

Localization of bioluminescence in the siphonophore *Nanomia cara*

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Abstract

The base of the tentacle of the developing physonect larva (*Nanomia cara*) has a bioluminescent region. The ability to produce light in the larva is transitory; this ability first appears at about two days of development and is disappearing by eight days, as the larva begins to feed. Subsequently paired bilaterally symmetrical bioluminescent organs are found on the nectophores and the bracts of the adult colony. In both the larva and the adult, bioluminescence is mediated by a calcium specific photoprotein. In all cases the photocytess lack a green fluorescent protein.

Introduction

Bioluminescence has been noted in a number of species of the order Siphonophora (Harvey, 1952; Nicol, 1958; Mackie, 1962; Morin, 1974). This order is divided into three sub-orders: the Cystonectae (3 genera), the Physonectae (19 genera) and the Calcophorae (30 genera) (Totton, 1965). Bioluminescence has not been observed in the Cystonectae; however, it has been observed in two, possibly three, genera of Physonectae and in seven genera of Calcophorae. Very little is known about the location of the cells that are responsible for light production in these animals. The only species that has been adequately studied in this respect is *Hippopodius hippocampus* of the suborder Calcophorae (Bassot *et al.*, 1978). Observations on this colony with an image intensifier indicate that the entire surface epithelium of the nectophores is capable of producing light.

The biochemical mechanisms responsible for bioluminescence have been studied in a number of species of cnidarians. In hydrozoans this mechanism involves the activation of a photoprotein by calcium to produce light (Shimomura, in press). In many hydrozoans, photocytess also contain a green fluorescent protein that acts as a sec-

ondary scintillatory transfer agent (Morin, 1974). The molecules responsible for light production in siphonophores have not been purified; however, Morin (1974) has unpublished data indicating that two genera of siphonophores contain both a calcium-activated photoprotein and a green fluorescent protein.

This study localizes the luminescent tissues of both the larva and the adult colony of the physonect siphonophore *Nanomia cara*. Mackie (1962) noted bioluminescence in this species: "*Nanomia* is bioluminescent and the regions emitting light are distributed in the nectophore and possibly elsewhere in a pattern resembling the color pattern by day". Péron and Lesueur (1807-16, cited by Totton, 1954) noted bioluminescence in the siphosome region of the colony. The current study also characterizes the biochemical mechanisms responsible for bioluminescence in the photocytess of this species and examines these cells for the presence of green fluorescent protein.

Materials and methods

The colonies of *Nanomia cara* used were collected at the Friday Harbor Laboratories. Freeman (1983) gave directions for collecting these colonies and for rearing the larvae from gametes produced by the colonies. For experiments in which larvae or parts of colonies were monitored for bioluminescence, the unit to be monitored was washed several times and maintained in millipore-filtered, pasteurized sea water to exclude the possibility of light production by other organisms in the sea water.

Light production was assayed qualitatively, in a dark room by a dark adapted observer (20 min in the dark), after the addition of 550 mM KCl to the preparation. In some experiments a solution of 10% Triton-X 100 in distilled water was used. Quantitative measurements of the amount of light produced were made with an integrating photometer. The recording chamber, photomultiplier tube, housing and shields used have been described in Ridgway

et al. (1977). One of the inner light tight covers of the photomultiplier was fitted with a bent 20 gauge hypodermic needle through which solutions could be added to the preparation. The integration of light production was achieved using a low leakage capacitor ($10 \mu\text{F} \pm 2\%$) whose voltage can be detected on an electrometer. Although an absolute primary calibration was not available for the photomultiplier, a secondary standard calibration was available through an assay device belonging to Dr. O. Shimomura. According to this standard, the assay system yielded 0.85×10^9 photons per volt. A three stage Noctron IV (Varo, Inc.) image intensifier was used to visualize sites of bioluminescence. The intensifier was coupled to the photography tube of a compound microscope. The output of the intensifier was coupled to an MTI (Dage) model 65 video camera. Video recordings were made on a cassette video recorder (model TLC 2001, Gyr Products).

Fluorescence microscopy was used to assay for green fluorescent protein in photocytotes. An excitation filter with transmittance in the UV range was used in conjunction with a chromatic beam splitter and a barrier filter reflecting light below ca 475 nm. The preparations were always examined with high numerical aperture objectives by a dark-adapted observer before they were considered not to have cells containing the green fluorescent protein.

