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Internal and secreted bioluminescence of the marine polychaete *Odontosyllis phosphorea* (Syllidae)

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Abstract. The syllid polychaete *Odontosyllis phosphorea* produces brilliant displays of green bioluminescence during mating swarms. We studied freshly collected individuals of *O. phosphorea* in the laboratory to understand the characteristics of its luminescent system. Light emission appeared as an intense glow after stimulation with potassium chloride, and was associated with secreted mucus. The mucus was viscous, blue in color, and exhibited a long-lasting glow that was greatly intensified by addition of peroxidase or ammonium persulfate. The emission spectrum of mucus-associated bioluminescence was unimodal, with a maximum emission in the green spectrum between 494 and 504 nm. The fluorescence emission spectrum was similar, but the fluorescence intensity was low unless it originated from mucus that had already produced light, suggesting that the oxidized product of the light production is the source of fluorescence. Individuals as small as 0.5–1.0 mm produced bioluminescence that was mainly internal and not secreted as mucus. The early occurrence of bioluminescence in the life cycle of members of *O. phosphorea* suggests that bioluminescence may be used for purposes other than attracting mates. The luminous system was functional at temperatures as low as −20°C and was degraded above 40°C. Mixing hot and cold extracts of the mucus did not result in reconstituting original levels of light emission. Additionally, mucus samples exposed to oxygen depletion by bubbling with argon or nitrogen were still able to produce intense bioluminescence. These results suggest that bioluminescence from the mucus may involve a photoprotein rather than a luciferin–luciferase reaction.

Additional key words: fire-worm, luminous mucus, fluorescence, photoprotein

In marine organisms, bioluminescence is used as a form of optical communication for attracting mates or prey, or for defense against predation (Herring 1978; Morin 1983). For the communication signal to be effective, the light should transmit well through ocean water and be detectable by the appropriate receiver. In general, the maximum emission of bioluminescence occurs in the blue-green region of the visible spectrum (460–490 nm) for open-ocean pelagic organisms, and at slightly longer wavelengths (480–510 nm) for organisms living in benthic or shallow coastal environments. Bioluminescence also has different intensities and temporal patterns (flash vs. glow), depending on the species and the function associated with the light signal (Morin 1983). Light production results from a chemical reaction involving the oxidation of a substrate luciferin as catalyzed by a protein luciferase. In some systems, e.g., ctenophores, cnidarians (except anthozoans), and some polychaetes, the luciferin is tightly bound to luciferase and oxygen, forming a stable molecule called a photoprotein. Light production is triggered when the photoprotein binds to calcium or some other co-factor, and occurs under anoxic conditions because oxygen is already incorporated into the photoprotein (Campbell 1988).

Syllid polychaetes in the genus *Odontosyllis* have long been known for their bioluminescence displays that occur with lunar periodicity during reproductive swarming (Galloway & Welch 1911; Crawshay 1935; Markert et al. 1961; Tsuji & Hill 1983; Gaston & Hall 2000). The worms inhabit shallow coastal areas and are benthic, except during reproductive swarming. Swarming is initiated by females that secrete luminous mucus, and that eventually release their gametes. The bright bioluminescence attracts males

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that release their gametes in the luminous cloud. Males also produce bioluminescence when approaching the luminous cloud; their light production is either secreted from the body as in females, or remains internal, occurring as a series of short flashes produced from the posterior section. Such a pattern is also observed in females that are mechanically stimulated when on the seafloor, suggesting a dual function for light production in *Odontosyllis* spp. in that it is used not only as a visual signal to attract mates but also as a startle mechanism against predation during nocturnal foraging (Fischer & Fischer 1995).

Although well known because of its bioluminescence display, the mechanism of bioluminescence of *Odontosyllis* spp. has been little studied because of practical difficulties in obtaining samples that are sufficiently large and pure for optical or biochemical investigations. The worms are relatively small in size, and difficult to collect from their benthic habitat; they are cryptic and live inside tubes made of a silk-like material during the day. The worms are primarily observed in the field by virtue of their bright green bioluminescence released during swarming nights. However, by that time, the worms have released most of their luminous material and are no longer suitable for studies of bioluminescence. An alternative approach, collecting samples of the luminous water, is also not effective because the samples contain gametes, and exhausted and dilute luminous material, in addition to microorganisms and various other substances found in seawater (Fischer & Fischer 1995). The lack of suitable material has been a significant limitation in studies of bioluminescence in *Odontosyllis* spp.

The bioluminescence in *Odontosyllis* spp. is thought to be due to a conventional luciferin–luciferase reaction, based on studies of the Bermuda fireworm *Odontosyllis enopla* (Harvey 1952). Tests using material collected from $>50{,}000$ individuals of *O. enopla* revealed that light production involves the oxidation of a substrate, has an emission maximum at 507 nm, is sensitive to pH, is optimal at seawater pH 7–8, and uses cyanide and/or magnesium as a co-factor (Shimomura et al. 1963, 1964). The emission spectrum of the luminescent reaction is similar to that of the luciferin fluorescence emission spectrum, peaking at 510 nm (McElroy 1960). This optical characterization was used to identify, isolate, and partially purify the (oxy)luciferin from worm extracts, although the chemical structure of the luminous compound could not be determined (Trainor 1979). No information is available on the luciferase or on the basic chemistry of the bioluminescence reaction once secreted from the worms.

The present study investigates the bioluminescence of freshly collected *Odontosyllis phosphorea* Moore 1909 and is unique in studying chemically stimulated light emission from both intact organisms and the luminous mucus secreted by worms. Bioluminescence was not reconstituted when hot and cold extracts of luminous mucus were mixed, and persisted under oxygen-depleted conditions, suggesting that the bioluminescence associated with the mucus involves a photoprotein.

Methods

Odontosyllis phosphorea swarms during the summer in the 2-m-depth area of De Anza Cove, Mission Bay, located in San Diego, CA (Tsuji & Hill 1983). After verifying that the luminous behavior was still occurring at nighttime in the cove, we collected worms during daytime by sampling the material covering the seafloor of the cove. Samples were obtained between May and October in 2004 and 2005, and consisted of the alga *Ulva* sp. and bryozoan *Zoobotryon verticillatum,* which were kept in jars with seawater for transport to the nearby research laboratory at the Scripps Institution of Oceanography. There, samples were distributed into different containers to allow small organisms, using the algae and bryozoans as protective substrates, to escape and swim into the water. This behavior was facilitated by gently swirling the substrate, sometimes after a brief (10 min) exposure to cold (4°C) seawater, which anesthetized the organisms and facilitated their separation from the substrate into the water. This technique yielded many small invertebrates, including copepods, amphipods, pycnogonids, and polychaetes. On a good collection day, dozens of specimens of *O. phosphorea* of various sizes, sexes, and maturation stages would be seen crawling on the walls of the containers, sometimes starting to build tubes, as also observed in *Odontosyllis enopla* (Fischer & Fischer 1995). Three size categories of worms were considered for further analyses: small worms (0.5–2.5-mm long), medium worms (5–8 mm), and large worms (12–15 mm). Worms were individually collected using a microprobe or a Pasteur pipette, and placed into a container filled with isotonic artificial seawater (ASW; Deheyn et al. 1997). Taxonomic identification was confirmed based on a microscope evaluation of the morphological features following Moore (1909) (L. Lovell & G. Rouse, pers. comm.). Worms were maintained at room temperature until used in experiments. Some worms were maintained for ≤ 9 months in aerated aquaria containing sand-filtered ambient temperature seawater that was changed

every other week. Worms in these aquaria were not otherwise fed. Each month, three worms were removed and tested for bioluminescence using potassium chloride (KCl) stimulation.

Each type of experiment was performed with a minimum of 12 independent replicates, spanning two summer collection seasons, and using different batches and quantities of worms. This variation in methods and seasons led to a high quantitative variation in results among replicates. However, the results for a given experiment were always qualitatively similar among replicates. Therefore, experimental data were not pooled together for descriptive statistical analysis; instead, the most representative datasets were used to illustrate common qualitative patterns.

