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Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm

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Abstract In most symbioses between animals and luminous bacteria it has been assumed that the bacterial symbionts luminesce continuously, and that the control of luminescent output by the animal is mediated through elaborate accessory structures, such as chromatophores and muscular shutters that surround the host light organ. However, we have found that while in the light organ of the sepiolid squid *Euprymna scolopes*, symbiotic cells of *Vibrio fischeri* do not produce a continuously uniform level of luminescence, but instead exhibit predictable cyclic fluctuations in the amount of light emitted per cell. This daily biological rhythm exhibits many features of a circadian pattern, and produces an elevated intensity of symbiont luminescence in juvenile animals during the hours preceding the onset of ambient darkness. Comparisons of the specific luminescence of bacteria in the intact light organ with that of newly released bacteria support the existence of a direct host regulation of the specific activity of symbiont luminescence that does not require the intervention of accessory tissues. A model encompassing the currently available evidence is proposed for the control of growth and luminescence activity in the *E. scolopes*/*V. fischeri* light organ symbiosis.

Key words Biological rhythm · Bioluminescence · Symbiosis · *Vibrio fischeri* · *Euprymna scolopes*

Abbreviations *CFU* colony-forming-unit · *LD* light-dark

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Introduction

Many fundamental processes that occur in plants and animals (e.g., cell division, metabolic activity, growth, etc.) exhibit regular fluctuations with a period length of approximately 24 h (Edmunds 1988; Hastings et al. 1991). These rhythms, which are often controlled by endogenous biochemical "clocks", represent mechanisms by which biological activities are coordinated with daily environmental changes in light and temperature. Although such daily rhythms have now been described in members of every eukaryotic phylum (Edmunds 1988, Hastings et al. 1991), the roles that these rhythms may play in controlling the symbiotic activities of animal-host-associated prokaryotes remain unknown. Thus, while photoperiodic control of cells within a genome has been extensively studied, such interactions between the genomes of two different, yet intimately associated organisms, have not yet been explored. Nevertheless, because bacterial symbionts are usually intimately associated with host tissues, and the two partners are often metabolically interconnected, it should not be surprising that the scope of their interactions might extend to patterns of diel activity.

The lack of definitive evidence for host control of symbiotic interactions via biological rhythms is in part due to the inability to adequately define and quantify the respective roles and activities of a given host and its symbiont in an intact association. However, in recent years the light organ association between the sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* has proven to be an experimentally tractable system by which to study several levels of symbiotic interaction (McFall-Ngai and Ruby 1991; Ruby and McFall-Ngai 1992). The natural association exhibits a strong species specificity and is initiated within hours after the juvenile squid hatches only if symbiotically competent *V. fischeri* cells are present in the ambient seawater (Ruby and Asato 1993;

Ruby and McFall-Ngai 1992; Wei and Young 1989). The bacterial symbionts remain extracellular and proliferate rapidly within the nascent epithelial crypts of the host light organ, which lies embedded within the ink sac in the center of the squid's mantle cavity (Montgomery and McFall-Ngai 1993). As the squid matures, the organ develops into a complex bilobed structure, with numerous accessory tissues that serve to allow the host to control the intensity of light output (McFall-Ngai and Montgomery 1990). These accessory tissues include a thick reflector, which serves to ventrally direct bacterially produced light, and diverticula of the ink sac, which can dynamically rotate around the bacteria-containing tissue to attenuate the level of light emitted from the animal (McFall-Ngai and Montgomery 1990; Montgomery and McFall-Ngai 1993, 1994). Typically, the host squid is buried in the sand during the day, emerging at dusk to forage in the water column (Moynihan 1983); while the pattern of host bioluminescence has not been studied in nature, the morphology of the light organ suggests that during this crepuscular activity the light organ is used for counterillumination, a camouflaging behavior not uncommon in bioluminescent marine animals active in low-light environments (McFall-Ngai 1991).

The first evidence that an additional, more direct, regulation of the specific activity of bacterial luminescence occurs in this light organ association came with the observation that, following the initial colonization of the juvenile light organ, excess symbionts are periodically expelled from the light organ (Ruby and Asato 1993). In adult animals this expulsion event (which removes over 95% of the light organ bacterial population) coincides with the onset of environmental light under a photic regime of alternating 12 h periods of light and darkness (Lee and Ruby 1994), and coincides with the time when the animal is buried in the sand. An average doubling time of 4.8 h was calculated to account for the "repopulation" of the adult light organ between each daily expulsion event, although it was not determined whether the symbionts grow slowly and continuously, or more quickly over a limited period, during the host's daylight quiescence (Lee and Ruby 1994). These observations have suggested that a diel expulsion is a primary mechanism by which the animal limits the maximum number of symbiotic cells present in its light organ.

