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Variation in Abundance of Subcuticular Bacteria in Florida Echinoderms

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Abstract

Subcuticular bacteria have been reported for echinoderms for a number of taxa from different parts of the world. Questions remain about the variation in their occurrence. Variation in the abundance of subcuticular bacteria (SCB) was examined in five species of Florida echinoderms from different locations and times. Direct counting of symbionts from hosts fixed immediately after field collection indicates much variation within a population sampled at a given place and time and among populations over both space and time. The highest mean SCB cell numbers were found in the ophiuroid *Ophiophragmus filograneus* (2.21×10^9 cells g^{-1} ash free dry weight) and lowest in the echinoid *Lytechinus variegatus* (3.67×10^7 cells g^{-1} ash free dry weight). This sample of *L. variegatus* exhibited the highest coefficient of variation (128.3%) These observations support the conclusion that SCB are widely distributed among echinoderms, but that considerable variation in abundance occurs. This variation indicates that sampling hosts over time and space is necessary to establish the amount of variation and for interpretation of infection, maintenance, and role of the bacteria.

Keywords: Symbiosis, cuticle, asteroidea, echinoidea, ophiuroidea

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1. Introduction

Subcuticular bacteria (SCB) are endosymbiotic, gram-negative bacteria found within the cuticle or between the overlying surface coats and the epidermal epithelium of echinoderms. They were overlooked until Holland and Nealson (1978) described them in 4 of the 5 extant classes (crinoids, asteroids, echinoids, and ophiuroids) of echinoderms. Soon thereafter, SCB were found in holothuroids (Féral, 1980). Since their initial discovery, SCB have been described morphologically (Féral, 1980; McKenzie and Kelly, 1994; Kelly et al., 1995; Kelly and McKenzie, 1995) and characterized physiologically (Lesser and Blakemore, 1990; Lesser and Walker, 1992), and their distribution among echinoderm taxa has been examined from several geographically distant regions (McKenzie and Kelly, 1994; Kelly et al., 1995; Kelly and McKenzie, 1995). Phylogenetic analysis of 16S ribosomal RNA suggests that SCB belong in the α subdivision of the purple non-sulfur bacteria (Cl. Proteobacteria) (Burnett and McKenzie, 1997), a subdivision that includes symbionts such as rhizobacteria, rickettsias, and important plant pathogens, the agrobacteria.

Over 60% of temperate echinoderm species may possess SCB (McKenzie et al., 1998). No correlation has been made between the presence of SCB and the ecology of their hosts. SCB are found in echinoderms from both shallow (Kelly et al., 1994) and deep water habitats (Roberts et al., 1991), in species with varying trophic strategies, and in individuals of various sizes and developmental states (Cameron and Holland, 1983; Walker and Lesser, 1989; Bosch, 1992; Cerra et al., 1997).

It is not unusual to find abundant SCB ($>10^9$ cells g^{-1} ash-free dry weight) in echinoderms (Kelly and McKenzie, 1995). Such bacterial counts per unit dry weight are comparable to that of vestimentiferan trophosomes (Cavanaugh et al., 1981), and therefore considered high enough to provide significant contributions to their hosts (Kelly and McKenzie, 1995). While there is no existing evidence that SCB contribute to host energetics, McKenzie and Kelly (1994) estimated that SCB could comprise up to 1% of the ash-free dry weight biomass of ophiuroid arms. Given these densities, SCB can represent a significant component of the epithelium. If SCB contribute in any way to host energetics, then their abundance suggests it may be substantial.

SCB numbers may vary between species by over an order of magnitude and intraspecific variation has been described as "considerable" (McKenzie and Kelly, 1994; Kelly et al., 1995). McKenzie et al. (2000) found that SCB counts fluctuated over the course of a year in the ophiuroids *Ophiothrix fragilis* and *Amphiura chiajei* but observed no seasonal trends in variation. Further understanding the variability that exists in SCB densities in various echinoderms over time and space will assist in ascertaining whether a particular host taxon truly possesses symbionts or not. Information about the

variation in occurrence and abundance of SCB is necessary for interpretation of infection, maintenance, and role of the bacteria. This study evaluates the variation in SCB densities in echinoderms from the coasts of Florida.

