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Epidermal lesions and mortality caused by vibriosis in deep-sea Bahamian echinoids: a laboratory study

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ABSTRACT: When significant mortality of the bathyal spatangoid echinoid *Paleopneustes cristatus* occurred under laboratory conditions, we investigated the cause and course of the disease by culturing and identifying internal pathogens, then experimentally infecting healthy urchins with isolates of the suspected disease organism. The pathogen was determined to be the Gram-negative halophilic bacterium *Vibrio alginolyticus*. This species was also recovered from frozen post-challenge specimens of *P. cristatus* and from moribund individuals of *Archaeopneustes hystrix*, another spatangoid reared under similar *in vitro* conditions. This is the first experimental study of bacterial disease in any deep-sea invertebrate.

KEY WORDS: Bathyal echinoids · Epidermal lesions · Vibriosis · Mortalities

INTRODUCTION

Diseases are known to be important sources of mortality for populations of some benthic marine invertebrates, but pathogens and the etiologies of marine invertebrate diseases have been investigated in relatively few non-commercial species. This is especially true for the deep sea; indeed, to our knowledge no pathogen has been isolated from any deep-sea invertebrate. Recent years have seen highly publicized outbreaks of coral diseases (Antonius 1995, Richardson 1997) and massive population-wide infections have been documented in both temperate and tropical sea urchins (Pearse et al. 1977, Boudouresque et al. 1981, Lessios et al. 1984, Scheibling & Stephenson 1984, Jones 1985, Jangoux 1990). Bang & Lemma (1962) observed that starfish traumatized by dredging suffered increased mortality in tanks because of heightened sensitivity to bacterial infections. Here we report an analogous finding, namely that bacterial disease can cause mortality of carefully collected deep-sea echinoids under culture conditions. The disease agent occurs commonly in shallow coastal waters and infects deep-sea urchins that are maintained in water containing the bacterium. The disease is exacerbated by even minimal mechanical damage to the epidermis. It is similar to bald-sea-urchin disease, a well-documented bacterial disease of shallow-water sea urchins (reviewed by Jangoux 1990).

Bald-sea-urchin disease is often marked by dark mucoid lesions on the epidermal tissue overlying the test (Maes & Jangoux 1984, 1985). Although similar lesions have been attributed to unsuccessful predation attempts by predatory snails (Eaton 1972), bacterial and protozoan disease organisms appear to be the causative agents in most cases. An ameoboid protist, *Paramoeba invadens*, apparently was responsible for mass mortality of the echinoid *Strongylocentrotus droebachiensis* on the southwestern coast of Nova Scotia, Canada (Scheibling & Stephenson 1984, Jones 1985). However, Roberts-Regan et al. (1988) found lesions containing the bacteria *Acinetobacter* sp. and *Alcaligenes* sp. in some individuals of an otherwise healthy population of *S. droebachiensis*, suggesting that bacteria as well as protozoans may play a role in the disease. Gillis & Pearse (1986) reported *in vitro* studies of a disease with similar symptoms in the sea urchin *S. purpuratus* from Monterey Bay, California, USA. They isolated 14 different strains of bacteria, including species of *Flavobacterium* and *Pseudomonas*, from epidermal lesions, but found that only

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Vibrio anguillarum and *Aeromonas salmonicida* could produce lesions experimentally. The widespread tropical echinoid *Diadema antillarum* experienced a catastrophic decline during 1983 and 1984 throughout the Caribbean and tropical western Atlantic Oceans. The suspected vector causing this mass mortality appears to have been a water-borne, host-specific bacterium (Bak et al. 1984, Bauer & Agerter 1988).

The external and internal prokaryotes of deep-sea echinoids have not been thoroughly characterized or catalogued. In this study, we document the natural flora of a bathyal irregular echinoid, *Paleopneustes cristatus*, and describe the etiology of a bacterial disease in *P. cristatus* and a second spatangoid urchin, *Archaeopneustes hystrix*. We provide evidence that the disease is a form of vibriosis that occurs following minor mechanical damage to the epidermal tissue layer. To our knowledge, this is the first experimental study of bacterial disease in any deep-sea invertebrate.