We demonstrate here that, consistent with the behavior of adult squids, the diel expulsion of bacteria by juvenile *E. scolopes* is a rapid and transitory event that occurs synchronously, coincident with, and perhaps triggered by, the appearance of daylight in the animal's environment. In addition to this diel expulsion, we show that within the light organ of *E. scolopes* the bacterial symbionts do not produce a continuous level of light emission, but instead exhibit a daily, cyclic fluctuation in the specific level of luminescence. Unlike the expulsion events, the cycles in luminescence intensity appear to be controlled by an intrinsic biological rhythm (driven by the ambient photic conditions) that is in many ways analogous to a circadian rhythm. Not only do these data suggest several layers of host regulation over *V. fischeri* luminescence activity, but also, to our knowledge, these observations represent the first direct evidence of an animal host controlling the number and activity of its extracellular bacterial population as part of a daily biological rhythm.

Materials and methods

Infection of juvenile squid hatchlings and manipulation of photic conditions

A modification of a previously described procedure (McFall-Ngai and Ruby 1991) was used to inoculate the nascent light organs of newly hatched juveniles of *E. scolopes* (approximately 1.5 mm in mantle length) with symbiotic bacteria. Briefly, cultured cells of *V. fischeri* strain ES114 (Boettcher and Ruby 1990) were suspended in 5 ml of seawater in 20-ml glass vials to a final concentration of approximately 10^3 cells per ml before the addition of a single hatchling squid. The hatchlings were then maintained in a variety of light regimes consisting of different periods of moderate light intensity (30 μEinsteins/m^2/s), dim light intensity (2 μEinsteins/m^2/s) or complete darkness. Changes in the light regimes were produced by controlling the room lights with an electronic timer. After colonization, light production from symbiotic juveniles was monitored at regular intervals (~ once every 2–3 h) over a 3- to 4-day period using either a sensitive photometer as previously described (Boettcher and Ruby 1990), or a Wallac-LKB RackBeta 1211 automated luminescence counter.

These laboratory studies were complemented with additional experiments performed with field-caught *E. scolopes*. Several juvenile animals (2.0 to 3.8 mm mantle length) were collected at night during February 1994 from Kaneohe Bay, Hawaii, as previously described (McFall-Ngai and Montgomery 1990). The animals were placed in vials of seawater and maintained outdoors at the ambient temperature (24° C) and natural environmental light conditions (the period between sunrise and sunset was approximately 11.5 h). At regular intervals the animals were transferred to fresh seawater, and their luminescence was determined photometrically.

Quantification of symbiotic *V. fischeri* expelled by juvenile squid

Newly hatched *E. scolopes* were placed in seawater containing *V. fischeri* cells as described above to initiate a colonization of their nascent light organs. After 3 h, the animals were transferred to fresh seawater containing no added *V. fischeri*. At subsequent intervals of 3 or 6 h the animals were transferred again to fresh seawater, and an aliquot of the spent seawater was spread on a plate of seawater-nutrient agar medium (Boettcher and Ruby 1990). The number of *V. fischeri* CFUs was determined after a 24-h incubation at 28° C, and the concentration of cells present in the spent seawater was calculated (Ruby and Asato 1993; Lee and Ruby 1994). Almost all the colonies that formed were *V. fischeri* as determined by their distinctive colony morphology (Lee and Ruby 1994).

Determination of in vivo and in vitro luminescence specific activities

Groups of juvenile squid displaying cyclic light emission were killed at times of elevated ("peak") or reduced ("valley") levels of

luminescence. Groups of arrhythmic animals, which exhibited no cyclic emission, were killed at various times over a given 24-h period. Animals were rinsed in 0.2-μm filter-sterilized seawater, and in vivo luminescence was determined photometrically. For the in vitro measurements, these same animals were each then immediately transferred to 700 μl of sterile seawater in a microcentrifuge tube and rapidly homogenized. Previous experiments have determined that no detectable luminous bacteria reside anywhere in the animal except within the light organ (Ruby and Asato 1993); therefore, it was unnecessary to dissect away the other tissues before homogenization. After vortexing for 30 s the light emission of the homogenate was measured photometrically, and dilutions of the homogenate were spread onto the surface of seawater-nutrient agar plates. Enumeration of the *V. fischeri* colonies arising on the plates after a 24-h incubation at 28 C provided an estimate of the number of bacterial symbionts present in each light organ (Boettcher and Ruby 1990; Ruby and Asato 1993). Luminescence measurements from the whole animal (in vivo) and homogenate (in vitro) were then divided by the number of bacterial cells present in that light organ to yield estimates of the specific activity of luminescence. The luminescence of juvenile squid with both expanded and contracted chromatophores was measured and no systematic difference in luminescence was observed due to the state of the chromatophores.

