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Abstract

Although relationships between marine invertebrates and epibiotic fungi, bacteria, microalgae, and protozoa have been documented, little literature exists describing the relationship between deep-sea nematodes and protozoan epibionts. This study utilized samples from sediment cores collected as part of a CO_2 sequestration experiment. The incidence of epibiont-containing nematodes was examined in these deep-sea samples and determined to be independent of both CO_2 treatment and vertical distribution. Epibiont-containing nematodes were categorized into two groups – desmoscolids and non-desmoscolids; the desmoscolids accounted for the majority of the epibiont-containing nematodes. Epibiont-containing nematodes in the non-desmoscolid group had more epibionts per nematode than the epibiont-containing desmoscolids. However, epibiont-containing desmoscolids contained more epibionts per unit of length. The location of protozoan epibionts throughout the length of the epibiont-containing nematodes was also examined. Epibionts on desmoscolids were found to be disproportionately more abundant on the posterior half of the nematode. Epibionts on non-desmoscolids were more evenly distributed as they did not exhibit a preference for one region of the nematode over another. From the observations, it was hypothesized that the protozoan epibionts selectively favor the epibiont-containing nematodes due to their cuticle ornamentation.

Introduction

The term "epibiosis" has been defined as a "non-symbiotic, facultative association between epibionts and basibionts" (Wahl, 1989). Specifically, the epibiont is the living organism found attached to the surface of the host, known as the basibiont (Wahl, 1989). In aquatic environments, the presence of epibionts on marine invertebrates has been well documented and identified to exist as a variety of organisms including fungi, prokaryotes, microalgae, and protozoans (Carman and Dobbs, 1997). While these epibiotic associations have been widely recognized, the ecological significance of such relationships

remains relatively unknown. The presence of suctorian (Protozoa, Ciliophora) epibionts on marine crustaceans has been well documented and reviewed (Fernandez-Leborans, 2000). Puckett and Carman (2002) demonstrated that protozoan epibionts on harpacticoid copepods influenced host susceptibility to environmental contaminates. While reports of protozoan epibionts on nematodes have been largely anecdotal and primarily in the taxonomic literature (see Fisher 2003 as an exception), symbiotic relationships between prokaryotic organisms and specific families of nematodes especially that of Desmodoridae have been documented (Schiemer et al. 1990). We are unaware of any previous reports of protozoan epibionts on deep-sea nematodes.

The phylum Nematoda is composed of a diverse range of animals described as round worms. They are characterized by their bilateral symmetry, cuticle, and psuedocoel. They are widely distributed and can be found in nearly every habitat. Nematodes are the most abundant animals in the world believing to constitute four out of every five multicellular animals on earth. Taxonomically, the phylum consists of 5 classes, 9 subclasses, 23 superorders, 39 orders, 52 suborders, 89 superfamilies and 241 families. While many of these nematodes are parasitic, those in this study are free-living species (Hodda, 2007).

Deep-sea nematodes are important organisms to study as they constitute the most abundant and diverse of the deep-sea metazoans (Thiel, 1979) and are typically the most abundant taxon in the meiobenthic community. Benthic fauna can be subdivided into three categories: macrobenthos, meiobenthos, and microbenthos (Giere, 2009 and Mare, 1942). Macrobenthos refer to the animals that are retained on a 1-mm sieve (Mare, 1942). Microbenthos defines the smallest of the fauna and includes organisms such as protozoans, diatoms, and bacteria. Finally, meiobenthos pass through a 1-mm sieve and are retained on a 32-µm sieve; this group includes organisms such as copepods, polychaetes, formanifera, and nematodes (Giere, 2009).

This study was conducted as part of an experiment designed to examine potential environmental impacts of deep-sea CO_2 sequestration (Carman et al., 2004 and Fleeger et al., 2006). The purpose of this paper is to investigate the prevalence of protozoan epibionts in nematode samples collected from the deep sea. Individual epibiont-containing nematodes were analyzed to determine the distribution of epibiont location along the length of the nematode. Differences in epibiont colonization of desmoscolid and non-desmoscolid nematodes were also investigated. Desmoscolids are nematodes belonging to a family that has distinctive cuticular armor (Vanaverbeke, 2004) characterized by thick, transverse rings along the length of the body (Shirayama, 1992). Further, desmoscloids are most prevalent in the upper 1-2 cm of deep-sea sediments and typically have a much smaller body length/diameter ratio (and thus are short and corpulent) compared to nematodes in other families (Soetaret et al., 2002).