Stimulation of bioluminescence

Spontaneous bioluminescence was rare in members of *O. phosphorea,* which produced light following mechanical or chemical stimulation. To analyze bioluminescence from worms and to trigger secretion of luminous mucus, individuals were transferred, using a microprobe with minimal extra fluid volume, into a known volume of fresh ASW and stimulated for light production by treatment with an equal volume of 400 mmol L^{-1} KCl in ASW (final concentration 200 mmol L^{-1}). At this concentration, KCl depolarizes cell membranes, triggers intracellular processes (Germain & Anctil 1988; Deheyn et al. 2000), and stimulates bioluminescence in some other luminous invertebrates (Widder et al. 1989; Deheyn et al. 1997). In *O. phosphorea,* light can be produced both in body tissues as well as secreted from the body. KCl treatment induced secretion of luminous mucus in medium and large individuals. Mucus secretion was accompanied by frenetic swimming and sometimes spawning when collecting the worms close to their normal swarming times periods (Tsuji & Hill 1983).

Mucus bioluminescence was expressed as a glow whose intensity was increased by treatment with 0.02% peroxidase or 10 mmol L^{-1} ammonium persulfate ($(NH_4)_2S_2O_8$) (APS; Sigma-Aldrich, St. Louis, MO, USA) in ASW (final concentrations). Working with luminous mucus thus presented a unique opportunity for assessing the optical and biochemical characteristics of the processes involved in light production by individuals of *O. phosphorea* under controlled laboratory conditions. In this study, all experiments assessing the possible effect of chemical treatment on light production were conducted using luminous mucus separated from the worms. Mucus samples were obtained from groups of 40 worms in a small volume of ASW (usually 0.5 mL) and transferred into an equal volume of 400 mmol L^{-1} KCl in ASW. The mucus was retrieved as the supernatant after several minutes of gentle centrifugation. The resulting mucus sample was then subdivided according to the experimental plan. Bioluminescence recorded by a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany) was stimulated by injection of the test chemical into the sample tube. Bioluminescence was expressed as relative light units produced per unit of time (RLU s^{-1}). The residual bioluminescence capacity of the mucus samples was measured by treatment with peroxidase. In some cases, a 2.0 neutral density filter was used to avoid saturation of the luminometer, in which case the light intensity values were multiplied by 100 before further data analysis.

Emission spectrum of light production

Emission spectra were measured using a Low Light Coupled Intensified SE200 Digital Spectrograph (Catalina Scientific, Tucson, AZ, USA). Using an intensified charge-coupled device detector, the instrument measures the 300–700 nm spectral range in one exposure, with no scanning necessary, and thus with no alteration of the spectrum when light intensity varies during the recording process. The measurements were made using exposures ≥ 0.2 s, depending on the light intensity and gain of the intensifier, resulting in a spectral resolution of ~ 1 nm. Spectra were smoothed using the built-in smooth function, which applies a triangle-function convolution filter with a 40-nm width to pixels, with intensity count values below a threshold of 1,000,000.

Bioluminescence emission spectra were measured from large worms after KCl stimulation, and from mucus treated with peroxidase or APS, as described in the previous section. Analyses were performed during the reproductive period of members of *O. phosphorea* (May through September: Tsuji & Hill 1983) using separate groups of medium and large specimens. Smaller individuals of the medium-size class were lightly pigmented, except for two black eyes at their head section, while larger individuals had dense black and yellow/brown pigmentation. Fluorescence emission spectra excited at 380 nm were measured from freshly collected mucus, and from mucus treated with peroxidase or APS. Exposure time and gain conditions for fluorescence measurements were optimized so that any residual bioluminescence was below the detection levels; thus, the measured light was predominantly due to fluorescence. Controls for bioluminescence and fluorescence measurements con-

sisted of ASW blanks to which KCl, peroxidase, or APS was added.

Variation with size in small worms

Preliminary observations suggested that when stimulated by KCl, small worms produced primarily internal bioluminescence. To determine whether these small worms secrete luminous mucus as do large worms when spawning, a total of 27 small worms were individually tested for secreted bioluminescence. Light emission was integrated for 2 min following addition of 50 μL KCl to the tube containing the worm in 50 μL of ASW. The specimen was then removed from the tube using a microprobe, and the bioluminescence of the remaining media (ASW+KCl+any mucus) was measured for an additional 2 min, with no further chemical stimulation. This protocol assessed variability in light production among individuals and differentiated between strictly internal production versus secreted luminous mucus. The variation of bioluminescence intensity with worm size was expressed for individual worms versus their corresponding mucus secretion, and the allometric variations were modeled by a Gompertz-like equation (Deheyn & Jangoux 1999): $B = B_\infty e^{-be^{(-kS)}}$, where B is the bioluminescence of an individual worm for a given body length S (in mm), B_∞ the asymptotic value of bioluminescence, b the ratio B_∞/B_0 in which B_0 is the bioluminescence of the smallest worm, and k the variation coefficient of the allometric model. Equation parameters were estimated from the original values of bioluminescence using the non-linear Quasi-Newton method, with the robustness and the significance of the relationship indicated by R^2 and p values, respectively (Zar 1996).

Fluorescence mapping is sometimes used to identify putative sources of bioluminescence based on autofluorescence of luciferins or proteins involved in the bioluminescence reaction (Brehm 1977; Germain & Anctil 1988; Hastings 1996). Small worms, which lack the dark-brown pigmentation of larger worms, were photographed using epifluorescence microscopy (Zeiss Axioskop with Spot mercury lamp, Diagnostic Instruments Inc., Melville, NY, USA) equipped with a Chroma GFP-Long Pass filter set (excitation at 470 nm; emission $\geq$500 nm) to identify the distribution of the potential areas of bioluminescence.

Hot/cold extracts of bioluminescent mucus

In bioluminescence systems that involve a conventional luciferin–luciferase reaction, hot extracts denature the luciferase and preserve the activity of the luciferin, while cold extracts exhaust the luciferin by oxidation and preserve the activity of the luciferase. Recombining the hot and cold extracts then reconstitutes a considerable amount of the light production produced by chemical stimulation. If light is not produced by combining hot and cold extracts, one may infer that the system does not involve a conventional luciferin–luciferase reaction. Harvey (1952) reported that mixing hot and cold extracts from whole individuals of *O. enopla* regenerated high levels of light, suggesting that light production in *Odontosyllis* spp. is due to a conventional luciferin–luciferase reaction. The goal of this experiment was to replicate Harvey's experiment but with mucus extracts from *O. phosphorea*.

Samples of luminous mucus were kept at room temperature (cold extract) or heated to 90°C (hot extract) until bioluminescence decreased to <1% of the original level. This decrease in bioluminescence took $\leq$90 min for cold extracts and 10–20 min for hot extracts. The hot extract was left on ice until the cold extract was ready for use, and re-checked for lack of spontaneous bioluminescence before starting the hot–cold cross-reactivity experiment. Samples were made in triplicate (six tubes of mucus sample from the same batch of worms) and with enough initial volume (1 mL for each mucus sample) so that the extracts could be combined as follows (200 μL for each extract tested): hot+cold, hot+hot, cold+cold, hot+ASW, and cold+ASW. Bioluminescence was measured from each combination for 1 min after addition of peroxidase. The experiment was replicated 12× over two collection seasons, using mucus from different numbers of worms, and worms of different sizes.

Bioluminescence under anoxic conditions

Light production in a conventional luciferin–luciferase system requires oxygenated conditions. On the other hand, because a photoprotein already contains oxygen pre-bound to luciferin and luciferase, light emission triggered by a co-factor can occur under oxygen-depleted conditions. Thus, bioluminescence activity under anoxic conditions can provide crucial information about the nature of the chemical system. Vials of fresh luminous mucus (300 μL samples) or individual worms in ASW were bubbled with argon or nitrogen for 10–30 min to deplete oxygen. The vials were then sealed with rubber stoppers and immediately measured for spontaneous bioluminescence for 1 min. A 300-μL volume of KCl for worm samples, or APS for mucus samples, was

then injected through the rubber stopper, and bioluminescence was recorded for an additional 1 min. The stock solutions of 20 mmol L^{-1} APS and 400 mmol L^{-1} KCl were bubbled with argon or nitrogen for 30 min before use. Controls consisted of samples and solutions bubbled with air, and of non-bubbled samples. The experiment was repeated several times using mucus from different numbers of worms >1 mm in size.

