Cytoskeletal Changes in Epithelial Cells During the Symbiotic Infection of *Euprymna scolopes* by *Vibrio fischeri*

Thesis

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by

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ABSTRACT

In the study of symbiotic relationships a system with only two species is ideal. The Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent gram-negative bacteria, *Vibrio fischeri,* establish such a relationship. *V. fischeri* infects the epithelial crypts of the light organ of *E. scolopes* within hours after hatching, and confers light emitting ability upon the squid. Changes of the cytoskeleton and volume increases of the epithelial cells lining the crypts are observed as a result of this infection. By using small, fluorescent probe molecules, characterization of cytoskeletal changes and quantitative measurement of the volume increases caused by *V. fischeri* infection of the epithelial crypt cells in *E. scolopes* was possible. These probes revealed microfilaments and microtubules in both infected and asymbiotic animals, including the circumferential microfilament bundles characteristic of epithelial cells and the axonemal cores of cilia. Also, tubulin-associated inclusions appeared in the apical regions of epithelial cells of infected crypts.

Previous literature on this symbiotic relationship has described the cell volume increase as cell swelling, an edematous response; however, this has not been empirically determined. As the light producing reaction of the bacteria directly consumes oxygen, it is possible that the bacteria cause local hypoxia during light emission and the cell volume increase of the epithelial crypt cells is a pathological response. An alternative explanation for the cell volume increase of infected epithelial crypt cells is a bacterial mediated hypertrophy. Therefore, I tested the hypothesis if cell volume increase and cytoskeletal reorganization are caused by hypoxia in infected squid, then a relationship should exist among cytoskeletal changes, cell swelling, light production, and oxygen consumption in

the light organ crypts. Oxygen uptake rates indicated that hypoxia could exist in the light organ crypts, however, the morphological change, as observed by transmission electron microscopy, was not typical of hypoxia-induced pathology. Epithelial crypt cells enlarged during the diel symbiotic cycle however, the increased volume did not appear as edematous swelling, but rather as hypertrophy of the cytoplasm and/or engorgement resulting from uptake of *V. fischeri*. The results did not support a role for hypoxia-induced cell swelling, and suggested the hypertrophic changes in the epithelial cells instead result from physiological interactions between the prokaryotic guest and the eukaryotic host.

INTRODUCTION

There are several eukaryotic/prokaryotic symbiotic systems known. Most systems, such as the mammalian intestinal tract and enteric bacteria, include mixed populations of prokaryotes. Multiple species confounds study of the system making it difficult to understand the eukaryotic/prokaryotic interaction. Therefore, studying a system with only two species is ideal for discerning cellular and morphological interaction.

The Hawaiian sepiolid squid *Euprymna scolopes* maintains a symbiotic relationship with a luminous bacterial partner, *Vibrio fischeri*. Although all animals have associations with microbes, this association is one of the few experimental models in which only two species are present. Upon hatching, the squid infect themselves with *Vibrio fischeri* by extracting the bacteria from surrounding seawater into a specialized light organ. The relationship begins within hours after hatching of *E. scolopes* (Ruby and Asato, 1993) when *V. fischeri* infects, colonizes, and persists in the light organ of the juvenile squid. This light organ is a bi-lobed structure located ventral to the ink sack in the center of the mantle cavity of the squid. There are two sets of three epithelial crypts, located laterally in the light organ, that house the bacteria. The crypts are lined with microvilli and each crypt opens to the outside of the squid through a pore. The squid is infected by *V. fischeri*, with the aid of two ciliated epithelial appendages (CEA), also located laterally. Once infected, the squid have a diel rhythm of venting 90-95% of the bacteria in the crypts at dawn (Lee and Ruby, 1994). This behavior not only allows for daily repopulation of bacteria in the crypts but also replenishes the supply of *V. fischeri* in the surrounding water for hatchling infection (Lee and Ruby, 1994; Boettcher *et al.*, 1996; Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998; McFall-Ngai, 1999). The

bacterial luminescence is used by the squid as counter-illumination camouflage when they feed at night near the ocean surface. By directing light downward from their ventral surface at a brightness equal to incident ambient light, they do not cast shadows; consequently, they become invisible to predators (McFall-Ngai, 1990).

It is known that cytoskeletal changes occur in the light organ and CEA of *E. scolopes* upon infection by *V. fischeri*. One such permanent change that occurs subsequent to infection is *V. fischeri*'s induction of apoptosis of the cells of the CEA of *E. scolopes* resulting in the reabsorption of this tissue (Foster and McFall-Ngai, 1998). A reversible change occurs in the cells lining the epithelial crypts of the light organ after infection. Epithelial cells in direct contact with the bacteria increase the density of microvilli on their apical surfaces and (appear to) swell four-fold in volume. Antibiotic treatment eradicating bacteria from the crypts of the light organ causes the microvillar density and the volume of the epithelial cells to be restored to that typical of unexposed host animals (Lamarcq and McFall-Ngai, 1998).

Previous work has pointed to oxygen, or the lack of it (hypoxia), as a possible signaling method by which the bacteria induce cell swelling (Visick *et al.*, 2000). If the cell volume increase of infected epithelial crypt cells is in fact cell swelling than this work would be supported by the many studies of the effects of hypoxia on muscle cells, particularly mammalian cardiac cells during episodes of hypoxia induced by ischemia (Koke *et al.*, 1982). As the luciferase reaction directly consumes oxygen, it is possible that the bacteria cause local hypoxia during light emission and the increase in cell volume is cell swelling, a pathological response. This could also explain the apparent necessity of expelling most of the bacteria every 24 hours to ensure healthy epithelial crypt cells.

Alternatively, other signaling pathways could be involved that result in cell hypertrophy, or enlargement of the cell without any loss of cell integrity, as opposed to cell swelling. Many such pathways have been characterized, such as the hypertrophy of parietal cells of the gastric mucosal glands caused by *Helicobacter pylori* infection (Rappel *et al.*, 2001). The infection of *Edwardsiella tarda* on the liver cells of the Japanese flounder *Paralichthys olivaceus* causes hypertrophy of these cells in the early stages of infection as well as preventing a decrease in liver cell number as compared to uninfected control starved fish (Miwa and Mano, 2000). The hypertrophy could be in preparation for provision of nutrients to the bacteria, and be necessary to support long periods of light production. Arguing in favor of this mechanism is the high degree of vascularity in the light organ, suggesting that light production is not oxygen limited (Boettcher *et al.*, 1996). The lack of oxygen probe molecules which could provide information about oxygen levels in the crypts of the light organ directly, and the lack of a simple and direct methods of measuring epithelial cell volumes in the light organ, have hampered clarification of these alternatives.

Therefore, I employed experiments to test the hypothesis that: if cell swelling and cytoskeletal reorganization occur and are caused by hypoxia in the light organ, then a relationship should exist among cytoskeletal changes, cell swelling, light production, and oxygen consumption. To test this hypothesis, I pursued the following aims:

1) development of staining methods using cytoskeletal-specific fluorescent probes for filamentous actin (microfilaments) and tubulin;

2) the use of these probes and confocal microscopy to characterize cytoskeletal changes and cell volume increases;

3) investigation of light production-dependent changes in morphology and oxygen consumption by comparison among asymbiotic squid (control), squids infected with wild type, *Vibrio fischeri* strain ES114, and squids infected with a luciferase-mutant *ΔluxA::erm Vibrio fischeri* strain KV150, unable to produce light and thus unable to use oxygen for the luciferase reaction; and

4) use of transmission electron microscopy (TEM) to determine if the apparent epithelial cellular volume changes are indeed swelling as previously described or are in fact hypertrophy. This distinction is important in determining the relevance of previous studies of the effects of hypoxia on cell structure, and bears directly on the hypothesis that hypoxia results in cell swelling in the light organ epithelium (Visick *et al.*, 2000).

MATERIALS AND METHODS

I. Squid and Bacteria.

A. Collection and Maintenance of Samples

Euprymna scolopes samples were provided by the Dr. M. J. McFall-Ngai research lab, and *Vibrio fischeri* strain ES114, wild type, and strain KV150, a *ΔluxA::erm* mutant, were provided by the Dr. E.G. Ruby research lab with the University of Hawaii, Pacific Biomedical Research Center (PBRC), Oahu, HI.

