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Enhanced Production of ALDH-Like Protein in the Bacterial Light Organ of the Sepiolid Squid *Euprymna scolopes*

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Abstract. We localized one or more aldehyde dehydrogenase (ALDH)-like proteins in the bacterially bioluminescent light organ of the sepiolid squid *Euprymna scolopes,* and determined the temporal changes in expression through normal light organ development. Our previous studies have revealed that 70% of the total protein in the light organ lens of adult animals is comprised of an ALDH-like protein, which we called L-crystallin. In the present study, antibodies raised to this protein were used in immunocytochemical analyses which showed that, in adult light organ lens cells, ALDH-like protein was localized to the cytoplasm, but not to the nuclei or mitochondria. Labeling in adult tissue was also found in moderate abundance in the ciliated duct epithelium, a tissue that is in direct contact with the bacterial symbionts.

To determine the spatial and temporal onset of expression of ALDH-like protein(s), we examined light organs from juveniles at developmental stages before and after the differentiation of lens cells, which begins approximately 7–10 days after hatching. In 5-day symbiotic juvenile light organs, ALDH-like protein was not detected at levels significantly above those in non-symbiotic tissue of the same animals. However, expression of ALDH-like protein began within 10 days after hatching, seen first in a few cells of the ciliated duct, adjacent to the symbiont-containing tissue and in a few differentiated cells of the anterior presumptive light organ lens. These data suggest that, during normal development, induction of one or more ALDH-like proteins occurs simultaneously in both the lens and ciliated duct soon after the differentiation of lens cells.

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Abbreviations: ALDH, aldehyde dehydrogenase.

Introduction

Mutualistic associations between prokaryotes and higher eukaryotes are widespread in many ecosystems (Margulis and Fester, 1991). Many of these associations are characterized by symbiosis-specific organs or tissues, such as nodules in leguminous plants, bacteriomes in several orders of insects, and light organs in some species of bioluminescent fishes and squids (*e.g.,* Hastings *et al.,* 1987; Herring, 1988; Ishikawa, 1990; Nap and Bisseling, 1991). Studies of plant/bacterial associations have shown that the morphogenesis of symbiotic organs involves both the evolution of new proteins that occur only in the symbiotic organ, as well as enhanced expression of genes already present in the host genome (*e.g.,* Sanchez *et al.,* 1991; Verma *et al.,* 1992). In contrast, little is known about such cellular and molecular integration and regulation between the partners of animal/bacterial symbioses during the infection and maintenance of the symbiotic state. Thus, studies directed toward an examination of symbiosis-induced changes in gene expression and the patterns of protein occurrence are essential to reveal the nature of communication that must occur in these highly orchestrated associations.

The symbiosis between the Hawaiian sepiolid squid *Euprymna scolopes* and its luminous bacterial partner *Vibrio fischeri* is well suited as a model association for the study of host-symbiont interactions at this level (McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). Both host and symbiont can be independently raised in the laboratory, a feature that permits the experimental examination of the influence of the symbiotic state on host and bacterial gene expression. The host squid harbors a culture of *V. fischeri* in a complex bilobed organ in the mantle cavity (McFall-Ngai and Ruby, 1991) and is

thought to use its bacterially produced luminescence in anti-predatory behaviors (Moynihan, 1983). The bacteria-containing tissue consists of a network of epithelial cells lining crypts that house the bacteria (Fig. 1a; McFall-Ngai and Montgomery, 1990). Ciliated ducts are contiguous with these crypts and connect them with the outside of the light organ (the mantle cavity) by two pores. The bacteria-containing tissue is surrounded by a reflector and the ink sac, which serve to direct light ventrally. Finally, a thick pad of transparent tissue overlies the ventral surface of the organ and functions as a lens to refract light from the bacterial source into the environment.

During normal development, newly hatched squids become infected by free-living *V. fischeri* within hours after hatching (McFall-Ngai and Ruby, 1991). At the time of hatching, all components of the light organ, except the light organ lens, are differentiated and are simply elaborated during the further maturation of the light organ (Montgomery and McFall-Ngai, 1993). In contrast, the light organ lens cells first begin to differentiate approximately 7 to 10 days after hatching (Montgomery, pers. obs.).

Previous studies of the biochemical nature of the light organ of the adult squid have revealed that a significant portion (approximately 50%) of the total protein occurs as a 54 kD species, which was designated L-crystallin (Montgomery and McFall-Ngai, 1992). We found that the vast majority of this protein occurs in the light organ lens, which is protein rich to refract bioluminescent light effectively. Sequences of tryptic peptides derived from this protein, which had been purified from light organ lens, had a high sequence similarity to human cytosolic aldehyde dehydrogenase (ALDH, EC 1.2.1.3), a member of a large group of related enzymes that catalyze the conversion of aldehydes to carboxylic acids. Although L-crystallin apparently lacks ALDH activity, a polyclonal antibody raised against L-crystallin cross-reacted with 54 kD proteins, a characteristic size for ALDH subunits, from both cephalopods and mammals (Montgomery and McFall-Ngai, 1992). Anti-L-crystallin is therefore specific to ALDH-like proteins and not L-crystallin alone.

The high abundance of ALDH-like protein(s) provides a dramatic example of a gene product whose production is enhanced in a symbiotic organ. In this study, to determine where immunoreactive protein occurs during the maintenance of the symbiotic state, we examined the spatial pattern of their production in the adult light organ. Further, to determine how this pattern is achieved, we examined the temporal nature of protein production during normal development. Our results indicate that one or more of these proteins occurs in abundance not only in the light organ lens, but also in the ciliated duct, a tissue directly in contact with bacterial symbionts. During development, increased levels of these ALDH-like proteins are first seen simultaneously in the ciliated ducts and in newly differentiating lens cells. By following the occurrence of this group of proteins through the establishment and maintenance of the association, we might ultimately better understand how bacterial infection affects host gene expression in the light organ.

Materials and Methods

Maintenance of experimental organisms

Specimens of *E. scolopes* were collected from Kaneohe Bay on Oahu, Hawaii, in May, July, and November of 1991. The animals were kept in running seawater tables at the Hawaii Institute of Marine Biology (University of Hawaii) in Kaneohe Bay before being transported to 24°C recirculating seawater aquaria at the University of Southern California in Los Angeles. The animals were fed a variety of freshwater shrimp daily and kept on a 12 h light/dark cycle.

Clutches of eggs laid by captive females were removed to small recirculating aquaria, also at 24°C. Immediately upon hatching, juveniles were placed in small bowls of seawater from aquaria containing adult animals which regularly shed light organ symbionts. In this water, the juveniles became infected with *V. fischeri* within 12 h (Wei and Young, 1989; McFall-Ngai and Ruby, 1991). Juveniles could be maintained in good condition with daily water changes for up to 6 days. At the end of an incubation period, animals were either processed immediately or frozen in liquid N_2 and stored at −80°C.

Tissue fixation and embedding

To localize the ALDH-like proteins in the *E. scolopes* light organ, organs from both juvenile and adult squids were prepared for immunocytochemistry. *E. scolopes* were anesthetized with a 2:1 0.37 *M* $MgCl_2$:seawater mix for 5 min before dissection or processing. In adults, which range in size from 12 to 20 mm in mantle length, the ventral mantle and funnel were slit open to reveal the light organ, which could be easily removed. Juveniles, which are smaller than 4 mm in mantle length, were treated whole. The fixation protocol was modified from Erickson *et al.* (1987). Tissues were fixed for 1 h at room temperature in 1% paraformaldehyde and 1% glutaraldehyde in 0.1 *M* sodium phosphate buffer containing 0.45 *M* sodium chloride, pH 7.2, washed three times for 10 min each in a 0.1 *M* sodium phosphate buffer containing 0.45 *M* sodium chloride, dehydrated in 15, 30, and 50% methanol (MeOH) for 10 min each. The samples were fixed in a 2% uranyl acetate, 70% MeOH solution for 1 h, and further dehydrated for 10 min each in 85, 95, and 100% MeOH. The light organs were infiltrated with a 1:1 ratio of 100% MeOH and LR White resin (Ted Pella

Inc.) for 12 h at 4°C on a turntable. Subsequently, the specimens were rotated in 100% LR White for 72 h, transferred to fresh 100% LR White in gelatin capsules and allowed to polymerize for two days at 52°C.

Some light organs were also fixed for transmission electron microscopy to provide micrographs of well preserved tissues for orientation. These light organs were fixed for 12 h in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate, 0.45 *M* sodium chloride (NaCl), pH 7.4. Samples were post-fixed in 1% osmium tetroxide in the fixative buffer and further processed for histology as described in McFall-Ngai and Montgomery (1990).