2. Methods

Collections

Five species of echinoderms were collected near shore or offshore during R/V *Bellows* cruises using SCUBA. *Mellita tenuis* Clark 1940 (Echinoidea: Clypeasterida) was collected from Egmont Key (Egmont= 27 35.0 N, 82 46.5 W) in September 1997 and February 1998. *Lytechinus variegatus* Lamarck 1816 (Echinoidea: Temnopleurida) was collected from Venice Beach (Venice= 27 06.0 N, 82 27.5 W) in October and December 1997, and from Station 5 in the Gulf of Mexico (Gulf5= 27 34.3 N, 82 49.3 W) in February 1998. *Arbacia punctulata* Lamarck 1816 (Echinoidea: Arbaciida) was collected from Venice Beach in October 1997 and from Station 2 (Gulf2= 28 07.2 N, 82 59.0 W) in February 1998. *Luidia clathrata* Say 1825 (Asteroidea: Paxillosida) was collected in Tampa Bay from the Courtney Campbell Causeway (Old Tampa= 27 58.0 N, 82 37.5 W) in September 1997 and January 1998 from Apollo Beach (Apollo= 27 46.5 N, 82 27.0 W) in November 1997 and from Station 5 in February 1998. *Ophiophragmus filograneus* Lyman 1875 (Ophiuroidea: Ophiurida) was collected from the Indian River (Indian= 28 24.0 N, 80 44.4 W) and Banana River (Banana= 28 12.6 N, 80 37.5 W) lagoons near Cape Canaveral and from Lower Tampa Bay at the Howard Frankland Bridge (Tampa= 27 53.5 N, 82 38.0 W) in September 1997.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to confirm the presence of SCB in all species studied except *Ophiophragmus filograneus*. Most samples were fixed immediately after collection. Samples for TEM were fixed following the procedure of Kelly and McKenzie (1995) using a 3:1 ratio of glutaraldehyde (4% in 0.1 M cacodylate buffer, pH 7.6) and osmium tetroxide (1% in filtered seawater). Calcareous tissues were decalcified in several changes of saturated EDTA. Samples were dehydrated in an ethanol series followed by transition to 100% acetone. Specimens were then infiltrated and embedded in low-viscosity epoxy resin (Spurr, 1969). Ultrathin (gold and silver) sections were obtained by sectioning on a Sorvall (PorterBlum) MTII ultramicrotome with glass knives. Sections were stained with ethanoic uranyl acetate and lead citrate (Reynolds, 1963) and observed using a Hitachi H500 TEM at 70kV. At least three samples per species were observed.

Quantification of SCB

SCB were counted using a technique adapted from Hobbie et al. (1977), Kelly and McKenzie (1992), and Largo et al. (1997). Specimens were fixed in 4% formalin in sterile, filtered artificial seawater. For echinoids, a portion of an ambulacrum and the adjacent peristomial membrane was dissected, taking care not to rupture the gut. Aboral segments of asteroid body wall and whole ophuiroid arms were used. Dissections were blotted dry, weighed, and repeatedly rinsed in filtered (>0.2 μm), autoclaved artificial seawater (FAASW). Tissue was homogenized (10% w/v) by grinding with a mortar and pestle in FAASW. Homogenates were diluted to optimal cell concentrations according to Largo et al. (1997); this dilution was typically 1:1000 (final concentration). Aliquots (10 ml) were stained with an equal volume of 0.01% acridine orange and filtered through hydrophilic, >0.2-μm polycarbonate (Nuclepore™) membrane filters stained with Sudan Black B in 50% ethanol and stored at 4°C. Filters were mounted on slides and examined at 600X on a Nikon Diaphot inverted-objective microscope equipped with a 100-W mercury lamp and a 430-nm filter cube.

Negative controls (blanks without tissue homogenates) were routinely examined for the presence of contaminants. The mean number of bacteria from blank preparations was used to correct each of the samples analyzed. Positive controls were prepared using cultures of *Escherichia coli* diluted and stained as above to establish the appearance of acridine orange-stained bacteria similar to SCB. SCB were identified by their morphology and tendency to fluoresce bright yellowish-green.

