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The role of sclerites in the defense against pathogens of the sea fan *Gorgonia ventalina* (Octocorallia)

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ABSTRACT

Sessile organisms such as octocorals cannot avoid threats such as predation, parasitism or fungal infections through evasive responses. Instead, they rely on strategies that deter or reduce the impact of these threats. One such strategy is the development of hard structures such as sclerites, which are minute calcium carbonate skeletal elements located on top of the ectoderm and within the mesoglea and gastrodermal tube walls. Sclerites have multiple roles, including structural support of the colony and protection from predators. The role of sclerites as a physical barrier that deters fungal infection, however, is unknown. This study examines the potential role of sea fan sclerites as protection from fungal infection. To accomplish this, four different fungi isolated from healthy sea fans were inoculated into 5 mL tubes filled with culture media and with sclerites from healthy and diseased fans placed on top. To determine whether chemical compounds in the sclerites impact infection, sclerites from healthy and diseased fans were extracted for several days with acetone prior to inoculation. Results showed that the sclerite layer delayed fungal infection from reaching the agar when compared to controls (tubes with agar but no sclerites). There was no difference between tubes with healthy and diseased sclerites, but there were differences among sclerites extracted with acetone. This study suggests that, in addition to the roles in structural support and predator deterrence, sclerites play a role as physical and chemical barriers against to fungal infection.

1. Introduction

Sessile marine invertebrates cannot avoid threats, such as predation or infection by pathogens, through evasive responses. As a consequence, these organisms have evolved other strategies to prevent, or at least reduce, the impact of those threats. One such strategy is the development of physical or structural barriers that prevent or deter the feeding of predators or infection by parasites. Physical barriers are usually found external to the epithelium of animals to protect their internal environment (Toledo-Hernández and Ruiz-Diaz, 2014a). In scleractinian corals and octocorals, for instance, the external mucus layer may serve as a physical barrier. This barrier can make the tissue unpalatable for predators, create an inhospitable environment for potential pathogens (Banin et al., 2001; Brown and Bythell, 2005) or reduce the effects of environmental challenges such as desiccation when corals are exposed to the air during a low tide in shallow waters.

Octocorals have evolved a physical barrier, sclerites, that provide structural support and also deter predation. Sclerites are minute calcium carbonate structures (Rahman and Oomori, 2008), which lay on top of the ectoderm and within the mesoglea and gastrodermal tube walls of the coral (Fig. 1; Lewis and Wallis, 1991). Sclerites vary in shape, from simple small rods to highly ornamented and larger spheres (Bayer, 1961). They vary both among species and in response to changes in water motion and predation pressure (West, 1998). Sclerites also vary in coloration, mostly from clear, light yellow or purple to dark purple. The darkening of sclerites is the result of carotenoid pigment deposition. However, it is not known whether these pigments are synthesized during sclerite formation or are translocated to the sclerites from an exogenous source such as endosymbiotic dinoflagellates (Leverette et al., 2008).

Multiple roles have been attributed to sclerites in octocorals. For instance, it has been suggested that they provide structural support to the colony by limiting the elasticity of the flexible axial skeleton (Ben et al., 2015; Lewis and Wallis, 1991). Sclerites also play a role in defense against predation. Several independent field assays have shown that when added to artificial feed, sclerites from different octocorals,

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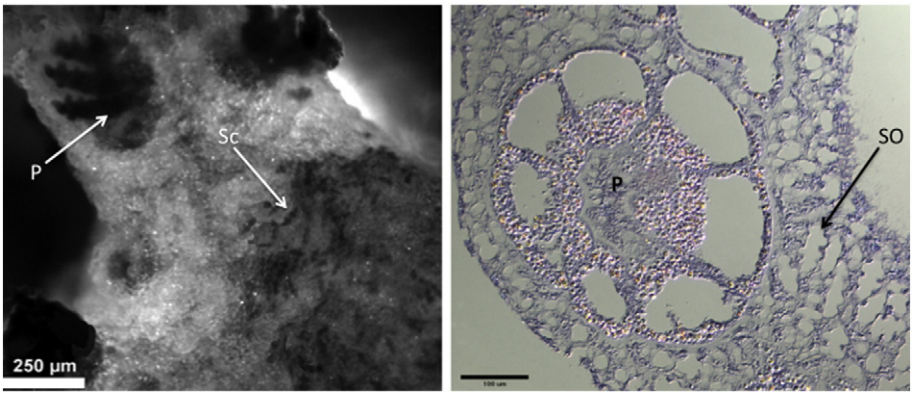

Fig. 1. Image showing (A) sclerites on top of the ectoderm of a *Gorgonia ventalina* colony; (B) Differential interference contrast (DIC) images of a cross section of a polyp and mesoglea of *G. ventalina* colony. Sc: sclerites on top of the ectoderm; SO: sclerites opening; P: polyps. (panel B modified from Toledo-Hernández et al., 2014b).

including *Gorgonia ventalina*, deter predation by reef gastropods and fishes (Alstyne and Paul, 1992; Harvell and Fenical, 1988; Koh et al., 2000; West, 1998).

