertebrates occurring on these reefs, sponges appear to be good candidates for inducible defenses. The use of secondary metabolites to deter predators has been well-documented in sponges (Bakus et al. 1986, Mc Clintock 1987, Schulte and Bakus 1992, Pawlik et al. 1995, Uriz et al. 1996, Wilson et al. 1999, Assmann et al. 2000, Burns et al. 2003, and others), including species occurring on SAB reefs (Ruzicka 2005, Freeman 2007, Ruzicka and Gleason 2008). In addition, there is evidence of qualitative and quantitative changes in sponge secondary metabolites following tissue wounding (Walker et al. 1985, Zea et al. 1999, Thoms et al. 2006) that enhance deterrence of feeding by predators (Ebel et al. 1997, Richelle-Maurer et al. 2003). To my knowledge, however, there has been

only one study addressing explicitly the potential for inducible chemical defenses in sponges and this possibility was dismissed because mechanical injury did not enhance predator deterrence over time (Swearingen and Pawlik 1998).

The goal of my study was to obtain an initial assessment of whether temperate reef sponges in the SAB employ inducible chemical defenses to deter predation. Based on the working hypothesis that sponges occupying SAB reefs employ inducible chemical defenses, I tested two predictions of this hypothesis. First, if inducible chemical defenses against predators are common in these sponges, then higher concentrations of secondary metabolites should be observed when potential sponge consumers (i.e., fishes) are most abundant. Second, for induction of chemical defenses to be favored, higher secondary metabolite concentrations should be more effective at deterring potential predators. To address these predictions, I quantified the concentrations of secondary metabolites from tissues of *Ircinia campana, I. felix,* and *Aplysina fulva* collected at different times during the year and conducted palatability assays with artificial food cubes containing different concentrations of sponge crude extracts fed to fish and sea urchins. Results from these two assays were then viewed within the framework of published fish abundance surveys conducted by other investigators in the SAB.

Methods

Study site

This study was conducted at J-Reef (31° 36.0 N, 80° 47.4 W), a temperate hard-bottom reef located approximately 35 km off the state of Georgia coast in North America. Unlike tropical coral reefs that are built by living hard corals, J-Reef consists of a tertiary bedrock outcropping, lined with a matrix of smooth sand and fossil scallop shell

inclusions that projects 2 m above the continental shelf at a water depth of 18-20 m (Garrison et al., in press).

Sponge species

The concentration of anti-predator compounds was quantified from tissues of the stinker vase sponge, *I. campana*, the stinker amorphous sponge, *I. felix,* and the rope sponge, *A. fulva* (Figure 1). These species were chosen because secondary metabolites of sponges belonging to these genera have been identified and have been shown to be predator-deterrent in previous studies (Pawlik et al 1995, Ebel et al. 1997, Tsoukatou et al. 2002, Freeman 2007, Ruzicka and Gleason 2008). In addition, these sponges were abundant and large enough to provide adequate sample sizes.

Tissues from sponges of the genus *Ircinia* lack spicules, are compressible, tough in texture, difficult to cut or tear, and emanate a sulfur-garlic odor when handled (Kelly-Borges and Pomponi 1992, Duque et al., 2001). These sponges produce several linear furanosesterterpene tetronic acids (FTAs) (Cimino et al 1972c, Cimino et al. 1975, Lumsdon et al. 1992, Capon et al 1994, Martinez et al. 1997) that exhibit antibiotic, antifouling, and predator-deterrent properties (Faulkner 1973, Pawlik et al.1995, Epifanio et al.1999, Tsoukatou et al. 2002). FTAs from *I. campana* and *I. felix* at J-reef were isolated and identified previously by Freeman (2007) as a mixture of the compounds: variabilin, felixinin, and strobilinin (Faulkner 1973, Martinez et al. 1995, Martinez et al. 1997).

Likewise, tissues from sponges of the genus *Aplysina* lack spicules, but are soft, fleshy, firmer, and easy to cut or tear. Defensive mechanisms in these sponges are thought to derive from the presence of brominated-tyrosine derivatives (Ebel et al. 1997,

Thoms et al. 2004, 2006) which, in addition to predator-deterrence, also have shown antimicrobial and cytotoxic activity (Teeyapant et al. 1993, Betancourt-Lozano et al. 1998). Freeman (2007) also isolated brominated-tyrosine derivatives from *A. fulva* at J-reef and identified a mixture of 9 different compounds. Of these 9 compounds, 8 have been previously classified as high molecular weight precursor compounds: aerophobin-1, aerophobin-2, aplysinamisin-1, hydroxyaerothionin, hydroxy-oxo-aerothionin, homoaerothionin, aerothionin, and fistularin-3 (Walker et al. 1985, Teepayant and Proksch 1993, Ciminiello et al. 1994, Ebel et al. 1997, Thoms et al. 2004, 2006). These compounds are known as precursors because they serve as starting molecules for the synthesis of the low molecular "activated" compound aeroplysinin-1 (Thoms et al. 2006).

Sponge tissue collection

Ten individuals from each species were haphazardly selected and marked by attaching a combination of metal tags and plastic construction tape to the adjacent substrata with masonry nails. Tissue samples were collected from these tagged individuals on 21 April, and again on 7 June, 25 August, and 6 October of 2006 (Table 1). An equal number of haphazardly selected individuals from the surrounding community also were sampled at each time period to serve as controls for any changes in chemical composition induced by repeated wounding of tagged individuals. These individuals were marked to keep them from being sampled more than once. To minimize the impacts of variability in chemical concentrations that might be present within individuals, 5 tissue samples (approximately 5 ml each) were removed from different regions (i.e., top, sides, and base) of each sponge using a dive knife. This protocol resulted in some regions of a sponge being sampled more than once. For example, in

I. campana and *I. felix* a typical sample set consisted of 1 tissue sample from the top, 2 from the same side, and 2 from the base on opposite sides, or 2 from the top on opposite sides, 1 from the side, and 2 from the bottom on the same side. This sampling regimen was feasible on both *I. campana* and *I. felix* because of their large surface areas as well as their vase and amorphous growth forms, respectively (as defined by Freeman et al. 2007). In contrast, *A. fulva* is a branching sponge, so I sampled tops, sides, and bases from different branches of the same individual. Once I collected the 5 tissue samples from each individual, these were combined and stored as a single sample set in a plastic bag. Upon surfacing, tissue samples were wrapped in aluminum foil and stored in liquid nitrogen (-196° C) for transport to the laboratory (transit time approximately 8 hours). In the laboratory, samples were transferred to an ultracold freezer (-70° C) and stored there until further processing.

Secondary metabolite extraction and quantification

Frozen sponge samples were lyophilized and homogenized using a mortar and pestle for *A. fulva*, and chopped into fine pieces with a razor blade for *I. campana* and *I. felix*. Lyophilized samples were weighed to the nearest 0.1 mg on an analytical balance (APX-60, Denver Instruments, Denver, CO). Approximately 1 g of each lyophilized sample was extracted 3 times in a 10 ml mixture of 1:1 dichloromethane (DCM) and methanol (MeOH) at 4° C for 24 hours. The 3 crude extract solutions were pooled, filtered (P8 coarse filter paper, Fisher Scientific Company L.L.C., Pittsburg, PA) and 10 ml portions decanted into 3 pre-weighed 30-ml scintillation vials. Excess solvent from crude extracts was removed by rotary evaporation (model SC210A-115, Thermo Electron Corporation, Somerset, NJ) and resulting material was stored at -70º C. One vial of the 3

obtained from each sponge sample was used for quantification of secondary metabolites via high performance liquid chromatography (HPLC) and the other 2 vials were used for food preparation for field and laboratory feeding assays.

The concentration of FTAs in *I. campana* and *I. felix* was quantified via HPLC following established techniques (Martinez et al. 1997, Freeman 2007). Vials containing *I. campana* and *I. felix* crude extract were lyophilized for approximately 12 hours to ensure anhydrous conditions before being acetylated, and reweighed to obtain the mass of crude extract. Immediately after freeze drying, 2 ml of pyridine were added and the vials were sonicated (FS20 Fisher Scientific Ultrasonic Cleaner) until complete dissolution was achieved. Once the sample was dissolved, I added 1 ml of acetic anhydride and stirred this solution with a magnetic stir bar for 6 hours. After stirring, the mixture was poured into a 50 ml beaker containing 30 ml of cold (9º C) deionized and filtered water, and stirred for 5 minutes. This mixture was extracted twice with 20 ml of ethyl acetate resulting in 2 distinguishable organic layers. The clear upper organic layer from each extraction was removed using a 5 ml pipette, combined, and dried by rotary evaporation (Brinkmann/Buchi Rotavapor® Collegiate, Eppendorf, Germany) at 55º-60º C until a thick yellow-brownish residue was all that remained. This residue was re-suspended in 1 ml of 100% MeOH per 20 mg of crude extract and sonicated to complete dissolution. To remove any particulates, samples were filtered through a PTFE 45 µm syringe filter directly into an auto-sampler vial for HPLC quantification. A volume of 9 µl was injected into a Shimadzu HPLC system fitted with a Phenomenex Gemini C-18 analytical column (4.6 x 250 mm). The mobile phase consisted of a 93:7 mixture of MeOH and H_2O at a flow rate of 1 ml min^{-1}. Detection of FTAs was at 270 nm.

Brominated-tyrosine derivatives in *A. fulva* were quantified via HPLC following techniques of Puyana et al. (2003) and Freeman (2007). Vials containing *A. fulva* crude extracts were lyophilized for at least 12 hours to ensure removal of water, and reweighed to obtain the mass of crude extract. Lyophilized samples were resuspended in 1 ml of 100% MeOH per 10 mg of crude extract, sonicated to complete dissolution, and filtered through a PTFE 45 μm syringe filter directly into an auto-sampler vial for HPLC quantification. A volume of 7 μl was injected into the HPLC system described above. Compounds were eluted with a solvent gradient of acetonitrile ($CNCH_3$) and H_2O buffered in 0.1% formic acid delivering 90% H_2O for the first 3 minutes with a gradual increase to 100% $CNCH_3$ over 28 minutes. The solvent flow rate was 1 ml min^{-1} with compound detection of brominated-tyrosine derivatives at 254 and 280 nm. Quantification of anti-predator compounds from both species was determined by comparing peak areas to corresponding standards of known concentrations.

Food preparation and feeding assays

Artificial food cubes containing sponge secondary metabolites were prepared for fish and sea urchin feeding assays following established techniques (Pawlik et al. 1995, Ruzicka 2005). Concentrated crude extracts were resuspended in 1 ml of MeOH and sonicated to complete dissolution. Artificial food cubes were prepared using a mixture of 2.1 g of powdered squid mantle, 0.84 g type I carageenan, 0.14 g of agar, and 42 ml of distilled water. All ingredients were thoroughly mixed and heated in a microwave until boiling. The heated mixture was added to a 50 ml beaker containing sponge crude extract dissolved in 1 ml of MeOH. This concoction was mixed thoroughly and poured into 1 x 1 x 1 cm cube molds and allowed to cool. Once cooled, the food cubes were carefully

removed and stored in plastic vials at -4º C. Control cubes were prepared in the same manner, but there was no sponge extract dissolved in the 1 ml of MeOH.

Artificial food cubes for fish were initially prepared using 6 different secondary metabolite concentrations that spanned the range of compound concentrations that I quantified in the J-reef sponge population. These concentrations were as follows: 11.0, 8.0, 5.0, 2.5, 0.6, 0.1 mgg^{-1} of sponge dry tissue for *I. campana,* 15.0, 10.0, 5.0, 2.0, 0.5, 0.05 for *I. felix*, and 27.0, 19.0, 12.0, 7.0, 2.0, and 0.6 of dry sponge tissue for *A. fulva*. To verify that the food cubes retained the original anti-predator compound concentrations, spare food cubes from *I. campana, I. felix,* and *A. fulva* were processed for quantification of secondary metabolites via HPLC in the same manner as in sponge tissue samples. This process revealed predictable and consistent degradation of secondary metabolites in food cubes prepared with crude extracts from *I. campana* and *A. fulva* (Figure 2). In contrast, attempts to quantify secondary metabolites in food cubes prepared with crude extracts from *I. felix* were not successful suggesting that these compounds may have degraded to the point that concentrations were below the detection limits of my HPLC instrumentation.