E. scolopes were sent via Federal Express either fixed in 4% paraformaldehyde (PF) in phosphate-buffered saline/0.45 M NaCl or as a clutch of eggs attached to rocks on half of a PVC pipe in Waikiki seawater. The latter were transferred to glass bowls of Instant Ocean, (Aquarium Systems, Mentor, OH), in deionized water to pH 8.2 and specific gravity 1.026, in a water bath at 25°C with aeration and an artificial diel light cycle of twelve hours light: twelve hours dark. Fresh Instant Ocean water (artificial seawater, ASW) was prepared every 2-3 days to replace the water in the hatching bowls. Hatching of the squid occurred between days 18-24 post-fertilization at the beginning of the dark cycle. Upon hatching, squid were transferred through three washes of ASW to prevent infection by residual bacteria associated with the egg clutch (McFall-Ngai and Ruby, 1991). The juvenile squid were held in ASW until an appropriate number of squid were hatched for infection by *V. fischeri* for each experiment.

V. fischeri strain ES114 and strain KV150 were received on Seawater Tryptone Agar (SWT-agar), a minimal medium (Ruby and Asato, 1993), streaked for isolation. For each strain, one colony was transferred to SWT-broth and incubated at room temperature

(21°C) overnight. Stock cultures were then prepared by adding 0.6 ml sterile 50% glycerol to 1.4 ml of the vortexed *V. fischeri* in SWT-broth to 2 ml cryo-vials and frozen at -80°C. To obtain working bacterial stocks the frozen stock was inoculated on SWT-agar and incubated at room temperature (21°C) a day before the egg clutch was scheduled to hatch. In the event the hatching was delayed, the bacteria were transferred to sterile SWT-agar plates every 2-3 days, not to exceed three transfers.

B. Infection of Nascent Light Organs

The following protocol was used for the experiments in section II. Confocal Microscopy and Morphometry, performed at the PBRC, and in section IV. Oxygen Consumption Determination, performed at SWTSU. The infection of squid for the experiment in section III. Cytoskeletal Structure of Crypt Cells as Viewed by Transmission Electron Microscopy (TEM), was prepared by Dr. S. Davidson at PBRC.

The protocol for infection of *E. scolopes* with *V. fischeri* was modified from a protocol previously described (McFall-Ngai and Ruby, 1991). In short, *V. fischeri* strain ES114 and strain KV150 were inoculated in SWT-broth and grown to log phase (6-9 hours). Just prior to the dark phase, squid were randomly pippetted into one of three hatching bowls, labeled control, ES114, and KV150, each containing 50 ml ASW. No more than 40 squid were placed into each hatching bowl because overcrowding will kill the squid. A 100-fold dilution of suspended bacterial cells in sterile ASW was brought to a concentration to seed 10^3 cells/ml ASW in each hatching bowl. It was imperative to dilute the SWT-broth in sterile ASW because a high concentration of media will kill the squid. The appropriate bacterial dilution was pippetted into the hatching bowls after the

dark phase began. Squid were considered infected after nine hours (Ruby and Asato, 1993; Davidson, 2000). Confirmation of infection of the squid was done for each experiment.

II. Confocal Microscopy and Morphometry

A. Sample Time Points and Fixation of Infected *E. scolopes*

4% PF (Electron Microscopy Sciences, Ft. Washington, PA) in 1X phosphate-buffered saline/0.50 M NaCl (PBS-S) was freshly prepared for fixation of the following sample groups:

1. Control - asymbiotic *E. scolopes*,
2. ES114 - *E. scolopes* infected with *V. fischeri* strain ES114, and
3. KV150 - *E. scolopes* infected with *V. fischeri* strain KV150, Δ*luxA::erm* mutant.

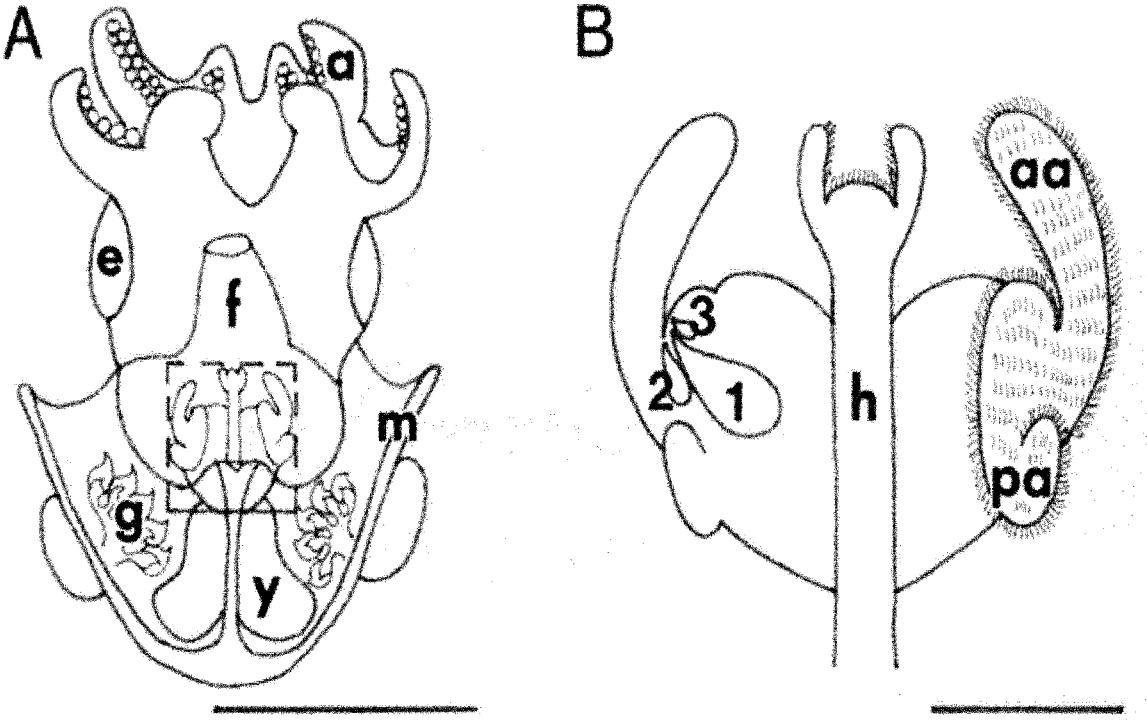
Five squid were fixed just prior to infection and labeled "Control, Time (T) 0." There were five squid fixed for each of the three sample groups for each of the following time points post-infection: T = 10 hours, 14 hours, 18 hours, 42 hours, 66 hours. Fixed symbiotic animals were stored in 4% PF in PBS-S in scintillation vials at 4°C. Because of the large sample size and because 4% PF in PBS-S is known to de-gas and lose fixation properties, frozen 4% PF in PBS-S was thawed once a week to replace fixative in unprocessed sample vials.

B. Dissection of Squid Light Organs

Each fixed, infected and control squid was placed in approximately 2 ml PBS-S in a watch glass under a dissecting microscope. The light organ of each squid was dissected out of the animal using an insect pin, forceps and micro-cutting tool (see Figure 1 for squid diagram). The micro-cutting tool was fashioned from a 4 mm section of one side

Figure 1

A diagram of the location of the light organ within *E. scolopes*. **A.** Ventral dissection shows the juvenile light organ (in dashed box) with the mantle (m) removed. An insect pin was placed anterior to the funnel (f) to hold the squid in place while forceps were used to make a vertical cut on the ventral surface of the mantle. The micro-cutting tool was used to cut away the tissue and free the light organ. Care was taken not to puncture the ink sac (located dorsal to the light organ), however, if this occurred the organ was washed with PBS-S and the ink was cleared away with a tuberculin syringe. Bar, 1 mm. a, anterior end; e, eye; g, gill; y, internal yolk sac. **B.** Morphology of pre-infected light organ (close-up of dashed box in A). Each set of anterior (aa) and posterior (pa) appendages is ciliated and collectively known as ciliated epithelial appendages (CEA). *V. fischeri* infect the light organ by entering pores leading to the crypts, numbered 1, 2, 3, which are located bi-laterally in the light organ. The numbers correspond to the crypts in the order they appear during embryonic development (Montgomery and McFall-Ngai, 1993). Bar, 150 μm. h, hindgut (Montgomery and McFall-Ngai, 1994).


A
a
e
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of a double edge razor blade, cut at an angle, inserted into a cut slot at one end of a broken cotton swab stick, and secured with nail polish (Ballinger, 1999).

Once all five light organs for each sample group were placed into PBS-S in micro-centrifuge tubes, they were washed three times with PBS-S for ten minutes each wash. The squid were either stained immediately or stored in PBS-S at 4°C for future use.

C. Staining and Mounting Protocol to View Crypt Epithelial Cell Volume Changes

The staining techniques described here were determined empirically through trial and error. The initial staining protocol to determine penetration of cytoskeletal stains employed Texas Red®-X phalloidin to stain actin instead of BODIPY® 650/655 phalloidin as listed below. Since Texas Red®-X phalloidin and propidium iodide (nucleic acid probe) are both seen in the red channel, the substitution was made to differentiate cell composition based on the LSCM channels available.

