ELSEVIER

Journal of Experimental Marine Biology and Ecology 322 (2005) 67–81

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules?

Adam C. Jones[a], James E. Blum[b], Joseph R. Pawlik[a,*]

[a]*Biological Sciences and Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC 28409, United States*

[b]*Mathematics and Statistics, University of North Carolina at Wilmington, Wilmington, NC 28403, United States*

Received 31 January 2005; received in revised form 12 February 2005; accepted 12 February 2005

Abstract

Chemical and physical defenses of sessile organisms against consumers are well described for both terrestrial and marine systems. However, previous studies have focused on chemical or physical defenses in isolation, and have not considered their interaction. Marine sponges provide a model system for testing this interaction. Some sponge species produce secondary metabolites that deter predation; they may also contain siliceous spicules, but previous studies have provided little evidence that spicules in isolation offer any defense against generalist fish predators. To determine whether the two components have an additive, antagonistic, or synergistic interaction, crude organic extracts and spicules from individuals of 8 Caribbean sponge species were isolated and tested in laboratory feeding assays. These included one chemically defended reef sponge (*Agelas clathrodes*) and seven known to be intermediately deterrent: six from reef habitats (*Cinachyrella alloclada*, *Clathria virgultosa*, *Cribrochalina infundibulum*, *Niphates digitalis*, *Svenzea zeai*, and *Xestospongia muta*) and one from mangrove habitats (*Tedania ignis*). Extracts and spicules were assayed at various concentrations, both individually and in combination, in laboratory feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*. A SAS based GENMOD procedure based on an isobolographic analysis model was used for statistical comparisons. Four sponges (*A. clathrodes*, *C. alloclada*, *C. virgultosa*, and one of three individuals of *X. muta*) showed evidence of synergisms. Of these, synergy in *C. alloclada*, *C. virgultosa*, and *X. muta* was caused by approximately natural concentrations of extracts and spicules. The extract of *A. clathrodes* was deterrent, but combination assays required nearly a 3-fold reduction in extract concentration and an 8-fold increase in spicule concentration to show the synergistic effect. Contrary to previous findings, spicules from *C. infundibulum* and two of three individuals of *X. muta* were deterrent at natural concentrations. Sponge spicules may be defensive in isolation, or may enhance chemical defenses against consumers, but the lack of synergisms for individuals in 4 of 7 species with intermediate levels of chemical defense suggests that defensive synergy is not the general rule and, when present, may be an example of an exaptation.

Keywords: Chemical defense; Predation; Secondary metabolites; Spicule; Sponge; Synergy

* Corresponding author. Tel.: +1 910 962 2377; fax: +1 910 962 2410.
E-mail address: pawlikj@uncw.edu (J.R. Pawlik).

0022-0981/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2005.02.009

1. Introduction

Chemical and physical defenses of sessile organisms have long been the subject of ecological research (e.g. Bridwell, 1918; Janzen, 1969; Pennings and Paul, 1992). In terrestrial ecology, specific theories have evolved regarding how plants resolve the dilemma of allocating enough energy for growth and reproduction, but at the same time successfully defending themselves against consumers, pathogens, and overgrowth (Levin, 1976; Herms and Mattson, 1992). When resources are limiting, optimal defense theory predicts that the amount of energy invested in defending tissue should commensurate with the value of the tissue, and that inducible defenses are more energy efficient than constitutive defenses when levels of herbivory are low (e.g. Zangerl and Rutledge, 1996). Recent evidence has also suggested that plants with variable resistance phenotypes as a result of induction may be more effective against herbivores than plants maintaining a mean constitutive level (Karban et al., 1997).

Defenses against predation employed by terrestrial plant species include the use of structural components such as spines (e.g. Cooper and Owen-Smith, 1986), thorns and resin ducts (e.g. Maxwell et al., 1972), and tissue toughness (e.g. Howard, 1988). Silica content in African grasses has been proposed to act as a defense against herbivory, while at the same time serving as a growth promoter. Silica could cause both mouthpart abrasion and function as structural support for the plant, allowing carbon to be used in other energy-requiring roles (McNaughton et al., 1985). Some marine plants are also characterized by comparable structural defenses, including surface cuticles (Gaines, 1985), tissue toughness (Pennings and Paul, 1992) and reduction in tissue nutritional quality available to consumers (Duffy and Paul, 1992). However, plant secondary metabolites (i.e., those compounds not involved in primary metabolic functions as defined in Berenbaum and Neal, 1985) may be the most important mechanism of defense against herbivory (e.g. Howard, 1988; Schultz, 1988). While physical and chemical defensive mechanisms in plants have independently been the topic of research, their activity in combination has received less attention.

In tropical marine environments, benthic sessile organisms use similar defensive strategies to those of terrestrial plants. In particular, many coral reef invertebrates are chemically defended by secondary metabolites (e.g. Pawlik, 1993). Secondary metabolites appear to be most common among benthic organisms that are vulnerable to high predation or grazing rates (e.g. Paul, 1992; Pawlik, 1993; McClintock and Baker, 2001). These organisms include sponges, soft corals, sea slugs, and tunicates, all of which lack obvious physical means of deterring consumers (Pawlik, 1993).

Marine sponges provide an interesting model system to test for the presence of defensive interactions because they differentially express physical and chemical defenses that could be used in isolation or combination. Sponges are important constituents of reef ecosystems and are among the most abundant and diverse groups of sessile invertebrates in the Caribbean (e.g. Schmahl, 1991). Sponges are a rich protein source (Chanas and Pawlik, 1995) and the soft, fleshy tissue of many species appears to make them targets in areas noted for high predation rates (e.g. Hixon, 1983). Relatively few spongivores have been identified, but they include some fishes (e.g. Randall and Hartman, 1968; Wulff, 1994; Dunlap and Pawlik, 1996, 1998), hawksbill turtles (Meylan, 1988) and invertebrates such as nudibranchs (Pawlik et al., 1988) and echinoids (Birenheide et al., 1993).

While sponges are soft-bodied organisms, many produce structural components as part of their skeleton. Structural components in Caribbean sponges include siliceous spicules (e.g. Hooper and Van Soest, 2002) and proteinaceous spongin fibers (Bergquist, 1978; Chanas and Pawlik, 1996). Koehl (1982) described how sponge spicules strengthen the sponge skeleton by increasing tissue rigidity. A positive correlation between sponge spicule density and ambient water flow has also been demonstrated (e.g. Palumbi, 1986; McDonald et al., 2002).

Sponges provide the greatest diversity of marine natural products (Blunt et al., 2004, and previous citations therein). Sponge secondary metabolites are complex molecules, and can be present in high concentrations. Classes of secondary metabolites include sterols, terpenoids, amino acid derivatives, saponins, and macrolides (Blunt et al., 2004). Secondary metabolites from sponges may be involved in allelopathic spatial competition (e.g. Engel and Pawlik, 2000), inhibition of bacterial colonization

and fouling (e.g. Kelly et al., 2003), and protection from ultraviolet radiation (Paul, 1992). However, more attention has been devoted to studying how these metabolites may offer protection against potential predators (e.g. Pawlik et al., 1995; Uriz et al., 1996; Pawlik, 1997).

Crude organic extracts from many sponges deter feeding by generalist fish predators and invertebrates. Pawlik et al. (1995) found that of 71 Caribbean sponges, 69% yielded extracts that, when incorporated into artificial foods at natural volumetric concentrations, deterred feeding of the bluehead wrasse *Thalassoma bifasciatum* in aquarium assays. In two separate studies, Waddell and Pawlik (2000a,b) demonstrated that crude organic extracts from several sponge species also deterred feeding by the hermit crab *Paguristes punticeps* and the sea stars *Echinaster echinophorus* and *Echinaster sentus*.

Levels of deterrence offered by sponge extracts can also vary among individuals of the same species. Chanas and Pawlik (1997) observed that 60 specimens of the barrel sponge *Xestospongia muta* yielded crude organic extracts that ranged in levels of palatability based on geographic location, and Swearingen and Pawlik (1998) noted significant variability in deterrence in the sponge *Chondrilla nucula*, based on geographic location and month of collection.