SCB counts were expressed as cell numbers per ash-free dry weight (AFDW) to allow general comparisons between taxa of varying skeletal calcite contents. Tissues were dried in a vacuum chamber over sulfuric acid, pulverized with a mortar and pestle, and ashed in a muffle furnace for 4 h at 500°C (Paine, 1971). Ash weight was subtracted from dry weight to calculate mean AFDW for 10 individuals.

In most instances, 10 individuals were sampled from each population. Bacteria within a 2.94 μm^2 area field of view were counted. For each individual, 20 haphazardly chosen fields of view were counted, averaged, and corrected for dilution to determine mean number of cells per gram AFDW.

Statistics

Mean SCB loads with standard errors (SE) were calculated and coefficients of variation were used to avoid assumptions about variances that merely reflect the magnitude of their means. All other statistical analyses were performed using SigmaStat for Windows© Version 2.0 (Jandel Corporation, 1995).

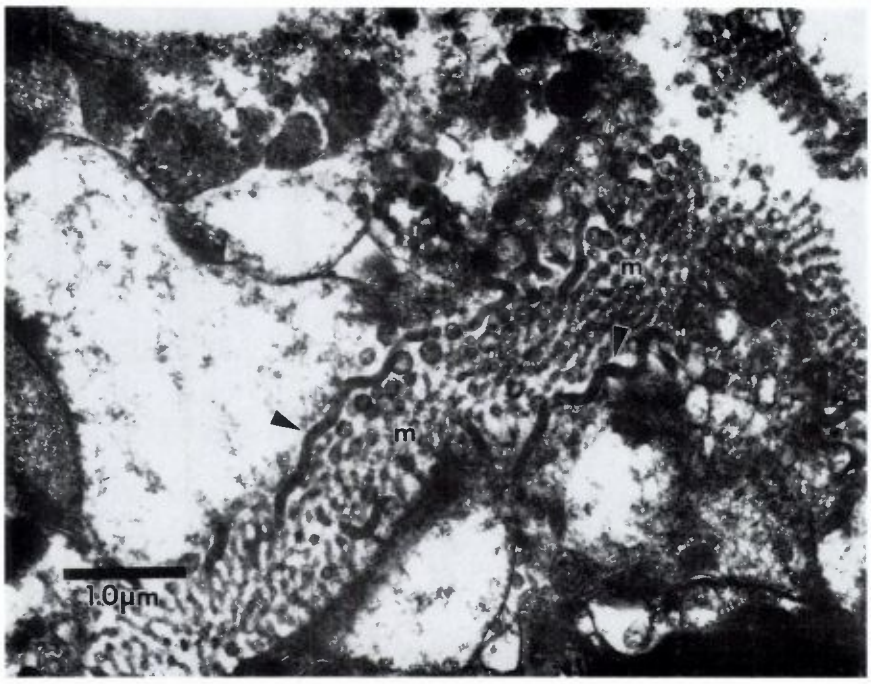

Figure 1. *Mellita tenuis*. Oblique section through cuticle showing tightly-kinked spiral T2 SCB (arrows) among host microvilli (m).

Normality was tested using Kolmogorov-Smirnov test. Differences in means were tested using parametric one-way and, where appropriate, multiway ANOVAs. Multiple comparisons between means were tested using the Student-Neuman-Keuls post hoc test (α=0.05). A Kruskal-Wallis One Way ANOVA with Dunn's post hoc test was used for testing differences in mean SCB counts among *Lytechinus variegatus* due to non-normality. The Mann-Whitney Rank Sum test was used to test for differences in means of *Arbacia punctulata*, due to heterogeneous variances.

3. Results

Using TEM, SCB were found to be present in the echinoids *Mellita tenuis* (Fig. 1) and *Arbacia punctulata* (Fig. 2), but not in the sampled tissues of *Lytechinus variegatus*. SCB were also observed in the asteroid *Luidia clathrata* (Figs. 3 and 4).