Materials and Methods

Samples used in this study were collected from a site off the coast of Monterey, CA, USA in Monterey Canyon (36°41.9'N 123°0.1'W) at a depth of 3100 m. The ROV *Tiburon* was operated from the R/V *Western Flyer* and used for all sampling and experimental manipulations. In December of 2004, roughly 20 L of liquid CO_2 was pumped into fifteen 48-cm-diameter PVC pipes embedded in the seabed and extending approximately 15 cm above the seabed. These "corrals" were injected with liquid CO_2 that slowly dissolved into the surrounding seawater. This resulted in a dense dissolution cloud enriched in CO_2 with a low pH that over time diffused from the corrals and permeated the surrounding sediment. Upon return 35 days later, no CO_2 was seen (the refractive index of CO_2 is different than that of water making its presence visible) in the corrals or on the seabed bordering the corrals. This CO2 release experiment corresponds to CO_2-4 described in detail by Barry et al. (2005).

We collected five 7-cm inner diameter cores from a site ~2 m away from a corral (treatment) and five cores from a site ~ 40m away from the nearest corral (control); also a core were taken from within two of the corrals. Immediately upon retrieval, cores were transferred to a controlled temperature room onboard ship and held at 4°C. Overlying water was aspirated from all cores and concentrated on a 32-µm sieve. A precision extruder was used to vertically section sediment cores into 0-5 mm, 5-10 mm, 10-20 mm, and 20-30 mm layers. Samples were fixed with 4% formaldehyde solution composed of artificial seawater at 35 psu and buffered to neutrality with borax. In the laboratory, samples were stained with Rose Bengal and extracted with LUDOX before being rinsed through a 32-µm sieve for the purposes of sorting. Nematodes were sorted and counted from both the supernatant and the pellet fractions of the sample using a stereo-dissection microscope.

Nematodes were placed on microscope slides containing two 15-mm-diameter etched wells. Fifty nematodes were added to each well in a drop of glycerin. Cover slips (25 x 25 mm) were placed on each well and then sealed with clear fingernail polish. An Olympus BX50 microscope was used to examine a maximum of 250 nematodes per sample. If a nematode was seen to have an epibiont on it, a picture was taken using a SPOT RT (model 7.2 Color Mosaic) camera using SPOT RT Software, v. 3.5 at a magnification varying from 5-100 x. After viewing five wells, if less than 250 nematodes were tallied, the sixth well was viewed to account for the remaining nematodes. Nematodes were not identified to species, but were classified as desmoscolids or non-desmoscolids.

IPLab, v. 3.6 software was employed to measure the nematode length and epibiont distance away from the head of each nematode. A hand-operated mouse was used to measure the length (excluding filiform tails), and the distance of epibiont attachment from the head of the nematode. Through software programming functions, these values were quantified after calibration to a known length and transferred into a spreadsheet.

The location of each epibiont was determined in relation to the length of nematode. Epibionts were classified as being located in the head region if they were on the anterior half of the nematode, and as being in the tail region if they were on the posterior half of the nematode.

A Two Way Analysis of Variation (ANOVA) was conducted to investigate the effects of depth, treatment, and their interaction on epibiont incidence. Two Way Repeated Measures ANOVA (One Factor Repetition) was used to investigate the relationship between nematode type (desmoscolids and non-desmoscolids) and the location of epibionts on each.

Results

Protozoan epibiont frequency of occurrence was evaluated in 21 deep-sea nematode core samples that were further divided into two depths (0-5 mm and 5-10 mm). The average nematode abundance per sample was 1831 ± 1053 (mean ± SD) nematodes. In the 0-5 mm depth samples an average of 742 ± 565 nematodes were found, and 1088 ± 648 nematodes were found in the 5-10 mm depth samples. Out of the total 10,184 nematodes examined in the 0-5 mm and 5-10 mm depths of the 21 samples, 100 epibiont-containing nematodes were found.

Two-way ANOVA revealed that neither depth ($p = 0.897$) nor treatment ($p = 0.372$) influenced the incidence of epibiont-containing nematodes. The interaction term was also insignificant ($p = 0.223$). Thus, neither depth, treatment, nor the interaction between the two factors significantly influenced the incidence of nematode-containing epibionts in deep-sea samples.

