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## Multiple defensive roles for triterpene glycosides from two Caribbean sponges

Received: 23 July 2001 / Accepted: 29 October 2001 / Published online: 14 December 2001  
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**Abstract** Despite their high nutritional value and a lack of physical defenses, most marine sponges appear to be minimally affected by predators, competitors, and fouling organisms, possibly due to sponge chemical defenses. In the last 15 years, several triterpene glycosides have been isolated from sponges, but their ecological or physiological roles are largely unknown. We tested triterpene glycosides from *Erylus formosus* and *Ectyoplasia ferox*, Caribbean sponges belonging to two different orders, in field and laboratory assays for effects on fish feeding, attachment by potential biofilm-forming bacteria, fouling by invertebrates and algae, and overgrowth by neighboring sponges. Formoside and other triterpene glycosides from *Erylus formosus* deterred predation, microbial attachment, and fouling by invertebrates and algae. Triterpene glycosides from *Ectyoplasia ferox* were found to be antipredatory and allelopathic. Thus, triterpene glycosides in these sponges appear to have multiple ecological functions. Tests with different triterpene glycosides at several concentrations indicated that small differences in molecular structure affect ecological activity. In order to establish whether triterpene glycosides could be involved in water-borne versus surface-mediated interactions, the presence of triterpene glycosides in the seawater surrounding live sponges was measured using two in situ sampling methods followed by HPLC and NMR spectral analysis. Water-borne triterpene glycosides were below detection limits for both species. However, top sponge

layers and swabs of the surfaces of both sponges contained sufficiently high concentrations of triterpene glycosides to deter bacterial settlement and fouling of *Erylus formosus* surfaces and overgrowth of *Ectyoplasia ferox* by neighboring sponges. Enemies of these sponges appear to be deterred by surface contact of triterpene glycosides rather than by water-borne interactions. The dual strategy of employing one group of compounds for multiple purposes and minimizing the loss of compounds into seawater suggests that these organisms utilize chemical defenses with efficiency.

**Keywords** Sponge · Predation · Fouling · Allelopathy · Chemical defense

### Introduction

Tropical reefs are diverse ecosystems, characterized by intense predation and spatial competition. Sponges are among the most abundant and diverse group of organisms on tropical coral reefs (Targett and Schmahl 1984). While many sponges lack physical defenses (Chanas and Pawlik 1995), their abundance and persistence is attributed to chemical defenses used to deter predation, inhibit bacterial attachment and fouling, and prevent overgrowth by neighboring organisms. There is evidence that sponge secondary metabolites play important roles in predator deterrence (e.g., Thompson et al. 1985; Pawlik et al. 1988; Herb et al. 1990; Pennings et al. 1994; Albrizio et al. 1995; Chanas et al. 1996; Becerro et al. 1998; Wilson et al. 1999; Assmann et al. 2000; Kubanek et al. 2000) and in competition for space (Sullivan et al. 1983; Thompson 1985; Thompson et al. 1985; Porter and Targett 1988; Henrikson and Pawlik 1995; Turon et al. 1996; Thacker et al. 1998; Engel and Pawlik 2000). In particular, protection of sponge surfaces against bacterial colonization and bio-film formation (Wahl 1989) has been suggested by the antimicrobial activities of some sponge compounds (e.g., Thompson et al. 1985; Bobzin and Faulkner 1992; Abarzua and Jakubowski 1995;

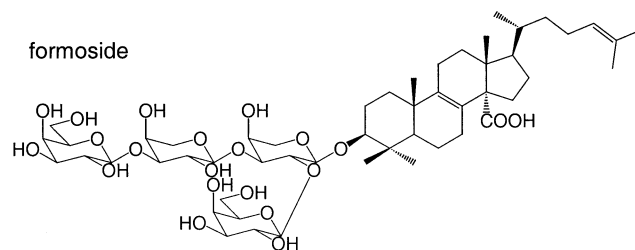
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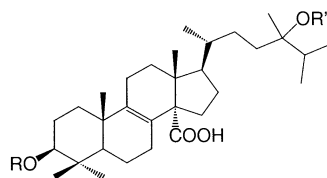
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**Fig. 1** Antipredatory triterpene glycosides from *Erylus formosus* (Jaspars and Crews 1994; Kubanek et al. 2000, 2001)



Mixtures A, B consist of hexasaccharides in which R and R' = saccharides:



Mixture A saccharides:

arabinose  
galactose  
N-acetyl glucosamine  
glucose

Mixture B saccharides:

arabinose  
galactose  
N-acetyl glucosamine  
unknown amino sugar

Newbold et al. 1999). Macrofoulers such as invertebrates and algae can also be deterred from settlement by sponge compounds (Nakatsu et al. 1983; Thompson et al. 1985; Walker et al. 1985; Davis et al. 1989; Pawlik 1992), and sponges may produce compounds that prevent overgrowth by neighboring organisms (Porter and Targett 1988; Turon et al. 1996; Thacker et al. 1998; Engel and Pawlik 2000).

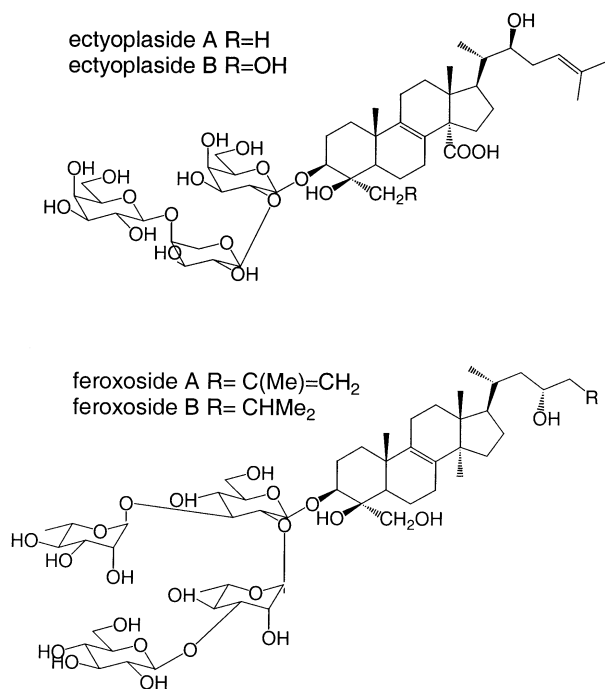
Optimization of defenses could lead to multiple roles for secondary metabolites. Molecules that function on receptors conserved across taxa or that function by generalized mechanisms such as membrane disruption might provide protection against diverse biotic threats. If costs of synthesizing and maintaining chemical defenses are high (Rhoades 1979) and constrain the widespread use of such defenses (Coley et al. 1985; Gulmon and Mooney 1986; Herms and Mattson 1992), then utilizing a compound for multiple purposes should increase the overall fitness of an organism relative to producing several compounds for several purposes. However, multiple uses for single compounds could limit adaptive change. For example, if a bacterial enemy acquires resistance to an antibiotic that also deters predators, then mutation-generated changes to the pathway for antibiotic production may result in loss of predator deterrence. Nevertheless, instances of multiple ecological roles for natural products are known, for example C<sub>11</sub> cyclic olefins that both deter herbivorous amphipods and attract conspecific gametes from brown algae (Boland 1995; Hay et al. 1998) and nordihydroguaiaretic acid from creosote bush that deters herbivorous insects and functions as an antifungal agent (Fernandez et al. 1979; Chapman et al. 1988).

