

Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*

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Abstract

The small neritic cephalopod *Euprymna scolopes* possesses a large glandular light organ that contains the symbiotic luminous bacterium *Vibrio fischeri*. Adult and immature *E. scolopes* were caught in the evening with dip nets in shallow water along the shore of Kaneohe Bay, Oahu, Hawaii, during late February 1984. The initiation of the symbiosis was investigated by rearing the cephalopods either in seawater taken from aquaria containing adult *E. scolopes* or in seawater with reduced bacterial concentrations due to filtration or due to absence of adults. Light production was measured during early development. Bioluminescence was not detected in *E. scolopes* immediately after hatching. Most individuals of *E. scolopes* that hatched into seawater containing, or previously exposed to, adults produced light within 24 h. Individuals that hatched into filtered seawater did not produce light. The data suggest that each generation acquires an infection from free-living bacteria rather than from the egg, and that light production is dependent on the nutritional state of the host. Access to an initial inoculum of free-living, luminous bacteria seems to be critical for establishing a successful symbiosis.

Introduction

Most bioluminescent oceanic cephalopods have intrinsic bioluminescent systems, whereas neritic species such as *Euprymna scolopes* usually employ a bacterial bioluminescent system (Morin 1983, Herring 1988). Unlike intrinsic light organs, all bacterial organs open to the exterior, either directly through a pore or through the gut lumen (Herring 1977). All hosts with bacterial light organs must contend with some similar, unique problems. These include restriction of the bacteria to the particular organ, culture mainte-

nance, and reinfection of successive generations with the appropriate strain of bacterium (Herring 1977, Hastings and Nealson 1981).

Euprymna scolopes is a nearshore, nocturnally pelagic cephalopod that contains symbiotic luminous bacteria within a pair of large, bilobed visceral light organs. Each light organ consists of a pair of lobular transparent lenses, a large bilobed reflector, and a sacculate crypt housing the symbiotic bioluminescent bacteria (Singley 1980). The symbiont of *E. scolopes* is *Vibrio fischeri* (Leisman, in Hastings and Nealson 1981). *E. scolopes* apparently controls bacterial light production mechanically by means of a shutter apparatus that exposes the bacterial chamber of the light organ when it is opened, as does *Sepiolo atlantica* (Herring et al. 1981). In laboratory culture, bacterial light emission starts in the middle of the log phase of growth and is triggered only when a bacterial colony reaches a critical density. Nealson (1977) has described the phenomenon of autoinduction, in which a specific concentration of an inducer produced by the bacteria is necessary for luminescence. The development of bacterial bioluminescence in newly hatched *E. scolopes* may therefore be a complex process involving both maturation of the light organ and luminescence induction of the symbionts.

The transfer of *Vibrio fischeri* to new generations of *Euprymna scolopes* could occur in either of two ways. First, the infection could be passed on through the egg. Alternatively, an inoculation might occur subsequent to hatching. Both potential methods of infection were investigated in the present study by rearing *E. scolopes* through hatching in the laboratory and monitoring the development of bioluminescence under different environmental conditions.

Materials and methods

Adult and immature *Euprymna scolopes* were caught in the evening with dip nets in shallow water along the shore of Kaneohe Bay, Oahu, Hawaii, during late February 1984. The cephalopods were kept in recirculating seawater aquar-

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ia containing subsand filters covered with several centimeters of coarse beach sand, at 23 °C. The salinity of the seawater was initially 35.37‰. Each cephalopod was fed several grass shrimp, *Leander debilis*, per day. Each of four females spawned one to three times within 2 mo of capture. All females produced eggs of uniform size in clutches, and coated the outermost eggs with grains of sand. Hatching began approximately 22 d after spawning, and all viable eggs in a clutch hatched within a 2 to 3 d period.

