

Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*

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SUMMARY

The mutualistic association between the squid *Euprymna scolopes* and the bacterium *Vibrio fischeri* is an emerging experimental system for the study of the influence of bacteria on animal development. Taking advantage of the ability to raise both this host and its microbial partner independently under laboratory conditions, we describe the effects of bacterial interactions on morphogenesis of the juvenile host symbiotic organ. Our results show that bacteria are essential for normal postembryonic development of the symbiotic organ, which involves changes in both the surface epithelium and the epithelial tissue within the organ where the bacterial culture will take up residence. Cell death induced by exposure to symbiotic *V. fischeri* results in the regression of a complex ciliated surface epithelium, a tissue that apparently functions to facilitate inoculation of the juvenile organ with the appropriate specific bacterial species. Regression of this tissue begins within hours of exposure to symbiosis-competent

bacteria and progresses over the next 5 days, at which time full regression is complete, resulting in a symbiotic organ whose epithelial surface resembles that of the fully mature organ. Moreover, symbiosis-competent bacteria induce modification of the epithelial cells of the crypts that will house these symbionts; these cells undergo significant changes in shape and size in response to interactions with symbiotic *V. fischeri*. In contrast, we find that when these tissues are not exposed to the proper bacterial symbionts they remain in a state of arrested morphogenesis, a condition that can be rescued by interactions with symbionts. The results of these studies are the first experimental data demonstrating that a specific bacterial symbiont can play an inductive role in animal development.

Key words: cell death, cell differentiation, *Euprymna*, *Vibrio*, induction, symbiosis

INTRODUCTION

Studies of animal developmental processes have typically focused on defining the differing yet complementary roles of cell-cell interactions and intrinsic cell factors in processes of determination and differentiation. With few exceptions, it has been assumed that all the information needed to complete the formation of a complex multicellular organism is contained within the genome of that organism and the structure of its egg. However, the developmental programs of most, if not all animals, include specific interactions with benign, essential microorganisms during embryonic and/or postembryonic periods of morphogenesis (Hungate, 1966; Schwemmler and Gassner, 1989; Saffo, 1992). Technical difficulties, however, have precluded the analysis of the interactions between the partners that lead to the establishment and maintenance of proper stable associations. In many instances, such as the cow rumen and mammalian hindgut, the microbial complement exists as a consortium, where it is difficult to discern the influence on the host of any one microorganism independent of the others. Or, if the association does involve the more experimentally tractable situation of one host and one

microbial partner, often the host and symbiont are not viable outside of the symbiosis and, thus, cannot be raised separately under laboratory conditions.

While animal experimental systems have been lacking for the study of animal/bacterial interactions in development, our knowledge of the influence of bacteria on plant development is comparatively sophisticated. The legume/nitrogen-fixing bacteria symbiosis has been studied under laboratory conditions for over 100 years, providing a wealth of knowledge that has described the cellular, biochemical and molecular language between the plant and microbe that characterizes the development of a stable association. Presently dozens of genes in each partner have been identified as essential to a complex 'conversation' between these two disparate genomes that results in a series of developmental changes which lead to a mature association (Long, 1989; Nap and Bisseling, 1990; Hirsch, 1992). The descriptions of tissues of animals raised in microbe-free environments have revealed vastly different morphologies from the normal condition (Kenworthy, 1967; Bartizal et al., 1984; Pabst et al., 1988), suggesting that in animals we should expect a no less complex dialogue between the animal and microbial complements of an association.

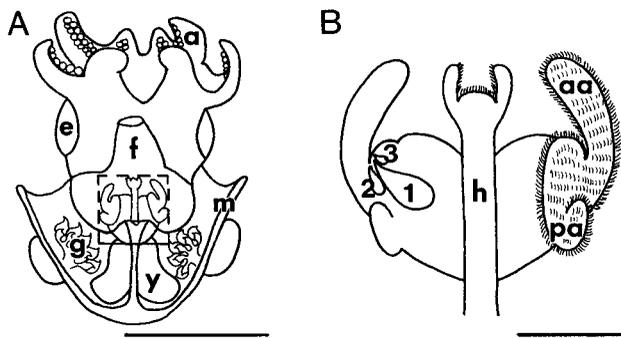


Fig. 1. Diagrammatic representations of the location and components of the *Euprymna scolopes* symbiotic organ. (A) Ventral dissection of a newly hatched *E. scolopes*. The light organ (within the dashed box) is situated largely within the funnel (f), through which seawater exits from the mantle (m). The orientation in this and in all subsequent figures is anterior at top, unless specifically noted. Bar, 1 mm. (B) Close-up of the immature light organ illustrated in A. Much of the surface of the organ is ciliated, including two anterior (aa) and two posterior (pa) appendages. Symbiotic bacteria present in seawater that passes over the surface of the organ enter pores that lead into three differently sized crypts (1, 2, 3). The crypts are numbered by convention according to the order in which they appear during embryonic development (Montgomery and McFall-Ngai, 1993). Bar, 100 μm . e, eye; g, gill; h, hindgut; y, internal yolk sac.

The association of the Hawaiian sepiolid squid *Euprymna scolopes* with its luminous bacterial symbiont *Vibrio fischeri* provides a model system to study the initiation and development of bacterial symbioses in higher animals, in particular the influence of bacteria on basic processes of development within symbiosis-specific host tissue. The *Euprymna/Vibrio* symbiosis has experimental advantages not found in other commonly studied animal/bacterial symbioses in that the association is highly specific, yet both partners can be grown independently of one another (McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). The squid maintains strains of the bacterium *V. fischeri* as light-emitting symbionts in a bilobed light organ partially embedded in the ventral surface of the ink sac. The mature organ is composed of several host tissues, including a core of branching epithelial crypts that house the symbionts, plus a reflector and a muscle-derived lens, which together with the ink sac function to direct the bacterial luminescence ventrally (McFall-Ngai and Montgomery, 1990). This configuration of adult tissues fosters maintenance of the symbiotic association and effective control of light emission. However, the incipient light organ of the newly hatched juvenile is very different from that of the adult in having a morphology that serves to ensure initiation of the symbiosis (Fig. 1; McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993). Juveniles hatch without *V. fischeri*, which are acquired within hours from the free-living population of the bacterium in the water column. Their immature light organs have ciliated epithelial appendages that appear to facilitate infection of the organ with the symbionts. Following inoculation with *V. fischeri*, the light organ undergoes a series of morphogenetic events that includes extensive tissue remodeling and differentiation. Preliminary experiments demonstrated that the ciliated epithelial appendages regressed within days of inoculation with symbiotic *V. fischeri*, but that the ciliated

structures remained in uninfected juveniles, suggesting that initiation of the symbiosis is a prerequisite for normal development of the host light organ (McFall-Ngai and Ruby, 1991).

We have characterized the influence of symbiotic bacteria on the early postembryonic development of the light organ of *E. scolopes*. We present evidence that cell death is the primary mechanism responsible for regression of the ciliated surface epithelium and that the onset of cell death is induced by infection of the light organ with symbiotic *V. fischeri*. We also demonstrate that infection with *V. fischeri* induces further cell differentiation in the epithelial cells that form the bacteria-containing crypts, causing these cells to change in size and shape. In contrast, uninfected juveniles do not undergo any of these early developmental events, indicating that *V. fischeri* has a profound effect on the developmental program of *E. scolopes*.