The effect of molecular oxygen on the light production process in *O. phosphorea* was also assessed chemically by exposing luminous mucus to sodium thiosulfate, a strong oxygen scavenger, and hydrogen peroxide, a strong oxidizer. Their effect on light production was addressed by comparing the bioluminescence intensity of mucus samples before and after addition of each chemical. The light intensity of an aliquot of 50 μL fresh, luminous mucus was measured for 10 s, after which an equal volume of sodium thiosulfate (20 mmol L^{-1} in ASW; Fisher Scientific, Fair Lawn, NJ, USA) or hydrogen peroxide (30%; ACS grade, Fisher) was added, and the light intensity was measured for an additional 10 s. Controls consisted of adding ASW or MilliQ water to the mucus aliquot. The experiments were repeated using 15 different batches of fresh luminous mucus; by varying the number of worms used to prepare the mucus from five to 40 individuals, different relative mucus concentrations were achieved. Bioluminescence was expressed as the ratio between the intensity following chemical treatment and the initial level of intensity.

Effect of temperature on bioluminescence

The dependence of light emission on temperature was assessed to further characterize the bioluminescence system. A conventional luciferin–luciferase system will produce light only within a specific temperature range based on enzyme kinetics. Light production is thus expected to be inactivated similarly beyond the lower and higher threshold of temperature range optimal for the metabolism and ecology of the luminous species (Herring 1978; Campbell 1988; Shimomura 2006). Members of *O. phosphorea* are found in coastal water ranging ~15°–25°C, which thus represents the expected range of maximal light production if the bioluminescence follows a luciferin–luciferase system.

Thermal breakdown of a photoprotein system is driven mainly by the denaturation of disulfide bonds, which is less sensitive to colder than to warmer temperatures; therefore, a photoprotein system will likely be active at lower temperatures while denatured at higher temperatures (Shimomura 2006). In the case of *O. phosphorea,* one would expect the bioluminescence to be preserved at low temperatures and be degraded rapidly above 25°C if the light production mechanism involves a photoprotein system.

Samples of luminous mucus were exposed to temperatures of −20°C, 4°C, 20°C, 40°C, 60°C, and 80°C for ≤30 min. A total volume of 350 μL was exposed at each temperature, and a sub-sample of 50 μL was removed for light measurement every 5 min. All samples were from the same stock of mucus and processed in parallel. Each bioluminescence measurement included 3× 10-s consecutive recordings of light production from the same sample. The first measurement represented light production from mucus, then from mucus+APS, and finally from mucus+APS+peroxidase. Controls that consisted of ASW ± APS ± peroxidase did not produce light. Mucus samples at −20°C never reached the frozen state while samples at 80°C showed some evaporation.

The decay of bioluminescence over time was modeled using a simple negative exponential model, $y = \alpha \times e^{-\beta x}$, where α is the initial value of light intensity and β the slope, which represents the rate at which bioluminescence decreases over time under the various temperature conditions; the greater the β value, the faster the decay. The coefficient of determination (R^2) of each model was tested for statistical significance by comparison with critical values (Zar 1996).

Results

Bioluminescence appeared immediately after KCl stimulation of whole medium and large worms, peaking within seconds but continuing at lower intensities as a glow that could last for minutes (Fig. 1A). Subsequent addition of APS or peroxidase triggered an immediate increase to a plateau level where light emission persisted for >60 min. Light intensity at the plateau level increased with the size of the tested individual. Bioluminescence from both medium and large worms generally followed this pattern with the intensity of produced light for both size ranges, reaching high-intensity values (10^8–10^{10} RLU s^{-1}), but showing clearly distinct kinetics. In large individuals, the kinetics profile was constant among samples, with the light intensity always increasing rapidly after stimulation, peaking ~1–3 s after KCl injection, and then followed by a rapid exponential decrease. About 60–70 s in the decay phase, the light intensity reached a steady level at ~3,000× greater than before stimulation. Addition of peroxidase then triggered an immediate increase to 100× greater intensity. In medium individuals, the kinetics profile

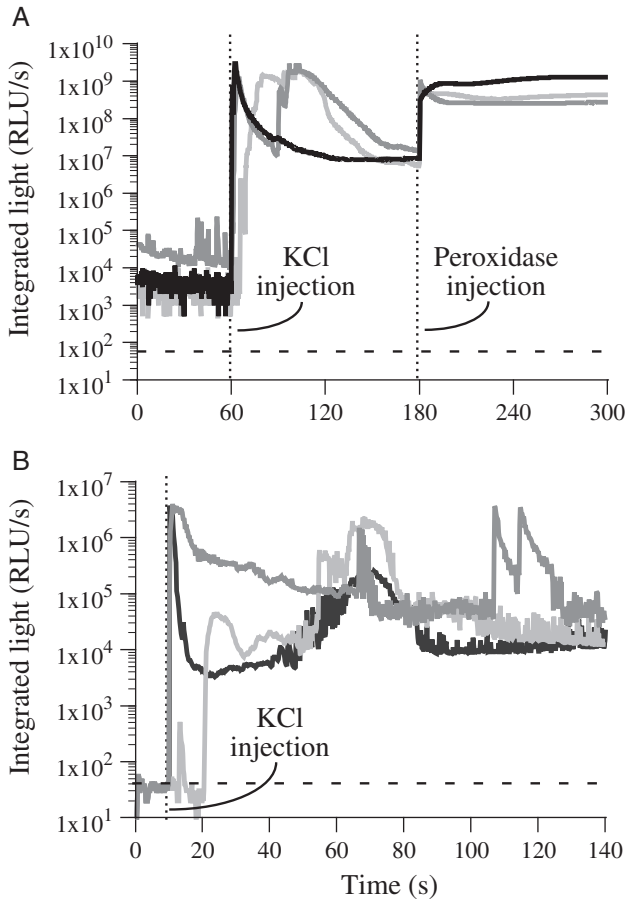

Fig. 1. Chemical stimulation of bioluminescence of individuals. **A.** Time course of emission from a large, yellow/brown individual (black line) and two medium, gray individuals (dark and light gray lines). The first 60 s of measurement was before chemical stimulation to assess spontaneous emission. Light production was stimulated at 60 s by potassium chloride (KCl), and at 180 s by peroxidase. **B.** Typical bioluminescence time course for 0.5 mm (light gray), 1.3 mm (dark gray), and 2.5-mm-long (black) individuals. KCl was injected at 10 s and light emission recorded for the following 130 s. In both panels, the horizontal dashed line is the instrument background level.

was more variable among samples, with a KCl peak similar to that of large individuals, followed by a second long-lasting peak during the exponential decay phase, or with only one peak that was reached later (10–20 s after stimulation) and lasted longer (30–50 s of a relatively steady light intensity) (Fig. 1A). These observations were based on a total of only six individuals (three of each size category). Medium and large worms kept in captivity beyond the summer collection showed bioluminescence characteristics similar to those collected during the summer time. The intensity and kinetics of KCl-stimulated light production in small worms showed much greater variability compared with medium and large individuals, having multiple modes and various times to reach the maximum intensity (Fig. 1B).

Laboratory observations showed that in medium and large worms, KCl-stimulated bioluminescence was accompanied by the secretion of mucus, to an extent that varied with the size of the individual and the stage of the reproductive cycle. The mucus was highly viscous, in particular when stimulating several worms in a confined volume. The mucus would develop as threads that stuck to the worms and walls of the vials (Fig. 2A).

Concentrated mucus had a blue tint and after centrifugation the mucus separated into two layers: an upper blue layer and a lower pale yellow/translucent layer (Fig. 2B). It was not possible to completely separate the two layers from each other, but our attempts showed that samples from both layers produced similar amounts of light. Within minutes of light production by KCl stimulation, both layers of the mucus became more translucent and less viscous, thus mixing back together. Once the distinction of layers based on color had disappeared, the less viscous mucus was collected and divided into aliquots for light production experiments.