A summary of mean SCB densities (as number g^{-1} AFDW) for all taxa is given

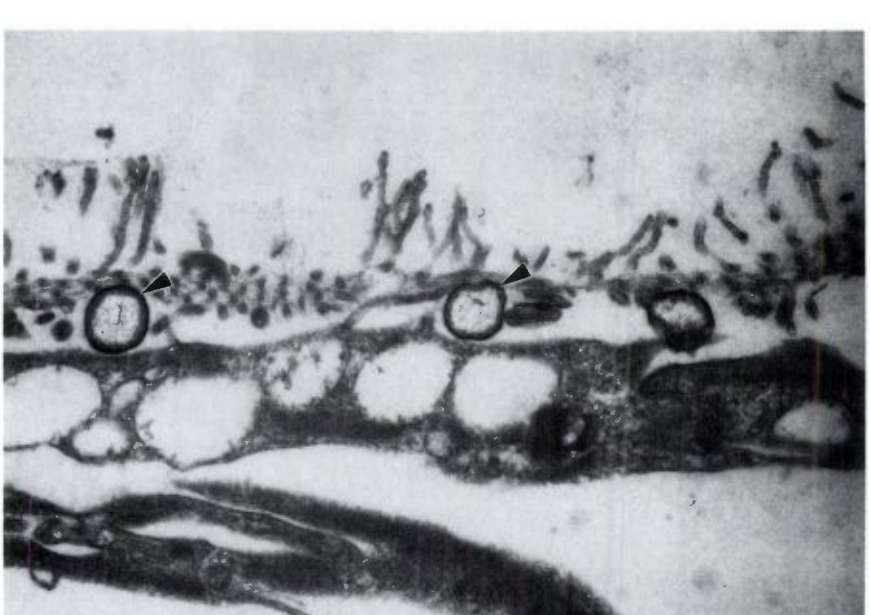

Figure 2. *Arbacia punctulata*. Cross-section through SCB (arrows) with sparse internal structure. The fibrous inner cuticle remains intact, revealing symbionts.

in Fig. 5. Of the 4 species examined over time, 2 had significantly lower mean densities of SCB in January and February than those sampled during September and October. Mean counts were highest for the ophiuroid *Ophiophragmus filograneus* from the Indian River Lagoon site (2.21×10^9 cells g^{-1} AFDW), and lowest for the regular echinoid *Lytechinus variegatus* from Station 5, with densities 2 orders of magnitude lower (3.67×10^7 cells g^{-1} AFDW) than the ophiuroid. Grouping samples by species, the mean SCB load of the *Ophiophragmus filograneus* was 1.79×10^9 g^{-1} AFDW. *Mellita tenuis* (7.93×10^8) had the second highest densities followed by *Luidia clathrata* (6.40×10^8), *Arbacia punctulata* (2.57×10^8), and *Lytechinus variegatus* (1.45×10^8), respectively.

Significant spatial variation was detected in mean SCB numbers in the ophiuroid *Ophiophragmus filograneus* among 2 of the 3 populations sampled in September 1997 (Table 1). Of these, the Banana River lagoon sample had significantly lower mean SCB densities than did those from the Indian River lagoon (ANOVA; $p<0.05$). There was no difference between either sample from