Even though the concentrations of secondary metabolites in food cubes made from *I. campana* and *A. fulva* were less than originally intended, they still spanned the range of mean concentrations that I detected in tissues of tagged sponges at J-reef. Ultimately, the ability of these secondary metabolites to deter feeding by fish was tested at the following concentrations: 3.0, 1.2, 0.7, 0.3, 0.1, 0.02 mgg^{-1} of dry sponge tissue for *I. campana*, and 18.0, 12.0, 7.0, 4.0, 1.5, and 0.4 mgg^{-1} of dry sponge tissue from

A. fulva. Concentrations of secondary metabolites in food cubes equaled that found in approximately 8 ml and 6 ml of sponge volume for *I. campana* and *A. fulva* respectively.

Fish feeding assays were conducted at J-reef during May 2007. A total of 20 food cubes for each secondary metabolite concentration and a control were stored in 50 ml centrifuge tubes and hand carried to the bottom in a mesh dive bag. Feeding trials proceeded by reaching into the mesh bag, grabbing a centrifuge tube haphazardly, and dispensing 2 food cubes sequentially to fish. Once observation of fish feeding behavior was complete, this tube was placed in a second mesh bag and the process was repeated until all concentrations had been tested and all the tubes were in the second bag. This process of haphazardly selecting tubes, testing the food cubes against fish feeding, and storing the most recently tested tube in a new mesh bag continued until all cubes had been dispensed. To reduce the potential for bias, the concentration of secondary metabolites in the food cubes being dispensed was unknown to the observer. This was accomplished by having an independent party select the tubes via lottery and label them alphabetically. Thus, each letter corresponded to a specific compound concentration, but this quantity was unknown to the observer carrying out the feeding assay.

Treatment and control cubes were dispensed haphazardly to generalist fish predators such as *Centropristus striata* (black seabass), *Haemulon aurolineatum* (tomtates), and *Diplodus holbrooki* (spottail pinfish). A food cube was considered unpalatable if it was rejected more than twice or if it sank to the bottom uneaten. Food cubes from a single sponge species were dispensed during one dive. Feeding assays with cubes containing extracts from *I. campana* and *A. fulva* were accomplished in a single day; whereas *I. felix* food cubes were tested 7 days later. Food cubes were generally

offered to groups of 10 or more fish initially, but as feeding assays progressed, more fish would congregate and participate. In an attempt to increase the number of naïve fish involved in these assays, 3 times during a dive I would swim a distance of approximately 15 m across the reef and resume feeding assays. *Centropristus striata* was most commonly involved in these feeding assays, whereas feeding by *H. aurolineatum*, and *D. holbrooki* was less frequent.

Sea urchin feeding assays were conducted in the aquarium room of the biology building at Georgia Southern University. Twenty-three individuals of the common sea urchin, *Arbacia punctulata*, were collected at J-reef and kept in 75.7 L aquaria with artificial seawater at a salinity of 32 ‰. Sea urchins were starved for 48 hours prior to feeding assays. Each sea urchin was used for a single feeding trial in one of the two sponge species.

Artificial food cubes for the sea urchin feeding assays were prepared in the same manner as for fish trials. However, only 3 secondary metabolite concentrations and a control were used because preliminary assays showed that sea urchins were able to sample this many treatments in a 32 hour period before decomposition of food cubes occurred. Degradation of secondary metabolites also occurred during food cube preparation and the mean percent degradation rates closely matched those from fish feeding assays (Table 2). The actual concentrations tested were: 3.0, 0.4, 0.02 mgg^{-1} of dry sponge tissue for *I. campana* and 17.0, 4.0, 0.4 mgg^{-1} of dry sponge tissue for *A. fulva***.** Food cube design was similar to that used by Hay et al. (1994) and Freeman (2007), where a small piece (approximately 7 x7 mm) of fiberglass window screening (1 x 2 mm open area) was inserted into each 1.5 cm^3 cube mold prior to the addition of

the heated food mixture. Once the artificial food mixture congealed, a small piece of monofilament line was threaded through the embedded screen for attachment to a larger support screen (approximately 140 x 130 mm). The purpose of this screen was to serve as a point of attachment to hold the food cube in place during the feeding assays.

Congealed food cubes were removed from their mold, blotted dry, and weighed. One food cube per secondary metabolite concentration and one control (4 cubes total) were attached to the larger support screen and were placed equidistant in a square arrangement. This design allowed a sea urchin placed in the middle of all food cube choices to explore all food types with its tube feet. Prior to introducing urchins to the treatments, attachment bases were secured to the bottoms of Glad© plastic containers (3.07 L) that had holes drilled in their sides to facilitate sinking when placed inside the aquaria. These containers were large enough for sea urchins to move freely when the lid was sealed. Six plastic containers holding food cubes prepared with crude extracts from the same sponge species were stacked into a 75.7 L aquarium.

Individual sea urchins were left to feed on all food cubes inside their feeding containers for approximately 32 hours. This amount of time was sufficient for sea urchins to sample all treatments before decomposition of food cubes occurred. Upon completion of the feeding assays, sea urchins were removed from their feeding containers and placed in a different aquarium. The support screens holding the food cubes were detached from every feeding container and individual food cubes were carefully removed from the screens, blotted dry, and weighed to determine the percent consumed. To ensure that loss of mass from food cubes was due entirely to consumption by sea urchins, additional food cube treatments were exposed to the same conditions but without sea urchins and

reweighed after 32 hours. In addition, I verified the concentration of secondary metabolites in these food cubes via HPLC in the same manner as in sponge tissue samples.

Statistical analyses

Differences in the concentration of FTAs from tissues of tagged individuals of *I. campana* and *I. felix* were compared across collection dates using a repeated-measures ANOVA. Sample sizes in tagged individuals decreased by 2 in *I. campana* after 25 August and by 1 in *I. felix* on 6 October. To maintain equal sample sizes, only those individuals that were present on all 4 collection dates were used in the repeated-measures ANOVA. In order to detect significant differences between pairs of collection dates, I made pairwise comparisons between collection dates that seemed to be most different using a paired t-Test. This test was used as an alternative to a Tukey Kramer means pairwise comparison because tagged individuals were not independent.

FTA concentrations from samples of untagged individuals of *I. campana* and *I. felix* were compared across collection dates using a one-way ANOVA because different sets of sponges were sampled on each date. This analysis was followed with a Tukey Kramer test for multiple comparisons ($\alpha = 0.05$). I also compared differences in FTA concentrations between tagged and untagged individuals of *I. campana* and *I. felix* with a two-way ANOVA using date of collection and sample type (tagged or untagged) as factors. Deviations from the assumptions of normality and equal variances were analyzed with a Shapiro-Wilk and a Levene test respectively. A $\log_{10}$ transformation was applied prior to all statistical analyses because FTA concentrations in *I. campana* and *I. felix* were not normally distributed (Shapiro-Wilk test: $W= 0.82$, $p < 0.0001$ and $W= 0.75$, $p < 0.001$

for tagged and untagged *I. campana* respectively, and W= 0.65, $p < 0.0001$ and W= 0.93, $p < 0.04$ for tagged and untagged *I. felix* respectively) (Sokal and Rohlf 1995).

Non-parametric tests were used to evaluate differences in the concentration of sponge secondary metabolites from all *A. fulva* because data did not meet normality assumptions despite a $\log_{10}$ transformation (Shapiro-Wilk test: W= 0.93, $p = 0.02$ and W= 0.78, $p < 0.0001$ for tagged and untagged respectively). For tagged individuals of *A. fulva* the chemical concentrations from individual samples were ranked within their respective collection period and compared using a Friedman's method for randomized blocks. Secondary metabolite concentrations from untagged individuals of *A. fulva* were ranked and compared across collection periods with a Kruskal-Wallis test (Sokal and Rohlf 1995).

Statistical analyses for deterrence of fish feeding by food cubes prepared with *I. campana* extracts were not warranted because all food cubes, both controls and treatments, were consumed. The same consumption pattern was observed for *I. felix* food cubes but verification of FTA concentrations in these cubes via HPLC was not possible. Being unable to determine whether production of food cubes from *I. felix* extracts had caused significant degradation of the antipredator compounds, I excluded this species from statistical analyses evaluating feeding deterrence for both fish and sea urchins. To compare deterrence of fish feeding by food cubes prepared with *A. fulva* crude extracts, I used a 7 x 2 contingency table with a G-test of independence (Sokal and Rohlf 1995).

Data from feeding by sea urchins on food cubes formulated with chemical extracts from *I. campana,* and *A. fulva* were expressed as % of weight loss from each

food cube, square root arcsin transformed, and analyzed using a repeated-measures ANOVA (Sokal and Rohlf 1995).

Results

Secondary metabolite concentrations in sponge tissues

If temperate reef sponges of the SAB are using an antipredator strategy based on inducible defenses, then I would expect the concentration of secondary metabolites to vary over time. More specifically, if these changes in concentration represent a response to fish predators, I would expect to see the highest concentrations of secondary metabolites in August when fish densities have been reported to be at their peak in the SAB (Sedberry and Van Dolah 1980).

Of all 4 sampling dates (21 April, 7 June, 25 August, and 6 October), tagged individuals of *I. campana* exhibited significantly higher concentrations of furanosesterterpene tetronic acids (FTAs) on 7 June only. In untagged individuals, FTAs were also significantly higher on 7 June when compared to 25 August and 6 October (Figure 3). *Ircinia felix* showed a similar pattern in terms of FTA concentrations. However, the values observed on 7 June were significantly higher only in tagged individuals and when compared to samples collected on 21 April and 6 October (Figure 4).

Variances in mean FTA concentrations extracted from tagged sponges were greater on 7 June than at any other date in both *I. campana* and *I. felix*. In general, FTA concentrations in these two species did not exceed 1.6 and 0.9 mgg^{-1} for *I. campana* and *I. felix*, respectively. The large variances in FTA concentrations observed on 7 June in each sponge species (see Figures 3 and 4) were driven primarily by a single sample with

an FTA concentration of 11 mgg^{-1} in *I. campana* and 14 mgg^{-1} in *I. felix.* However, removing these outliers (Grubb's test: $z = 2.3$, $p < 0.05$ for *I. campana,* and $z = 2.6$, $p < 0.05$ for *I. felix*) from statistical comparisons of mean FTA concentrations across collection dates did not affect the statistical outcomes reached for *I. campana* (Figure 5). In *I. felix,* FTA concentrations sampled on 7 June were significantly higher than 21 April and 6 October, and also 25 August (Figure 6).

Although higher brominated-tyrosine derivatives were observed in *A. fulva* on 21 April and 7 June for both tagged and untagged sponges, the large variance in secondary metabolite concentrations among individuals on these dates resulted in no significant differences among sample periods (Figure 7).

In addition to temporal variation in secondary metabolite concentrations, I predicted that there would be significant differences in chemical concentration between tagged and untagged sponges if mechanical damage induces up-regulation of antipredator defenses. Counter to this prediction, mechanical damage of *I. campana,* as represented by repeated sampling of the same individuals, appeared to have no long term (i.e., several months) impacts on FTA concentrations. Specifically, a two-way ANOVA showed that there was no significant difference in FTA concentrations between tagged and untagged individuals and that differences observed were strictly a function of collection date (Table 3). In *I. felix,* FTA concentrations differed significantly between tagged and untagged individuals (Table 4), but this result was a function of the differences in concentration observed on a single date, 7 June. FTA concentrations in tagged and untagged *I. felix* were similar on the other three sample dates with tagged individuals showing slightly lower, but non-significant mean concentrations on two of these dates (i.e., 21 April and

6 October). Again, these results are inconsistent with the prediction that mechanical damage causes increases in secondary metabolite concentrations for an extended period.

Feeding assays

If temperate reef sponges of the SAB are using an antipredator strategy based on inducible defenses, then I would also predict that higher concentrations of secondary metabolites should be more effective at deterring potential predators. Fishes that consumed food cubes prepared with crude extracts from all 3 sponge species were primarily *Centropristus striata* (black seabass), and occasionally *Haemulon aurolineatum* (tomtate), and *Diplodus holbrooki* (spottail pinfish). In *C. striata,* an entire food cube was sucked in and either consumed entirely or ejected all at once. In contrast, both *H. aurolineatum* and *D. holbrooki* either ingested small portions until the entire food cube was gone, or avoided the cube after the first bite.

Ircinia campana extracts did not deter feeding by fish and both control and treatment food cubes were consumed completely. The same result was observed with food cubes containing *I. felix* extracts. In *I. felix*, it is possible that compound degradation during food preparation resulted in these food cubes containing extremely low concentrations of FTAs. Evidence for this possibility is provided by the fact that I was unable to detect these compounds via HPLC after re-extracting them from the food cubes.

