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# Significance of immune responses in *Diadema antillarum* to Caribbean-wide mass mortality

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**Abstract.** Mass mortality of the grazer *Diadema antillarum*, probably caused by a water-borne pathogen, was a major factor leading to algal domination of Caribbean reefs. Recovery of *Diadema* in St. Croix allows us to addresses questions on how recovery may be influenced by immunological processes. What are the basic features of immunological responses in *Diadema*? How does strength of immune responses in *Diadema* compare to other urchins that did not die (*e.g., E. lucunter*, or *E. viridis*)? We used coelomic fluid and coelomocytes of urchins from several locations on St. Croix to test their immune responses. All urchins released humoral defense molecules when stimulated by various agents – with one exception. *Diadema* did not respond as efficiently as other urchins did to lipopolysaccharide. This suggests a defect in immune response that is specific to *Diadema* and independent of stressors associated with particular environments. These studies may provide information required to understand whether a weakened immune response was responsible for the mass mortality, and whether strengthening of immunity has occurred since. Effective management of a recovery may depend on knowing whether diseases continue to impact *Diadema* population growth due to weak immune responses, and whether a recovered/restored population could experience another die-off.

**Keywords:** Comparative Immunology, Echinoderm, Ecology, Evolution, Immunity

## Introduction

*The Black-spined Sea Urchin and Coral Reef Ecology*

In 1983 the black-spined sea urchin (*Diadema antillarum*) began to vanish from the Caribbean Sea, disappearing first from coral reefs close to the Panama Canal and eventually from almost every coral reef in the Caribbean by 1984. This mass mortality, which wiped out more than 97% of the Caribbean-wide *Diadema* population, was one of the most devastating mortalities ever recorded for a marine animal. This die-off is a major factor leading to a phase shift from coral-dominated to algae-dominated communities that has occurred on many Caribbean reefs during the past 20 years (Lessios 1988; Hughes 1994). In St. Croix, the density of *Diadema* before the 1983 die-off was estimated to be ≈6.4 individuals/$m^2$. In 1984, densities plummeted to <0.1 individuals/$m^2$, and in 2001 there were ≈0.17 individuals/$m^2$ (Lessios 1988). Decades after the mass mortality event, *Diadema* was still rare, with very low recruitment rates (Lessios 1988; Edmunds and Carpenter 2001).

It is thought that the demise of *Diadema* was caused by a water-borne pathogen. Indeed, plots of surface currents in the Caribbean Sea coincide significantly with the spread of the *Diadema* die-off (Lessios 1988; Miller et al. 2003). In addition, several species of bacteria capable of killing *Diadema* have been associated with dying urchins in the laboratory, but bacteria have not been detected in field-collected individuals (Bauer and Agerter 1987).

Recent recovery of *Diadema* has been associated with reductions in algal cover and increased coral recruitment success in Jamaica (Edmunds and Carpenter 2001), and with reductions in algal cover in St. Croix (Miller et al. 2003), where *Acropora palmata* colonies are reappearing in areas with high *Diadema* densities, but are largely absent where *Diadema* are absent or uncommon (unpublished). Recovery of *Diadema* may be critical to restoration of coral communities, particularly where reefs have few herbivorous fishes due to heavy fishing (Hay 1984), as in the USVI (Garrison et al. 1998). Many factors may be influencing *Diadema* recovery, including predation, competition from other herbivores, and effects of rarity [low reproduction and recruitment stemming from low population density (*e.g.,* Lessios 1988; Miller et al. 2003)]. Another factor that must be considered is possible immunological weakness of this *Diadema* population which would allow continuing morbidity or mortality due to the original mass mortality pathogen or other diseases. Continuing recovery of *Diadema* populations in St. Croix (Miller et al. 2003) has allowed study of how recovery dynamics of *Diadema* may be influenced by basic

immunological and ecological processes.

Our studies are aimed at uncovering basic features of the immunological responses of *Diadema*: What are the basic features of the immunological responses of *Diadema*? How does strength of immune responses in *Diadema* compare to other Caribbean urchins that did not die off (*e.g., Tripneustes ventricosa, Echinometra lucunter,* or *Echinometra viridis*)? Are there immunological differences in local populations of *Diadema* on St. Croix that could be related to site-specific speeds of recovery?