Emission spectrum of light production

The bioluminescence emission spectrum of living individuals of *O. phosphorea* after KCl stimulation appeared as a unimodal peak with the maximum emission at 494–504 nm and a full-width at half-maximum (FWHM) of ~50–60 nm (Fig. 3), with no apparent difference in emission spectra between small and large specimens (data not shown). Bioluminescence emission spectra of mucus stimulated with peroxidase or APS peaked between 494–498 and 494–504 nm, respectively, and had an FWHM similar to worm KCl-stimulated bioluminescence (Fig. 3).

The fluorescence emission spectrum of freshly KCl-stimulated mucus was unimodal and broad, peaking at 495–515 nm with an FWHM of ~70–80 nm (Fig. 3). Fluorescence after stimulation with peroxidase or APS had a slight short-wavelength shift in peak emission to 488–503 nm (Fig. 3).

Variation with size in small worms

The smallest individuals collected were 0.5 mm in size, with only several segments visible, but clearly visible cirri. At this size, most of the bioluminescence was internal and not secreted (Fig. 4A), although the range of light intensity was similar to that of

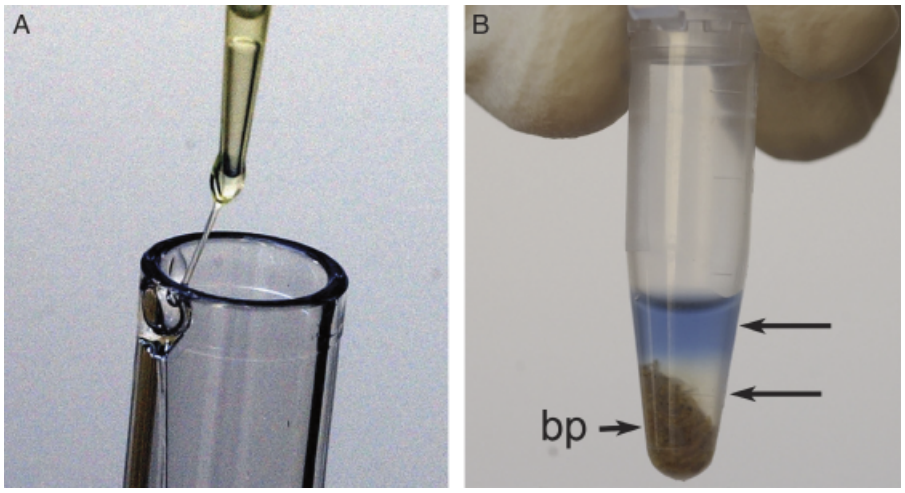

Fig. 2. Secreted mucus from 20 large individuals. **A.** The mucus was highly viscous and developed threads when collected with pipette. **B.** After centrifugation, the mucus separated into two layers (arrows), the upper layer blue and the lower translucent. The brown pellet (bp) contained the worms.

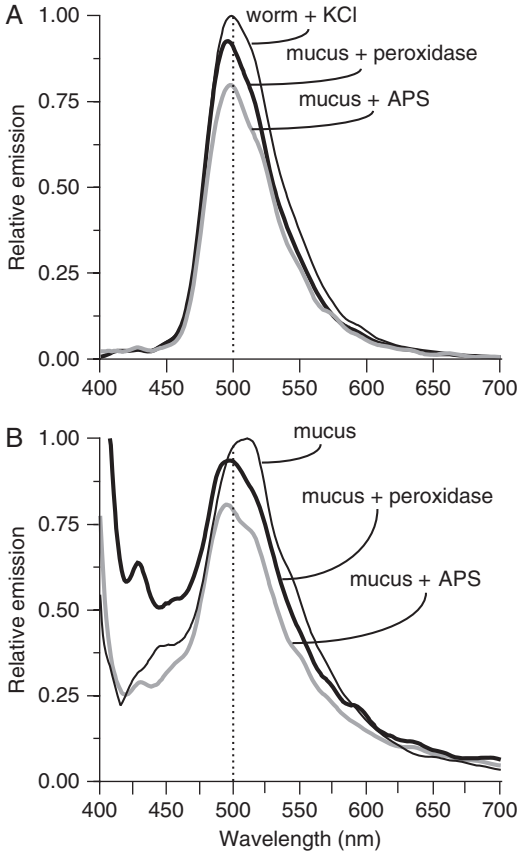

Fig. 3. Relative intensity emission spectra of bioluminescence and fluorescence of a large individual and its secreted mucus. The intact worm was treated with potassium chloride (KCl; thin black line), while the mucus was treated with peroxidase (thick black line) and ammonium sulfate (APS; gray line). Dotted line marks 500 nm for reference. **A**. Bioluminescence emission spectra, which peak at 494–504 nm. **B**. Fluorescence emission spectra with peaks between 488 and 515 nm, based on 380 nm excitation.

2.5-mm-long individuals known to secrete bioluminescent material. Total integrated bioluminescence was similar across the size range investigated and was not correlated with specimen size ($R^2 = 0.002$, $p > 0.05$). Light production varied by a factor of $\sim 50\times$ between the smallest and the largest individuals for "worm-free" samples that contained only secreted mucus (Fig. 4B). The allometric variation of bioluminescence intensity in ASW that had contained a stimulated worm followed the relationship: $B = 6.3 \times 10^7 e^{-4740e^{-4.7S}}$, with $R^2 = 0.51$ and $p = 0.005$ (Fig. 4B). This relationship shows that the level of bioluminescence was low in seawater containing KCl-stimulated worms $\leq$1-mm long, and was higher in seawater containing >1-mm-long individuals.

In small worms, green fluorescent sources were spread over most of the worm body surface (Fig. 5A–D). Fluorescence was observed from inside epidermal cells, probably gland cells described by Harvey (1952), which were homogeneously distributed from one segment to the other, and from around the head. Fluorescence originated from small vesicles that coalesced upon exposure to the excitation light. Eventually, the larger vesicles burst to the surface of the epidermal cells, releasing fluorescent mucus that drained under the cover slip (Fig. 5E,F). This phenomenon was observed from epidermal cells of various parts of the worm. The eyes, cirri, antenna, and palp did not show any fluorescence. Fluorescence was also particularly bright posteriorly, where it was confined to a region deep in the body. This fluorescence was yellow rather than green in color and probably originated from other compounds of the worm's digestive and/or reproductive tissues.

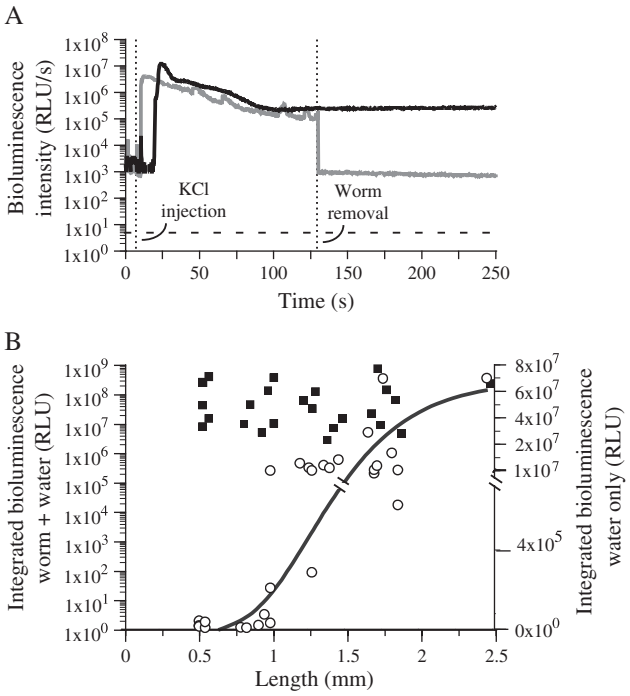

Fig. 4. Representative records of potassium chloride (KCl)-stimulated bioluminescence from worms of various sizes. KCl was injected at 10 s and light emission recorded for 120 s. **A.** Typical time course for <1 mm (light gray) and ≥ 1-mm-long (black) individuals. When the worm was removed from the tube at time 130 s, there was an immediate decrease in bioluminescence intensity for worms <1 mm, indicating that the worm was the main source of light, and only a slight decrease for worms ≥ 1 mm, indicating that the emission was not internal to the worm and was found mainly in the fluid. Horizontal dashed line is the instrument background level. **B.** Bioluminescence as a function of body size for worms <2.5-mm long. Filled squares represent samples containing worm and seawater; open circle and black line (model) represent the corresponding samples from which the worm was removed, thus leaving mucus as the only possible source of bioluminescence in the sample. The dual scaling of this axis is for better viewing of the large allometric variation of intensities that was fitted with a double exponential model. RLU, relative light units.