Aplysina fulva extracts were more deterrent to fish than controls at every secondary metabolite concentration tested (Table 5). A regression analysis of the percent of feeding deterrence as a function of secondary metabolite concentration resulted in a power function showing that the net gain in terms of enhanced feeding deterrence decreases rapidly once brominated-tyrosine derivative concentrations are >8 mgg^{-1}

(Figure 8). Interestingly, the highest mean concentration of brominated tyrosine derivatives measured in the population fell just below this value (see Figure 7).

In the sea urchin feeding assays, control food cubes lost more weight than treatment food cubes in both *I. campana* and *A. fulva,* but this was only significant in *A. fulva* (Figure 9). No significant differences were observed in the percent weight loss between the 3 treatment food cubes in either sponge species when controls were excluded from the analysis (repeated-measures ANOVA: $F = 0.10$, $df = 2, 33$, $p = 0.9$ for *I. campana,* and $F = 0.48$, $df = 2, 30$, $p = 0.62$ for *A. fulva*). All *I. campana* food cubes were consumed in similar proportions as the control (Figure 9), including *I. campana* cubes containing secondary metabolite concentrations comparable to the highest FTA mean (2.7 mgg^{-1}) found in the population (see Figure 3). By contrast, control food cubes from *A. fulva* feeding trials were significantly favored over treatment food cubes to include those containing the lowest secondary metabolite concentrations (0.4 mgg^{-1}) found at J-reef (see Figure 7).

Food cubes exposed to the same aquarium conditions but without sea urchins showed a mean weight loss of 0.03 g ± 0.002 S.E. (n = 32). This value was approximately 13 times lower than the minimum amount of weight loss observed in food cubes exposed to sea urchins suggesting that the loss of weight from treatment food cubes was mostly due to consumption by sea urchins.

Discussion

Marine plants and sessile animals may employ one or more strategies when allocating limited resources to antipredator defenses (reviewed in Cronin 2001). Constitutive defenses may be favored when predation rates are constant and predictable

(Harvell 1986, Adler and Harvell 1990). In contrast, inducible defenses occur when levels of predation are spatially or temporally variable and when cues emitted by predators are good predictors of future attacks (Harvell 1990). Accordingly, an inducible defense that is meant to confer protection against consumers should be maximized during periods of greater consumer abundance and activity (Rhoades 1979, Havel 1987, Harvell 1990). Results presented here showed temporal variation in the concentration of antipredator secondary metabolites in two (*I. campana* and *I. felix*) out of three species of sponges from the SAB; a result consistent with a strategy based on inducible defenses. Interestingly, however, variation in the concentration of these compounds appears to have no measurable impact on feeding by generalist fish or urchin predators. In contrast, the third sponge species investigated, *A. fulva*, showed no significant temporal variation in the concentration of secondary metabolites, but had deterrent effects on generalist fish predators even at low concentrations. These results are supportive of a constitutive chemical defense in *A. fulva*.

Chemical defenses in *I. campana* and *I. felix*

To further investigate the relationship between chemical defenses in *I. campana* and *I. felix* and the abundance of potential spongivorous fishes, I compared the temporal variation in sponge secondary metabolite concentrations I observed with temporal patterns of reef fish abundances reported in the literature. The most comprehensive assessment of seasonal trends in fish abundances in the SAB comes from Sedberry and Van Dolah (1984). These investigators surveyed fish populations from 9 different hard bottom reefs scattered throughout the SAB during winter (January through March) and summer (August through September) months of 1980. The data used in this study

includes surveys from Grays Reef National Marine Sanctuary (31º 23.6 N, 80º 53.1 W), a hard bottom reef that is 23 km from J-Reef (31° 36.0 N, 80° 47.4 W). Both reefs have similar topographical features, depth and temperature profiles, as well as sponge and fish species (Ruzicka and Gleason 2008). Fishes surveyed by Sedberry and Van Dolah (1984) included representatives from both generalist (i.e., *C. striata* and *H. aurolineatum*) and spongivorous species, such as angelfishes and trunkfishes (i.e., *Holacanthus bermudensis,* and *Acanthostracion quadricornis*). These investigators found fish densities to be substantially higher during summer compared to winter (Figure 10). These higher fish abundances during summer, specifically during the month of August, correspond with lower concentrations of secondary metabolites in *I. campana* and *I. felix* found in this study. Acknowledging that there are significant limitations in comparing data on fish abundances and sponge secondary metabolite concentrations that are from single and different years, this analysis suggests that *I. felix* and *I. campana* from the SAB do not possess defenses induced by fish predation.

Further evidence supporting the contention that the temporal changes in secondary metabolites observed in *I. campana* do not represent an inducible defense against predators comes from the *in situ* and laboratory feeding assays with fish and urchins, respectively. Results from feeding trials with generalist fish predators showed that FTAs in *I. campana* were unable to deter feeding by fish at the highest mean FTA concentrations (3.0 mgg^{-1}) found in the J-reef population (Figure 3). These results contrast with similar studies conducted at J-reef where approximately 35% of food cubes prepared with *I. campana* extracts were rejected by the same fish species (Ruzicka 2005, Freeman 2007). It should be noted that the concentrations of FTAs contained within food

cubes in these earlier studies were not determined and there was no attempt by these investigators to mimic the secondary metabolite levels occurring in sponge tissues. Rather, secondary metabolites were extracted in bulk using large volumes of sponge tissue and may have resulted in unnaturally high concentrations of chemicals within food cubes. In contrast, the concentration of secondary metabolites in my food cubes was manipulated to match that found in 1 g of dry sponge tissue (approximately 8 ml). Though the FTA levels in *I. campana* food cubes were much lower than originally intended because of some degradation during processing, I was able to test deterrence at concentrations that were representative of those found in the J-reef population. Clearly, these differences in concentration could account for the disparate results among studies. Further investigation is needed to determine if natural concentrations of secondary metabolites deter feeding by generalist and spongivorous fish species.

In tropical regions, predation on sponges is restricted to a few specialized groups of consumers, notably angelfishes, trunkfishes, filefishes, and parrotfishes, as well as hawksbill turtles, starfish, sea urchins, and nudibranch molluscs (Randall and Hartman 1968, Vance 1979, Meylan 1988, Paul 1992, Birenheide et al. 1993, Wulff 1994, Wulff 1995, Dunlap and Pawlik 1998, Leon and Bjorndal 2002). Gut content analyses on angelfishes of the genera *Pomacanthus*, *Holocanthus,* and filefishes of the genus *Cantherines* have shown that these sponge predators consume *Ircinia sp.* (Randall and Hartman 1968). Ruzicka and Gleason (2008), using a combination of data they collected on SAB reefs and published information from Florida/Caribbean sites, investigated latitudinal variation in spongivorous fish densities and feeding deterrence of conspecific sponge crude extracts from temperate and subtropical localities. They concluded that, in

general, sponges in the SAB are less deterrent to fishes and are exposed to lower predation pressure than their subtropical conspecifics. The results of my feeding assays with *I. campana* extracts, showing lack of deterrence of fishes, are consistent with the prediction that predation pressure is low enough to allow minimal investment into chemical defenses.

The laboratory feeding trials conducted here corroborate previous findings (i.e. Freeman 2007) that the sea urchin, *A. punctulata,* is able to tolerate food cubes containing chemical extracts from *I. campana* that are equivalent to the highest mean levels observed in the J-reef population (Figure 9). While laboratory feeding assays do provide useful information, how these results relate to the field is uncertain. For example, it is unknown currently whether mobile invertebrates at J-reef represent a significant threat to sponge fitness. At any rate, it does not appear that at least one common invertebrate predator, *A. punctulata*, is driving the temporal differences in secondary metabolite concentrations observed for *I. campana* on J-reef.

Given that *I. felix* and *I. campana* do not possess inducible chemical defenses against predators, what might explain the temporal fluctuations in secondary metabolite concentrations observed in this study? One possibility is that temporal patterns of production in sponges represent a response to other biotic factors that co-vary with water temperature (Green et al. 1985, Turon et al. 1996, Duckworth et al. 2004, Page et al. 2005, Abdo et al. 2007). In particular, seasonal increases in secondary metabolites may be an inducible defensive strategy against biofouling agents (i.e., bacteria, fungi, algae, and invertebrate larvae of non-sponge species) that become more abundant as water temperature rises (Green et al. 1985, Wahl 1989, Duckworth and Battershill 2001). At

J-reef, however, there does not appear to be a strong positive relationship between temperature and secondary metabolite concentrations in *I. felix* and *I. campana*. Temperatures measured hourly during 2006 were highest in late July and August and corresponded with low secondary metabolite concentrations (Figure 11). Thus, it is unlikely that the high concentrations I observed in June in *I. felix* and *I . campana* represent either direct or indirect effects of temperature fluctuations.

While not addressed in this study, it should be noted that two other factors have been hypothesized to contribute to seasonal differences in the concentration of secondary metabolites in the tissues of sessile, benthic invertebrates such as sponges: food availability and reproductive state. Most taxa of sessile, benthic invertebrates (including Porifera) that have been investigated in cold temperate seas show annual cycles in secondary metabolite production with highest concentrations in tissues during spring and summer when nutrient levels are highest (Hughes 1989, Coma et al. 2000). On SAB reefs, seasonal differences between warm, low-density surface waters and cold, high-density waters peak during the month of June (Blanton et al. 2003). This water column stratification is thought to promote particle sinking and may enhance nutrient concentrations towards the bottom (Nybakken 2001) where sponges occur. Thus, these excess nutrients may provide *I. campana* and *I. felix* with additional energetic resources for the production of expensive secondary metabolites.

Layered upon these cycles of food availability are differences in sponge reproductive state. Gametogenesis, an energetically costly process in itself, results in a partial loss of feeding activities of the choanocyte chambers in sponges and may lead to reduced energy reserves (Fromont and Bergquist 1994, Ilan 1995). All species of *Ircinia*

are brooders with breeding cycles lasting up to 8 months (Hoppe 1988). For example, the Caribbean sponge, *I. strobilina*, bears reproductive structures (i.e. spermatic cysts and oocytes) and developing larvae from September through April (Hoppe 1988). While not yet investigated, if *I. campana* and *I. felix* in the SAB exhibit similar reproductive periodicity then the higher levels of FTAs observed in June may reflect reallocation of energy reserves in the interval between reproductive cycles (see Turon et al. 1996).

While most of the hypotheses outlined above assume that the production of secondary metabolites is costly, it should be recognized that this may not be the case. It has been suggested that secondary metabolites that are ineffective predator deterrents may represent (1) accumulations of side products from synthetic pathways, (2) waste or detoxification products, or (3) vestigial products that played a role in the past against predators that have now gone extinct (Haslam 1986, Pawlik 1993, Hay and Steinberg 1992). Additionally, the possibility exists that some of these metabolites may still be produced because they are tied to the synthesis of a functional metabolite or because costs are not sufficiently intense to discontinue production (Pawlik 1993).

Chemical defenses in *A. fulva*

Previous studies have shown that the bromotyrosine derivatives in *Aplysina* sp. are stored within tissues at relatively high concentrations and serve as precursors for rapid bioconversion into more potent "activated" compounds (Thoms et al. 2006). These activated compounds, rather than deterring predators, are thought to provide immediate protection against invading pathogens, such as bacteria, in wounded sponges (Thoms et al. 2006). In contrast, the precursor bromotyrosines are much more effective than the activated compounds at deterring predation by fish (Thoms et al. 2004, Thoms et al.

2006). Thus, these bromotyrosine derivatives appear to serve a dual function: first as a constitutive defense against predation and second as an inducible defense against infection.

Aplysina fulva occurring on tropical reefs are consumed by angelfishes of the genus *Pomacanthus,* and at a disproportionately high rate by the trunkfish, *Acanthostracion quadricornis* (Wulff 1994), which is also found on SAB reefs (Sedberry and Van Dolah 1984, pers. obs.). Though predation rates on *A. fulva* inhabiting temperate reefs have not been investigated, specialization by *A. quadricornis* on *A. fulva* may extend to the temperates and exert the selective pressure necessary for the evolution of constitutive chemical defenses. This hypothesis is consistent with fish feeding assays conducted in both temperate and tropical regions, where bromotyrosine derivatives in *A. fulva* from both localities have been demonstrated to be highly deterrent (Pawlik et al. 1995, Ruzicka 2005, Freeman 2007, Ruzicka and Gleason 2008).