Results

Patterns of light emission in symbiotic hatchlings

Soon after inoculation with *V. fischeri* cells, juvenile *E. scolopes* that were maintained in a constant 12:12 h light-dark (LD) photic cycle produced an increasing level of bioluminescence (Fig. 1A) concomitant with symbiotic colonization. Thereafter, the level of intensity

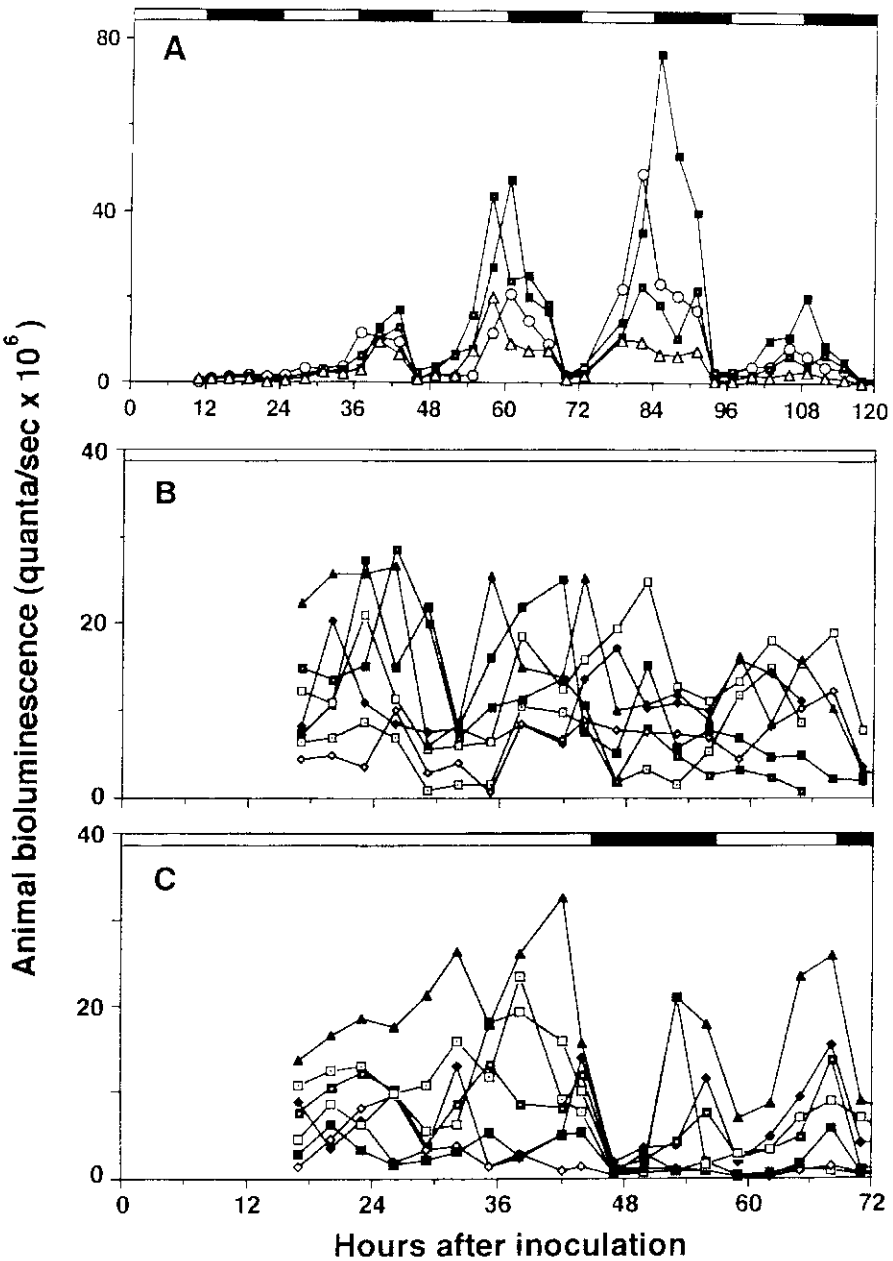

Fig. 1A–C Intensities of light emission detected from symbiotic juvenile *E. scolopes* maintained in environments of either: **A** 12:12 h LD cycle of moderate light intensity, **B** constant dim light, or **C** constant dim light followed by a 12:12 h LD cycle, as indicated by the *bar at the top of each panel*. Each series of points represents the behavior of an individual animal; between 10 to 15 animals were examined for each condition. *Black bars* indicate periods of darkness, and the *light bars* represent 30 μEinsteins/m^2/s in panel **A**, and 2 μEinsteins/m^2/s in panels **B** and **C**

of light emission followed a cyclic pattern, reaching the highest values in the hours preceding the onset of darkness, and becoming between 10- and 100-times lower throughout the remaining hours of the daily cycle. On rare occasion, juveniles can be maintained for over 72 h, and these animals show a persistence of this rhythmic pattern (Fig. 1A). There was no evidence of such cyclic luminescence when animals were exposed to either constant dim (2 μEinsteins/m^2/s) illumination (Fig. 1B), constant moderate (30 μEinsteins/m^2/s) illumination (data not shown), or constant darkness (data not shown); however, the partial establishment of a rhythmic cycle could be induced in arrhythmic animals by restoring them to a normal sequence of 12:12 h LD periods (Fig. 1C).