Hot/cold extracts of bioluminescent mucus

Addition of a hot mucus extract, expected to contain functional luciferin, to a cold mucus extract, expected to contain functional luciferase, and vice versa, yielded only small increases in bioluminescence, with light levels >1 min only 3–10$\times$ greater compared with the background before addition. Control treatments consisting of mixing cold+cold, hot+hot, cold+ASW, and hot+ASW extracts resulted in only small increases in light emission, only 1.2–3.0 $\times$ greater than the background (Fig. 6). The intensity of reconstituted light from recombining hot and cold extracts always remained $<3\%$ of the light production from fresh mucus. As the hot extract is expected to contain reduced luciferin, then addition of peroxidase is predicted to trigger intense light production due to oxidation of the luciferin. However, additional light production after peroxidase treatment of hot extracts was not consistently observed (Fig. 6).

Bioluminescence under anoxic conditions

Worms exposed to air bubbling occasionally produced bioluminescence, perhaps from mechanical stimulation when entrained in the bubbles. These worms, however, produced orders of magnitude higher levels of bioluminescence following KCl stimulation, although with a high interworm variability. Following argon or nitrogen bubbling, worms sometimes produced a spontaneous glow, again perhaps due to mechanical stimulation from bubble entrainment. Although the worms exposed to argon or nitrogen bubbling were sometimes moribund after treatment, stimulation with a KCl solution pre-treated with argon or nitrogen bubbling always resulted in a large and rapid increase in the bioluminescence intensity, with light production stabilizing at a level with a range less than one order of magnitude for all treatments (Fig. 7A). Thus, oxygen depletion did not result in a striking difference in KCl-stimulated bioluminescence compared with the oxygenated condition.

Luminous mucus exposed to bubbling with any gas still produced spontaneous bioluminescence, and yet with levels different between treatments and experiments. The bioluminescence intensity from argon- and nitrogen-bubbled mucus was 1.2–10 $\times$ lower than that of the still control and 10–90 $\times$ greater than that of the air-bubbled samples (Fig. 7B). Mucus still produced additional light when stimulated with APS, increasing to levels that were $\sim 60\times$ greater than the spontaneous level in still control samples, 3 $\times$ greater than air-bubbled samples, and 1.5–15 $\times$ greater than argon- and nitrogen-bubbled samples, respectively (Fig. 7B).

The addition of sodium thiosulfate to luminous mucus resulted in a decrease in light intensity to 50–90% of the pre-treatment intensity, with a median value of 65% ($n = 15$ replicates). The decrease was inversely proportional to the number of worms used to make the initial stock of fresh mucus, such that the decrease was greater as the number of worms diminished.

The addition of hydrogen peroxide to luminous mucus resulted in almost complete inhibition of light production in all tested samples, with the light

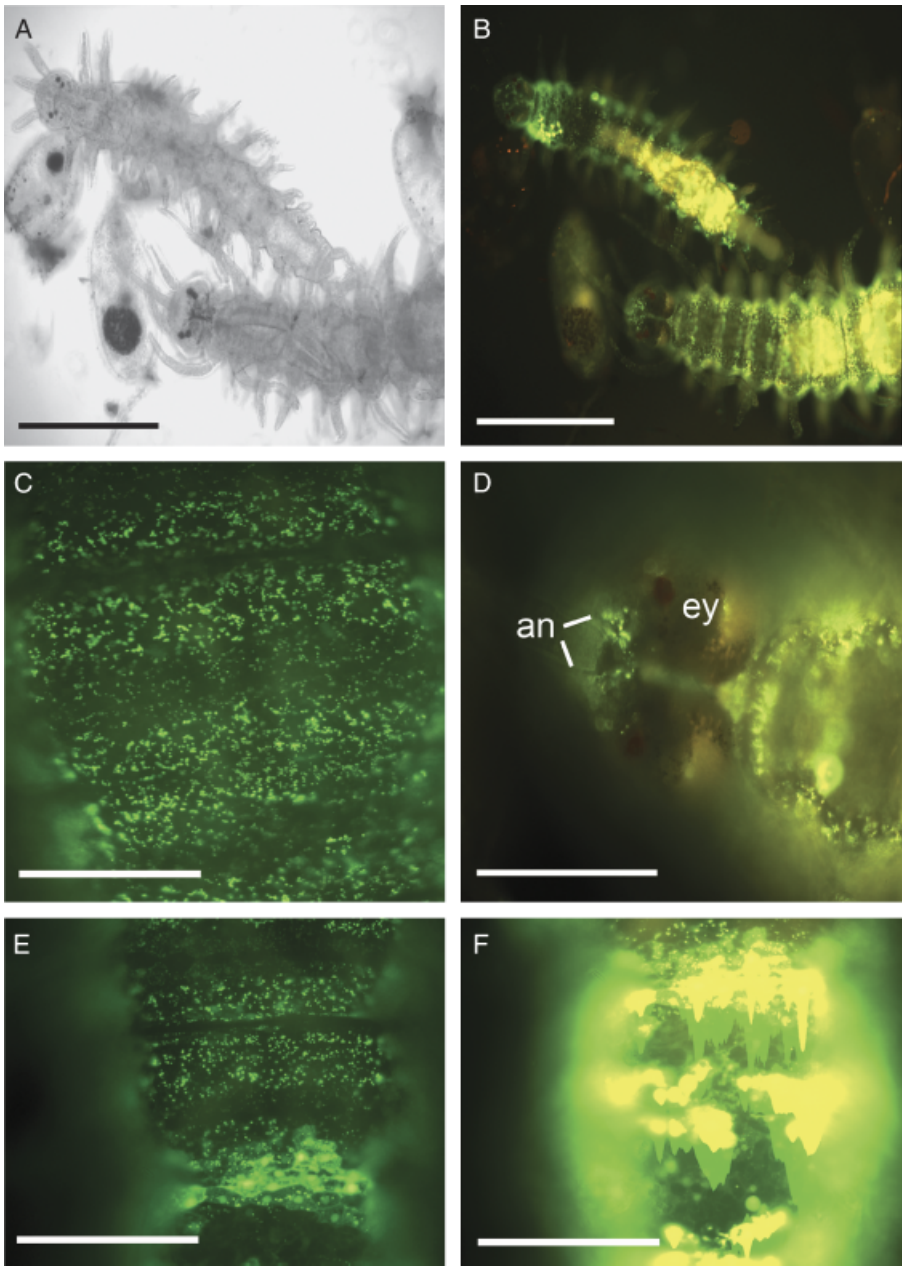

Fig. 5. View of two small individuals using bright field and epifluorescence microscopy. **A.** Bright field view. **B.** Same field of view as in (A), using blue-excited fluorescence, showing green and yellow emission, the latter probably not associated with the bioluminescence system. **C.** Green fluorescence, presumably representing sites of bioluminescence because of the same color of the light production, originated from epidermal cells spread over the body segments. **D.** Fluorescence from the head was not associated with appendages like eyes (ey), antenna (an), cirri and palps (not shown). **E.** Coalescing of green fluorescent organelles in epidermal cells. **F.** Secretion of green fluorescent mucus from the epidermal cells a few seconds later than in (E). Scale bars, 0.5 mm in A, B; 0.05 mm in C–F.

intensity ranging between 0.5% and 2.0% of that for pre-treated mucus. Controls involving addition of ASW or MilliQ water resulted in light intensity ranging between 70% and 120% that of the pre-treated mucus, with median values of 85% for ASW and 90% for MilliQ water treatment.