*Invertebrate Immunity*

Animals posses both cellular and secreted protein (humoral) mechanisms for distinguishing self from non-self. Potential invaders include viruses, bacteria, protozoa, fungi and even protein molecules. The distinction between "self" and "nonself" is achieved by even the most primitive protozoans (Beck and Habicht 1996; Flajnik and DuPasquier 2004). Evolutionary forces have given rise to two arms of the immune system in animals: acquired (also known as adaptive or specific) and innate (or non-specific) immunity. Acquired immunity is only found in vertebrates. Invertebrates have innate immune systems, characterized by activation of immune effector cells, mediation by circulating coelomocytes. Invertebrates have many of the innate host defense mechanisms vertebrates do, but lack the specificity of the vertebrate immune response. The position in phylogeny of echinoderms makes them an important invertebrate group for comparative studies, including those addressing the evolution of the immune system. Although echinoderms efficiently clear bacteria from the coelomic fluid (CF) and mount an innate type immune response to pathogens and their products by coelomic fluid cells (coelomocytes), little is understood of the molecular mechanisms and genes that are used by these cells in response to a non-self challenge.

Phagocytosis [the engulfment of foreign material (*i.e*., pathogens) by specialized cells (*i.e*., phagocytes)] is the predominant cellular defense mechanism in vertebrates and invertebrates (Beck and Habicht 1996; Flajnik and DuPasquier 2004). Humoral-based defenses include: antimicrobial peptides (AMPs) and proteins (*i.e.,* lysozyme), phenoloxidase, complement-like proteins (Beck and Habicht 1996; Flajnik and DuPasquier 2004; Raftos et al. 1992; Beck et al. 1996; Lin et al. 2001), and reactive oxygen intermediates [ROI; *e.g.*, superoxide ($O_2^{\cdot-}$)] (Meier 2001; Babior et al. 1973; Clifford and Repine 1984; Beck et al. 2001). The ability of invertebrates to combat infections is dependent on the release of ROI (a group of inorganic compounds that are used by phagocytes in the formation of more toxic radicals to destroy pathogens).

**Materials and Methods**

*Reef surveys:* At each back-reef site at each sampling time, SCUBA divers counted *Diadema* in 14 randomly located 50m × 2m transects, and *Diadema* test diameters were recorded as small [<40mm; mean size of 1 yr. old *Diadema* = 48.6 mm (Karlson and Levitan 1990)], medium (41-60mm) or large (>60mm).

*Collection of sea urchin coelomocytes:* Urchins were collected from three study sites around St. Croix and processed within 2 hrs. A syringe containing ice-cold anticoagulant solution (99.8g EDTA and 33.5 ml 2-mercaptoethanol to 1L in seawater) was inserted into the coelom through the soft tissue surrounding the urchin mouth structure (Aristotle's lantern) to extract coelomocytes (Beck and Habicht 1991). These cells were washed two times and resuspended in saline at a concentration of $1 \times 10^6$ cells/ml. Cells were counted in a hemocytometer (Lin and Beck 2007). Proteins in the CF were quantified by the Bradford assay (Beck and Habicht 1991; Lin and Beck 2007). Sodium dodecycl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the CF was performed as previously described (Lin et al. 2001; Beck and Habicht 1991).

*Determination of reactive oxygen intermediates (ROIs):* The ROIs produced by phagocytes were assayed using a spectrophotometric assay to measure $O_2^{\cdot-}$. When phagocytes engulf a pathogen, a burst in oxidative metabolism results in the generation of ROIs (Beck et al 2001; Arumugam et al. 2000; Anderson et al. 1992; Vidya et al. 2007; Bettencourt et al. 2007). A simple and direct method of detecting this metabolic change is the nitro-blue tetrazolium (NBT) reduction assay [wherein the yellowish NBT is reduced to an insoluble, purplish precipitate in the cells (Arumugam et al. 2000; Anderson et al. 1992)]. Two hundred fifty µl cells ($10^6$ cells/ml), 250 µl of 0.1% NBT, 10µl stimulator [*e.g.,* peptidoglycan, lipopolysaccharide in phosphate buffered saline (PBS)]) or PBS alone (control) were mixed and incubated at 22ºC. (Beck et al 2001; Arumugam et al. 2000; Anderson et al. 1992; Vidya et al. 2007; Bettencourt et al. 2007). After 1 hr the cells were separated from the supernatant by centrifugation in a microfuge, and reduced NBT in the pellet was determined spectrophotometrically at an optical density at 630 nm (in quadruplicate). The production of $O_2^{\cdot-}$ was monitored over time.