To determine whether the bioluminescence patterns observed in newly hatched animals inoculated and maintained in the laboratory were also characteristic of those exhibited by animals in nature, we measured the luminescence of juvenile *E. scolopes* collected and maintained in Hawaii under a natural day/night photic cycle. Such animals also exhibited luminescence rhythms that fluctuated over orders of magnitude in intensity, generally becoming most intense as darkness approached (Fig. 2). However, the periods of elevated luminescence in these animals were longer than those observed in laboratory-maintained animals and persisted for much of the ensuing night. Nevertheless, while the squid exhibited these qualitative differences, perhaps in response to whether the transitions from light to dark conditions were rapid or gradual, the cyclic nature of bioluminescence was a characteristic of both laboratory- and field-maintained animals.

Daily expulsion of symbiotic bacteria from the light organ

A dramatic peak in light emission was observed when the luminescence of symbiotic juvenile squid was measured within 90 min after the onset of the photic period (Fig. 3). This peak was significantly greater than the normal elevated luminescence levels measured in the hours preceding darkness, and was transient (i.e., not detected at the subsequent time point). Periodic analyses of the seawater itself both for luminescence, and for the presence of *V. fischeri* (Fig. 4), indicated that the peaks were due to an expulsion into the ambient water of about 10^6 cells, or between 50 and 95% of the bacterial population of the juvenile light organ. Because such a peak was not observed in animals maintained under constant illumination (data not shown), this synchronized expulsion event appears to be a direct behavioral response to the transition from dark to lighted conditions. The decrease in the number of symbionts resulting from the expulsion may contribute to a decrease in the potential for animal luminescence; however, it is unlikely to directly account for the daily fluctuations in light emission observed (Figs. 1A and 2) because the expulsion occurs approximately 12 h after the decrease in luminescence of 12:12 h LD-entrained animals (Fig. 3).

Comparison of in vivo and in vitro luminescence specific activities

A possible mechanism by which the host could mediate the observed fluctuations in its light emission is to

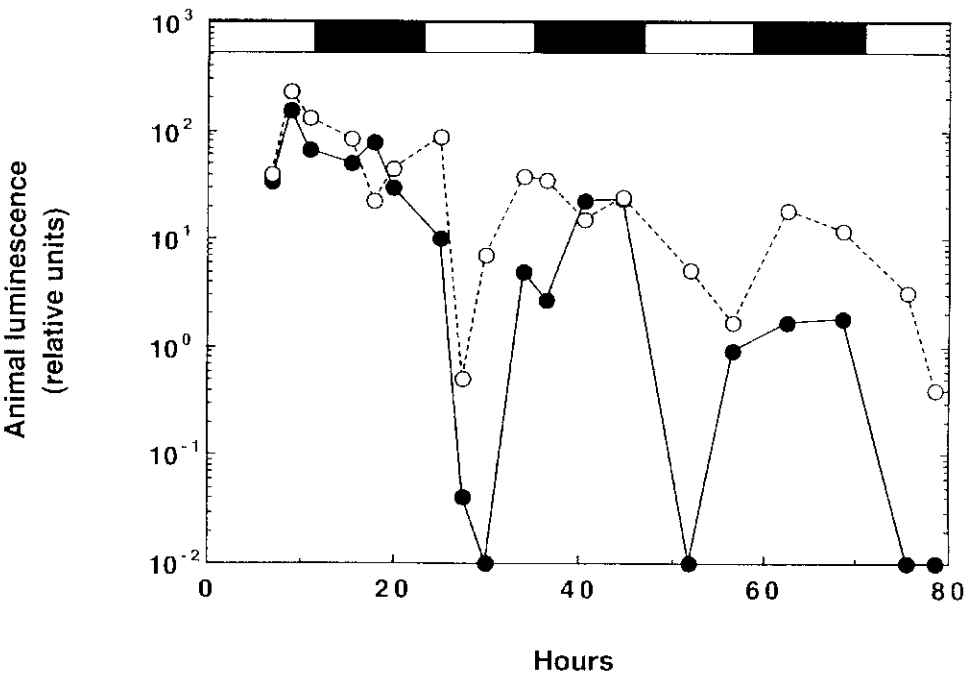

Fig. 2 Intensities of light emission detected from field-collected symbiotic juvenile *E. scolopes* maintained under natural lighting. Juvenile animals collected in Hawaii were left in the ambient daylight and night conditions, and their luminescence was measured periodically over 3 days. The official times of sunrise and sunset are indicated by the *bar at the top of the graph*. The relative intensities of luminescence emitted by either a 2.0-mm animal (●), or a 3.8-mm animal (○), approximately 1 week-old and 1 month-old respectively, were determined photometrically. While the larger animal produced levels of luminescence approximately 50-fold higher than that of the smaller squid, for graphical convenience the maximum value of light emission for each animal is normalized to approximately 100 relative luminescence units and the data have been plotted on a log scale