The defensive role of physical structures in sessile benthic reef invertebrates is controversial. Harvell et al. (1988) found that purified calcitic sclerites from the gorgonian *Pseudopterogorgia acerosa* inhibited feeding, but O'Neal and Pawlik (2002) tested natural volumetric concentrations of sclerites from 32 Caribbean gorgonian species and discovered that 30 of the species (including *P. acerosa*) were palatable in aquarium assays with *T. bifasciatum*. Sponge spicules have been found in gut and intestinal content analyses of those spongivores listed previously (Dunlap and Pawlik, 1998), and Chanas and Pawlik (1995) found that spicules from eight demosponge species did not deter feeding in laboratory assays with *T. bifasciatum*, or in field assays with a natural assemblage of reef fishes. Conversely, Burns and Ilan (2003) did find that spicules from 4 Caribbean sponges and 2 Red Sea sponges deterred feeding with the Red Sea wrasse *Thalassoma klunzingeri*, and that assays combining natural concentrations of crude organic extract and spicules from the sponge *Crella cyatophora* were significantly more deterrent than either the extract or spicules by themselves. Beyond the issue of spicules in isolation, the relationship between secondary metabolites and spicules in sponges remains to be explored.

Interactions between physical or chemical agents can be defined as additive, synergistic, or antagonistic, depending on the net effect resulting from their combination. Interactions are additive if the joint action of different agents does not differ from the sum effect of each agent in isolation (Berenbaum, 1988), while synergistic and antagonistic interactions exist when the joint action of agents is significantly greater or less than their sum effect, respectively. Synergisms exist in any case in which one compound elevates the biological activity of another, such as when one defense makes another more potent (Wilkinson, 1973).

Resources designated for growth in plants via leaf production need to be diverted to create chemical and structural defenses (e.g. Fagerström, 1989), which cause defenses to have a "high opportunity cost" (Herms and Mattson, 1992). Therefore, any method of reducing defensive investment would be beneficial to maintaining fitness. In many cases, plants can accomplish this reduction by using secondary metabolites in primary roles, including pollinator attraction (e.g. Rhoades, 1979), storage of nutrients (e.g. Poulton, 1990), structural support (e.g. Haslam, 1988), and in relationships with bacterial symbionts (e.g. Lynn and Chang, 1990). Synergistic interactions between chemical or structural components in plants could also offset resource allocation to defense. If sponges follow similar tradeoffs between growth and defense to those proposed in plants (Fagerström, 1989) evidence of interactions between secondary metabolites and spicules that elevate defense levels could imply that less energy in sponges is being allocated to secondary metabolite construction, and would allow for faster growth and reproduction rates.

Synergy has received limited attention in the field of chemical ecology. Berenbaum and Neal (1985) found that myristicin, a methylenedioxyphenyl containing phenylpropene compound in plants of the family Umbelliferae, synergistically interacted with the furanocoumarin xanthotoxin to elevate toxicity against the corn earworm *Heliothis zea*. In marine systems, Hay et al. (1994) detected defensive synergy

when semipurified secondary metabolites from the calcified green seaweeds *Rhipocephalus phoenix* or *Udotea cyathiformis* were combined with finely powdered calcium carbonate ($CaCO_3$). The findings of Hay et al. (1994) were subject to some debate: Pennings (1996) argued that a multiplicative null hypothesis was more appropriate than the additive null hypothesis used by Hay et al. (1994) and that synergisms may have been incorrectly defined, but Hay (1996a) responded that limitations identified by Pennings were not relevant to the Hay et al. (1994) study, and that synergy was present.

Statistical approaches to analyze data for evidence of interactions have varied depending on the field of study. Isobolographic analyses (Fraser, 1870–1871) are a common method for examining interactions in pharmacology and pathology (e.g. Berenbaum, 1988; Gessner, 1988; Tallarida et al., 1997). In an isobolographic analysis, the composition of dose mixtures of two agents are expressed graphically using rectangular coordinates, with the doses being defined by reference to the abscissa and ordinate (Gessner, 1988). A line connects the intercepts of defined potencies for each agent (Z_1* and Z_2*), which represents all of the possible isoeffective combinations. A second, radial line denotes all possible coordinates of the chosen proportions for the experimental mixture (Z_{mix}), and the intersection of these two lines has the coordinates [(Z_1/Z_1*), (Z_2/Z_2*)]. The location of the coordinates from Z_{mix} determines the overall effect of the combination. If the coordinates are not significantly different from the line, the mixture is additive; if below or above the line, the mixture is synergistic or antagonistic, respectively (Tallarida et al., 1997; Nelson and Kursar, 1999).

Other statistical models for comparing interactions, including ANOVA, the Case and Bender test (Case and Bender, 1981), and multiplicative tests (Wootton, 1994) are usually targeted at interactions between species but, as Hay (1996b) noted, may also be applicable to situations involving potential synergisms. Billick and Case (1994) stated that intended comparisons using these models may not be achieved because statistical analyses can be difficult to implement properly, and can be applied to interactions that are inappropriately assumed to be relevant. There are several characteristics of the isobolographic model that are preferable to tests such as ANOVAs when analyzing dose–response data. No assumptions are made about the interaction, and data do not have to be linear or limited to a normal distribution (Nelson and Kursar, 1999). The interaction between components can also be easily depicted and interpreted using the graphic isobole. The isobole allows for identification of maximum synergy or antagonism, and to isolate specific combinations that may yield anomalous effects (Berenbaum, 1977). To our knowledge, only one attempt to apply isobolographic analysis to ecological data has been made (Nelson and Kursar, 1999) in which the results of Berenbaum and Neal (1985) were verified.

The isobole itself can be viewed as a contour of a response surface; a projection from three dimensions down to two. For feeding assay data, the response, the probability of consumption, is modeled as a continuous function of two quantitative predictors, the concentrations of structural and chemical defenses. Once a model is fit, a horizontal slice through any particular value of the response gives a contour, showing the relation between the predictors at that value of the response. Thus, it is very important to choose an appropriate model for the response surface. For the binary nature of the response in this situation, the generalized linear model (GLM), and the logistic regression model in particular, are typically appropriate. All GLMs have three components: the random component, the systematic component, and the link. The random component identifies a response variable Y and it assumes a particular distribution. The systematic component identifies the explanatory variables, which are used as model predictors. The link is the functional relationship between the mean of the random component and the systematic component. Fisher and Yates (1938) proposed $\log [\pi^*(1-\pi)-1]$ as a binomial parameter transformation for binary data analysis, with Berkson (1944) later referring to this as the "logit" transformation. So, while a GLM with a normal distribution (and an identity link function) is equivalent to models used in traditional ANOVA and regression, using the binomial distribution with a logit transformation is appropriate for examining the probability of success or failure in a set number of trials. Therefore, in this study, we applied the binomial distribution to feeding assays to predict predator responses to food items containing

chemical and structural components in varying concentrations. The GENMOD Procedure in SAS (version 8.2, 2001, SAS Institute, Cary, NC) was used to construct the response surfaces and isoboles for the relationship between secondary metabolites and spicules in providing antipredatory defenses to individual sponges from the Florida Keys and Bahamas. Because our past feeding experiments indicated that spicules alone had little or no deterrent effect against fish predators, we examined whether the addition of spicules to assay foods containing crude organic extracts resulted in an interaction that changed the level of deterrency of secondary metabolites in aquarium feeding assays using the bluehead wrasse, *T. bifasciatum*.

2. Materials and methods

2.1. Sponge collection and identification

Sponges for this study were collected from South Abaco (25°98′N, 77°29′W) and San Salvador (24°03.095′N, 74°32.372′W), Bahamas in May 2003, and Jewfish Creek (25°11.24′N, 80°23.27′W), North Dry Rocks (25°07.850′N, 80°17.521′W) and Pickles Reef (24°59.07′N, 80°24.97′W), Florida Keys in October 2003, at depths of 10–30 m using SCUBA. The sponge species used were based on feeding assay data reported by Pawlik et al. (1995): one chemically defended species (*Agelas clathrodes*) and seven species shown to have intermediate levels of defense: *Cinachyrella alloclada* (=*Cinachyra alloclada*), *Clathria virgultosa* (=*Rhaphidophlus juniperinus*), *Cribrochalina infundibulum* (=*Cribrochalina vasculum*), *Niphates digitalis*, *Svenzea zeai* (=*Calyx podatypa*), *Tedania ignis*, and *X. muta* (taxonomy and taxonomic authorities in Zea, 1987; Hooper and Van Soest, 2002). *T. ignis* was collected in mangrove habitats, while all other sponges were found on reefs. Although one individual of each species was used in all procedures, two additional individuals of *X. muta* were used that were both collected at South Abaco, Bahamas, and these had notably different morphologies. Portions of sponges were obtained either by gentle tearing or cutting tissue with a scalpel, and were frozen at −20 °C until used for extractions and spicule collection.

