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Isolated and synergistic effects of chemical and structural defenses of two species of *Tethya* (Porifera: Demospongiae)

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ABSTRACT

Sponges are an important source of many interesting secondary metabolites with multiple ecological roles. Sponges can also use their spicules as a means of deterring consumers. The present study investigated the importance of chemicals and spicules as defensive strategies against predation for two congeneric sponge species from the Brazilian coast, *Tethya rubra* and *Tethya maza*. Crude extract and spicules differed somewhat in their effectiveness between these sponge species, with *T. maza* better defended than *T. rubra* against predation by the hermit crab *Calcinus tibicen* and synergistic effects stronger in *T. rubra*. These results show that defensive strategies may be similar between sponge species possessing monophyletic origin, and reveal the importance of research on congeneric species to understand the ecology and evolution of defensive strategies.

1. Introduction

Sponges are conspicuous organisms in marine benthic communities, even in coral reefs, in which they can establish large populations in spite of the intense predation existing in these ecosystems (Alcolado, 1991; Ribeiro and Muricy, 2004; Sarmento and Correia, 2002). Marine sponges occur worldwide in a great diversity of habitats, probably because of their effective defense mechanisms. However, some animals are known to eat sponges, including fish (Dunlap and Pawlik, 1998; Randall and Hartman, 1968; Wulff, 1994), sea stars (McClintock et al., 1994; Schiebling, 1982; Sheild and Witman, 1993), polychaetes (Pawlik, 1983), echinoids (Birenheide et al., 1993; Santos et al., 2002), turtles (Leon and Bjorndal, 2002; Meylan, 1988, 1990), nudibranchs (Knowlton and Highsmith, 2005) and crabs (Hazlett, 1968, 1981; Schembri, 1982).

Several species of marine sponges have chemical defense mechanisms that help protect them against certain bacterial infections, predator attacks, biofouling, and overgrowth by other sessile organisms (see McClintock and Baker, 2001; Paul and Puglisi, 2004; Paul et al., 2006). However, the development of chemical defenses by sponges seems to be closely related to predation pressure in the environment. For example, transplantation experiments revealed that mangrove sponges were highly vulnerable to predation when transplanted to the coral-reef habitat, while coral-reef sponges remain resistant to predation when transported to mangrove areas (Pawlik, 1998; Ruzicka and Gleason, 2009).

Physical defenses have been formally known in plants since the 1970s, through studies on spines (Cooper and Owen-Smith, 1986), thorns and resin ducts (Maxwell et al., 1972), siliceous phytoliths (Kaufman et al., 1981), lignified fibers (Coley, 1983) and tissue toughness (Howard, 1988). In the marine environment, physical defenses have been studied in several kinds of organisms, including algae, corals and sponges. Some marine macroalgae have calcified tissues which may act as a physical defense (Hay et al., 1994). Gorgonians and soft corals have calcareous sclerites in their tissues, which are important structural components (Lewis and von Wallis, 1991) and also act as deterrents against some generalist predators (Harvell et al., 1988). In sponges, however, physical defenses are a controversial topic. Chanas and Pawlik (1995) found that spicules from eight highly spiculose species of Demospongiae (*Cribrochalina vasculum*, *Geodia neptuni*, *Mycale laevis*, *Neofibularia nolitangere*, *Xestospongia muta*, *Agelas clathrodes*, *Chondrilla nucula*, and *Ectyoplasia ferox*), representing different spicule types, did not deter feeding by fish in laboratory or field assays. In addition, Waddell and Pawlik (2000a) found that spicules had no deterrent effect against predation by the hermit crab *Paguristes punticeps*. Nonetheless, Burns and Ilan (2003) found that spicules of four sponge species from the Red Sea (*Diacarnus erethrianus*, *Hemimycale* sp., *Crella cystophora*, and *Suberites clavatus*) and two from the Caribbean Sea (*C. nucula* and *G. neptuni*) deterred feeding by fish. Spicules from the temperate-zone sponges *Cliona*

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celata and *Halichondria bowerbanki* significantly inhibited feeding by the hermit crab *Pagurus longicarpus* (Hill et al., 2005), and spicules from the tropical species *Cribrochalina infundibulum* and *X. muta* were an effective deterrent against the fish *Thalassoma bifasciatum* (Jones et al., 2005).

The simultaneous presence of chemical and physical defenses against predation was observed in several marine sponges, as well as in other marine organisms, i.e., macroalgae, soft corals, mollusks and ascidians (Burns and Ilan, 2003; Harvell and Fenical, 1989; Hill et al., 2005; Pawlik et al., 1995). Indeed, studies have revealed important aspects associated with the variability of these defenses, such as intraspecific variation due to geographical location or month of collection (i.e., *X. muta*; Swearingen and Pawlik, 1998), and variation within the same individual (i.e., *Oceanapia* sp.; Schupp et al., 1999).

Despite these studies, the variability in defensive strategies (chemical and physical) between congeneric species remains little explored in the literature on marine chemical ecology (Davis and Wright, 1989; Pawlik et al., 1995; Roper et al., 2009). In general, the presence of certain classes of compounds is conservative within sponge groups, constituting a basis for taxonomic treatments (Bergquist and Wells, 1983; Braekman et al., 1992). However, the relationship that may exist among antipredation responses in a group of sponges with the same class of compounds is not yet well known. For example, do sponges with a similar chemical profile show different responses to predators?