Ideally, a defensive compound should provide enough protection from predation to enhance significantly individual survival while simultaneously minimizing resource investment into maintenance of this compound (Schulte and Bakus 1992). Carrying out fish feeding assays with food cubes containing a range of *A. fulva* secondary metabolite concentrations allowed assessment of the relationship between quantities of bromotyrosine derivatives in sponge tissues and the effectiveness of deterrence. This analysis led to two important outcomes relevant to the evolution of antipredator secondary metabolites in this species (Figure 8). First, possessing bromotyrosine derivatives at any level within the tissues is better than none at all; as evidenced by the fact that even the lowest concentrations (1 mgg^{-1}) deter feeding by fish approximately

20% of the time. Second, in terms of deterrence of fish predators, the added advantage of maintaining brominated-tyrosine derivatives drops precipitously at concentrations >8 mgg^{-1}. Given these outcomes, I would predict that the optimal concentrations of brominated-tyrosine derivatives in the *A. fulva* population at J-reef should be between 2 and 8 mgg^{-1}. In fact, on all 4 sample dates mean concentrations measured in the population were within or just below this range (Figure 7). Thus, I hypothesize that these brominated-tyrosine concentrations allow *A. fulva* to maintain a balance between fitness gains and cost of secondary metabolite production.

Recommendations and conclusions

My study, like most others in this area, conducted palatability assays using a flavored, heat-treated polysaccharide mixture that is added to sponge crude extracts (Epifanio et al. 1999). Several secondary metabolites, including FTAs, contain highly reactive compounds that may suffer decomposition when exposed to excessive heat treatment (Epifanio et al. 1999, Mark Hamann pers. comm.). Until this study, the magnitude of degradation in bioactive compounds from *I. campana* and *A. fulva* exposed to heat-treated artificial food recipes was unknown. I found that secondary metabolites exposed to high levels of heat may degrade up to 86% and 43% in *I. campana* and *A. fulva* respectively (Table 2a, b), and that the rate of degradation is predictable. These findings underscore the importance of quantifying compounds in prepared food treatments because decomposition or alteration of bioactive compounds during chemically-mediated processes used in artificial food recipes may impede realistic assessments of sponge defensive chemistry (see Epifanio et al. 1999). Furthermore, matching the chemical concentrations in food cubes with those found in the natural

population provides for a better understanding of the evolution of these chemicals, as shown in my analysis of *A. fulva*.

Previous studies conducted on colonial marine invertebrates have suggested that induction of structural defenses, rather than chemical, is more common (reviewed in Harvell 1999). Examples include production of spines and stolons in bryozoans, changes in sclerite size and density in gorgonian corals, and alterations in polyp behavior and nematocyst density in scleractinian corals (Osborne 1984, Harvell 1984a, Gaulin et al. 1986, Harvel 1990 and all references therein, Harvell 1992, Harvell 1999, West 1997, Iyengar and Harvell 2002, Gochfeld 2004). Some investigators have hypothesized that the lack of chemical induction in marine systems may be due to one or both of the following: 1) an actual rarity of this response (Hay and Steinberg 1992), and 2) insufficient appropriate and relevant experimental designs (Harvell 1999, Pavia and Toth 2000). I specifically designed this study to obtain a preliminary assessment of the potential for inducible chemical defenses in 3 of the most abundant sponge species of the SAB (Ruzicka 2005).

Although I identified temporal variability in the concentration of antipredator chemistry in two of the three species, the results do not provide strong support for induced chemical defenses against predators in these sponges. Specifically, the temporal shift in fish densities observed at SAB reefs does not appear to provide the appropriate selective pressure to favor the evolution of predator-induced chemical defenses in these sponges. Future studies should build on these findings by investigating the role played by other spongivorous predators, including small cryptic invertebrate forms that reside in

sponge tissues (Freeman 2007), in allocating resources to antipredator chemical defenses in temperate reef sponges.

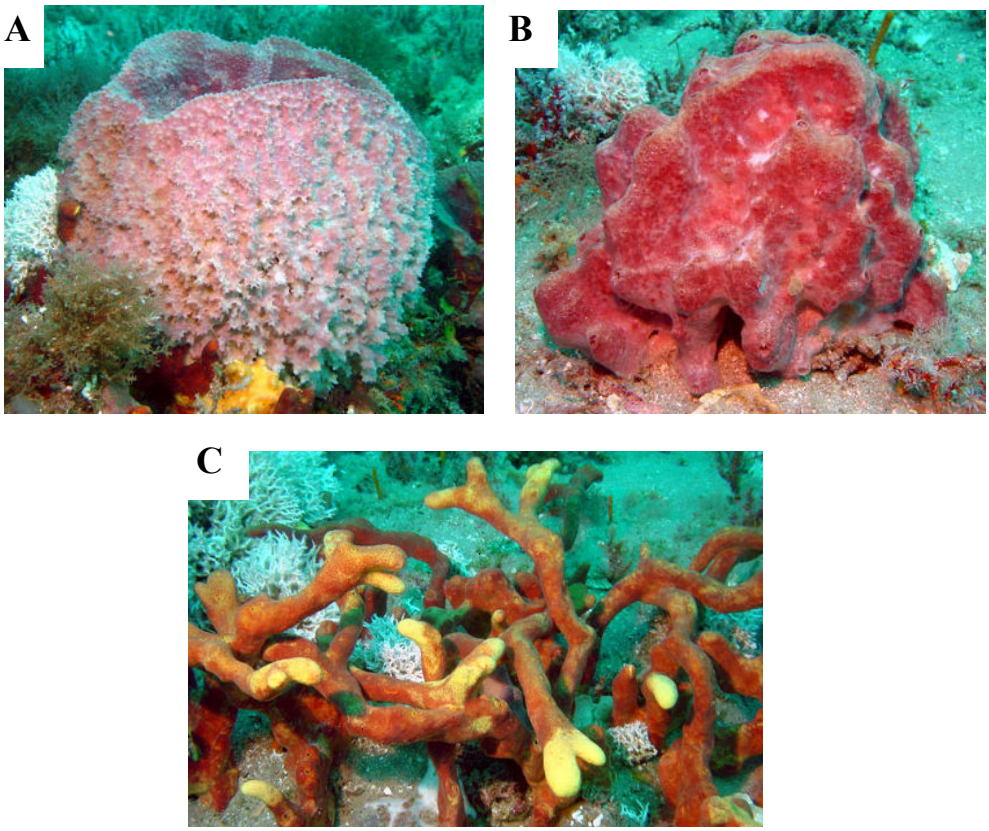

Figure 1. The sponge species *I. campana* (A), *I. felix* (B) and *A. fulva* (C) from J-reef. Photographs courtesy of Greg McFall.

Table1. Dates and number of individuals sampled from each sponge species at J-reef. Two tagged *I. campana* individuals were missing from the reef during the last 2 collection dates and 1 tagged *I. felix* individual was missing on the last collection date. All samples were collected during 2006.

Species	Date	Tagged	Untagged
I. campana	21 Apr	10	10
	7 Jun	10	10
	25 Aug	8	10
	6 Oct	8	10
I. felix	21 Apr	10	10
	7 Jun	10	10
	25 Aug	10	10
	6 Oct	9	10
A. fulva	21 Apr	10	10
	7 Jun	10	10
	25 Aug	10	10
	6 Oct	10	10

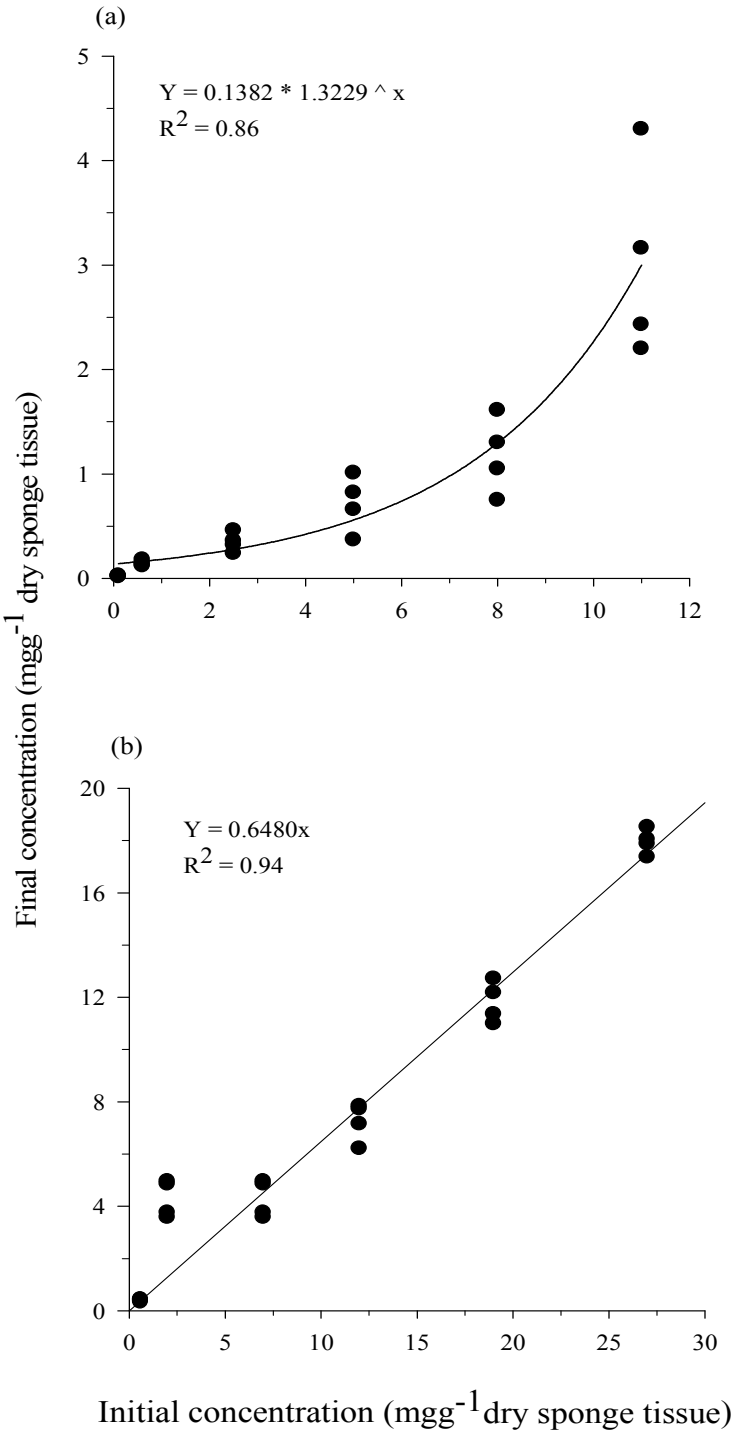

Figure 2. Secondary metabolite concentrations of *I. campana* (a) and *A. fulva* (b) before and after preparation of food cubes used for fish feeding assays. Initial concentration refers to secondary metabolites from sponge tissues and final concentration refers to secondary metabolites from food cubes.

Table 2. Mean % (± S.E.) degradation of secondary metabolites (mgg^{-1} dry sponge tissue) following preparation of food cubes for fish (a, n = 4) and sea urchin (b, n = 3) feeding assays. Quantification of secondary metabolites in post-treated food cubes via HPLC showed that FTAs from *I. campana* used in fish and urchin feeding assays degraded an average of 80.9% and 78.2% respectively. Degradation of brominated-tyrosine derivates in *A. fulva* was lower at 34.9 % and 37.0 % for food cubes used in fish and urchin feeding assays respectively.