MATERIALS AND METHODS

Collection and maintenance of echinoids. *Paleopneustes cristatus* were collected in February and October 1997, between 500 and 700 m depths from Goulding Cay, near the western tip of New Providence Island, Bahamas. *Archaeopneustes hystrix* were collected in June 1998 at 600 m off Paradise Island, Bahamas. Specimens were individually collected by manned submersible (Johnson-Sea-Link) from fine sandy sediments on a steep slope, using either a closing 'clam-bucket' scoop or a gentle suction device attached to the manipulator arm. Because the urchins are too large to pass through the suction tube, those collected by suction were picked up by gently sucking on the aboral test surface, then dropping them into a Plexiglas container of seawater. This caused apparently minor damage to the fine spines and the epidermis, where the round suction tube contacted the specimen. The clam bucket scoop, which completely surrounds the urchin and some underlying sediment, generally collects specimens in perfect condition. However, spines on the sides of the urchins are sometimes abraded.

On the ship, sea urchins were maintained in large aerated tanks of chilled (15°C) seawater in walk-in incubators. Following the cruise, urchins were transferred into 150 l seawater tanks, which were part of a 1937 l chilled, recirculating seawater system (see Fig. 1A) at Harbor Branch Oceanographic Institution. Seawater (35 psu) for these tanks was collected from the seawater intakes of the St. Lucie Nuclear Power Plant on South Hutchinson Island, Florida, USA, midway between Ft. Pierce and St. Lucie Inlets and 20 km away from any potential sewage input from metropolitan areas. The echinoids were fed weekly on a diet of the planktonic diatom *Chaetoceros gracilis*, which had been flocculated with chitosan to resemble phytodetritus that the urchins might ordinarily encounter in the field. The algae were cultured on Guillard's media using standard protocols.

Isolation of the *in situ* bacterial flora in healthy individuals. Some individual sea urchins were dissected soon after the submersible arrived on deck to obtain samples of the natural internal bacterial flora. Because the urchins were exposed to surface waters (and associated flora) during the ascent, we cultured only the internal organs and body cavities. Samples of coelomic fluid (2 ml), mid-gut (1 cm long pieces) and gonadal tissues (1 cm) were taken using sterile transfer pipetes, syringes and surgical scalpel blades and placed in tubes of 3 different culture media: (1) 9 ml of chopped meat with glucose, hemin and vitamin K (hereafter labelled CMG); (2) 8 ml trypticase soy broth agar with 6.5% sodium chloride (TSBSC); and (3) 10 ml of thioglycollate with vitamin K and hemin (THIO) (Bauer & Agerter 1987). All media were purchased from BBL Microbiology Systems, Cockeysville, MD, USA.

Culture and identification of potential disease organisms. The epidermal lesions and the internal organs of the diseased bathyal urchins *Paleopneustes cristatus* from the experimental tanks were dissected and cultured as described above. In addition, samples were cultured from lesions and coelomic fluid of 2 moribund *Archaeopneustes hystrix*. For the latter species, a 2 ml sample of coelomic fluid was removed from each specimen using a sterile syringe.

Isolation procedures for both the normal bacterial flora and the diseased specimens used 100 mm plates consisting of TSB agar with 5% sheep blood (BA), chocolate agar (CA) and MacConkey agar (MA). The recovery of marine vibrios was enhanced by using selective plated media thiosulphate citrate bile salt (TCBS) agar and tubed TSBSC. Bacteria isolated from both field and laboratory specimens were identified using 2 commercial systems: Auto Microbic Systems Microscan (Baxter: Baxter Healthcare Corporation, West Sacramento, CA, USA), and Vitek (bio Merieux Vitek, Inc., Hazelwood, MO, USA). These systems provide automated identification to the species level of facultative anaerobic and aerobic Gram-negative bacilli, both fermentative and non-fermentative. The API 20E (bio Merieux) was also used as a manual system for confirmation of Gram-negative identifications. The endospore-forming Gram-positive rods were identified using the Microscan Rapid Anaerobe Identification Panels and the API An-Ident System (bio Merieux).

Virus culture—isolation and identification. Virus cultures were taken from epidermal lesions (lesion swabs in sterile screw-cap tubes with Multi-Microbe media [M4-3], Micro Test Inc., Snellville, GA, USA), coelomic fluid (fluids inoculated in sterile screw-cap containers with Multi-Microbe media) and midgut (sterile containers containing transport medium without fixatives) of 6 diseased *Paleopneustes cristatus* specimens in May 1997 and December 1997. These fluids and tissues were evaluated by a commerical laboratory using the tissue cultures and immuno-fluorescence assay.