In some experiments preparations were assayed for light production in Ca^{++} -free sea water (432 mM Na^+ , 9 mM K^+ , 47 mM Mg^{2+} , 505 mM Cl^- , 1 mM EGTA buffered with 20 mM TES to pH 7.8 with NaOH). In one set of experiments the calcium ionophore A23187 was used to elicit light production. It was made up as a stock solution with 1 mg ionophore/1 ml DMSO and used at a concentration of 1 $\mu\text{g}/\text{ml}$. Larvae and persons in colonies with photocytotes were homogenized in a solution containing 26 mM EGTA, 43 mM TES and 32 mM PIPES at pH 7.2 (KOH) to obtain a photoprotein extract. The calcium solution used to assay these extracts for light production contained 50 mM calcium acetate and 66 mM PIPES at pH 6.5.

Results

Bioluminescence in the larvae

The process of embryogenesis which generates the *Nanomia cara* physonect larva has been described by Freeman (1983). After cleavage and gastrulation, the embryo elongates along what will be its aboral-oral axis and the ectoderm thickens on what will be its ventral side. The aboral end of the embryo differentiates into a gas-filled pneumatophore, while the oral end differentiates a mouth which frequently has pigment cells associated with it. About half way down from the aboral end of the embryo, the ventral thickening differentiates to form one or two unbranched tentacles. At the base of the tentacle cnidobands differentiate and migrate down the tentacle.

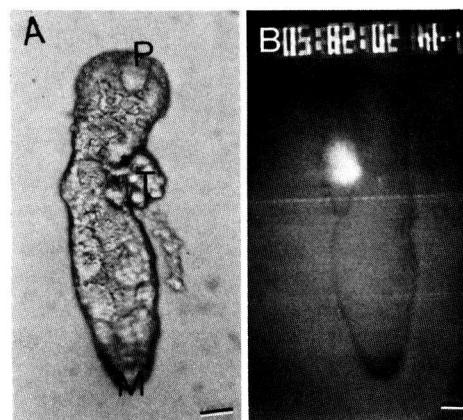


Fig. 1. *Nanomia cara*. Physonect larva: (A) Lateral view of four and a half day larva. (B) Image intensifier record of five day larva producing light. The bar indicates 50 μm . P: pneumatophore rudiment; T: tentacle rudiment; M: mouth rudiment. The image intensifier prints in Figs. 1, 4, and 5 were made by making a negative of a video tape frame showing the object with background lighting; the lights were turned off and bioluminescence was induced with KCl; a negative was then made of part of the video tape showing light production. The two negatives were printed in register to give a composite photograph

Fig. 1a shows a larva. Fig. 1b shows a similar larva viewed with an image intensifier with low background lighting following the addition of KCl. The region which is producing light is at the base of the tentacle of the larva. Five light production records of larvae ranging in age from 2.5 through 8 d of development were obtained in this way; in every case the only light-producing region was at the base of the tentacle.

The ontogeny of bioluminescence has been followed in two cohorts of eggs from different colonies by measuring the amount of light produced by each embryo in a sample every 12 or 24 h during development (Fig. 2). This graph shows that initially these embryos do not have the ability to produce light. This ability first appears at about two days of development, increases for the next two or three days, and then declines. This decline begins before the embryo has differentiated a mouth and before cnidobands migrate down the tentacle. The graph also indicates that the amount of light produced by different batches of eggs on a given day of development can vary considerably.

Bioluminescence in larvae appears to be a variable trait. A number of embryos (10 to 40) from cohorts of eggs from nine different colonies were assayed for bioluminescence at developmental stages when one would expect light to be produced. In one cohort none of the developing larvae were bioluminescent, in four cohorts only a minority (less than 20%) of the larvae were bioluminescent, while all of the larvae in the remaining four cohorts were bioluminescent.