2.2. Crude organic extract isolation

Frozen sponge portions were chopped into 1 cm^3 pieces and 200 ml of sponge tissue was measured by volumetric displacement of water in a graduated cylinder. Crude organic extracts were prepared from the sponge tissue using the successive extraction protocol described in Pawlik et al. (1995). The resulting 200 ml crude extract was partitioned into 20 ml scintillation vials for use in laboratory feeding assays.

2.3. Spicule isolation

Spicules from each sponge were isolated using methods based on Chanas and Pawlik (1995). In order to determine an average amount of spicule content per ml of sponge tissue, each 200 ml equivalent of tissue was placed in an inverted 2 l bottle after extraction. The bottom of the bottle was removed and the opening was fitted with a valve. Chlorine bleach (sodium hypochlorite, 6%) was added to the tissue, and as the tissue oxidized spicules collected at the mouth of the bottle. Several bleach additions were usually needed to oxidize all of the tissue. After the bleach had stopped bubbling the spicules that collected in the bottle were drained through the valve through 52 μm mesh. The material collected on the mesh was removed and allowed to dry on wax paper. To collect microscleres (very small spicules) the filtrate was collected and filtered again through a 5 μm polycarbonate Millepore isopore membrane filter and rinsed with acetone. Large spicules and microscleres were recombined, rinsed again in bleach to dissolve any remaining tissue and moved to a plastic centrifuge tube. The bleach was then removed using a pipet, and the pellet was rinsed three times in distilled water. The pellet was treated with a 1 M solution of sodium thiosulfate for approximately 15 min to neutralize any remaining bleach. The pellet was rinsed twice more in distilled water, and then suspended in a small amount of acetone. The solution was filtered a final time through another 5 μm filter, and the pellet was broken up into small pieces and allowed to dry. The mass of the pellet was obtained for each sponge, and spicule content was standardized to 200 ml of tissue.

2.4. Feeding assays

The use of the bluehead wrasse, *T. bifasciatum*, in aquarium bioassays has been detailed previously (Pawlik et al., 1987, 1995). It is one of the most abundant fishes on Caribbean reefs, and is a generalist carnivore that feeds on a wide diversity of invertebrate species (Randall, 1967). *T. bifasciatum* were used in this study because generalist predators would be less likely than specialists to have evolved ways of avoiding defenses, and invertebrate defense mechanisms would be directed against them in particular because of their abundance and predatory impact. Bioassays used in the present study are based on the methodology described by Pawlik et al. (1995).

To each vial containing crude organic extract prepared from 200 ml of sponge tissue, a composite matrix of 5 g freeze dried, powdered squid mantle, 3 g high viscosity alginic acid (sodium salt), and 100 ml water was added using a graduated syringe. The volumetric amounts of crude extract and squid matrix were varied to create different concentrations; for example, 2.5 ml of squid matrix added to a 2.5 ml crude extract equivalent would constitute a natural concentration of secondary metabolites, while 5 ml of squid matrix added to a 2.5 ml crude extract equivalent would create a concentration one-half that found in the sponge. The mixture was vigorously stirred with a metal spatula to homogenize and suspend the extract into the matrix. The matrix was viscous enough to prevent any settling of assay ingredients. A control mixture was prepared in the same way, but without the addition of the crude extract. Both the control and treatment mixtures were treated with minimal amounts of food coloring until they matched in color. Each mixture was then separately loaded into a 3 ml syringe, and extruded into a 0.25 M solution of calcium chloride to form a long, spaghetti-like strand. After a few minutes, the hardened strands were removed, and chopped into 4 mm long pieces with a razor blade. Food pellets containing spicules were created using weighed spicule amounts proportional to the volumetric densities established using the bleaching process. Squid matrix was extruded into the vial containing the spicules, and the contents were gently stirred to distribute the spicules homogeneously while avoiding breakage. On occasion, sample pellets were bleached again and spicules were examined for any damage incurred during mixing.

Aquarium assays were performed onboard the *RV Seward Johnson* and in the wet laboratory at the Center for Marine Science, University of North Carolina at Wilmington. Control and treated pellets were presented to groups of 2 to 5 yellow- or blue-phase *T. bifasciatum*. For each group, the fish were given a control pellet. If the control pellet was consumed, a treatment pellet containing either crude extract or spicules was offered. If the fish rejected the treatment pellet, another control pellet was given to determine if the fish had ceased to feed. A pellet was considered rejected if the pellet was not eaten (swallowed and spit out) after a total of three attempts by one or more of the fish in that particular group. Fish that did not eat control pellets were not used, and at least 10 groups of fish were required for each individual assay. Feeding assays were conducted until concentrations of crude extract and spicules were found that yielded intermediate levels of deterrency (or ED_{50}, for 50% effective dose). In practice, approximate ED_{50} concentrations used in assays ranged from 3 to 7 pellets out of 10 eaten. In all but a few instances, assays at every concentration were replicated at least twice using different sets of assay fish.

A series of additional, combination assays using both crude extract and spicules in a range of concentrations was performed. All combination assays used concentrations of each ingredient that were equal to or less than the ED_{50} values. For preparation of the food pellets, the squid matrix was added to a dried extract sample and vigorously mixed. Food coloring was then added, and spicules were added last and stirred gently to avoid breakage. For each assay, the ratio of squid matrix added to both the extract and spicules was designed to create different concentrations of each ingredient. Before conducting the combination assays, assays with pellets containing predetermined ED_{50} concentrations of either crude extract or spicules were conducted to verify that the assay fishes being used were not starving or satiated. Again, in all but a few instances, combination assays were replicated at least twice using different sets of assay fish.

2.5. Statistical procedures

The GENMOD Procedure in SAS (version 8.2, 2001, SAS Institute, Cary, NC) was used to fit a generalized linear model to the data collected for all feeding assays conducted. This model was constructed using a binomial probability distribution and a logit link function (Agresti, 1996), with the systematic component consisting of linear terms for both spicule and extract concentrations and a first order interaction between them. Tests of significance (significant difference from zero) for all parameters can be constructed; however, the test of the interaction parameter is the test of interest because it is a test of synergy or antagonism versus a purely additive effect. It should be noted that synergy between spicule and extract concentrations might actually be of a more complex form than this first order interaction; hence, for what follows, statements of significant synergistic or antagonistic effects refer to this level of complexity. In addition, all models were checked for goodness of fit via the deviance statistic (Agresti, 1996) and the Hosmer and Lemeshow test (Hosmer and Lemeshow, 2000), with the logistic regression model being judged as satisfactory in all cases where synergism was detected. Three- and two-dimensional plots were created based on the isobolographic model (e.g. Greco et al., 1995; Tallarida et al., 1997; Nelson and Kursar, 1999) by plotting the logit model, and its ED_{50} contour, after incorporating the parameter estimates.

The X and Y intercepts on the 3-D plot represented the ED_{50} values for crude organic extract and spicule concentrations as determined by the model. A plane intersecting the ED_{50} values was also included as a reference. 2-D plots were created to obtain an additional view of the function/plane interface from the 3-D plot. Each 2-D plot included the interaction function (IF), an additive line that connected the model-derived ED_{50} values (MA), and another additive line that connected the ED_{50} values determined from assay results (EA).

The type of relationship between crude extract and spicules for each individual sponge was assessed using the results from the logistic regression model, and the shape of the function curve in relation to the additive line. Where the interaction parameter was judged as significant, a synergistic or antagonistic interaction was present, with the sign of the parameter indicating the type of interaction. An interaction parameter significantly greater than zero was indicative of a synergistic interaction, while an interaction parameter significantly less than zero indicated an antagonistic interaction. A plot of a synergistic interaction would create a concave curve below the additive line, and a plot of an antagonistic interaction would create a convex curve above the additive line. If synergisms were present, comparisons were also made to determine the difference in the ED_{50} value of crude extract alone and the ED_{50} value of crude extract combined with a natural concentration of spicules.

