Optical and Physicochemical Characterization of the Luminous Mucous Secreted by the Marine Worm *Chaetopterus* sp.

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ABSTRACT

Bioluminescence of the marine worm *Chaetopterus variopedatus* was first investigated several decades ago mainly using tissue extract. Light production of the worm, however, originates from a secreted mucus only. Here, we report the optical and physicochemical properties of the luminous mucus. We show that the produced light occurs as a long glow in the blue range (455 nm), which is an unusual color for a shallow benthic invertebrate. We also show that the light originates from a photoprotein whose light production is independent of molecular oxygen yet somewhat related to the physicochemical (rheological) characteristics of the mucus itself. Indeed, the mucus seems to polymerize and become more viscous on exposure to H_2O_2, which in turn seems to inhibit the light production. Ferrous iron was not associated with any strong stimulatory effect. This is in contrast to past studies on worm tissues showing that the light production is strongly stimulated by H_2O_2 and ferrous iron. Overall, our results highlight the fact that working on the luminous mucus only (vs. worm tissues) provides the ability to study its chemical properties possibly involved in the fine control of light production—as well as its rheological properties—and identify the possible interactions between these two properties.

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Introduction

Bioluminescence, the production of visible light by living organisms following a biochemical reaction, is a biological property found in many organisms from terrestrial and marine environments (Harvey 1924; Haddock et al. 2010). The light production is associated with an ecological function and is therefore produced with specific kinetics, intensity, and color. This is reflected in the fact that in most cases, bioluminescence is produced as bright flashes with yellow-orange color in terrestrial environments, greenish color in shallow-water environments, and bluish color in deep-sea environments, which corresponds to the wavelengths that propagate best in these media (Harvey 1924; Morin 1983; Haddock et al. 2010).

Bioluminescence always involves a chemical reaction where a substrate (generically luciferin) reacts with an enzyme (generically luciferase). The reaction systematically uses molecular oxygen and possibly other oxidation-related transformations related to cofactors (proteinic and/or ionic) involved in the molecular control of the light-producing reaction (McCapra 1990; Mager and Tu 1995; Rees et al. 1998). Bioluminescence following a typical luciferin-luciferase reaction thus requires and consumes oxygen, except when the luciferin-luciferase first reacts with molecular oxygen in a precursor phase, forming a so-called photoprotein. In this case, the molecular oxygen is already bound to the reagents involved in the light production (Shimomura 1985). The photoprotein then releases light without concomitant consumption of oxygen but following exposure to a specific cofactor; another characteristic is a direct 1 : 1 ratio between the amount of protein and the level of produced light (Shimomura 1985).

The intriguing biology of the marine worm *Chaetopterus variopedatus* (commonly known as parchment tube worm) has been the subject of several studies, most performed 30–40 yr ago. The worm has a worldwide distribution and is found from shallow to several hundred meters deep environments, building tubes in which it lives on the seafloor. The worm is a polychaete showing specific anatomical adaptation to a tube-dwelling lifestyle and produces from various parts of its body a bright luminescent mucus that is generated in abundance and somewhat constantly on stimulation (Nicol 1952*a*; Johnson 1959); the mucus can be produced in such abundance that when squeezed underwater during scuba diving, a cloud of light is noted puffing out of the tube into the water (D. D. Deheyn, personal observation).

Several studies have listed different functions of the luminous mucus, such as serving as a medium to trap food, attracting small living organisms (MacGinitie 1939; Nicol 1957; Flood and Fiala-Médioni 1982), deterring organisms from settling in

the burrow (Morin 1983), and/or building the tubes itself (Enders 1909). However, it is not clear at this stage whether different body parts of the worm secrete different types of mucus or the same mucus with different abilities over time, producing light first and then building the tube as the light production is spent. From the bioluminescence aspect, light production from the mucus is a long-lasting glow of bluish color whose chemistry remains unknown (Shimomura 2006*a*, 2006*b*). Using extracts from ground whole individuals, chemical analysis yielded evidence of a photoprotein system with unique characteristics, having a 1 : 1 relationship between protein content and levels of produced light yet showing sensitivity to molecular oxygen and hydrogen peroxide, while also depending on ferrous iron (Shimomura and Johnson 1966, 1968).

