A COMPARISON OF THE LIGHT ORGAN DEVELOPMENT OF SEPIOLA ROBUSTA NAEF AND EUPRYMNA SCOLOPES BERRY (CEPHALOPODA: SEPIOLIDAE)

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ABSTRACT

Light organ development was studied in the sepiolid squid Sepiola robusta Naef and compared to that of Euprymna scolopes Berry. Both species form obligate mutualisms with luminous bacteria from the environment. The embryonic period of S. robusta, which is at least double to that of E. scolopes at similar temperatures, produced additional features not reported to occur in the E. scolopes light organ, including an extended ciliated ridge at the base of the light organ, as well as additional crypt spaces to house symbiotic bacteria. Accessory structures, which are used to modify the bacteria-produced light, are not under the induction of symbiotic bacteria and appear in S. robusta before hatching, whereas in E. scolopes these tissues form post-hatching. At hatching the light organs of both species respond to symbiotically competent bacteria and undergo similar developmental remodeling. Specifically, the ciliated epithelial fields on the surface of the light organ undergo a program of cell death and regression that spans 4 to 5 d in E. scolopes, and over 9 d in S. robusta. Although the timing of embryogenesis differs between these two species, the results of these studies demonstrate that the influence of Vibrio fischeri, the specific symbiont, appears to be restricted to the initial stages of post-hatching development of both light organs.

The family Sepiolidae (Cephalopoda) contains 50–60 species arranged in 14 genera that occur across a wide geographic distribution (Nesis, 1982; Bello, 1995). These species are most often benthic, nocturnal predators, burying by day in the sand or mud substrate, and emerging in the evening to forage in the water column for prey (Moynihan, 1983). Six of the genera (Sepiola, Euprymna, Inioteuthis, Rondeletiola, Semirossia, and Heteroteuthis) have members with bacterial light organs (Pierantoni, 1918; Boletzky, 1970; Nesis, 1982; Herring et al., 1981; Herring, 1988). The organs are typically bilobed, kidney-shaped structures embedded in the ink sac. In adult animals, a set of labyrinthine epithelial crypts (Pierantoni, 1918; Kishitani, 1932; McFall-Ngai and Montgomery, 1990) supports an extracellular culture of luminous bacteria of the genus Vibrio (McFall-Ngai and Ruby, 1991; Fidopiastis et al., 1998). This epithelial tissue is surrounded by accessory tissues that serve to modify the light for its use by the animal in its behavior. A thick reflector directs the luminescence out of the ventral aspect of the squid; diverticula of the ink sac dynamically rotate around the bacteria-containing epithelium to modulate luminescence intensity; and, a ventral, muscle-derived lens diffuses the light. The resultant diffuse glow that is emitted by the animal is presumed to be used as a counterillumination, antipredatory strategy during the nocturnal activity of the host (Herring et al., 1981; Moynihan, 1983; McFall-Ngai, 1990).

The symbiosis of the Hawaiian sepiolid squid Euprymna scolopes has been investigated for several years (McFall-Ngai, 1999). The light organ of this species is populated anew each generation with its specific symbiont, Vibrio fischeri, which occurs as a member of the bacterioplankton in the habitats surrounding host populations (Wei and Young, 1989; McFall-Ngai and Ruby, 1991). During embryogenesis, which averages 20 d at 23–24°C (Arnold et al., 1972), a rudimentary light organ develops in the hindgut-ink sac.
complex (Montgomery and McFall-Ngai, 1993). At hatching, the light organ exhibits a morphology that is quite different from the adult host (for review see McFall-Ngai and Ruby, 1998). The most conspicuous feature of the juvenile-specific organ is a superficial ciliated, microvillous field on each lateral surface that appears to potentiate the inoculation of the organ with *V. fischeri*. During the inoculation process, this field entrains the bacteria into the vicinity of three pores on each side of the juvenile light organ. Once inside the pores, the *V. fischeri* move through ciliated ducts and colonize a set of epithelia-lined crypts, three on either side of the organ. In addition to these characters that are unique to the juvenile organ, the organ of a newly hatched *E. scolopes* lacks a number of features that are present in the adult host (Montgomery and McFall-Ngai, 1998). The juvenile light organ is ovoid, rather than bilobed, and the accessory tissues that modify the luminescence are poorly developed or lacking. Specifically, the reflector is only a few cell layers thick, the ink sac diverticula have not formed, and the lens is entirely absent.