Although multiple functions for individual secondary metabolites may be common in the marine environment, only a handful of studies have considered this subject (Thompson et al. 1985; Bobzin and Faulkner 1992; de Nys et al. 1995; Schmitt et al. 1995, 1998; Becerro et al. 1997; Kjelleberg et al. 1997; Hay et al. 1998; Maximillien et al. 1998; Newbold et al. 1999). To date, more attention has been paid to antipredatory and antiherbivory effects

of secondary metabolites than to defensive roles against fouling and overgrowth or to intraspecific functions such as mating attractants (reviewed in Hay and Steinberg 1992; Paul 1992; Pawlik 1993; Hay 1996). The potent and varied biological activities of marine plant and animal extracts suggest that some compounds may fulfill several functions for their host organisms. However, demonstration that compounds are involved in multiple interactions requires that we know the identity of compounds, and that we test purified compounds rather than whole organisms or crude extracts. Therefore, rarely have discrete metabolites been tested for their capacity to fulfill multiple ecological roles.

Recently, triterpene glycosides of the Caribbean sponge *Erylus formosus* were found to be fish feeding deterrents (Kubanek et al. 2000, Fig. 1). Although triterpene glycosides and steroidal glycosides are common in echinoderms (Burnell and ApSimon 1983; Stonik and Elyakov 1988), and have long been ascribed defensive roles in terrestrial plants (Applebaum and Birk 1979), their presence in sponges is a relatively recent discovery (Kitagawa et al. 1987; Carmely et al. 1989; Kobayashi et al. 1991; D'Auria et al. 1992; Antonov et al. 1994, 1998; Gulavita et al. 1994; Jaspars and Crews 1994; Cafieri et al. 1999; Stead et al. 2000; Kubanek et al. 2001; Campagnuolo et al. 2001) and their natural functions are largely unknown (except Kubanek et al. 2000).

In this study, we tested the hypothesis that sponge triterpene glycosides fulfill multiple ecological functions, acting as defenses against: (1) predatory attack, (2) attachment by potential biofilm-forming bacteria, (3) fouling by invertebrate larvae and algae, and (4) overgrowth by other sponges. We focused this study on two sponges belonging to different orders from which triterpene glycosides have previously been isolated: *Erylus formosus*, which contains metabolites that deter fish predation (Kubanek et al. 2000), and *Ectyoplasia ferox*, known to contain ectyoplasides and feroxosides (Fig. 2, Cafieri et al. 1999; Campagnuolo et al. 2001), but for which there was no knowledge of the ecological function of these



**Fig. 2** Triterpene glycosides known from *Ectyoplasia ferox* (Cafieri et al. 1999; Campagnuolo et al. 2001)

metabolites. We employed laboratory and field assays using purified and partially purified triterpene glycosides at concentrations bracketing natural concentrations to test for the functions named above. We measured the relative concentrations of these metabolites across different portions of sponges, including at sponge surfaces and in surrounding seawater. This enabled us to predict whether interactions between sponges and organisms that merely contact sponge surfaces or that approach sponges without making contact are likely to be affected by secondary metabolites.

## Materials and methods

### Compound isolation and characterization

Triterpene glycosides from *Erylus formosus* were initially isolated using bioassay-guided fractionation for antipredatory chemical defenses during a previous study (Kubanek et al. 2000, 2001). Additional quantities of purified and semi-purified compounds were isolated and identified by the same methods from sponges collected at the following locations in the Bahamas Islands, then frozen before lyophilization and extraction: Highborn Cay (23°53.30'N, 75°83.30'W); Sweetings Cay (26°33.721'N, 77°52.973'W); Black Rock (26°33.852'N, 77°41.374'W); Samana Cay (23°23.380'N, 73°42.986'W); Chubb Cay (25°01.509'N, 80°23.604'W); Egg Island (25°20.79'N, 76°53.82'W); Little San Salvador (24°32.90'N, 75°56.01'W); and Cat Cay (24°08.37'N, 75°22.50'W). The whole sponge concentrations of compounds in *Erylus formosus* from the Bahamas were the following: formoside (5.8 mg compound/ml sponge); mixture A of two closely related triterpene glycosides with molecular weights of 1,453.7 g/mol and 1,483.7 g/mol (0.39 mg/ml); mixture B of two closely related triterpene glycosides with molecular weights of 1,495.7 g/mol and 1,526 g/mol (0.23 mg/ml). Sponges collected at later dates (i.e., for the dissec-

tion and extractions experiments described below) showed some variation in metabolite concentrations, although the concentrations listed here are typical for Bahamian specimens of this species (Kubanek et al. 2000). In the assays used in this study, whole sponge volumetric concentration refers to the concentrations listed above (either tested as a mixture of total triterpene glycosides prior to HPLC purification, or tested as individual compounds or fractions after HPLC purification, depending on the assay). Formoside purified by HPLC was used as an HPLC standard for quantification of this compound from *Erylus formosus*. Semi-preparative HPLC was performed using UV absorbance detection at 210 nm or 260 nm, coupled with refractive index detection. Analytical HPLC was performed with diode array UV detection (210–400 nm). HPLC columns used were semi-preparative Zorbax C<sub>18</sub> silica and Vydac 201TP C<sub>18</sub> silica, and analytical Zorbax C<sub>18</sub> silica.