MATERIALS AND METHODS

Animals

Adult *Euprymna scolopes* were collected from shallow sandflats in Oahu, Hawaii, and transported to the University of Southern California, Los Angeles, where they were maintained in recirculating seawater aquaria. Eggs and juveniles were handled in our laboratory as previously described (Montgomery and McFall-Ngai, 1993). All the juveniles in a single experiment were hatched in filter-sterilized seawater (FSW) within 1 hour from the same clutch. Immediately upon hatching, juveniles were placed into one of four treatment groups as follows (see below for detailed descriptions): (1) axenic (bacteria-free environment), (2) inoculated with symbiotic *Vibrio fischeri* under sterile conditions, (3) aposymbiotic (symbiont-free environment), and (4) inoculated with symbiotic *V. fischeri* under natural conditions. (1) Axenic juveniles were rinsed three times immediately upon hatching in FSW and transferred into sterile Petri plates containing 50 ml FSW; the juveniles were transferred into fresh FSW every 4 hours. To verify that the axenic juveniles were free of bacteria, one juvenile was homogenized for dilution plating at the end of each experiment. Aliquots of the homogenate were spread on a seawater-based agar medium and the number of colony forming units was counted the following day. In each experiment the axenic juvenile tested contained undetectable numbers of bacteria (<10). Similar testing was performed on the FSW from the final incubations at the end of each experiment. Again, in each experiment the incubation water contained undetectable numbers of bacteria. (2) Symbiotic juveniles used in comparison with axenic juveniles were inoculated with *V. fischeri* ES114, a representative symbiotic strain previously isolated from an adult specimen of *E. scolopes* (Boettcher and Ruby, 1990). Juveniles were treated as described in (1) except for the initial inoculum with *V. fischeri* at 5×10^3 cells ml^{-1} FSW. (3) Aposymbiotic juveniles were placed in unfiltered California coastal seawater (CSW), which contains only symbiosis non-competent strains of *V. fischeri* (i.e., strains of *V. fischeri* that are incapable of initiating a symbiosis with *E. scolopes*, see McFall-Ngai and Ruby, 1991). (4) Symbiotic juveniles used in comparison with aposymbiotic juveniles were infected with symbiotic *V. fischeri* by exposure to seawater from aquaria containing adult *E. scolopes* which regularly shed light organ symbionts (Wei and Young, 1989; Ruby and Asato, 1992). After an initial 4 hour exposure, juveniles were transferred to CSW.

In an experiment to determine whether arrested development of the light organs of uninfected juveniles could be 'rescued' by the presence of bacterial symbionts, 2-day axenic juveniles were exposed to symbiotic *V. fischeri* as described in (4) and maintained as symbiotic juveniles for an additional 3 days. In all treatments with symbiotic juveniles, the successful infection of the juvenile light organ could be measured by monitoring the onset of luminescence as previously

described (McFall-Ngai and Ruby, 1991). In all symbiotic juveniles tested, luminescence was first measurable between 6 and 8 hour post-inoculation, indicating that the light organs of these juveniles were infected almost immediately upon exposure to the symbiotic bacteria.

Juveniles were maintained on a 12 hour light/dark cycle at 24°C. Juveniles were not fed during these experiments; the animals can survive for several days post-hatch utilizing internal yolk stores as an energy source.

Scanning electron microscopy

Juveniles were fixed in 5% formaldehyde in FSW for 12 hours and processed for SEM as previously described (Montgomery and McFall-Ngai, 1993). Unless specifically noted, the orientation of all figures is the same as that shown in Fig. 1 (ventral view, anterior at top).

Cell death determinations

Beginning with newly hatched juveniles and at 2 hour intervals through the first 24 hours post-hatch, five symbiotic and five axenic juveniles were fixed in 5% formaldehyde in FSW. In addition, five aposymbiotic juveniles each were fixed at 3 time points (12, 18, and 24 hours post-hatch). Juveniles were anesthetized in a 1:1 solution of 0.37 M MgCl₂:seawater; their mantles and funnels were removed prior to fixation. To visualize the nuclei of the surface cells of the light organs, juveniles were rinsed in FSW, stained with 100 µg ml⁻¹ bisbenzimidazole (a DNA-binding stain), and viewed under epifluorescence. Pycnotic nuclei, an indicator of cell death, present on the ciliated surface epithelium of the light organs, were counted from one side of five light organs for each time point and treatment. Most pycnotic nuclei were recognized at a late stage, after the nucleus had begun to fragment. Several fragments were counted as one nucleus if the total area of the fragments was equal to or less than that of an average healthy nucleus. For permanent data collection and verification of cell counts, all light organs examined were filmed with video microscopy. Not all of the cells of the ciliated surface epithelium could be visualized by this method because of the presence of the ciliated epithelial appendages. Neither the most dorsal surface of the anterior appendage nor the relatively small areas of the ciliated ridge covered by the appendages could be viewed. Thus, the numbers of pycnotic nuclei reported are the numbers observed rather than the absolute numbers present over the entire ciliated surface epithelium. Nonetheless, the majority of cells (i.e., ≥ 75%) could be viewed and counted by this method.

Acridine orange (AO) was also used to identify cells in the last stages of death. Juveniles were dissected in FSW containing 1 µg ml⁻¹ AO and examined under epifluorescence. Whereas bisbenzimidazole staining was used for cell counts (see below) and to quantify the numbers of dying cells through the first 24 hours post-inoculation, AO staining was used to examine the general pattern of cell death in symbiotic light organs through the first 5 days post-hatch.

Cell counts

Newly hatched juveniles and 24 hour, 48 hour, and 72 hour symbiotic juveniles were fixed as described above under *Cell death determinations*. To obtain accurate counts of the numbers of cells making up the ciliated epithelial appendages, the light organs were removed from the mantle cavity of the juveniles, rinsed in FSW, stained with bisbenzimidazole, and flattened under a coverslip prior to viewing with epifluorescence illumination. Most, if not all, cells of both the anterior and posterior appendages could be viewed and counted by this method. The nuclei in the anterior and posterior appendages of one side of three light organs were counted for each time point. The light organs of three 72 hour axenic juveniles were also examined.

Light microscopy and three-dimensional reconstructions

Three symbiotic and three aposymbiotic juveniles were fixed in 5% formaldehyde in 0.5 M sodium phosphate buffer, pH 7.4 and processed for light microscopy as previously described (Montgomery

and McFall-Ngai, 1993). Three-dimensional reconstructions from serial 1.5 µm sections of these light organs were constructed using a computer program (PC3D Three-Dimensional Reconstruction Software, Jandel Scientific, Corte Madera, CA). The volumes of light organ tissues, including the crypt epithelia and ink sac were determined from the three-dimensional reconstructions as previously described (Montgomery and McFall-Ngai, 1993). Cell sizes of the crypt epithelia were measured from representative sections of each specimen. Results for symbiotic and aposymbiotic treatments were compared with two-tailed paired *t*-tests.

Bromodeoxyuridine incorporation and visualization

To examine host mitotic activity, three 24 hour symbiotic and three 24 hour aposymbiotic juveniles were exposed to 50 µM bromodeoxyuridine (BrdU) in FSW for 48 hours each. Following BrdU exposure and prior to fixation, the juvenile squids were rinsed in FSW for 60 minutes. Juveniles were fixed for immunocytochemistry similar to that described in Weis et al. (1993). Briefly, whole juveniles were fixed for 2 hours at room temperature in 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer containing 0.45 M sodium chloride, pH 7.4. Control juveniles, which had not been exposed to BrdU, were also fixed. The specimens were then rinsed and dehydrated as previously described (Weis et al., 1993). The specimens were infiltrated with a 1:1 ratio of 100% methanol and LR White resin (Ted Pella, Inc., CA) for 12 hours at 4°C on a rotating table. Subsequently, the specimens were rotated in 100% LR White for 8 hours, transferred to fresh 100% LR White in gelatin capsules and allowed to polymerize for 2 days at 52°C.

The incorporated BrdU was visualized using immunocytochemistry. Histological cross-sections, 1 µm in thickness, were cut with glass knives on a Porter Blum MT2-B ultramicrotome and dried onto gelatin-coated glass slides. The antibody incubation procedure was modified for light microscopy from the methods of Erickson et al. (1987). All incubations were carried out at room temperature. First, to denature the DNA, sections were treated with 4 N HCl for 10 minutes followed by a neutralizing step in 0.1 M sodium borate as described by Migheli et al. (1991). Sections were incubated for 30 minutes in a blocking solution consisting of a 1:50 ratio of non-immune goat serum (Sigma Chemical Co.) to a 10 mM sodium phosphate buffer, containing 150 mM NaCl, 0.05% sodium azide and 0.5% bovine serum albumin (PBS/BSA). The sections were rinsed with PBS/BSA and then incubated overnight in a 1:50 dilution of primary (1°) antiserum, monoclonal anti-BrdU (Becton-Dickinson, CA) in PBS/BSA. The sections were rinsed with PBS/BSA and incubated for 1 hour in the secondary (2°) antibody solution, consisting of a 1:50 dilution of goat anti-mouse IgG complexed to 5 nm colloidal gold spheres (Ted Pella Inc., CA) in PBS/BSA. The immune complex was visualized for light microscopy using silver enhancement (Sigma). Finally, the sections were counterstained with 1% acid fuchsin for 20 minutes, rinsed in water, dried and mounted in heavy immersion oil.