Light microscopy

The antiserum used to locate ALDH-like proteins was made against a purified fraction of the light organ lens, designated L-crystallin, by Montgomery and McFall-Ngai (1992). The antiserum cross-reacts with ALDH-like proteins from both cephalopods and mammals.

Histological cross sections of light organs were cut with glass knives for light microscopy and dried onto gelatin-coated glass slides. The antibody incubation procedure was modified for light microscopy from the methods of Erickson *et al.* (1987). First, to block non-specific adsorption and binding, the 1-μm sections were incubated for 30 min in a blocking solution consisting of a 1:50 ratio of non-immune goat serum (Sigma, the same species as the secondary antiserum without the antibody) to a 10 m*M* sodium phosphate buffer, containing 150 m*M* NaCl, 0.05% NaN_3 and 0.5% bovine serum albumin (PBS/BSA). The slides were gently rinsed with PBS/BSA and then flooded with a 1:2000 dilution of primary (1°) antiserum: rabbit anti-L-crystallin in PBS/BSA for 2 h. The sections were rinsed twice in PBS/BSA for 10 min, then flooded with the secondary (2°) antibody solution, consisting of a 1:50 dilution of goat anti-rabbit IgG complexed to 15 nm colloidal gold spheres (Ted Pella Inc.) in PBS/BSA. The sections were then rinsed with deionized water (DH_2O) and stained with a silver enhancement kit (Sigma), which allows for the visualization of the immune complex with light microscopy due to the precipitation of silver granules in the presence of colloidal gold. Finally, the sections were counterstained with 1% acid fuchsin for 20 min, rinsed in water, dried, and mounted in heavy immersion oil.

In controls, to determine the degree of non-specific binding of the 2° antibody solution, a 1:2000 dilution of a pre-immune serum, obtained from the same rabbit as the immune serum before injection with L-crystallin, was substituted for the 1° antibody incubation. These sections were designated pre-immune preparations. Some untreated sections were stained with Richardson's stain (Richardson *et al.*, 1960) to provide companion histological sections for orientation and comparison.

Electron microscopy

Ultrathin sections of the light organs were cut with a diamond knife using a Porter Blumm MT2-B ultramicrotome and placed on nickel grids. For all incubations, grids were floated section-side down in solutions in 500 μl wells. The incubation procedure was the same as that described above for 1 μm sections except that grids were rinsed dropwise with PBS/BSA between antibody incubations. After the 2° antibody incubation and DH_2O rinse, grids were stained in 3% uranyl acetate and Reynolds lead citrate for 10 min each. As with the 1 μm sections, pre-immune grids were prepared to control for non-specific binding of the 2° antibody solution.

Sections of light organs were examined at 20,000× to look for the presence of 15 nm gold spheres on a JEOL 100CX transmission electron microscope at 80 kV. To quantify gold spheres in the tissues of the light organ from both immune and pre-immune incubations, spheres were counted in micrographs taken at 40,000× (n = 5; area = 4 μm^2) of each tissue.

Electrophoresis and immunoblotting

To look for the onset of ALDH-like protein production in *E. scolopes*, light organs from newly hatched juveniles and 5-day symbiotic juveniles were dissected and prepared for electrophoresis. Light organs and mantles from approximately 50 previously frozen juveniles per treatment were removed under a dissection microscope and homogenized in a Wheaton ground glass micro-tissue homogenizer in 50 μl of 50 m*M* sodium phosphate, 0.1 *M* NaCl, pH 7.0. Homogenates were spun at 15,000 × *g* for 15 min at 4°C in a Sorvall superspeed RC-5B centrifuge. The supernatants were decanted and kept at 4°C. A homogenate from adult light organs was used as a positive control for the presence of ALDH-like protein (Montgomery and McFall-Ngai, 1992). Concentration (mg/ml) of the soluble protein in each homogenate was determined spectrophotometrically (Whitaker and Granum, 1980).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out using a 12.5% resolving gel on a Hoefer SE 250 slab gel apparatus (techniques modified from Laemmli, 1970). Gels were run at a constant current (20 mA/gel) and stained with Coomassie blue. Eight μg of protein were loaded for all juvenile samples and a range of quantities, from 1 to 200 ng, was loaded for the adult light organ lens positive control.

For immunoblotting, proteins were electrophoretically transferred from unstained gels onto nitrocellulose membrane in a Hoefer TE 22 transfer apparatus for 2 h at 12°C at a constant current (200 mA) in a 25 m*M* Tris, 192 m*M* glycine, 20% methanol, 0.1% SDS buffer, pH 8.3. (modified from Towbin *et al.*, 1979). Immunoblots were performed using an amplified, streptavidin/biotin,

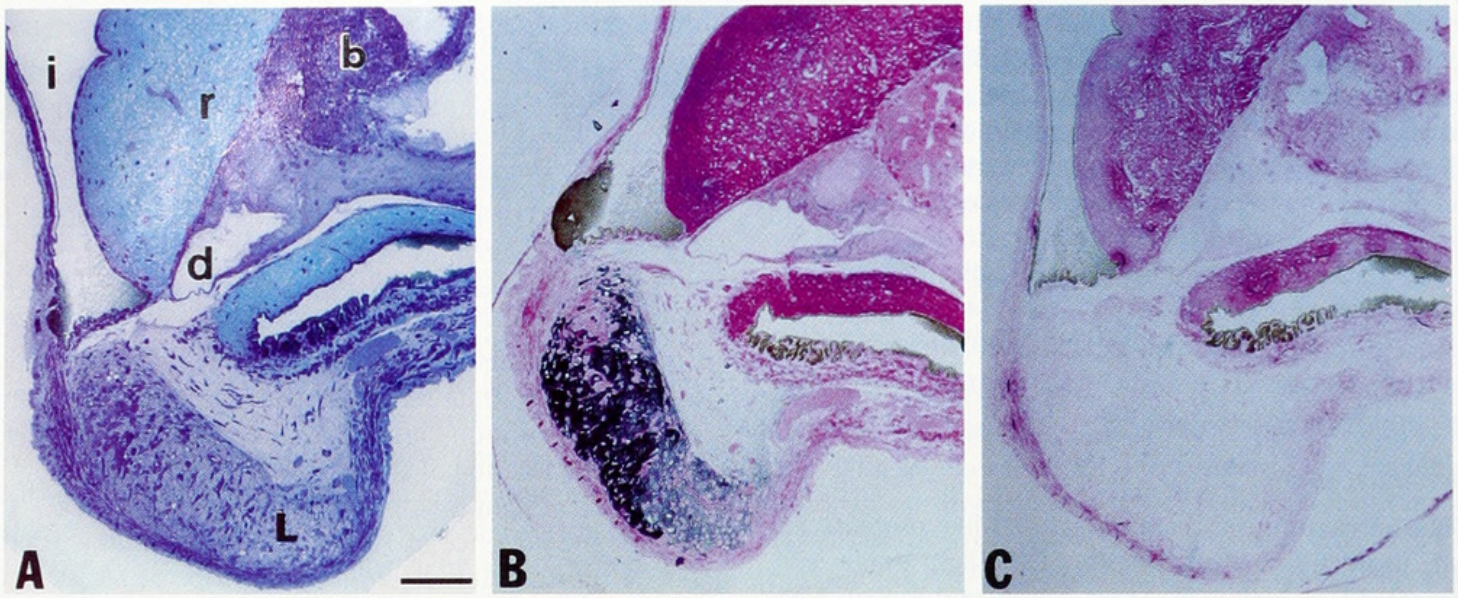

Figure 1. Cross sections through an adult *Euprymna scolopes* light organ. (A) Richardson's stained section: b, the inner bacteria-containing epithelium; d, the ciliated duct with its surrounding epithelium (duct does not reach the pore in this section); r, the reflector; i, the ink sac; and L, the light organ lens. Bar = 50 μm. (B) Silver-enhanced immune section, counter-stained with acid fuchsin. The silver granules indicate the presence of L-crystallin in the light organ lens (dense black) and of an ALDH-like protein in the ciliated duct epithelium (gray). The ink in the ink sac appears brown. (C) A pre-immune section lacks any dense black stain in the lens or duct epithelium.

alkaline phosphatase immune blot kit (BioRad), which was designed to detect as little as 10 pg of antigen. The membrane was incubated in a 1:1000 dilution of the 1° antiserum for 2 h and subsequently incubated and developed according to the manufacturer's directions.

Results

Localization of ALDH-like protein in adult light organs

To determine the location of ALDH-like protein in the adult *E. scolopes* light organ, immunocytochemistry, using anti-L-crystallin, was performed on cross sections of light organs at both the light microscopic and electron microscopic levels. In the 1 μm thick sections, L-crystallin was localized in very high concentrations in the lens of the light organ as evidenced by the dense black silver nitrate granules visible against the pink acid fuchsin background stain (Fig. 1b, see Fig. 1a for orientation). No such black precipitate was present in the pre-immune incubation (Fig. 1c). Residual ink, present in the ink sac, appeared brown in the immune and pre-immune sections. Although not as dramatic as the staining in the lens, silver nitrate granules were also present in the epithelial cells of the ciliated duct in the immune (Fig. 1b) but not in the pre-immune (Fig. 1c) preparations. In some sections, the medial portion of the lens stained more lightly than the lateral portion (Fig. 1b), however in other sections the lens appeared to stain uniformally along this axis.