Studies of *E. scolopes* have shown that the bacteria participate in some portions of the postembryonic development of the host’s light organ (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995; Foster and McFall-Ngai, 1998; Lamarcq and McFall-Ngai, 1998). The bacteria induce marked morphogenic changes in the organ during early post-hatching development, the most dramatic of which is the loss of the superficial ciliated, microvillous field over the first 4 to 5 d of the symbiosis (Montgomery and McFall-Ngai, 1994). Ultrastructural, biochemical and molecular analysis of this process revealed that bacteria-induced apoptosis of the cells in this field is responsible for some, or all of its morphogenesis (Foster and McFall-Ngai, 1998). In contrast to these early, bacteria-dependent events, additional studies of the development of the *E. scolopes* light organ have demonstrated that late developmental events, i.e., changes in the light organ shape and elaboration of accessory tissues, are not under the induction of the bacterial symbionts (Montgomery and McFall-Ngai, 1998; Claes and Dunlap, 2000).

In the present study, we compared the development of the *E. scolopes* light organ to that of the Mediterranean squid *Sepiola robusta*, which has an embryonic period over twice as long as that of the Hawaiian species at comparable temperatures. Early studies of *Sepiola* spp. demonstrated that the juveniles have a ciliated surface similar to that described in juveniles *E. scolopes* (Pierantoni, 1918). However, the development of the accessory structures and the influence of bacteria on the development of the organ have not been described. Our studies indicate that the difference in the length of the embryonic period, which reflects the greater embryo size, influences the extent to which various components of the light organ are developed at hatching. In addition, our data show that the bacterial symbionts participate in similar aspects of light organ morphogenesis in these two species.

**Materials and Methods**

The *S. robusta* Naef samples used in this study were obtained during benthic trawling, at depths averaging 50 m, in the Mediterranean Sea off the coast of Banyuls-sur-Mer, France. Eggs of *S. robusta*, attached to various hard surfaces, were maintained in running 18°C seawater aquaria at the Laboratoire Arago in Banyuls-sur-Mer, or transferred to small mesh bags and then moved to the University of Southern California, where they were incubated for the remainder of their embryonic period. Specimens of *E. scolopes* Berry were collected with dipnets on the shallow reefs of Oahu, Hawaii and brought to the University of Southern California, Los Angeles, California, where they were maintained in running sea water aquaria at 23°C. The animals were mated approximately...
once a week and the subsequent clutches of eggs were kept in smaller aquaria for the duration of their embryonic period. Upon hatching, the juveniles of both species of squid were kept in California seawater, which does not contain infective strains of *Vibrio fischeri* (McFall-Ngai and Ruby, 1991). To inoculate the hatching squid, cultures of *V. fischeri* ES114, a light organ isolate, were grown to log-phase in a seawater tryptone medium and diluted in seawater to a final concentration of approximately 10^6 cells ml⁻¹. Successful colonization of the squid was monitored using a sensitive photometer (Luminescence Photometer, Model 3600, Biospherical Instruments, San Diego, California).

To prepare animals for scanning electron microscopy, embryos and newly hatched squid were anesthetized in 7.5% magnesium chloride in seawater and then placed in 5% formaldehyde in seawater for 12 h. After fixation, the animals were rinsed 3 times in seawater, for 15 min each rinse, and then dehydrated with a graded ethanol series. After dehydration, the animals were either critical point dried or treated with hexamethyldisilazane (HMDS). The dried specimens were then mounted on stubs and examined with either a Cambridge 360 scanning electron microscope or a Hitachi S-800 field emission scanning electron microscope.

Juvenile squid were fixed for light-level microscopy by placing them directly into 2.5% glutaraldehyde/2.5% paraformaldehyde in a buffer of 0.1 M sodium cacodylate with 0.45 M NaCl, pH 7.4, at 23°C for 12 h. The specimens were rinsed three times for 15 min each rinse in the same cacodylate/NaCl buffer then dehydrated in a graded ethanol series. The animals were then infiltrated with a 1:1 ratio of 100% ethanol and propylene oxide for 15 min, followed by an additional incubation in 100% propylene oxide for 15 min. The specimens were then exposed to a 1:1 ratio of 100% propylene oxide and unaccelerated Spurr (Spurr, 1969) for 12 h. The animals were infiltrated with 100% accelerated Spurr for 3 days at room temperature, and embedded in fresh 100% accelerated Spurr at 60°C for 24 h. Thick sections (~0.1 μm) were cut and placed on slides treated with a 1% gelatin/0.1% chromium potassium sulfate. The sections were then stained with a solution of toluidine blue at 37°C for 2 min and rinsed with deionized water.