Staining techniques were performed under dark conditions, as fluorescent probes are light sensitive. (See Table 1. for sources and working dilutions.)

1. PBS-S was removed from sample micro-centrifuge tubes with a tuberculin syringe and discarded. Care was taken to avoid sucking up light organs into syringe. Paclitaxel, BODIPY® FL conjugate (subsequently referred to as Paclitaxel) was pippetted to cover the five light organs in each tube. All sample tubes were covered in aluminum foil and stored at 4°C for 18-24 hours.
2. The light organs were washed with PBS-S, three times for 10 or more minutes each wash.

3. The working solution of BODIPY® 650/655 phalloidin (Subsequently referred to as phalloidin) was pippetted to cover the five light organs in each tube. All sample tubes were covered in aluminum foil and stored at 4°C for 4-8 hours.
4. The light organs were washed with PBS-S, twice for 10 or more minutes each wash.
5. The working solution of propidium iodide was pippetted to cover the five light organs in each tube. All sample tubes were covered in aluminum foil and stored at room temperature (21°C) for 10-20 minutes.
6. The light organs were washed with PBS-S, twice for 10 or more minutes each wash. The samples were mounted immediately or stored in PBS-S at 4°C.
7. Just prior to observation of light organs by scanning laser confocal microscopy, each light organ was mounted in 100% glycerol on a standard microscope slide, covered with a cover slip and gently pressed between a paper towel.

D. Laser Scanning Confocal Microscopy

An Olympus IX-70/Bio-Rad MRC 1024 Laser Scanning Confocal Microscope (LSCM, Hercules, CA) was used to collect images of squid light organs stained with fluorescent probes. Image acquisition was done using Bio-Rad's Lasersharp software. Images were written to CD-ROM for storage. From the CD-ROM, image processing and analysis of cell volume was done using NIH Image (NIH, Bethesda, MD; http://rsb.info.nih.gov/nih-image/) software on a Macintosh G4 computer (Apple Computer, Cupertino, CA). Final image processing and printing were done using Adobe Photoshop software running on a PC clone and driving an Epson Stylus 800 color ink-jet printer.

Table 1. Fluorescent Probes used. All probes are light sensitive. All working and stock solutions were stored at –20°C.

Fluorescent Probes (Catalog #)	**Working Dilution**	**Stock Dilution**	**Marker for**	**Manufacturer**
Paclitaxel, BODIPY® FL conjugate (BODIPY® FL Taxol) (# P-7500)	40 µM in PBS-S*	10µg in 125 µL DMSO	tubulin	Molecular Probes, Eugene, OR
BODIPY® 650/655 phalloidin (#B-12382)	1:40 in PBS-S**	300U in 1.5 ml methanol	filamentous actin (microfilaments)	Molecular Probes, Eugene, OR
Texas Red®-X phalloidin (#T-7471)	1:40 in PBS-S**	300U in 1.5 ml methanol	filamentous actin (microfilaments)	Molecular Probes, Eugene, OR
Propidium iodide (# P-1304)	1000X in PBS-S	1.5mM in PBS-S	nucleic acid	Molecular Probes, Eugene, OR

*Paclitaxel working solution: 41.6 µL stock solution was pippetted into a micro-centrifuge tube with 125 µL PBS-S. Each manufacturer vial yielded three tubes of 40 µM working solution. Each tube was used up to three times with freezing at –20°C between each use.

**phalloidin working solutions: One part stock solution was pippetted into a micro-centrifuge tube. The tube was left uncapped and covered with foil. After evaporation of phalloidin, 40 parts PBS-S was added. Each tube was used up to three times with freezing at –20°C between each use.

E. Morphometry

In the crypt epithelium, initial results showed the microfilament cytoskeleton essentially unchanged throughout the diel cycle. The microtubular cytoskeleton was limited to the sub-membrane cortex and thus provided a convenient label for the epithelial cell boundary. A large "dark" bounded by tubulin appeared to increase in volume during the diel cycle, and this area – which initially was thought to represent the nucleus – stained positive with propidium iodide. The Paclitaxel fluorescence, which stained tubulin, was recorded in a separate channel from the propidium iodide fluorescence.

Confocal image stacks from each specimen were loaded into NIH Image and the separate tubulin (Paclitaxel) and propidium iodide images were compared side by side. Five to ten cells were selected from crypt epithelia for volume measurements. The cells were assumed to be ellipsoidal solids and their major and minor axes determined in the XY plane at the center of the cell. The image stack was then re-sliced in the Z direction to permit measurement of the cell thickness, and volume calculated by the product of XYZ. Other volume determining algorithms were tried but none gave significantly more consistent results. The total cell volume and the propidium iodide-positive volume were both determined.

Measurements were grouped by experimental time, meaning that all values from several squids at each time point were combined. Therefore, N = number of epithelial cells, not the number of squid. Significance of differences was determined by single factor analysis of variance (ANOVA) and ANOVA post hoc Fisher's PLSD for Volume, as appropriate. A "P" of 0.05 or less was taken to indicate significance of difference.

III. Cytoskeletal Structure of Crypt Cells as Viewed by Transmission Electron Microscopy (TEM)

To determine if increases in cell volume resulted from swelling or hypertrophy, and to possibly identify the nature of the propidium iodide-positive volume in enlarged cells, some squids were fixed for TEM. After hatchling infection, as described in section B, symbiotic and asymbiotic squid were fixed in freshly prepared 2% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) /2% PF (Electron Microscopy Sciences, Ft. Washington, PA) (Foster and McFall-Ngai, 1998), in PBS-S at 24 hours post-infection. After fixation of more than 24 hours at 4°C, the light organs were washed in PBS-S and then dissected. The light organs were post fixed in 1% osmium tetroxide, then processed routinely for TEM. After preliminary sectioning of samples embedded using the standard protocol for Spurr's epoxy resin (SER) (components from Electron Microscopy Sciences, Ft. Washington, PA) failed because the resin was too soft, the protocol was modified to achieve a harder resin. The amount of DER 736 Resin used was five grams instead of six grams per the standard protocol and the amount of ERL 4206 (vinyl cyclohexene dioxide) used was eleven grams instead of ten grams per the standard protocol. Thin sections were stained with 2% aqueous uranyl acetate for two hours and examined in a JEOL 1200 EX TEM (Boston, MA). Micrographs were taken of representative samples.

IV. Oxygen Consumption Determination

Dissolved oxygen measurements were made using a Traceable Digital Oxygen Meter (Control Company, Friendswood, TX). Values were recorded as mg/L using the auto-calibrate function of the meter against room air. All measurements were made at room

temperature (21°C). Measurements of *V. fischeri* and squids were made in a small polyethylene cup that fit tightly around the oxygen probe tip and prevented contact with room air. A small magnetic stir bar was included in the cup to maintain fluid flow across the probe tip during oxygen measurements.

All measurements were performed on 2.5 ml volumes in triplicate. Measurements of oxygen uptake on *V. fischeri*, strains ES114 and KV150 were made on cells from stationary phase cultures at a density of $6.9x10^8$ and $9.7x10^9$, cells/ml respectively. Measurements on squids were made on groups of 10 squid/2.5 ml of ASW.

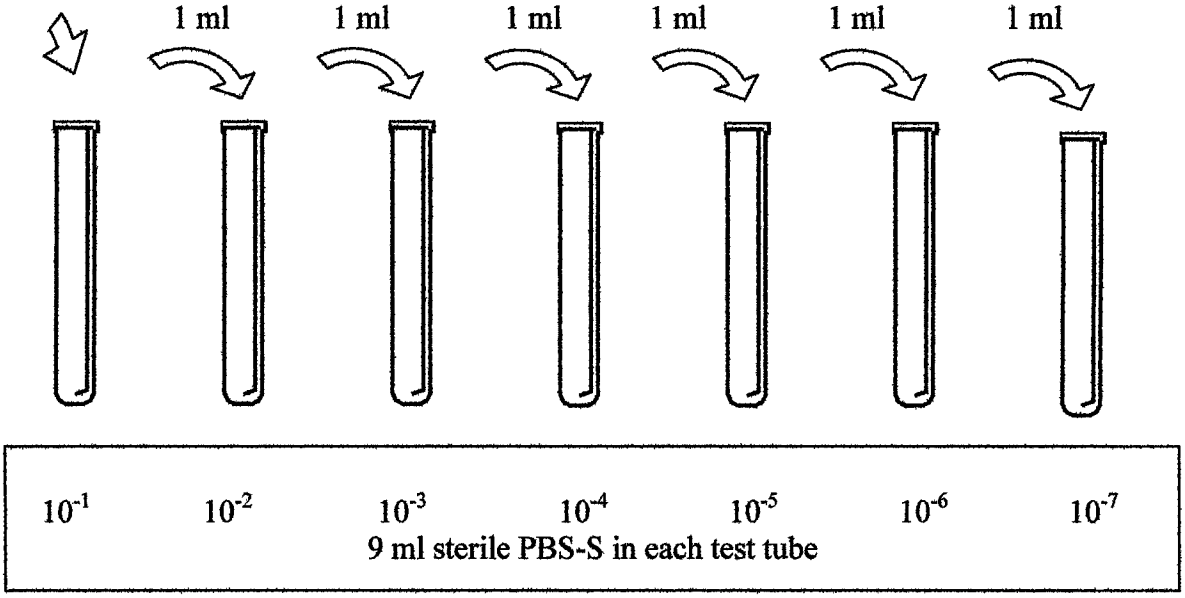
Preliminary experiments showed oxygen uptake by both squids and bacteria under these conditions to be linear with respect to time for 60 to 90 seconds. The rate of oxygen consumption was therefore determined in each case by subtraction of the 60-second oxygen concentration level from the initial measured level and then expressed as mg/L/minute/organism.