Silver nitrate granules only precipitated over the cytoplasm of lens cells that stained lightly with Richardson's stain (Fig. 2a, b). The outer epithelial layer surrounding the lens, and muscle cells, shown in longitudinal section running through the lens and in cross section on the edge of the lens, lacked silver granules. Higher magnifications of ciliated duct showed the silver nitrate granules only in the cytoplasm of the epithelial cells, not in the cell nuclei or in the duct itself (Fig. 2c, d).

Ultrastructural examination of the *E. scolopes* light organ confirmed the patterns described in the histological sections. Colloidal gold spheres were found in high numbers in the lens of the light organ, in intermediate numbers in the ciliated duct, and in very low numbers (not significantly above background levels) in the ink sac, reflector, and bacteria-containing epithelial tissue (Table I).

Lens tissue is comprised largely of cells containing nuclei, mitochondria, and a diffuse cytoplasm containing numerous, thinly aligned elements, but lacking any fibrous muscle structure, obvious endoplasmic reticulum or Golgi complexes (Fig. 3a; McFall-Ngai and Montgomery, 1990). It was these cells in which high concentrations of colloidal gold spheres were found in immune (Fig. 3b) compared to pre-immune (Fig. 3c) preparations. The spheres were localized only in the diffuse cytoplasm, not in the mitochondria (Fig. 3d) or cell nuclei (Fig. 3e). There was no gradient of sphere numbers throughout the center of the lens or on the outside margin where gold spheres evenly covered the diffuse lens cells but were absent from muscle cells immediately adjacent to them (Fig. 4a). However, the cells at the inside margin contained a patchy distri-

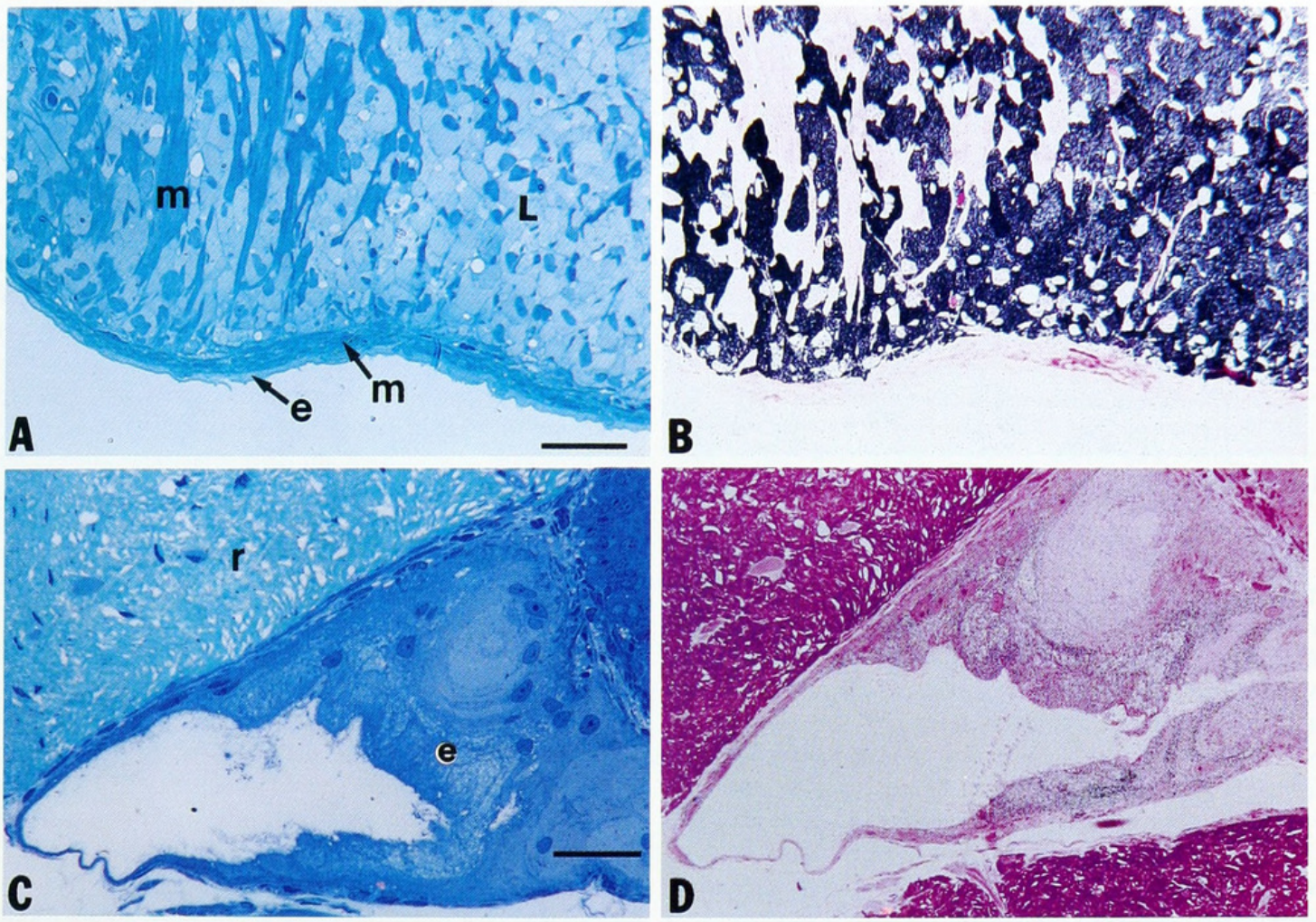

Figure 2. Detail of cross sections from Figure 1. (A) Richardson's stained light organ lens: e, outer epithelial layer; L, lightly staining lens cells comprising the majority of the lens; m, muscle cells running through the lens in longitudinal section and in cross section at the arrow. (B) Immune-treated light organ lens counter-stained with acid fuchsin. Only the cytoplasm of lens cells contains silver granules. Lens cell nuclei, and muscle and epithelial cells lack the black precipitate. (C) Richardson's stained area around the ciliated duct: e, epithelial cells lining the duct and r, reflector tissue. (D) Immune-treated ciliated duct epithelium counter-stained with acid fuchsin. The cytoplasm of the epithelium contains some silver granules. Bars = 20 μm.

bution of spheres where large areas of the diffuse cytoplasm were unlabeled (Fig. 4b).

The epithelial cells lining the duct, which connects the bacteria-containing crypts to the outside of the light organ, contain several cilia and prominent root hairs per cell (Fig. 5a, b). As in the lens cells, immune preparations of epithelial cells in the ciliated duct contained high numbers of gold spheres in the cytoplasm (Fig. 5c) compared to pre-immune preparations (Fig. 5d). Only background levels of spheres were found on the cilia, in the cell nuclei, and in the duct itself.

Localization of ALDH-like protein in E. scolopes *through development*

To determine the onset of ALDH-like protein production and to examine the pattern of production through development, light organs from animals of different sizes were tested for the presence of these proteins. Juvenile animals, collected from the field, with mantle lengths of 4.2, 3.0, 2.3, 2.0, and 1.8 mm were examined. In the 4.2 mm and 3.0 mm animals, the staining pattern was the same as it had been in adult light organs: the developing lens tissues stained a very dense black and the ciliated duct epithelial cells stained a light, more diffuse gray (data not shown). In the 2.3 and 2.0 mm animals, both the lens and the ciliated duct epithelium were positive for these proteins, but the lens, consisting of only a thin layer of differentiated cells at this stage (Fig. 6a), stained gray instead of a dense black (Fig. 6b). In some cases, staining in the ciliated duct was actually more intense than in the developing lens cells.

In the anterior ventral portion of the light organ of the 1.8 mm juvenile, a few dozen lens cells appeared to have

Table I

Number of colloidal gold spheres present in different tissues of the adult E. scolopes *light organ*

Cell type	Number of spheres per micrograph		*P* value
	immune	pre-immune	
Lens	314.2* (74.4)	2.4 (0.9)	<.001
Ciliated duct epithelium	157.4* (24.4)	1.6 (1.1)	<.0005
Bacteria-containing tissue	1.2 (0.8)	1.0 (1.0)	>.1

* Number of spheres in lens and ciliated duct are different by $P < .05$.