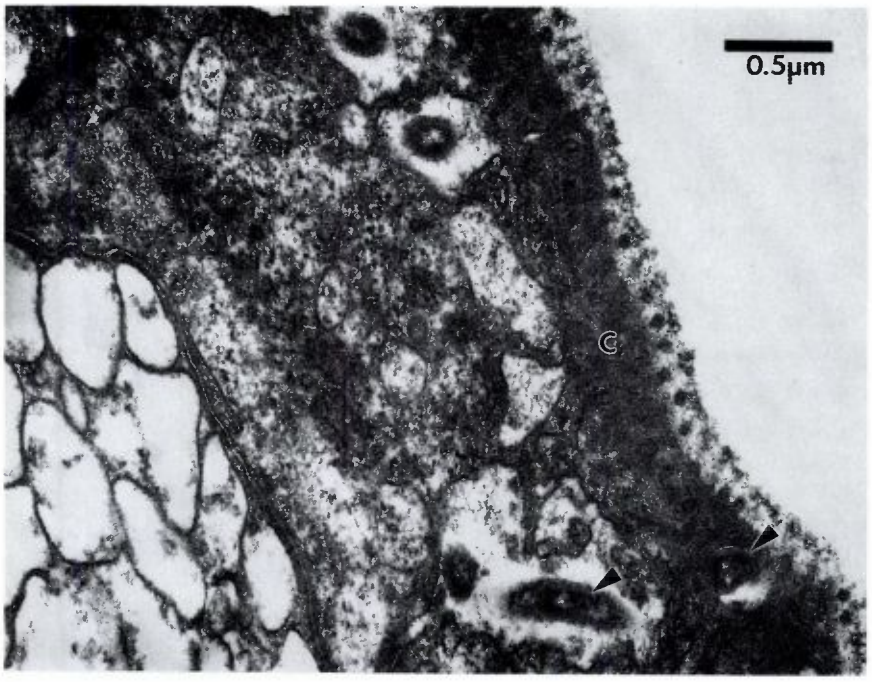

Figure 3. *Luidia clathrata*. Curved T2 rods (arrows) located within the subcuticular space and embedded within the cuticle (c). Specimen fixed after three days in aquarium.

the east coast and that from Lower Tampa Bay. Variation in SCB densities of individuals ranged over an order of magnitude from 2.80×10^8 to 2.78×10^9 cells g^{-1} AFDW. The greatest abundance was in an individual from the Lower Tampa Bay site.

The SCB abundance among *Luidia clathrata* (n=10 for all samples) was lower than that of the ophiuroid, ranging from $1.92 \times 10^8 - 1.77 \times 10^9$. Significant variation was not evident among this species sampled two months apart (Table 2) as no difference was detected between samples taken from Courtney Campbell Causeway in September and Apollo Beach in November (ANOVA; $p=0.22$). Similarly, the SCB densities were not significantly different (ANOVA; $p=0.72$) between the Courtney Campbell Causeway and Station 5 populations sampled in January and February, respectively.

Samples of *Luidia clathrata* collected in January–February had both lower mean SCB counts and coefficients of variation than those taken in September and November (Table 2). While no significant differences were detected between sites, mean SCB counts of the September and November samples

Figure 4. *Luidia clathrata*. High magnification view of SCB (arrows) embedded within the cuticle (c) showing double membranes.

differed significantly (Multiway ANOVA; $p<0.05$) from the January–February samples among the 4 collections examined. The Student-Neuman-Keuls multiple comparison test showed a difference ($p<0.05$) between September and January samples from Courtney Campbell Causeway and differences ($p<0.05$) between Apollo Beach and the January–February samples. The mean observed SCB load (n=10) of *L. clathrata* from the Courtney Campbell Causeway decreased by 52% from 7.87×10^8 to 3.76×10^8 between September 1997 and January 1998. Coefficients of variation were greater among the January–February samples from both Station 5 (90.9%) and the Courtney Campbell Causeway (80.0%) than September and November samples.

Among *Lytechinus variagatus*, low sample sizes (n=5) for the October and December samples from Venice Beach resulted in large variances. February's sample (n=10) exhibited the greatest coefficient of variation (128.3%).

Observed SCB densities from *Arbacia punctulata* from Station 2 in February, 1998 were 83% lower than Venice Beach SCB densities October 1997, a highly significant difference (Mann-Whitney Rank Sum Test; $p<0.001$). Abundance of