Sections were examined using light microscopy and all labeled and unlabeled nuclei for each pair of crypts on a section were counted. At least six sections, each 10 µm apart, containing a total of at least 200 crypt cells were examined for each juvenile. Cells that had incorporated BrdU, as revealed by the presence of heavy silver staining of the nucleus, were expressed as a percentage of the total number of nuclei counted for each pair of crypts.

RESULTS

The initiation of symbiosis induces cell death in the ciliated epithelial surface of the light organ

The ventral surface of the incipient light organ of newly hatched *Euprymna scolopes* is largely composed of a ciliated

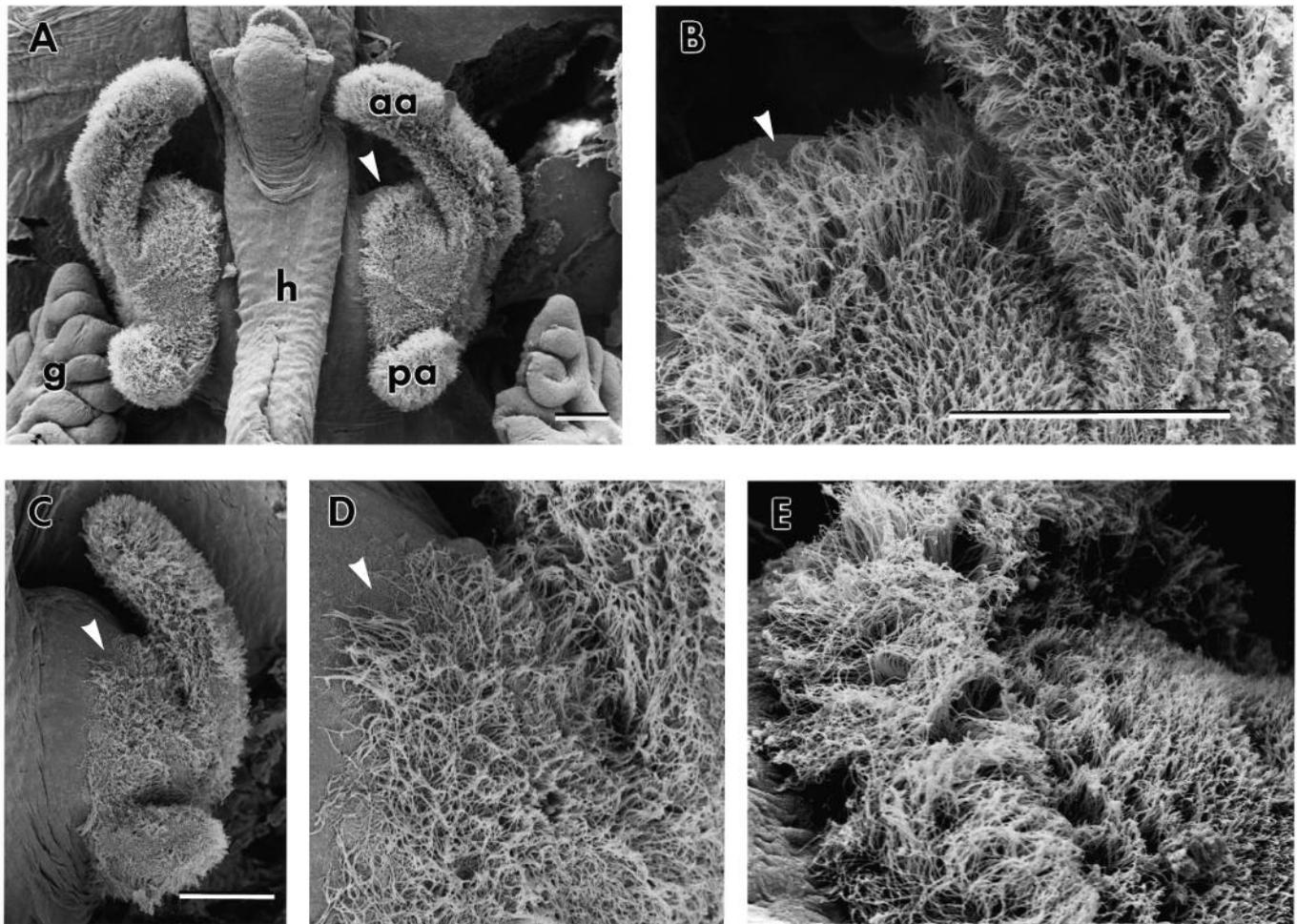


Fig. 2. Scanning electron micrographs of the light organs of juvenile *E. scolopes* revealing the regression of a ridge of cilia following infection with symbiotic *Vibrio fischeri*. (A) Ventral view of the light organ of a newly hatched juvenile. In addition to the long anterior (aa) and shorter posterior (pa) ciliated epithelial appendages, a ridge of elongated cilia (arrow) rings the border between the ciliated and non-ciliated surface epithelium of the light organ. (B) Higher magnification view of the region indicated by the arrow in A. (C,D) Views of one side of the light organ of a 24 hour symbiotic juvenile inoculated with *V. fischeri* immediately upon hatching. The ridge of cilia (arrow) is almost completely regressed and is replaced by non-ciliated epithelium. (E) The ridge of cilia remains present in a 24 hour axenic *E. scolopes*, a condition unchanged from that of a newly hatched juvenile. Magnification in D and E same as in B. Bar, 50 μm . g, gill; h, hindgut.

epithelium with a pair of anterior and a pair of posterior appendages (Fig. 2A) (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993). Another feature of this ciliated epithelium is a pronounced ridge of elongated cilia along the border between the ciliated columnar epithelium and the non-ciliated squamous epithelium of the surface of the light organ (Fig. 2A,B). Within 24 hours of inoculation with symbiotic *V. fischeri* the ciliated ridge was almost completely regressed and replaced with non-ciliated epithelium (Fig. 2C,D). Juveniles that were maintained in an axenic condition (i.e., sterile) for 24 hours, however, had light organs indistinguishable from those of newly hatched juveniles. The ciliated ridges in these 24 hour axenic juveniles were very prominent (Fig. 2E).

To determine if cell death functioned in the regression of the ciliated surface epithelium, symbiotic juvenile light organs were examined for the presence of pycnotic nuclei every 2 hours over the first 24 hours post-inoculation period. Pycnotic nuclei were first observed as early as 4 hours post-inoculation

in the anterior ciliated epithelial appendage and 6 hours post-inoculation in the posterior ciliated epithelial appendage and ciliated ridge (Fig. 3). The numbers of dying cells peaked at 14 hours post-inoculation and then decreased to low levels by 20 hours post-inoculation. As many as 25 pycnotic nuclei were observed in the anterior appendage of one light organ at 14 hours post-inoculation. Not a single pycnotic nucleus was observed in the light organs of axenic juveniles examined during this same period and throughout the experiment.

