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VARIABILITY IN THE CHEMICAL DEFENSE OF THE CARIBBEAN REEF SPONGE *XESTOSPONGIA MUTA*

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ABSTRACT

Tissue from 60 specimens of the Caribbean loggerhead sponge, *Xestospongia muta*, yielded crude organic extracts that had a variable capacity to deter the feeding of a predatory reef fish in aquarium assays. This intraspecific variability in chemical defense was not correlated to sponge size or to the sterol composition in sponge tissue, but sponges collected from one site in the Bahamas were more deterrent than those collected in Florida, and sponges collected on deep reefs (>15 m) were more deterrent than those collected on shallow reefs. For two specimens of *X. muta* that yielded very deterrent extracts, there was no difference in the deterrency of the heavily pigmented, cyanobacterially rich exterior tissue and the white inner tissue of the sponge. Crude extracts of *X. muta* assayed at natural concentrations deterred feeding of a natural assemblage of reef fishes in the field. Both laboratory and field assays revealed that the deterrent capacity was limited to the polar constituents of the crude extract. Although the patterns of chemical defenses of *X. muta* are not easily explained, the variability may reflect site-specific differences in levels of predation.

INTRODUCTION

Sponges are the single richest source of marine secondary metabolites, accounting for about a third of the natural products isolated to date from marine organisms (Faulkner 1996, and previous reviews by the same author). Compounds isolated from sponges vary widely in structural complexity (Sarma et al. 1993) and concentrations of secondary metabolites in sponge tissues can be quite high (Rogers and Paul 1991). Only recently have the ecological functions of sponge secondary metabolites been investigated (Paul 1992); they have been implicated in the mediation of overgrowth in sponge-coral allelopathic interactions (Sullivan et al. 1983), inhibition of the settlement of fouling organisms (Henrikson and Pawlik 1995; Hirota et al. 1996), and protection against microbes (Bergquist and Bedford 1976). But interest in the function of sponge secondary metabolites as defenses against potential predators has predominated (reviewed in Pawlik 1993).

Sponges are sessile and generally soft-bodied, and thus appear to be particularly vulnerable to potential predators. In addition, Caribbean demosponges represent a rich source of protein (Chanas and Pawlik 1995) in an environment noted for intense feeding activity by fishes (Hixon 1983). Despite their nutritional value, very few fish species are known to feed on sponges (Randall and Hartman 1968; Wulff 1994). Other predators, such as invertebrates and turtles also feed on sponges (Pawlik 1983; Meylan 1988; Wulff 1995), but predatory fish may have a greater influence on Caribbean sponge distributions and abundances (Pawlik in press). Several feeding studies have demonstrated that sponge secondary metabolites deter consumption by predatory fishes (Thompson et al. 1985; Herb et al. 1990; Duffy and Paul 1992; Albrizio et al. 1995; Chanas et al. in press). Surprisingly, the skeletal components of sponges do not appear to offer any physical defense against predatory fishes (Chanas and Pawlik 1995; 1996).

In a recent survey of the chemical defenses of Caribbean demosponges, over 69% of the crude organic extracts of 71 sponge species were found to deter feeding by the bluehead wrasse *Thalassoma bifasciatum* in aquarium assays (Pawlik et al. 1995). In that study, crude extracts from seven sponge species had a high degree of intraspecific variation of feeding deterrency, and one of these was *Xestospongia muta*. Commonly known as the loggerhead sponge, *X. muta* was chosen for further study because it is conspicuous and abundant on Caribbean reefs. The very large (up to 2 m) brown barrel-shaped colonies of *X. muta* are found at depths from 9 m to > 40 m and have been reported to cover > 9% of the surface of some Colombian coral reefs (Zea 1993). The species has been divided into 3 distinct chemotypes based on the abundance pattern of 14 different sterols found in the tissue of the sponge (Kerr and Kelly-Borges 1994).