Specimens of *Ectyoplasia ferox* were collected at Key Largo, Florida (25°07.5'N, 80°25.5'W) and in the Bahamas at San Salvador (23°01.11'N 74°32.68'W), Berry Islands (25°01.509'N, 80°23.604'W), Sweetings Cay (same coordinates as for *Erylus formosus* above), Egg Island (above), and Little San Salvador Island (above). The samples were immediately immersed in seawater to determine volumetric displacement, then frozen at –20°C and lyophilized. *Ectyoplasia ferox* is known to contain triterpene glycosides, including ectyoplaside A and B and feroxosides A and B, and fractionation procedures followed published methods (Cafieri et al. 1999; Campagnuolo et al. 2001), which yielded a mixture of triterpene glycosides isolated from whole sponges at a concentration 4.4 mg/ml, consistent with the spectral data of ectyoplasides A and B and feroxosides A and B (Cafieri et al. 1999; Campagnuolo et al. 2001). <sup>1</sup>H and <sup>13</sup>C NMR spectra were acquired on a Bruker Avance DRX 400 MHz spectrometer in deuterated methanol, referenced to CHD<sub>2</sub>OD (<sup>1</sup>H δ 3.31, <sup>13</sup>C δ 49.0). <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz) δ 0.64 (~3H, s), 1.1–2.1 (br m), 1.98 (~3H, s, possibly grease impurity), 2.30 (br d, 1H), 2.45 (br m, 1H), 3.2–4.2 (br m), 4.43 (br d, 1H), 4.54 (m, 1H), 4.63 (br d, 1H), 5.23 (br m, 1H). Low resolution mass spectrometry (MALDI-TOF) confirmed a complex mixture, with two of the molecular weights matching those of feroxosides (1,077 g/mol and 1,079 g/mol; Campagnuolo et al. 2001). This mixture was used in ecological assays and as a standard for HPLC quantification of metabolites, using a gradient solvent system of methanol and water that eluted triterpene glycosides at a common retention time.

### Predator deterrence assays

Aquarium assays using a generalist predatory reef fish, the bluehead wrasse *Thalassoma bifasciatum*, were conducted as previously described (Pawlik and Fenical 1992; Pawlik et al. 1995; Kubanek et al. 2000). Sponge compounds or extracts dissolved in a minimum of methanol were incorporated into pellets containing squid paste, alginate, and water. Control pellets were made without the addition of sponge extracts or compounds but with solvent and occasionally with the addition of food coloring to both treatment and control pellets to control for coloration of sponge extracts. Control and then treatment pellets were offered in that order to each of ten cells containing three to six fish. A food pellet was considered rejected if not eaten after a minimum of three attempts by one or more fish to take it into their mouth cavity, or if the pellet was approached and ignored after one such attempt. The significance of differences in the consumption of treated versus control pellets was evaluated with the Fisher exact test (Zar 1984). Triterpene glycosides at each concentration (including whole sponge natural concentration) were tested using this assay on three separate occasions using different fish. Effects on feeding treatments relative to controls were determined using one-tailed paired *t*-tests of the data from the three assays. In all figures and text, data are shown as means±1 SE.

Field assays were conducted on shallow reefs (<15 m) at various locations in the Bahamas following the method used in Hay et al. (1987) (assay design), and Pawlik and Fenical (1992) and Chanas and Pawlik (1995) (assay food preparation). Extracts or compounds

from sponges were incorporated at natural whole sponge concentrations into carageenan or phytagel strips that contained squid mantle for nutritional value (Pawlik et al. 1995). Approximately 20 replicate ropes, each with a treatment and control strip attached, were placed on the reef and offered to the natural assemblage of fishes until half or more of one food strip from each was eaten. Changes in strip length were measured, and data were analyzed using the Wilcoxon paired sample test (one-tailed, Zar 1984) after excluding pairs for which either all or none of both strips had been consumed.

#### Bacterial attachment assays

Bacteria were isolated from a seawater sample collected at Sweetings Cay, Bahamas, using standard plating techniques. Motile colonies were identified by examining seawater wet mounts with phase contrast microscopy (1,250 $\times$ ). The motile, attaching marine bacterium that was isolated and used in the assay was identified as *Vibrio harveyi* by fatty acid methyl ester analysis (Microbial ID, Newark, Del., using Sherlock Microbial ID system). Bacteria grew on nutrient-agar plates containing yeast extract (1 g/l seawater), peptone (1 g/l seawater), and agar (16 g/l seawater). Various concentrations of sponge extract or triterpene glycoside were suspended in 500  $\mu$ l methanol and added to 10 ml molten agar (60°C). The homogeneous extract-agar mixture was placed into a sterile agar plate using a pipette, and allowed to solidify. Control-agar blocks were made with 500  $\mu$ l methanol added to account for possible solvent artifacts. Five 1-cm<sup>2</sup> blocks were aseptically cut and removed from the extract-agar plate. Each block was set in an individual well of a six-well microtiter plate, with each well containing 3 ml sterile filtered seawater and 25  $\mu$ l bacteria culture in liquid medium. Concentrations of bacteria were standardized before each inoculation using a spectrophotometer (0.227 absorbance units at 660 nm).

One hour after bacteria were inoculated into the wells, bacteria were stained using 30  $\mu$ l 4,6-diamidino-2-phenylindole. After 10 min, 300  $\mu$ l of 37% formaldehyde was added to fix bacteria. Blocks were then rinsed in a Petri dish containing sterile, filtered seawater to remove unattached bacteria. Control and treated blocks were mounted on slides and examined with an Olympus BH-2 epifluorescence microscope under UV excitation. Bacteria were quantified using epifluorescent direct counting (Kepner and Pratt 1994). Ten random fields of 0.64 mm<sup>2</sup> were counted for each block. Means taken from each of the five blocks were compared to means of control blocks using the Mann-Whitney *U*-test (Zar 1984). The effects of sponge compounds or extracts on bacterial attachment is represented as a percentage of the total mean value of all five treated blocks compared to the total mean value of the five control blocks, whereby smaller percentages reflect greater inhibition of bacterial attachment by extracts or compounds.

#### Fouling assays

Assays with sponge compounds and extracts were conducted between March and June in the Intracoastal Waterway at Wrightsville Beach, North Carolina. Fouling rates in this location are very high permitting short assay times and many of the fouling organisms represented are the same as those found in Caribbean waters (Sutherland and Karlson 1977). Antifouling effects were examined using the technique of Henrikson and Pawlik (1995), in which sponge extracts were incorporated into stable gels that served as a settlement substratum for field assays. Mixtures of phytagel and water were heated to boiling, then added to sponge compounds dissolved in methanol and poured into Petri dishes to harden. Control gels were made in the same fashion with solvent but without sponge compounds. Because of the loss of compounds from the gel over time in the field (Henrikson and Pawlik 1995; Engel and Pawlik 2000), the starting concentration for treated gels was chosen to be twice the whole sponge volumetric concentration. When surface concentrations of compounds were later determined (see section on Quantification below), we tested formoside purified by HPLC from *Erylus formosus* in the antifouling assay at a concentration based on surface and first layer concentrations of formoside. Treated gels

contained 2.12 mg/ml of formoside, the mean concentration of formoside found in the top 1 mm of the sponge. Once gels had hardened, 1.06 mg formoside was dissolved in 500  $\mu$ l MeOH, pipetted onto the 26.4-cm<sup>2</sup> gel, and the methanol was allowed to dry by evaporation, corresponding to 0.040 mg/cm<sup>2</sup>.