Experimental infections. The suspected pathogen was used to challenge healthy specimens of *Paleopneustes cristatus* under controlled laboratory conditions. The limited number of specimens available prevented the determination of lethal dosages using conventional LD_{50} testing. Instead, the suspected pathogens were injected into the coelom at 2 different doses and then survival was compared to uninjected controls and to control individuals given intracoelomic injections of sterile 0.85% saline.

The dosages in units of cells ml^{-1} were determined using the McFarland method, which compares a liquid bacterial suspension to known turbidity standards (Baron et al. 1994). Turbidity was assayed with a Microscan Turbidity Meter (Baxter), which measures the % absorbance at 670 nm. For the experimental treatments, we adjusted the low dose to a McFarland standard number of 0.5, which represents an approximate cell density of 1.5×10^8 ml^{-1}, and adusted the high dose to a McFarland standard number of 1.0, which is equivalent to 3×10^8 ml^{-1}. Sixteen apparently healthy *Paleopneustes cristatus* were removed from the experimental tanks and placed into 16 individual 11.3 l plastic containers in a temperature-controlled (15°C) walk-in refrigerator, where they were aerated continuously for 9 d. Each specimen was randomly assigned to 1 of 4 treatments: (1) *Vibrio alginolyticus* low dose, (2) *V. alginolyticus* high dose, (3) saline-injected control or (4) uninjected control. Saline solution (0.85%, PML Microbiologicals, Tualitin, OR, USA) and pathogens were injected into the coelomic cavity near the mouth region, using a 1 cc sterile tuberculin syringe with a $22 \times 1^1/_2$ needle. Each urchin was examined daily to determine if it was still alive, as indicated by spine movement. Six dead post-challenge urchins were frozen (–60°C) and cultured later for the possible recovery of the infecting pathogen.

RESULTS

Bacterial flora of healthy individuals

During February 1997, 3 specimens of *Paleopneustes cristatus* were dissected on board ship, immediately after collection. Cultures of the midgut using the medium CMG produced bacterial isolates consisting of the Gram-positive anaerobes *Clostridium perfringens* and *C. paraputrificum*. *Pseudomonas* sp. was cultured from the gonad and midgut with TSBSC. No bacterial isolates were recovered from the coelomic fluid.

Description and etiology of the disease

Healthy *Paleopneustes cristatus* (Fig. 1B) were maintained in the chilled recirculating seawater systems from February to July 1997, at which time 2 urchins developed dark mucoid lesions on their bodies (Fig. 1C). All spines were lost in the region of the lesions. We attempted to treat this disease by moving several of the affected urchins into tanks with tetracycline (2 g/11.3 l), but this was not successful and the urchins died in a matter of several weeks. Additional specimens developed lesions over the next several months, until all urchins had died (Fig. 2). In-line UV sterilizers were subsequently installed in the recirculating seawater systems of our deep-sea culture laboratory. *P. cristatus* collected on 2 later cruises were maintained successfully for many months in UV-irradiated seawater, with no epidermal lesions developing.

We noted that many of the lesions appeared in a perfect circle on the aboral side of the test (Fig. 1C), where the round suction tube had been in contact with the urchins during collection. It was therefore hypothesized that the epidermal layer on the aboral surface had been compromised during collection, creating a route by which pathogens could infect the urchins. In subsequent cruises, we avoided use of the suction tube in collection, instead using a clam bucket scoop. Eventually, lesions also appeared in individuals collected by this latter method, but the lesions were not located in a perfect circle around the aboral surface. Instead, the most common location of lesions was near the base, presumably where the urchins has been scraped by the clam bucket scoop during the collection process. These observations lent further credence to the hypothesis that epidermal lesions caused by mechanical abrasion provide a route for infectious organisms to enter (possibly through eroded or damaged test plates) into the coelomic compartment, where massive internal infection eventually leads to death.

Archaeopneustes hystrix also developed lesions on the body, but the lesions tended to be less distinct than those of *Paleopneustes cristatus*. In many urchins, the disease was manifest by localized loss of spines rather than distinct epidermal lesions (Fig. 1D).