Effect of temperature on bioluminescence

The bioluminescence of mucus was rapidly affected by temperature. For example, after 15 min of treatment, the spontaneous bioluminescence intensity at −20°C and 4°C was similar to that at the acclimation condition of 20°C, while it was reduced to 0.1% at 40°C and to 0.0001% at 60°C and 80°C (Fig. 8). Mucus bioluminescence intensity increased upon treatment with APS as well as on subsequent treatment with peroxidase. The relative increase in intensity with APS stimulation was greater for lower temperatures, being $\leq 90\times$ the level of spontaneous bioluminescence at −20°C, and $8\times$ at 40°C, and yet was similar at 60°C and 80°C; the bioluminescence intensity increased $1.2–5\times$ after peroxidase treatment (Fig. 8).

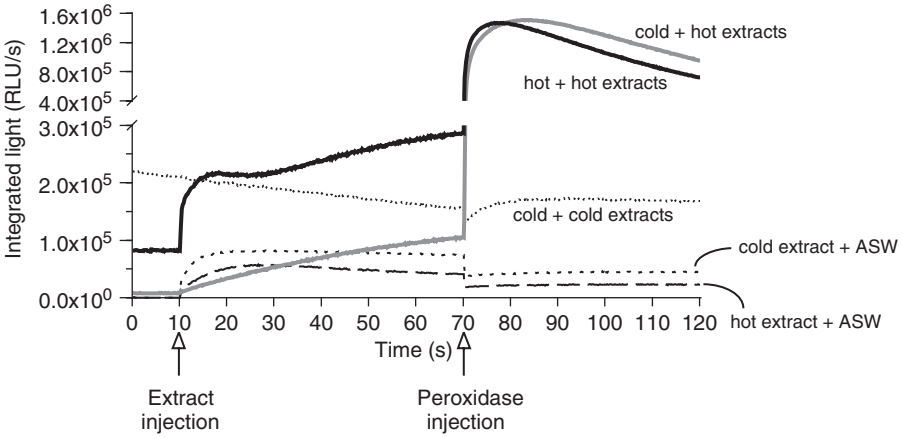

Fig. 6. Results from a representative experiment recombining hot and cold extracts of the mucus. Spontaneous bioluminescence of an extract was measured for 10 s at which time the complementary extract was added. Peroxidase was added to the mix 60 s later. Control experiments consisted of adding a similar extract as the one already being measured, or adding artificial seawater (ASW). RLU, relative light units. Different lines represent various combinations of extracts as indicated in the figure.

The intensity of spontaneous bioluminescence from mucus decreased over time for all temperatures, reaching ~80% of the pre-treatment level after 30 min at −20°C, 45% when at 4°C, and 10% at 20°C; the intensity reached <1% in 20 min at 40°C, and within 5 min at 60°C and 80°C (Fig. 9A). The temperature effect on the decrease in the intensity of APS and peroxidase-stimulated bioluminescence was similar to that of spontaneous bioluminescence (unpubl. data). The change in intensity with temperature always followed a simple exponential decay (Table 1). For each type of treatment, the decay rate increased with temperature; the greatest changes in decay rate were observed within the range of 20°–60°C (Fig. 9B).

The various mucus treatments (mucus, mucus+APS, and mucus+APS+peroxidase) showed similar temperature effects on the decay rate although the values for untreated mucus samples were always lower than for the other treatments, except at −20°C (Fig. 9B), indicating that light intensity following APS and peroxidase treatment decreased faster. The initial bioluminescence intensity (the α coefficient) was greater in APS and peroxidase-treated mucus than for untreated mucus, and was the lowest at temperatures of 60°C and 80°C (Table 1).

Discussion

This study addresses the characteristics of bioluminescence in the marine polychaete *O. phosphorea* using whole individuals freshly collected in the field and luminous mucus freshly secreted by worms after chemical stimulation. Individuals of *O. phosphorea* were, for the first time, experimentally stimulated to secrete luminous mucus, providing a unique framework for studying this bioluminescence system. The findings provide an insight into the possible ecological roles of light production and add to earlier assessments of the chemistry of the bioluminescence reaction, which were based on extracts from whole organisms and were not conclusive in identifying the luminous reagents in *Odontosyllis* spp. (Shimomura et al. 1963, 1964).

The high viscosity of the mucus suggests that it contains high concentrations of carbohydrates and/or has a high degree of sulfation (Takano 2002; Suzuki et al. 2003). There are, however, no reports of sulfated polysaccharides or carbohydrates involved in a bioluminescence reaction; the most similar system would be the luminous reaction of bacteria that involves aldehydes from carbohydrate cycling (Tu & Mager 1995).

The mucus of *O. phosphorea* was blue when freshly secreted but changed to pale/translucent yellow as it aged. In *Odontosyllis enopla,* partially purified luminous reagents had different colors, including yellowish and pink (Shimomura et al. 1963), but not blue. A blue color is usually associated with the presence of copper and/or iron, which could indicate their role as co-factors in the bioluminescence reaction (Harvey 1952; Herring 1978; Shimomura 2006). Additional evidence supporting the possible involvement of copper and/or iron in bioluminescence includes the stimulation of light production in *O. enopla* by potassium ferricyanide, a source of iron (Shimomura et al. 1964), and the fact that copper and iron are secreted in high concentrations in the mucus of *O. phosphorea* (D. Deheyn, unpubl. data).

Members of *O. phosphorea* produce green light (495–515 nm), with similar emission spectra for bioluminescence, chemiluminescence, and fluorescence, indicating that oxidation of the chromophore did not alter its spectral properties. These results are consistent with the properties of the partially purified luminescence system of *O. enopla,* which has peak

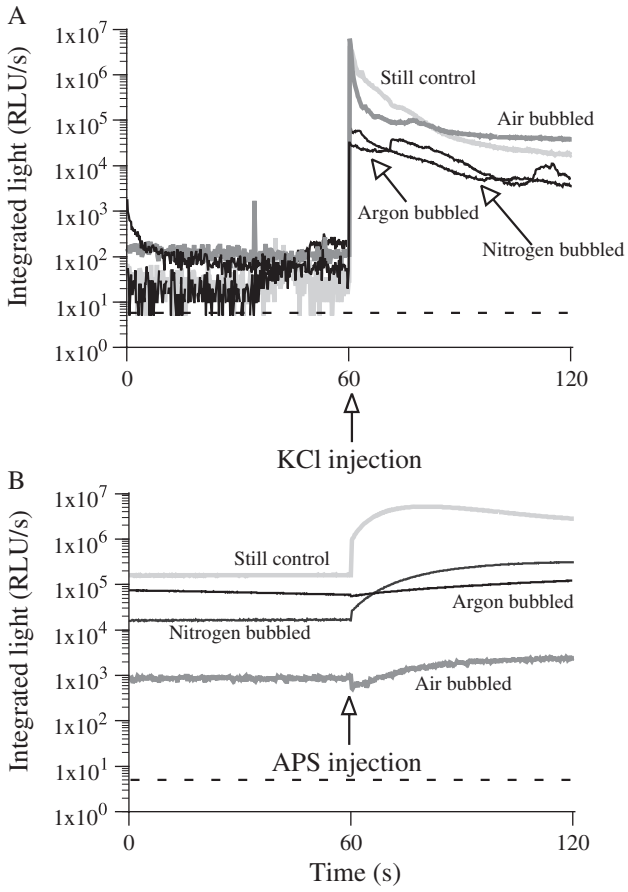

Fig. 7. Representative results of the effect of gas bubbling on bioluminescence. Treatments included the still control (light gray thick line), air bubbling (dark gray thick line), and argon or nitrogen bubbling (black lines). Chemical stimulation occurred after 60 s measurement of spontaneous light emission. **A.** Individual worms treated with potassium chloride (KCl). **B.** Mucus treated with ammonium persulfate (APS). KCl and APS solutions were pre-treated with argon or nitrogen accordingly. Horizontal dashed line is the instrument background level. RLU, relative light units.