For each antifouling assay, four treated and control gel replicates were made. Weighted gels were left in the field for an average of 20–30 days. Total percent cover (including algae and invertebrates) was recorded at 4-day intervals beginning with the first day of observable fouling (typically 1 week). Settlement of algae and invertebrates was measured using an overlay grid with 60 random points marked within the area of the gel (Henrikson and Pawlik 1995, 1996). Total percent cover was calculated by dividing settlement points by the total number of possible points, and was compared between treatments and controls using a one-tailed paired *t*-test on arcsine transformed data using an alpha level of 0.05 (Zar 1984).

#### Sponge overgrowth assays

To examine the potential allelopathic function of sponge triterpene glycosides, natural concentrations were incorporated into gels and allowed to be overgrown by a sponge, *Tedania ignis*, in the field as per Engel and Pawlik (2000). Previously, *T. ignis*, along with *Haliclona hogarthi* and *Lyssodendoryx isodictyalis*, chosen because of their rapid growth, were used to examine the allelopathic properties of 20 Caribbean sponge extracts (Engel and Pawlik 2000). In this previous study, all three sponges responded similarly to all 20 sponges' extracts, so the overgrowth of *T. ignis* in the experiment likely parallels other sponge overgrowth patterns.

Acrylic plates were used to construct a square assay plate containing four gel wells surrounding a central plate upon which the overgrowth organism was later fixed (Engel and Pawlik 2000). Two opposing wells were used for extract-treated gels, while the remaining two were used for control gels. Gel mixtures of phytagel and water were heated to boiling, then added to sponge compounds dissolved in methanol and poured into two wells. Control gels were prepared identically, but without sponge compounds.

Overgrowth assays were conducted in a high-flow creek branching off from Dusenbury Creek, Key Largo, Florida. Lobes of *T. ignis* were collected from nearby mangrove roots and secured with cable ties to the center square of each assay plate, adjacent to two treated and two control gels. Each plate was left in the field for 21 days allowing the *T. ignis* to laterally overgrow paired treated and control gels. After removal from the field, the plates were scored by placing window screen over each gel and counting the number of squares in which the overgrowth organism appeared. Percent overgrowth was calculated by dividing the number of growth squares by the total number of squares. A one-tailed paired *t*-test, performed on arcsine transformed data, was used to assess the significance of the difference in mean overgrowth on extract-treated versus control gels.

#### Distribution of metabolites in and around sponges

##### *Measurement of exudation of metabolites*

Two methods were used to quantify water-borne triterpene glycosides around live specimens of *Erylus formosus* and *Ectyoplasia ferox*:

1. In situ manual pump and organic extraction by column using techniques previously described (Coll et al. 1982; Schulte et al. 1991; Slattery et al. 1997; Nishiyama and Bakus 1999). Four specimens of *Erylus formosus* were analyzed for exuded formoside using this method, at Sweetings Cay, Little San Salvador (two specimens), and Cat Cay in August 2000. Using scuba at depths of 4–15 m, we located a sponge measuring between 30 and 200 ml and placed a clear plexiglas cylinder (12 cm diameter and 16 cm height) over it without contact between the cylinder and sponge. This cylinder was fitted at the top end with a plastic funnel leading to a column containing 50 ml Diaion HP-

20 resin, connected by tubing to a 60-ml syringe. Over a 30-min time period, 5,400 ml seawater (3 times the volume of the chamber) was passed through the column by repeatedly pulling water through the syringe. Sponges were collected at the end of the experiment, and their volumes quantified by seawater displacement in a graduated cylinder. Organics adsorbed to the column resin were eluted with methanol (1,000 ml) and analyzed by reversed-phase TLC against standards previously isolated and by analytical HPLC (see next section). Further structural identification was accomplished by  $^1\text{H}$  NMR spectroscopy. In order to be sure that triterpene glycosides would be detected if they were in the water column, and to establish the efficiency of this method, we performed three positive controls in the field. The chamber was placed on solid substrate on the reef, and formoside (15.5 mg dissolved in 0.5 ml methanol and diluted in 60 ml seawater) was delivered into the chamber over a 30-min period using a second syringe and tube, while water was passed through the column as above. In addition, a negative control was performed to confirm that triterpene glycosides were not found in the water column in the absence of these sponge species, by performing the experiment on solid substratum where no *Erylus formosus* was found.

- Use of a gas-permeable/water-impermeable bag. A second assay was devised in order to measure exudation of ectyoplasides by *Ectyoplasia ferox* and by *Erylus formosus* in the water column. Experiments were performed by scuba at 7–18 m depth in March 2001. Two specimens of *Ectyoplasia ferox* were collected from Cat Cay (70 ml) and from Berry Islands (240 ml), and one clone of *Erylus formosus* from Little San Salvador, Bahamas (157 ml). Sponges were chiseled off non-living substrate and gently placed in a sealed teflon bag (fluorinated ethylene propylene, 58 cm by 45 cm; American Fluoroseal). This bag allowed carbon dioxide and oxygen exchange but prohibited water flow and loss of dissolved organics. The bag was anchored with a weight and left on the reef for 24 h, then it was brought to the surface and the specimen was carefully removed from the bag. Water from the bag (2,000 ml) was immediately pushed through a reversed-phase Sep Pak (ENVI-18, 60 ml, 10 g; Supelco). Organic compounds were eluted with methanol, concentrated, and analyzed by analytical HPLC (see next section). Confirmation of compound identities was performed by  $^1\text{H}$  NMR spectroscopy.

#### Dissection and extraction of sponges

Four samples of *Erylus formosus* were collected from the Bahamas: one from Egg Island, one from Little San Salvador, and two from Sweetings Cay in August 2000. Four samples of *Ectyoplasia ferox* were collected, one from each of Sweetings Cay, Egg Island, Little San Salvador, and San Salvador, Bahamas in August 2000. Sponges were collected with minimum disturbance to sponge tissue by chiseling the substratum around each sponge, and then sponges were placed in a plastic bag and brought to the surface. Each sponge was immediately removed from its bag, and water that dripped from sponges (5–30 ml) was collected. Sponge surfaces were gently swabbed with a gauze pad, which was extracted with methanol (“surface swab”). Using texture and color differences between layers as a gauge for layer thickness, four layers were dissected with a scalpel from each sample of *Erylus formosus* and three layers from each *Ectyoplasia ferox*. Both sponges have firm consistencies and are relatively dry, facilitating dissection. Layer thickness was measured with a ruler and volumes quantified by volumetric displacement. Surface swabs and the tissue of each layer were extracted 3 times in methanol and the extracts combined and concentrated in vacuo. Water collected along with sponges were concentrated in vacuo. Each extract was then suspended in deionized water and washed with *n*-hexane (1:1) to remove non-polar compounds, and then partitioned against *n*-butanol (1:1). The presence of triterpene glycosides in the *n*-butanol partition and their absence in the *n*-hexane and water partitions was confirmed by reversed-phase TLC against triterpene glycoside standards from *Erylus formosus* and *Ectyoplasia ferox*. *N*-butanol was removed from the samples in vacuo and the samples were analyzed by HPLC (see below).