	I. campana			*A. fulva*	
a	Pre-treatment	Post-treatment (mean %, ± s.e.)	*a*	Pre-treatment	Post-treatment (mean %, ± s.e.)
	11.0	73.0 (± 4.2)		27.0	33.5 (± 0.8)
	8.0	85.2 (± 2.2)		19.0	37.8 (± 2.0)
	5.0	85.7 (± 2.7)		12.0	39.7 (± 3.0)
	2.5	86.2 (± 1.8)		7.0	37.9 (± 5.2)
	0.6	75.8 (± 2.2)		2.0	25.7 (± 3.5)
	0.1	79.5 (± 2.7)		0.6	34.9 (± 3.5)
b	11.0	73.2 (± 1.9)	*b*	27.0	35.0 (± 1.4)
	2.5	84.7 (± 0.3)		7.0	43.4 (± 2.7)
	0.1	76.8 (± 1.4)		0.6	32.8 (± 2.6)

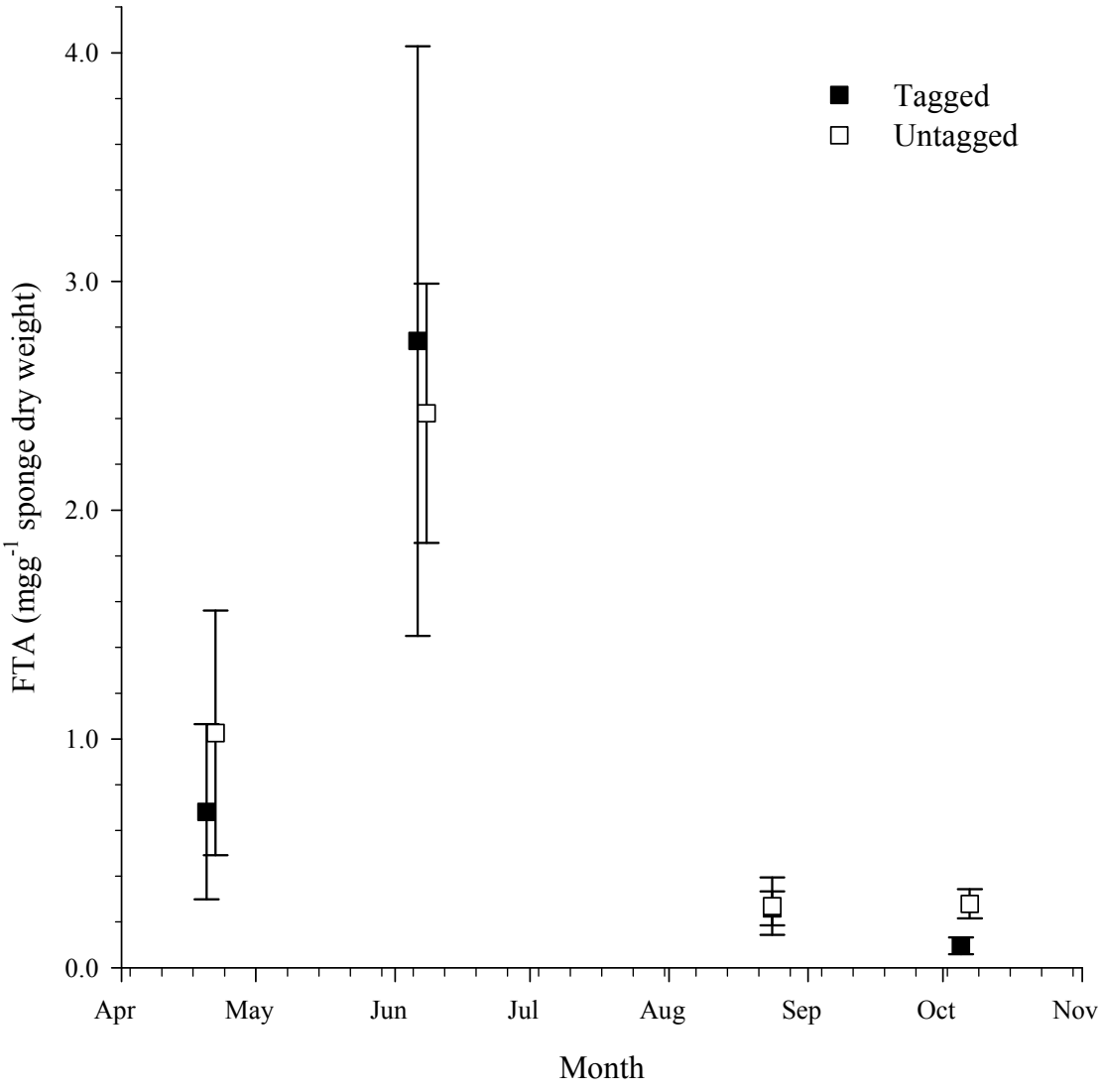

Figure 3. Mean (± S.E.) FTA concentration in *I. campana.* Tagged samples analyzed by repeated measures ANOVA: $F = 19.7$, $df = 3, 21$ $p < 0.0001$, $n = 8$ for all dates. FTA concentrations from samples collected on 7 June were compared to remaining collection dates with a paired t-Test: $t = 2.54$, $df = 14$, $p = 0.01$ for 7 June vs. 21 April, $t = 4.06$, $df = 14$, $p < 0.001$ for 7 June vs. 25 August, and $t = 5.72$, $df = 14$, $p < 0.0001$ for 7 June vs. 6 October. Untagged samples analyzed by ANOVA: $F = 10.6$, $df = 3$, $p < 0.0001$, $n = 10$ for all dates. A Tukey Kramer pairwise comparison ($\alpha = 0.05$) showed significant differences in FTA concentrations between 7 June and 25 August, and 7 June and 6 October..Each minor tick on the *x*-axis represents one week starting on a Monday

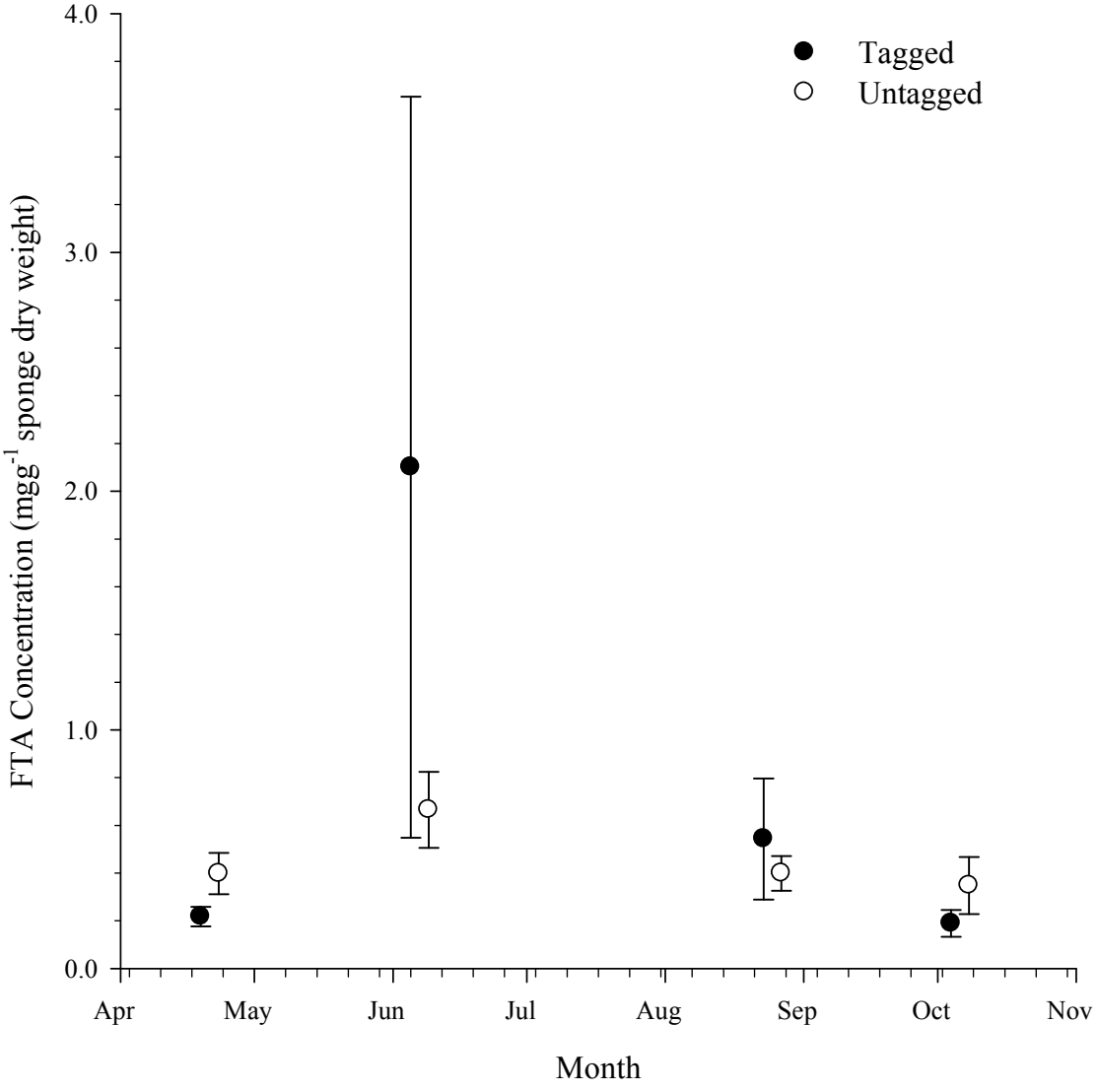

Figure 4. Mean (± S.E.) FTA concentration in *I. felix.* Tagged samples analyzed by Repeated Measures ANOVA: $F = 4.7$, $df = 3, 24$, $p < 0.01$, $n = 9$ for all dates. FTA concentrations from samples collected on 7 June were compared to remaining collection dates with a paired t-Test: $t = 2.45$, $df = 16$, $p = 0.01$ for 7 June vs. 21 April, $t = 1.48$, $df = 16$, $p = 0.07$ for 7 June vs. 25 August, and $t = 2.93$, $df = 16$, $p < 0.01$ for 7 June vs. 6 October. Untagged samples analyzed by ANOVA: $F = 1.2$, $df = 3$, $p = 0.3$, $n = 10$ for all dates. Each minor tick on the *x*-axis represents one week starting on a Monday.

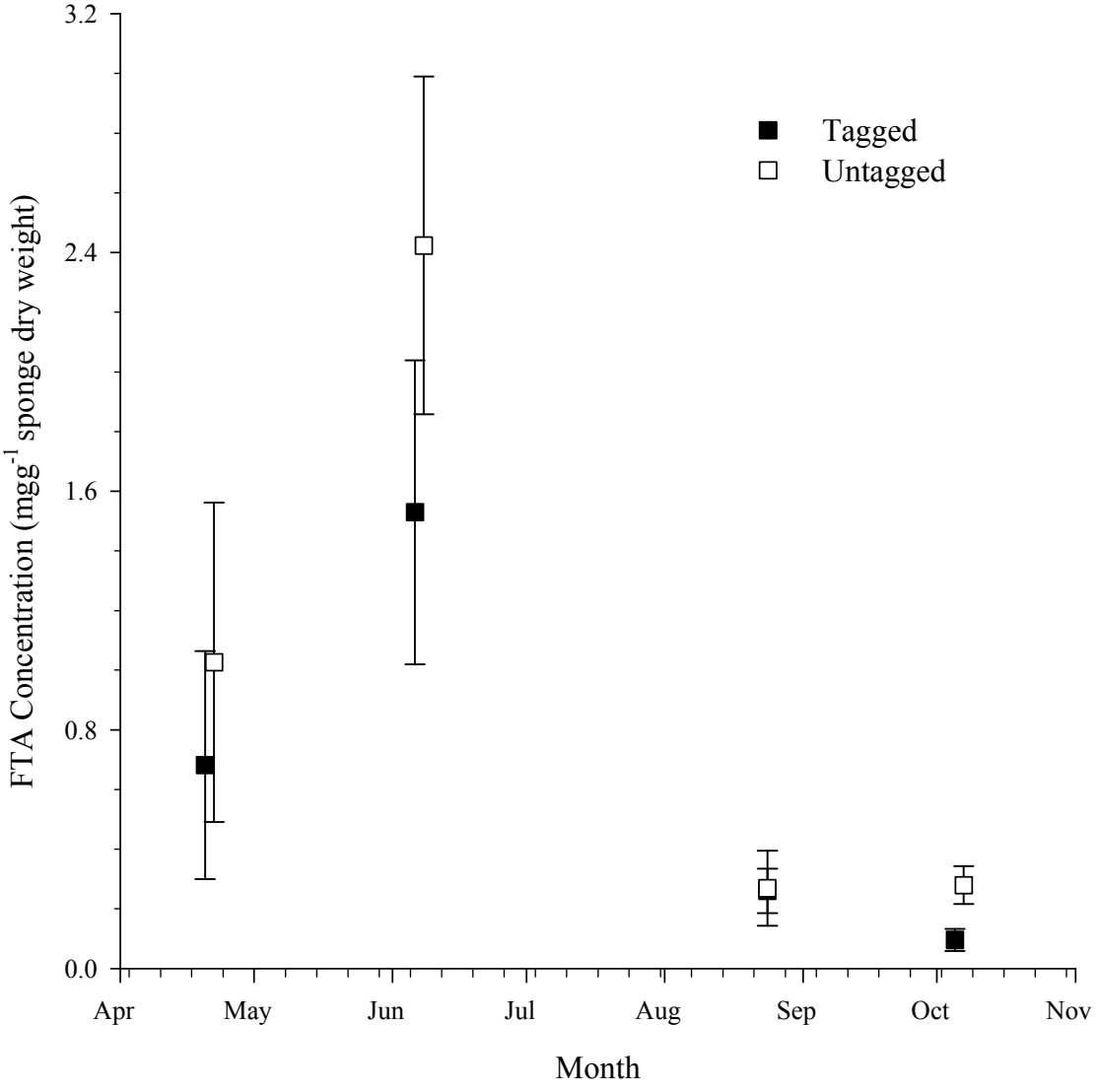

Figure 5. Mean (± S.E.) FTA concentration in *I. campana* excluding highest FTA value. Sample size of tagged sponges in each collection date does not include the sample containing the highest concentration of FTAs (11 mgg^{-1}) recorded on 7 June. Tagged sponges were analyzed by repeated-measures ANOVA: $F = 18.00$, $df = 3, 18$, $p < 0.0001$, $n = 7$ for all dates. FTA concentrations from samples collected on 7 June were compared to remaining collection dates with a paired t-Test: $t = 2.04$, $df = 12$, $p = 0.03$ for 7 June vs. 21 April, $t = 4.75$, $df = 12$, $p < 0.001$ for 7 June vs. 25 August, and $t = 6.62$, $df = 12$, $p < 0.0001$ for 7 June vs. 6 October. Analysis for untagged samples is the same as Figure 3.