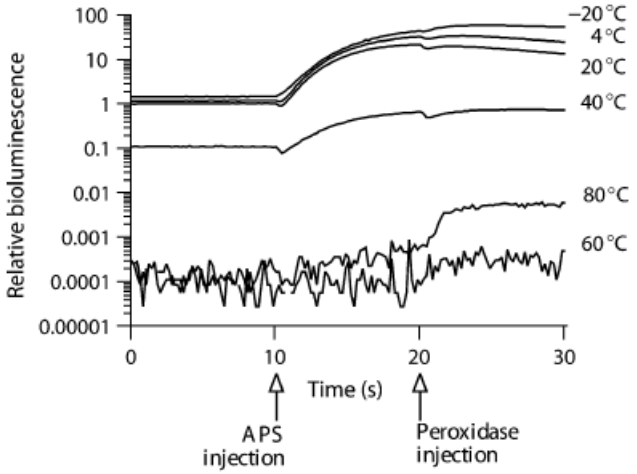

Fig. 8. Representative results of temperature effect on bioluminescence of mucus after 15 min of treatment. Spontaneous bioluminescence was measured before stimulation with ammonium persulfate (APS) at 10 s followed by stimulation with peroxidase at 20 s. Data are expressed in relative values compared with the first time point for the 20°C treatment.

emission for both bioluminescence and fluorescence at 507 nm (Shimomura et al. 1963). In the case of *O. enopla,* the partially purified system was not fluorescent before producing bioluminescence, indicating that the oxyluciferin, the oxidized form of luciferin after bioluminescence, is the fluorescent molecule (Shimomura et al. 1963).

Multiple functions of bioluminescence

Assigning specific functions to bioluminescence displays is usually not straightforward and requires a combination of field observations and experimental analyses (Morin 1983; Hastings & Morin 1991). In *O. phosphorea,* the bioluminescence is related to an intraspecific behavior related to swarming, but might also serve other functions. Internal bioluminescence associated with possible startle defensive functions has been reported to occur during foraging behavior of adults and following mechanical stimulation in the closely related species *O. enopla* (Fischer & Fischer 1995); flashes of light may also be used as an aposematic signal, considering that predators of *Odontosyllis luminosa* were observed to regurgitate freshly ingested worms (Gaston & Hall 2000). The results of the present study also supported the dual role of bioluminescence in interspecific defensive and intraspecific mate attraction behaviors.

Worms <1-mm long are not involved in mating swarms (Tsuji & Hill 1983; D. Deheyn, unpubl. data), and yet produced intense, primarily internal bioluminescence. Accordingly, nighttime collection of the bryozoan *Zoobotryon verticillatum* triggered a myriad of intense flashes of light due to the mechanical stimulation. Examination by light microscopy and a low light video system suggested that the observed bioluminescence originated from individuals of *O. phosphorea* found crawling on the bryozoan's surface, and not the bryozoan (data not shown). Because the metabolic cost associated with bioluminescence production is usually high (Herring 1978), it is presumed that these bright displays serve an ecological role. The most common function of bright

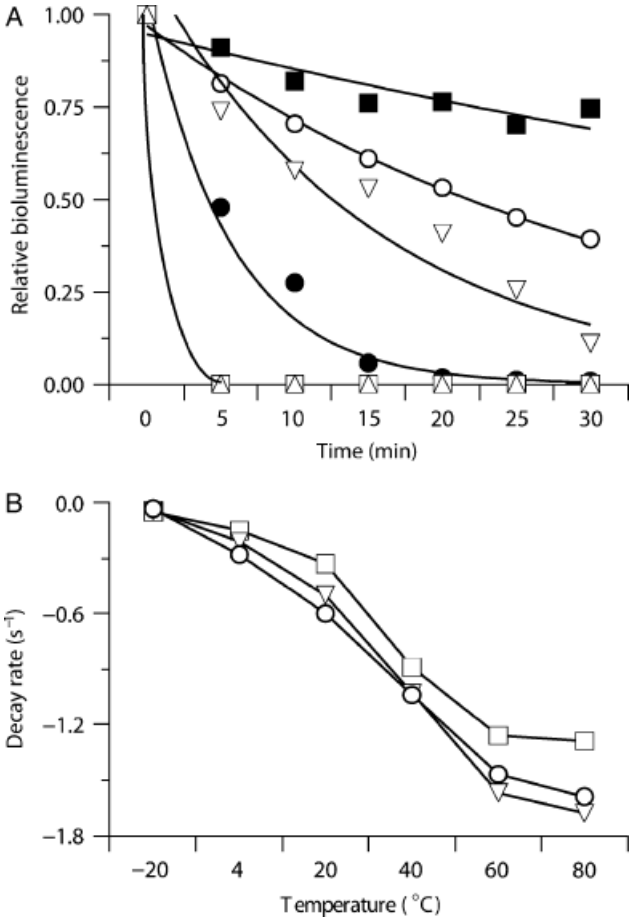

Fig. 9. Representative results of temperature effect on bioluminescence of individuals. **A.** Spontaneous light emission of mucus; filled square for −20°C, open circle for 4°C, inverted triangle for 20°C, filled circle for 40°C, open square for 60°C, and open triangle for 80°C. **B.** Exponential decay rate (β) of bioluminescence from untreated mucus (square), mucus+ammonium persulfate (APS, triangle) and mucus+APS+peroxidase (circle), measured >30 min.

Table 1. Parameters of the model for exponential decay of bioluminescence, $y = \alpha \times e^{(-\beta x)}$, where α is the intercept and β the decay rate, for various temperature treatments of mucus, mucus+ammonium persulfate (APS), and mucus with APS+peroxidase. R^2 is the coefficient of determination, in bold for $p \leq 0.05$.

Stimulation	T (°C)	Model parameters		
		α	β	R^2
None (spontaneous)	−20	3.42×10^5	0.053	**0.83**
	4	3.87×10^5	0.152	**0.99**
	20	5.37×10^5	0.325	**0.90**
	40	8.81×10^5	0.893	**0.97**
	60	0.17×10^5	1.261	0.56
	80	0.22×10^5	1.293	0.60
APS	−20	4.68×10^6	0.042	0.59
	4	6.35×10^6	0.207	**0.99**
	20	11.01×10^6	0.499	**0.94**
	40	8.70×10^6	1.027	**0.95**
	60	0.09×10^6	1.568	0.53
	80	0.24×10^6	1.684	**0.65**
Peroxidase	−20	9.69×10^6	0.033	0.32
	4	13.61×10^6	0.276	**0.95**
	20	23.61×10^6	0.604	**0.95**
	40	15.56×10^6	1.043	**0.96**
	60	0.13×10^6	1.468	0.50
	80	0.95×10^6	1.592	**0.72**

flashes is for predator deterrence (Herring 1978), and so the intense flashes of mechanically stimulated worms are suggestive of an interspecific defensive function.

The results of this study suggest that the kinetics of light production differ with individual size, supporting earlier field observations on light production in *O. enopla* (Fischer & Fischer 1995). During the swarming period, large individuals produce long-lasting glows while medium individuals produce mainly flashes. However, during non-swarming periods when the worms are benthic, individuals of both sizes can produce light in reaction to mechanical stimuli (Fischer & Fischer 1995). In this study, small individuals produced highly variable patterns of bioluminescence, with multiple emission peaks that were sometimes reached several minutes after KCl stimulation; light production also did not significantly increase following repeat stimulation with APS or peroxidase (data not shown). This pattern was very distinct from that of larger worms; here, light emission was always unimodal and responded rapidly to KCl, APS, and peroxidase treatments. The differences in response patterns with worm size suggest multiple ecological functions for the bioluminescence (Morin 1983).

KCl treatment to trigger light production showed that in the 0.5–2.5-mm size range, there was a transition from internal bioluminescence to the production of luminous mucus, so that most of the bioluminescence from the largest individuals in this size range originated from mucus. This indicates that in the smallest individuals, the bioluminescence was mainly internal, while it was both internal and secreted for larger individuals, thus also supporting the idea that there are multiple functions associated with light production in *O. phosphorea*. Integrated bioluminescence was independent of size in the 0.5–2.5 mm range, suggesting that the capability to

produce light is innate to post-metamorphic worms and not acquired during subsequent growth, development, and sexual maturation (Tsuji & Hill 1983; Fischer & Fischer 1995).

Multiple functions of bioluminescence were also supported by observations made by keeping individuals of *O. phosphorea* isolated in aquaria. Individuals maintained in flow-through aquaria for ≤ 6 months past the swarming period were found within tubes, as reported from field observations for *O. enopla* (Fischer & Fischer 1995). Worms gently removed from their tubes still showed similar levels of KCl-induced bioluminescence as freshly collected specimens (data not shown). The freshly emptied tubes also showed various levels of spontaneous bioluminescence, indicating that light production is an integral component of the mucus, whether it is secreted by pelagic or benthic worms.