Inter- and intraspecific variability of chemical defenses has been the subject of considerable theoretical and experimental work among terrestrial chemical ecologists, and has recently been addressed by marine ecologists (see Paul 1992). The purpose of this study was to build on our observation that the chemical defenses of *X. muta* were variable (Pawlik et al. 1995) by determining, with a much larger sample size, whether deterrency is correlated with sponge size, site of collection, depth of collection, or sterol chemotype. We also wished to discover whether there was intra-colony variability in deterrency by assaying extracts of the pigmented, cyanobacterially rich surface tissue (ectosome) of *X. muta* separately from the white interior tissue (endosome). Finally, we began the process of isolating the deterrent secondary metabolites from extracts of *X. muta*, using both laboratory and field assays.

MATERIALS AND METHODS

Tissue from replicate colonies of *Xestospongia muta* was collected from 10 different sites in the Florida Keys and the Bahamas. Of the collection sites in the Florida Keys, three were within 15 km of one another (Molasses, Pickles, Conch), while the fourth was approximately 100 km further south (Long). Collection sites in the Bahamas were within approximately 300 km of one another (Grand Bahama southeast to Acklins). Collection depth was recorded at all sites. Colony height, a simple measure of sponge size, was noted for specimens collected from 3 Florida sites (Conch, Pickles, Molasses). Unless extracted immediately, sponge tissue was frozen and stored at -20°C until extracted.

Laboratory aquarium assays were performed to determine the deterrency of crude organic extracts and fractions (for details, see Pawlik et al. 1995). For each assay, a 10 cm^3 piece of ectosomal sponge tissue was macerated and extracted in 40 ml of 1:1 dichloromethane:methanol (DCM:MeOH) and methanol (MeOH) at 4°C for 24 h and 1 h, respectively. The extracts were filtered, combined, and concentrated by evaporation under vacuum to yield an organic residue with residual water (< 2 ml). The crude extract was mixed to a final volume of 10 ml with alginate-based food (0.5 g homogenized lyophilized squid mantle and 0.3 g alginic acid in 10 ml distilled water) until all organic and water-soluble components were distributed uniformly in the matrix. Food coloring was added to both treated and control foods to avoid differences in consumption due to extract color. The alginate food was then dispensed with a 10 ml syringe into a 0.25 M calcium chloride solution and the resulting strand allowed to harden for 2 min. The hardened food was rinsed in filtered seawater and cut into 3 mm pellets. Control pellets were prepared identically but without the addition of crude extract. Feeding assays were performed with groups of blue-head wrasse, *Thalassoma bifasciatum*, using previously described methods of assay scoring and statistical analyses (Fisher exact test; see Pawlik et al. 1995).

To determine whether deterrency varied with collection site, data from aquarium assays of crude extracts were analyzed using a one-way ANOVA and Bonferroni pairwise comparisons (Zar 1984). Data from these assays were also divided into two groups based on depth at collection: shallow reefs (9-15 m) and deep reefs (15-30 m); a two-

sample t-test was used to determine if mean deterrency differed between groups (Zar 1984). Collections from some sites were entirely from deep or shallow reefs, precluding a two-factor ANOVA of the effects of site and depth (Zar 1984). As an estimate of sponge size, the height of the sponges was recorded at three Florida sites (Conch, Molasses, and Pickles) and height was compared with deterrency (linear regression, Zar 1984). To determine whether deterrency varied within a colony, aquarium assays were performed with crude extracts from exterior (within 1 cm of surface) and interior (3 cm or more from surface) tissues of 2 colonies that had already been determined to have deterrent tissues in an earlier assay. Crude extracts of 9 colonies from each of 3 Florida sites (Conch, Molasses, and Pickles) and 5 colonies from a fourth Florida site (Long Key) tested in aquarium assays were also used to determine colony chemotype based on sterol fingerprinting techniques of Kerr and Kelly-Borges (1994). Data from aquarium assays of crude extracts were compared with sterol type using a one-way ANOVA and Bonferroni pairwise comparisons (Zar 1984).