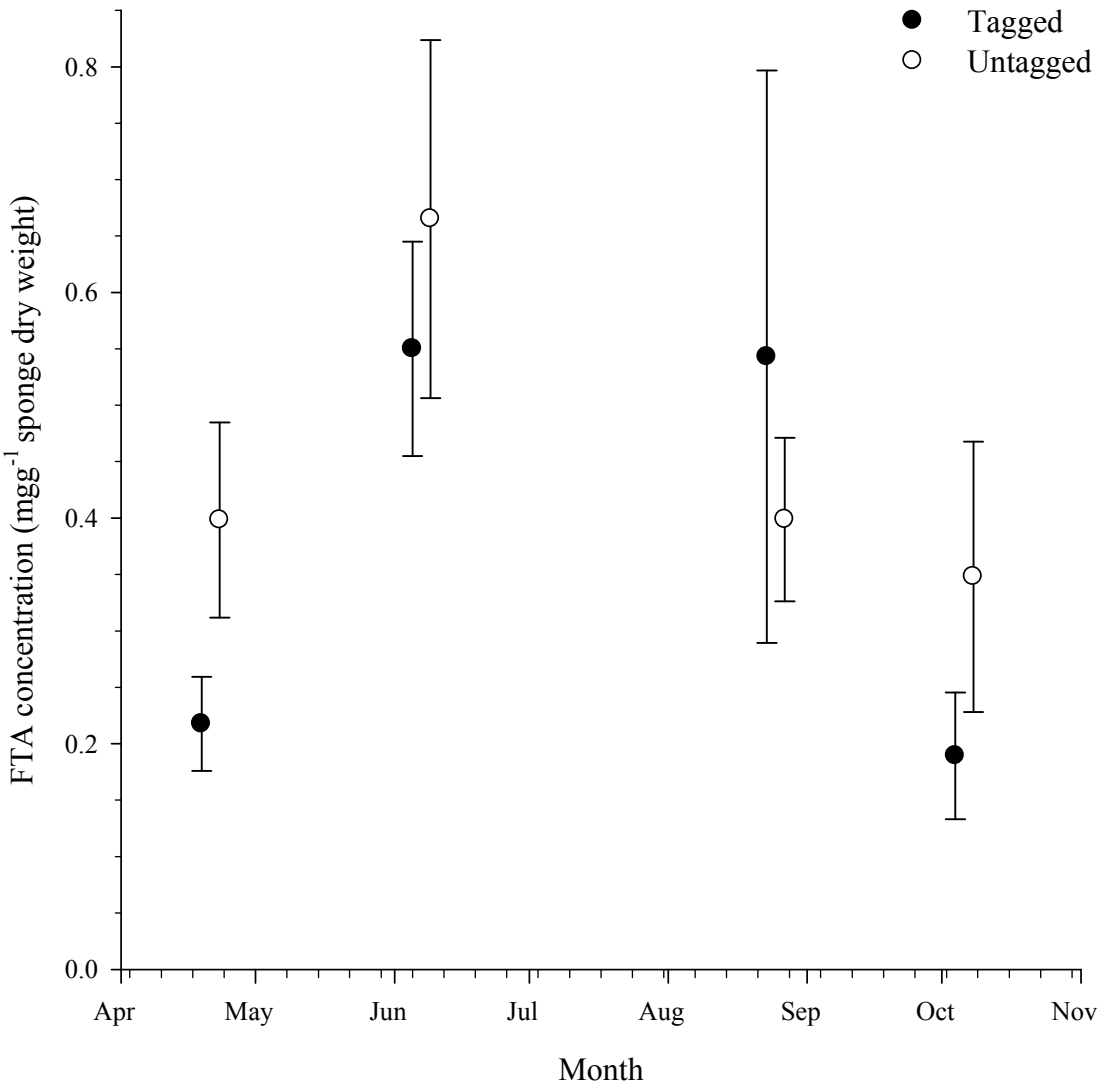

Figure 6. Mean (± S.E.) FTA concentration in *I. felix* excluding highest FTA value. Sample size of tagged sponges in each collection date does not include the sample with the highest concentration of FTAs (14 mgg^{-1}) recorded on 7 June. Tagged sponges were analyzed by repeated-measures ANOVA: $F = 3.24$, $df = 3, 21$, $p = 0.04$, $n = 8$ for all dates. FTA concentrations from samples collected on 7 June were compared to remaining collection dates with a paired t-Test: $t = 2.17$, $df = 14$, $p = 0.02$ for 7 June vs. 21 April, $t = 1.81$, $df = 14$, $p = 0.04$ for 7 June vs. 25 August, and $t = 3.15$, $df = 14$, $p < 0.003$ for 7 June vs. 6 October. Analysis for untagged samples is the same as Figure 4.

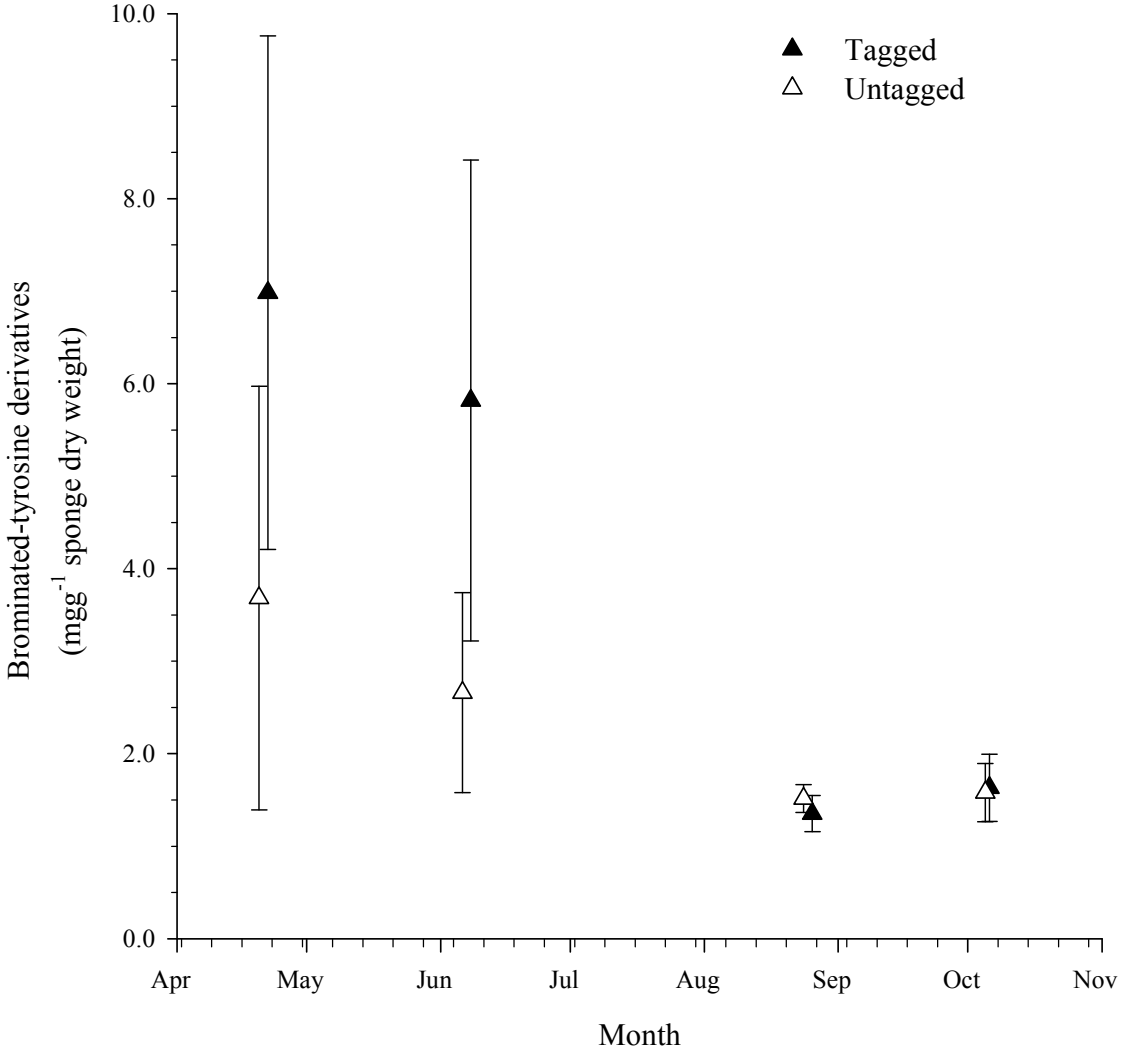

Figure 7. Mean (± S.E.) concentration of brominated-tyrosine derivatives in *A. fulva.* Tagged samples analyzed by Friedman's test: χ^2= 5.26, df= 3, p= 0.15, n = 10 for all dates. Untagged samples analyzed by Kruskal Wallis: H= 1.3, df= 3, p= 0.7, n = 10 for all dates.

Table 3. Two-way ANOVA for concentrations of FTAs in *I. campana* between sample type (tagged and untagged sponges) and among dates of collection.

Source	SS	d.f.	*F*	*P*
Sample type (St)	0.52	1	1.54	0.30
Collection date (Cd)	13.04	3	12.90	0.03
St x Cd	1.01	3	1.78	0.15
Error	12.08	64		
Total	26.43	71		

Table 4. Two-way ANOVA for concentrations of FTAs in *I. felix* between sample type (tagged and untagged sponges) and among dates of collection.

Source	SS	d.f.	*F*	*P*
Sample type (St)	1.13	1	46.43	0.006
Collection date (Cd)	2.53	3	34.60	0.007
St x Cd	0.07	3	0.13	0.93
Error	12.13	68		
Total	15.83	75		

Table 5. Food cubes prepared with crude extracts from tissues of *A. fulva* consisted of 6 different secondary metabolite concentrations and a control that were offered to natural assemblages of fish in the field. Data were analyzed using a 7x2 contingency table ($G = 6.9$, $df = 6$, $p < 0.0001$), n = 20 and ♦ n = 19.