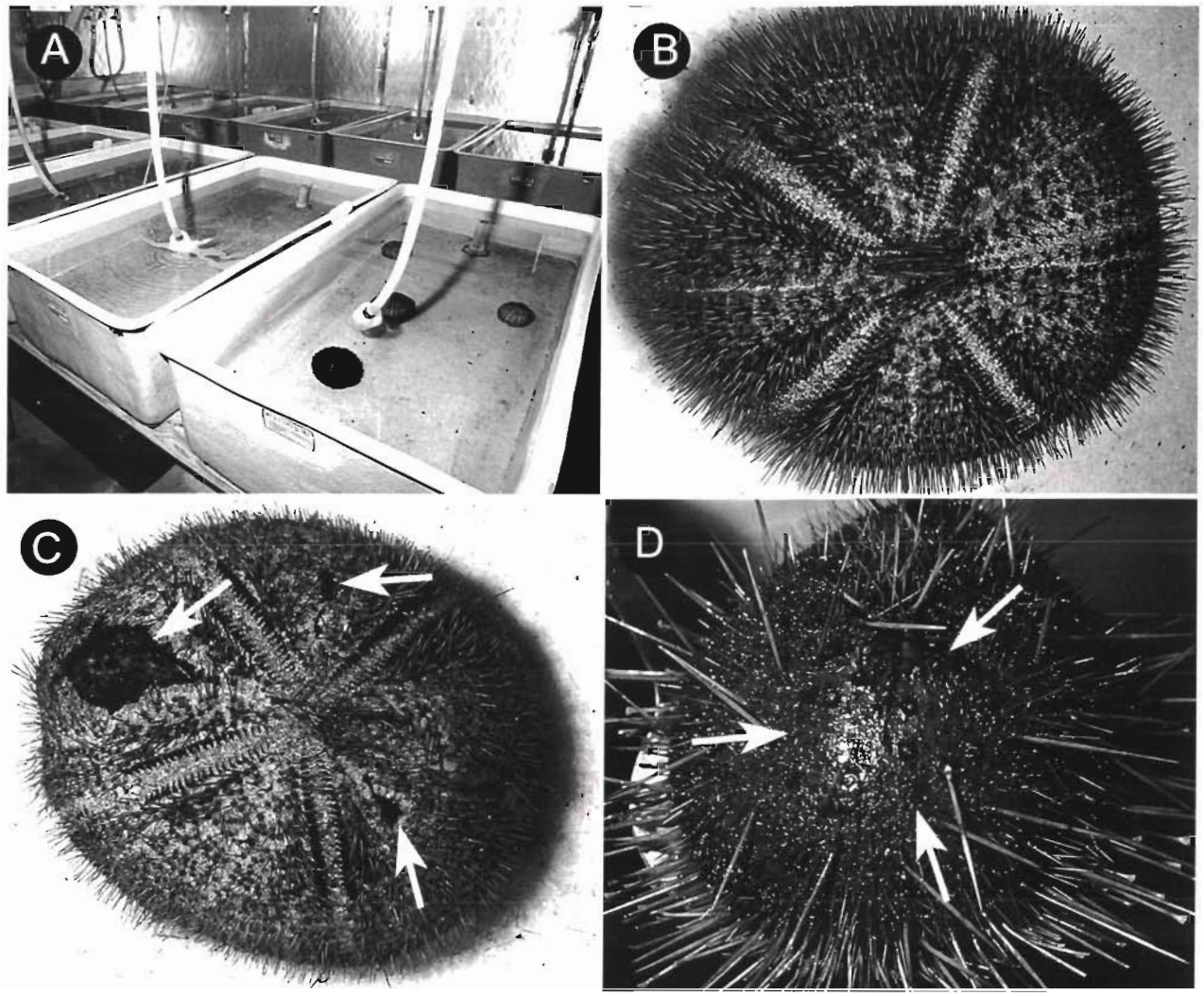

Fig. 1. (A) Refrigerated, recirculating seawater system in which spatangoid echinoids were maintained. (B) Aboral view of healthy *Paleopneustes cristatus*. (C) Aboral view of diseased *P. cristatus*, showing dark epidermal lesions (white arrows) arrayed in a circular pattern. (D) Diseased *Archaeopneustes cristatus*, showing localized region (circumscribed by white arrows) with loss of spines

Bacteria and viruses isolated from dying urchins

Cultures taken from the lesions and midgut of dead and dying *Paleopneustes cristatus* yielded the following bacteria: *Bacillus* sp., *Clostridium* sp., *Pseudomonas putida* and *Vibrio alginolyticus* (Table 1). Only *Bacillus* sp. was isolated from the gonad and only *V. alginolyticus* was found in the coelomic fluid. *Bacillus* spp. are known to be ubiquitous saprophytes and *Clostridium* sp. was also found in the guts of newly collected healthy individuals, so we suspected that the disease organism might be *V. alginolyticus*, which was found in cultures from all tissues and fluids of diseased urchins, but not in newly collected specimens. *V. alginolyticus* and *Vibrio* sp. (with an affinity to *V. alginolyticus*) were isolated from the internal organs of 6 frozen post-challenge specimens of *P. cristatus*. Also, *V. alginolyticus* was the only bacterium cultured from the coelomic fluid of *Archaeopneustes hystrix* which were dying with symptoms similar to those of *P. cristatus* (Table 1). We were not able to culture or isolate any viruses from infected sea urchins.