Bioluminescence in the adult colony

The physonect larva transforms into a colony by forming two budding zones between the pneumatophore and the

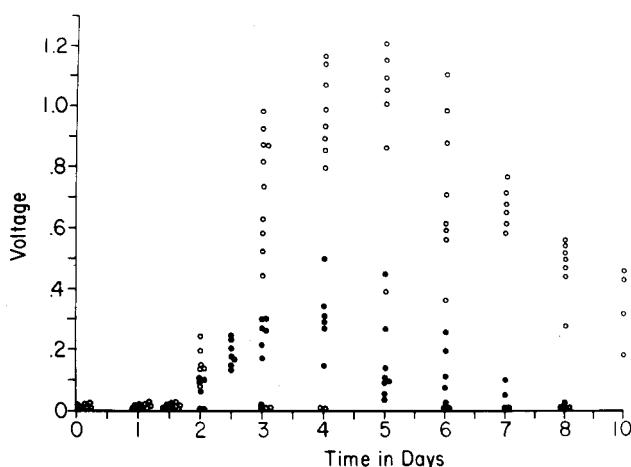


Fig. 2. *Nanomia cara*. The total amount of light produced (volts) as a function of age for developing larvae obtained from spawnings of two individuals (filled circles – larvae from Individual 1, open circles – larvae from Individual 2). Each point is one assay. An assay was done by placing a single developing larva in 0.5 ml of pasteurized sea water; light production was initiated by injecting 0.5 ml of KCl. This treatment elicits most of the light production, but the preparation was routinely given one or two additional treatments (0.5 ml) with detergent to make sure that the photoprotein was exhausted

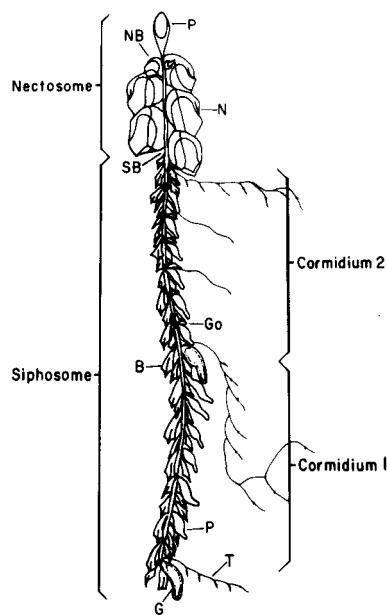


Fig. 3. *Nanomia cara*. Diagrammatic view of a colony. A stem runs the length of the colony. The nectosome consists of a pneumatophore (P), a nectophore budding zone (NB) and 5 functional nectophores (N). The siphosome begins with a siphosome budding region (SB). It consists of two complete cormidia. The gastrozooid (G) of Cormidium 1 is derived from the oral region of the larva. The tentacle (T) of the gastrozooid is short and lacks or has very short tentilla. The gastrozooid of Cormidium 2 has a tentacle with side branches. Male and female gonads (Go) are beginning to develop in association with the palpons (P) of the cormidium. Bracts (B) envelope the various members that make up the siphosome. (Drawing adapted from Mackie, 1964)

tentacle (Fig. 3). This region elongates to become the stem. At the top of the stem, just under the pneumatophore, is the budding zone which gives rise to the nectophores. The number of nectophores in a colony is variable. The pneumatophore and the nectophores with the region of the stem that they are attached to is referred to as the nectosome. New nectophores are continually being generated in the budding zone and being lost at the bottom of the nectosome region. At the base of the nectosome there is another budding region on the stem which generates the part of the colony known as the siphosome. At the end of the stem is the tentacle and the gastric region derived from the oral half of the physonect larva, which now makes up the terminal gastrozooid of the colony. The persons along the stem which make up this part of the colony are budded off in repeating units called cormidia. Each cormidium in *Nanomia cara* consists of a basal gastrozooid with a tentacle containing side branches (tentilla) with cnidobands surrounded by bracts; further up the stem there are a number of repeating groups of palpons with unbranched tentacles. Associated with each group of palpons are a set of male and female gonophores and a set of bracts. The stem of the siphosome region has from part of one cormidium to several cormidia. When one examines a set of cormidia, progressing from the terminal end of the stem to the budding zone at the base of the nectosome, the size of the persons in each cormidium becomes smaller and they are less well developed. If the terminal cormidium is present, it differs from the other cormidia because the tentacle of the gastrozooid lacks any or has very short branches (tentilla) and the cormidium has no gonads. All of the persons that make up the siphosome originate at the budding zone with the exception of the terminal gastrozooid and the bracts. The bracts are probably continuously formed all along the stem because bracts of various sizes and at various stages of development are found all along the stem regardless of the degree of growth and maturation of the cormidia that they are in. Large bracts are regularly shed from the colony.