Female No. 1 was kept isolated in a 190-liter aquarium. She laid two clutches of eggs and died 2 d prior to hatching of the second clutch. Clutch No. 1 consisted of more than 100 eggs scattered on a plastic partition and on the side of the tank. The partition was broken in half approximately 10 d after spawning. One half was placed in a 30.4-liter aquarium without adult *Euprymna scolopes*. The other half was left, with Clutch No. 2, in the large aquarium with the adult female. Female No. 2 was kept in a 38-liter aquarium with two coral heads and a mature male. Three small clutches were laid on the coral at unobserved times, and only one was used in this study. Each egg mass had been cemented within a crevice at the base of a coral and completely covered with sand. The clutch of Female No. 2 examined was left on the coral head in the tank with the two adults until just before hatching. Then, the egg mass was transferred from the coral head to a jar of filter-sterilized seawater. Female No. 3 was kept in a 76-liter tank. She spawned only once, and the egg mass was transferred to filtered seawater 10 d later. Only three individuals from this clutch were successfully induced to hatch. Female No. 4 was caught at a juvenile stage and reared in the laboratory in a 57-liter aquarium with five other immature *E. scolopes*. Eggs were laid in the corner of the tank and were immediately transferred to the 30.4-liter tank without adult *E. scolopes*. Shortly before hatching, the egg mass was transferred to filtered seawater.

Either 0.22 or 0.45 μm Nuclepore filters were used to remove luminous bacteria to obtain filtered seawater conditions. Ruby et al. (1980) found that these pore sizes were equally effective in removing luminous bacteria. There were three types of hatching conditions: (1) actively induced hatching (AI); (2) passively induced hatching (PI); (3) natural hatching (N). Eggs that were actively induced to hatch were separated from the attachment site when embryonic development was near completion. Eggs that were passively induced to hatch were gently disturbed without touching the egg capsules; this often occurred when the egg mass was moved or temporarily held out of the water. In both AI and PI hatching, the exact time of hatching is known. During natural hatching, only the approximate time of hatching is known.

The three sets of environmental conditions for the eggs are summarized in Table 1. From all three environment types, 14 groups, containing 2 to 16 individuals per group ($x=9$), were included in this study. The groups were separated and placed in autoclaved jars to monitor the development of bioluminescence under specified hatching conditions. Only on one occasion did a group contain individuals from more than one clutch. On this occasion, the three offspring of Female No. 3 were transferred to individual jars of filtered seawater for a 24 h time-series conducted in conjunction with separated individuals from Female No. 2. The length of time that the eggs were exposed to filtered seawater prior to hatching in that seawater type depended upon the specific group of individuals, but the duration did not seem to have any effect. Therefore, the filtered seawater type refers to hatching but not necessarily incubation in filtered seawater.

Light production was measured by pipetting each individual hatchling plus some of its seawater into a vial and inserting the vial into an ATP-photometer (Model 3000, SAI Technical Co. instrument). Prior to examining all individu-

Table 1. *Euprymna scolopes*. Summary of hatching and post-hatching environmental conditions. Numbers of groups from all females (F) within each hatching type are listed. AI: actively induced hatching; PI: passively induced hatching; N: natural hatching

Female used	Egg source	Seawater type	Adults present	Hatching:		
				AI	PI	N
Environment 1 F 1 } F 4 }	Half of Clutch No.1	Unfiltered	No	2 ^a	1	1
Environment 2 F 1 } F 1 }						
	Half of Clutch No. 1 } Clutch No. 2 }	Unfiltered	Yes	1 ^b	1	3 ^b
Environment 3 F 2 } F 3 } F 4 }	Clutch No. 1 } 3 individuals } Clutch No. 1 }	Filtered	No	3	1	1

^a One AI group of Female No. 4 was hatched into seawater that had contained six adult *E. scolopes* until 1 wk before hatching. Early development of this group was identical to groups of Environment Type 1

^b One AI group and one N group of Female No.1, Clutch No. 2, were hatched and reared in seawater that had contained the adult female until 2 d before hatching. Early development of these groups was identical to groups of Environment Type 2

