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The Perfect Microbial Symbiosis Hotel: Marine Sponges

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Specific symbiotic bacteria likely key to Mediterranean fruit fly control

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Tephritidae, or the true fruit flies, contain members that threaten agriculture worldwide. *Ceratitis capitata*, or the Mediterranean fruit fly (medfly), is considered by many researchers to be the world's most devastating agricultural pest. For example, if medfly became established in California, and Japan alone refused importation of citrus from California, statewide loss of income would be approximately $618 million. In addition, if US national quarantine implementation occurred, Californian families would lose approximately $3.6 billion of income (www.cdfa.ca.gov).

Medflies, and other tephritids destroy the marketability of a variety of hosts, notably fruits. Fruits provide a safe and nutritious haven for eggs, deposited by gravid females, which develop into voracious larvae. Eventually, the larvae will exit a rotten fruit, pupate, and later emerge as an adult that will begin the cycle again. Intense and expansive efforts are made on a daily basis to prevent medfly from entering regions at risk and to control populations in regions where medfly already exists.

Effective control of medfly and other tephritid pests is predicated on extensive knowledge of fruit fly biology and behavior. While much is known about fruit fly biology and behavior, little is known about the association these pests have with microorganisms, particularly with bacteria, and the possible roles bacteria play in their life history. The study of bacteria-tephritid interactions may provide new avenues of prevention and control, yet these studies come with numerous challenges associated with the complexities of understanding and manipulating multitrophic interactions within microbial ecology.

Two dominant bacteria

We (my students, colleagues, and I) know that certain species within the family Enterobacteriaceae are important life history symbionts of pest tephritids, *Enterobacter (Pantoea) agglomerans* and *Klebsiella pneumoniae* (Lauzon, 2003) The realization of the importance of *Enterobacter agglomerans*, in particular, came from observations I had in the late 1980's wherein several pest tephritids were attracted to this bacterium when the bacterium was put on fly traps (MacCollom *et al*, 1992, 1994). I isolated the bacterium originally, often in pure culture, from oviposition sites in apples where the tephritid, *Rhagoletis pomonella*, or the apple maggot fly, deposited several eggs. At the time, we imagined that the presence of this bacterium was a consequence of defecation; however, when we witnessed the affinity of this bacterium compared to others found in tephritid frass to wild apple maggot flies, we soon realized there may be more to the story.

Other researchers at the time were beginning to look at *E. agglomerans* for ideas to increase the power of a fruit fly trap. In subsequent work, we found that not all *E. agglomerans* were equally attractive to pest tephritids (Lauzon et al, 1998) and those that are attractive possess an enzyme that degrades uric acid (Lauzon *et al.* 2002). Uric acid is found in bird feces, a main source of nitrogen for fruit flies in nature. At this point, we found a link between attraction and a possible role for *E. agglomerans*. Fruit flies cannot catabolize uric acid. Consequently, *E. agglomerans* could be functioning in this role within the fruit fly gut and/or in bird feces. Interestingly, if you add antibiotics to bird feces, fruit flies are no longer attracted to the material (Prokopy et al, 1993). Volatile chemical analysis of bird feces (Epsky *et al*, 1997) and *E. agglomerans* on a uric acid medium (Epsky *et al*, 1998) showed that the "attractive *E. agglomerans*" emits odors that are protein cues and fruit-like (Epsky *et al*, 1998; Robacker and Lauzon, 2002; Robacker *et al*, 2002). Therefore, attraction to *E. agglomerans* may be a reflection of a need by the fly to find nitrogen and/or host fruit for egg laying and mating.

Nitrogen role?

Where does *Klebsiella pneumoniae* fit in? Over the years, we have surveyed thousands of fruit fly guts from all over the world for the presence of aerobic and anaerobic heterotrophic bacteria. We have also surveyed sources where fruit flies consume microorganisms, such as bird and other animal feces, leaf and fruit surfaces, and aphid honeydew. Despite the consumption of numerous genera of bacteria and fungi, we consistently, often only, recover *E. agglomerans* and *Klebsiella* spp. *Klebsiella* species routinely isolated are *K. pneumoniae, K. oxytoca, K. terrigena*, and *K. trevisanii. Klebsiella* spp. possess the ability to catabolize urea to ammonia. Therefore, when a fly ingests uric acid, we envision *E. agglomerans* and *K. pneumoniae* (for example) jointly catabolizing uric acid. First, *E. agglomerans* degrades uric acid to urea, and then urea is degraded to ammonia by *Klebsiella sp.* Ammonia is a useable form of nitrogen for the fly whereas uric acid is not.