The ability of various parts of the colony to produce light was tested by cutting the colony up into various units and testing the units for their ability to produce light. When a colony was divided into a nectosome and a siphosome, both parts produced light when tested. The nectosome was then dissected to produce a number of nectophore units and a unit consisting of a stem and the pneumatophore. When this dissection is done, one has to be careful to remove the nectophore buds from the budding zone of the stem. The nectophores produce light but the stem and pneumatophore do not. The siphosome was cut up into units the size of cormidia and all of the bracts were removed; great care was taken to remove even the smallest bracts. The bracts produce light but the remainder of the cormidia, which consists of the stem with its gastrozooid and tentacle, the palpons and the gonads, do not produce light. Because the base of the tentacle of the physonect larva is bioluminescent, the terminal gastrozooid and tentacle derived from the larva were tested. In

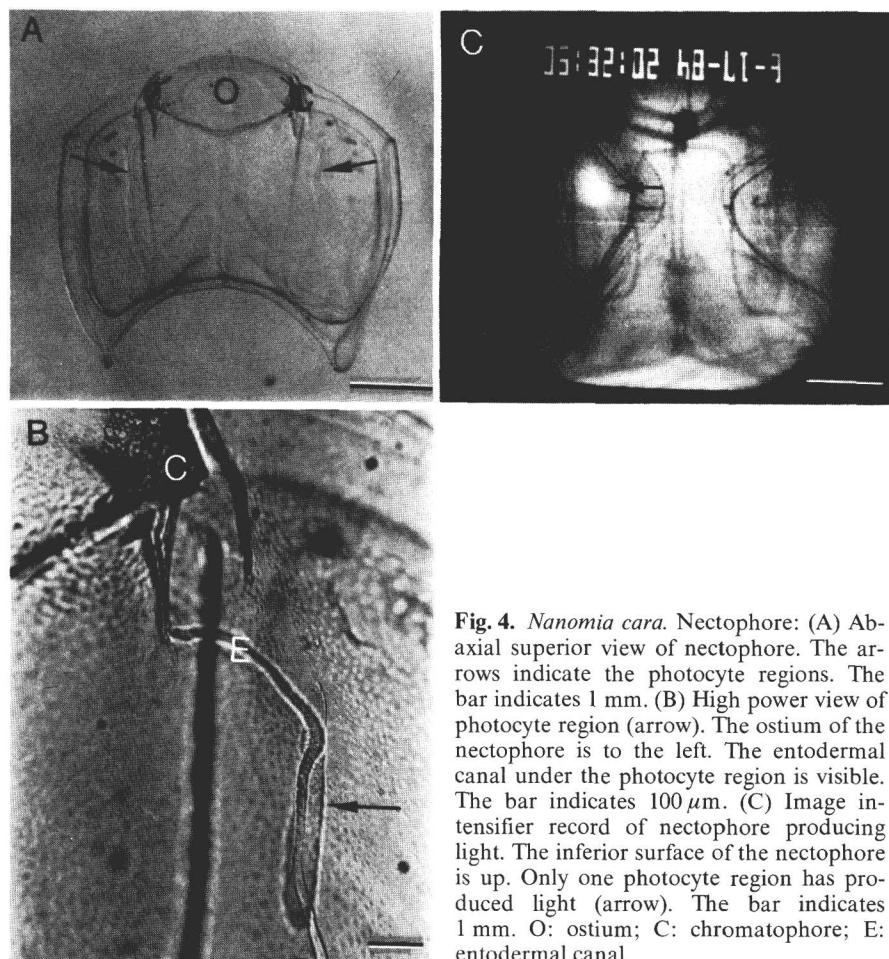


Fig. 4. *Nanomia cara*. Nectophore: (A) Abaxial superior view of nectophore. The arrows indicate the photocyte regions. The bar indicates 1 mm. (B) High power view of photocyte region (arrow). The ostium of the nectophore is to the left. The entodermal canal under the photocyte region is visible. The bar indicates 100 μ m. (C) Image intensifier record of nectophore producing light. The inferior surface of the nectophore is up. Only one photocyte region has produced light (arrow). The bar indicates 1 mm. O: ostium; C: chromatophore; E: entodermal canal

large colonies the terminal region is frequently absent. It has presumably been lost as a consequence of predation or some kind of accident. However, in small colonies with 3 to 5 nectophores, it is frequently present. Five of these gastrozooids with their tentacles were tested. None produced light. This suggests that the larval photocytes are lost as the colony develops from the larva, and is consistent with the decline shown in Fig. 2.