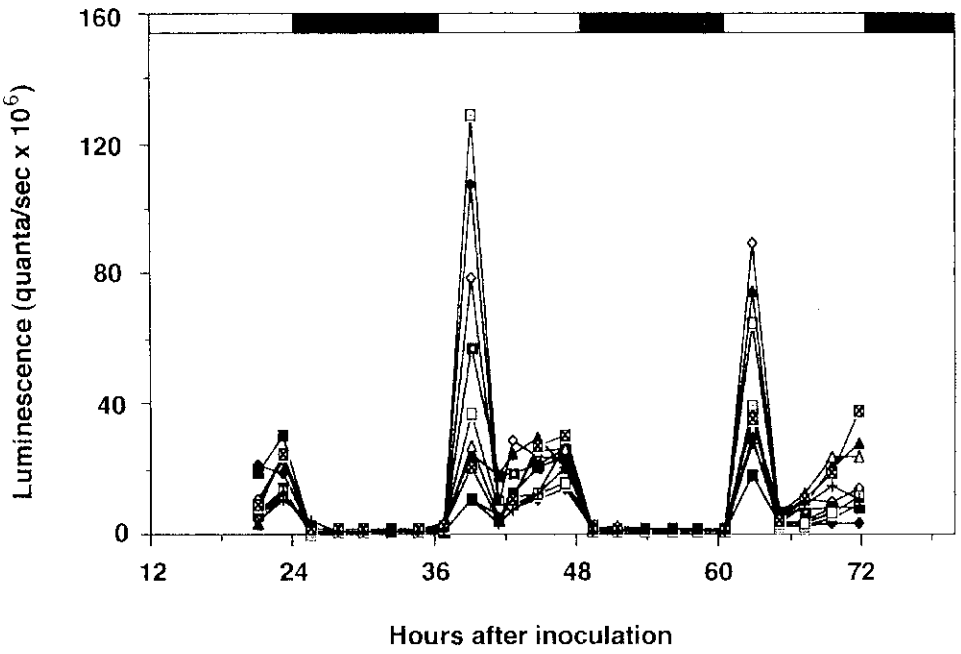

Fig. 3 Levels of light detected from water containing juvenile *E. scolopes* under the same conditions as described in Fig. 1A. A sharp transient peak in luminescence was observed when light measurements were taken within 90 min after the onset of photic conditions (periods of light and darkness are indicated by the *bar at the top of the graph*). Analyses of CFUs revealed that these peaks resulted from the expulsion into the surrounding seawater of approximately 4×10^5 bacteria (per animal). The smaller shoulders following these peaks represent the typical daily increases in animal light emission, the intensities of which remain well below those resulting from symbiont release