Each micrograph, taken at a magnification of 40,000× corresponds to 4 μm^2. Numbers are means ± (S.D.), n = 5 for each treatment. *P* values are results of two-tailed paired *t*-test.

differentiated from a pad of mesodermal cells (presumptive lens). In light microscopic immune preparations, these cells stained a dark gray, whereas the ciliated duct and posterior presumptive lens were completely free of silver nitrate precipitate. Examination at the ultrastructural level revealed that the number of colloidal gold spheres per 4 μm^2 of these differentiated lens cells was 146.4 ± 58.5 (n = 5) whereas pre-immune preparations averaged only 1.6 ± 1.1 (n = 5; averages different by *t*-test, $P < 0.001$). The level of ALDH-like protein occurrence in these recently differentiated lens cells is significantly lower than that in adult lens cells (*t*-test, $P < 0.05$), and is more similar to protein occurrence in adult ciliated duct (see Table I). Low numbers of colloidal gold spheres (27.0 ± 3.2 per 4 μm^2, n = 5) were detected in a few duct epithelial cells upon examination at the ultrastructural level; but these numbers were significantly above those found in pre-immune preparations (2.6 ± 0.6 per 4 μm^2 n = 5; immune and pre-immune averages different by $P < 0.001$). These cells were located at the interface between the bacteria-containing non-ciliated crypts and the ciliated duct (Fig. 7). Cells in the more distal portion of the duct contained only background levels of spheres.

To further examine the temporal pattern of ALDH-like protein occurrence in early juvenile development and its relation to infection by symbiotic bacteria, newly hatched animals, 5-day symbiotic juveniles (average mantle length = 1.5), and a field caught juvenile measuring 1.6 mm in mantle length were tested for the presence of immunoreactive protein. The size and morphology of this field-caught juvenile suggests that it was between three and five days old at the time of fixation. Ultrastructural examination of the light organ of this animal showed it to be infected with bacterial symbionts. In all of these juveniles, both at the histological and ultrastructural levels, no ALDH-like protein was detected either in the ciliated duct or in presumptive lens tissue (data not shown). No cells were present that resembled the large, lightly staining cells typical of adult lens and which stained positive for these proteins in the adult and older juveniles. Further, in all of these animals, including the 1.6 mm field-caught juvenile, three separate pores opened into three ciliated ducts on each side of the light organ; none of the epithelial cells in these ducts appeared to express ALDH-like protein at this stage of development. The presence of three ducts per side is typical of newly hatched and very young juveniles (Montgomery and McFall-Ngai, 1991). The slightly larger and older juvenile, 1.8 mm in mantle length, contained only one duct on each side of its light organ; the size of this juvenile and the presence of one duct indicate that this animal was approximately one week to ten days old at the time of fixation.

Because detection of the ALDH-like protein induction in a small area of the juvenile light organ might be missed with immunocytochemistry, anti-L-crystallin immunoblots of juvenile light organs were compared to further search for a temporal pattern of induction and its relation to bacterial infection. Light organs and mantles from newly hatched and 5-day symbiotic juveniles were compared, and in all cases only very slight bands at 54 kD, the apparent molecular mass of denatured L-crystallin (Montgomery and McFall-Ngai, 1992) were detected (data not shown). Further, the 54 kD bands in the light organ lanes were weaker than those in mantle lanes, which suggests that levels of the proteins in the light organs were not significantly above baseline levels expressed in all tissues. The amplified immunoblot kit was able to detect L-crystallin in adult light organ lens, which is comprised chiefly of L-crystallin (Montgomery and McFall-Ngai, 1992), when as little as 1 ng of protein was loaded. Therefore, if the very slight positive band in both juvenile preparations represents approximately 1 ng of total soluble protein, and 8 μg of protein were loaded, ALDH-like protein comprises less than 0.02% of the juvenile light organ soluble protein compared to approximately 50% in the adult organ (Montgomery, unpub. data).

Discussion

Our investigation localizes ALDH-like proteins in the adult *E. scolopes* light organ: (1) in high abundance in the cytoplasm of lens cells and (2) in moderate abundance in the cytoplasm of the epithelial cells lining the ciliated duct. Further, we explore the onset of ALDH-like protein occurrence in juvenile light organs and show that these proteins: (1) are not detectable in light organs from either newly hatched or 5-day symbiotic juveniles; (2) are first expressed simultaneously in ciliated duct epithelium and in newly differentiating lens

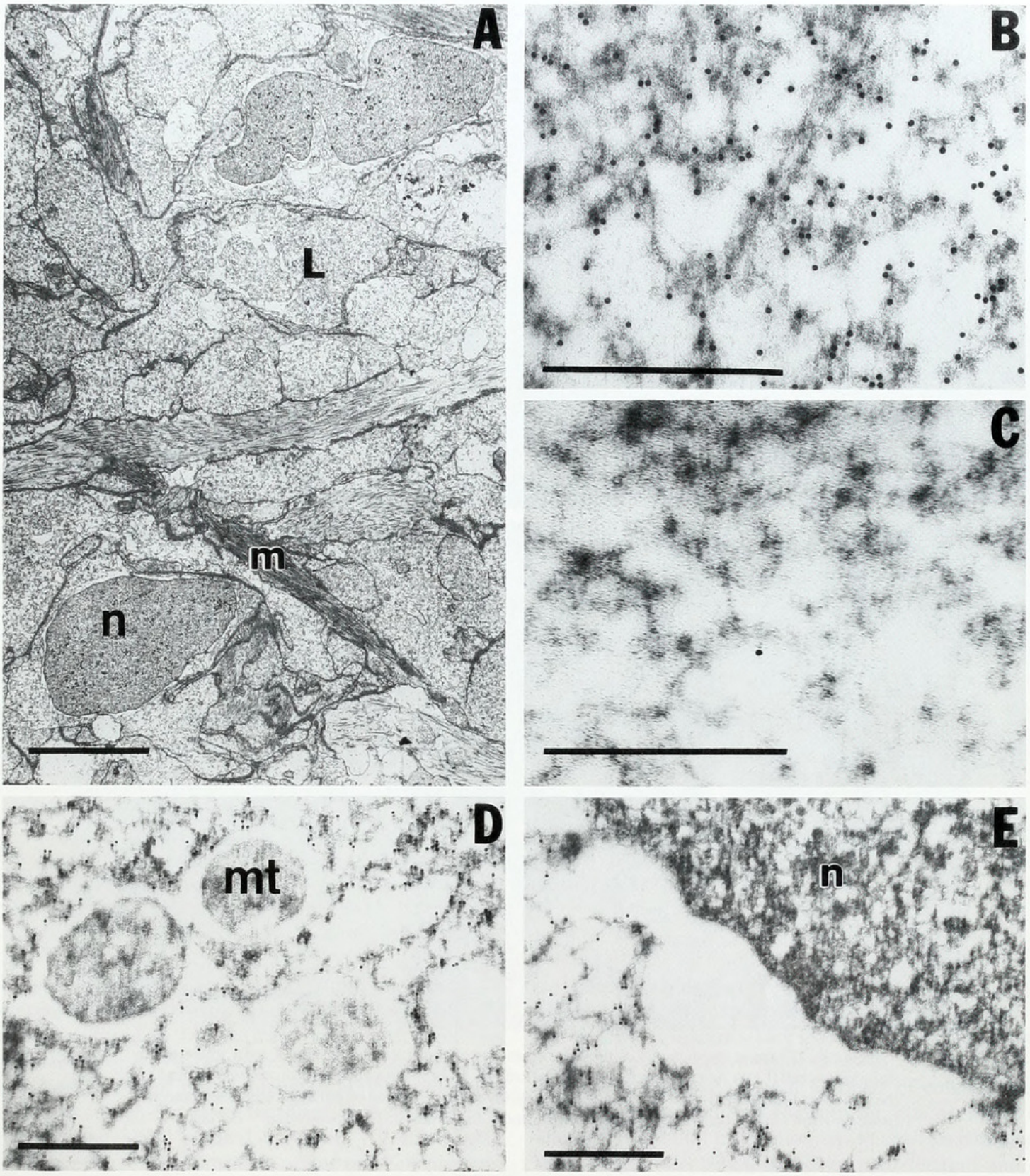

Figure 3. Electron micrographs of immune and pre-immune treatments of the light organ lens. (A) Low magnification of the light organ lens showing that the lightly staining lens cells comprise the majority of the lens (fixed for TEM, not for immunocytochemistry, as described in Methods). L, lens cells with diffuse cytoplasm and lacking any fibrous muscle structure; m, longitudinal section of a muscle fiber; and n, nucleus of a lens cell. Bar = 5 μm. (B) High magnification of immune-treated lens cell cytoplasm revealing numerous 15 nm gold spheres. Bar = 0.5 μm. (C) High magnification of pre-immune-treated lens cell cytoplasm revealing few 15 nm gold spheres. Bar = 0.5 μm. (D) High magnification of immune-treated lens cell cytoplasm containing 3 mitochondria (mt). Gold spheres are present in the cytoplasm but not in the mitochondria. Bar = 0.5 μm. (E) High magnification of immune-treated lens cell cytoplasm and nucleus (n). Gold spheres are present in the cytoplasm but not in the nucleus. Bar = 0.5 μm.