Fig. 4 shows a typical nectophore. There is a special population of cells that form two elongated structures on the upper abaxial epithelial face of the nectophore on either side of the ostium. When the nectophore is viewed with an image intensifier, following the addition of KCl, this is the only region that produces light. These two groups of cells will be referred to as nectophore photocyte regions. Nine nectophores were dissected so that nectophore photocyte regions were removed (Fig. 6a). When the nectophores were tested for bioluminescence after this operation they did not produce light, while the photocyte regions produced light when they were tested. This indicates that the nectophore photocyte region is the only light-producing area in the nectophore.

Nectophores originate in the budding zone at the base of the pneumatophore and slowly move down the necosome region of the stem until they are lost. When

developing nectophores from the budding region, in which the ostium has not yet formed, are tested for bioluminescence they produce light. This suggests that photocytes differentiate as the nectophore forms. When one examines the functional nectophores of a freshly collected colony with several nectophores, both photocyte regions in each nectophore look very much like the regions shown in Fig. 4 in the nectophores on the top third of the stem. The elongated photocyte region is frequently broken up into a series of smaller regions as a consequence of missing photocyte areas in the nectophores from the middle third of the stem. The nectophores from the bottom of the stem frequently do not have photocytes or only a small patch of photocytes. These observations suggest that photocytes are lost either by death or by being shed from the nectophore as the nectophore ages. When older nectophores without photocytes are tested for bioluminescence they do not produce light (6 cases). On each side of the ostium of the nectophore there are pigment cells (Mackie, 1962); these cells also disappear as the nectophore ages.

Fig. 5 shows a typical bract. There is a special population of cells that form two circular structures on the upper epithelial face on either side of the midline of the bract. When the bract is viewed with an image intensifier,