3. Results

Volumetrically derived spicule densities for each of the ten sponges used in this study are given in Table 1. Synergistic interactions between crude organic extracts and spicules were noted for four species: *A. clathrodes*, *C. alloclada, C. virgultosa*, and one individual of *X. muta*. In most cases, ED_{50} values for crude extract and spicules for each sponge found by conducting assays were similar to those predicted by the logit model. Experimental and model derived ED_{50} values, total number of assays conducted, and deviance for each model are given in Table 2. Comparisons between combination assays and those assays using only crude extract and spicules were

Table 1
Spicule density from each volumetric (200 ml) sponge tissue sample, listed in order of smallest to largest

Sponge	Spicule density (g ml^{-1})
Agelas clathrodes	**0.0052**
Niphates digitalis	0.0094
Clathria virgultosa	**0.0102**
Svenzea zeai	0.0116
Tedania ignis	0.0146
Xestospongia muta, Bahamas soft morphology	0.024
Cinachyrella alloclada	**0.0276**
Cribrochalina infundibulum	0.0294
***Xestospongia muta*, Florida Keys hard morphology**	**0.0397**
Xestospongia muta, Bahamas hard morphology	0.0692

Sponges in which synergy was detected are shown in bold.

Table 2
Comparison of experimental and model derived ED_{50} values for extract and spicule concentrations, total number of assays conducted per sponge, and deviance (scaled by degrees of freedom) with p-value from Hosmer and Lemeshow test for lack of fit for each model

Sponge	Experimental ED_{50} [extract]	Model ED_{50} [extract]	Experimental ED_{50} [spicule]	Model ED_{50} [spicule]	Total assays	Deviance/ *df*	Fit *p*-value
Agelas clathrodes	**0.5×**	**0.37×**	**8×**	**8.6×**	**22**	**1.34**	**0.1082**
Cinachyrella alloclada	**0.5×**	**0.40×**	**2×**	**1.7×**	**19**	**2.11**	**0.1064**
Clathria virgultosa	**1×**	**1.1×**	**4×**	**5.5×**	**25**	**1.77**	**0. 0938**
Cribrochalina infundibulum	4×	4.0×	1×	0.91×	16	2.33	0.0054
Niphates digitalis	1×	2.9×	2×	2.7×	23	0.925	0.9485
Svenzea zeai	2×	1.9×	2×	2.0×	25	2.77	0.0063
Tedania ignis	2×	2.2×	1×	1.3×	17	2.52	0.1360
***Xestospongia muta*, Florida Keys hard morphology**	**1×**	**0.80×**	**1×**	**0.93×**	**17**	**1.27**	**0.9725**
Xestospongia muta, Bahamas hard morphology	1×	1.1×	1×	1.1×	23	2.52	0.0020
Xestospongia muta, Bahamas soft morphology	1×	0.75×	4×	4.1×	28	1.45	0.3899

Sponges in which synergy was detected are shown in bold.

made using the IF and MA plots on the 2-D graphs. The percentages of food pellets accepted at natural conditions for each sponge in terms of extract, spicule, and combination concentrations are shown in Table 3. There was no obvious spicule breakage in assay pellets that were bleached for inspection after assays. Combination assays for *A. clathrodes* replicated twice composed 18% of the total assay data for the sponge. For *C. alloclada*, they composed 21% of the data set, and for *T. ignis* and the Florida Keys *X. muta* they composed 35% of total data. At least 40% of the total data for all other sponges was composed of replicated combination assays.

Table 3
GENMOD logistic model predictions of percentage of food pellets accepted per assay for each sponge at natural concentrations

Sponge	Extract=1×	Spicule=1×	Extract=1×, spicule=1×
Agelas clathrodes	**1.0**	**91**	**<1.0**
Cinachyrella alloclada	**15**	**62**	**<1.0**
Clathria virgultosa	**54**	**80**	**12**
Cribrochalina infundibulum	83	45	19
Niphates digitalis	70	69	57
Svenzea zeai	67	68	45
Tedania ignis	71	60	37
***Xestospongia muta*, Florida Keys hard morphology**	**46**	**48**	**<1.0**
Xestospongia muta, Bahamas hard morphology	55	53	12
Xestospongia muta, Bahamas soft morphology	34	84	22

Sponges in which synergy was detected are shown in bold.

The crude organic extract of *A. clathrodes* at natural concentration exhibited the highest level of deterrence of all sponges tested. This was in accordance with crude extract assays conducted by Pawlik et al. (1995), who observed a mean of 0 out of 10 pellets consumed at natural concentration. The plots show that the ED_{50} for crude extract was approximately 0.37× while the ED_{50} for spicules was approximately 8.6×. A synergistic interaction between crude extract and spicules was noted (GENMOD, $p<0.01$; $IF=(-6.97x+2.58)*(0.30+1.88x)^{-1}$; $MA=-23.07x+8.55$).

The model ED_{50} values for *C. alloclada* were approximately 0.40× crude extract and 1.7× spicules. Synergism was also observed in this case (GENMOD, $p=0.02$; Fig. 1). According to the 2-D plot, when combined with 1× spicules, approximately 0.10× crude extract is required to create an ED_{50}. The crude extract ED_{50} value for *C. virgultosa* was close to natural concentrations (1.1×), while the spicule ED_{50} was 5.5×. The graphs show that a combination of a natural concentration of spicules with a reduced extract concentration (0.55×, slightly more than half of that needed in isolation) was also an ED_{50}. This interaction was synergistic (GENMOD, $p<0.01$; Fig. 2).