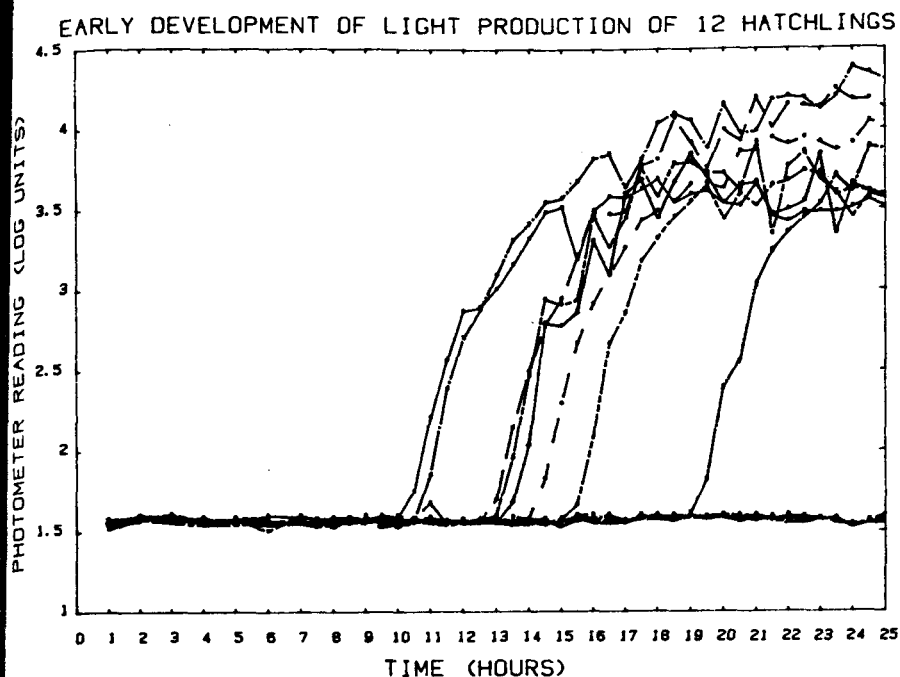


Fig. 1. *Euprymna scolopes*. Light production measured at 30 min intervals from 0 to 24 h for twelve individuals of Clutch No. 2 of Female No. 1. Hatching and rearing occurred in seawater that had contained the adult female until 2 d before hatching. Three individuals did not begin light production until after 24 h, and one individual never developed bioluminescence. (Light production = amount of irradiance detected and is shown as log ATP-photometer units here and in all subsequent figures)

als of a group at a developmental time point, the pipette and vial were rinsed with fresh water and then flushed with seawater taken from the jar of individuals to be measured. This procedure ensured that no luminous bacteria from a previous group contaminated the next group, since the light of marine, luminous bacteria disappears in fresh water and cannot be revived upon adding salt (Harvey 1952). For most experimental groups, the hatchlings were not fed and death presumably resulted from starvation. Maximum light production was estimated based upon a cross-calibration of the Model 3000 ATP-photometer with a Model 2000 ATP-photometer (both SAI Technical Co. instruments), using a ^{14}C light standard. The Model 2000 ATP-photometer had been calibrated with phototubes and known light intensities from a tungsten light source and neutral-density filters. The level of bioluminescence measured was below the eye's detection. The instrument detects irradiance on the phototube due to light emission from the light organ relative to the distance between the phototube and the light organ. Therefore, maximum light production was estimated in units of photons $\text{cm}^{-2} \text{s}^{-1}$.

The hatchlings were kept in a lighted room for at least 30 min prior to measuring light production. The vial was filled to less than one fourth with seawater to reduce the cephalopod's movement, because both its position and the state of chromatophore expansion could affect the photometer reading. The average of four consecutive readings was used as the estimate of light production. For unknown reasons, readings decreased slightly with time spent in the ATP-photometer. Occasionally, after long periods of use, increased thermionic emission by the ATP-photometer caused readings to increase above the standard background. Therefore, the background was checked by periodically measuring filtered seawater or fresh water. The background photome-

ter readings varied between 1.5 and 1.6 (log units) most of the time, and reached a value as high as 2.1 during periods of extended observation.

Results

No bioluminescence was detected in unhatched (e.g. Time 0 on Fig. 3A) or freshly hatched *Euprymna scolopes* (Fig. 1–4). *E. scolopes* that hatched into seawater taken from tanks containing adults, or which hatched directly into those tanks, began to produce light at approximately 10 to 20 h after hatching (Fig. 1). The time of the onset of bioluminescence varied between individuals, but almost all individuals which became luminescent did so within 24 h. Of the 12 individuals whose development is shown in Fig. 1, one did not luminesce but also died very early (between 49 and 69 h after hatching). Three individuals did not produce light until 24 to 48 h after hatching, but these may have been premature hatchlings.