After recording oxygen uptake rates in squids, the animals were removed from the chamber and representative squid were aseptically dissected (dissecting tools were autoclaved for 15 minutes at 121°C, 15 psi) to remove the light organs for enumeration of the *V. fischeri* content associated with the light organ. Each dissected organ was placed in a sterile stomacher bag (Fisher Scientific, Pittsburgh, PA) with one ml sterile PBS-S with 0.1% Polyoxyethylene-Sorbitan Monolaurate, Tween 20 (Sigma, Saint Louis, MO). The light organ was macerated in the bag and then sonicated for five minutes in a tabletop ultrasonic cleaner, model FS60 (Fisher Scientific, Pittsburgh, PA). A 1:10 serial dilution was performed in sterile PBS-S water blanks; homogeneous suspension of the bacterial cells was achieved by using a tabletop vortex (Scientific Industries, Inc., Springfield,

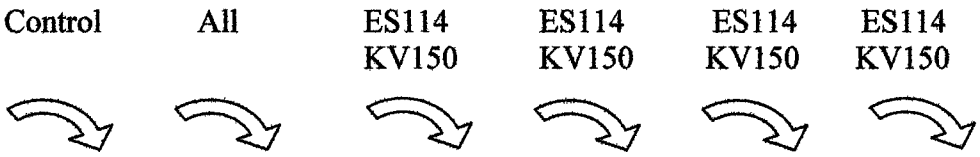
MA) for 10 seconds between each dilution, and plating in triplicate on SWT-agar (See Figure 2 for dilution scheme). Plates were incubated at room temperature (21°C) for a minimum of 48 hours but not more than four days. Colony forming units (CFU) of *V. fischeri*, identified on the basis of colony morphology, were counted and recorded.

As it was not possible to measure oxygen levels in the light organ in situ, the rate of oxygen uptake by *V. fischeri* in the light organ was estimated by multiplying the number of CFU recovered from the squid by the oxygen consumption rates measured in pure cultures of *V. fischeri* strains ES114 and KV150. This method is based on the assumption that all the *V. fischeri* associated with the squid are in the light organ and that CFU determination counts 100% of the *V. fischeri* in the light organ. It was not possible to independently verify these assumptions, however, sufficient differences among experimental groups were observed to determine statistical significance. Statistical analysis was performed as described above for morphometric analysis.

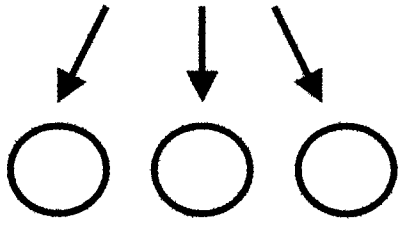
Figure 2. Dilution scheme for serial dilution to enumerate *V. fischeri*.



Sample sets plated (Control, ES114, KV150):



0.1 ml spread on each plate



Triplicate SWT-agar plates were spread for dilutions 10^{-2} through 10^{-7}

RESULTS

A. Cytoskeletal Staining

Initial staining of *E. scolopes* light organs using the small probe molecules Texas Red®-X phalloidin which stained filamentous actin (microfilaments) and Paclitaxel, BODIPY® FL conjugate which stained tubulin, revealed microfilaments and microtubules in both infected and asymbiotic animals, including the circumferential microfilament bundles (CMB) characteristic of epithelial cells and the axonemal cores of cilia (Figure 3).

B. Crypt Epithelial Cell Volume Changes

To track epithelial cell volume changes of the crypt epithelial cells of infected and asymbiotic squid, Paclitaxel, BODIPY® FL conjugate and propidium iodide were used and were allowed characterization of cytoskeletal changes in association with *V. fischeri* infection of the epithelial crypts in *E. scolopes*.

Epithelial cell swelling was apparent in infected crypts (Figure 4), and tubulin-associated inclusions appeared in the apical regions of these cells (Figure 5).

Figure 3

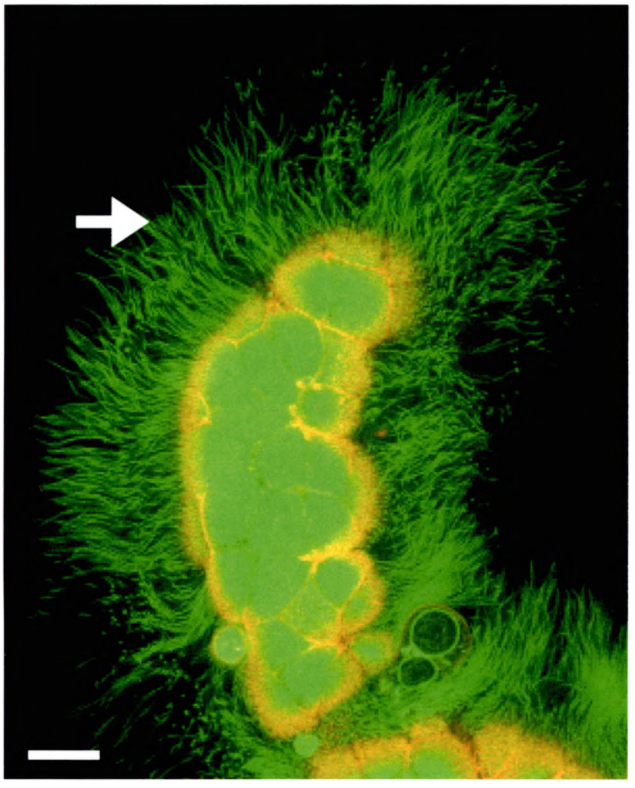
Laser Scanning Confocal Microscopy images of parts of the *E. scolopes* light organ. Paclitaxel, BODIPY® FL conjugate stained tubulin (green) and Texas Red®-X phalloidin stained filamentous actin (microfilaments) (red). All bars, approximately 40 μm.

A. Uninfected *E. scolopes*, CEA, Paclitaxel, BODIPY® FL conjugate stained tubulin, showing axonemal cores of cilia (arrow). Texas Red®-X phalloidin stained actin.

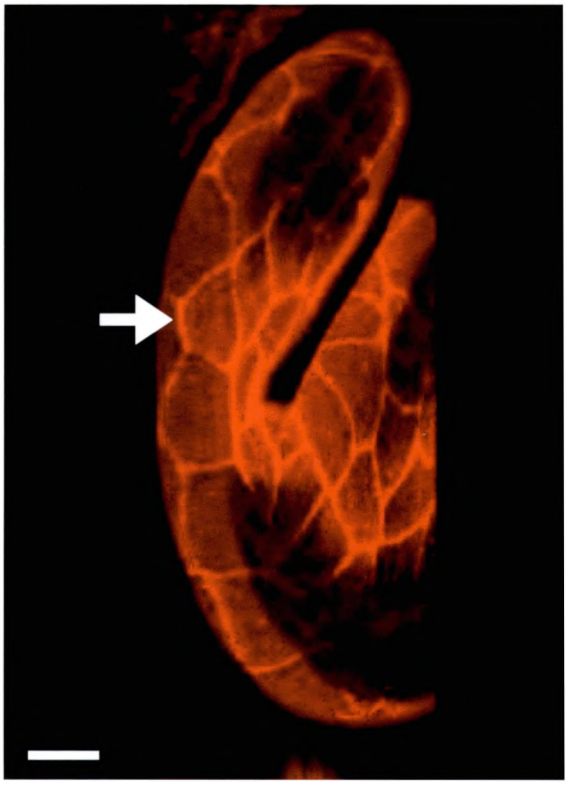
B. Uninfected *E. scolopes*, CEA, Texas Red®-X phalloidin stained actin only. Circumferential microfilament bundles (CMB) (arrow).

C. Uninfected *E. scolopes,* duct leading to crypt space. Texas Red®-X phalloidin stained actin only. Center duct space shows microvilli. Circumferential microfilament bundles (CMB) (arrow).