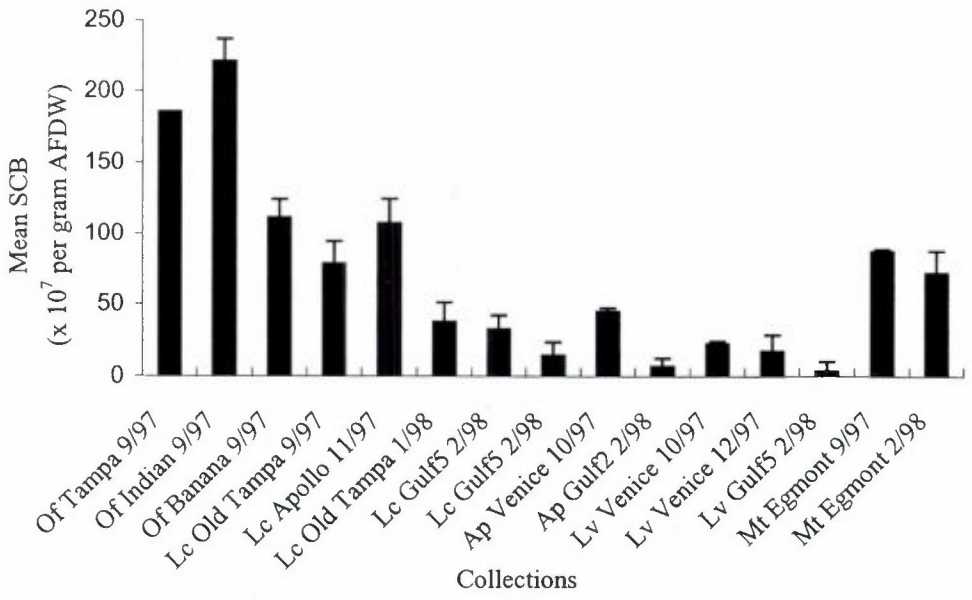

Figure 5. Mean SCB counts of all populations sampled. Error bar = standard error. MT = *Mellita tenuis*; LV = *Lytechinus variagatus*; AP = *Arbacia punctulata*; LC = *Luidia clathrata*; OF = *Ophiophragmus filograneus*.

SCB in the February sample was low relative to all other samples, while the coefficient of variation was among the highest (78.7%). SCB were undetectable in two of ten *L. variagatus* and one of ten *Arbacia punctulata* from this month. SCB were never detected in specimens from Station 5 and Station 2 from the Gulf of Mexico. The sand dollar *Mellita tenuis* showed no difference in SCB load between September (n=10) and February (n=10) from Egmont Key (see Fig. 5).

4. Discussion

Incidence and density of SCB

SCB have been discovered in echinoderms from a wide variety of geographic locations: Florida, USA (this study), northeastern Pacific and Atlantic (McKenzie and Kelly, 1994; Kelly and McKenzie, 1995), northwestern Atlantic (Walker and Lesser, 1989), Australia (McKenzie, 1992) and the southwestern Pacific (Kelly et al., 1995). This suggests that SCB are globally distributed symbionts of echinoderms. However, failure to find SCB in a given taxon is not

Table 1. Spatial variation of SCB densities (as cells g^{-1} AFDW) among *Ophiophragmus filograneus* from three sites in September, 1997.

Site	Tampa	Indian	Banana
Mean ($\times 10^8$)	20.5	22.1	11.1
Sample Size	8	7	8
Range ($\times 10^8$)	14.1	8.6	13.3
Standard error ($\times 10^8$)	1.6	1.3	1.6
Coefficient of variation (%)	24.7	16.0	41.6

Table 2. Spatial and temporal variation in SCB densities (as cells $^{-1}$ AFDW) among *Luidia clathrata* over a six month sampling period.

Site	Old Tampa	Apollo	Old Tampa	Gulf5
Month, year	Sept. 1997	Nov. 1997	Jan. 1998	Feb. 1998
Mean ($\times 10^8$)	7.9	10.7	3.8	3.3
Sample size	10	10	10	10
Range ($\times 10^8$)	17.6	15.0	10.0	10.0
Standard error ($\times 10^8$)	1.7	1.4	0.9	0.9
Coefficient of variation (%)	71.2	40.8	80.0	90.9

definitive evidence of their absence, as individuals may lack them in the tissues sampled. Reasons for the apparent absence of SCB may include, but are not limited to: (1) discontinuous distribution of symbionts over the body surface and (2) variability of symbiont numbers determined by both the time and the location of sampling.

The SCB counts in this study were similar to those of Kelly et al. (1995) and McKenzie et al. (2000), in which the highest densities were on the order of 10^8 to 10^9 cells g^{-1} AFDW. The generally lower counts found in this study may have resulted from repeated washing with filtered artificial seawater during preparation. The goal was to remove contaminant surface bacteria should they be present, although this is never completely reliable (Stephens, 1988). Alternatively, lower densities may truly be present in the species examined in this study. Bacterial densities were higher in the ophiuroid studied than in the echinoids and asteroid, but comparing densities between species may not be valid because of differences in epithelial architecture and composition of sampled tissue and taxon. Similarly, Kelly et al. (1995) found 4.96×10^9 g^{-1}

AFDW in the ophiuroid *Amphipholis squamata*, but slightly fewer SCB per unit mass in the echinoid *Pseudechinus* and fewer still in the asteroid *Asterodon miliaris*.