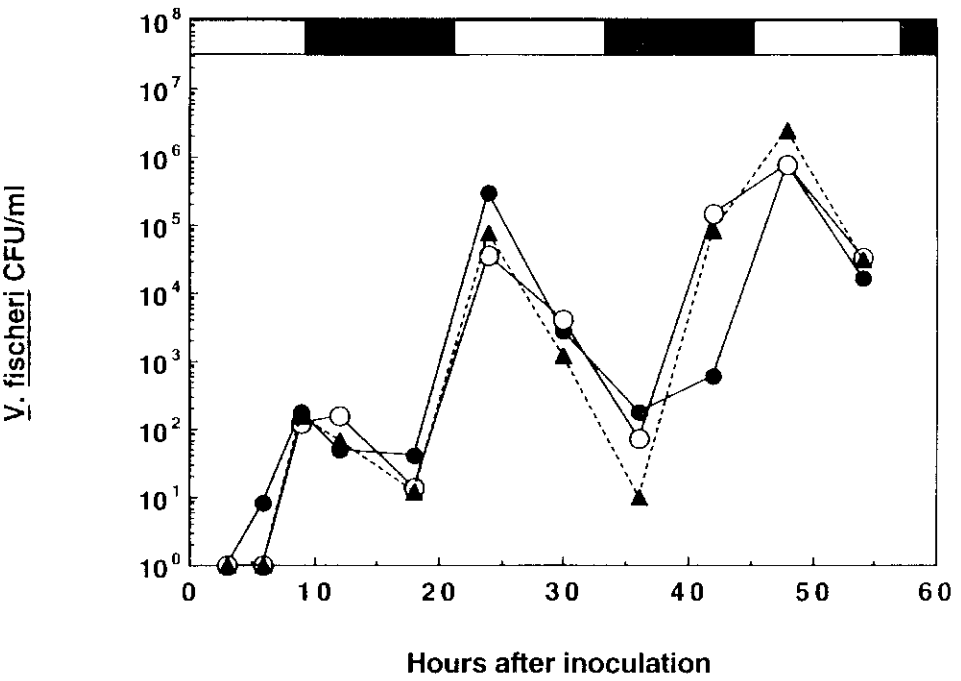

Fig. 4 Appearance of *V. fischeri* cells expelled from the light organs of symbiotic juvenile squid. Three animals in individual vials of seawater were monitored at the times indicated. The number of *V. fischeri* CFUs accumulating in the seawater during the time interval since the previous measurement (and seawater change) are graphed as a function of time after an initial 3 h exposure to an inoculum of bacteria. The periods of ambient light and darkness experienced by the animals are indicated by the *bar at the top of the graph*

directly regulate the level of luminescence of the cells in the symbiont population. To examine this possibility we determined the amount of light produced per bacterial cell, both in the intact squid light organ (in vivo measurements), and from whole animal homogenates (in vitro measurements) of squid examined at different phases of the 12:12 h LD photic cycle. Because there is significant individual variation in the absolute number of bacterial symbionts present in any given juvenile hatchling [Ruby and Asato 1993; this study (data not shown)] we calculated the average amount of luminescence produced per bacterial cell (i.e., specific luminescence) in vivo and in vitro. The ratio of in vivo luminescence to in vitro luminescence provided a direct

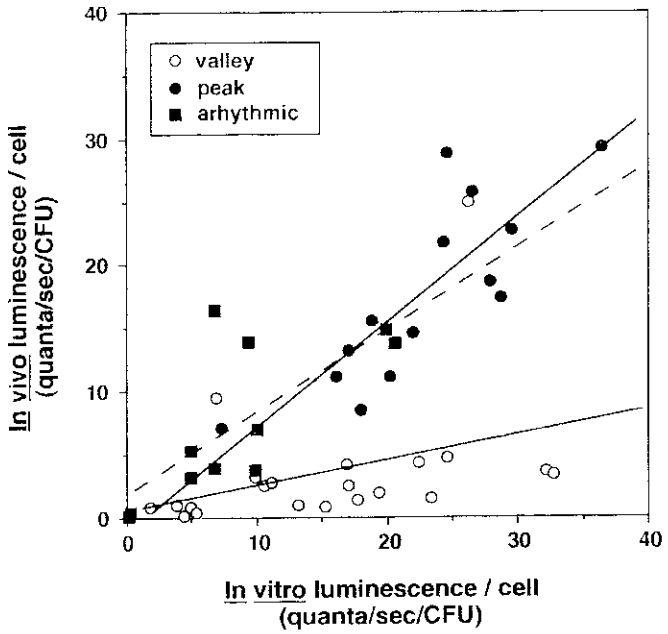

Fig. 5 Specific activity of in vivo and in vitro luminescence by symbiotic *V. fischeri* cells. Each point represents the amount of light (in quanta/s) per bacterial cell detected in an intact animal (in vivo) relative to the amount of light per cell detected immediately after artificial release from the animal (in vitro). (See Materials and methods for further details.) The linear least-squares regression method was used to identify trends in the data (*solid lines* are drawn through both the "peak" and "valley" data sets; the *dashed line* corresponds to the data from arrhythmic animals). The approximate 1:1 ratio of in vivo to in vitro luminescence of bacteria from brightly luminescent animals ("peak" animals) suggested that the *V. fischeri* cells were capable of producing relatively high levels of light emission in the light organ at these times. However, similar comparisons of in vivo and in vitro luminescence of bacteria from animals producing relatively low levels of light ("valley" animals) reveal that during these periods the bacterial symbionts in the light organ were not expressing their actual capacity for light production. Bacteria obtained from arrhythmic animals (see Fig. 1B) consistently resembled those from the brightly luminescent "peak" animals, suggesting that under a regime of constant luminescence the host squid do not regulate the light emission of their symbionts

measurement of the differences between actual and potential light emission activity by the symbionts at times of either elevated or reduced levels of animal bioluminescence.

During the elevated ("peak") period, produced at the onset of darkness by animals maintained in the experimentally controlled 12:12 h LD photic cycle (Fig. 1A), the level of in vivo luminescence closely approximated in vitro luminescence (Fig. 5); that is, the actual level of light emission of symbionts in the organ was similar to their maximum level of emission when released from the organ. However, during the reduced ("valley") period, the in vivo luminescence was diminished by a factor of about 10, while the specific activity of luminescence of the released symbionts remained high. These differences between levels of in vivo luminescence suggest that under laboratory conditions in a 12:12 h LD regime the animal actively suppresses light emission of its bacterial symbionts during much of the 24 h cycle. Support for this conclusion comes from an examination of squid that are maintained under constant photic conditions and, thus, exhibiting arrhythmic light production. In these animals, the specific activity of in vivo luminescence was equivalent to the in vitro light production regardless of the time of measurement (Fig. 5). Thus, these animals appeared to behave as if they were LD-entrained animals that were suspended in the elevated phase of their light emission. At no time did the arrhythmic animals appear to inhibit the luminescence of their bacterial symbionts.