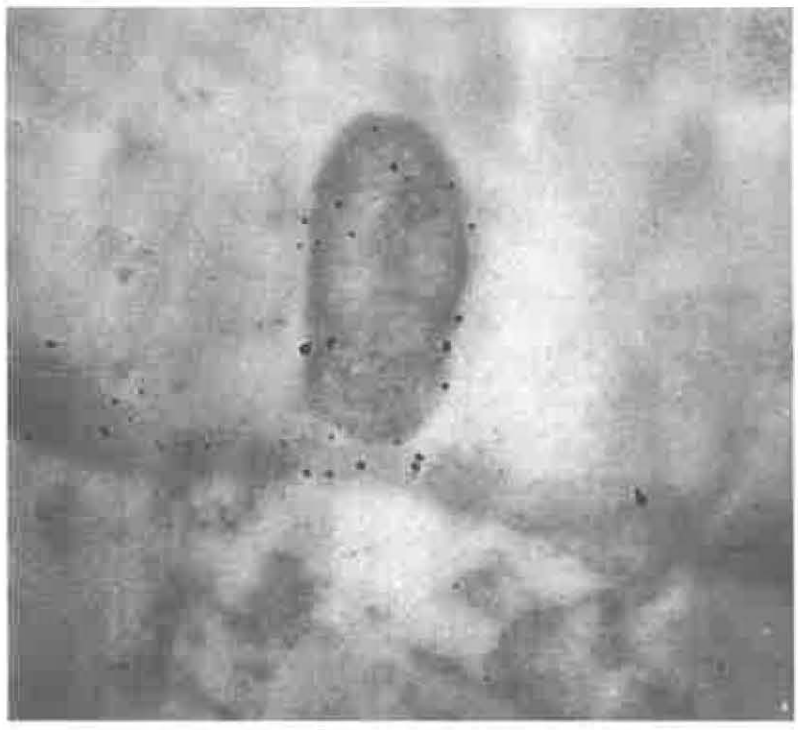

Figure 1. Ammonia export: *TEM of a bacterium attached to the peritrophic membrane of a medfly gut. Black dots are ammonia/ammonium compounds emitted from the bacterium and were made electron dense for purposes of visualization.*

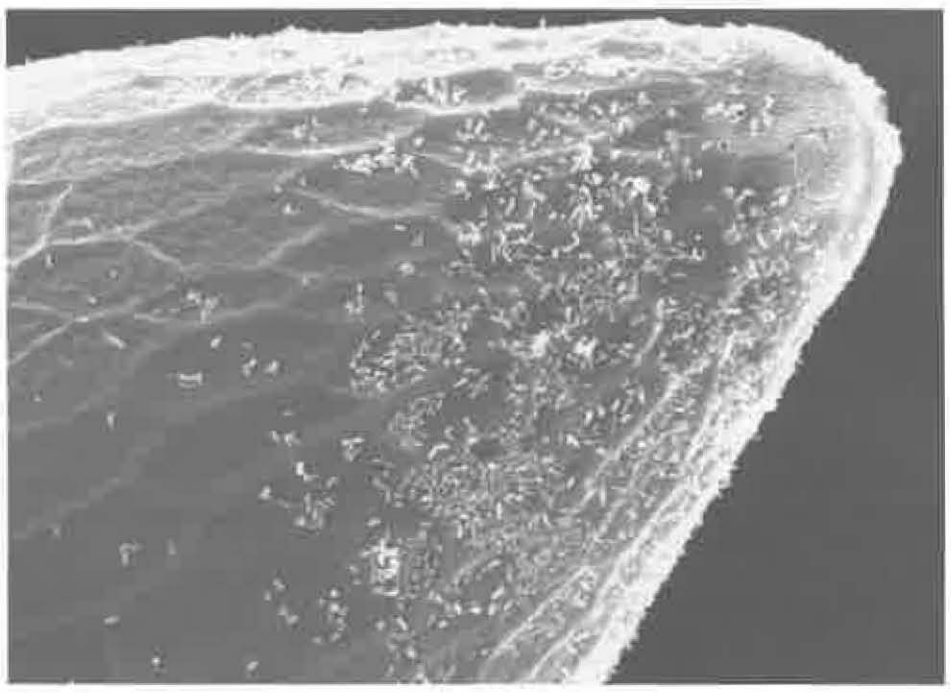

Figure 2. Egg biofilm: *Bacteria, identified as* E. agglomerans *and* K. pneumoniae, *attached to the apical end of an egg dissected from the ovaries of a female medfly.*