A.



B.



C.

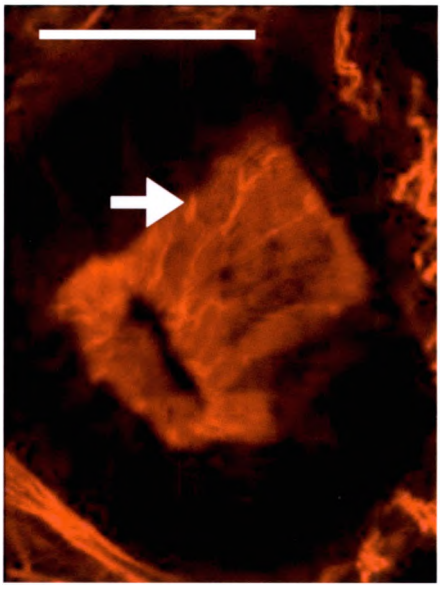


Figure 4

LSCM image of infected *E. scolopes* crypt epithelial cells, approximately 18 hours post infection. Paclitaxel, BODIPY® FL conjugate stained tubulin (green) and Texas Red ®-X phalloidin stained filamentous actin (microfilaments) (red). Microvilli (blue arrow), and CMB (yellow arrow), composed of actin, stained red. Tubulin associated inclusions (white arrow) stained green.

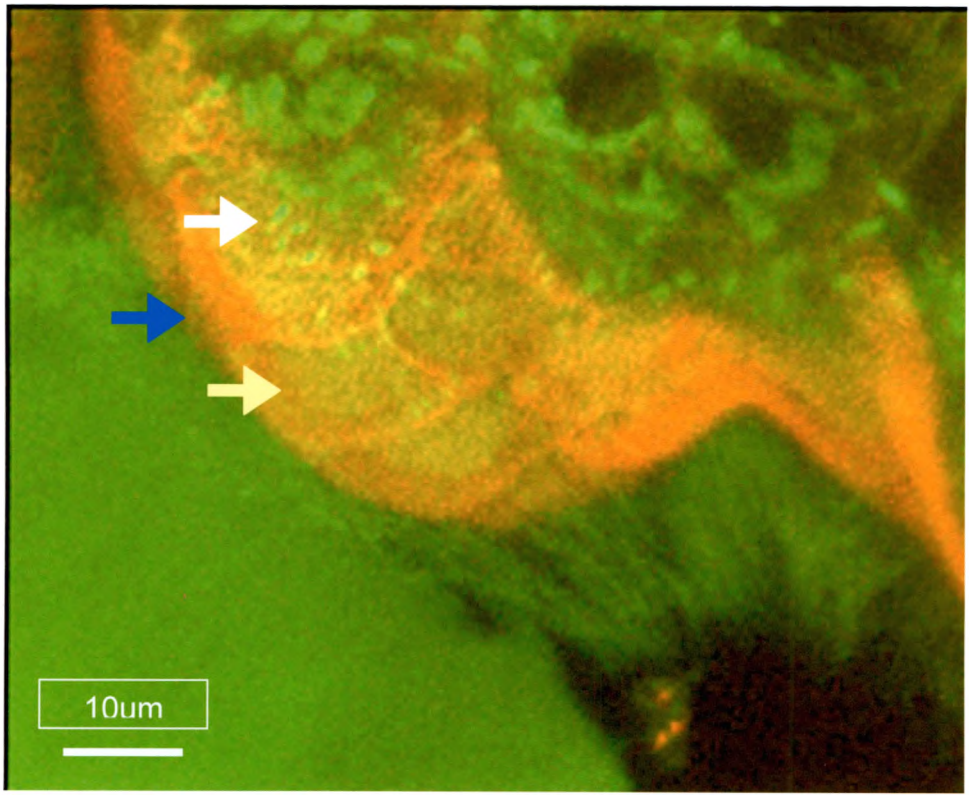

10um

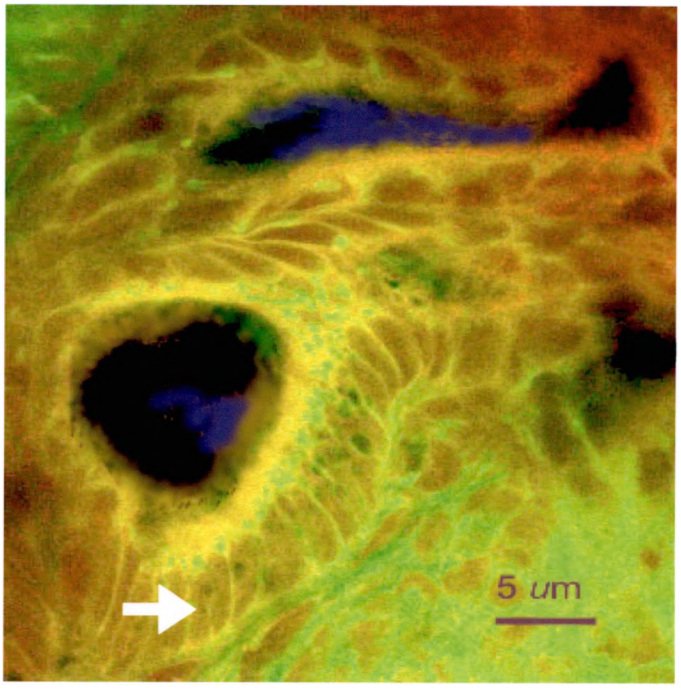
Figure 5

LSCM images of infected *E. scolopes* crypt epithelial cells, 18 hours post infection. Paclitaxel, BODIPY® FL conjugate stained tubulin (green) and Texas Red®-X phalloidin stained filamentous actin (microfilaments) (red).

A. Uninfected *E. scolopes* epithelial crypt cells. Cell shape is columnar (arrow).

B. *V. fischeri* ES114 infected *E. scolopes* epithelial crypt cells. Cell shape is ellipsoidal. Tubulin associated inclusions in apical region of the cell (white arrow) stained green. Microvilli brush border of crypt (blue arrow) stained red.

A.



B.

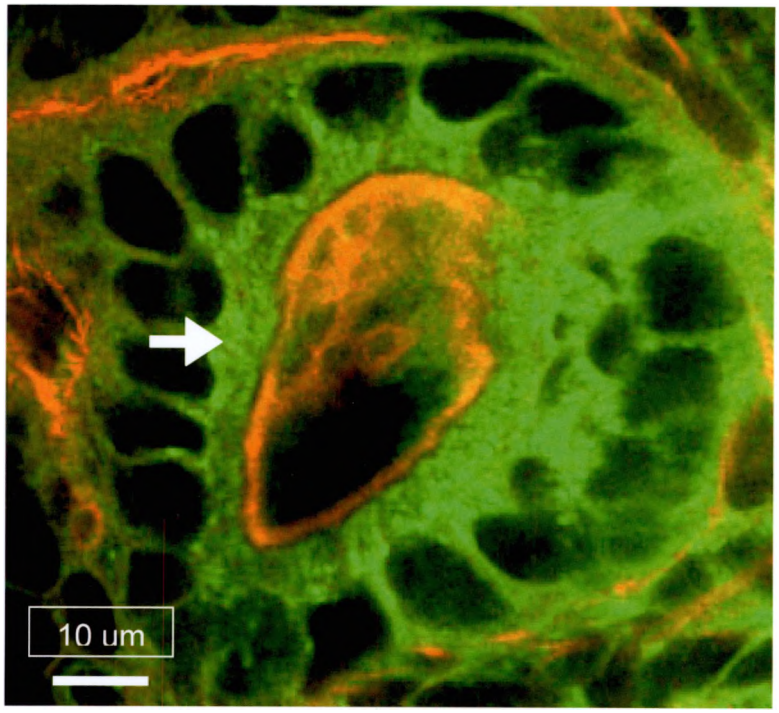


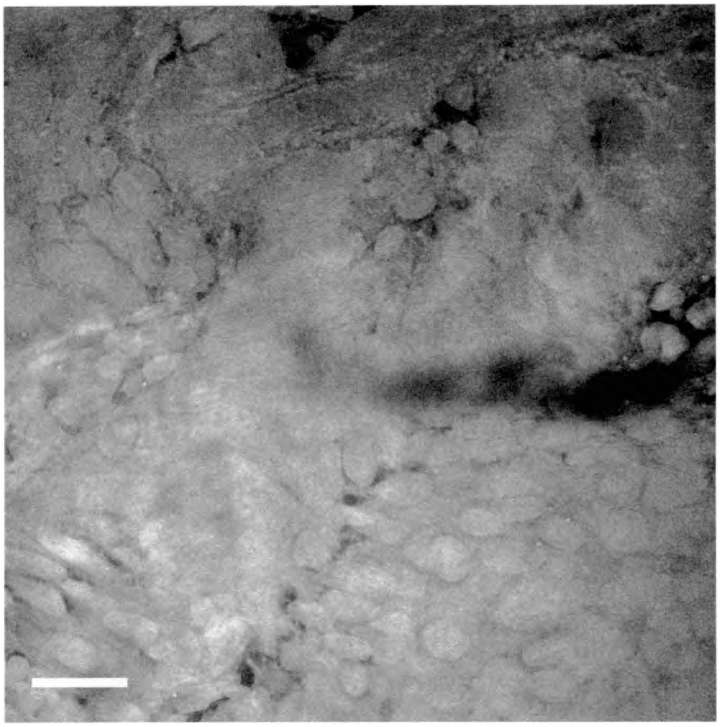
Figure 6

LSCM image with NIH Imaging software. Epithelium of the crypt of *E. scolopes* infected by *V. fischeri* ES114, 14 hours post-infection. Both images are approximately the center slice of the stack. Gray scale, light area represents stain. Bars = 40 μm.