In the samples examined, a total of 100 epibiont-containing nematodes were found containing 269 epibionts. The average number of epibionts per epibiont-containing nematode was 2.69 ± 1.66 epibionts. Of the 100 total epibiont-containing nematodes found, 38 were found in the 0-5 mm depth, and the

remaining 62 nematodes were located in the 5-10 mm depth (Table 1). The epibiont-containing nematodes were divided into two groups: desmoscolids and non-desmoscolids. A total of 62 desmoscolids were found containing 128 epibionts, and 38 non-desmoscolids were found containing 141 epibionts. The average number of epibionts per epibiont-containing nematode was 2.06 ± 1.11 for desmoscolids and 4.41 ± 2.20 for non-desmoscolids. As desmoscolids are known to be much shorter than the average nematode, the total number of epibionts per epibiont-containing desmoscolid was expected to be lower than that of the non-desmoscolids. However, epibiont-containing desmoscolids contained more epibionts per unit of length averaging one epibiont per 191.42 ± 130.75 µm. Non-desmoscolids averaged only one epibiont per 587.01 ± 543.7 µm.

Table 1

Distribution of the epibiont-containing nematodes (n=100) organized by nematode type (desmoscolid and non-desmoscolid) and depth (0-5 mm and 5-10).

Depth	Desmoscolids	Non-desmoscolids	Total
0-5 mm	23	15	38
5-10 mm	39	23	62
Total	62	38	100

A Two Way Repeated Measures ANOVA, which failed both the normality and equal variance tests, revealed a significant difference between epibionts located in the head region and epibionts found in the tail region ($p = 0.030$). The epibionts favored the posterior end of the nematode. Also, the type of nematode (desmoscolids v. non-desmoscolid) was found to influence epibiont location ($p = 0.004$). The interaction between these two factors was also significant ($p = 0.001$). Interaction effects were further examined using Tukey's test. The location of the epibionts on desmoscolids was significant ($p = < 0.001$),

while the location of epibionts on non-desmoscolids was not significant ($p = 0.495$). Thus, when considering the two nematode types collectively (desmoscolids + non-desmoscolids), epibionts occurred disproportionately in the tail region; epibionts on desmoscolids followed this trend, however, the occurrence of epibionts in non-desmoscolids did not differ between anterior and posterior regions (Fig. 1).