## Results

*Enumeration of Diadema on the Reefs of St. Croix*

We asked whether the initial recovery of *Diadema* that we have observed on critically affected coral reefs of St. Croix (Miller et al. 2003) is continuing? To determine relative rates of population recovery in different parts of St. Croix, U.S.V.I., *Diadema* were counted and sized twice at each of six locations (Fig. 1): Two south shore back-reef sites (TH & RB), two north shore back-reef sites (POW & SOL), and two north shore patch reefs in Tague Bay (TB), following the methods of Miller *et al.* (Miller et al. 2003). As can be seen in Fig. 2, recovery of *Diadema* on St. Croix continues to be slow, or has stalled out entirely.

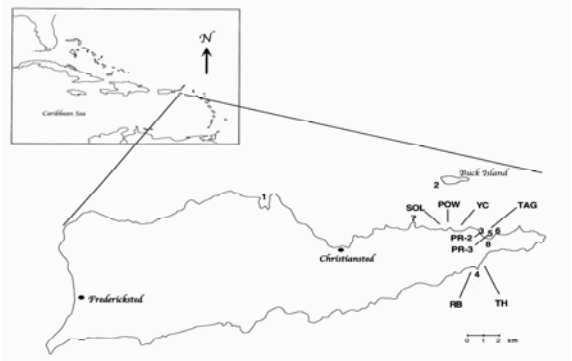


Figure 1: Map of St. Croix, USVI showing the 6 study sites. SOL = Solitude Bay, POW = Pow Point, YC = Yellowcliff Bay, TAG = Tague Bay, TH = Turner Hole, RB = Rod Bay. (From 4)

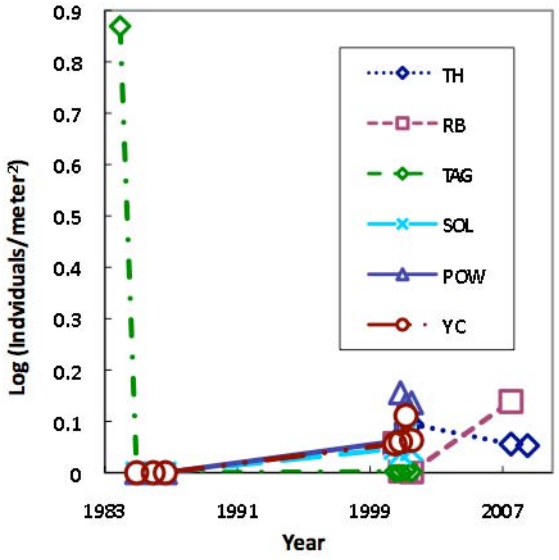


Figure 2: The number of *Diadema* found at study sites on St. Croix from 1984 to 2008. The study sites are the same as described in Fig. 1.

*Gross Protein Profiles and Cellular Components of Coelomic Fluid in the Urchins*

Protein profiles and cellular components of the coelomic fluid varied substantially among the four urchin species (Fig. 3). *Diadema* differs significantly from *Tripneustes* and the two *Echinometra* species in the counts for both red blood cells and total coelomic cells, but in both cases the values for *Diadema* are in the middle of the pack and not at either extreme. On the other hand, as determined by the Bradford assay, *Diadema* has significantly more protein in the CF than any of the other urchins.