For staining with acridine orange, a fluorochrome that labels nucleic acids (Delic et al., 1991), animals were anesthetized in 7.5% magnesium chloride in seawater, then incubated for 1 min in a 5 ng ml⁻¹ solution of acridine orange. The mantles and the funnels of the squid were dissected away exposing the light organ, and the specimens were examined with a Nikon HFX-11 epifluorescence microscope or a Zeiss LSM 510 confocal microscope.

**RESULTS**

**DEVELOPMENTAL EVENTS INDEPENDENT OF BACTERIAL INDUCTION.**—The egg sizes differ between *E. scolopes* and *S. robusta* and are correlated to the different body sizes of the hatchling squid. The eggs and hatchlings of *S. robusta* are three-fold larger in volume than those of *E. scolopes*. Although different in size, upon fertilization and continuing on over the embryonic period, the light organ development of these two species is remarkably similar when eggs are maintained at 23°C. The organ initially forms as a thickening of the surface of the visceral complex. Scanning electron microscopy (SEM) revealed that at stages A26/NXVI the lateral surface epithelium of the light organ rudiment extends outward, producing the first of a pair of appendages (Fig. 1; Montgomery and McFall-Ngai, 1993). [Stages are according to Arnold et al. (1972) for *E. scolopes* and Naef (1928) for *S. robusta*. The difference in stage numbers is due to an additional 10 stages during blastulation in Arnold’s system, so that A26 corresponds to NXVI; thus the hatching stage A30 corresponds to NXX.] In addition, at this stage, the surface epithelium begins to invaginate in both species, a process that eventually leads to the production of the first crypt. Over the next several days, the epithelium on each lateral surface of the organ develops into a complex ciliated field of cells. In both species, each field is comprised of
a posterior appendage, in addition to the first-formed anterior appendage, and a set of cells adnate to the main body of the light organ that ends medially with a row of elongated cilia.

Although the early embryogenesis of the light organ is similar in these two species, because of the difference in the length of the embryonic period, the maturity of the light organ at hatching is markedly dissimilar. At hatching, *E. scolopes* juveniles average 1.4 mm in mantle length (ML) and their light organs average 300 μm in anterior-posterior length, whereas *S. robusta* hatchlings average 4.5 mm in ML and their light organs average 800 μm in anterior-posterior length. The light organ of newly hatched *E. scolopes* is more ovoid (Fig. 2A,B) and, over the first few weeks following hatching, it develops the characteristic bilobed shape of the mature organ (Fig. 2C). In contrast, during the final weeks of embryogenesis, the *S. robusta* light organ develops this bilobed morphology, such that at hatching it has the mature shape (Fig. 2D). In both species, the light organ continues to grow proportionally to the body of the squid (data not shown) maintaining the bilobed shape that is achieved 5–6 wks following fertilization.

Whereas the shape of the organs is different at hatching, both have the complex, ciliated field of cells on the organ surface, although the *S. robusta* field is more elaborate (Fig. 3). In hatchling *S. robusta*, the ciliated appendages are longer and the ridge along the medial edge of the ciliated field is comprised of not only the elongated cilia, but also an outgrowth of cells, producing a ruffled appearance of the ciliated ridge.

Once the bacteria become entrained by the ciliated fields of cells on the light organs of *E. scolopes* and *S. robusta*, they enter pores on the surface of the organ. These pores lead...
Figure 2. The juvenile light organs of the host squid *E. scolopes* and *S. robusta*. A. Ventral dissection of hatchling *E. scolopes*, arrow indicates the position of the light organ in the center of the mantle cavity. bar = 500 μm. B. Higher magnification of a ventral dissection of the *E. scolopes* light organ. The rounded light organ is invested in the ink sac diverticula. The ciliated appendages and ridge are transparent and cannot be seen in this dissection. bar = 100 μm. C. A 20-d aposymbiotic *E. scolopes* that was reared in the laboratory. The shape of the light organ has been significantly altered, now resembling the adult bilobed morphology. The yellow pigmentation of the digestive gland (dg) surrounding the light organ is the result of the animals feeding on a diet of mysids and zooplankton. bar = 350 μm. D. Ventral dissection of hatchling *S. robusta* showing the mature shape of the light organ. bar = 350 μm.
to blind-ended sacs, or crypts, lined by epithelial cells, where the bacteria reside and continue to grow throughout the symbiosis (Fig. 4). In *E. scolopes*, three pores on either side of the light organ lead to three independent crypt spaces (Fig. 4A,C). In contrast, the extended embryonic period of *S. robusta* results in the formation of four pores on either side of the organ through which the bacteria can enter and colonize four independent crypt spaces (Fig. 4B,D).