Regardless of the time of onset, once bioluminescence had become detectable, light intensity increased exponentially with time in a logarithmic pattern (Fig. 1). The lag phase individually varied in length, but the exponential phase was uniform in duration (approximately 5 h) for all hatchlings. A relatively stationary phase of maximal light production ensued. Maximum light production was estimated to be 2.4×10^4 photons $\text{cm}^{-2} \text{s}^{-1}$. Bioluminescence gradually diminished over the next few days in the unfed individuals (Fig. 4).

Bioluminescence became completely extinguished at 3 to 4 d, before death of the organism at 5 to 6 d. Attempts to feed mysids and *Artemia* sp. to some hatchlings were ineffective for their survival. One individual lived for 9 d and an-

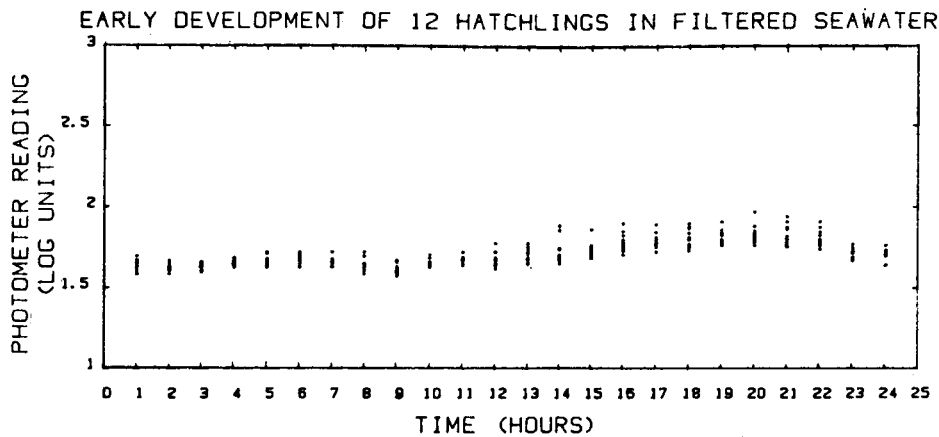


Fig. 2. *Euprymna scolopes*. Light production measured at 30 min intervals from 0 to 24 h for nine individuals of Female No. 2 and three individuals of Female No. 3. Hatching and rearing occurred in filtered seawater

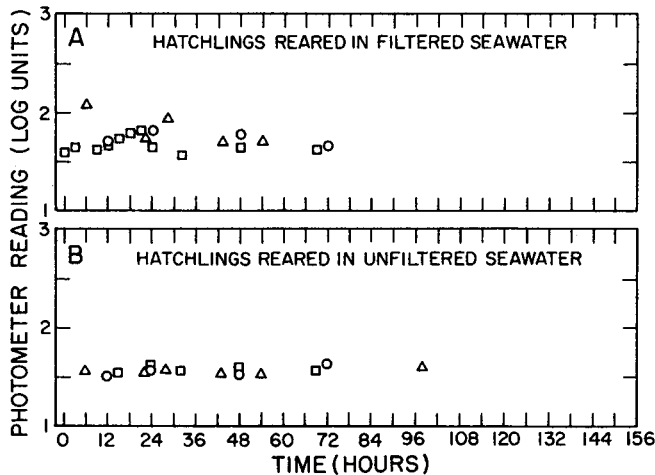


Fig. 3. *Euprymna scolopes* (A) Light production of groups hatched into and reared in filtered seawater taken from tanks without adults; data point on ordinate at time = 0 h represents eggs containing live but unhatched *E. scolopes*. (B) Light production of groups hatched into and reared in unfiltered seawater taken from the tanks without adults. Here, and in Fig. 4, light production by groups of individuals was measured at specific times after hatching. Symbols represent median for all individuals in one or more groups measured at a given time, under specific hatching conditions; \square = actively induced hatching; Δ = passively induced hatching; \circ = natural hatching. For natural hatching, time intervals of 0–12 h, 12–24 h, 24–48 h, 48–72 h, and 72–132 h were chosen, and symbols are plotted at the end of each relevant interval; all naturally-hatched groups whose known age-range fit anywhere within a chosen interval are therefore plotted together at the end of that interval

other lived for 11 d. These apparently fed on some of the mysids. Several individuals displayed a secondary increase in light production to the stationary phase, after having shown a tendency to decline (data not shown). This may have resulted from feeding, but persistent feeding attempts were not made. The light output of dead individuals was negligible compared to the background reading except in the case of one individual which died very early, probably of a cause other than starvation.