Concentration (mgg^{-1})	Food cubes rejected
18.0	16
12.0	15 ♦
7.0	13
4.0	11
1.5	6 ♦
0.4	3
Control	0

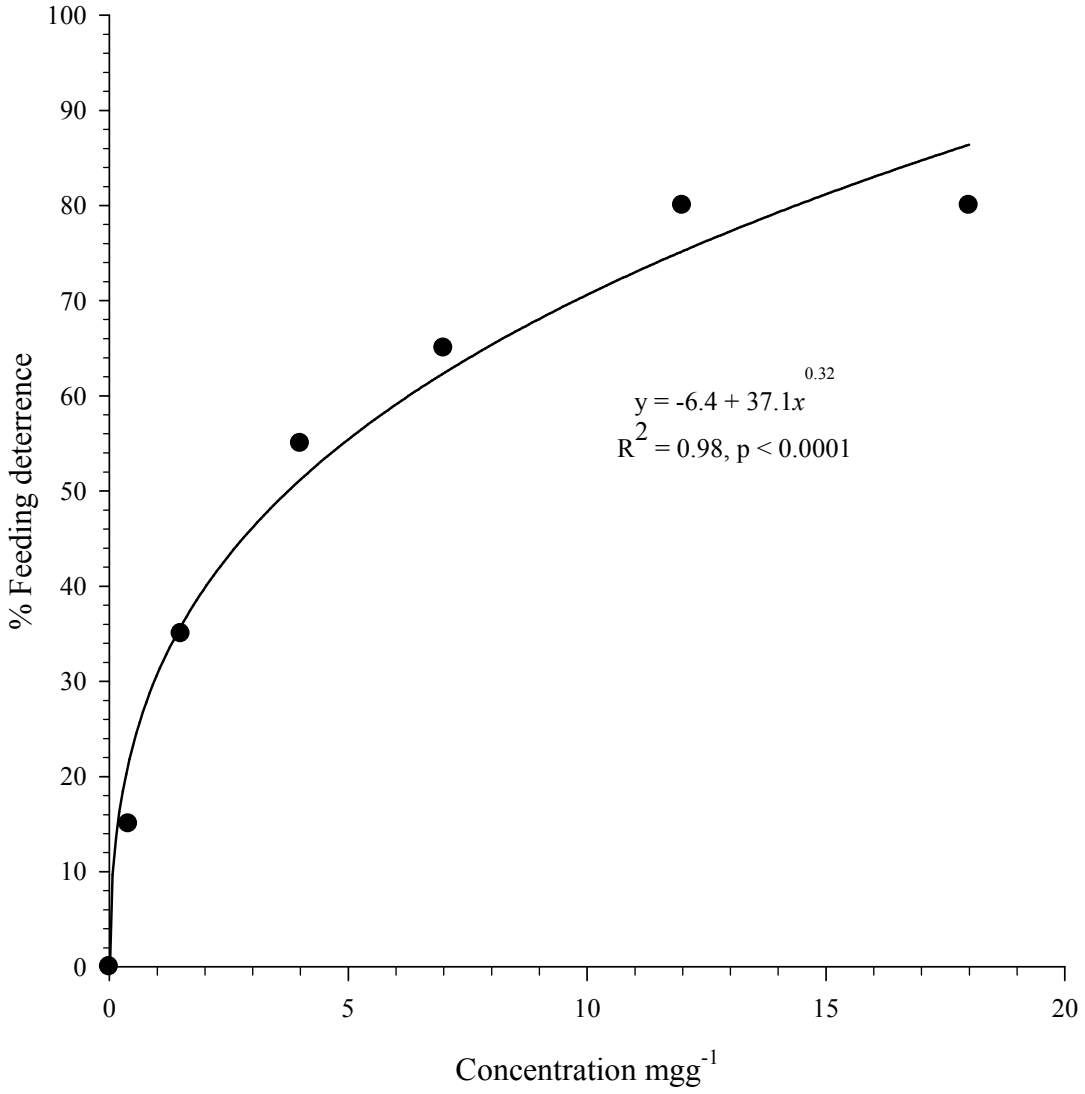

Figure 8. The relationship between *A. fulva* secondary metabolite concentrations in food cubes and deterrence of feeding by generalist fish predators. The regression equation for the functional relationship between the concentration of brominated-tyrosine derivatives and % of feeding deterrence was generated from data collected during fish feeding assays. ● Represents treatment food cubes used in the field

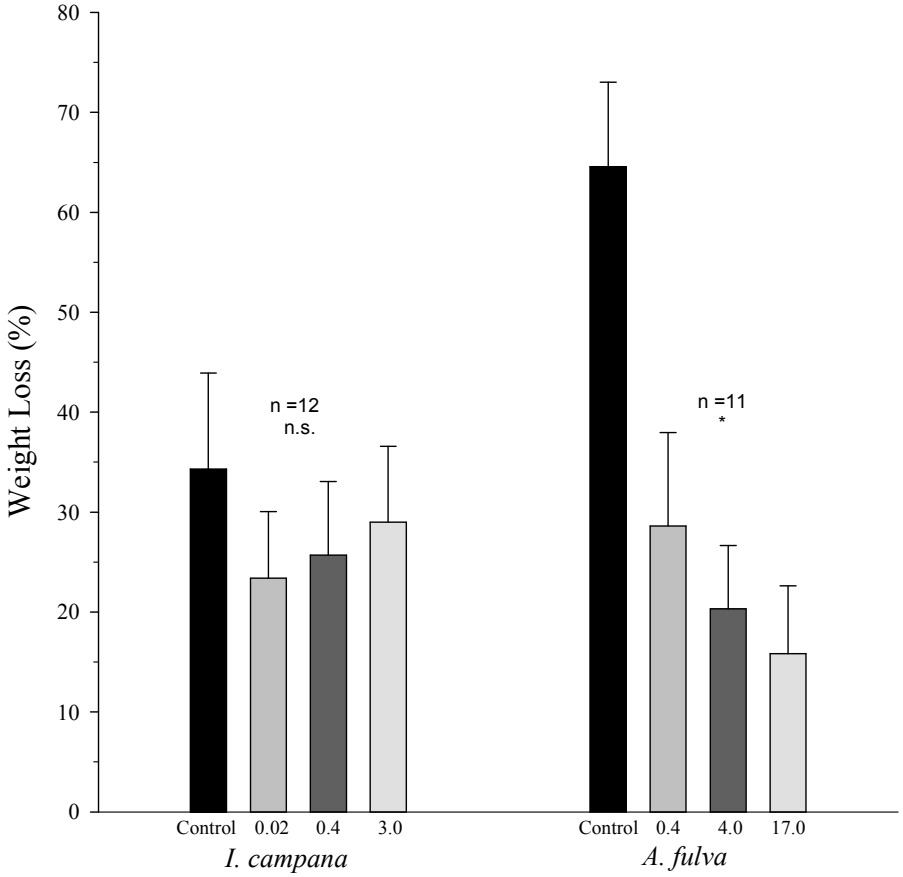

Figure 9. Mean (± S.E.) percent weight loss from food cubes prepared with crude extracts with different concentrations of secondary metabolites (mgg^{-1}) from *I. campana* and *A. fulva* that were fed to the sea urchin *A. punctulata.* Control food cubes contained no sponge crude extracts. Data were analyzed using a repeated measures ANOVA: $F = 6.79$, $df = 3, 40$, $*p < 0.001$, n.s.= not significant.

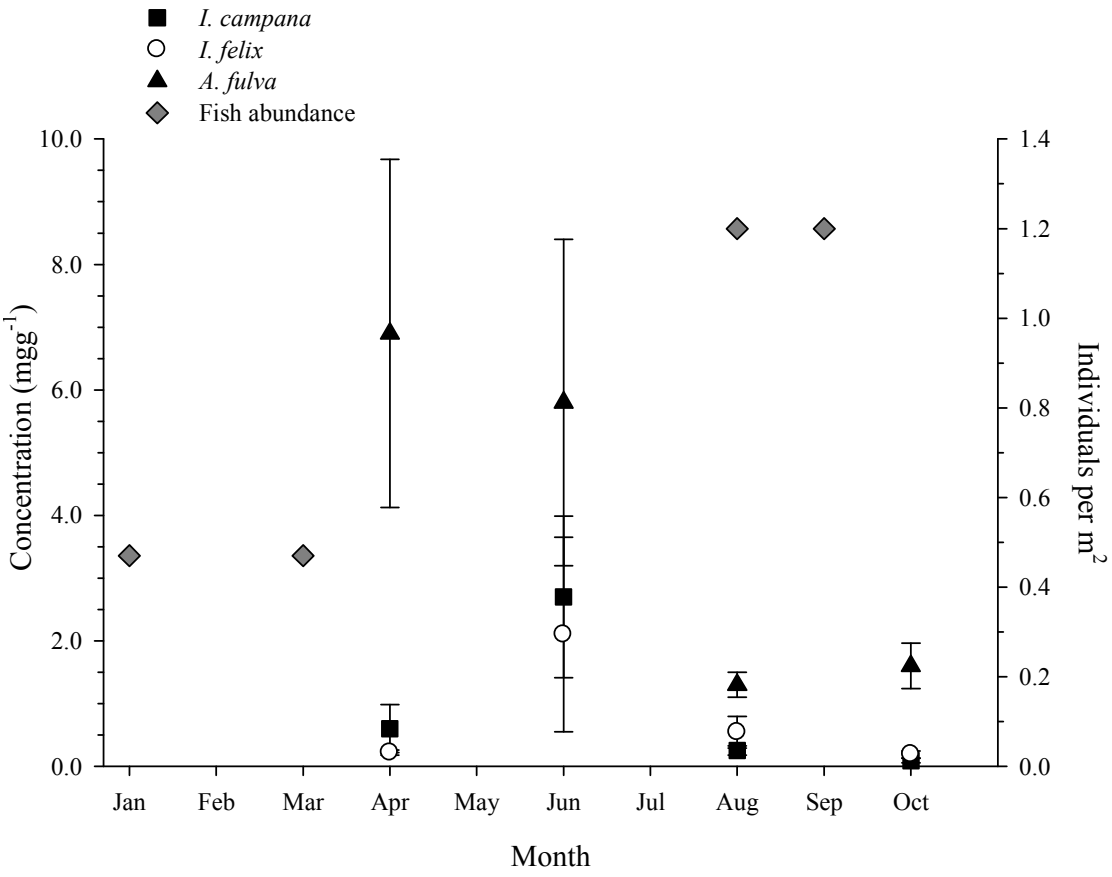

Figure 10. Mean (± S.E.) concentration of secondary metabolites from tissues of tagged individuals of *I. campana, I. felix,* and *A. fulva* collected during my study period and mean density of fish at Grays Reef National Marine Sanctuary surveyed by Sedberry and Van Dolah (1980).

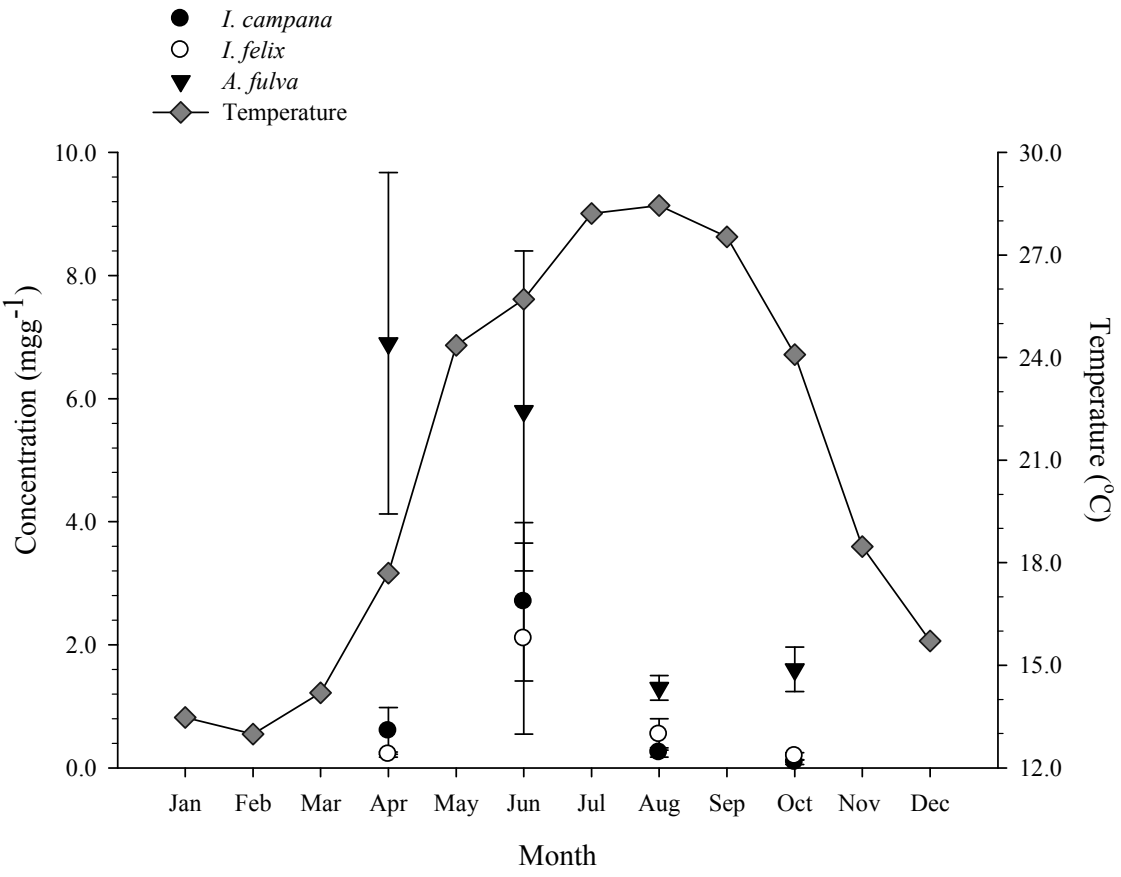

Figure 11. Mean (± S.E.) concentration of secondary metabolites from tagged individuals of *I. campana, I. felix,* and *A. fulva* collected in April, June, August, and October 2006 and monthly mean temperatures recorded at J-reef during the same time period. Temperatures recorded hourly with an Onset Stowaway Tidbit temperature logger.