Histological analyses of the accessory structures of the hatchling organ confirmed what was evident by examination of the superficial morphology; i.e., the accessory tissues that surround the crypts are markedly more developed in *S. robusta* at hatching. Whereas in *E. scolopes*, the reflector is only a few platelet-layers thick and is confined principally to the dorsal surface of the organ at this time (Montgomery and McFall-Ngai, 1993), in *S. robusta* hatchlings, the reflector is several layers thick and wraps around the crypt tissue. In addition, the diverticula of the ink sac more completely surround the crypts of the hatchling *S. robusta*. The lens tissue of the *S. robusta* light organ is more mature than that of *E. scolopes* by two criteria. Firstly, the thickening of the tissue in the area just ventral to the crypts is much more pronounced than in *E. scolopes* hatchlings. In addition, immuno-
tochemical analyses of this tissue have revealed high levels of the ALDH-like protein, which is the signature protein of the light organ lens tissue (Weis et al., 1993). In *E. scolopes*, ALDH-like protein is not detected in presumptive lens tissue until approximately 10 d post-hatching (Weis et al., 1993), whereas in the *S. robusta* light organ lens tissue levels of ALDH-like protein were detectable at hatching (data not shown).

**BACTERIA-INDUCED MODIFICATIONS OF LIGHT ORGAN MORPHOLOGY.**—Within hours of exposure to competent strains of symbiotic bacteria, the light organs of both *E. scolopes* and *S. robusta* undergo a morphological remodeling. The cells of the ciliated ridge and appendages, used to promote the initiation of the symbiosis, begin a program of cell death and regression. What differs between these two species is the time frame in which this restructuring takes place. In *E. scolopes*, the bacteria initiate a program of apoptosis approximately 6 h post-inoculation, peaking at 14 h after exposure (Montgomery and McFall-Ngai, 1993; Foster and McFall-Ngai, 1998). The ciliated epithelial field then flattens out and recedes over the next 4 d (Fig. 5A). In *S. robusta*, although there was a bacteria-induced cell death pattern comparable to that of *E. scolopes*, the regression of the ciliated field was not complete, even after 9 d of exposure to *V. fischeri* ES114 (Fig. 5B). A
portion of the anterior appendage and ciliated ridge remained active (Fig. 5C), continuing to circulate water over the pores of the light organ. Where no competent strains of *V. fischeri* were present in their environment, no initiation of apoptosis and no regression of superficial ciliated field were noted in either species of host.

**DISCUSSION**

The results of this comparative study (Fig. 6) of the light organs of *S. robusta* and *E. scolopes* revealed that: (1) differences exist in the extent of light organ development during embyogenesis, most likely the result of the disparity in the length of the embryonic period between these two species, which in turn is related to different egg sizes leading to different body sizes of hatchlings; and (2) the role of bacteria in light organ morphogenesis is similar in these two species, and is restricted to the early phases of post-hatch development.
Both species begin the process of light organ formation at similar stages in their embryonic periods. These similarities include the formation of deeply invaginated crypt spaces in succession on the surface of the light organ. Upon formation of the third crypt space, the embryonic period of *E. scolopes* ends and the animal hatches. The embryonic period in *S. robusta*, however, continues producing additional structures, including a fourth pore leading to a fourth crypt space, features that are not found in its counterpart *E. scolopes*. Upon hatching, the light organs of both species undergo a series of morphological changes in response to symbiosis-competent bacteria. The ciliated epithelial field (CEF) on the surface of the light organ is lost through a process of cell death and regression. The length of this bacteria-induced development differs between the two species. In *E. scolopes* the program occurs over the first 4 d of the symbiosis, whereas in *S. robusta* the loss of the CEF takes over 9 d to complete. (* refers to number of days post-hatching).

Both species begin the process of light organ formation at similar points in their embryonic development. The superficial field of epithelial cells that extends outward comprising the anterior and posterior appendages, forms within two weeks of fertilization in both species. The similarities continue as the crypts begin to form in succession as invaginations on the epithelial surface of the nascent light organ. After the appearance of the third pair of crypts in *E. scolopes*, the animal hatches, but embryogenesis continues in the *S. robusta*. The protracted embryogenesis of the Mediterranean species produces a larger light organ with more elaborate superficial structures, including an expansive cili-
ated field with a more pronounced ridge of elongated cilia, as well as an additional crypt on each side of the organ.