A. Propidium iodide stained nuclear inclusions initially thought to be swollen nuclei. (See TEM results).

B. Paclitaxel, BODIPY® FL conjugate stained tubulin. Measure of X, Y, and Z axes taken within dark bound cell area.

A.



B.

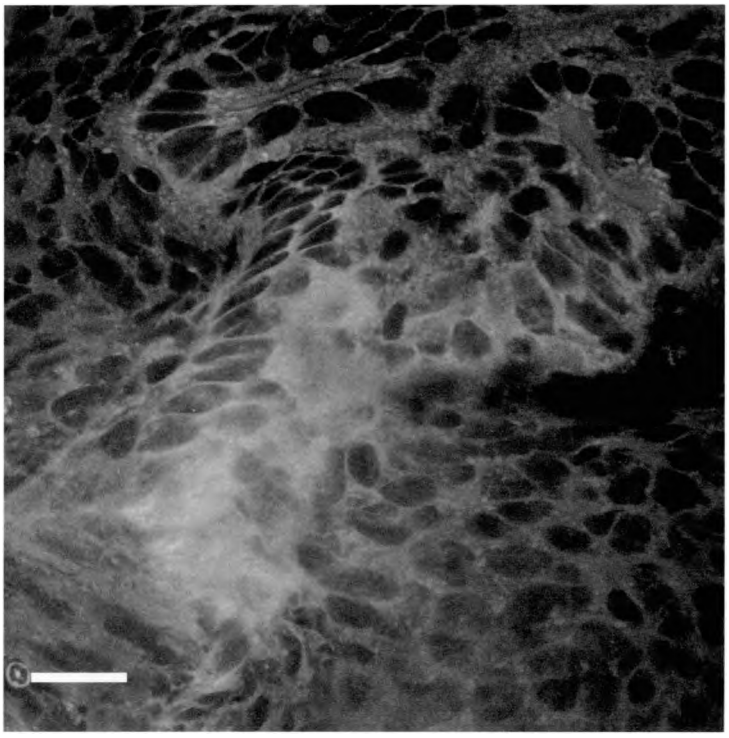


Figure 7

Bar graph of average crypt epithelial cell volumes for control, *V. fischeri* ES114, and *V. fischeri* KV150 infected sample groups, N=10 cells. Error bars in the figures represent Standard Error of the Mean (SEM).

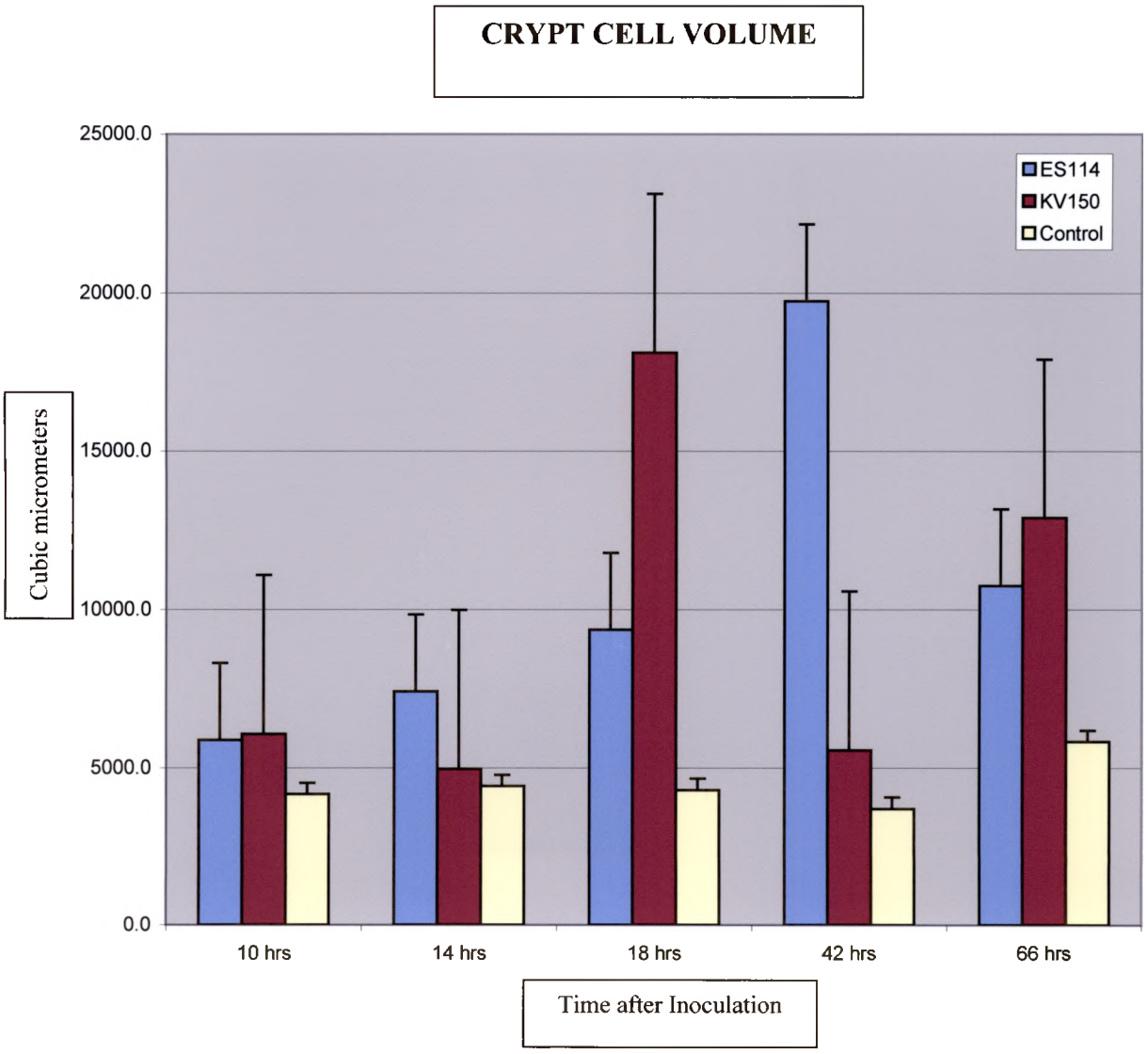

CRYPT CELL VOLUME
25000.0
20000.0
15000.0
10000.0
5000.0
0.0
Cubic micrometers
ES114
KV150
Control
10 hrs
14 hrs
18 hrs
42 hrs
66 hrs
Time after Inoculation

Table 2. ANOVA Post Hoc, Fisher's PLSD for Volume

P-values for each sample group compared to each of the other sample groups is listed below. An "*" denotes a significant difference ≤ 0.05.

Sample Groups	**10 hrs.**	**14 hrs.**	**18 hrs.**	**42 hrs.**	**66 hrs.**
Control ES114	0.0169 *	0.0007 *	<0.0001*	<0.0001*	0.0056 *
Control KV150	0.0083 *	0.4872	<0.0001*	0.0775	0.0002 *
ES114 KV150	0.7656	0.0042 *	<0.0001*	<0.0001*	0.1992

C. Cytoskeletal Structure as Shown by TEM

Electron microscopy of light organs revealed striking differences among the three sample groups. *V. fischeri* ES114 and KV150 infected light organs were compared with light organs from squids never exposed to bacteria (control). Epithelial cells of control animals were uniform in size, appeared mononucleate, and generally unremarkable. In comparison, cells from squids infected with *V. fischeri* ES114 appeared substantially enlarged and contained a cytoplasm full of vesicles consistent in size and appearance with remnants of bacteria or badly fixed mitochondria. These features were not present in sections of control or *V. fischeri* KV150 infected animals (Figures 8 and 9).