Discussion

The results of the present study show that after onset of symbiosis, juvenile *E. scolopes* squid begin to temporally modulate their bioluminescence such that they emit relatively elevated levels of light during the hours preceding ambient darkness. The resulting pattern exhibits several characteristics of a circadian rhythm (for a comprehensive treatment of biological rhythms, see reference Edmunds 1988 and Hastings et al. 1991). First, changes in the levels of bioluminescence cycled with a 24-h periodicity when animals were maintained under conditions of alternating 12-h exposures to light and darkness. In laboratory experiments animals decreased their light emission shortly after the onset of darkness (Fig. 1A), although among animals exposed to more natural (i.e., daylight) photic conditions the elevated luminescence persisted throughout the night (Fig. 2). In addition, the rhythm was driven by (and not merely a response to) the ambient light regime. Because of the presumed role of bioluminescence in the crepuscular activity of *E. scolopes*, we were not surprised that animals maintained under natural daylight conditions (i.e., where dusk precedes darkness) exhibited elevated light emission as the sun began to set (Fig. 2). However, even in the laboratory, where the transition from light to dark was an abrupt one, animals anticipated the onset of darkness by increasing the intensity of their light emission (Fig. 1A).

Circadian clocks are entrained by 24-h environmental cycles (usually light) (Hastings et al. 1991), and we observed that arrhythmic squid maintained in constant dim light for 48 h partially established a normal rhythm when restored to a 12:12 h LD regime (Fig. 1C). However, even though *E. scolopes* eggs were maintained in a 12:12 h LD regime prior to hatching, a cyclic pattern of luminescence was not observed in the absence of a photic cue after hatching and inoculation with *V. fischeri* cells (Fig. 1B). There was a coordinate decrease in light emission at 32 h in these animals (Fig. 1B), but no clear trend in light emission became established. Unfortunately, we are not as yet able to conduct experiments that directly address

the ability of an established rhythm to "free run" (i.e. to persist after the removal of a photic cue) because of the sharp decline in the health of laboratory-maintained juveniles that occurs as soon as 72 h after hatching. Thus, at this time, we must conclude that the daily bioluminescence rhythm of juvenile *E. scolopes* cannot be termed a true circadian rhythm. Nevertheless, the observed similarities suggest that the squid luminescence cycle shares functional analogies with such rhythms.

We considered several morphological bases for the cyclic pattern of luminescence, including the possible modulation of light either by accessory tissues of the light organ or by chromatophores in the mantle tissue. Histological examination of adult *E. scolopes* light organs have revealed several accessory tissues (i.e. ink sac, lens and reflector) that are believed to modify the intensity of the ventrally directed light (McFall-Ngai and Montgomery 1990). However, these structures are either not present or are not functionally developed in juvenile animals (McFall-Ngai and Montgomery 1990; Montgomery and McFall-Ngai 1993, 1994), and therefore cannot explain the observed fluctuations in luminescence. Further, contraction and expansion of chromatophores on the animal's surface did not significantly affect light output (data not shown).

We also investigated the possibility that the changes in bioluminescence emission from symbiotic squid were a direct result of changes in the absolute number of bacterial symbionts present in the light organ, and found that there is a daily synchronous expulsion of bacterial symbionts by the juvenile squid. However, while dramatic, the expulsion events appeared not to account for the observed cyclic changes in levels of luminescence because it occurred as much as 12 h after the onset of the observed decrease in luminescence of 12:12 h LD-entrained animals in the laboratory (Fig. 3). The release of symbionts into the surrounding seawater by a 12:12 h LD-entrained squid was detected both as the sudden appearance of *V. fischeri* CFUs in, and as a dramatic increase in light produced by, samples of the seawater itself (Figs. 3 and 4) immediately after the onset of lighted conditions. In contrast to light emission from symbiotic light organs, these peaks of luminescence by released symbionts are, as expected, very short-lived; laboratory-cultured *V. fischeri* cells rapidly become dim when they are diluted into seawater (Nealson 1977; Boettcher and Ruby 1990; Ruby and Asato 1993). The phenomenon of symbiont expulsion has been previously reported for adult *E. scolopes* (Lee and Ruby 1994), and appears to be precipitated by the transition from conditions of darkness to those of light, rather than by an intrinsic biological rhythm. We hypothesize that the expulsion of symbionts at sunrise serves a dual purpose in nature: (i) to control the absolute number of symbiont cells, thereby limiting the potential for both overgrowth of the light organ and unnecessary metabolic expense to the host; and, (ii) to facilitate the infection of the nascent light organs of subsequent generations of newly hatched squids by contributing to the abundance of symbiosis-competent *V. fischeri* in the seawater of *E. scolopes* habitats (Lee and Ruby 1994).