When *S. robusta* hatches, the light organ has the shape and anatomical structure that it will have throughout the host’s life history. This stage of development is reached only several weeks after *E. scolopes* has hatched, when the host has achieved a similar mantle length as the newly hatched *S. robusta*. Perhaps this heterochronic shift in the time point of maturation reflects the size at which a fully functional light organ is essential for the counterillumination, antipredatory behavior for which the bacterial luminescence is used. However, both species have elaborate structures to potentiate infection of the organ, and when bacteria are presented to hatchlings at concentrations similar to those in the field, the animals always become infected within minutes to hours (Ruby and Asato, 1993; pers. observ.) These data would suggest that inoculation of the light organs immediately upon hatching has evolved as a critical aspect of the progression of these symbioses. Further, since development of the mature light organ shape and the accessory tissues is not under bacterial induction, the presence of these features in *S. robusta* at hatching may simply be the result of the extended embryonic period in this species.

Although different in their morphological appearance at hatching, the developmental response to symbiosis-competent bacteria in *S. robusta* is similar to that of *E. scolopes*. Most notably, the bacteria trigger the loss of the ciliated field of cells on the light organ surface of the juvenile. The presence of an elaborate tissue, the sole function of which is to potentiate the formation of a symbiosis, appears to be unique to the squid-bacteria symbioses; i.e., in other symbiotic associations, the bacteria take advantage of already existing anatomical features as a means to achieve host tissue colonization. For example, nitrogen-fixing rhizobia use the root hair as the site of entry in the legume-rhizobia symbioses (Niner and Hirsch, 1998), and the hydrothermal-vent tube worm *Ridgea* spp. uses a transient gut to ensure colonization of the trophosome with sulfur-oxidizing symbionts (Southward, 1988). The selection for a structure dedicated to the colonization process underscores the potential importance of immediate colonization of the tissue upon hatching of the squid host.

The presence of a light organ within the sepiolids may be a primitive or a derived condition; species of the sister genus of *Sepiola*, *Sepietta* (Nishiguchi et al., 1998), do not have light organs (Fig. 3C). However, the light organ location, anatomy, and morphology is similar not only in the geographically distant *Euprymna* spp. and *Sepiola* spp., but also in the distantly related loliginid squid species that have bacterial light organs (Okada, 1927; Fukasawa and Dunlap, 1986; Pringgenies and Jorgensen, 1994), although they harbor *Photobacterium leiognathi* (Fukasawa and Dunlap, 1986). The morphology of the loliginid light organ is virtually indistinguishable from that of the sepiolid light organ. A determination of whether the light organs are primitive or independently derived will require a more thorough understanding of the relationship among these species and the patterns of light organ occurrence.

The closest analogue, and perhaps homologue, to the light organ is the accessory nidamental gland (ANG), which is composed of a series of epithelia-lined tubules that harbor a consortium of bacteria (Bloodgood, 1977). This gland, which is present in the females of a variety of squid species, including members of the Sepiolidae and Loliginidae, only develops as the female comes to sexual maturity. Both the ANG and the light organ are situated close to the ink sac. Further, the immature ANG has a ciliated epithelium that also appears to potentiate the colonization of the gland with symbionts. In addition, after
colonization, the cilia are lost from the ANG epithelium and are replaced by microvilli (Kaufman et al., 1998). Because of these similarities with the light organ, some authors have posited that the light organ is derived evolutionarily from the more widely occurring ANG (see review in Montgomery and McFall-Ngai, 1993). Alternatively, the two organs evolved independently and the tissues around the ink sac are unusually susceptible to interactions with bacteria.

The onset of late light organ development is independent of symbiont control in both *E. scolopes* (Montgomery and McFall-Ngai, 1998; Claes and Dunlap, 2000) and *S. robusta* (this study). This pattern of development, in which some portions of the program are ‘hardwired’ whereas others require interaction with the symbionts, contrasts sharply with the developmental pattern of root nodules in legume-rhizobia symbioses. In the root-nodule symbioses, which are the only other associations where development has been extensively experimentally manipulated, roots do not have well-developed tissues awaiting colonization with symbionts (Niner and Hirsch, 1998). Instead, nodules form only after direct interaction with rhizobia in the soil, and each stage in nodule maturation requires interaction with the symbionts. This difference may reflect the fact that the legume-rhizobia symbioses are facultative, occurring only under conditions of nitrogen limitation. In contrast, *Sepiola* and *Euprymna* species never occur without light organs, and the organs have always been found to have bacterial symbionts (M.M.-N., pers. observ.). This observation would suggest that, whereas the animals can be raised without the bacteria under laboratory conditions, the symbiosis is obligate for these animals in the field.

The direct comparison between these two host species of sepiolids has revealed that the role of bacteria in early and late light organ development is conserved, despite a significant difference in the length of their embryonic development. This difference in embryonic duration results in morphological differences in light organ appearance at hatching, but it does not affect the eventual appearance and function of the organ.

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