Field assays were performed to assess the deterrency of crude organic extracts and fractions against a natural suite of predatory reef fishes (for details, see Chanas and Pawlik 1995). For the crude extract assay, a 60 cm^3 piece of exterior (ectosomal) sponge tissue was extracted sequentially with 1:1 DCM:MeOH and MeOH at 4°C for 24 h and 1 h, respectively. The 1:1 DCM:MeOH and MeOH extracts were filtered, combined, and concentrated by evaporation under vacuum. The concentrated crude extract (or fraction) was dissolved in a minimal volume of MeOH and combined with a preheated carrageenan-based food (3 g of homogenized lyophilized squid mantle and 1.5 g of type I carrageenan in 60 ml of distilled water) to a final volume of 60 ml. Food coloring was added to both treated and control foods to avoid differences in consumption due to the color of the extract. The mixture was then poured into molds containing cotton string and allowed to harden. After hardening, 20 string-embedded strips were cut from the molds. Control strips were prepared identically but without the addition of crude extracts. Field assays were conducted on shallow water reefs (Key Largo, FL) using previously described methods regarding deployment, retrieval, and statistical analyses (Wilcoxon pairwise comparisons; see Chanas and Pawlik 1995).

In an attempt to isolate the compounds responsible for feeding deterrency, approximately 500 ml of tissue of a deterrent colony of *Xestospongia muta* was macerated and extracted sequentially in MeOH (x3), 1:1 DCM:MeOH (x3), and DCM (x1) for 24 h in each solvent. The extracts were filtered, concentrated by evaporation under vacuum, and recombined to yield a green extract. An aliquot of crude extract representing 100 ml of tissue was separated by vacuum flash chromatography over normal phase silica gel using DCM in a MeOH gradient (DCM, 98:2, 90:10, 85:15, 75:25, 50:50, MeOH) with 7 fractions collected (100 ml each). Fractions were monitored using thin-layer chromatography (TLC) (SiO_4 in 9:1 DCM:MeOH; developed using sulfuric acid and heat). Both laboratory and field assays were performed on recombined fractions.

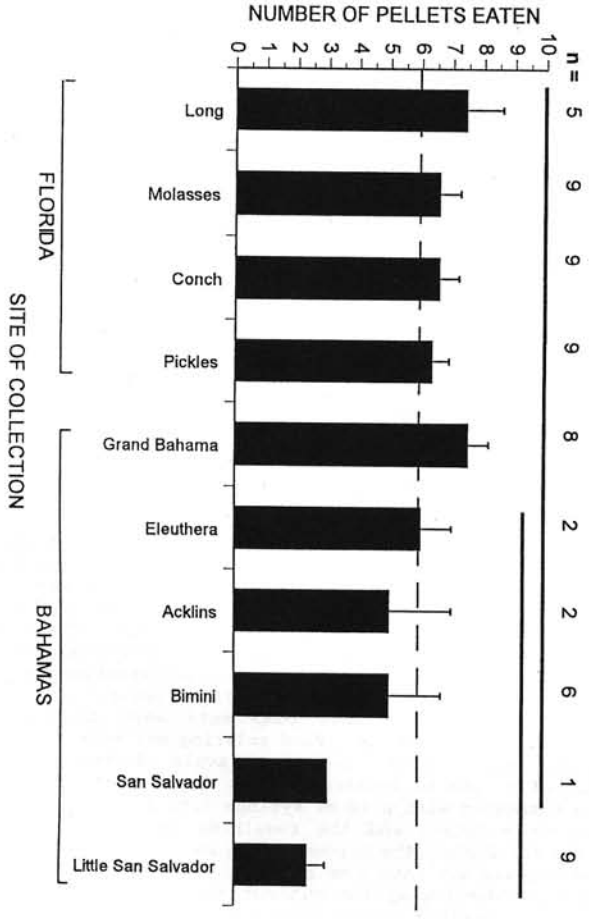

Fig. 1 Consumption by *Thalassoma bifasciatum* of food pellets (mean + SE) containing crude extracts of *Xestospongia muta* from sites in Florida and the Bahamas. N = number of replicate samples per site. For any individual aquarium assay, extracts were considered deterrent if the number of pellets eaten was less than or equal to 6 ($p \leq 0.043$, Fisher Exact test, 1-tailed), as indicated by the dashed line on the graph. Lines to the right of histograms indicate groups for which means were not significantly different (ANOVA, Bonferroni).