To determine if this hypothesis is plausible, we conducted a cytochemical assay in conjunction with transmission electron microscopy in an attempt to visualize bacteria and their catabolic byproducts or wastes in the gut of medflies. We found that indeed bacteria do produce ammonia and ammonium-like compounds in the midgut lumen (Fig. 1). In concurrent studies, we transformed *E. agglomerans* and *K. pneumoniae* to express two different fluorescent proteins (Lauzon *et al* unpublished; Peloquin *et al*, 2002) so we could visualize their establishment and arrangement within the fruit fly gut. Those studies showed that these bacteria assemble as a biofilm (Fig. 3). Biofilms are complex assemblages of microorganisms that generally suggest coordinated and efficient metabolism. While our intention became focused on understanding how this digestive biofilm was working for the fly,

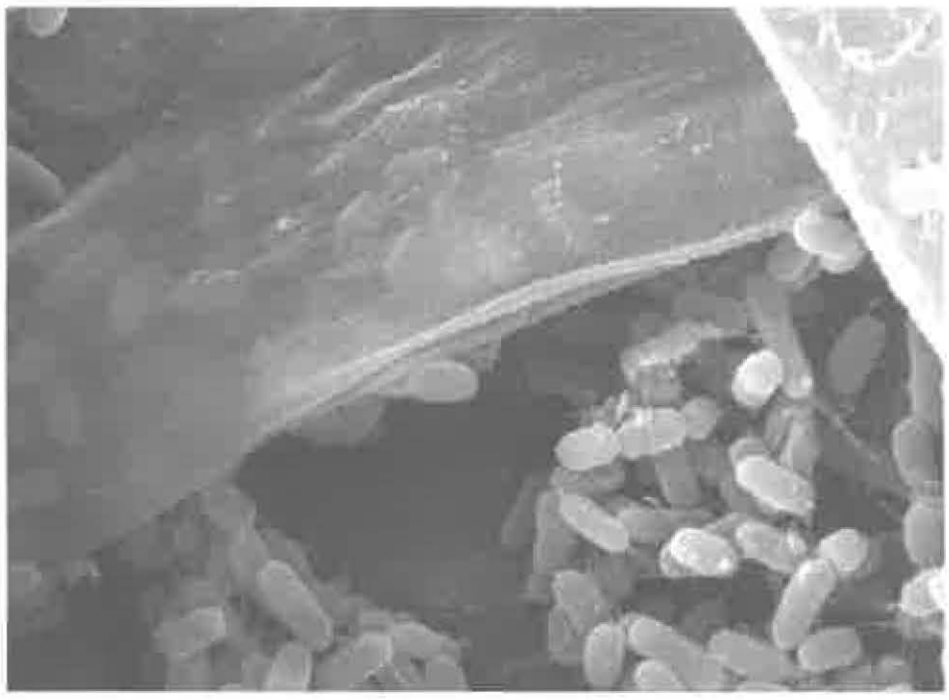

Figure 3. Gut biofilm/nonirradiated: *SEM of a medfly that has not gone through the irradiation process to render it sterile. The top sheath is the peritrophic membrane (that covers microvilli, not shown) and bacteria are in a biofilm that extends from the peritrophic membrane into and throughout the lumen.*

we observed a smaller biofilm on the apical end of developed and developing eggs within females (Fig. 2). Ingested *E. agglomerans* and *K. pneumoniae* found their way to the eggs where no other bacteria are present. In fact, it has been reported that females slather an antibacterial peptide on the surface of her eggs that is effective against *E. coli* (Marchini *et al* 1997) *E. agglomerans* and *K. pneumoniae*, close relatives to *E. coli*, should also be affected by this compound, but they are not. Moreover, the vertical transmission of these two bacteria strongly suggests their importance in the life history of fruit flies.