In addition, cells of *V. fischeri* ES114 infected animals had extensive areas of rough endoplasmic reticulum and the nuclei contained one or more large, very well developed inclusions that may or may not be nucleoli (Figure 10). In some cases, it appeared that one or more of the epithelial crypt cells had fused, creating large, multinucleate structures. While cells of the *V. fischeri* KV150 infected light organs appeared slightly enlarged in thin sections, neither these cells or control cells showed the nuclear or rough endoplasmic reticular changes that were obvious in cells of *V. fischeri* ES114 infected animals (Figure 10).

The volume increase apparent in cells of *V. fischeri* ES114 infected light organs did not appear to be a result of edematous cell swelling, as the entire intracellular volume appeared filled more or less uniformly with cytoplasmic structures. Clear voids, as seen in cells subject to edematous swelling, were not present.

Figure 8

TEM micrograph of control epithelial crypt cells of *E. scolopes*. The nucleus (N) is of normal size, 5 μm. There is no indication of edematous swelling by holes in the cytoplasm (Cy). Cr, crypt space; mv, microvilli.

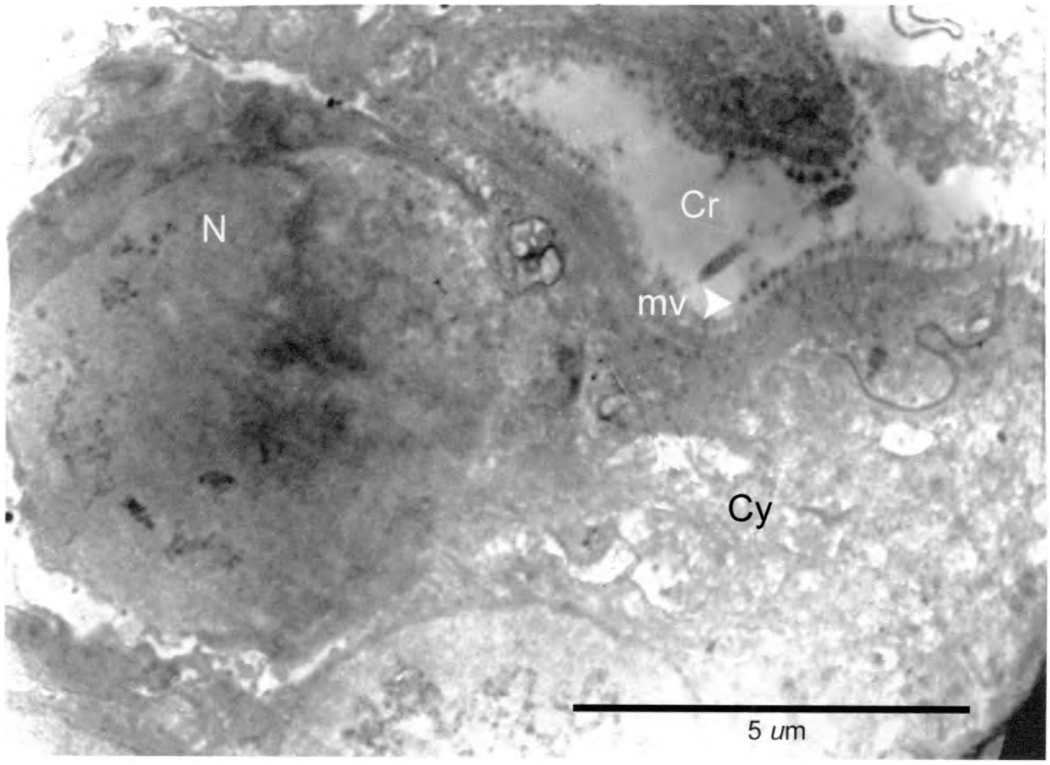

N
Cr
mv
Cy
5 um

Figure 9

TEM micrograph of epithelial crypt cells of *E. scolopes* infected by *V. fischeri* KV150. The nucleus (N) is of normal size. There is no indication of edematous swelling by holes in cytoplasm. n, nucleolus.

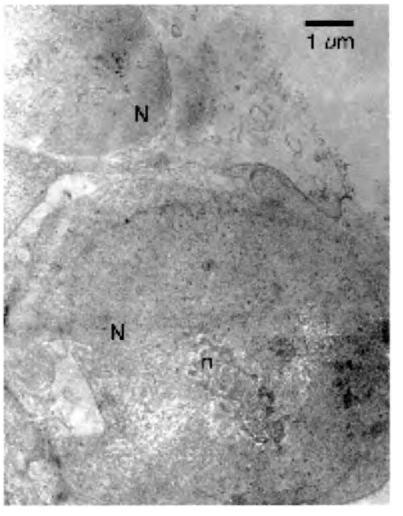
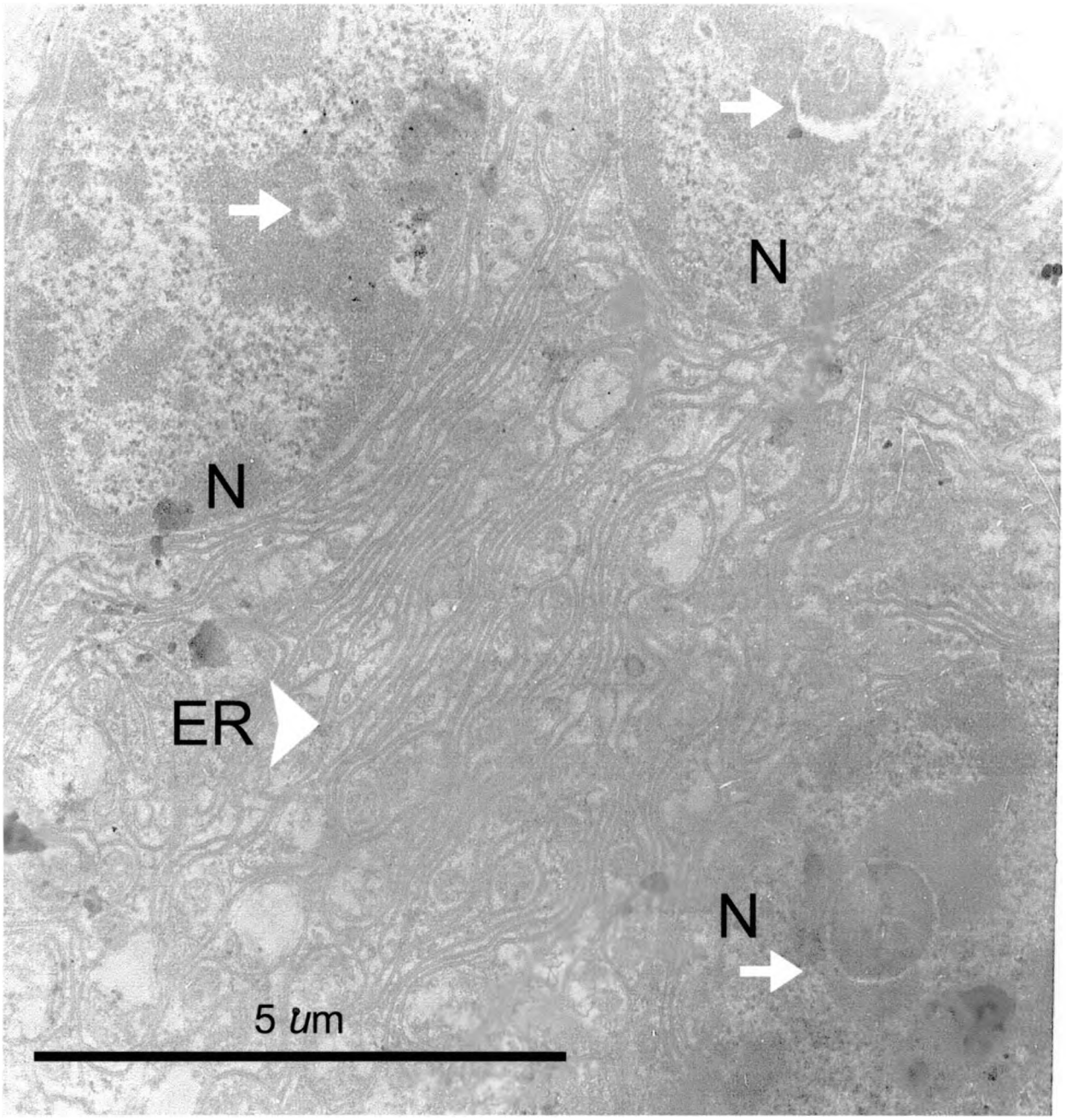

1 um
N
N
n

Figure 10

TEM micrograph of epithelial crypt cells of *E. scolopes* infected by *V. fischeri* ES114. There is no indication of edematous swelling by holes in the cytoplasm. There is more rough endoplasmic reticulum (ER) found in this sample group than in the control or *V. fischeri* KV150 sample groups. The abnormal inclusions in the nuclei (N) are indicated by arrows.