RESULTS

Variability of chemical defense

Crude organic extracts from tissue from 60 colonies of *Xestospongia muta* from sites in Florida and the Bahamas were assayed for their capacity to deter feeding of *Thalassoma bifasciatum* in aquarium assays. Of these 60 replicates, 16 were included in Pawlik et al. (1995). The mean of these 60 replicates was 5.8 pellets eaten out of 10, with a range of 0 to 10 pellets eaten and a high level of variation (SD = 2.7). For any single aquarium assay, an extract was considered to be deterrent if 6 (or less) of the 10 treated food pellets were eaten (Fisher exact test; see Pawlik et al. 1995). Between collection sites, crude extracts from *X. muta* from Little San Salvador in the Bahamas were significantly more deterrent than any of the extracts from sites in Florida or the site at Grand Bahama Island (ANOVA, $p = 0.002$, Bonferroni pairwise comparison, Fig. 1). For all sites, tissue from sponges collected on

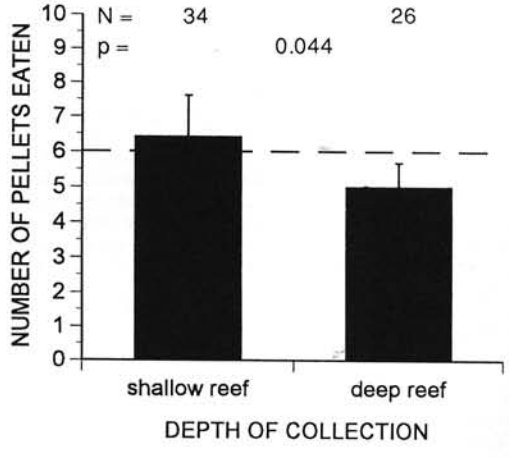

Fig. 2 Data from Fig. 1 sorted by depth of sponge collection. Shallow reefs were at 9-15 m depth, deep reefs were 15-30 m depth. N = number of replicate samples per depth group. Dashed line as in Fig. 1. Crude extracts of *X. muta* from deep reefs were significantly more deterrent in aquarium assays (t-test).

deep reefs (15-30 m) yielded extracts that were significantly more deterrent than tissue from sponges collected on shallow reefs (9-15 m) (Fig. 2; t-test, $p=0.04$). There was no relationship between the height of specimens of *X. muta* and the deterrency of crude extracts from their tissues ($r^2=0.04$, Fig. 3).

The sterol fingerprinting technique of Kerr and Kelly-Borges (1994) was performed on extracts of 32 tissue samples of sponges from Florida. There was no difference in the deterrency of the crude extracts of sponges when sorted by chemotype (ANOVA, $p = 0.81$, Fig. 4). Two specimens of *X. muta* from Florida were found to have tissues that yielded crude extracts that were strongly deterrent in the aforementioned assays. Additional tissue samples of these two specimens were assayed, and there was no difference in the deterrency of the pigmented surface tissues (0 ± 0 pellets eaten) and the white interior tissues (1 ± 0.5 pellets eaten) of these sponges.

Isolation and identification of deterrent activity

When assayed at natural concentrations, the crude extract of the tissues of a colony of *X. muta* that was demonstrated to deter feeding in aquarium assays also deterred feeding of a natural suite of reef fishes in a field assay (Fig. 5). Fishes observed sampling food strips during field assays included wrasses (*Thalassoma* and *Halichoeres* sp.), snappers (*Ocyurus chrysurus*), parrotfishes (*Scarus* sp.), and sergeant majors (*Abudefduf* sp.).