Juveniles that hatched into either filtered seawater (Figs. 2, 3 A) or unfiltered seawater without adults (Fig. 3 B) failed to develop bioluminescence. The slight increase in light

production shown in Fig. 2 and variation around the background level of approximately 1.5 (Fig. 3) were due to thermionic emission of the ATP-photometer.

Removal of adult *Euprymna scolopes* 2 d before hatching did not inhibit the development of bioluminescence, whereas removal of adults 1 wk before hatching prevented most hatchlings from producing light. The seawater of two groups that were hatched and reared in Environment Type 2 had contained an adult until 2 d before hatching (see footnote "b", Table 1). Of the 21 individuals in these two groups, 20 developed bioluminescence (Figs. 1 and 4). The seawater of one group that was hatched and reared in Environment Type 1 had contained adults until 1 wk before hatching (see footnote "b", Table 1). None of the 10 individuals in this group developed bioluminescence (Fig. 3 B). Another group of 10 individuals was hatched into filtered seawater and was transferred after 5 h to the seawater that had contained adults 1 wk earlier; only one member of this group developed bioluminescence (additional data).

Discussion

Newly hatched *Euprymna scolopes* developed bioluminescence when reared in seawater taken from tanks containing adults. Bioluminescence did not develop before death (from starvation) when luminous bacteria were removed from seawater by filtration. These findings support the hypothesis that a bacterial inoculum from seawater is necessary in order for *E. scolopes* to develop bioluminescence.

The development of bioluminescence must rely upon both functionality of the light organ and presence of the symbiont. The induction of bioluminescence may have been delayed for individuals that were actively induced to hatch during a premature developmental stage. Passively induced hatching should have caused the least amount of individual variation within a group while still ensuring that all members of the group hatched simultaneously at a known time.

Neither bacteria attached to the egg cases nor seawater which lacked recent contact with adult *Euprymna scolopes* was sufficient as a source of inoculum. Although the concentrations of *Vibrio fischeri* in the seawater were not known, they were likely to have been reduced or absent in unfiltered