Radiation damage

While working on the large question of symbioses and fruit flies, reports emerged that revealed the poor performance of reproductively sterile male medflies that are used to interrupt the life cycle of any invading female medfly into California. This strategy, known as Sterile Insect Technique, is part of the Preventative Release Program implemented by the United Stated Department of Agriculture and the California Department of Food and Agriculture. Each week, millions of sterile male medflies are released into Southern California in hopes that the over-flooding ratio of sterile males to fertile males prevents any infestation. In fruit fly-rearing facilities around the world, males are irradiated during development and rendered sterile. We wondered if the radiation used in this process was affecting the gut to a point where these flies existed in a state of dysbiosis. We found that the gut is severely damaged by radiation and a probiotic diet consisting of *E. agglomerans* and *K. pneumoniae* improved the gut condition and performance of these flies (Niyazi *et al*, 2004). Symbiosis was restored.

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The marine sponge Leiodermatium sp.

The perfect microbial symbiosis hotel: Marine sponges

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Compared to the myriad oddly shaped, decorated, fast and nimble, or bioluminescent swimmers, floaters and predators that inhabit the oceans, marine sponges may not be the first animal to evoke the greatest excitement about ocean life. However, upon closer inspection, sponges can indeed hold their own. They not only comprise the oldest phylum among the metazoa and yield the widest array of cytotoxic natural products in the sea, but also offer a treasure, perhaps unique to themselves— vast, diverse microbial communities.

For example the bacterial biomass of some sponge species can exceed 50% and include a wide taxonomic diversity of resident bacteria. A bacterial cell count as high as 109 cells/ml sponge tissue has been recorded. (Interestingly, the marine species, *Crambe crambe*, appears to have no true microbial associates or symbionts, similar to freshwater sponges.) Perhaps the beckoning entrances and exits (e.g. ostia and oscules, respectively) and inner canals which are lined with relentlessly beating choanocytes, act as the archetypal hotel "vacancy" sign that certain microbes cannot refuse.

Microbial diversity and species richness in marine sponges has been studied via various microbiological culturing, fluorescent *in situ* hybridization (FISH), and culture-independent molecular biology techniques (such as PCR). These previous studies have revealed the presence of phototrophic cyanobacteria (in shallower hosts), deep lineage Planctomycetes and green non-sulfur bacteria, psychrophilic crenarchaea, along with a large number of heterotrophic aerobes, facultative and phototrophic anaerobes, and fungi.

In fact, a new microbial phylum, "Poribacteria," associated with sponges, has just been proposed by Hentschel et al, 2003 and Fieseler et al, 2004. Therefore, sponge taxa may embody "oases" of (microbial) species richness, in contrast to oases of biomass, which is the perception often associated with hydrothermal vents and their pivotal microbial communities. Nevertheless, despite potentially specific symbioses between bacterial species and sponge taxa, there are few published works that survey and correlate the relationship of eubacterial and fungal species to sponge (invertebrate) taxa, depth, geographical location, or natural products biosynthesis.

Biochemical diversity

In this context, our laboratories in the Division of Biomedical Marine Research (DBMR) at Harbor Branch Oceanographic Institution (HBOI) in Ft Pierce, Florida, have been exploring biochemical diversity in marine invertebrates and their microbial associates for nearly two decades (http://www.hboi.edu/dbmr/dbmr_home.html). Within DBMR, I (a molecular biologist) have teamed with microbiologist Dr. Peter McCarthy, to characterize a cross-section of the > 17,000 heterotrophic eubacteria and fungi derived from marine invertebrate specimens, primarily deep-sea marine sponges, for an NSF-funded "Biotic survey and inventory." These isolates comprise the unique HBOI Marine Microbial Culture Collection or HBMMCC. Molecular taxonomic methods included the standard techniques of restriction fragment length polymorphism (RFLP) and sequence analyses of the small subunit (SSU) 16S and 18S-like rRNA genes. Because these genes evolve relatively slowly over time (each 1% of difference in sequence comparisons is clocked to represent ~50 million years), they are a major tool for resolving deep phylogenies . The results to date support previous findings of wide phylogenetic diversity within certain microbial communities, as well as culturing of unexpected new isolates.