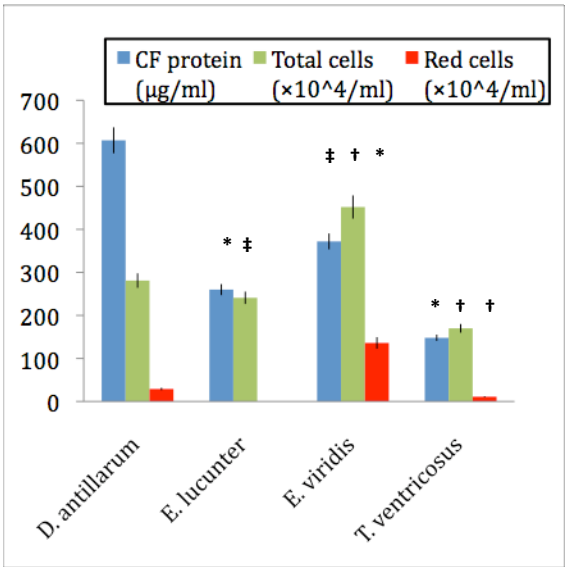


Figure 3: Analysis of CF and coelomocytes of 4 urchins found in the coastal waters of St. Croix (mean ± SD for 6 urchins of each species). **P*< 0.001 when compared to *Diadema* alone, †*P*< 0.01 when compared to *Diadema* alone, ‡*P*< 0.05 when compared to *Diadema* alone.

*Differences in Protein Profiles of Coelomic Fluids of Diadema from Different Sites (SDS-PAGE)*

When CF proteins from *Diadema* collected from 3 different sites on St. Croix (SOL, RB, and TH) were subjected to SDS-PAGE we could see no readily apparent differences in their crude protein profile (Fig. 4).

*Changes in the Humoral Responses of the Urchins*

The ability of invertebrates to combat infections depends, in large part, on the release of reactive oxygen intermediates, groups of inorganic compounds that inhibit vital enzymes of pathogens and are used by phagocytes in the formation of more toxic radicals (Arumugam et al. 2000; Anderson et al. 1992; Vidya

et al. 2007; Bettencourt et al. 2007). As seen in Fig. 5 all the urchins were capable of releasing $O_2^{\cdot-}$ when stimulated by various agents. There was one statistically significant exception: The response of *Diadema* to stimulation with lipopolysaccharide (LPS) is low compared to the other urchins. There is no geographic component to the lack of this response to LPS since *Diadema* from all sites were equally unresponsive (unpublished).

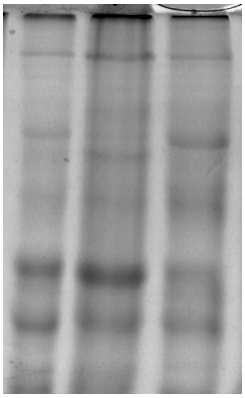

Figure 4: SDS-PAGE (7.5% SDS-PAGE gel stained with Coomassie Blue R-250) of CF from *Diadema* collected from 3 different locations on St. Croix.

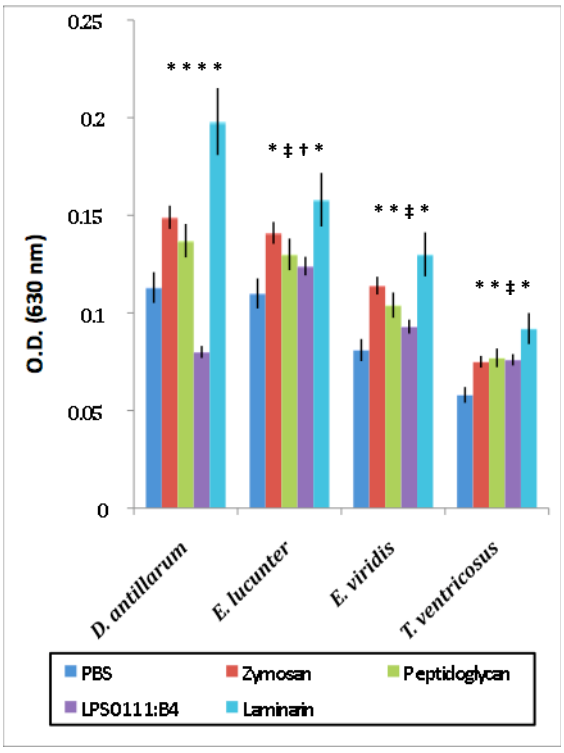


Figure 5: Stimulation of sea urchin coelomocytes with different agents. Data are presented as mean ± SD from six experiments. *$P$< 0.001 when compared to PBS control, †$P$< 0.01 when compared to PBS, ‡$P$< 0.05 when compared to PBS.