#### Quantification of triterpene glycosides from sponge exudates and dissected sponge layers

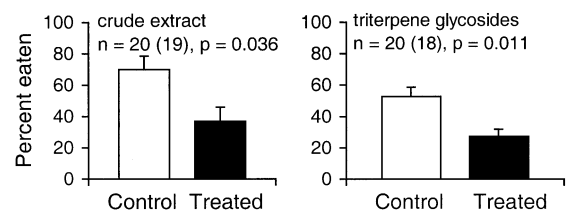
Analytical diode-array HPLC was used to quantify triterpene glycosides from dissected samples and extracted seawater. Because formoside accounts for >90% of triterpene glycosides in *Erylus formosus* found in the Bahamas (Kubanek et al. 2000), only this compound was measured using previously purified formoside as a standard. Because the mixture of triterpene glycosides from *Ectyoplasia ferox* was found to be complex and possessed common chromatographic properties, a fraction that was partially purified by HPLC, accounting for >90% of triterpene glycosides in this sponge, was used as a standard for extracts of this species. HPLC standard curves were generated for formoside ( $R^2=0.9971$ ) and for the *Ectyoplasia ferox* mixture ( $R^2=0.9895$ ). Concentrations of triterpene glycosides in sponge layers, surface swabs, and seawater were determined by comparison of integrated UV peak areas (at 210 nm for *Erylus formosus*; 225 nm for *Ectyoplasia ferox*) at the retention time of the appropriate standard with the standard curve. For each of the dissected layers of one specimen of each species, confirmation that the HPLC peak consisted of triterpene glycosides of >95% purity was accomplished by  $^1\text{H}$  NMR spectroscopy. Spectral data were compared with formoside for *Erylus formosus* (Jaspars and Crews 1994) and ectyoplasides/feroxosides for *Ectyoplasia ferox* (Cafieri et al. 1999; Campagnuolo et al. 2001). Triterpene glycosides were standardized to volume of each layer and concentrations were compared across all layers using a Kruskal-Wallis test. Differences between layers were tested through non-parametric multiple comparison for equal sample sizes (Zar 1984).

## Results

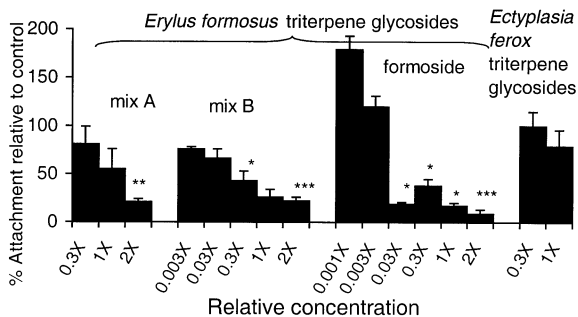
### Ecological roles of triterpene glycosides

#### Predation

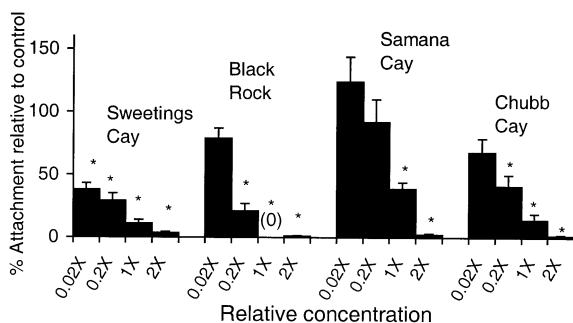
Crude extracts and partially purified triterpene glycosides from *Ectyoplasia ferox* deterred feeding by fishes in field assays at whole sponge volumetric concentration (Fig. 3). The same triterpene glycosides also showed antipredatory activity in aquarium assays. At 1× whole sponge volumetric concentration, 3.7±0.3 treated pellets out of ten were eaten, compared with 10±0 control pellets ( $P=0.001$ ), and at 4× whole sponge volumetric concentration, 0±0 treated pellets out of ten were eaten, compared to 10±0 control pellets ( $P<0.0001$ ) ( $n=3$  assays each). Antipredatory effects of *Erylus formosus* triterpene glycosides were previously reported in Kubanek et al. (2000).



**Fig. 3** Results of field assays assessing antipredatory effects of sponge compounds from *Ectyoplasia ferox* against natural assemblages of reef fishes in the Bahamas. Error bars indicate  $\pm 1$  SE. *P*-values are for one-tailed paired *t*-test. [Assays using crude extracts and triterpene glycosides from *Erylus formosus* indicated similarly deterrent effects (Kubanek et al. 2000)]



**Fig. 4** Bacterial attachment assay results of different triterpene glycosides. Concentrations are expressed as numbers of times whole sponge volumetric concentration. In each case, counts of bacteria stained with 4,6-diamidino-2-phenylindole (DAPI) on treated agar blocks ( $n=5$ ) were compared with bacterial counts from control blocks ( $n=5$ ). The mean control count of each test was normalized to 100% (and is therefore not shown) and counts from treatments are reported relative to 100% control counts. Error bars indicate  $\pm 1$  SE. \* $P \leq 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  (Mann-Whitney  $U$ -test)

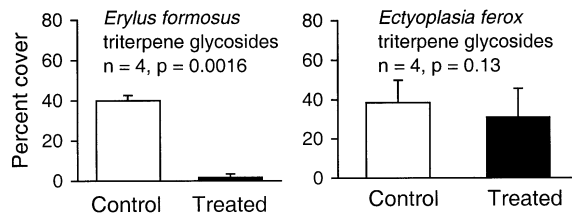


**Fig. 5** Bacterial attachment assay results of total triterpene glycosides from *Erylus formosus* from different sites. Concentrations are expressed as numbers of times whole sponge volumetric concentration. In each case, counts of bacteria stained with DAPI on treated agar blocks ( $n=5$ ) were compared with bacterial counts from control blocks ( $n=5$ ). The mean control count of each test was normalized to 100% (and is therefore not shown) and counts from treatments are reported relative to 100% control counts. Error bars indicate  $\pm 1$  SE. \* $P \leq 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  (Mann-Whitney  $U$ -test)

### Bacterial attachment

Triterpene glycosides isolated from *Erylus formosus* deterred bacterial attachment (Fig. 4). These triterpene glycosides were tested in three groups: mixture A (deterrent at  $2\times$  whole sponge volumetric concentration), mixture B (deterrent at  $0.3\times$  and  $2\times$  natural concentration), and formoside (deterrent at four different concentrations from  $0.03\times$  to  $2\times$  natural concentration, i.e., at concentrations  $\geq 0.1$  mg/ml). Triterpene glycosides from *Ectyoplasia ferox* did not inhibit attachment at  $0.3\times$  and  $1\times$  whole sponge volumetric concentration.