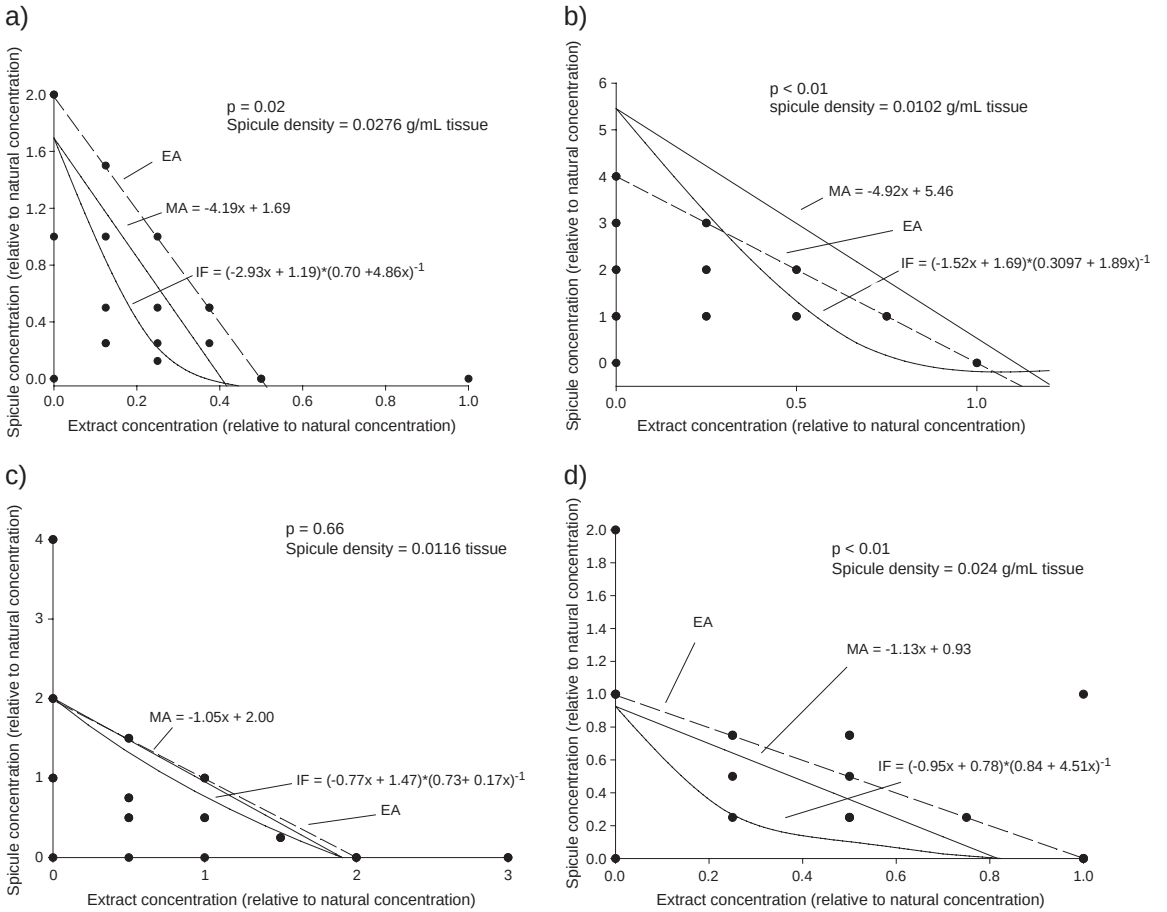

Fig. 1. Two-dimensional isobolograms for (a) *C. alloclada*, (b) *C. virgultosa*, (c) *S. zeai*, and (d) *X. muta*, Florida Keys. EA=Experimental additive line, MA=model derived additive line, IF=interaction function. Filled circles indicate combination assays. The p value is determined by comparing the square of the parameter estimate for the interaction function divided by its standard error with the χ^2 distribution.

Additive interactions (no significant relationship) between crude extract and spicules were found for *C. infundibulum* (GENMOD, $p=0.18$; IF$=(-0.53x+2.10)*(2.31+0.72x)^{-1}$; MA$=-0.23x+0.91$), *N. digitalis* (GENMOD, $p=0.92$; IF$=(-0.44x+1.27)*(0.48-0.08x)^{-1}$; MA$=-0.92x+2.67$), *S. zeai* (GENMOD, $P=0.66$), and *T. ignis* (GENMOD, $p=0.84$; IF$=(-0.75x+1.65)*(1.25+0.31x)^{-1}$; MA$=-0.60x+1.32$). Results varied considerably among the three individuals of *X. muta*. The structural morphology of each individual appeared in the field to be different: one collected at South Abaco, Bahamas was rigid and required cutting with a scalpel to obtain it, while another about 5 m away was soft and was collected easily with gentle tearing. The third, collected in the Florida Keys, resembled the more rigid sponge found in the Bahamas.

All of the *X. muta* appeared to be healthy and none exhibited signs of bleaching. The spicule densities varied from 0.024 g ml^{-1} tissue in the Bahamas soft morphology to 0.0692 g ml^{-1} tissue in the Bahamas hard morphology (Table 1). The ED_{50} values for the crude extracts were similar (Bahamas soft *X. muta*=0.76×, Bahamas hard *X. muta*=1.1×, Keys *X. muta*=0.80×) while the spicule concentration required by the Bahamas soft *X. muta* was higher (4.1×) than the Bahamas hard *X. muta* (1.6×) or Keys *X. muta* (0.9×). However, only the Keys *X. muta* was synergistic (GENMOD, $p<0.01$) while the others (Bahamas hard *X. muta*: GENMOD, $p=0.47$;

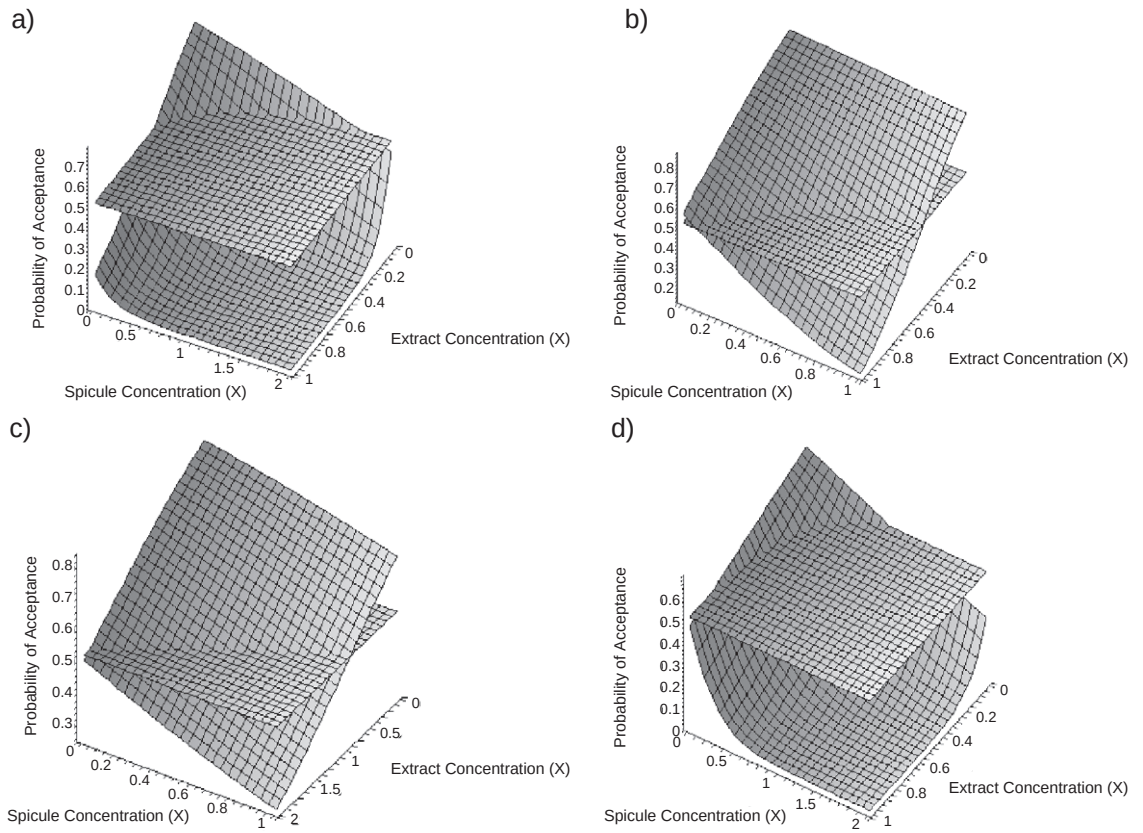

Fig. 2. Three-dimensional isobolograms for (a) *Cinachyrella alloclada*, (b) *Clathria virgultosa*, (c) *Svenzea zeai*, and (d) *Xestospongia muta*, Florida Keys. A plane at the ED_{50} response level is included as a reference. Concentrations are given relative to natural concentration (X).

$IF = (-1.48x + 1.66) * (1.53 + 0.65x)^{-1}$; MA = $-0.97x + 1.09$; Bahamas soft *X. muta*: GENMOD, $p = 0.91$, IF = $(-2.86x + 2.19)*(0.54 + 0.04x)^{-1}$; MA = $-5.29x + 4.05$) were additive. Approximately 0.25× concentrations of crude extract and spicules in combination were required in the Florida Keys *X. muta* to reach the ED_{50} level.

4. Discussion

Synergistic interactions between crude organic extracts and spicules as antipredatory defenses against the generalist fish predator *T. bifasciatum* were discovered for the tissues of individuals of four Caribbean sponge species: *A. clathrodes*, *C. alloclada*, *C. virgultosa*, and *X. muta* from the Florida Keys. For the last three of these sponges, synergy was detected at approximately natural concentrations of crude extracts and spicules. For *A. clathrodes*, combination assays required nearly a 3-fold reduction in extract concentration and an 8-fold increase in spicule concentration to show a synergistic effect. Contrary to the findings of Chanas and Pawlik (1995), spicules from *C. infundibulum* and two of three individuals of *X. muta* were deterrent at natural concentrations. Spicules from *C. alloclada* and *T. ignis* were deterrent at near-natural concentrations. Sponges with higher spicule densities required less manipulation of their spicule concentrations in laboratory assays to achieve ED_{50} levels, but neither synergy nor chemical defense was correlated with spicule density. These findings indicate that for some sponges, structural elements may be antipredatory defenses in isolation, or may serve to enhance chemical defenses against consumers.