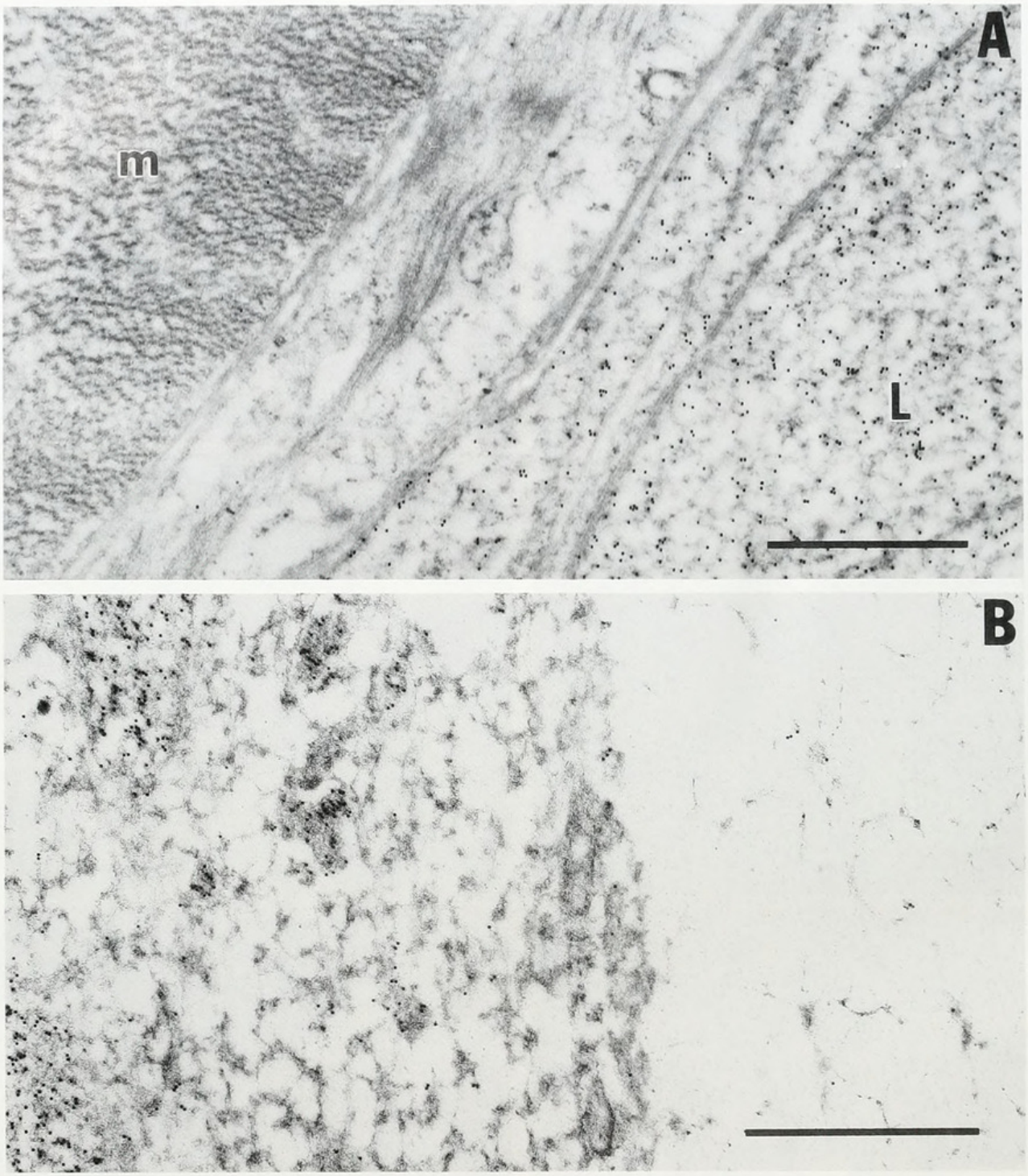

Figure 4. Electron micrographs of the light organ lens margins. (A) Outer lens margin where lightly staining lens cell (L) on right abuts a muscle cell (m) on left. Gold spheres are evenly distributed throughout the lens cell cytoplasm. (B) Inner lens margin where lightly staining lens cell on left abuts acellular connective tissue on right. Gold spheres appear in loose aggregations in the lens cell cytoplasm. Bar = 0.5 μm.

cells in juveniles; and (3) are only expressed in lens cells after lens cell differentiation from mesodermal tissue.

ALDH-like protein occurrence in adult light organ lens

Like the transparent epidermally derived ocular lens tissue of vertebrates and cephalopods, which are comprised chiefly of one or several lens crystallins, the muscle-derived light organ lens has a simple protein profile, in which L-crystallin accounts for 70% of the soluble protein (Montgomery and McFall-Ngai, 1992). Further, L-crystallin is located in the cytoplasm of the large, lightly staining cells, which comprise the vast majority of the lens, but is absent from lens epithelium and muscle (Figs. 1,

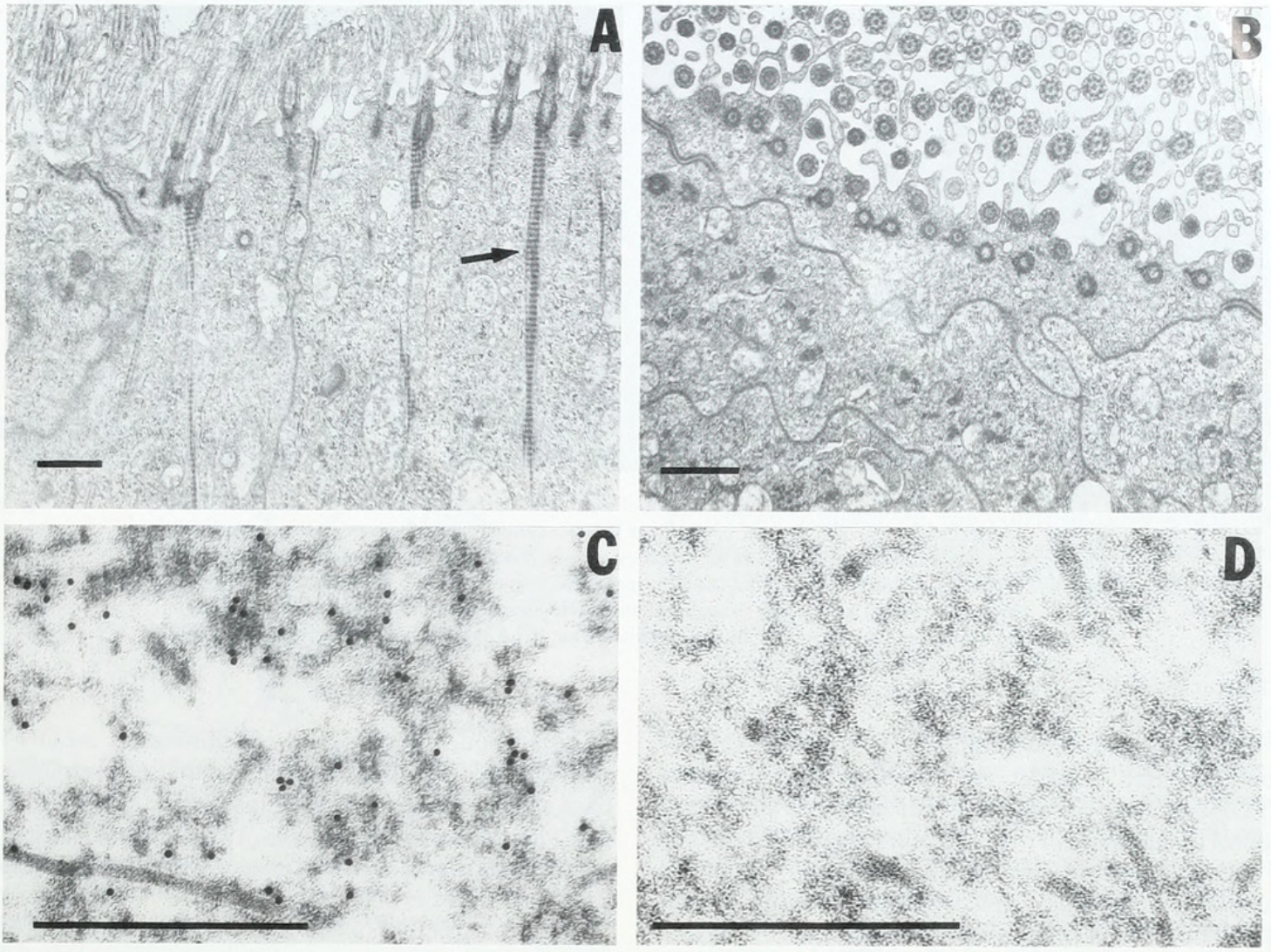

Figure 5. Electron micrographs of immune and pre-immune treatments of ciliated duct epithelium. (A & B) Low magnification of the ciliated duct epithelium in: (A) longitudinal section, and (B) cross section, showing numerous cilia and ciliary root hairs (arrow) (fixed for TEM, not for immunocytochemistry, as described in Methods). Bars = 1 μm. (C) High magnification of immune-treated duct epithelium cytoplasm revealing numerous 15 nm gold spheres. Bar = 0.5 μm. (D) High magnification of pre-immune-treated duct epithelium cytoplasm revealing few 15 nm gold spheres. Bar = 0.5 μm.