Photoprotein-like system of mucus

The classic "hot–cold" mixing experiment is useful in making inferences on whether light production results from a luciferin/luciferase reaction versus a photoprotein system. The mixing of hot and cold extracts of mucus from members of *O. phosphorea* did not lead to reconstitution of the light activity, suggesting that the bioluminescence may not be produced by a conventional luciferin/luciferase reaction. An alternative explanation is that the mucus contains inhibitors of luciferase activity or oxidized luciferin, in which case the hot–cold extract recombination tests might result in increased emission only after dilution or dialysis of the cold extract to separate the oxidized luciferin or other inhibitors from the luciferase (Shimomura et al. 1963). These possibilities were not tested in the present study.

Working with *O. enopla,* Harvey (1952) made the qualitative observation that "a good luminescence" was obtained by mixing hot and cold extracts of worms without dialysis. This observation does not support the inhibitor hypothesis for the *Odontosyllis* system, and suggests that a luciferin/luciferase reaction is responsible for light production. However, the results of the present study suggest that the mucus-associated bioluminescence of *O. phosphorea* is not a luciferin/luciferase system. The discrepancy between these recombination test results may be due to the use of whole worms by Harvey versus luminous mucus in this study. This discrepancy, and the fact that the bioluminescence exhibits two clearly distinct patterns (internal flashes and secreted mucus), may also indicate that several mechanisms are involved in light production in *O. phosphorea*. In luminous metazoans, patterns of light production are usually species specific and depend on physiological pathways under neural or hormonal control, as well as the physicochemical environment in which the light production takes place (Hastings & Morin 1991). Within a species, differences in the kinetics pattern of bioluminescence can be observed with age, sexual maturity, and/or gender (Morin 1983; Wilson & Hastings 1998). There are also numerous additional factors that might control the pattern of light production. These include stimulatory co-factors, such as calcium for some photoproteins and ATP for the firefly luciferin/luciferase reaction; luciferin-binding proteins as in the anthozoan *Renilla* or dinoflagellate *Lingulodinium* systems; and/or inhibitory agents (Campbell 1988), all of which could be involved in the flash versus glow bioluminescence in *O. phosphorea*.

The failure of mixtures of hot and cold extracts to reconstitute bioluminescence is evidence for a photoprotein-like system in *O. phosphorea*. Photoproteins are stable intermediates of luciferin that have already bound to luciferase and molecular oxygen, but for which the light production is blocked. The presence of a specific co-factor allows stereoscopic changes of the pre-packed system, thus allowing the photoprotein to produce light. Light can therefore be produced in the absence of oxygen, is not dependent on an enzymatic reaction, and is not reconstituted by hot/cold experiments (Shimomura 1985). Photoproteins are common in many luminous organisms including coelenterates and polychaetes (Campbell 1988), with the calcium-activated aequorin from the hydromedusa *Aequorea* (Ohmiya & Hirano 1996) serving as a model system.

Additional evidence supports the involvement of a photoprotein in bioluminescence in *O. phosphorea*. Mucus bioluminescence persisted under anoxic conditions. In addition, 10 mmol L^{-1} sodium thiosulfate, a strong oxygen scavenger, inhibited no more than 50% of light production even though such a high concentration is predicted to fully inhibit bioluminescence if light production is strictly oxygen dependent (Wang et al. 2002). The inhibitory effect of sodium thiosulfate was found to be greater with a lower concentration of mucus. Within the framework of a photoprotein hypothesis, these data suggest a direct inhibitory interaction between sodium thiosulfate and the photoprotein-like material rather than an inhibition due to the lack of oxygen in the medium.

An alternative hypothesis to the photoprotein-like system is that the bioluminescent process is efficient at very low oxygen concentrations. Although this possibility has not been directly tested in this study, it is not supported by the results of the treatment by

hydrogen peroxide, a strong oxidizer commonly used to oxidize the substrate of light production in many luminous systems (Shimomura 2006). Hydrogen peroxide inhibited rather than stimulated bioluminescence in *O. phosphorea,* as would be expected if oxygen were directly involved in the light production process. The inhibition of bioluminescence by hydrogen peroxide suggests that the bioluminescence system in *O. phosphorea* is already strongly pre-oxidized; it also suggests that the photoprotein-like system involves an inhibitory factor. This factor may be a compound that would be non-inhibitory in a reduced form but strongly inhibitory to the production of bioluminescence once oxidized, as proposed in earlier studies (Shimomura et al. 1963).

The conclusion that mucus bioluminescence in *O. phosphorea* involves a photoprotein rather than a luciferin/luciferase system contradicts previous results with extracts from members of *O. enopla,* showing that oxygen is necessary for light production (Shimomura et al. 1963). While it is possible that the two species (*O. phosphorea* and *O. enopla*) could have distinct bioluminescent chemistries, the discrepancy may again originate from the different types of samples used, as fresh luminous mucus was used in our study while the study of Shimomura et al. (1963) involved fractions of the luminescence system partially purified from hundreds of worms.

A luciferin–luciferase system follows the general rules of an enzyme-catalyzed reaction and shows activity within a specific range of temperatures, losing activity at temperatures lower and higher than the range of temperatures typically experienced by the species (Campbell 1988; Shimomura 2006). The range of temperatures of a luciferin–luciferase system is therefore optimized to the environmental conditions experienced by the luminous organism (Harvey 1952; Herring 1978; Hastings 1996). On the other hand, a photoprotein system regulated by a co-factor is not enzymatically driven, and so it is expected to be active at very low temperatures but degraded at higher temperatures when denaturation of disulfide bonds between the apo-protein and the substrate takes place (Ohmiya & Hirano 1996). Thus, a photoprotein system shows a different pattern of activity related to temperature than a luciferin–luciferase system. For example, many organisms with luciferin–luciferase systems involving coelenterazine have maximum bioluminescence at $\sim 35^{\circ}$C, with light production reduced by 75% in both colder ($\sim 15^{\circ}$C) and warmer ($\sim 45^{\circ}$C) temperatures (Shimomura 2006). On the other hand, the cnidarian photoprotein aequorin maintains bioluminescence activity down to -75°C, and yet is rapidly inactivated above 30°C (Shimomura 1985). The results of the temperature experiment with mucus from individuals of *O. phosphorea* were consistent with a photoprotein-like system. There was a direct relationship between increasing temperature and decreasing light production, with maximum bioluminescence at -20°C that was rapidly inactivated above 30°C, similar to the aequorin system (Shimomura 1985).

Moreover, addition of APS resulted in a 10–15-fold increase in light intensity for temperatures $\leq 40^{\circ}$C (Fig. 8; Table 1). Assuming that APS acts only through protein–protein interactions (Fancy & Kodadek 1999; Denison & Kodadek 2004), these results suggest that the luminescent source in *O. phosphorea* is associated with a protein or an apo-protein that is degraded by an elevated temperature and is yet well preserved at temperatures as low as -20°C. The increase in the rate of decay of light production with increasing temperature, especially between 20°C and 60°C, suggests that the light production in *O. phosphorea* involves a reagent (e.g., apo-protein, substrate, or co-factor) that degrades rapidly, is inhibited, or is uncoupled from the light production process at higher temperatures.

Conclusions

Although the chemistry of the bioluminescence system in *Odontosyllis* is as yet uncharacterized, it is different from that of other luminous organisms (Shimomura 2006). The present study using the luminous mucus from members of *O. phosphorea* provides a new methodological approach to assess bioluminescence in these organisms. Light production occurs as a long glow of intense green light and is associated with green fluorescence. The production of flashes in small worms, and the occurrence of both internal and secreted bioluminescence in larger worms, suggest multiple functions associated with light production. The chemistry appears to involve a photoprotein based on the evidence from mixing hot and cold extracts, temperature treatments, anoxic conditions, and chemical treatments. These results are encouraging for pursuing further studies on the purification and identification of compounds involved in the bioluminescence of *Odontosyllis* spp.

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