For example, at least 220 unique RFLP patterns (presumably representing distinct operational taxonomic units or "species") of the 16S rRNA gene were observed from >2300 isolates derived from about 40 invertebrate hosts. The microbial taxa surveyed from the cultured collection encompass at least six major subdivisions: 37% a-Proteobacteria (835 isolates), 1% b-Proteobacteria (25 isolates), 34% g-Proteobacteria (767 isolates), 5% CFB (122 isolates), 14% Gram+ High GC Content (327 isolates), and 9% Gram+ Low GC Content bacteria (198 isolates). Representative database queries indicated best matches to microbes such as *Neptunomonas naphthovorans, Mesonia algae, Sphingobacter mizutae* and *Bacteroidetes bacterium* , or "previously unclassified" eubacteria.

Novel microbial taxa

The predominance of the Alpha proteobacteria clade in this survey matched results from a recent albeit much larger, marine mega-sequencing project of Dr. Craig Venter. Furthermore, our efforts revealed at least 19 eubacterial taxa that were only < 93% similar to the closest sequence match in the GenBank database. This indicates a likely discovery of novel microbial taxa, while molecular "culture-independent" studies based on PCR amplification of 16S rRNA sequences directly from sponge metagenomic DNA samples are ongoing and will likely reveal even wider phylogenetic diversity. The survey is now also exploring fungal isolates. These results will be posted on a new public website: http://www.hboi.edu/dbmr/dbmr_hbmmd.html.

With diverse culturable isolates of the HBMMCC, it will be interesting to empirically address deeper questions in microbial ecology, evolution and natural product biosynthesis in the future. For example, what are the functional roles of so many diverse symbionts , and how do they benefit the host? Perhaps many microbes comprise food for grazing sponge cells, or transients staying for the proverbial night, while others are permanent residents. All in all, the number of possible symbiotic associations between sponge and microbe appears to be astronomical as well as ancient, and can be studied for many years to come.

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Primary database data was generated through the following work, soon to be published:

Gunasekera, A., K.A. Sfanos, P.J. McCarthy, J.V. Lopez, HBMMD: An enhanced database of the microorganisms associated with deep water marine invertebrates. *Applied Micro. Biotech. In Press.*

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Cellular and molecular aspects of cnidarian - algal mutualisms: A report from the Weis lab

In my (Virginia Weis) laboratory at Oregon State University, we are investigating the cellular and molecular interactions underlying mutualistic symbioses between cnidarians, such as corals and anemones, and their photosynthetic dinoflagellate symbionts. We are interested in the establishment, maintenance and breakdown of these associations that form the trophic and structural foundation of the coral reef ecosystem. We use several model associations in our studies, including a Hawaiian stony coral, *Fungia scutaria*, a tropical sea anemone *Aiptasia pallida*, and a temperate sea anemone found on the Oregon coast, *Anthopleura elegantissima.*

We are examining the dynamic process of the onset of symbiosis during the cnidarian host larval stage. Most species must acquire symbionts anew with each generation and therefore must engage in a complex recognition and specificity process that results in the establishment of a stable symbiosis. We are using confocal microscopy and *in situ* hybridizations to examine the dynamics of first infection, such as the location and mechanism of algal uptake by host larvae. In addition we are examining changes in gene expression of host larvae that occur during symbiosis onset.

We are interested in identifying genes that are up-regulated specifically as a function of the symbiotic state in host cnidarians. We are employing a variety of techniques, including subtractive libraries, cDNA microarrays and RNAi to identify and characterize host "symbiosis" genes. We are most interested in their function in the association. To date we have identified several host symbiosis genes using these approaches.

Recently, we have begun investigating the breakdown of the symbiosis and the role of apoptosis in this process. Coral bleaching is caused by the breakdown of the host animal/algal symbiont association. Bleaching is a growing global environmental problem and can cause the destruction of the entire reef ecosystems. My lab is investigating links between coral bleaching, oxidative and heat stress and apoptosis. We are examining the role of nitric oxide in bleaching, and we are characterizing apoptosis genes and apoptotic pathways in host animals, one of the first investigations of these critical cellular mechanisms to be performed on lower metazoans. To date we have identified caspase and bcl2 homologs and are in the process of searching for an apaf-1 homolog using a yeast two-hybrid system.