The total triterpene glycoside fraction (fraction containing the full suite of triterpene glycosides from each sponge) of four individual *Erylus formosus* collected at different locations in the Bahamas deterred bacterial attachment at  $1\times$  and  $2\times$  whole sponge volumetric concen-



**Fig. 6** Antifouling assay results of triterpene glycosides from Caribbean sponges tested at twice whole sponge volumetric concentration. Percent cover of invertebrates and algae were measured after 27 days and 22 days in the field, for *Erylus formosus* and *Ectyoplasia ferox*, respectively. Error bars indicate  $\pm 1$  SE.  $P$ -values are for one-tailed paired  $t$ -test

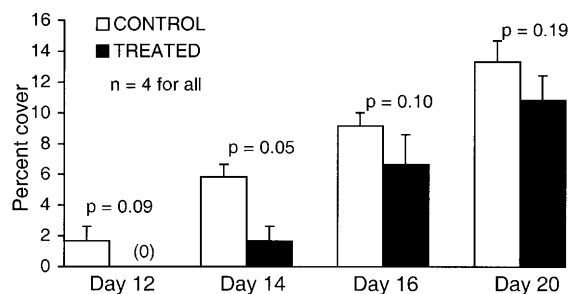
tration (Fig. 5). In addition, triterpene glycosides from *Erylus formosus* collected at Black Rock and Chubb Cay significantly deterred attachment down to  $0.2\times$  natural concentration, and the metabolites from *Erylus formosus* collected at Sweetings Cay were deterrent down to  $0.02\times$  natural concentration. Crude extracts were also tested at whole sponge volumetric concentrations, yielding the following attachment results (normalized to 100% attachment of control data,  $n=5$  for treatments and controls): *Erylus formosus* treated  $0.19 \pm 0.19\%$ , control  $100 \pm 13\%$ ,  $P=0.0097$ ; *Ectyoplasia ferox* treated  $44 \pm 9\%$ , control  $100 \pm 11$ ,  $P=0.022$ .

### Fouling

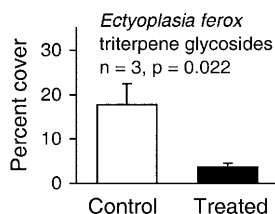
At starting concentrations of twice whole sponge volumetric concentration, neither crude extracts nor triterpene glycosides (Fig. 6) of *Ectyoplasia ferox* significantly deterred fouling by invertebrates and algae. Gels treated with crude extracts of *Ectyoplasia ferox* were  $61 \pm 14\%$  covered with invertebrates and algae after 21 days, compared to control gels that were  $90 \pm 4\%$  covered ( $n=3$ ,  $P=0.26$ ). In contrast, triterpene glycosides from *Erylus formosus* strongly deterred fouling (Fig. 6). Crude extracts of this sponge were not tested due to limited availability of material. In addition, antifouling assays were also run with formoside from *Erylus formosus* at the natural surface concentration of formoside as measured from the surface swabs and first layer of this sponge. Over a 20-day period, total percent cover increased on both treated and control gels, with a significant difference between treatments and controls detected at day 14 (Fig. 7).

### Overgrowth by neighboring sponges

Crude extracts of *Ectyoplasia ferox* inhibited overgrowth of *Tedania ignis*: treated gels were  $5.0 \pm 5.3\%$  overgrown whereas control gels were  $12.2 \pm 4.2\%$  overgrown after 21 days ( $n=6$ ,  $P < 0.05$ ). Triterpene glycosides isolated from the same collection of *Ectyoplasia ferox* also inhibited growth of *T. ignis* in sponge overgrowth assays (Fig. 8). The experiment using total tri-



**Fig. 7** Antifouling assay results of formoside from *Erylus formosus* tested at realistic surface concentration calculated from surface swab and first layer of dissected sponges (see Table 1). Error bars indicate  $\pm 1$  SE. *P*-values are for one-tailed paired *t*-test



**Fig. 8** Sponge overgrowth assay results using *Tedania ignis* as overgrowing sponge, and whole sponge volumetric concentration of triterpene glycosides from *Ectyoplasia ferox*. Error bars indicate  $\pm 1$  SE. *P*-values are for one-tailed paired *t*-test

terpene glycosides from *Erylus formosus* was inconclusive because in two of the three replicates, the overgrowing sponge died. However, on the one plate that was successfully scored after 21 days in the field, growth was less over the two treated gels (0, 4%) than over the two control gels (17, 24%). Crude extracts of *Erylus formosus* were not tested.

#### Distribution of triterpene glycosides in and around sponges

Triterpene glycosides were not found in the seawater surrounding either *Erylus formosus* or *Ectyoplasia ferox*, using both a manual pump that passed water through a

column, followed by elution of organics (detection limit 90  $\mu\text{g}/\text{l}$  seawater), and collection of seawater in a waterproof bag (detection limits of 5  $\mu\text{g}/\text{l}$  and 50  $\mu\text{g}/\text{l}$  seawater for *Erylus formosus* and *Ectyoplasia ferox*, respectively). Although HPLC peaks corresponding to the retention times of triterpene glycosides were observed from seawater extracts,  $^1\text{H}$  NMR spectroscopy confirmed that other co-eluting compounds were responsible for these peaks (including phthalate esters from the tubing of the pumping apparatus). Negative controls (pumping of seawater when no sponge was present) revealed similar HPLC peaks and no triterpene glycosides. Positive controls (pumping of seawater with pure formoside added to the chamber via syringe) revealed that collection efficiency was  $20 \pm 4\%$  ( $n=3$ ).

Within dissected portions of *Erylus formosus*, the concentration of formoside in the top layer was found to be lower than in the third layer (Table 1;  $q=3.78$ , critical value=3.63 for  $\alpha=0.05$ ,  $df=3$ ,  $n=4$ ), but other layers were not significantly different from each other. In each specimen, this first layer had a distinctive leathery texture and black color, and was easily peeled from live sponges. In contrast, the concentration of triterpene glycosides was greater in the top layer of *Ectyoplasia ferox* than in the inner third layer (Table 1;  $q=3.47$ , critical value=3.31 for  $\alpha=0.05$ ,  $df=3$ ,  $n=4$ ) and differences between other layers were not significant. Surface swabs of both sponges contained triterpene glycosides at low concentrations (Table 1). Seawater that was collected along with individual sponges (5–30 ml) contained no triterpene glycosides.