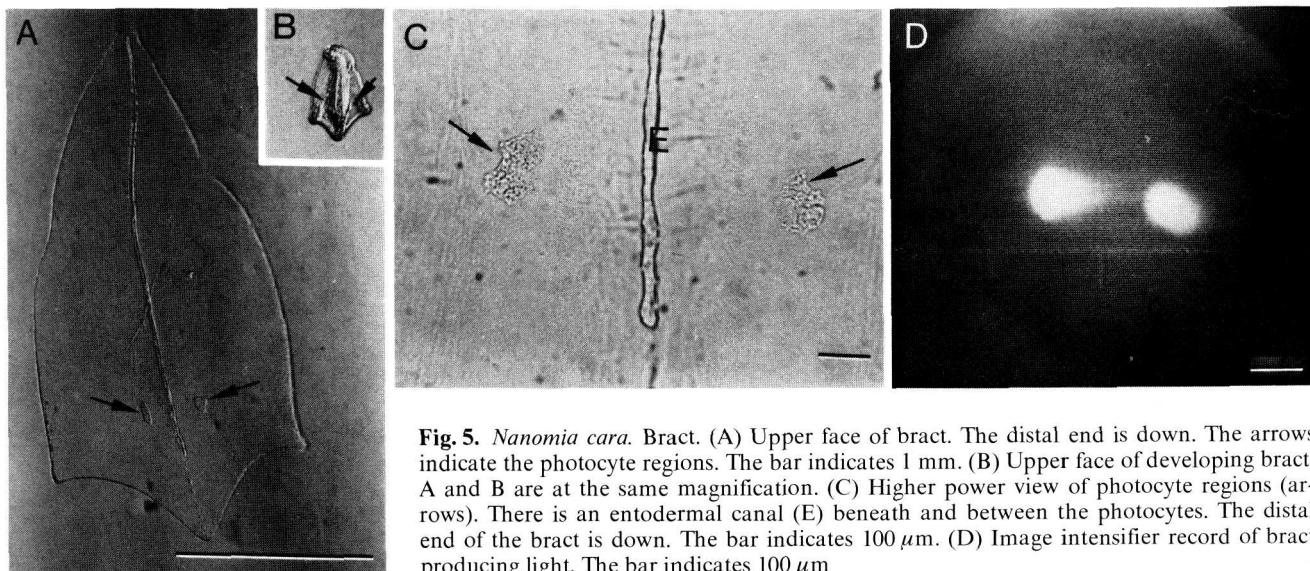


Fig. 5. *Nanomia cara*. Bract. (A) Upper face of bract. The distal end is down. The arrows indicate the photocyte regions. The bar indicates 1 mm. (B) Upper face of developing bract. A and B are at the same magnification. (C) Higher power view of photocyte regions (arrows). There is an endodermal canal (E) beneath and between the photocytes. The distal end of the bract is down. The bar indicates 100 μ m. (D) Image intensifier record of bract producing light. The bar indicates 100 μ m

following the addition of KCl, these cells are the only ones that produced light. They will be referred to as the bract photocyte regions. Eight bracts were cut into two parts two-thirds of the way down their length so that the top part did not contain the photocyte regions while the bottom third did (Fig. 6 b). When the top part of the bract was tested for bioluminescence it was negative, while the bottom third of the bract produced light when tested.

When small developing bracts were tested for bioluminescence they invariably produced light. If one brings a freshly collected colony into the lab, some large bracts will fall off the colony. When these bracts are examined they usually do not have photocyte regions. Bracts without photocytes do not produce light when tested for bioluminescence. Fig. 7 indicates whether both, only one or no photocyte regions are present on bracts as a function of bract length for a set of bracts removed from two cormidia of a freshly collected individual. Most small- and medium-sized bracts have both photocyte regions; as bracts get larger the photocyte regions are lost on one or both sides of the bract. These bracts frequently lack the nematocysts along their dorsal midline at the bract tip and show other evidence of wear. These observations suggest that photocytes are also lost as a function of age in bracts.

Both bracts and nectophores have been examined with Nomarski optics in order to find out if their photocyte regions are innervated. There are no nerves in bracts. While there are nerves in nectophores (Mackie, 1964), there is no indication that nerves innervate the photocyte regions. However, because of the thickness of the nectophore, Nomarski optics cannot be used under optimal conditions.

Chemical basis for bioluminescence

The photocytes of the larvae, and the, nectophores and bracts of the adult colony were examined for green

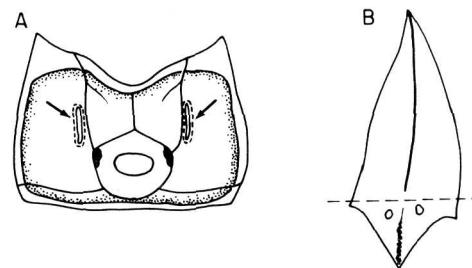


Fig. 6. *Nanomia cara*. Diagram of the operations used to remove the photogenic regions from the nectophores (A) and bracts (B). The dashed line indicates the operations done to remove the photogenic area. (Note: the drawings are not to scale)

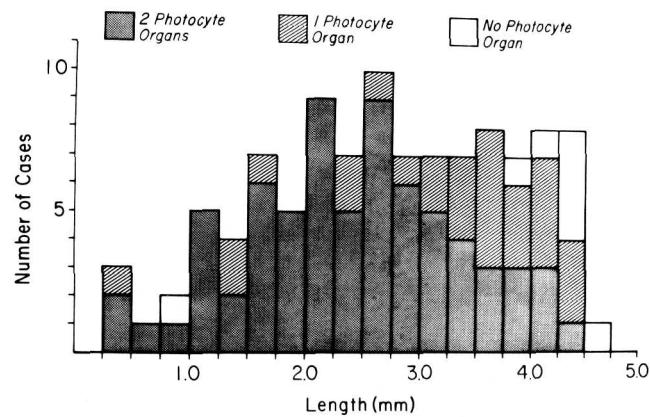


Fig. 7. *Nanomia cara*. Histogram showing the number of bracts in each length class for all bracts from the terminal most cormidium of a freshly collected sexually mature colony (The terminal cormidium did not contain the larval gastrozooid.). The number of photogenic regions per bract is plotted as a function of bract length. While a low percentage of the small bracts lack one or both photogenic regions, the majority of the large bracts lack these regions