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APPENDICES

APPENDIX A: EXPERIMENTAL ASSESSMENTS OF PREDATION PRESSURE

To determine if the temporal increase in predator abundance observed during the summer at J-reef represented an increase in attack rates on sponges, and thus, an increase in the concentration of sponge chemical defenses, I attempted to measure predation pressure through manipulative experiments in the field. These experiments were carried out within a few days of sponge sampling intervals. I assessed predation intensity by recording changes in tissue volumes from transplanted sponge tissues that were either protected from (caged), or left exposed to (uncaged) predators for 1 week. These surveys however, were inconclusive.

Field Experimental Design

Ten reinforcement bars, which served as cage anchors, were pounded into the reef approximately 6 m apart and 1 m from the ledge border following the contour of the reef. Ten cylindrical cages (20 cm diameter x 40 cm length), that served as exclusion cages, built from hard plastic netting (4 cm mesh size), cable ties, and monofilament nylon netting (1 cm mesh size) were secured onto the reinforced bars with cable ties. These exclusion cages were raised approximately 30 cm from the bottom of the reef to further inhibit accessibility to sponge samples by benthic predators. Ten smaller cylindrical cages (6 cm diameter x 40 cm length) were built with the same materials, and served to hold sponge samples that were exposed to predators. One small cage was placed in between each pair of reinforced bars and secured to the reef with cable ties and masonry nails (Figure A1).

Collection, measurement, and handling of sponge tissues

I collected 2 large tissue samples from 10 haphazardly selected individuals of *I. campana, I. felix* and *A. fulva*. Paired tissue samples from the same sponge were stored together in plastic bags and immediately placed in coolers containing aerated seawater upon surfacing. The volume of each tissue sample was determined by displacement of water in a 100 ml graduated cylinder to the nearest 0.5 ml. Paired sponge samples were separated and individually selected for either caged or uncaged treatments. Tissue samples (1 from each species) were attached to a strip (8 cm width x 30 cm length) of hard plastic netting, consisting of the same material used to build the cages, with cable ties. Once the 3 sponge tissue samples were attached to the plastic strip they were immediately stored in coolers containing aerated seawater pending relocation to the caged and uncaged treatments underwater.

To ensure that sponge volume loss in caged and uncaged tissue samples was not due to differences in water motion, I measured dissolution rates in pre-weighed clod cards made with plaster of Paris (calcium sulfate hemi-hydrate). These clod cards were designed following a similar technique by Dotty (1971). A 2:1 combination of Plaster of Paris (Kids Kreations by Creative Crafts, Inc.) and water were added into a large bowl and thoroughly mixed until the batter was smooth enough to pour into ice cube molds. The plaster cubes were left to dry for 24 hours after which they were removed from the molds and glued onto small pieces of plexiglass cards (approximately 6 x 4 cm). Finished clod cards were weighed and cable tied to every plastic netting strip containing sponge samples.

Plastic netting strips with attached sponges and clod cards were affixed to either the upper, outside surface of small cages (predator exposed treatments) or to the bottom, inside surface of large cages (predator exclusion treatments) (Figure A2). Once the plastic strips were secured inside the predator exclusion cages, these were sealed at the end by tying the monofilament mesh shut. All plastic netting strips were collected at the end of one week. Final sponge volumes were recorded by displacement of water, and clod cards were left to dry and then reweighed.

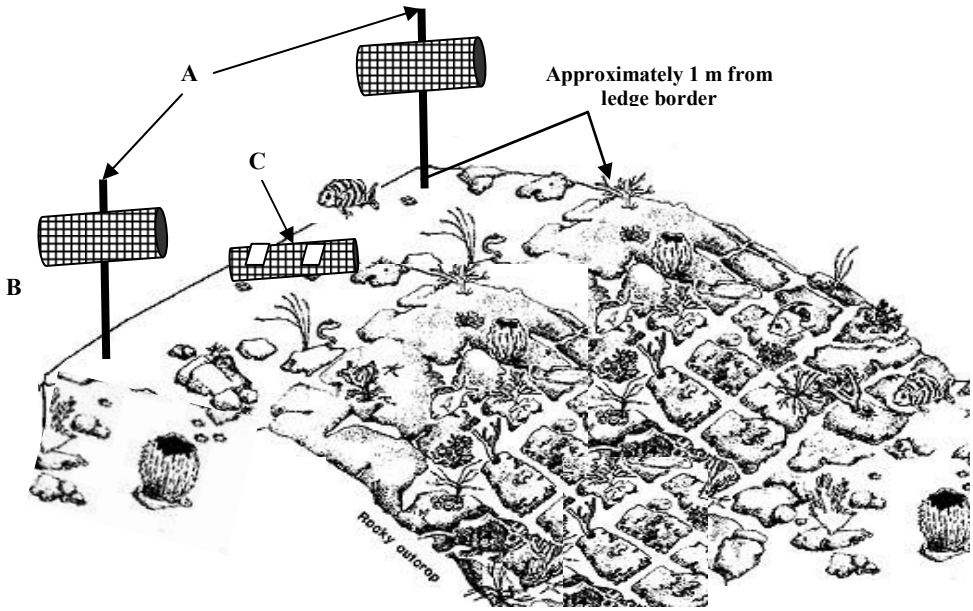

Figure A1. Predatıon pressure experiment set up on the ledge at J-reef. Ten reinforcement bars (A) placed at 6m intervals were used to secure exclusion cages (B). Smaller cylindrical cages (C) were placed in between reinforcement bars and were used as feeding platforms. Diagram adapted from Carol Johnson.

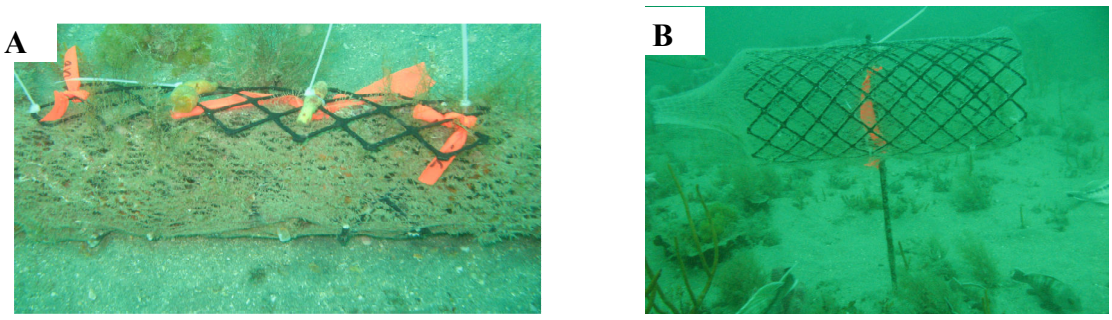

Figure A2. Strips of plastic netting with sponge samples exposed to predators (A) were attached to the top of the small cylindrical cages with cable ties. Exclusion cages (B) were pushed through the reinforcement bars, raised 30 cm from the bottom, and secured onto the reinforcement bars with cable ties. Both large and small cylindrical plastic netting cages were wrapped in a monofilament nylon netting (1 cm mesh size). The excess monofilament netting that protruded from one side of the exclusion cages (B) served as an opening that facilitated access to the inside of the cages. This netting was tightly shut with cable ties once samples were secured inside their cages.

Predation pressure surveys

During the first predation pressure experiment, carried out from 6 to 11 June 2006, I found that *I. campana* and *I. felix* tissue samples used in caged and uncaged treatments exhibited pronounced degradation in < 5 days. Thus, accurate final volumetric measurements in these species were not possible. All *A. fulva* tissue samples exhibited some localized necrosis, specifically, in tissue areas that were pressed against the cable ties used for attachment to the plastic netting strips. The next predation pressure experiments were conducted on 23 to 29 June 2006. During this survey, I replaced *I. campana* and *I. felix* with *Desmapsamma anchorata,* because this sponge was successfully used in previous sponge tissue transplantation experiments conducted at J-reef (Ruzicka 2005). A third attempt to survey predation pressure was expected to take place during the early fall season, but was delayed until early December 2006 due to weather conditions. Although I was able to place another set of sponge samples from *A.*

fulva and *D. anchorata* on the reef in December, retrieval of these samples was not possible due to weather conditions and unavailable transportation from shore out to J-reef. My predation pressure assay was designed primarily to evaluate intensity of predation within the same time frame in which sponge sampling was carried out and, because this was not possible, I chose to omit this assay from my research and continued on to address feeding deterrence.

Results and discussion

Water motion appeared to be the same in both caged and uncaged environments as no significant differences in the change in weight between caged and uncaged clod cards were observed (one-way ANOVA: F: 0.9, df = 1, p = 0.3, n = 10). Thus, any significant changes in sponge tissue volume were attributed to grazing by predators.

Predation intensity measured as the percent loss in volume between caged and uncaged sponge tissues was only significant in *D. anchorata* (one way ANOVA: F = 6.4, df = 1, p = 0.02, n = 9). Intense predation on *D. anchorata* was further evidenced by the grazing scars observed on predator-exposed sponge tissues. In contrast, predation on *A. fulva* appeared to be consistently low (one-way ANOVA: F = 0.9, df = 1, p = 0.3, n =10 for 6 June, and F = 2.5, df = 1, p = 0.1, n = 9 for 23 June). These results showed that tissue samples of *A. fulva* were significantly less palatable than those of *D. anchorata* (one-way ANOVA: F = 6.47, p = 0.02, df = 1). Though these results provided no information on the potential relationship between the temporal variation in predation pressure and induction of sponge chemical defenses, they did offer relevant support for the high feeding deterrence properties in *A. fulva* as these experiments were conducted under field conditions.

APPENDIX B: FEEDING ASSAYS AND FOOD CUBE PREPARATION TECHNIQUES

Once my initial field feeding trials with crude extracts from *Ircinia campana* and *I. felix* failed to deter feeding by fish, I conducted a second set of trials with food cubes prepared in the same manner as my initial ones. These additional trials were conducted to confirm that the initial results were repeatable, and indeed they were. As indicated in the body of the thesis (pages 20 and 28), the inability of these food cubes to deter predators may have been a function of degradation in the concentration of secondary metabolites that resulted when food cubes were heated during the preparation process (see Figure 2). In an effort to produce food cubes without the incorporation of heat, I tried several iterations of the preparation technique. While these attempts were unsuccessful, I am outlining them below for the benefit of other researchers that may attempt such studies in the future.

Food cube preparation techniques

One artificial food preparation technique that does not require heat has been described by Burns et al. (2003), where sponge crude extracts are incorporated into a calcium alginate-based food pellet. This food mixture consists of a combination of 0.3 g of alginic acid, 0.5 g of lyophilized squid mantle, and sponge crude extracts dissolved in 10 ml of distilled water. This mixture is then vigorously mixed and loaded into a 10 ml syringe. The tip of the syringe is then submerged in a 0.25 M solution of calcium chloride and the mixture is slowly unloaded into the medium. Presumably, calcium chloride hardens this mixture relatively quickly to a consistency that allows it to be sliced into food pellets. However, I never observed this effect. I sought assistance from faculty in the

Department of Chemistry at Georgia Southern University (L. Shannon Davis Ph.D., and James M. LoBue Ph.D.), but their suggestions did not provide satisfactory results.

Next, I formulated a recipe that consisted of the same carageenan-based food cube, but without the addition of agar or heat. Instead, the crude extract was incorporated into the soft carageenan mixture and poured into 1 x 1 x 1 cm plastic square mold. The mold containing the food mixture was stored in the freezer (- 70 ºC), and lyophilized. Lyophilized food cubes were removed from their molds and covered in a hot mixture of agar and distilled water, but this hot mixture did not penetrate the food cube. The end result was an enveloped crude extract mixture that was protected from water and direct exposure to heat. While this last recipe seemed feasible, it did not produce satisfactory results in field experiments because fish seemed to reject the hardened exterior of the food cube. Treatment and control food cubes prepared with this technique were all rejected by fish during field feeding experiments. Perhaps one alternative to improving this technique is to envelop the lyophilized food cube with a less concentrated hot mixture of agar and distilled water that still conveys enough protection from water and is also more palatable to fish (i.e. thin and flavored outer shell).