Variation in SCB counts

SCB loads may be highly variable within populations, although much of this could be attributed to sampling artefact. This is evident by the large standard errors associated with samples. Data from 4 species sampled at different times and sites were largely inconclusive as a result of this variation. 2 of the 4 species examined showed a decrease in SCB numbers during January–February but cannot be attributed solely to season. Spatial variation in SCB in *O. filograneus* and temporal variation in SCB in *L. clathrata* were relatively great.

As a statistic, the coefficient of variation is useful in comparing relative variation between populations, regardless of the magnitude of their means. Coefficients of variation are not constant over time either within or between taxa, so that by using these methods variations in mean SCB densities in given taxa remain largely unpredictable. Calculations based on homogenate counts of Kelly et al. (1995) show coefficients of variation ranging from 87% (*Ophiocoma bollonsi*) to as low as 17% (*Amphipholis squamata*). The highest coefficient of variation in the present study was even larger, found in *Lytechinus variegatus* from Station 5 in February (128%). The lowest CV was 16% in *Ophiophragmus filograneus* from the Indian River lagoon. Conversely, the coefficient of variation of *Leptosynapta bergensis* (Kelly and McKenzie, 1995), again derived from homogenate counts, was 75%. This species' mean SCB load was reported as the lowest of the study and had the highest coefficient of variation of the 4 species examined. It is difficult to interpret the causes of such variation within populations, but clearly significant intraspecific variation is possible. This alone suggests that substantial effort must be taken to establish that a single population of a particular taxon does not possess SCB.

The relationship between SCB and their hosts is dynamic in that densities of SCB within their hosts may differ spatially and/or temporally. Two populations of the ophiuroid *O. filograneus* in adjacent estuaries differed in the density of SCB although neither differed significantly from the population on the west coast collected at the same time. It is possible that a normal distribution would have resulted if more sites were sampled at one time. SCB counts of echinoderms sampled in January–February were lower in two of the four species examined over time, suggesting that the potential for significant variation over time exists. Using direct counting, Fong and Mann (1980) found seasonal variation in the numbers of nitrogen-fixing bacteria in the gut of

Strongylocentrotus droebachiensis. Bacteria decreased in number between November and April. These workers documented an inverse relationship between bacterial abundance and the nitrogen content of the host's food. However, in annual monitoring of SCB densities, McKenzie et al. (2000) found no such seasonality in the ophiuroids *Ophiothrix fragilis* and *Amphiura chiajei* although SCB loadings fluctuated cyclically.

Potential sources of variation

Newton and McKenzie (1995) found lowered symbiont numbers after hydrocarbon insult. The same group (Kelly and McKenzie, 1992; Newton and McKenzie, 1998) observed decreased numbers of bacteria among individuals kept in static seawater. Newton and McKenzie (1998) considered these animals stressed, having found significantly lower SCB densities compared to individuals from through-flow systems over all durations sampled. If SCB decline in abundance in aquaria-kept individuals, it raises intriguing questions as to the mechanism for SCB loss. It is possible that SCB become moribund and disintegrate or are phagocytosed by the host under these conditions. Further testing of this hypothesis is required.

Although this study suggests that SCB abundance may vary within taxa sampled at different times and sites, it is possible that the methods used are unsuited to accurately assess SCB numbers. It was not uncommon, for example, to find a mixture of both green-fluorescing and red-fluorescing cells on the filters, particularly in the January–February samples. Acridine orange staining may appear yellow-green in the presence of DNA and red-orange in the presence of RNA (Hobbie et al., 1977). In a study of acridine orange as a nucleic acid-binding fluorochrome, this shift was detected as bacterial division rates changed from log phase growth, which fluoresced red, to steady states, which fluoresced green (Back and Kroll, 1991). For consistency with earlier studies, only yellowish-green fluorescing cells were counted in the present study. Thus, it is possible that DNA/RNA ratios in SCB may change, resulting in the appearance of varying abundance. It is clearly important to use caution when interpreting data from fluorescence microscopy observations alone. Additional quantitative measures such as the lipopolysaccharide assay, electron microscopy image analysis techniques, or molecular probes would be of assistance in more precisely determining SCB densities. To ensure a given species lacks SCB, it is desirable to examine that species from more than one location and at various times. Direct counting may be used for this purpose, but may be inadequate to accurately determine abundance over time and space. Improved methods to quantify SCB may show the overall incidence of SCB among echinoderms to be higher than is currently represented.

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