## Discussion

Triterpene glycosides from the two Caribbean sponges studied, *Erylus formosus* and *Ectyoplasia ferox*, appear to serve more than one defensive function. Realistic concentrations of triterpene glycosides from *Erylus formosus* deterred predatory fishes (Kubanek et al. 2000), bacterial attachment (Figs. 4, 5), and fouling propagules (Figs. 6, 7), while similar compounds from *Ectyoplasia*

**Table 1** Distribution of major triterpene glycosides within sponges ( $n=4$ , collected at different sites in the Bahamas). Means are shown  $\pm 1$  SE

Sponge species	Dissected portion	Depth from surface (mm)	Concentration (mg triterpene glycosides/ml sponge)
<i>Erylus formosus</i> <sup>a</sup>	Surface swabs	–	0.04 $\pm$ 0.01 <sup>c</sup>
	First (top) layer	0–1	2.1 $\pm$ 0.4
	Second layer	1–3	6.3 $\pm$ 0.8
	Third layer	3–7	7.5 $\pm$ 1.4
	Fourth (innermost) layer	7–14	6.4 $\pm$ 1.9
<i>Ectyoplasia ferox</i> <sup>b</sup>	Surface swabs	–	0.4 $\pm$ 0.2 <sup>c</sup>
	First (top) layer	0–2	7.8 $\pm$ 0.6
	Second layer	2–6	4.0 $\pm$ 0.6
	Third (innermost) layer	6–14	3.7 $\pm$ 0.8

<sup>a</sup> Formoside was measured

<sup>b</sup> A mixture of triterpene glycosides including ectyoplasides and feroxides was measured

<sup>c</sup> Surface swab concentrations are per area (mg/cm<sup>2</sup>) not per volume

*ferox* deterred predatory fishes (Fig. 3) and overgrowth by neighboring sponges (Fig. 8). The compounds from *Ectyoplasia ferox* were ineffective antibacterial and antifouling agents (Figs. 4, 6). Thus, it appears that some marine sponges have developed effective multifunctional chemical defenses.

Formoside, which accounts for more than 90% of the triterpene glycosides in *Erylus formosus* from the Bahamas (Jaspars and Crews 1994; Kubanek et al. 2000, 2001), deterred attachment of bacteria at concentrations ranging from 3% to 200% of natural whole sponge levels (Fig. 4). Because the concentration of formoside at the surface and in the 1-mm-thick "skin" of *Erylus formosus* falls within this range (Table 1), this compound could effectively deter bacteria from attaching to live sponges. Mixtures of triterpene glycosides from *Erylus formosus* collected at four different locations in the Bahamas all showed similar antibacterial effects (Fig. 5), suggesting that these effects may be widespread. In the current study, one potentially attaching bacterium, *Vibrio harveyi*, was used. This microbe was isolated from a majority of seawater samples and sponges collected in the Bahamas as part of a separate study (S. R. Kelly et al., in preparation). The ubiquity, motility, and ready attachment of this bacterium to biotic surfaces in the laboratory made it an excellent test organism for this assay, although it remains to be determined whether other marine bacteria are similarly affected by sponge triterpene glycosides.

In many cases, microfouling organisms can condition surfaces for the settlement of macrofoulers (Kirchman et al. 1982; Wahl 1989; Leitz and Wagner 1993; Holmstrom and Kjelleberg 1994). It is possible that inhibition of bacterial attachment onto *Erylus formosus* retarded or excluded the settlement of subsequent propagules, resulting in the reduction of fouling on gels containing compounds from *Erylus formosus*. Therefore, suppression of bacterial film formation may be a good indication of the antifouling potential of a compound. In support of this, metabolites of *Ectyoplasia ferox* neither deterred attaching bacteria nor macrofouling organisms, whereas metabolites of *Erylus formosus* deterred both attaching bacteria and macrofouling organisms (Figs. 4, 6, 7).

In the fouling assays, because loss of materials from gels was found to be approximately 50% of total mass of sponge crude extract over 3 weeks (Henrikson and Pawlik 1995; Engel and Pawlik 2000), compounds were incorporated into gels at twice their whole sponge volumetric concentration, in order to start with a sufficiently high concentration that would allow for loss of compounds over 21 days. However, the exudation rate of these triterpene glycosides could be different from other extract constituents, and processes such as oxidation and UV damage could differentially reduce concentrations of compounds over time. Thus, we also tested formoside from *Erylus formosus* incorporated into gels at the natural surface concentration as measured from live sponges (Table 1). An ideal experimental system would have allowed for constant replenishing of compounds lost by

leaching or degradative processes, as occurs in natural sponges. Because in our assay no replenishing was possible, the concentration of formoside a few days after the start of the assay was certainly less than occurs on live sponge surfaces. Nevertheless, after 14 days there was 3.5 times more cover on control than treated gels ( $P=0.05$ , Fig. 7), suggesting that formoside plays a role in keeping the surface of *Erylus formosus* unfouled. Measurements on days 18 and 20 revealed cover on treated gels to be gradually catching up to controls, which may have been due to the loss of these compounds from gels over time.

The allelopathic effect of *Ectyoplasia ferox* triterpene glycosides was demonstrated using one overgrowing sponge at a realistic surface concentration (Fig. 8, Table 1). In a previous study using the same assay technique, crude extracts of this sponge prevented overgrowth by three sponges, *Tedania ignis*, *Lissodendoryx isodictialis*, and *Haliclona hogarhi* (Engel and Pawlik 2000). Thus, it appears likely that triterpene glycosides from *Ectyoplasia ferox* function as defenses against encroachment by several sponges. The current study did not use bioassay-guided fractionation to arrive at triterpene glycosides as the active components in *Ectyoplasia ferox*. Thus, it is possible that other constituents also function as allelopathic agents in this sponge. However, because the allelopathic effect of *Ectyoplasia ferox* triterpene glycosides was greater than the effect of crude extracts (4 times versus 2.5 times more growth over control than treated gels, respectively) triterpene glycosides are probably responsible for most if not all of the allelopathic effect (Results section, Fig. 8). In a study investigating interspecific overgrowth interactions between sponges, S. Engel and J. R. Pawlik (in preparation) reported that *Ectyoplasia ferox* was neither observed to be overgrown nor did it overgrow other sponges in the field. *Ectyoplasia ferox* was not in contact with any other sponges in 92% of cases ( $n=229$ ), which places this sponge in the "loneliest" 20 percentile of sponges observed. Thus, this sponge appears to be highly spatially competitive, possibly due to its triterpene glycosides.

The allelopathic potential of triterpene glycosides from *Erylus formosus* is unclear. When a natural mixture of triterpene glycosides from *Erylus formosus* was tested, two of the three overgrowing sponges died. It is possible that the triterpene glycosides of *Erylus formosus* were toxic to the overgrowing sponges, although other unknown environmental factors may have been responsible for this occurrence. For the one replicate sponge that survived, overgrowth was lower over treated gels than over control gels after 3 weeks in the field. These treated gels contained a whole sponge volumetric concentration of triterpene glycosides from *Erylus formosus*, which is higher than the natural surface concentration of triterpene glycosides in this sponge (Table 1). Further work is needed to assess the possibility that *Erylus formosus* utilizes triterpene glycosides as allelopathic chemical defenses.