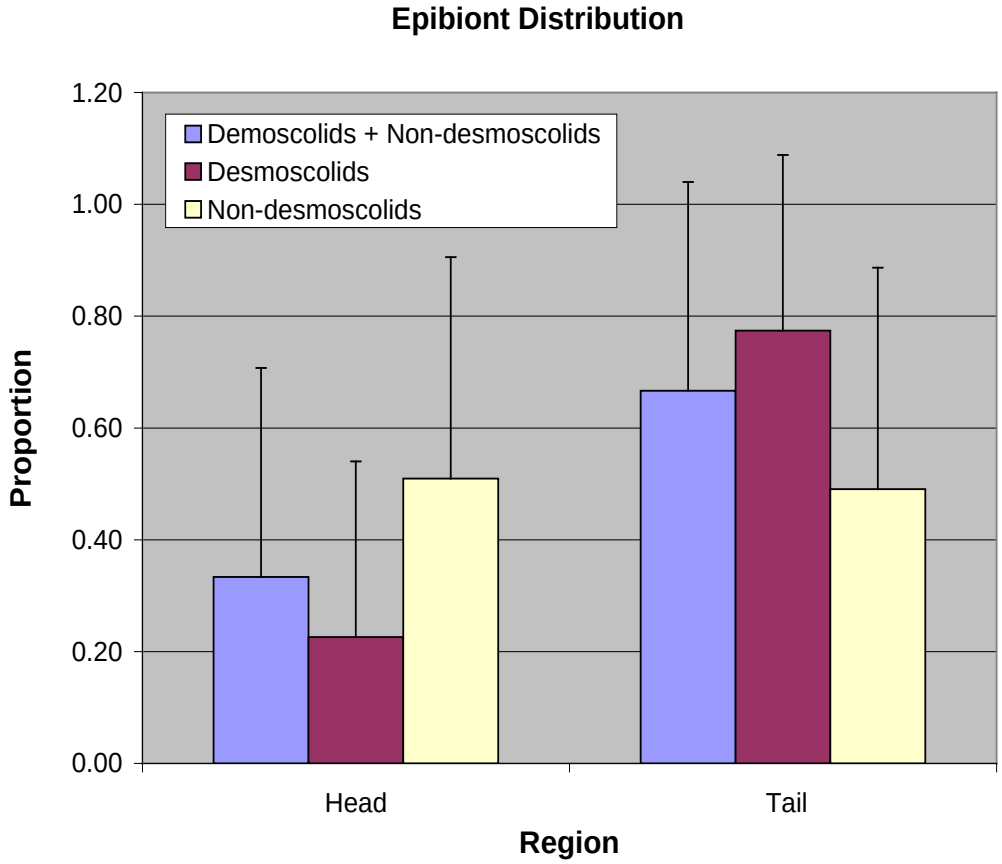

Fig. 1. Protozoan epibiont distribution expressed by proportion found in the head and tail region for desmoscolids + non-desmoscolids (n=100), desmoscolids (n=62), and non-desmoscolids (n=38).

Discussion

The objective of this study was to examine the presence of protozoan epibionts on deep-sea nematodes. The samples for this study were collected from locations consisting of three different CO_2 treatments. The first treatment served

as the control as samples were taken from areas free of any sequestered CO_2. In the second location, samples were taken from the corrals which were saturated with CO_2. Finally, samples were also taken from a location just outside the CO_2 ring. It was hypothesized that CO_2 treatment would have an affect on the incidence of epibiont-containing nematodes as treatment could potentially make individual nematodes more susceptible as a host (Puckett and Carman 2002). Our findings did not support this hypothesis, as we observed that CO_2 treatment did not have an affect on the incidence of epibiont-containing nematodes. An alternative hypothesis could be made proposing that epibionts colonized only dead nematodes. Barry et al. (2005) suggested that free-living single-celled animals, including nematodes, were killed by CO_2. Therefore, if this hypothesis were valid, samples from the corrals and outside the CO_2 ring would be expected to have a greater incidence of epibiont-containing nematodes; they did not, however, disproving this hypothesis.

The 0-5 mm and 5-10 mm layers of each sample were examined independently in order to determine if vertical distribution had an effect on epibiont-containing nematode incidence. Sediment depth did not have an effect on the prevalence of epibiont-containing nematodes. Also, it was observed that the interaction between depth and CO_2 treatment did not influence the incidence of epibionts on nematodes. This was a significant finding as previous experiments have shown that meiofaunal organisms, including nematodes, migrate between different depths to find the most environmentally favorable location (Carman et al., 1987; Thistle and Levin, 1998).

Previous findings have revealed the tendency of protozoan epibionts to attach to the posterior region of nematode hosts found in tropical sea grass meadows suggesting epibionts benefit from the host's excretions and secretions (Fisher, 2003). Our observations are generally consistent with those of Fisher (2003) in that epibionts tended to be more abundant on the posterior region of nematodes. However, when the epibiont-containing nematodes were sub-divided into two groups (desmoscolids and non-desmoscolids), we observed that epibionts on

desmoscolids follow the previously mentioned trend of posterior attachment, but epibionts on non-desmoscolids did not. This finding suggests that epibionts do not necessarily attach to the nematode to feed on excretions and secretions for if they did, epibiont location would be expected to be consistent on both types of nematodes. However, this finding may also suggest that the mucous secreting glands on nematodes vary depending on the type of nematode.

While epibiont prevalence on the deep-sea nematodes examined in this study was relatively low (< 1%), the relationship may be ecologically significant. Nematodes are of great importance due to their abundance in the benthic environments, and they act as indicators of environmental stresses (Coull and Chandler, 1992). Therefore, if the growth of protozoan epibionts on nematodes reflects a vulnerability of nematodes this could be an important indication of an ecological disturbance. Because there was no significant difference between the incidences of epibiont-containing nematodes found in the control and CO_2 treatments, we tentatively conclude that epibionts do not influence susceptibility of nematodes to potentially adverse environmental conditions that might occur as the result of elevated CO_2 concentrations.

Previous studies have found protozoan epibionts to favor nematodes of the desmodorid and desmoscolecid families, suggesting some sort of selection by the epibiont (Fisher, 2003). In the samples examined in this study, epibionts exhibit a preference for desmoscolid nematodes. Epibiont-containing nematodes from the non-desmoscolid group appear to primarily be from the family Desmodoridae. These non-desmoscolid nematodes exhibit cuticle ornamentation that may provide sites of attachment for protozoan epibionts that are analogous to desmoscolids. Thus, these findings along with previous research suggest that the cuticle ornamentation on the surface of the desmodorids and desmoscolecids nematodes provide a more secure area of attachment for the epibionts (Fisher 2003).

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