In aquarium assays of the flash column fractions, polar fractions 5, 6, and 7 deterred feeding in aquarium assays (1 of 10 pellets eaten) while non-polar fractions 1, 2, 3, and 4 did not (8 of 10 pellets eaten). Results were the same for field assays: combined non-polar fractions 1-4 did not deter feeding, but feeding was deterred by combined polar fractions 5-7 (Fig. 5). Using analytical TLC of the deterrent fractions, the compound responsible for the deterrent activity was characterized as moderately polar and UV-active. Isolation and identification of the deterrent compound is the focus of continuing research.

DISCUSSION

Crude organic extracts of *Xestospongia muta* deterred feeding in aquarium and field assays, suggesting that secondary metabolites of this species act to deter feeding by generalist predatory reef fishes (Pawlik et al. 1995). We have previously demonstrated that the siliceous spicules that pervade the tissues of *X. muta* do not deter feeding by reef fishes when assayed at natural concentrations in laboratory and field experiments (Chanas and Pawlik 1995, 1996). Despite high concentrations of spicules, (mean ash mass values ≈100 mg/ml), tissue of *X. muta* was previously found to be high in soluble protein (15-20 mg/ml; Chanas and Pawlik 1995), suggesting that the tissue of this sponge could provide a high-quality food source, if not for the chemical defense. But despite its chemical defense, we have observed that *X. muta* are frequently grazed by parrotfishes, primarily of the genus *Sparisoma*, which appear to scrape only the surface tissue (1-2 mm deep) from select individual sponges. The importance of parrotfish spongivory has only recently been described (Dunlap and Pawlik 1996). Parrotfishes may choose individuals of *X. muta* that have lower levels of deterrent chemistry, hence the selectivity of the observed grazing.

Samples of *X. muta* from the Bahamas tended to be more chemically deterrent than samples from Florida (Fig. 1), although only samples from Little San Salvador Island were significantly more deterrent than those from the 4 Florida sites and Grand Bahama Island. This pattern did not result because collections from Little San Salvador were all from

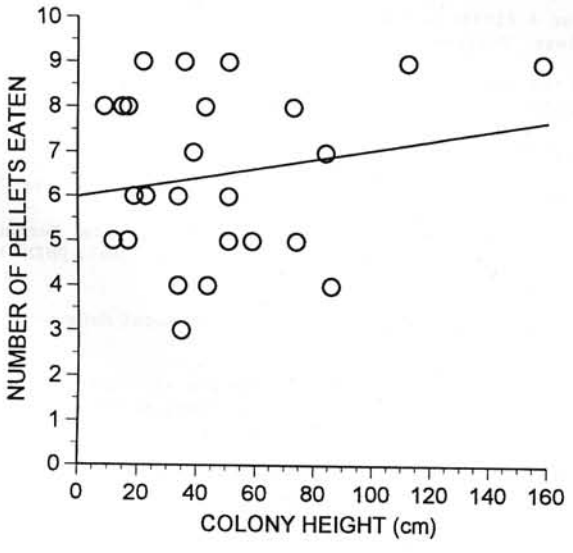

Fig. 3 Data for three Florida sites from Fig. 1 (Molasses, Conch, Pickles) plotted as a function of the height of the assayed sponge. $r^2 = 0.04$.

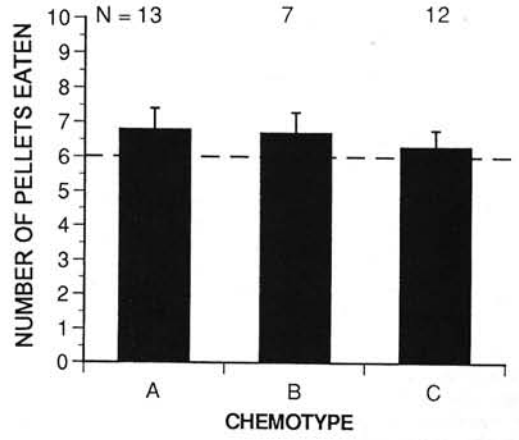

Fig. 4 Data for Florida sites from Fig. 1 sorted by sterol chemotype of the sponge tissue. N = number of replicate samples per chemotype. Dashed line as in Fig. 1. There was no significant difference in the palatability of the crude extracts from the three sponge chemotypes (ANOVA).