Table 1. Effect of the ionic environment on light production by intact photocytes and photocyte homogenates

Source of photocytes	Intact (I) or homogenized (H)	Medium	Treatment	Light production
Larvae	I	Sea water	KCl	+
			detergent	+
	H	Ca-free buffer	KCl	-
			detergent	-
Bracts	I	Sea water	Ca ²⁺	+
			KCl	+
	H	Ca-free sea water	Ca ionophore	+
			Ca ionophore	-
		Ca-free buffer	KCl	-
			Ca ionophore	-
Nectophores	I	Sea water	Ca ²⁺	+
			KCl	+
	H	Ca-free buffer	Ca ²⁺	-
			KCl	-

At least three preparations were tested in each experiment

fluorescent protein. It was not detected. With the filters and optics used, green fluorescent protein was easily detected in the medusa of *Aequorea victoria* and the polyp of *Phialidium gregarium*.

The role of calcium in bioluminescence was examined by comparing light production by intact photocytes and photocyte homogenates (Table 1). When intact photocytes in sea water are treated with KCl, which presumably depolarizes these cells allowing calcium to enter, or with agents that disrupt the cell membrane (detergent) or with calcium ionophore which permeabilizes cells to calcium, light is produced. When extracts of photocytes are prepared in Ca⁺⁺-free buffer, treatment of the extracts with these agents does not cause light production, therefore these agents do not cause light production *per se*. However, adding calcium ions to these homogenates causes light production, indicating that bioluminescence is calcium mediated.

Discussion

The photocyte regions in the ectoderm of *Nanomia cara* have been figured previously; Totton (1954) identified this region as an ectodermal patch of nematocysts; Mackie (1964) included this region in his drawings of ectoderm but did not identify it. While Mackie noted that the ectoderm of *N. cara* is bioluminescent and realized that the photocyte regions described here produce light (personal communication), his statement (1962) describing the distribution of bioluminescent sources is ambiguous. Bioluminescent regions in bracts and in larvae have not been previously described for siphonophores.

It is not clear if the photocytes in the larvae are ectodermal or entodermal derivatives. The photocytes on

both the ectoderm and bracts are ectodermal derivatives. In *Hippopodius hippocampus*, the ectoderm of the ectoderm is also ectodermal derivatives (Bassot *et al.*, 1978). In all other coelenterates which have been examined photocytes are entodermal derivatives (Morin, 1974). The fact that photocytes are lost from mature ectoderm and bracts probably reflects the fact that these cells get little or no nutrition once the ectoderm and bracts have completed development. No food particles are seen in the entodermal canals of mature ectoderm and bracts. The fact that bioluminescence is only seen in larvae from some batches of eggs, the low and variable light levels, and the fact that larval photocytes do not persist in adult colonies suggest that this trait is either just appearing or that it is disappearing as part of the phenotype of this species.

The experiments with photocyte extracts suggest that bioluminescence in both the larva and the adult colony is mediated by calcium-specific photoproteins. In order to put this suggestion on a firmer footing the bioluminescent system will have to be purified. Many coelenterate photocytes contain green fluorescent protein; it is not clear why this protein is not present in *Nanomia cara*.

I have examined two colonies of *Nanomia cara* in the dark in an aquarium filled with millipore-filtered sea water so that the only source of light is the colony. Under these conditions long periods of time can elapse without light production. When light is produced, the colony seems to have bumped into the side of the aquarium or the air water interface. It appears that individual ectoderm and bracts produce light only upon physical contact; there was no indication of a coordinated light production response by the whole colony.

Acknowledgements. I want to thank Dr. D. Willows, the director of the Friday Harbor Laboratories for facilitating

my work there. Drs. G. Mackie, M. Martindale and R. Satterlie kindly provided me with colonies of *Nanomia cara*. Dr. E. B. Ridgway let me use his photomultiplier. This work was supported by grant GM20024 from the National Institutes of Health.

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Date of final manuscript acceptance: August 20, 1986.
Communicated by J. M. Lawrence, Tampa