The genus *Tethya* is widely distributed along the Brazilian coast (Ribeiro and Muricy, 2011), but no ecological study has been done with Brazilian species. The aim of the present study was, therefore, to investigate and compare the chemical and structural defensive strategies produced by two *Tethya* species from the Brazilian coast, *Tethya maza* Selenka, 1879 and *T. rubra* Ribeiro and Muricy, 2004.

2. Materials and methods

2.1. Organism collection

Specimens of *T. maza* (1.5–2.0 cm in diameter) were collected in the intertidal zone at Tarituba Beach (Paraty, 23°02.729′S–44°35.725′W, Rio de Janeiro State, Brazil) by free diving in April and September 2004. Individuals of *Tethya rubra* (2.5–3.5 cm in diameter) were collected in the intertidal zone of Todos os Santos Bay (Salvador, 12°58′250″S–38°29′100″W, Bahia State, Brazil) in June 2004. Specimens of the hermit crabs *Calcinus tibicen* (Herbst, 1791) were collected at Itaipu Beach (Niterói, 22°58′S–43°04′W, Rio de Janeiro State, Brazil) and returned to the same location at the end of the study. As sponges and crabs were collected in distinct places, we employed an allopatric model in this study, assuming that *C. tibicen* has had no exposure to *Tethya* species over evolutionary time and would not be expected to have co-evolved adaptations of resistance to chemical defenses produced by these sponges.

2.2. Extraction procedures and analyses of extracts

Before extraction, the volume of fresh material (for each *Tethya* species) was determined using a graduated cylinder filled with natural sea water, in which we measured the volume displaced by a determined amount of sponge. Sponges were cut into small pieces and extracted three times with pure acetone at room temperature. The three extracts obtained were combined and passed through filter paper to remove sponge debris. The solvent was removed by rotary evaporation (Büchi R-114, Waterbath B-480 coupled to vacuum pump Thomas Scientific Pump, Model DOA-P104E-AA) after a period of one month of extraction. The crude extracts obtained were analyzed and compared by thin layer chromatography (TLC), ^{1}H, ^{13}C (APT), nuclear magnetic resonance (NMR) and high resolution gas chromatography coupled with a mass spectrometer (HRGC-MS).

Silica gel GF254 (Merck) was used for TLC analysis. ^{1}H and APT (300 MHz) spectra were recorded on a Varian Unity Plus 300 spectrometer using $CDCl_3$ as solvent and TMS as internal standard. Chemical shifts were recorded in δ (ppm).

Aliquots of the extracts were diluted with an appropriate volume of ethyl acetate and analyzed by HRGC–MS on a HP 6890 series GC system, coupled to a HP 5973 mass selective detector in the electron impact mode (70 eV), equipped with a HP-1 MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The injector and detector temperatures were set at 270 °C and 290 °C, respectively. The temperature program was set at 160 °C, then programmed to rise to 260 °C at a rate of 4 °C/min and finally raised at a rate of 15 °C/min to 290 °C for 15 min. Hydrogen was the carrier gas at a flow rate of 1 mL/min. Diluted samples were injected manually in the split mode (1/10 or 1/20).

The chemical components of each extract from the two sponge species were identified based on comparison of their mass spectra with those of standards, literature data, and Wiley 275 library data of the HRGC/MS system. Quantitative information concerning sterols was obtained from Frd area percent data.

2.3. Isolation of spicules

Spicules from *T. maza* and *T. rubra* were isolated by immersing fragments of these sponges in boiling nitric acid. Dissociated spicules in acid were washed by passing through a Büchner funnel with perforated plate linked to a kitasato coupled with a vacuum pump. The natural concentration of spicules was calculated as the mean of spicules obtained from five fragments of different portions of sponge, including the choanosome and ectosome, from a known volume.

2.4. Feeding assays

For preparation of artificial food using the method described previously (Waddell and Pawlik, 2000a), extracts and spicules were obtained from the equivalent of 9 mL of *T. maza* and *T. rubra*. To prepare 9 mL of artificial food, we used 0.09 g of carrageen, 0.45 g of powdered squid, and 8.55 mL of distilled water. Before these components were mixed, powdered squid was immersed in crude extract diluted in 2 mL of dichloromethane and, evaporated for use in treatment-foods. In control-foods, only the powdered squid in the solvent was evaporated. Then, carrageen and distilled water were mixed and heated in a microwave oven to the boiling point (about 20 s at high power). After that, powdered squid (control or treatment) was added and vigorously mixed. That mixture was left in a strip mold placed on a mesh square. When cooled, the artificial food was joined to the mesh, which was cut in strips (size 10 × 10 or 5 × 10 squares, each ca. 1.0 mm or 0.5 mm on a side, depending of high or low disponibility of extract or spicules, respectively).

Each replicate consisted of a small translucent closed box, with small round openings to allow water flow, one hermit crab, a strip of control-food, and the treatment. In each treatment, the component to be tested (spicules, crude extract, or both) was added to the artificial food. All replicates were placed in an aquarium filled with 100 L of natural sea water, submitted to constant water flux, temperature and aeration, under natural light (day/night cycle). The food eaten was quantified by counting squares without food, and the value was converted to percentage of consumption. Before the assays, the hermit crabs were fed with powdered squid or seaweed. The food was stopped 2 or 3 h before the assays began, and the experiments were carried out for 24–48 h.

For each sponge species, three independent experiments were performed to test the palatability of: (1) crude extracts, (2) spicules, and (3) crude extracts plus spicules. Natural concentrations of the natural component were used, and were calculated based on volume. The exception was the natural concentration of spicules in *T. rubra*,