While this study provides a novel technique for assessing synergistic interactions between defensive components using laboratory feeding experiments, we have not yet used the technique to investigate the variability in defensive synergisms within any one sponge species, beyond the three individuals of *X. muta*. For 7 of the 8 sponge species investigated, all of the data were collected from the tissue of a single sponge. The effort involved in generating the analyses shown in Figs. 1 and 2 was considerable, and precluded a more thoroughly replicated study in which tissue from several individuals of each species were subjected to the panel of assays. The alternative of assaying a composite sample of tissue from several individuals of one sponge species was rejected, because homogenization of spicules and secondary metabolites from several samples was likely to obscure the concentration-dependent relationship present in any individual sponge. In fact, for the one species subjected to replicate assays, *X. muta*, two individuals from different locations that had comparable spicule densities and crude extract palatabilities differed in interaction type; one was synergistic, the other was not. In lieu of a more thoroughly replicated study of within or between population variation in defenses for one sponge species, we chose to investigate the variation between individuals of 8 species, with a focus on 7 that had previously been demonstrated to have intermediate levels of chemical defenses (Pawlik et al., 1995; Chanas and Pawlik, 1997), because these would be the most likely to show a synergistic relationship between chemical and structural defenses. However, individuals of only 3 of these 7 sponge species exhibited defensive synergy at approximately natural concentrations of metabolites and spicules, suggesting that, while synergisms may exist, they are not the general rule for species elaborating intermediate levels of chemical defenses.

Why were spicules from *C. infundibulum* and from 2 of 3 individuals of *X. muta* found to be deterrent in this study when assayed alone, but not in previous investigations (Chanas and Pawlik, 1995; Burns and Ilan, 2003)? The reasons for this are probably methodological or a consequence of intraspecific variation. Chanas and Pawlik (1995) conducted laboratory and field assays with spicules from 8 sponge species, including 3 of the species investigated in the present study (*A. clathrodes*, *C. infundibulum*, and *X. muta*), and found that none of the spicule samples deterred feeding. Chanas and Pawlik (1996) also found that spiculated spongin skeleton with spicules oriented in their natural conformations was palatable in similar assays, but that spicules were deterrent when incorporated into artificial foods containing reduced protein concentrations. Contrarily, Burns and Ilan (2003) reported that natural concentrations of spicules from 4 Red Sea sponges and 2 Caribbean sponges (*C. nucula* and *Geodia neptuni*) were deterrent in feeding assays with the Red Sea wrasse *T. klunzingeri*, but that spicules from *C. infundibulum* were not deterrent. The findings presented herein may differ from these previous studies for two reasons. First, both Chanas and Pawlik (1995) and Burns and Ilan (2003) bleached small (10 ml) volumetric equivalents of sponge tissue for spicule collection. In preliminary trials conducted in the present study, five 10 ml volumetric equivalents of *A. clathrodes* tissue were bleached and treated following the protocol described in Chanas and Pawlik (1995), and significant variations were found between the dry weights of spicules collected from each equivalent. This may have been due to an unequal distribution of spicules throughout the sponge tissue, possibly in relation to the position of the sponge relative to current or wave action in the field. We avoided this problem by bleaching a larger amount (200 ml) of tissue. As discussed by both Chanas and Pawlik (1995) and Burns and Ilan (2003), while the spicule arrangement in the tissue of different sponges varies, spicule orientation is haphazard when food pellets are created using this assay technique. However, because Chanas and Pawlik (1996) found no feeding deterrence even when spicules were offered in their natural orientation, this variable should not have influenced the difference in results.

Intraspecific variation may provide an explanation for the disparate assay results for *X. muta*, individuals of which were observed to have notably different morphologies and spicule densities (Table 1). “Hard” and “soft” morphologies were first described for the Great Barrier Reef sponge *Xestospongia testudinaria* by Fromont (1988), who found that the hard form had complete tracts of spicules encased by spongin fibers, while the soft form had spicule tract junctions bound by spongin fiber development. Therefore, in addition to the difference in spicule isolation technique

discussed above, the tissue samples of *X. muta* used by Chanas and Pawlik (1995) could have been of a softer morphology than the two hard forms (the Florida Keys and hard Bahamas individuals), and may have not contained sufficient spicule densities to be deterrent. Chemical defenses in *X. muta* can vary significantly based on geographic location (Chanas and Pawlik, 1997), but although the *X. muta* collected in the Florida Keys exhibited a synergistic interaction while those from the Bahamas did not, all three individuals yielded crude extracts with comparable palatability. Spicules from each of the three were measured using an ocular micrometer on a compound microscope at 400× and were not significantly different ($n=10$; ANOVA, $p=0.97$). The fact that the tissue components of one *X. muta* exhibited a synergistic defensive interaction while the other two were additive could be due to a difference in the mixture of secondary metabolites present in each of the individuals.

The advantage of synergistic interactions between different components is considerable, because less energy could be designated to achieve the same degree of defense and these components could be effective against a wider range of competitors. Berenbaum and Neal (1985) found that a terrestrial plant secondary metabolite became significantly more toxic when a second, nontoxic compound was produced that acted as a competitive inhibitor of microsomal mixed-function oxidases against predatory insects. In the study of Hay et al. (1994), the proposed synergistic relationship between algal secondary metabolites and $CaCO_3$ was changed when a lower quality test food was substituted, suggesting that nutritional value was also interacting to affect herbivory. The mechanism by which secondary metabolites and spicules interact is still unknown. Spicules might abrade oral and digestive surfaces and facilitate the intake of secondary metabolites. Synergisms could then be dependent on the type of spicules present, if those of a particular size or shape are more effective in causing abrasion. Spicules pass through the guts of turtles (Meylan, 1988), fish (Randall and Hartman, 1968) and invertebrates (Birenheide et al., 1993) and do not appear to cause digestive problems. In the present study, assay fishes were observed for weeks after assays were performed and did not appear to be harmed by spicule consumption. Chanas and Pawlik (1996) showed that spicules deterred predation if the nutritional value of food items is sufficiently lowered. Again, the rapid acceptance or rejection of assay pellets was probably due to either variation in taste, texture, or digestive surface abrasion because the fishes could not visually distinguish between control and treatment pellets. Siliceous spicules are inert, as opposed to $CaCO_3$ sclerites or granules that may alter the pH of acidic guts, and therefore do not act as an inorganic chemical defense (see Hay et al., 1994; Hay, 1996b).

Most experimental evidence indicates that the primary function of structural components in sponges and other organisms such as gorgonians and ascidians is skeletal support. Koehl (1982) described the mechanical design of spicule-reinforced connective tissue and how it enhances tissue rigidity. Spicule concentrations in tissue increased in density in response to wave force and to sponge tissue damage (Palumbi, 1986; McDonald et al., 2002). However, it is unknown if spicules in some sponges are used specifically to augment chemical defense, or if increases in defense are only a side-product of structural reinforcement. Walters and Pawlik (2005) found that chemically undefended sponges healed wounds at faster rates than those that were chemically defended, and proposed that energy may be allocated either to faster healing and growth rates or chemical defenses and slower growth rates. They also studied the material created by sponges to initially cover the wounds and found it to be interlaced with spicules, which may be attributable to spicules being used to add stability to the afflicted area. It may be difficult to distinguish whether spicule densities reflect structural support relative to flow fields or defensive interactions with chemical components. Flow rates at the sites where sponges were collected for this study were not measured. One way to examine these relationships would be to replicate the analysis presented herein on several individual sponges of one species that are subject to very different flow environments.

Coley et al. (1985) proposed an evolutionary correlation in terrestrial plants between resource availability and levels of anti-herbivore defense. When nutrients are limiting in the environment, plants with slower growth rates are favored, and they can invest substantial amounts of energy into defenses while minimizing nutrient loss. Ample nutrient amounts

contribute to faster growth, since photosynthesis and respiration rates can increase and plants can tolerate rapid leaf turnover in order to maintain high levels of energy intake. Defensive compounds may also be the product of selection based on their construction costs and metabolic effort to maintain their concentrations; slower growth favors high construction costs but low maintenance, with the opposite observed in faster growing plants. The fact that coexisting reef sponges vary in both levels of chemical defense and the presence or absence of synergisms implies that resource availability is probably not the primary factor governing variation in sponge defensive mechanisms. Spongivorous fishes have a considerable affect on reef sponge distribution and abundance and confine several sponge species to cryptic refugia or mangrove habitats (Pawlik, 1997). If faster growth rates are needed to persist in the absence of strong chemical defenses, synergisms between secondary metabolites and spicules in sponges with intermediate levels of defense could increase the amount of energy available for growth and reproduction. As indicated above, it is interesting that synergy was found in only 3 of 7 sponge species with intermediate levels of chemical defense in this study.