Changes in sclerite coloration also are associated with stressful conditions. In sea fans from the genus *Gorgonia*, it has been shown that sclerite coloration changes from light to dark purple in response to infections, also known as tissue purpling. In fact, tissue purpling has been used to diagnose sea fan aspergillosis (Nagelkerken et al., 1997). Tissue purpling has also been documented during nonself interactions between conspecific individuals (Toledo-Hernández et al., 2013) and tissue regeneration (Ruiz-Diaz et al., 2016). Very few studies, however, have examined if the purpling process itself could play a role in the immune system of sea fans.

This study tests if sclerites can function as a barrier that prevents or delays infection by parasitic fungi in sea fan colonies by addressing three fundamental questions: 1) can sclerites deter the penetration of fungi into the internal environment of sea fans? 2) are sclerites from diseased sea fans (dark purple sclerites) more efficient at deterring fungal infection of sea fans than sclerites from healthy corals (light purple)? 3) do sclerites have associated chemical compounds capable of delaying fungal growth? To answer these questions a model system using agar was developed to mimic the sea fan internal environment, and inoculated the models with fungi cultivated from wild sea fans, with and without sclerites from both diseased and healthy sea fans. To control for associated chemical compounds, a separate group of sclerites were extracted with a polar solvent to eliminate metabolites that could provide chemical barriers to fungal growth. This study shows that sclerites do indeed inhibit fungal penetration and may therefore play a role in protection from fungal infection, in addition to the structural and antipredator roles previously described.

2. Materials and methods

2.1. Tissue collection

A total of 40 *Gorgonia ventalina* colonies, 20 healthy and 20 diseased 400–600 cm^2 in size, were collected from El Escambrón Beach, San Juan, Puerto Rico (18°28′00″ N, 66°05′12″ W) at a depth of 1.5–2.0 m. Healthy colonies had no lesions or purpled tissue. Diseased colonies, by contrast, had at least one tissue lesion usually overgrown by algae and a with ring of purple tissue surrounded it. Tissue samples of 4 cm^2 were cut from the center of each healthy colony to isolate sclerites. In the case of diseased colonies, sclerites were collected from the purpled ring surrounding the lesion. In previous studies, the protocol of cutting tissues from healthy and diseased sea fans did not cause fatalities, nor induce disease in the manipulated fans (Toledo-Hernández et al., 2013). Therefore, sampled sea fans were expected to readily recover within a few months after manipulation (Ruiz-Diaz et al., 2016; Toledo-Hernández et al., 2009). All tissue samples were collected under permit 2012-IC-086 issued to CRD by the Puerto Rico Department of Natural and Environmental Resources.

2.2. Collection of sclerites

In the laboratory, five randomly chosen healthy tissue samples were cut into two fragments; one was used for extraction of sclerites and the other for fungal isolation (see below). All diseased fragments and the remaining healthy fragments were used for extraction of sclerites. To extract sclerites, 2–3 cm^2 of tissue were individually placed in 15 mL vials with 3 mL Clorox (5.25% NaClO) for 8 h. Once sclerites disassociated from the soft tissue, they were rinsed several times with distilled water, transferred to new, labeled 1.5 mL vials, and dried at 75 °C for 2–3 days. Once dry, sclerites were divided into 0.02 g samples. Half of these samples (10 healthy and 10 diseased samples) were autoclaved and stored until used. The remaining samples (10 healthy and 10 diseased) were extracted using 1 mL of acetone per vial for 10 days, replacing the acetone at two day intervals. During the final day of extraction, acetone was discarded and sclerites were rinsed several times with distilled water, placed in new, labeled 1.5 mL vials, autoclaved and stored until use.

2.3. Fungal isolation and identification

Fungi used in this study were isolated from the healthy tissue used for collection of sclerites. Each fragment was surfaced-sterilized in 70% ethanol for 30 s, and rinsed in sterilized seawater three times (Toledo-Hernández et al., 2007). Then, tissues were cut into smaller fragments to increase the odds of fungal isolation (Toledo-Hernández et al., 2007), plated on Potato Dextrose Agar (PDA made with filtered seawater) and incubated at 25 °C for 3–10 days. As fungi began to emerge from the tissue samples, they were isolated in pure culture and were identified by morphological characteristics. In total, 12 morphospecies were identified. Once isolated, spores from each fungus were re-plated on GPYA, a standard culture medium for marine fungi (3 g glucose, 0.3 g peptone, 0.3 g yeast extract, and 20 g agar L^{-1} filtered seawater) and the time taken for each fungus to germinate and grow was recorded. Two criteria were used to select the fungi used in this study: 1) speed of germination and growth on GPYA; and 2) presumed pathogenicity. Based on these criteria, the following four fungi were chosen: *Trichoderma harzianum*, *Penicillium citrinum*, *Aspergillus tubingensis* and *A. fumigatus*. Many members of these fungal genera are opportunistic pathogens of animals, plants and fungi. Moreover, in previous studies, these fungal species were among the most common fungi isolated from healthy and diseased *Gorgonia ventalina* colonies (Toledo-Hernández et al., 2007, 2008; Zuluaga-Montero et al., 2010).