Other possible mechanisms for the cyclic nature of light emission by juvenile squid included: (i) changes in the luminescence potential of each bacterial cell; and/or, (ii) variations in the amount of light actually produced by each bacterium. These two hypotheses were jointly addressed by estimating the specific light emission of bacterial symbionts, both while they were in the organ and immediately after release from the host, and comparing these values during periods of elevated and reduced animal luminescence (Fig. 5). Regardless of the level of light emission from the animal, the luminescence potential of the symbionts (measured within seconds of their release) was relatively high. The increased luminescence of bacterial cells released from dim animals occurred too quickly to be the result of either transcriptional induction of the enzymes responsible for luminescence, or changes in the levels of physiological effectors [e.g., cAMP, iron, and osmolarity (Dunlap 1985, 1991; Dunlap and Greenberg 1985; Haygood and Nealson 1985)] that can induce bacterial luminescence: these changes require at least 10 min to exert their effect on bacterial light emission (Meighen and Dunlap 1993). Similarly, the effect of autoinducer [a positive transcriptional coregulator of the *lux* operon (Kaplan and Greenberg 1985; Eberhard et al. 1981)] requires at least 20 min to initiate an increase in luminescence (Nealson 1977), and recent evidence has shown that the concentration of autoinducer present in the light organs of *E. scolopes* is at least 40-times higher than necessary for elevated luminescence in culture (Boettcher and Ruby 1995). Thus, it appears most likely that, in the light organs of dim animals, the concentration of one (or more) components of the luminescence reaction itself is restricted, and that upon release this component becomes rapidly available, immediately unleashing the luminescence potential of the bacteria.

Light production by *V. fischeri* is catalyzed by bacterial luciferase, which uses molecular oxygen to oxidize $FMNH_2$ and tetradecanal, resulting in the release of a photon of light (Engebrecht and Silverman 1984; Meighen 1988). $FMNH_2$ and tetradecanal are internally regenerated within the bacterial cell, and release from the organ into seawater is unlikely to increase their availability; however, oxygen is a substrate that must be continuously supplied to the bacteria from the external environment. Thus we propose that, in the animal, the rate of delivery of molecular oxygen to the bacterial symbionts is host-controlled, and that reduced oxygen availability results in periods of decreased light emission by the symbionts. Upon release (either through the natural process of expulsion (Fig. 4), or artificially by tissue homogenization), oxygen instantly becomes available to these

bacteria in amounts sufficient to relieve the limitation, resulting in a 10-fold increase in specific luminescence (Fig. 5). Host-control of symbiont luminescence by regulating the availability of oxygen is a mechanism previously reported for the light organ of leiognathid fishes (McFall-Ngai 1991), suggesting the general feasibility of such a control. However, while these fishes were shown to have the potential to regulate the luminescence of their symbionts by changing the gas mixture in their gas bladders, it has not yet been reported that such a control of luminescence behavior occurs naturally, or that it is used to produce a cyclic modulation of the light emission of their symbionts.

We propose the following model for the regulation of symbiont growth and luminescence activity by *E. scolopes*. At dusk, high levels of oxygen are delivered to the light organ symbionts via diffusion from the vascularized epithelial tissue (Montgomery and McFall-Ngai 1993), stimulating them to produce the elevated levels of luminescence used for the animal's counterillumination behavior while hunting; additional tissues, present in the adult light organ, can also serve to rapidly modulate the intensity and direction of light emission (McFall-Ngai and Montgomery 1990). The symbiont population is maintained throughout the rest of the night (Fig. 2) but immediately after sunrise, the majority of the now unnecessary bacterial symbionts is expelled into the surrounding sea water (Fig. 3). In anticipation of the approach of the ensuing nightfall, the remaining cells are provided with the oxygen and nutrients required for recolonization, and an exponentially increasing expression of light production is again detected from the juvenile animals (Figs. 1A and 2).

Many aspects of this model have not been directly demonstrated (e.g., the presumed role of oxygen in the rhythm), and further investigation is warranted before a biological function for the luminescence cycle can be definitively ascribed. However, it will be of interest to now examine other light organ symbioses to determine whether similar rhythms in bioluminescence do in fact occur in these hosts. Due to the diverse array of functions for bacterially derived luminescence (Nealson and Hastings 1979; McFall-Ngai and Dunlap 1983; Nealson and Hastings 1991; Morin et al. 1975) such investigations might provide evidence regarding the evolution of this type of host control of bacterial symbionts. We may find that this phenomenon is a direct result of intrinsic circadian regulation of other aspects of the hosts' physiology. Alternatively, it is possible that this system may represent an example of a biological rhythm that has coevolved with the development of tissues that are exclusively designed to house bacterial symbiont populations.

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