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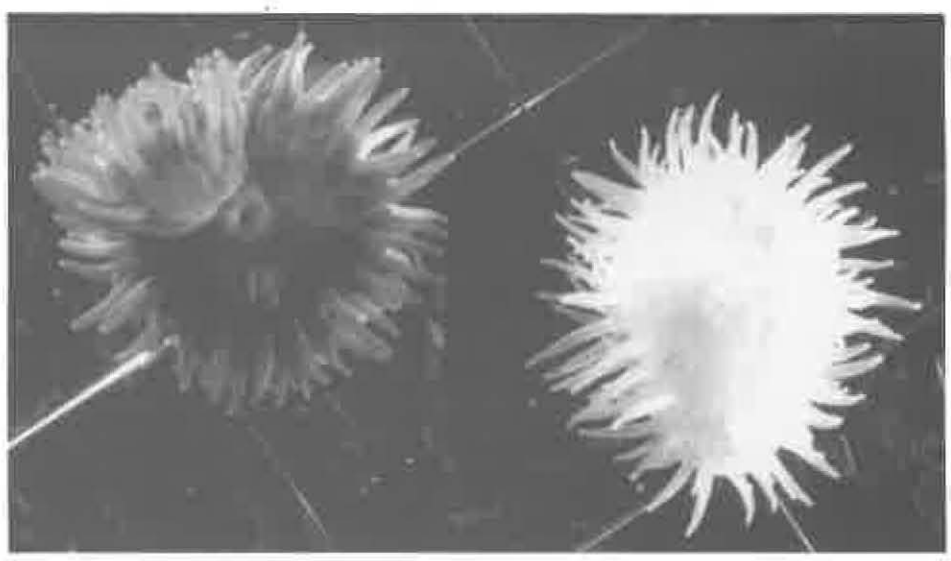
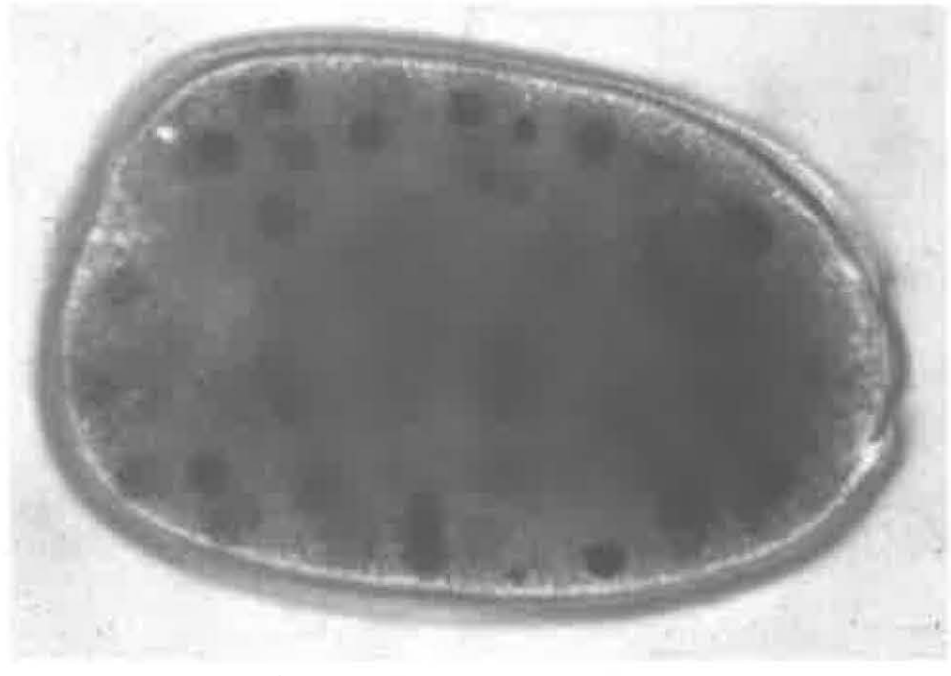


In high light environments, the anemone Anthopleura elegantissima *occurs naturally in the symbiotic state (shown on the left) with a large population of* brownish Symbiodinium sp. *In contrast, in low light, such as in caves and under rocks, anemones are found in the aposymbiotic state (shown on the right).*



A symbiotic planula larva of the coral Fungia scutaria. *Algae are evident as spheres inside the larva. Planulae are initially aposymbiotic but acquire algae during feeding.*