In the light organs of 14 hour axenic juveniles the nuclei of the surface epithelium appeared healthy and orderly (Fig. 4A). In 14 hour symbiotic juveniles, fragmented remains of several nuclei were evident over much of the surface epithelium (Fig. 4B). Whereas the ciliated ridge remained prominent in axenic juveniles (Fig. 4C), numerous dying cells were evident by 14 hours post-inoculation in infected animals as the ridge began to flatten and ciliated columnar cells were replaced by non-ciliated squamous epithelial cells (Fig. 4D). Similarly, no dying cells were found in the posterior appendage of axenic

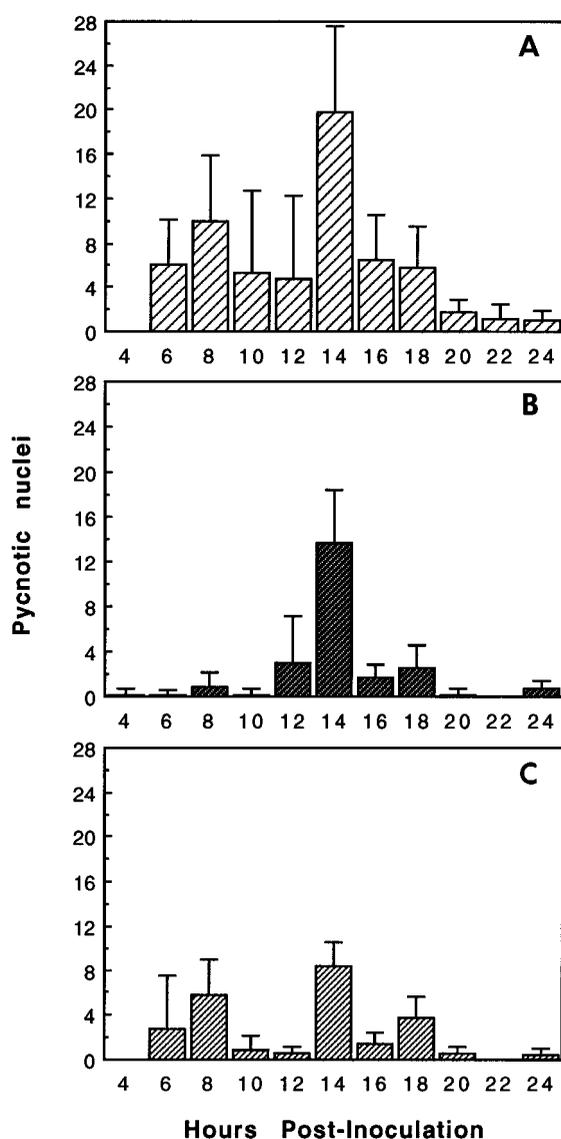


Fig. 3. Cell death in the ciliated surface epithelium of the juvenile light organ over the first 24 hour post-inoculation. The mean numbers (\pm s.d.) of pycnotic nuclei observed in the ciliated ridge (A), anterior appendage (B), and posterior appendage (C) of bisbenzimidazole-stained light organs during the first 24 hour post-inoculation in symbiotic juveniles. The pycnotic nuclei from one side of the light organ of five individuals were counted for each time point.

juveniles (Fig. 4E), but many fragmented nuclei were present in the posterior appendage of infected light organs (Fig. 4F). Changes in the shape of the anterior appendage also began to take place at this time as relatively large numbers of cells die and the appendage narrows in width. Pycnotic nuclei in the anterior appendage were often observed above the plane of focus of surrounding cells, suggesting that some of these dying cells may be extruded into the mantle cavity rather than phagocytosed by neighboring cells. Pycnotic nuclei in the posterior appendage were most often observed at the distal tip of the appendage, particularly in the first 12 hours post-inoculation.

Light organs from symbiotic juveniles that ranged from 24

to 48 hours old were also examined with bisbenzimidazole to determine whether the pattern observed in the first 24 hours was maintained. No peak in the numbers of pycnotic nuclei similar to 14 hours post-inoculation was observed over the second 24 hour period. The total number of pycnotic nuclei counted on the ciliated surface epithelium at any time point between 24 and 48 hours post-inoculation averaged $4.2 (\pm 3.2)$. Thus, after the initial peak at 14 hours and the following drop by 20 hours, the numbers of pycnotic nuclei, and presumably the rate of cell death, remained at a relatively low and steady rate.

The regression of the ciliated epithelial appendages is accompanied by a loss in cell number

To determine if cell death played a prominent role in the regression of the epithelial appendages, it was necessary to quantify the rate of cell loss in these surface structures. Both the anterior appendage and posterior appendage of symbiotic juvenile light organs exhibited an exponential decrease in cell number over time (Fig. 5). The light organs of 72 hour axenic juveniles, however, showed no such loss in cell number; the numbers of cells in both the anterior and posterior appendages of the aposymbiotic juveniles did not differ significantly from those of newly hatched juveniles ($P > 0.2$ and $P > 0.5$, respectively; Fig. 5).

Light organs of symbiotic juveniles stained with AO revealed that the process of cell death continues throughout the first 5 days post-inoculation. AO-stained fragments first appeared between 10 and 12 hours post-inoculation. AO-stained fragments were first present along the ciliated ridge and throughout the anterior and posterior appendages. However, beginning at about 24 hours post-inoculation, small AO-stained fragments were also found in the center of the ciliated surface epithelium in the vicinity of the pores; these fragments may represent the remains of dead cells that were in the process of being phagocytosed (Fig. 6A). 1-day old aposymbiotic juveniles showed little or no selective staining with acridine orange (Fig. 6B). The light organs of 2-, 3-, 4-, and 5-day old symbiotic juveniles also demonstrated selective staining with AO; although, only a few dozen AO-stained fragments were present on the light organs of 5-day old animals.

Regression of the ciliated epithelium through time

To document the time course of the regression of the ciliated epithelial surface, we processed juvenile *E. scolopes* at different stages of development for SEM (Fig. 7). Juveniles maintained axenically for 3 days (Fig. 7A) had light organs indistinguishable from those of newly hatched juveniles (see Fig. 2A). Juveniles inoculated with a symbiotic strain of *V. fischeri* and then maintained under the same sterile conditions as the axenic animals showed substantial regression of the ciliated surface epithelium by 3 days; both the anterior and posterior appendages were markedly reduced although still present (Fig. 7B). In comparison, the ciliated epithelial appendages of juveniles inoculated with symbiotic *V. fischeri*, but maintained in unfiltered seawater for 3 days, were slightly more reduced in size; the posterior appendages appeared completely absent in all of these juveniles (Fig. 7D); these results indicate that CSW has no inhibitory effect on either establishment of the symbiosis or induction of morphogenesis. Juveniles maintained axenically for 2 days and then exposed to symbiotic *V. fischeri* were still competent to undergo light