Traits that have evolved for one purpose that later become used for additional purposes are called exaptations (Gould and Vrba, 1982; Arnold, 1994). An "addition exaptation" is one in which the trait has initial functional advantages, and a new use becomes added to the first (Arnold, 1994). A genus of neotropical vines, *Dalechampia* (Euphorbiaceae) has several defensive traits that appear to be examples of exaptations, including bracts that function both as a floral advertisement system and as protection of staminate and pistillate flowers, and resins that evolved from being pollinator rewards to defending ovaries, seeds, leaves, and shoot tips (Armbruster, 1997). The dual role of spicules as support for the sponge skeleton and as an enhancement of chemical defenses may be an example of an addition exaptation, in which some sponge species are able to gain defensive advantages through the use of a component provided by evolution for the original purpose of structural support. This could explain why sponge spicules are in most cases not deterrent at natural concentrations by themselves in laboratory bioassays, but combination assays can show the synergistic effect.

The statistical procedure used in this study was inspired by two different analytical approaches, potentially leading to a novel way of examining defensive synergism. The isobolographic model is preferred by pharmacologists because of its flexibility and lack of assumptions about interaction mechanisms (Nelson and Kursar, 1999) as well as its graphical format (e.g. Berenbaum, 1977). It also serves as an alternative to additive or multiplicative designs that introduce complications or limitations in data analysis (Billick and Case, 1994). The generalized linear model is a common statistical procedure used for categorical data analysis that can expand upon the predictive abilities of the traditional linear model. Using attributes of these two methods in concert has considerable potential for ecological investigations. All assay data collected in procedures designed to measure interactions can be included in the analysis, rather than being limited to the inclusion of ED_{50} values with the isobolograph alone. The assay fish *T. bifasciatum* provides consistent feeding assay results and can give accurate ED_{50} values from which to gauge the concentrations used in combination assays. Using the method employed in the present study to reexamine data collected in investigations that found no evidence of ecological interactions could reveal synergisms or antagonisms that were previously overlooked.

Acknowledgements

This work was part of an MS thesis completed at the University of North Carolina at Wilmington by ACJ. The research was funded by the Biological Oceanography Program of the National Science Foundation (0095724, including UNOLS funding of ship time aboard *RV Seward Johnson*) and the National Undersea Research Center at UNCW (NOAA grant NA96RU-0260). The authors wish to thank Shobu Odate, Kyle Walters, Timothy Henkel, Jonathan Cowart, and Tricia Meredith for assistance with laboratory work and field collections. Comments of anonymous reviewers led to important improvements of the manuscript. We thank the captain and crew of the *RV Seward Johnson* and the staff of the National Undersea Research Center in Key Largo, Florida, for their cooperation. We are grateful to the

government of the Bahamas for permission to perform research in their territorial waters. **[SS]**

References

Agresti, A., 1996. An Introduction to Categorical Data Analysis. Wiley, New York.

Armbruster, W.S., 1997. Exaptations link evolution of plant–herbivore and plant–pollinator interactions: a phylogenetic inquiry. Ecology 78, 1661–1672.

Arnold, E.N., 1994. Investigating the origin of performance advantage: adaptation, exaptation and lineage effects. In: Eggleton, P., Vane-Wright, R. (Eds.), Phylogenetics and Ecology. Academic Press, London, pp. 123–168.

Berenbaum, M.C., 1977. Synergy, additivism, and antagonism in immunosuppression: a critical review. Clin. Exp. Immunol. 28, 1–18.

Berenbaum, M.C., 1988. Isobolographic, algebraic, and search methods in the analysis of multiagent synergy. J. Am. Coll. Toxicol. 7, 927–938.

Berenbaum, M., Neal, J.J., 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J. Chem. Ecol. 11, 1349–1358.

Bergquist, P.R., 1978. Sponges. University of California Press, Los Angeles.

Berkson, J., 1944. Application of the logistic function to bio-assay. J. Am. Stat. Assoc. 39, 357–365.

Billick, I., Case, T.J., 1994. Higher order interactions in ecological communities: what are they and how can they be detected? Ecology 75, 1529–1543.

Birenheide, R., Amemiya, S., Motokawa, T., 1993. Penetration and storage of sponge spicules in tissues and coelom of spongivorous echinoids. Mar. Biol. 115, 677–683.

Blunt, J.W., Copp, B.R., Munro, M.H., Northcote, P.T., Prinsep, M.R., 2004. Marine natural products. Nat. Prod. Rep. 21, 1–49.

Bridwell, J.C., 1918. Notes on the Bruchidae and their parasites in the Hawaiian Islands. Proc. Hawaii. Entomol. Soc. 3, 465–505.

Burns, E., Ilan, M., 2003. Comparison of anti-predatory defenses of Red Sea and Caribbean sponges: II. Physical defense. Mar. Ecol. Prog. Ser. 252, 115–123.

Case, T.J., Bender, E.A., 1981. Testing for higher order interactions. Am. Nat. 121, 887–891.

Chanas, B., Pawlik, J.R., 1995. Defenses of Caribbean sponges against predatory reef fish: II. Spicules, tissue toughness, and nutritional quality. Mar. Ecol. Prog. Ser. 127, 195–211.

Chanas, B., Pawlik, J.R., 1996. Does the skeleton of a sponge provide a defense against predatory reef fish? Oecologia 107, 225–231.

Chanas, B., Pawlik, J.R., 1997. Variability in the chemical defense of the Caribbean reef sponge *Xestospongia muta*. Proceedings of the 8th International Coral Reef Symposium, vol. 2, pp. 1363–1368.

Coley, P.D., Bryant, J.P., Chapin III, F.S., 1985. Resource availability and plant antiherbivore defense. Science 230, 895–899.

Cooper, S.M., Owen-Smith, N., 1986. Effects of plant spinescence on large mammalian herbivores. Oecologia 68, 446–455.

Duffy, J.E., Paul, V.J., 1992. Prey nutritional quality and the effectiveness of chemical defenses against tropical reef fishes. Oecologia 90, 333–339.

Dunlap, M., Pawlik, J.R., 1996. Video-monitored predation by Caribbean reef fish on an array of reef and mangrove sponges. Mar. Biol. 126, 117–123.

Dunlap, M., Pawlik, J.R., 1998. Spongivory by parrotfish in Florida mangrove and reef habitats. P.S.Z.N. I, Mar. Ecol. 19, 325–337.

Engel, S., Pawlik, J.R., 2000. Allelochemical activities of sponge extracts. Mar. Ecol. Prog. Ser. 207, 273–281.

Fagerström, T., 1989. Anti-herbivory chemical defense in plants: a note on the concept of cost. Am. Nat. 133, 281–287.

Fisher, R.A., Yates, F., 1938. Statistical Tables. Oliver and Boyd, Edinburgh.

Fraser, T.R., 1870–1871. An experimental research on the antagonism between the actions of physostigma and atropia. Proc. R. Soc. Edinb. 7, 506–511.

Fromont, J., 1988. Aspects of the reproductive biology of *Xestospongia testudinaria* (Great Barrier Reef). Proceedings of the 6th International Coral Reef Symposium, Australia, vol. 2, pp. 685–691.

Gaines, S.D., 1985. Herbivory and between-habitat diversity: the differential effectiveness of defenses in a marine plant. Ecology 66, 473–485.

Gessner, P.K., 1988. A straightforward method for the study of drug interactions: an isobolographic analysis primer. J. Am. Coll. Toxicol. 7, 987–1012.

Gould, S.J., Vrba, E.S., 1982. Exaptation—a missing term in the science of form. Paleobiology 8, 4–15.

Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: a critical review from a response surface prospective. Pharm. Rev. 47, 331–469.