Experimental induction of the disease

After the experimental dosages were administered to healthy urchins, mortality began within 2 d in the high dose urchins and after 3 d in the low dose treatments (Fig. 2). Within 8 d all high dose specimens had died and half of the low dose urchins had also died. The experiment was terminated after 9 d, 1 d after the

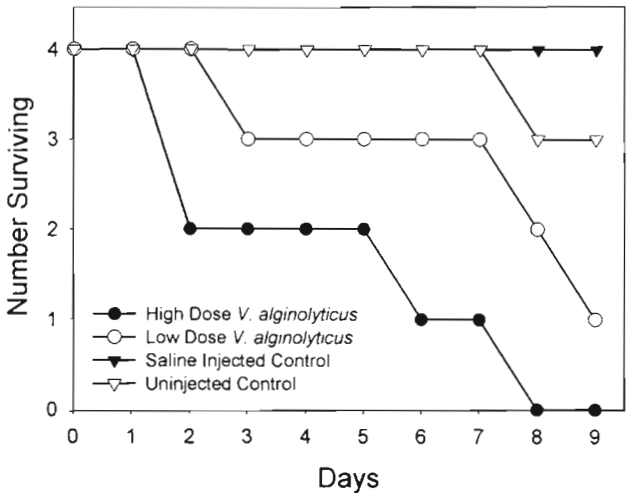

Fig. 2. *Paleopneustes cristatus*. Survivorship of individuals experimentally exposed to high and low doses of *Vibrio alginolyticus*, of control individuals injected with saline, and of control individuals that were not injected

first uninjected control died. By this time, only 1 low dose specimen remained alive and none of the saline-injected control specimens had died. Both of the tested dosages of *Vibrio alginolyticus* were lethal to *Paleopneustes cristatus* and degraded both mucus and tissues of the internal organs, with only remnants of the gut remaining in the dead specimens.

DISCUSSION

The suspected disease microorganism in this study, the Gram-negative halophilic bacterium *Vibrio alginolyticus* (Miyamoto et al. 1961), requires at least 3% NaCl and can tolerate up to 10% NaCl for growth (Carnahan & Kaplan 1995). Seawater is an important vehicle of transmission of this species and other vibrios, which accounts for their cosmopolitan range and the possible spread of vibriosis to marine invertebrates. Of the 4 major *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*), *V. alginolyticus* is the only one which rarely causes human diseases (Carnahan & Kaplan 1995). It occurs commonly in the Florida marine environment and does not indicate pollution from human waste products. Gram-negative aeromonads, flavobacteria, pseudomonads and vibrios plus both obligate and facultatively anaerobic, endospore-forming Gram-positive rods have been cultured from the Florida coastal environments (Bauer & Agerter 1995). The vibrios *V. alginolyticus* and

Table 1. Bacteria isolated from diseased individuals of *Paleopneustes cristatus* and *Archaeopneustes hystrix*. –: no isolates found. 0: not cultured

Date	Medium	Lesions	Bacterial isolates		
			Gut	Gonad	Coelomic fluid
Paleopneustes cristatus (n = 10 ind.)					
May 1997	CMG	–	–	–	0
	THIO	*Bacillus* sp. *Pseudomonas putida*	–	–	0
	TSBSC	*Bacillus* spp. *Vibrio alginolyticus*	–	– –	0
Jun 1997	CMG	–	–	–	0
	THIO	*Bacillus* sp.	–	–	0
	TSBSC	*Vibrio alginolyticus*	–	–	0
Jul 1997	CMG	–	*Clostridium* sp.	–	–
	THIO	*Bacillus* sp. *Vibrio alginolyticus*	*Bacillus* sp. *Vibrio alginolyticus*	*Bacillus* sp.	–
	TSBSC	*Bacillus* sp. *Vibrio alginolyticus*	*Bacillus* sp. *Vibrio alginolyticus*	–	*Vibrio alginolyticus*
Nov 1997	CMG	*Bacillus* sp.	–	–	–
	THIO	*Clostridium* sp.	*Bacillus* sp.	–	–
	TSBSC	–	*Vibrio alginolyticus*	–	*Vibrio alginolyticus*
Archaeopneustes hystrix (n = 2 ind.)					
Jul 1998	CMG	–	0	0	*Vibrio alginolyticus*
	THIO	*Pantoea*[a] *agglomeran*	0	0	–
	TSBSC	*Vibrio alginolyticus* *Comamonas*[b] *acidovorans*	0	0	*Vibrio alginolyticus*