2). Such a high abundance of L-crystallin together with an apparent reduction in the production of proteins typical of a muscle tissue, such as actin and myosin (Montgomery and McFall-Ngai, 1992) and a lack of subcellular structure (Fig. 3; McFall-Ngai and Montgomery, 1990) in this predominant cell type suggests that these cells do little else than synthesize and retain L-crystallin. As with ocular lens tissue, the over-production of one or a few proteins in specialized lens cells is thought to result in a tissue with a high refractive index, which results in increased transparency (Bloemendal, 1981).

L-crystallin abundance appears to vary along the medial/lateral axis in some sections (Fig. 1b), but not in others. The lighter staining lateral portion of the lens could indicate a differential abundance of L-crystallin along this axis in some areas of the lens, or could be due to uneven fixation of the tissue. L-crystallin occurrence is uniform through the lens along the dorsal/ventral axis, except for the dorsal lens margin where lens cells appear to give way to acellular connective tissue (Fig. 4b). The pockets of cytoplasm that contain high numbers of colloidal gold spheres, surrounded by areas with no spheres, might be sites of L-crystallin production. However, this packaging effect might also be explained by poor fixation and/or infiltration in the far interior of the lens. From these localization results, it is not possible to determine the location of continued lens cell differentiation or new L-crystallin production in the adult lens. In continuing studies, we have been able to isolate only minute quantities of poly A+ RNA from light organ lens tissue from adults (Weis and McFall-Ngai, unpub. data), which suggests that both L-crystallin turnover and synthesis in the adult lens are very low.

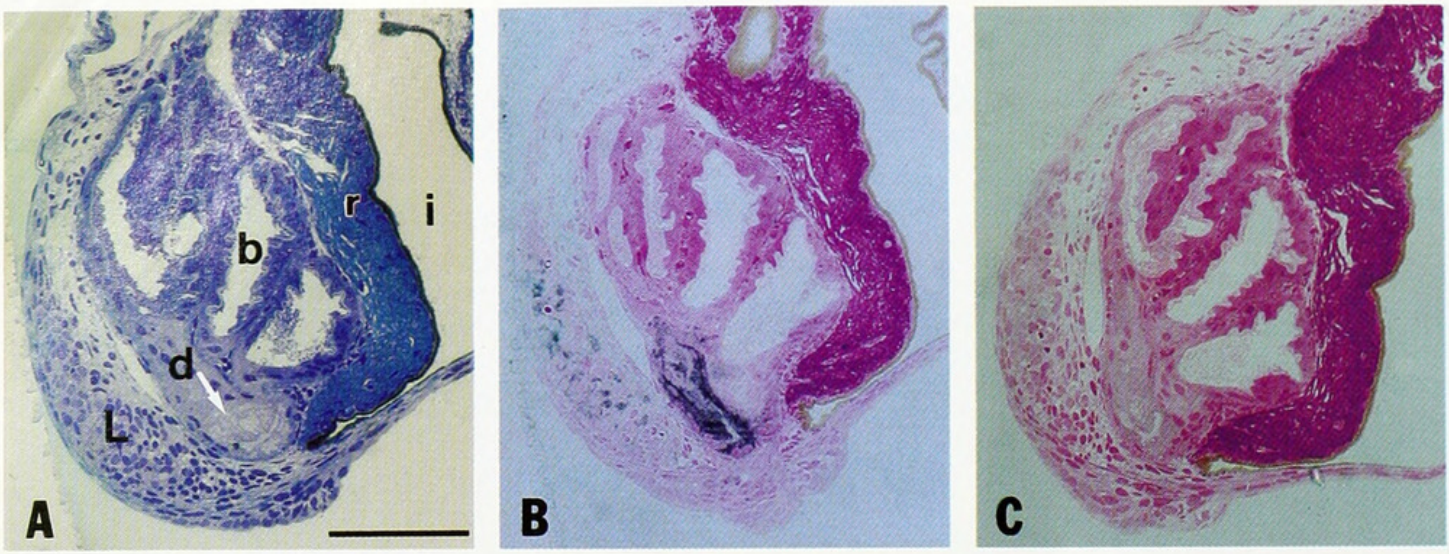

Figure 6. Cross sections through the light organ of a juvenile *Euprymna scolopes* (2.0 mm in mantle length). (A) Richardson's stained section: b, the inner bacteria-containing epithelium; d, the ciliated duct with its surrounding epithelium (duct does not reach the pore in this section); r, the reflector; i, the ink sac; and L, the light organ lens. Bar = 100 μm. (B) Silver-enhanced immune section, counter-stained with acid fuchsin. The silver granules indicate the presence of an ALDH-like protein in the ciliated duct epithelium and the light organ lens. The ink in the ink sac appears brown. (C) A pre-immune section lacks any dense black stain in the lens or duct epithelium.

We have found a 54% amino acid sequence identity between three peptides of L-crystallin and human ALDH1, an enzyme that has been localized in the cell cytosol of mammalian liver and other organs (Kitabatake *et al.*, 1981; Montgomery and McFall-Ngai, 1992). Because of its substantial sequence identity to a known enzyme, L-crystallin has been added to the list of enzyme/crystallins, a group of structural proteins in ocular lenses of vertebrates and cephalopods that have a high sequence identity to various enzymes (Piatigorsky and Wistow, 1991). Although L-crystallin and ALDH1 share a common location within the cell, and ALDH1 cross-reacts with the polyclonal antiserum to L-crystallin, they do not show high enough sequence identity to suggest that they are closely related isozymes.

ALDH isozymes have been localized in a wide variety of tissues using several techniques. In addition to its occurrence in the light organ as L-crystallin, we have found an immunoreactive ALDH-like protein in the *E. scolopes* eye lens in moderate levels, using immunoblot analysis (Montgomery and McFall-Ngai, 1992). This protein species likely functions as an enzyme/crystallin. Like L-crystallin, the ALDH enzyme/crystallins, which have been described in the eye lenses of elephant shrew (η-crystallin, Wistow and Kim, 1991) and octopus (Ω-crystallin, Tomarev *et al.*, 1991) are presumed to be present in the cell cytosol, based on immunoblot analyses. Many studies in various other mammalian tissues localize ALDH to various subcellular fractions using differential centrifugation (*e.g.*, Kitabataki *et al.*, 1981; Lindahl and Evecs, 1984; Weiner *et al.*, 1987). In addition, there are ample examples of histochemical localization of mammalian ALDH in several tissues including liver, brain, retina, and respiratory tract which employ both immunological (*e.g.*, Maeda *et al.*, 1988; McCaffery *et al.*, 1991, 1992) and activity staining techniques (*e.g.*, Lindahl *et al.*, 1983; Watabiki *et al.*, 1989; Motavkin *et al.*, 1990).

The ultrastructure of lens cells and the location of the lens crystallins in ocular lenses of vertebrates and the light organ of *E. scolopes* share some common features. Crystallins in vertebrate eye lenses are highly expressed in terminally differentiated fiber cells, which lack nuclei, mitochondria, endoplasmic reticulum, and Golgi complexes (Maisel *et al.*, 1981). Similarly, although light organ lens cells retain their nuclei and mitochondria, they too lose other organelles upon differentiation (Fig. 4; McFall-Ngai and Montgomery, 1990). Further, like L-crystallin in the *E. scolopes* light organ lens, crystallins in vertebrate ocular lenses are localized in the cell cytosol (Bloemendal, 1981).

ALDH-like protein occurrence in adult ciliated duct epithelium

ALDH-like protein was also localized in moderate abundance in the epithelial cells lining the ciliated duct (Figs. 1, 2c, d; Table I). Because lens and ciliated duct are unrelated ontogenetically (Montgomery and McFall-Ngai, 1993), it is unlikely that duct epithelium expressing one or more of these proteins is the source of ALDH-like proteins in the muscle-derived lens cells or visa versa. It is possible that the protein species in the duct epithelium is functioning as an enzyme rather than a structural protein.