## Conclusions

Recovery of *Diadema* on St. Croix continues to be slow, or has stalled out entirely. This recovery, with its beneficial effects on corals, is neither universal nor complete in the Caribbeanmand is progressing even more poorly in the Florida Keys (Miller et al. 2003; Carpenter and Edmunds 2006; personal observation). A principle recommendation of the recent *Diadema* Workshop, hosted by the Nature Conservancy, the National Fish and Wildlife Foundation, and the Rosenstiel School of Marine and Atmospheric Sciences in the summer of 2004, was to study key aspects of *Diadema* biology and ecology. Efforts to restore *Diadema* in the Florida Keys by seeding small areas with juvenile urchins have been unsuccessful because juveniles suffered severe mortality after they were introduced to the reefs (Rosov 2002). Efforts to reverse the algae-coral phase shift on coral reefs of the Caribbean and Florida Keys through restoration/recovery of *Diadema antillarum* can succeed only through improved comprehension of the recovery process. Managing a more rapid recovery may depend on understanding the competitive and predator/prey relationships between fishes and urchins, and how increasing adult *Diadema* densities affect recruitment and juvenile survival. It is also critical to understand whether weakened immune systems in *Diadema* were partly responsible for the mass mortality, and how much strengthening of immune systems has occurred since the die-off.

We used a preliminary broad-based "shotgun" approach (*e.g.,* coelomic fluid and coelomocyte population characterization) in an attempt to quantify any differences in host defense responses between several Caribbean sea urchin species. Our experiments suggest that the strength of the humoral immune response in *Diadema* appears to be weaker than in other urchin species of St. Croix. In addition, in both humoral assays and SDS-PAGE experiments we have observed that there are no differences among *Diadema* from different St. Croix locations. The inability of *Diadema* to respond as efficiently to LPS –a conserved structural component of gram-negative bacterial cell walls– as other urchins do could be a clue to a defect in a host defense response to a pathogen. One possible explanation for this decreased responsiveness could be a defect in Toll-like receptors (TLRs). The TLRs are a family of essential cell surface proteins of the innate immune system of vertebrates and invertebrates (Bochud et al. 2007; Akira et al. 2006; Beutler 2004). Their extracellular domains are capable of recognition of different microbial-associated molecular patterns (MAMPS). For example, TLR-4 detects LPS and mannan, while TLR-2 detects peptidoglycan and other β-glycans

(Bochud et al. 2007; Akira et al. 2006; Beutler 2004). This apparent *Diadema* lower response to LPS could be due to a defect in TLR-4 or a second polypeptide (MD-2) that binds to the extracellular domain of TLR-4 and enables it to bind to the conserved inner region of LPS. Is there a defect in LPS signaling in *Diadema* [*e.g.,* TLR-4 and/or MD-2 defects, a TLR-4/MD-2 signaling defect)? One could argue that not all *Diadema* TLRs are defective as *Diadema* responds appropriately (when compared to the other urchins) to peptidoglycan and zymosan (TLR-2 ligands) (Fig. 5).

Whatever the molecular basis for a weakened immune response in *Diadema antillarum*, the weakness could have consequences for the future of Caribbean coral reefs. The recovery dynamics of *Diadema* have been patchy in St. Croix waters, with some localities experiencing earlier, and/or more rapid recovery than others. Since *Diadema* from all parts of St. Croix seem to be showing the same degree of immunological weakness, we cannot associate local differences in recovery with local differences in immune response. Efforts to reverse the algae-coral phase shift on coral reefs of the Caribbean and Florida Keys through restoration/recovery of *Diadema* can succeed only through improved comprehension of the recovery process. It is critical to understand whether a weakened immune response in *Diadema* was responsible for the mass mortality, how much strengthening (if any) of immune systems has occurred since, whether continuing weakness may be interfering with *Diadema* recovery, and whether a recovered population could experience another epidemic. However slow, continuing recovery of Caribbean *Diadema* and the existence of populations of *Diadema antillarum* and related species that were not involved in the die-off affords an opportunity to look at how recovery and post-recovery dynamics of *Diadema* may be influenced by the development of immunological resistance.

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