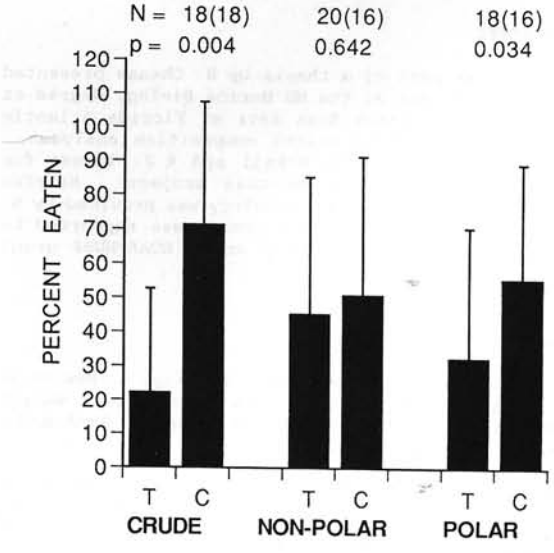

Fig. 5 Consumption by reef fishes of paired control food strips (C) and strips containing crude extracts or partitioned extracts of *X. muta* at natural concentrations (T) (mean + SD). N = number of paired strips deployed (number of paired strips used in statistical analyses). Probability values calculated using the Wilcoxon paired-sample test for each pair.

deeper water (i.e., the effects of site were not confounded by depth); 2 of 9 samples from Little San Salvador were taken from shallow reefs, and extracts from both of these samples scored 1 of 10 pellets eaten. Interestingly, we have observed greater levels of intraspecific deterrency in Bahamas vs. Florida collections for another sponge species we have studied, *Chondrilla nucula* (Swearingen 1996). It is unclear why sponges in the Bahamas should tend to have higher levels of defensive chemicals. One possibility is that predation rates on sponges are higher in the Bahamas than in Florida, but there were no noticeable differences in the abundances of generalist predatory or spongivorous fishes in the two locations.

Tissue collected from *X. muta* on deep reefs was significantly more deterrent than tissue from sponges on shallow reefs (Fig. 2). A similar situation was reported by Harvell et al. (1993) in their study of the variability of the chemical defense of the Caribbean gorgonian *Briareum asbestinum*. This pattern of deterrency is interesting because the abundance and diversity of omnivorous browsing fishes is generally thought to be greater on shallow than on deep reefs (Harvell et al. 1993). Depth differences in chemical defenses may reflect differences in the production of compounds by endosymbiotic microorganisms (Faulkner et al. 1993) or the greater accumulation of deterrent metabolites in sponge tissues that grow slower at greater depths.

Patterns of deterrency of crude tissue extracts from *X. muta* revealed no relationship with colony height or sterol type (Figs. 3,4). The distribution of sterol chemotypes in the populations of *X. muta* sampled for this study were comparable to patterns seen by Kerr and Kelly-Borges (1994). Chemical defense of *X. muta* does not appear to be dependent on sponge size (and presumably, sponge age), nor are deterrent metabolites any more likely to be found in one chemotype over another.

While the compounds responsible for the chemical defense of *X. muta* have yet to be identified, work to date suggests that they are moderately polar; all non-polar components (mostly cyanobacterial pigments) did not deter feeding. Although the inner and outer tissues of only two deterrent sponges were compared, chemical deterrency of crude extracts did not vary within a sponge. These data suggest that (1) there is no partitioning of defensive chemicals to the part of the sponge that is more likely to be attacked by potential predators, and (2) that deterrent compounds are not specifically associated with the pigmented microorganisms that give *X. muta* its brown color.

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