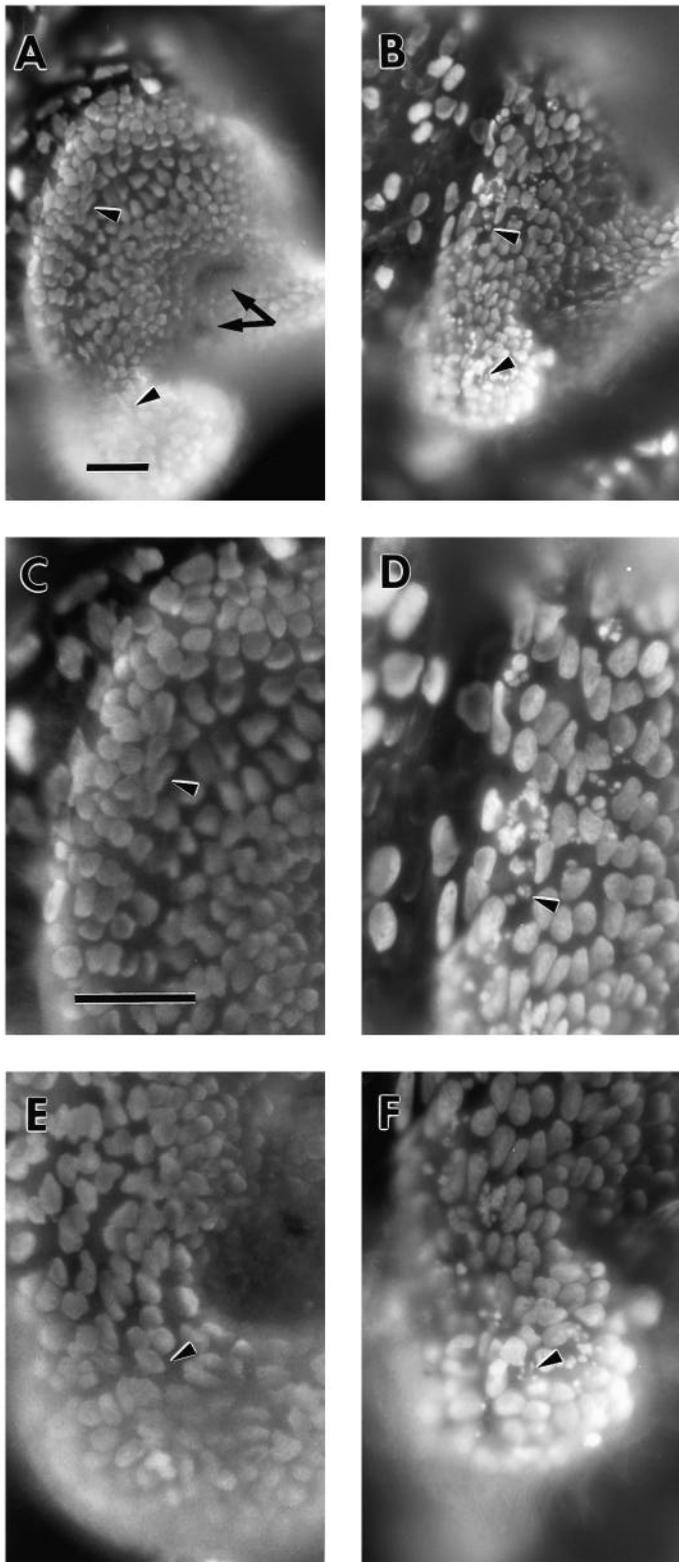


Fig. 4. Cell death evident in the light organs of symbiotic juvenile *E. scolopes* (right) but absent in axenic juveniles (left). (A) Bisbenzimidate-stained light organ of a 14 hour axenic juvenile (only one side shown). Arrowheads indicate cells viewed under higher magnification in C and E. Two of the pores that lead into the crypts of the light organ are evident (arrows). (B) Large numbers of pycnotic nuclei shown in higher magnification in D and F. (C) In the axenic light organ no pycnotic nuclei are present along the ciliated ridge, a transition area between columnar ciliated epithelial cells on the right and squamous epithelium on the left. (D) Several dying cells, evidenced by fragmented nuclei, are present all along the ridge in infected light organs. Similarly, no pycnotic nuclei are evident in the posterior appendage of the axenic juvenile (E) whereas several pycnotic nuclei are present in the regressing appendage of the symbiotic juvenile (F). A and B are the same magnification; C through F are the same magnification. Bars in A and C, 20 μ m.

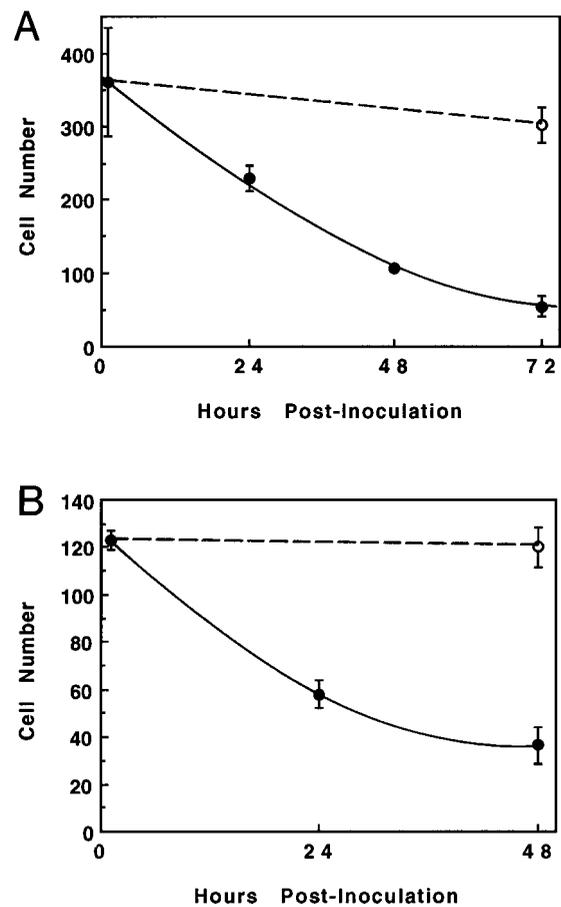


Fig. 5. The mean numbers of cells making up the ciliated epithelial appendages of symbiotic and axenic juveniles. The mean numbers (\pm s.d., $n=3$) decrease exponentially in symbiotic *E. scolopes* (solid line) in (A) the anterior appendage over the first 72 hours post-inoculation and (B) over the first 48 hours in the posterior appendage. The numbers of cells in axenic juveniles (open circles, dashed line) are not significantly different from those of newly hatched juveniles ($P>0.2$ and $P>0.5$, respectively).

organ morphogenesis. The light organs of these 5-day old juveniles (Fig. 7C), axenic for the first 2 days and symbiotic for the final 3 days, were very similar to the light organs of 3-day old symbiotic juveniles (compare with Fig. 7D). Five-day old aposymbiotic juveniles maintained in unfiltered seawater had light organs in which the ciliated surface epithelium was

largely intact, although some regression was evident (Fig. 7E). The ciliated ridge was not especially prominent and the anterior appendage had become somewhat reduced in size. In contrast, the ciliated surface epithelium in 5-day old symbiotic

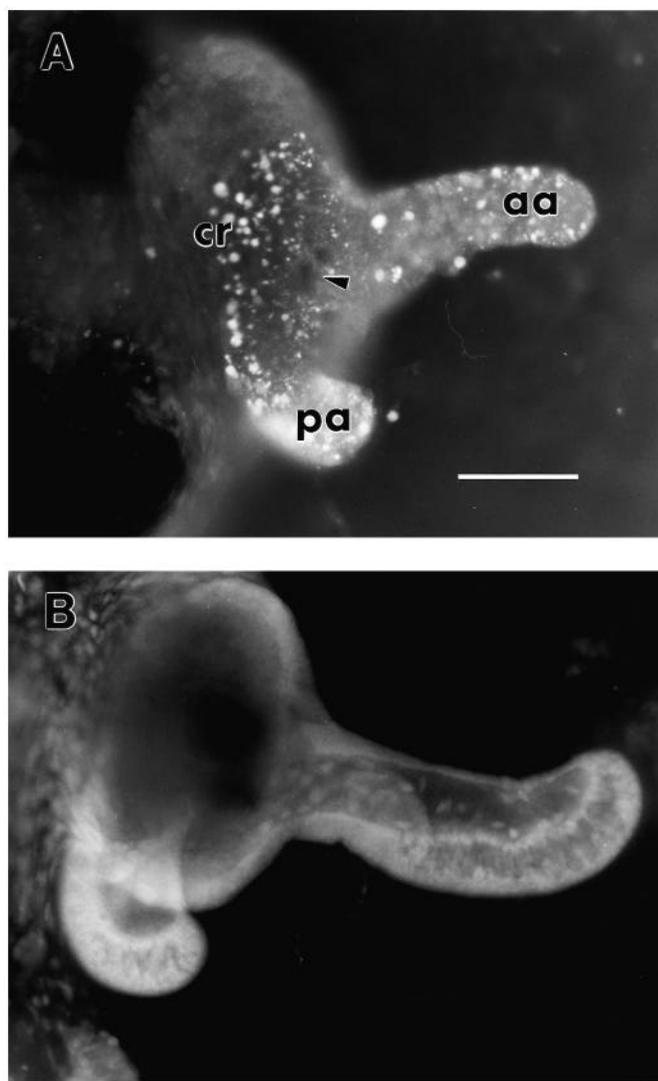


Fig. 6. The pattern of cell death in the juvenile light organ. (A) Cell death visualized with acridine orange (AO) in the light organ of a 24 hour symbiotic juvenile (one side shown). AO-stained cells and fragments are present along the ciliated ridge (cr), the anterior epithelial appendage (aa), and the posterior epithelial appendage (pa). AO-stained fragments are also present near the pores (arrowhead) that lead from the surface of the light organ into the bacteria-containing crypts. (B) AO-stained 24 hour aposymbiotic juvenile demonstrating no selective staining. Bar, 50 μm .

juveniles maintained in unfiltered seawater had almost completely regressed, both appendages being completely absent (Fig. 7F).