N
N
ER
N
5 um

D. Oxygen Measurements

Measurements of oxygen uptake by squid and bacteria, taken individually and in symbiosis, indicated that the production of light is correlated with a substantial metabolic cost (Table 3). By comparison of squids infected with wild type *V. fischeri* ES114 to *V. fischeri* KV150, Δ*luxA::erm* mutants, it was possible to estimate an oxygen cost of making light. For squid 18 hours post-infection (pre-dawn), the average difference in oxygen uptake due to *V. fischeri* ES114 and KV150 was 8.99×10^{-3} mg O_2/L/minute/squid. This corresponds to 4% of the total oxygen uptake of *V. fischeri* ES114 infected squids but only 0.2% of that of *V. fischeri* KV150 infected squids. Thus, it appears possible that oxygen uptake by *V. fischeri* could cause hypoxic conditions within the light organ.

Table 3. Oxygen uptake.

Organism	N	Average mg O_2/minute/ organism	Variance	P for ES114 vs. KV150
ES114/squid	6	0.00947	0.000024	
KV150/squid	6	0.00049	0.000059	*0.001
Squids alone	6	0.23	0.00818	
Squids + ES114	6	0.24	0.00124	
Squids + KV150	6	0.25	0.00269	0.86
		Percent total		
% of total oxygen uptake ES 114	6	4.055	3.995	
% of total oxygen uptake KV150	6	0.203	0.010	*0.008

*Values indicate significant differences.

DISCUSSION

The research performed to test the hypothesis: if cell swelling and cytoskeletal reorganization are caused by hypoxia in the light organ, then a relationship should exist among cytoskeletal changes, cell swelling, light production, and oxygen consumption, produced some conflicting results. Specifically, the oxygen consumption data indicates hypoxia may be occurring in the crypt spaces, which would cause cell swelling; however, the TEM data suggests that the cell volume enlargement is due to hypertrophy not cell swelling. The following sections examine each stage of this research.

A. Cytoskeletal Staining

There were several ways to approach the problem of staining cytoskeletal structures in the epithelial crypt cells of *E. scolopes*. One method to examine changes in the cytoskeletal structure of these cells would have been to use antibody-conjugated labels, however, the antibody conjugates are too large to penetrate the crypt space for adequate identification. Although small fluorescent probes have been utilized in staining cytoskeletal structures of *E. scolopes* light organs (Lamarcq and McFall-Ngai, 1998), the ability to view cytoskeletal structures within the crypt epithelial cells using Laser Scanning Confocal Microscopy (LSCM) had not yet been achieved. It was determined that the staining protocol developed in this study using the small probe molecules, BODIPY 650/665 phalloidin to stain filamentous actin (microfilaments) and Paclitaxel-BODIPY FL to stain tubulin, allowed penetration into the epithelial crypt cells to characterize cytoskeletal changes. By staining fixed light organs with these probes separately, the time of staining with Paclitaxel (18-24 hours) could be increased to ensure equal penetration into the epithelial crypt cells as that of phalloidin (4-8 hours).

C. Crypt Epithelial Cell Volume Changes

Differences of epithelial crypt cell volumes occurred among the time points post infection within both sample groups ES114 and KV150. The uninfected, control *E. scolopes* epithelial crypt cells showed no significant cell volume increase among all the sample time points, whereas infected light organs with *V. fischeri* ES114 showed differences in the cell volume increase of epithelial crypt cells through 42 hours post infection. There was a decrease in cell volume between *V. fischeri* ES114 infected cells between time points 42 and 66 hours post infection. This may be a result of the stage of the diel light cycle that the samples were collected (two hours earlier at 66 hours then at 42 hours) and the amount of bacteria housed in the crypt space at that time. Squid infected with *V. fischeri* KV150 showed a cell volume increase from 14 to 18 hours post infection, a decrease from 18 to 42 hours post infection and then an increase from 42 to 66 hours post infection. A previous study of *V. fischeri* KV150 showed no persistence of infection of the mutant strain in the crypt space after two days (Visick *et al.*, 2000). This could explain the decrease in cell volume of epithelial crypt cells of those squid infected with *V. fischeri* KV150 from 18 to 42 hours post infection.

The observed epithelial cell volume increases in the crypts of wild type *V. fischeri* ES114 infected squid in previous experiments have now been confirmed to be significantly different from that of the uninfected, control squid. The epithelial cell volume increases of the *V. fischeri* ES114 infected squid were significantly different from the control squid for all time points, therefore, infection of the light organ by *V. fischeri* ES114 causes cell volume increases in the epithelial crypt cells.

Alternating time points showed significant differences between epithelial crypt cell volumes of squid infected with *V. fischeri* KV150 as compared to the control and the middle three time points showed significant differences between epithelial crypt cell volumes of squid infected with *V. fischeri* KV150 as compared to *V. fischeri* ES114. However, there does not appear to be a definitive pattern of the effects of infection for these groups. Further research would have to be done to better understand this relationship.

C. Cytoskeletal Structure as Shown by TEM

The use of TEM to determine if the apparent epithelial cellular volume changes of infected crypts was due to cell swelling as previously described indicated that hypertrophy of these cells occurred at 24 hours post infection. Before this can be used to dispute previous studies which describe the cell volume increase as cell swelling (Visick *et al.*, 2000), further research must be done to determine if hypertrophy is present in these cells at all stages of infection.

The TEM micrographs revealed that the nuclei of all sample groups are of normal size. In the *V. fischeri* ES114 infected crypt epithelium, the apparent nuclear swelling that propidium iodide staining suggested as viewed by confocal microscopy indicates this staining is something other than nuclei. The unidentified vesicles in the TEM micrographs of *V. fischeri* ES114 infected crypt epithelium may be phagocytic vesicles containing bacteria. This would explain the large central area of epithelial cells stained by propidium iodide. This would be a novel finding once confirmed, as *V. fischeri* has been described as an extracellular symbiont to *E. scolopes* (McFall-Ngai, 1999).

The apparent increase in the amount of rough endoplasmic reticulum in the *V. fischeri* strain ES114 infected epithelial crypt cells suggests that the bacteria is signaling the squid to produce a protein to be excreted for their use. However, the identity of the protein and whether it is directly used for the luciferase reaction is not known.

D. Oxygen Consumption

The finding of hypertrophy, by TEM, in infected epithelial crypt cells bears directly on the hypothesis that hypoxia in the crypt space will result in cell swelling in the light organ epithelium. Since cell swelling was not apparent in the infected epithelial crypt cells whether hypoxia in the crypt space would cause cell swelling is irrelevant. It can be inferred from the oxygen consumption data that at 18 hours post infection, *V. fischeri* ES114 indirectly account for 4% of the oxygen consumed by the symbiotic squid whereas *V. fischeri* KV150 indirectly account for 0.2%. Whether this twenty-fold increase is due to light production by *V. fischeri* ES114 cannot be ascertained by this study.

E. Conclusions and Future Work

The cell volume measurement data showed a significant epithelial crypt cell volume increase of wild type *V. fischeri* infected squid, however the decrease in cell volumes of *V. fischeri* ES 114 and KV150 infected squid at later time points post infection suggest that cell volume corresponds to the stage of bacterial infection. The latter three time points tested in this study were all approximately six hours after dawn, when the squid purge the crypts of bacteria. It would be interesting to measure the volume of epithelial crypt cells of late time period, infected squid at different stages in the diel light cycle to correlate the influence of the amount and stage of bacterial growth on the cell volume.

The oxygen consumption data is wanting for lack of a direct method to measure available oxygen in the crypt spaces of uninfected and infected *E. scolopes.* Research toward the development of a light organ tissue culture would greatly improve the ability to directly measure oxygen in the crypt spaces. To accurately relate oxygen consumption to light production a mutant *V. fischeri* would have to be engineered that does not produce light, but does infect and persist in the crypt space and consumes an appreciable amount of oxygen as the luciferase reaction.

The apparent hypertrophy of the infected epithelial crypt cells as seen in the TEM study provides opportunity for future research. Further TEM work at different stages of infection during the diel light cycle would need to be performed to accurately identify the extent of the hypertrophy and whether it persists through all stages of infection. A quantitative measure of density differences of the epithelial crypt cells among the different sample groups could be performed by densitometry to definitively confirm hypertrophy. A TEM study could also include immuno-gold labeling with a *V. fischeri* antibody conjugate to determine whether the vesicles in the *V. fischeri* strain ES114 infected squid crypt epithelial cells contain the bacteria.

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