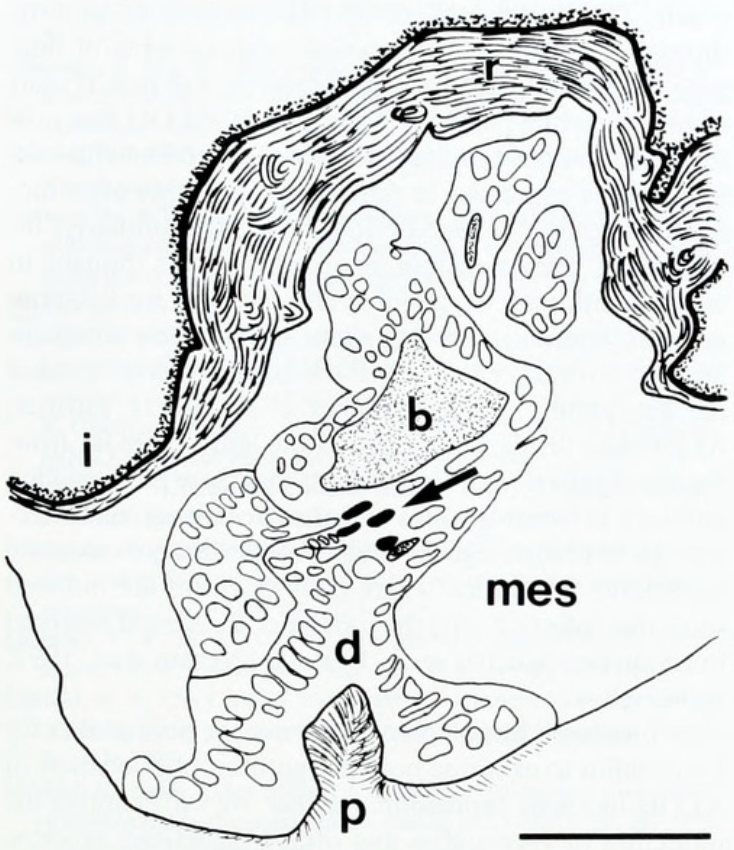

Figure 7. Diagrammatic reconstruction of a light organ from a 1.8 mm juvenile (estimated to be between 7 and 10 days old), illustrating the area where ALDH-like protein is first detected in the ciliated duct epithelium. Arrow points to darkened nuclei, representing cells within the ciliated duct epithelium which contain colloidal gold spheres, immediately adjacent to non-ciliated epithelium, lining the bacteria-containing crypts. All other epithelial cells, represented by clear nuclei, both in the bacteria-containing inner area and in the ciliated duct, lack colloidal gold spheres. b, bacteria-containing crypts; d, ciliated duct epithelium; i, ink sac; mes, mesodermal tissue, the site of the presumptive lens; p, the pore connecting the ciliated duct with the outside of the light organ; and r, reflector. Bar = 50 μm.

We have tested light organ lenses extensively for ALDH activity and found none (Montgomery and McFall-Ngai, 1992), but we have detected very low activities in whole light organs (Montgomery, unpub. data). Activity stains (see above) in the light organ might be a useful technique to further explore the function of the ALDH-like protein species in the ciliated duct epithelium. A positive stain in this tissue would suggest an enzymatic role for this protein.

Unlike lens cells, ciliated duct epithelium comes in direct contact with symbiotic bacteria and possibly other bacteria that enter the light organ through the pores. Some ALDH isozymes function in the detoxification of various biogenic aldehydes that are produced under certain conditions, such as during lipid peroxidation (Holmes *et al.*, 1989) and oxidative stress (Jedziniak *et al.*, 1987; Abedinia *et al.*, 1990). Such conditions have been proposed for several symbioses (Dykens and Shick, 1982; Blum and Fridovich, 1984). It is possible that an enzymatically active ALDH, and thus a form that is biochemically distinct from L-crystallin, could be functioning in a similar capacity in the ciliated duct to detoxify substances from foreign pathogenic microbes or even from symbiotic *V. fischeri* especially in juvenile light organs during the establishment of a stable mutualism.

From our studies, we cannot determine the relation of L-crystallin in the lens with the ALDH-like protein found in the ciliated duct epithelium. Anti-L-crystallin cross-reacts with several ALDH-like proteins, all with 54 kD subunits, from both cephalopods and mammals, but not with other proteins at other molecular masses (Montgomery and McFall-Ngai, 1992). Therefore, anti-L-crystallin is specific to ALDH-like proteins, and not to L-crystallin alone. It can therefore be used to identify ALDH-like proteins in general, but not L-crystallin specifically. Further, we have no sequence information from other ALDH-like proteins in other squid tissues, such as ocular lens and ciliated duct epithelium, with which to infer lineages between the squid ALDH isozymes.

ALDH-like protein localization in juvenile light organs

The examination of ALDH-like protein in juvenile light organs revealed both qualitative and quantitative changes in occurrence through time. Levels of the protein(s) were not significantly above baseline levels, present in all cells, in newly hatched and 5-day symbiotic juveniles and in the 1.6 mm field-caught animal, as shown both with immunocytochemistry and immunoblots. These findings suggest that ALDH-like proteins are neither constitutively expressed in high quantities at the time of hatching nor immediately induced upon infection by bacteria. Based on the size of the 1.8 mm field-caught animal, which was the smallest to stain positive for ALDH-like proteins, we estimate that the age of first induction in juvenile light organs occurs between 7 and 10 days, according to a study by Singley (1983) which correlates size with age in juvenile *E. scolopes* maintained in the laboratory. By comparison, animals hatched under laboratory conditions average 1.6 $\pm$ 0.1 mm mantle length (Montgomery and McFall-Ngai, 1993).

Immunolocalization of field-caught juveniles revealed that ALDH-like protein occurrence in the ciliated duct epithelium and anterior lens precedes that in other portions of the light organ. Further, the onset of expression seems to be related to fundamental changes in light organ morphology. Each side of the newly hatched juvenile light organ possesses three ciliated ducts that lead into three separate crypts. These ducts ultimately coalesce into a single branching duct that leads to a single large pore in symbiotic animals that are more than one week old (Montgomery and McFall-Ngai, 1991). Our data suggest that ALDH-like protein is not expressed in ciliated duct

epithelium until the single duct is formed. Immunoreactive protein was not found in any of the duct epithelial cells in juvenile light organs still containing three ducts per side. However, it was found in a few cells in the duct epithelium in the 1.8 mm juvenile, which had only one duct and pore on each side of the light organ. Because of the small number of cells expressing ALDH-like protein and the relatively low number of spheres within these positive cells, less than one fifth that of adult duct epithelial cells, it appears that these proteins were only just beginning to be synthesized in these cells at this stage of development. Further, expression first occurs in duct epithelial cells that are immediately adjacent to non-ciliated epithelial cells that line the bacteria-containing crypts (Fig. 7). It is in the juvenile light organ that the process of recognition and establishment of symbiotic bacterial strains occurs (McFall-Ngai and Ruby, 1991). High expression of ALDH-like proteins in a tissue in close proximity to the bacteria could indicate that their function is related to bacterial infection.

L-crystallin in developing lens tissue was only detected when clearly differentiated lens cells were present, which suggests that lens cell differentiation occurs before L-crystallin expression. This situation is again analogous to the epidermally derived vertebrate eye lenses where expression of some types of crystallin genes in fiber cells begins only after terminal differentiation (Ramaekers and Bloemendal, 1981). For this reason, the production of certain crystallins is used as a differentiation marker in lens cell tissue culture (Ramaekers and Bloemendal, 1981). Light organ lens cells appear to first differentiate in the anterior portion of the light organ. In the smallest juvenile in which differentiated lens cells were apparent, L-crystallin was found present only in lens cells anterior to the ciliated duct and several cell layers ventral to the crypts. L-crystallin was present in these cells at levels about one half that found in adult lens cells, which suggests that these cells had probably just recently differentiated. The lens cells appear to differentiate from a pad of mesodermal cells that are present ventral and lateral to the bacteria-containing crypts.

Patterns of host gene expression in prokaryote/eukaryote mutualisms

Despite the prevalence of prokaryote/eukaryote symbioses, very little is known about their complex cellular and molecular integration and regulation. One exception is the root nodule symbiosis between the nitrogen-fixing bacterium *Rhizobium* and leguminous plants. There are now many studies that have examined questions of regulation and communication in this highly integrated mutualism. There are a number of plant gene products, nodulins, that are induced or enhanced in the symbiotic organ. The comparison of ALDH-like protein patterns through light organ development with patterns of host gene expression described in symbiotic legumes reveals some interesting parallels. For example, ALDH-like protein is not induced immediately upon infection with bacteria, but is expressed in developing lens only after mesodermal cells differentiate into lens cells. Similarly, induction of leghemaglobin, a nodulin that is thought to aid in creating an oxygen-free environment for bacterial nitrogen fixation, does not occur immediately upon infection, but rather later after the bacteria have invaded the developing nodule (Sanchez *et al.*, 1991). Further, ALDH-like protein induction in the lens occurs far from the site of infection, which suggests that there is a signalling pathway between the lens and the site of bacterial infection. In symbiotic legume roots, similar distances separate the nodule primordia, where early nodulins are induced soon after infection, and the root hairs, the sites of bacterial infection (see Sanchez *et al.*, 1991, and Verma *et al.*, 1992, for reviews).