Comparison of the light organ crypts of symbiotic and aposymbiotic juveniles

The proposed function of the ciliated surface epithelium is to facilitate the inoculation of the incipient light organ with symbiotic bacteria (McFall-Ngai and Ruby, 1991). The bacterial symbionts enter pores on the surface of the light organ and populate the extracellular lumens of three pairs of epithelial crypts; the largest pair of crypts is the first to begin development during embryogenesis whereas the third, and smallest,

pair of crypts is the last to arise (Figs 1 and 8) (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993). Each crypt is composed of two major cell types: non-ciliated microvillous cells that directly interact with the bacterial symbionts, and ciliated cells that form a duct leading to the surface of the light organ. We observed changes in the size and morphology of some of the non-ciliated crypt cells following infection with symbiotic *V. fischeri* (Fig. 8). In newly hatched and 3-day old aposymbiotic juveniles, most of the cells of the crypts appeared columnar, including the ciliated cells of the ducts, which resemble the ciliated cells of the surface epithelium. Because the first pair of crypts has the highest number of easily identifiable non-ciliated microvillous cells, we chose to compare the sizes of these cells in symbiotic and aposymbiotic juveniles. In 3-day old aposymbiotic juveniles, the non-ciliated epithelial cells of crypt 1 that are located in the medial portions of the light organ (at least a distance of 4 cells from what appeared to be the distal end of the ciliated duct) averaged 11.4 μm (± 3.2) in height and 5.6 μm (± 1.5) in width ($n = 24$), as measured from histological sections. In contrast, similarly located cells in 3-day symbiotic juveniles were more cuboidal, averaging 12.9 μm (± 1.9) in height and 11.1 μm (± 2.9) in width ($n = 24$). The difference in the widths of these two cell types was statistically significant ($P < 0.01$). Interestingly, no bacteria were evident in any of the third pair of crypts of the symbiotic juveniles; we do not know whether this was due to bacteria being lost during the fixation procedure or whether crypt 3 was never populated with bacteria. The ciliated cells of the ducts of all three crypts appeared similar in size and shape in both symbiotic and aposymbiotic juveniles.

From three-dimensional reconstructions we also compared, in these same light organs, the volumes of the crypt epithelia; these volumes were normalized to ink sac volume to control for differences in overall light organ size between individuals. There was a statistically significant difference between the average normalized volumes of the first pair of crypt epithelia of 72 hour symbiotic and aposymbiotic juveniles ($P < 0.05$) (Fig. 9). There was no statistically significant difference between the volumes of the second and third pairs of crypt epithelia ($P > 0.2$ and $P > 0.1$, respectively) (Fig. 9).

To determine whether the larger crypt sizes in symbiotic juveniles might also be due to increased rates of cell division, we compared the percentage of crypt epithelial cells incorporating BrdU over a 48 hour period in 3-day old symbiotic and aposymbiotic juveniles. There was no statistically significant difference in the rate of BrdU incorporation in the light organ crypts of symbiotic and aposymbiotic juveniles ($P > 0.1$) (Fig. 10). There are differences, however, between the different pairs of crypts. There was little incorporation in the first pair of crypts, whereas the second pair exhibited a moderate level of BrdU incorporation, and over 60% of the cells examined in the third pair were labeled.

DISCUSSION

By comparing the early light organ morphogenesis of juvenile *Euprymna scolopes* infected with symbiotic *Vibrio fischeri* with that of uninfected juveniles, we have determined that the presence of the symbiotic bacteria induces specific morphological changes by affecting such fundamental developmental

processes as cell death and cell differentiation. Infection with symbiotic *V. fischeri* induces regression of the ciliated surface epithelium, a structure whose apparent function is to facilitate inoculation of the juvenile light organ with symbiotic bacteria (McFall-Ngai and Ruby, 1991). We found that cell death plays a prominent role in the removal of ciliated epithelial cells from the surface of infected light organs. Infection with symbiotic *V. fischeri* also induces further differentiation in the layer of epithelial cells that directly interact with the bacterial symbionts, resulting in changes in cell size and shape.

Initiation and establishment of the symbiosis of *E. scolopes* with *V. fischeri* presumably entails complex signaling events between the two partners. During this process the squid host must receive an inductive signal that signifies a successful infection and further development of the light organ. There are examples in plant-microbial associations in which the development of certain plant structures requires inductive interactions with specific microbes. The soil bacterium *Rhizobium* induces the development of nitrogen-fixing root nodules in leguminous plants (Nap and Bisseling, 1990). Upon initiation of the symbiosis, both plant and bacterial cells undergo dramatic morphological and physiological changes due to a cascade of interspecies gene signaling (Long, 1989). Although not as well understood as in the plant-microbial associations, microbial symbionts also influence developmental events in animal hosts. Many vertebrate tissues, such as those associated with the immune system and the digestive tract, develop normally only in the presence of a consortium of microbes (Kenworthy, 1967; Bartizal et al., 1984; Pabst et al., 1988). The presence of microbial symbionts is also often necessary for the normal development of many species of invertebrates. Infection with intracellular symbiotic algae is necessary for the completion of the life cycle of the scyphozoan, *Cassiopea* (Colley and Trench, 1985). Similarly, infection with luminescent bacterial symbionts is required for completion of the life cycle in certain pathogenic nematodes (Nealson, 1991). The abdomen of the leafhopper does not develop at all in the absence of the insect's endocytobionts, resulting in the formation of head-thorax only embryos (Schwemmler, 1989). In *E. scolopes* interactions with its specific bacterial symbiont are important for at least two early postembryonic developmental processes, the death of cells that form the ciliated surface epithelium of the light organ and the further differentiation of the epithelial cells of the bacteria-containing crypts.

Cell death is a fundamental process in normal development, affecting aspects of morphogenesis in virtually all animals. We found that cell death plays a primary role in the early postembryonic development of the juvenile light organ of *E. scolopes*. Within hours of infection with symbiotic *V. fischeri*, the cells of the ciliated surface epithelium of the