Harvell, C.D., Fenical, W., Greene, D.H., 1988. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.): I. Development of an in situ feeding assay. Mar. Ecol. Prog. Ser. 49, 287–294.

Haslam, E., 1988. Plant polyphenols (syn. Vegetable tannins) and chemical defense—a reappraisal. J. Chem. Ecol. 14, 1789–1805.

Hay, M.E., 1996a. Defensive synergisms? Reply to Pennings. Ecology 77, 1950–1952.

Hay, M.E., 1996b. Marine chemical ecology: what's known and what's next? J. Exp. Mar. Biol. Ecol. 200, 103–134.

Hay, M.E., Kappel, Q.E., Fenical, W., 1994. Synergisms in plant defenses against herbivores: interactions of chemistry, calcification, and plant quality. Ecology 75, 1714–1726.

Herms, D.A., Mattson, W.J., 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. 67, 283–335.

Hixon, M.A., 1983. Fish grazing and community structure of coral reefs and algae: a synthesis of recent studies. In: Reaka, M.L. (Ed.), The Ecology of Deep and Shallow Coral Reefs, Symposia Series for Undersea Research. NOAA/NURP, Washington, DC, pp. 79–87.

Hooper, J.N.A., Van Soest, R.W.M. (Eds.), 2002. Systema porifera: a guide to the classification of sponges, Introductions and Demospongiae, vol 1. Plenum Publishers, New York.

Hosmer, D., Lemeshow, S., 2000. Applied Logistic Regression. Wiley, New York.
Howard, J.J., 1988. Leafcutting and diet selection: relative influence of leaf chemistry and physical features. Ecology 69, 250–260.
Janzen, D.C., 1969. Seed eaters versus seed size, number, toxicity and dispersal. Evolution 23, 1–27.
Karban, R., Agrawal, A.A., Mangel, M., 1997. The benefits of induced defenses against herbivores. Ecology 78, 1351–1355.
Kelly, S.R., Jensen, P.R., Henkel, T.P., Pawlik, J.R., 2003. Effects of Caribbean sponge extracts on bacterial attachment. Aquat. Microb. Ecol. 31, 175–182.
Koehl, M.A.R., 1982. Mechanical design of spicule-reinforced connective tissue: stiffness. J. Exp. Biol. 98, 239–267.
Levin, D.A., 1976. The chemical defenses of plants to pathogens and herbivores. Annu. Rev. Ecol. Syst. 7, 121–159.
Lynn, D.G., Chang, M., 1990. Phenolic signals in cohabitation: implications for plant development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 497–526.
Maxwell, F.G., Jenkins, J.N., Parrott, W.L., 1972. Resistance of plants to insects. Adv. Agron. 24, 187–265.
McClintock, J.B., Baker, B.J., 2001. Marine Chemical Ecology. CRC Press, New York.
McDonald, J.I., Hooper, J.N.A., McGuinness, K.A., 2002. Environmentally influenced variability in the morphology of *Cinachyrella australiensis* (Carter 1886) (Porifera: Spirophorida: Tetillidae). Mar. Freshw. Res. 53, 79–84.
McNaughton, S.J., Tarrants, J.L., McNaughton, M.M., 1985. Silica as a defense against herbivory and a growth promotor in African grasses. Ecology 66, 528–535.
Meylan, A., 1988. Spongivory in hawksbill turtles: a diet of glass. Science 239, 393–395.
Nelson, A.C., Kursar, T., 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9, 81–92.
O'Neal, W., Pawlik, J.R., 2002. A reappraisal of the chemical and physical defenses of Caribbean gorgonian corals against predatory fishes. Mar. Ecol. Prog. Ser. 240, 117–126.
Palumbi, S.R., 1986. How body plans limit acclimation: responses of a demosponge to wave force. Ecology 67, 208–214.
Paul, V.J. (Ed.), 1992. Ecological Roles of Marine Natural Products. Comstock Publishing, Ithaca, New York.
Pawlik, J.R., 1993. Marine invertebrate chemical defenses. Chem. Rev. 93, 1911–1922.
Pawlik, J.R., 1997. Fish predation on Caribbean reef sponges: an emerging perspective of chemical defenses. Proceedings of the 8th International Coral Reef Symposium, vol. 2, pp. 1255–1258.
Pawlik, J.R., Burch, M.T., Fenical, W., 1987. Patterns of chemical defense among Caribbean gorgonian corals: a preliminary survey. J. Exp. Mar. Biol. Ecol. 108, 55–66.
Pawlik, J.R., Kernan, M.R., Molinski, T.F., Harper, M.K., Faulkner, D.J., 1988. Defensive chemicals of the Spanish dancer nudibranch *Hexabranchus sanguineus* and its egg ribbons: macrolides derived from a sponge diet. J. Exp. Mar. Biol. Ecol. 119, 99–109.
Pawlik, J.R., Chanas, B., Toonen, R.J., Fenical, W., 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrency. Mar. Ecol. Prog. Ser. 127, 183–194.
Pennings, S.C., 1996. Testing for synergisms between chemical and mineral defenses—a comment. Ecology 77, 1948–1950.
Pennings, S.C., Paul, V.J., 1992. Effect of plant toughness, calcification, and chemistry on herbivory by *Dolabella auricularia*. Ecology 73, 1606–1619.
Poulton, J.E., 1990. Cyanogenesis in plants. Plant Physiol. 94, 401–405.
Randall, J.E., 1967. Food habits of reef fishes of the West Indies. Stud. Trop. Oceanogr. 5, 665–847.
Randall, J.E., Hartman, W.D., 1968. Sponge-feeding fishes of the West Indies. Mar. Biol. 1, 216–225.
Rhoades, D.F., 1979. Evolution of plant chemical defense against herbivores. In: Rosenthal, G.A., Janzen, D.H. (Eds.), Herbivores: their Interaction with Secondary Plant Metabolites. Academic Press, Orlando, pp. 3–54.
Schmahl, G.P., 1991. Community structure and ecology of sponges associated with four southern Florida coral reefs. In: Rutzler, K. (Ed.), 3rd International Sponge Conference, New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington, DC, pp. 376–383.
Schultz, J.C., 1988. Many factors influence the evolution of herbivore diets, but plant chemistry is central. Ecology 69, 896–897.
Swearingen III, D.C., Pawlik, J.R., 1998. Variability in the chemical defense of the sponge *Chondrilla nucula* against predatory reef fish. Mar. Biol. 131, 619–627.
Tallarida, R.J., Stone Jr., D.J., Raffa, R.B., 1997. Efficient designs for studying synergistic drug combinations. Pharmacol. Lett., Life Sci. 61, 417–425.
Uriz, M.J., Turon, X., Becerro, MA., Galera, J., 1996. Feeding deterrence in sponges: the role of toxicity, physical defenses, energetic contents, and life-history stage. J. Exp. Mar. Biol. Ecol. 205, 187–204.
Waddell, B., Pawlik, J.R., 2000a. Defenses of Caribbean sponges against invertebrate predators: I. Assays with hermit crabs. Mar. Ecol. Prog. Ser. 195, 125–132.
Waddell, B., Pawlik, J.R., 2000b. Defenses of Caribbean sponges against invertebrate predators: II. Assays with sea stars. Mar. Ecol. Prog. Ser. 195, 133–144.
Walters, K.D., Pawlik, J.R., 2005. Is there a trade-off between wound-healing and chemical defenses among Caribbean reef sponges? Int. Comp. Biol. 45, 136–142.
Wilkinson, C.F., 1973. Insecticide synergism. Chemtech 3, 492–497.
Wootton, J.T., 1994. Putting the pieces together: testing the independence of interactions among organisms. Ecology 75, 1544–1551.
Wulff, J.L., 1994. Sponge feeding by Caribbean angelfishes, trunkfishes, and filefishes. In: Van Soest, R.W.M., Van Kempen, T.M.G., Braekman, J.C. (Eds.), Sponges in Space and Time. Balkema, Rotterdam, pp. 265–271.
Zangerl, A.R., Rutledge, C.E., 1996. The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. Am. Nat. 147, 599–608.
Zea, S., 1987. Esponjas del Caribe Colombiano. Editorial Catalogo Cientifico, Santa Marta, Colombia.