In future studies we plan to use nucleic acid probes for L-crystallin to examine both the patterns and location of ALDH-like gene expression. Further, we will examine the induction of L-crystallin and other ALDH-like proteins in the juvenile *E. scolopes* light organ. Experimental data will reveal whether (1) over evolutionary time, the symbiosis has resulted in a developmental timetable that includes induction of ALDH-like protein at a prescribed time, regardless of exposure to symbionts or (2) induction of these proteins is a direct result of interactions with the symbiotic bacteria during the infection process.

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Literature Cited

Abedinia, M., T. Pain, E. Algar, and R. Holmes. 1990. Bovine corneal aldehyde dehydrogenase: the major soluble corneal protein with a possible dual protective role for the eye. *Exp. Eye Res.* **51(4):** 419–426.

Bloemendal, H. 1981. The lens proteins. Ch. 1 in *Molecular and Cellular Biology of the Eye Lens,* H. Bloemendal, ed. John Wiley and Sons, New York.

Blum, J., and I. Fridovich. 1984. Enzymatic defenses against oxygen toxicity in the hydrothermal vent animals *Riftia pachyptila* and *Calyptogena magnifica*. *Arch. Biochem. Biophys.* **228(2):** 617–620.

Dykens, J. A., and J. M. Shick. 1982. Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. *Nature* **297:** 579–580.

Erickson, P. A., D. H. Anderson, and S. K. Fisher. 1987. Use of uranyl acetate en bloc to improve tissue preservation and labeling for post-

embedding immunoelectron microscopy. *J. Elec. Micros. Technol.* **5:** 303–314.

Hastings, J. W., J. Makemson, and P. V. Dunlap. 1987. How are growth and luminescence regulated independently in light organ symbionts? *Symbiosis* **4:** 3–24.

Herring, P. J. 1988. Luminescent organs. Pp 449–489 in *The Mollusca,* Vol 11. Academic Press, London.

Holmes, R., B. Cheung, and J. VandeBerg. 1989. Isoelectric focusing studies of aldehyde dehydrogenases, alcohol dehydrogenases and oxidases from mammalian anterior eye tissues. *Comp. Biochem. Physiol.* **93B:** 271–277.

Ishikawa, H. 1990. A synthesis: the types of interaction system between bacteria and insects. Pp 355–360 in *Endocytobiology IV: 4th International Colloquium on Endocytobiology and Symbiosis,* P. Nardon, V. Gianinazzi-Pearson, A. Grenier, L. Margulis and D. Smith, eds. INRA Service des Publications, Versailles, France.

Jedziniak, J., M. Arredondo, and U. Andley. 1987. Oxidative damage to human eye lenses. *Curr. Eye Res.* **6:** 345–349.

Kitabatake, N., R. Sasaki, and H. Chiba. 1981. Localization of bovine liver aldehyde dehydrogenase isozymes and their immunological properties. *J. Biochem.* **89:** 1223–1229.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lindahl, R., R. Clark, and S. Evecs. 1983. Histochemical localization of aldehyde dehydrogenase during rat hepatocarcinogensis. *Cancer Res.* **43:** 5972–5977.

Lindahl, R., and S. Evecs. 1984. Comparative subcellular distribution of aldehyde dehydrogenase in rat, mouse and rabbit liver. *Biochem. Pharmacol.* **33:** 3383–3389.

Maeda, M., Y. Hasumaura, and J. Takeuchi. 1988. Localization of cytoplasmic and mitochondrial aldehyde dehydrogenase isozymes in human liver. *Lab. Invest.* **59:** 75–81.

Maisel, H., C. V. Harding, J. Alcala, J. Kuszak, and R. Bradley. 1981. The morphology of the lens. Ch. 2 in *Molecular and Cellular Biology of the Eye Lens,* H. Bloemendal, ed. John Wiley and Sons, New York.

Margulis, L., and R. Fester. 1991. *Symbiosis as a Source of Evolutionary Innovation.* MIT Press, Cambridge, MA.

McCaffery, P., M. Lee, M. Wagner, N. Sladek, and U. Drager. 1992. Asymmetrical retinoic acid synthesis in the dorsoventral axis of the retina. *Development* **115:** 371–382.

McCaffery, P., P. Tempst, G. Lara, and U. Drager. 1991. Aldehyde dehydrogenase is a positional marker in the retina. *Development* **112:** 693–702.

McFall-Ngai, M., and M. Montgomery. 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae). *Biol. Bull.* **179:** 332–339.

McFall-Ngai, M., and E. Ruby. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* **254:** 1491–1494.

Montgomery, M., and M. McFall-Ngai. 1991. The influence of bacterial symbionts on early morphogenesis of the light organ of the sepiolid squid *Euprymna scolopes. Am. Zool.* **31:** 15A.

Montgomery, M., and M. McFall-Ngai. 1992. The muscle-derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. Evidence for recruitment of aldehyde dehydrogenase as a predominant structural protein. *J. Biol. Chem.* **276(29):** 20999–21003.

Montgomery, M., and M. McFall-Ngai. 1993. Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. *Biol. Bull.* **184:** 296–308.

Motavkin, P., V. Okhotin, O. Konovko, and S. Zimatkin. 1990. Localization of alcohol- and aldehyde dehydrogenase in the human spinal cord. *Neurosci. Behav. Physiol.* **20:** 79–84.

Moynihan, M. 1983. Notes on the behavior of *Euprymna scolopes* (Cephalopoda:Sepiolidae). *Behavior* **85:** 25–41.

Nap, J. P., and T. Bisseling. 1991. Developmental biology of a plant-prokaryote symbiosis: the legume root nodule. *Science* **250:** 948–954.

Piatigorsky, J., and G. Wistow. 1991. The recruitment of crystallins: new functions precede gene duplication. *Science* **252:** 1078–1079.

Ramaekers, F., and H. Bloemendal. 1981. Cytoskeletal and contractile structures in lens cell differentiation. Ch. 3 in *Molecular and Cellular Biology of the Eye Lens,* H. Bloemendal, ed. John Wiley and Sons, New York.

Richardson, K. C., L. Jarrett, and E. H. Finke. 1960. Embedding in epoxy resin for ultrathin section in electron microscopy. *Stain Technol.* **35:** 313–323.

Ruby, E., and M. McFall-Ngai. 1992. A squid that glows in the night: development of an animal-bacterial mutualism. *J. Bacteriol.* **174(15):** 4865–4870.

Sanchez, F., J. E. Padilla, H. Perez, and M. Lara. 1991. Control of nodulin genes in root-nodule development and metabolism. *Ann. Rev. Plant Physiol.* **42:** 507–528.

Singley, C. 1983. *Euprymna scolopes.* Pp 69–74 in *Cephalopod Life Cycles, Vol. 1.* Academic Press, London.

Tomarev, S., R. Zinovieva, and J. Piatigorsky. 1991. Crystallins of the octopus lens. Recruitment from detoxification enzymes. *J. Biol. Chem.* **266:** 24226–24231.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci.* **76(9):** 4350–4354.

Verma, D., C.-A. Hu, and M. Zhang. 1992. Root nodule development: origin, function and regulation of nodulin genes. *Physiol. Plant.* **85:** 253–265.

Watabiki, T., T. Tokiyasu, N. Ishida, and K. Ogawa. 1989. Histochemical localization of aldehyde dehydrogenase activity in the mouse liver by the copper ferrocyanide method. *Acta Histochem. Cytochem.* **22:** 397–400.

Wei, S., and R. E. Young. 1989. Development of a symbiotic bacterial bioluminescence in a nearshore cephalopod *Euprymna scolopes. Mar. Biol.* **103:** 541–546.

Weiner, H., M. McMichael, E. Lindahl-Hellstrom, and G. Tu. 1987. Use of polyclonal antibodies to study properties of mammalian aldehyde dehydrogenase. *Alcohol. Alcohol.* **Suppl. 1:** 187–191.

Whitaker, J., and P. Granum. 1980. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal. Biochem.* **109:** 156–159.

Wistow, G., and H. Kim. 1991. Lens protein expression in mammals: taxon-specificity and the recruitment of crystallins. *J. Mol. Evol.* **32:** 262–269.