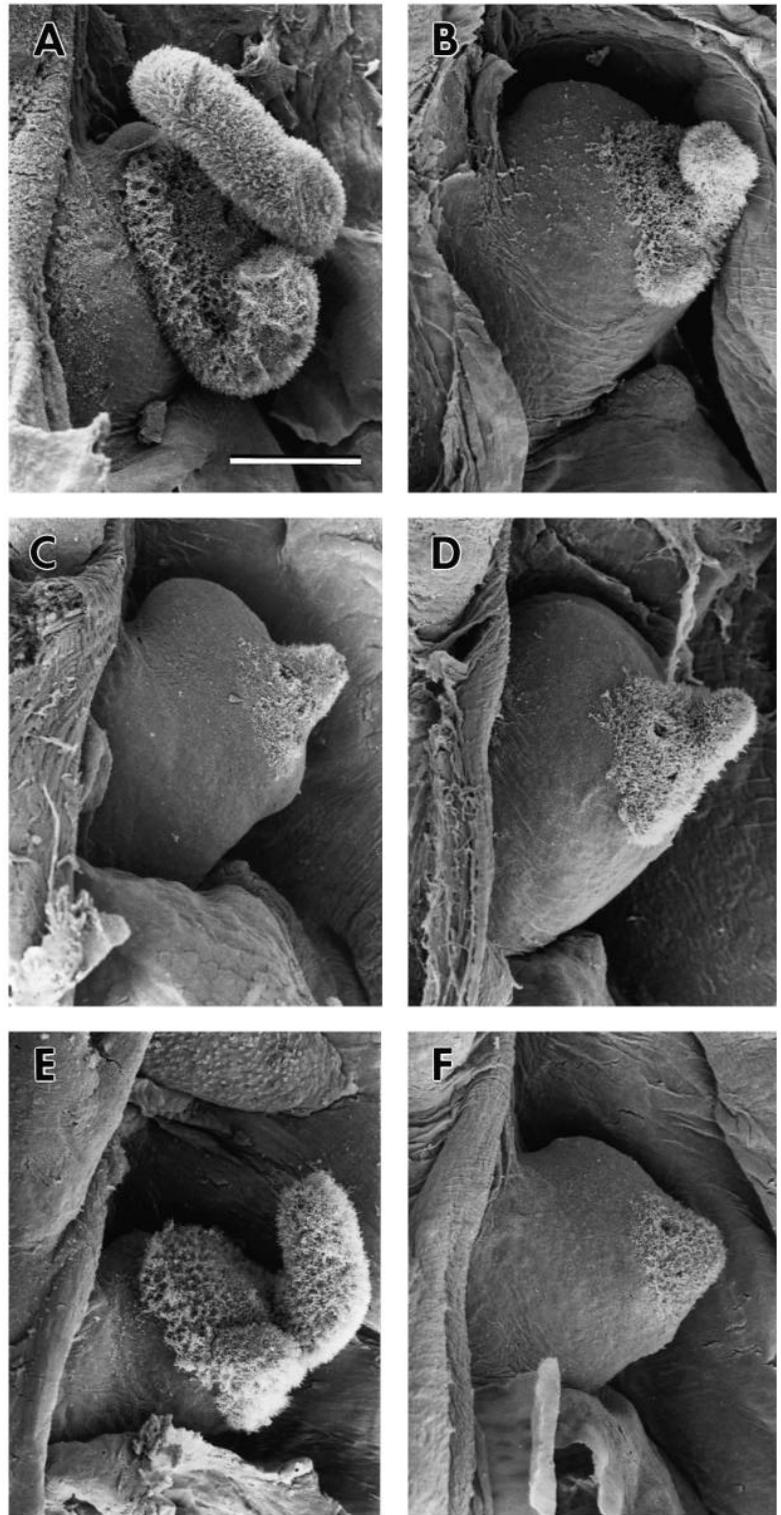


Fig. 7. Scanning electron micrographs demonstrating the effect of bacterial symbionts on the regression of the ciliated surface epithelium of the juvenile light organ. One side of the light organ of a (A) 3-day old axenic juvenile, (B) 3-day old symbiotic juvenile inoculated with *V. fischeri* ES114, (C) 5-day old juvenile maintained axenic for 2 days followed by 3 days exposure to symbiotic *V. fischeri* in natural seawater, (D) 3-day old symbiotic juvenile inoculated with symbiotic *V. fischeri* in natural seawater, (E) 5-day old aposymbiotic juvenile maintained in natural seawater, (F) 5-day old symbiotic juvenile inoculated with symbiotic *V. fischeri* in natural seawater. Bar, 200 μ m.

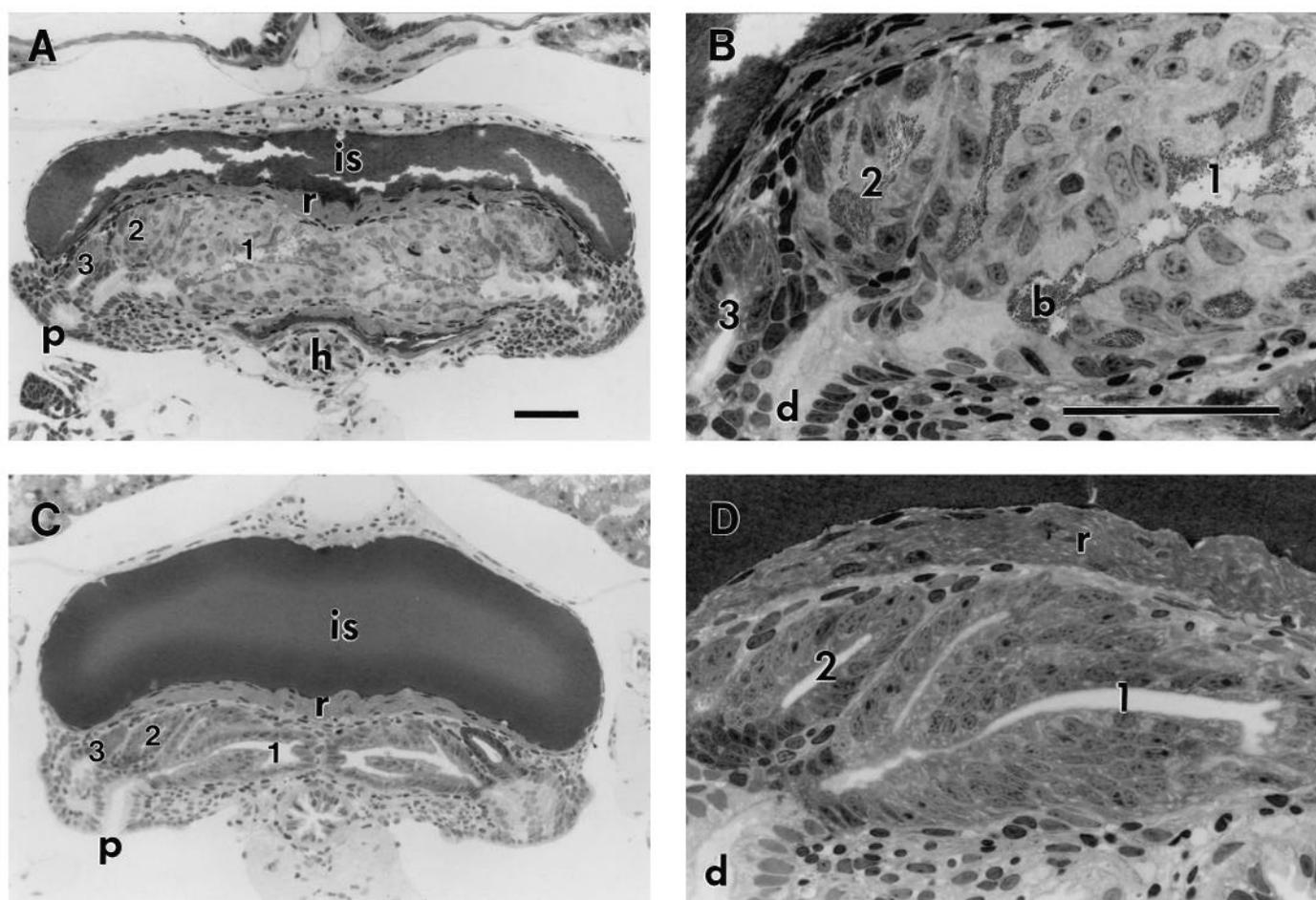


Fig. 8. Histological cross-sections (1.5 μm) through the light organs of a 72 hour symbiotic and aposymbiotic juvenile. (A) On each side of the light organ three pores (p, one visible in this panel) open into three ciliated ducts that lead to three differently sized sacculate crypts (1, 2, and 3), which are populated by bacteria (b) in this 72 hour symbiotic juvenile. Dorsal to the crypts are a thin reflector (r) and the ink sac (is), which together function to direct the bacterially produced light ventrally. (B) Higher magnification view of the crypt epithelial cells shown in A. The bacteria (b) appear as the black granules within the extracellular lumen of crypts 1 and 2; no bacteria are evident in crypt 3, which is largely ciliated duct at this stage. The non-ciliated epithelial cells that form crypts 1 and 2 are larger and more cuboidal than those in newly hatched and aposymbiotic juveniles. The ciliated cells that line the duct (d), however, as well as the cells in crypt 3, remain columnar in the symbiotic juvenile. (C,D) 72 hour aposymbiotic juvenile. The epithelial cells of the crypts have remained similar in size and shape to that of the newly hatched juvenile. A and B same magnification; C and D same magnification; bar, 50 μm . h, hindgut; dorsal at top.