[a]Formerly *Enterobacter*; [b]formerly *Pseudomonas*

V. damsela have both been isolated from the Florida near-shore waters and each species represents approximately 8% of the total bacterial isolates in the tropical west Atlantic Ocean (Bauer & Agerter 1995). *V. damsela* and *V. alginolyticus* have similar temperature and NaCl requirements (Holt et al. 1994). Although *V. damsela* was isolated from diseased *Paleopneustes cristatus* (Table 1), it could not be recovered from internal organs, so no deleterious effects of this bacterium can be inferred.

The process of collecting urchins with a submersible scoop often caused localized mechanical damage to the epidermis. This damage appears to have been a key factor in the observed infections, as lesions were often centered at damage sites. Maes & Jangoux (1984, 1985) documented a bacterial infection ('bald-sea-urchin disease') that caused lesions in the shallow Mediterranean echinoid *Paracentrotus lividus*. In this species, lesions could be experimentally induced by removing a few spines, scarring the epidermis slightly, then painting the damaged region with necrotic tissues from the lesions of a diseased urchin (Maes & Jangoux 1984). Bald-sea-urchin disease was not fatal unless enough of the skeleton was destroyed by lesions to permit entry of bacteria into the coelomic cavity. A similar phenomenon was observed by Bang & Lemma (1962), who found that starfish collected by dredging (and experiencing epidermal damage) were highly susceptible to bacterial disease. Gaffkemia disease of commercially exploited lobsters is also exacerbated by breaks in the integument (Steward et al. 1969).

Although the use of near-shore seawater in the recirculating experimental tanks at Harbor Branch Oceanographic Institution proved to be lethal for 2 species of spatangoid bathyal echinoids, several other deep-sea species maintained in the same tanks over the same period of time were not affected by the pathogen. These included *Stylocidaris lineata*, *Araeosoma fenestratum*, *Calocidaris micans* and *Aspidodiadema jacobyi*. The reasons for this differential susceptibility are unknown. In healthy sea urchins, the coelomic fluid is sterile (Bang & Lemma 1962, Wardlaw & Unkles 1978, Kaneshiro & Karp 1980), with sterility apparently maintained both by bactericidal properties of the coelomic fluid (Wardlaw & Unkles 1978, Turton & Wardlaw 1987) and by coelomocytes that phagocytize foreign organisms and particles (Smith 1981, Bang 1982, Dybas & Fankboner 1986). Perhaps the coelomocytes of the relatively less vulnerable regular sea urchins are either more numerous or more effective than those of the irregular urchins that were highly susceptible to the disease. An alternative hypothesis is that the larger, more fragile irregular urchins were more susceptible to damage during collection than were the regular urchins. These alternatives can be distinguished in future work by injecting pathogens into many deep-sea species.

Evidence currently at hand suggests that a main line of defense against vibriosis lies within the epidermal tissues. Bryan et al. (1994) screened body-wall extracts of 22 species of echinoderms against 19 different bacteria and fungi to assess possible antimicrobial activity. Not all extracts were effective against all bacteria, but antimicrobial activity was found in the epidermal extracts of many species. In bald-sea-urchin disease (Maes & Jangoux 1984, 1985) bacterial lesions eventually penetrate the skeleton, resulting in an infection that cannot be successfully fought by coelomocytes or bactericidal compounds (Maes & Jangoux 1984). Our experimental evidence likewise indicates that vibriosis is fatal when it involves massive coelomic infection.

The deaths of the bathyal urchins we studied were presumably caused by unknown and irreversible biochemical changes or by tissue lysis within the affected sea urchins. Additional research is needed at the cell and tissue levels to determine the nature of the process that causes lesions and mortality. This will be important information in preventing bacterial infections in future laboratory experiments with deep-sea animals.

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