Marine triterpene and steroidal glycosides have long been known from sea cucumbers (Holothuroidea) and sea stars (Asteroidea) where they are hypothesized to play defensive roles. However, experimental evidence for their function is slight and mostly related to seasonal fluctuations in metabolite levels and varying concentrations in different echinoderm tissues (Burnell and ApSimon 1983; Stonik and Elyakov 1988). Bingham and Braithwaite (1986) demonstrated the toxicity of holothurian triterpene glycosides against fishes, but reported no behavioral effects on predators in feeding assays. Lucas et al. (1979) showed that planktivorous fishes were deterred by steroidal glycosides from the crown-of-thorns sea star (*Acanthaster planci*) and its larvae. Despite limited experimental evidence, marine steroidal and triterpene glycosides have been widely assumed to play a defensive role. The current study and that of Kubanek et al. (2000) confirm that triterpene glycosides are important chemical defenses for two sponges from different orders within the class Demospongiae, but that their effects on predators and competitors are not universal; they show specificity of function related to molecular structure and concentration. Because these sponge triterpene glycosides were active against some enemies but not others (current study) and different triterpene glycosides from *Erylus formosus* showed different potencies as antipredatory defenses (Kubanek et al. 2000), broad generalizations regarding the functions of these compounds are inappropriate. As triterpene glycosides are increasingly identified from such diverse organisms as soft corals (e.g., Kittakoop et al. 1999) and amphipods (N. Lindquist, personal communication), further studies clarifying the dependence of ecological function on molecular structure will be possible.

The physiological mechanisms by which sponge triterpene glycosides negatively affect predatory fishes, settling propagules, bacteria, and other sponges are unknown. Triterpene glycosides erylosides E and F from *Erylus* spp. have been found to cause cellular calcium release (A. Wright, personal communication), which could potentially negatively affect consumers or competitors. Steroidal and triterpene glycosides from plants, known as saponins, are believed to cause digestive problems in large herbivores such as cattle by altering the surface tension of stomach contents, trapping gas produced by bacterial fermentation (Applebaum and Birk 1979). Sponge triterpene glycosides possess the same soapy character as their terrestrial analogs. However, soapiness in itself cannot explain the multiple ecological functions revealed in this study, because while all triterpene glycosides tested in this study were soapy, their activities differed.

Dissection of *Erylus formosus* followed by extraction and metabolite quantification by HPLC indicated that the majority of formoside (accounting for >90% of triterpene glycosides in *Erylus formosus*) is located below the first millimeter of the sponge surface (Table 1). In contrast, triterpene glycosides in *Ectyoplasia ferox* are highly concentrated in the first two millimeters (Table 1).

This difference suggests that triterpene glycosides may function differently in different species. Optimal defense theory (Rhoades 1979) would suggest that compounds are localized where they are most crucial and not wasted where they are not needed. This hypothesis is supported by localization of defensive metabolites in exposed portions of the Micronesian sponges *Oceanapia* sp. (Schupp et al. 1999) and *Cacospongia* sp. (Becerro et al. 1998), although Swearingen and Pawlik (1998) saw no evidence of this occurrence in the sponge *Chondrilla nucula*. The localization of triterpene glycosides in *Ectyoplasia ferox* is consistent with the allocation of defensive metabolites to areas that are vulnerable to enemies (i.e., to the surface for allelopathy, to the first few millimeters below the surface for the deterrence of small predators whose bites are shallow). Although the localization of triterpene glycosides in *Erylus formosus* did not follow this pattern, surface levels of triterpene glycosides were sufficient to deter microbial attachment and fouling by propagules (Figs. 4, 7, Table 1), and the below-surface concentration increased steeply enough to deter even small-mouthed predatory fishes (Fig. 3, Table 1, and Kubanek et al. 2000). It is also possible that triterpene glycosides fulfill additional, still unknown, functions inside *Erylus formosus* such as protection against internal fouling or microbial attack, and that their localization is consistent with these functions.

Exudation of triterpene glycosides from these two sponges into the seawater did not occur at detectable levels, as measured using a pumping and a bagging method. However, with both of these sponges, triterpene glycosides were detected from swabs of live sponge surfaces (Table 1). Thus, although compounds are not released into the seawater at measurable levels, they are present at the sponge surface where they can affect other organisms. Triterpene glycosides are amphiphilic: they possess a lipophilic core (the triterpene aglycone) and hydrophilic saccharides. This dual property might assist in keeping these compounds at the interface of sponge and seawater, where they can target potential predators and competitors. With this strategy, loss of compounds to the surrounding seawater and thus costs associated with biosynthesis, storage, etc. should be minimized. Surface deployment may be more efficient than release into the water column when facing enemies who either: (1) come into contact with the surface, sense surface chemistry, and then depart without causing tissue damage as might settling propagules (Wahl 1989), or (2) take small bites, then quickly learn to avoid unpalatable foods as do fishes (Lindquist and Hay 1995). The value per molecule is further enhanced by the multiple functions that these compounds play.

The dependence of ecological activity on triterpene glycoside concentration observed in the current study, and the relatively high concentrations of these compounds in the two sponge species studied (approximately 4–7 mg total triterpene glycosides/ml sponge tissue in both species or 0.4–0.7% by wet weight) might suggest that these compounds fit Feeny's (1976) definition of

quantitative chemical defenses from “apparent” (wide-spread, sessile, long-lived) organisms. However, the sponge triterpene glycosides were not effective against all enemies tested, and so it is likely that the modes of action of triterpene glycosides are more complex than those proposed for other possible quantitative chemical defenses (e.g., digestibility reduction as proposed by Rhoades and Cates 1976). Because this study involved only two sponge species and ecological interactions with a handful of potential consumers and competitors, further work will be needed to determine general patterns. The increasingly frequent discovery of triterpene glycosides in many diverse organisms offers the opportunity to test general models of chemical ecology.

**Acknowledgements** Paul Jensen at SIO provided expertise in the design of the bacterial attachment assay. David Bostwick at Georgia Tech ran MALDI mass spectra on the *Ectyoplasia* compounds. We thank Jeff Wright at the Center for Marine Science University of North Carolina at Wilmington (UNCW) for the use of two HPLC systems and Pam Seaton at UNCW for assistance with NMR spectroscopy. Greg McFall, Will O’Neal, Monica Puyana, and Robyn Palmer all helped with field experiments. This work was supported by NSF OCE-9711255 to J. R. P. and by a Life Sciences Research Foundation O’Donnell fellowship (2000–2001) and Natural Sciences and Engineering Research Council of Canada fellowship (1998–2000) to J. K. 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. La Que Center for Corrosion Technology in Wrightsville Beach, North Carolina, allowed us to perform antifouling assays on their property. This is contribution no. 260 of UNCW’s Center for Marine Science.

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