immature light organ begin to die. Only light organs infected with symbiosis-competent *V. fischeri* undergo this massive cell death event. Although cell death is responsible for much of the regression of the ciliated surface epithelium of the light organ, it is unclear if it is the sole responsible mechanism. An average of 125 out of 360 cells are lost from the anterior appendage within the first 24 hours post-inoculation. The average number of dying cells observed using bisbenzimid staining over this same 24 hour period totals 25. Similarly, an average of 60 out of 125 cells are lost from the posterior appendage, at least 25 of which undergo cell death, within the first 24 hours post-inoculation. It is not known how long it takes for a cell in the light organ to die and how long its pycnotic nucleus remains visible, although observations of cell death in the anterior appendage suggest that the process takes less than 2 hours. The number of pycnotic nuclei observed peaks at 14 hours post-inoculation, but relatively few pycnotic nuclei are observed either 2 hours before or 2 hours following this massive cell death event. Pro-

grammed cell death has been extensively studied in the nematode *Caenorhabditis elegans*, in which 131 cells of the 1090 generated during development of the hermaphrodite undergo cell death, a process completed in less than 1 hour (Sulston and Horvitz, 1977; Sulston et al., 1983). If the process of cell death in the light organ of *E. scolopes* takes significantly less than 2 hours, the total number of cells lost through cell death as measured with bisbenzimid staining may be severely underestimated. Alternatively, some cells from the ciliated surface epithelium may escape a cell death fate by being recruited into either the non-ciliated squamous epithelium or the ducts and crypts of the interior of the light organ. Observations of light organs stained with AO, however, lend additional support to the conclusion that cell death is the primary mechanism by which the ciliated surface epithelium regresses. Although few pycnotic nuclei were observed in symbiotic light organs stained with bisbenzimid at 24 hours post-inoculation, AO staining revealed the remains of dozens of dead cells and

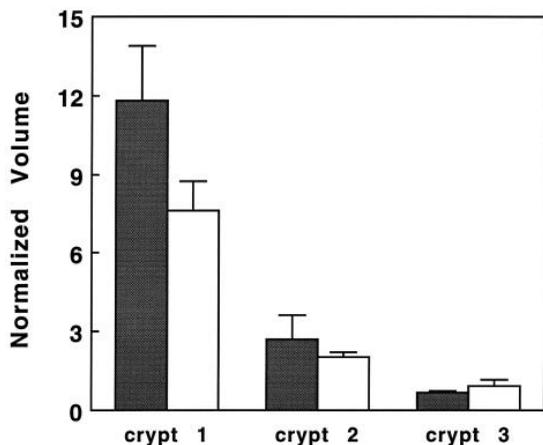


Fig. 9. Crypt sizes of the light organs of 72 hour symbiotic (stippled) and aposymbiotic (open) juvenile *E. scolopes*. The tissue volume of crypt epithelia (\pm s.d., $n=3$) was derived from three-dimensional reconstructions and normalized to ink sac volume (see text).

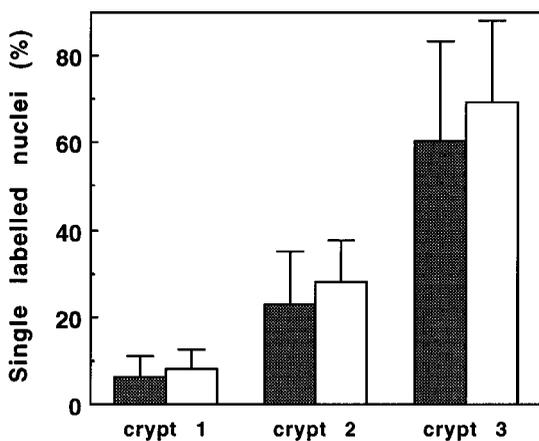


Fig. 10. Incorporation of BrdU in the light organs of 72 hour symbiotic (stippled) and aposymbiotic (open) *E. scolopes*. Six 24 hour juveniles (3 symbiotic, 3 aposymbiotic) were exposed to 50 μ M BrdU for 48 hours. Each bar represents the average percentage of labeled nuclei in each pair of crypts (\pm s.d., $n=3$).

hundreds of cell fragments (Fig. 6). Further, AO-staining fragments could be observed throughout the 5-day period during which the ciliated surface epithelium continues to regress.

Large numbers of AO-stained fragments appear after 12 hours post-inoculation, indicating massive cell death at about the same time we observed the largest percentage of fragmented nuclei. These results indicate that the breakdown of selective permeability in dying cells may not occur until very late in the cell death process, which is consistent with studies of programmed cell death in *Drosophila* (Wolf and Ready, 1991). In programmed cell death, as opposed to necrosis, cells fated to die activate a genetically encoded suicide program that requires RNA and protein synthesis (Ellis and Horvitz, 1986; Oppenheim et al., 1990). The loss of selective permeability typically does not occur until the end of this death process, at which time the cell breaks down into several fragments that are

often phagocytosed by neighboring cells. In the developing *E. scolopes* light organ, however, cell death does not occur as a strict maturational event, but requires a signal that results from the infection of the light organ with symbiotic bacteria. Whether this signal activates such a 'suicide program' or works through a different mechanism is unknown.

Infection with symbiotic *V. fischeri* appears to induce the further differentiation of epithelial cells that form the bacteria-containing crypts, causing these cells to increase in size and change their morphology from columnar to cuboidal. Interestingly, the epithelial crypt cells of a field-caught juvenile 2.5 mm in mantle length (approximately 2-weeks old and naturally infected with symbionts) fixed under the same conditions as the experimental animals in this study are of a similar size and morphology as the non-ciliated, microvillous cells of the largest crypts in 3-day symbiotic juveniles (data not shown). Concurrent with this increase in size of the epithelial cells in the first pair of crypts of 3-day old symbiotic juveniles is an apparent increase in the volume of this tissue (Fig. 9). In contrast, the volumes of the epithelia of crypts 2 and 3 in symbiotic juveniles are not significantly different from those of aposymbiotic juveniles. The largest pair of crypts is the first pair to form embryonically; the second and third pair of crypts form relatively late in embryonic development and are consequently smaller (Montgomery and McFall-Ngai, 1993). Whereas the majority of cells in the first pair of crypts are of the non-ciliated microvillous type with the ciliated duct accounting for a relatively small proportion of the cells, the third pair of crypts is composed almost exclusively of ciliated duct with few non-ciliated cells. Thus, there are fewer non-ciliated microvillous cells, both in absolute number and in proportion to ciliated duct cells, in crypt 2 and even fewer in crypt 3. Because the volumes of the epithelial tissues of crypts 2 and 3 are small, and fewer cells in these crypts are of the cell type that undergoes further differentiation, any volume differences between infected and uninfected juveniles may be hard to detect by 72 hours post-hatch. Experiments using BrdU incorporation to study cell proliferation in juvenile light organs indicated that a majority of the non-ciliated cells in crypt 1 did not incorporate BrdU, suggesting that these cells have ceased dividing and are undergoing further or terminal differentiation. Because these epithelial cells directly interact with the bacterial symbionts and are presumably involved in the maintenance and regulation of the symbiont population, it is not surprising that they might need to undergo further differentiation to perform specialized physiological functions. In contrast, many of the cells in crypts 2 and 3 continue to proliferate at relatively high rates during the first 3 days post-hatch (Fig. 10); the morphology of the non-ciliated cells in crypt 3 is consistent with that of a population of rapidly proliferating cells. Possibly, many of the cells of the second and third pair of crypts are not yet fully competent to undergo further differentiation, even in the presence of symbiotic *V. fischeri*. It is even possible that the cells of the third pair of crypts in 3-day old juveniles in this study have not yet differentiated enough to initiate and/or sustain a bacterial infection, thus explaining the absence of bacterial symbionts in the fixed tissues. Interestingly, the third pair of crypts in the slightly more developed field-caught animal described earlier did contain bacterial symbionts as well as non-ciliated microvillous cells similar in size and shape to those of crypt 1. Thus crypt epithelial cells may need to

develop to a certain stage before they are competent to respond to any inductive signal sent out by the bacterial symbionts.

In conclusion, we have presented evidence that the postembryonic development of the light organ of *E. scolopes* requires specific interactions with its bacterial symbionts *V. fischeri*. The symbiotic bacteria influence such fundamental processes as cell death and cell differentiation in the host light organ. Whether symbiotic *V. fischeri* induce postembryonic morphogenesis of the light organ by affecting each of these processes directly or by affecting the expression of a host master regulatory gene is unknown. Further studies of this animal-bacterial mutualism should reveal the underlying genetic regulation of postembryonic light organ development.

We thank R. Zimmer for assistance with video microscopy and for helpful discussions and suggestions. We thank K. Boettcher and E. G. Ruby for providing *V. fischeri* ES114 strains, and S. Bottjer and K. Moses for helpful comments on the manuscript. This research was supported by NSF grant IBN 9220482 and the ARCS Foundation. This publication is HIMB contribution no. 937.

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(Accepted 24 March 1994)