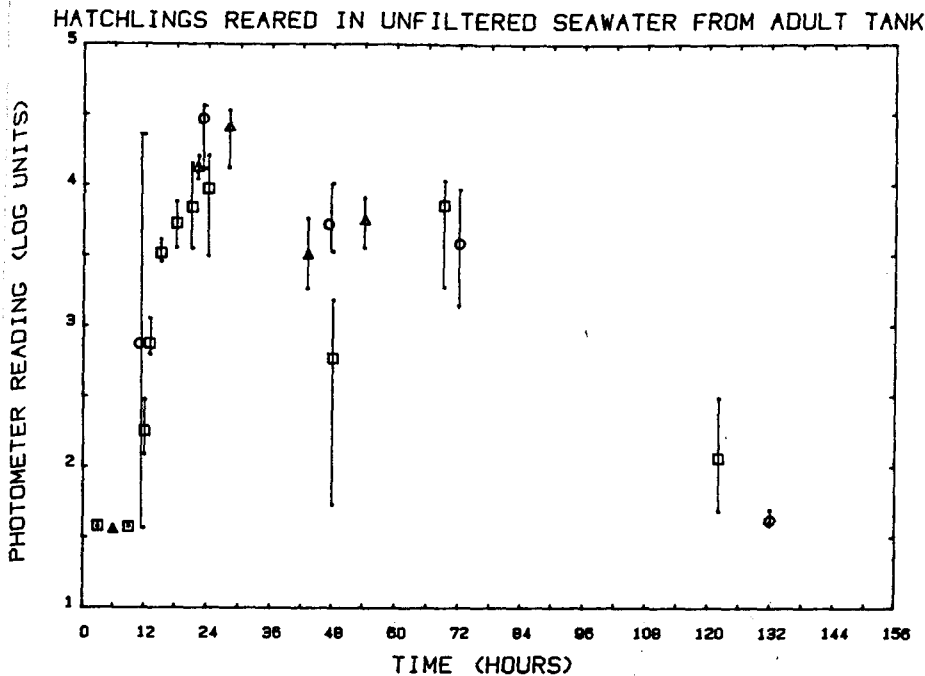


Fig. 4. *Euprymna scolopes*. Light production of groups hatched into and reared in unfiltered seawater taken from tanks containing adult cephalopods. Bars indicate 95% confidence intervals around medians of light production, except where interval is smaller than symbol size. Entire range is shown for the three naturally-hatched individuals at 12 h time point, since, when there are six or fewer individuals, 95% confidence limits cannot be determined. Individuals for which development was monitored separately over 24 h (Fig. 1) are here adjusted to a starting time set at 12 h, in order to reduce 95% confidence intervals for time points prior to 24 h, where individual variation in lag-phase length occurred. Further details as in legend to Fig. 3

seawater that had not contained adults relative to seawater that had recently contained adults. Both the monocentrid fish *Cleidopus gloriamaris* (e.g. Tebo et al. 1979) and the sepiolid *Sepiolo atlantica* (Herring et al. 1981) are known to release copious quantities of bacteria from the light organ into the medium. The difference in the development of light production of *E. scolopes* reared in unfiltered seawater that had contained adults until either 2 d or 1 wk before hatching suggests that adult *E. scolopes* release large numbers of *V. fischeri* into seawater.

In nature, the numbers of luminous bacteria may be too greatly reduced for the development of a symbiotic bacterial bioluminescent system to rely upon chance encounters between the newly hatched hosts and sparsely scattered bacteria. Symbionts isolated from a host are sometimes not the most abundant luminous bacteria in the seawater in the area (Reichelt et al. 1977, Orndorff and Colwell 1980). One possible explanation for the existence of the symbiosis is that the host's environment may be patchy with respect to the specific bacteria (e.g. the benthic area near the egg clutch may contain higher concentrations of the bacteria).

Metabolic integration of host organisms and symbiotic luminous bacteria has been described by numerous investigators. In the present study, the stationary growth phase appears to represent a fully functional bioluminescent system that is being maintained at a stable intensity. Tebo et al. (1979) demonstrated that 10 to 15% of *Vibrio fischeri* cells are in a dividing stage when growing in culture as opposed to 2% undergoing cell division within the photophore of the fish *Monocentris japonica*. Ruby and Nealson (1976) proposed a speculative model of symbiosis between *V. fischeri* and *M. japonica*. According to this model, the maintenance of a low ambient-oxygen concentration in the light organ is believed to produce maximal luminescence with a minimal

commitment to bacterial growth. The blood of marine fish, which contains glucose as its principal nutrient, is believed to support the metabolic demands of the bacteria. The symbiosis model proposed by Ruby and Nealson may apply to the system of *Euprymna scolopes* as well.

The decline in bioluminescence in *Euprymna scolopes* was probably a result of host starvation, and a few bacteria may have still been present in the light organ of *E. scolopes* after bioluminescence ceased, at 3 to 4 d after hatching. Similarly, the shallow-water fish *Anomalops katoptron* ceased to emit light more than 1 wk before death due to starvation, and some bacteria were still present within the organ even though light had become extinguished (Meyer-Rochow 1976). A reduced number of bacteria probably causes light emission to cease, but it may be revivable if nutrients become available. Some of the feeding observations made during this study suggest this idea.

Although the selection mechanism remains uncertain, the probable method of initiation of the symbiosis between *Vibrio fischeri* and *Euprymna scolopes* has been determined. The development of bioluminescence in successive generations of *E. scolopes* relies on exposure to a source of free-living, luminous bacteria. Light intensity increases exponentially during early development and appears to reach a maintenance level that depends on the nutritional state of the host. *E. scolopes* provides an accessible system for future studies of symbiotic bacterial bioluminescence due to the ease of capture, rapid spawning and embryonic development, and well-established rearing techniques (Arnold et al. 1972).

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