This study, in contrast to all the former work, aims to focus on the mucus itself, once secreted from the worm body. We provide fundamental descriptions of the light production in terms of intensity, kinetics, and spectra while also performing key experiments that address the nature of the biochemical reaction leading to light production. Rheology of freshly secreted mucus was also investigated in order to determine whether light production from the mucus is affected by changes in physical or material properties inherent to the mucus.

Material and Methods

All the worms used in this study originated from the La Jolla submarine canyon, located in San Diego, California. The *Chaetopterus* species in Southern California is usually described as *Chaetopterus variopedatus*, yet current molecular phylogeny studies indicate that this is not the case (G. Rouse, unpublished manuscript). For this reason, *Chaetopterus* sp. will be used throughout the text. Light production in the species occurs as a long glow, and this study mainly looked at changes in kinetics of the bioluminescence (i.e., changes in the output rate of photons per second). Therefore, the data are presented as representative profiles of raw kinetics measurements in order to better describe typical range and rapidity of some of the changes induced by the tested experimental conditions. When indicated, notes are made throughout the text about replicability of specific observations or data collected among independent samples of luminous mucus from different worms ($N = 3$–15, depending on the measurement/experiment). All data were processed with Excel (Microsoft) and figures put together with Deltagraph 5.0 (RedRock Software).

Worm Collection

Chaetopterus sp. lives in U-shaped tubes that each individual builds gradually from the inside. The tips of the tubes are above the seafloor sediment, and many tubes are usually interlaced together, with a thriving population thus generating a compact cover of tubes sticking up several centimeters from the seafloor. Bundles of tubes were hand collected by scuba at 20–30-m depth from 2008 through 2011 and transported in collecting bags to a surface boat, where they were maintained in 5-gal buckets filled with seawater. The buckets were brought back to the Marine Biology Experimental Aquarium Facility at Scripps Institution of Oceanography and kept with circulating seawater at ambient temperature until used for experimentation, usually within 4–6 wk. Some bundles of worms were also shipped overnight to Georgetown University (for rheological studies only), where they were kept in a closed-circuit aquarium with cold (15°C) artificial seawater (ASW; made using Instant Ocean sea salt).

The worms were left to grow in their tubes with no addition of artificial food. A majority of tubes rapidly showed new growth, indicated by a much paler discoloration, demonstrating that the stock of worms was thriving in holding conditions, although some tubes were also found without any resident worm. One to a few specimens (depending on the analysis) were collected freshly before each experiment by tearing open the tube along its length, transferring the exposed worms into a petri dish with seawater, and transporting the worms to the laboratory, where they were kept on ice.

Worm Dissection

Initial analyses were performed from mucus collected from the entire worm. However, it became rapidly apparent that during certain months (especially spring through summer), the mucus collection process would also induce the abundant release of gametes from the tail together with the mucus, thus interfering with further optical and biochemical analyses. *Chaetopterus* sp. shows four distinct anatomical sections, which we labeled as the head, middle (anterior and posterior), and tail (fig. 1). We investigated the potential of each section to produce luminous mucus by separating each apart from the others, using fine dissecting scissors, after about 15 min on ice (hypothermal anesthesia) and treating each section separately for mucus production. Ultimately, all analyses were performed with mucus produced from the main body section only (thus combining the head, middle anterior, and middle posterior sections), unless otherwise noted.

Mucus Preparation and Kinetics Analysis

Individual worms were removed from their parchment tube and placed into a glass petri dish with fresh, isotonic ASW (Deheyn et al. 1997). Body length was measured to the nearest centimeter, and worms were cut into three sections (head, middle, and tail). Each body section was placed into a plastic tube with a known volume of ASW to which potassium chloride (KCl; 400 mM in ASW) was added (vv) to stimulate mucus secretion. Mucus secretion was also generated from the main body section (head, middle anterior, middle posterior) and tail following the same mucus secretion process. After 5 min, the liquid (mucus mixed with KCl) from each body region was transferred into Eppendorf tubes. Unless otherwise indicated, mucus samples were always kept on ice during the mucus preparation process and until used for further experimental analysis. For some initial samples, the kinetics of light production were recorded for 90 s from each body part immediately after dis-

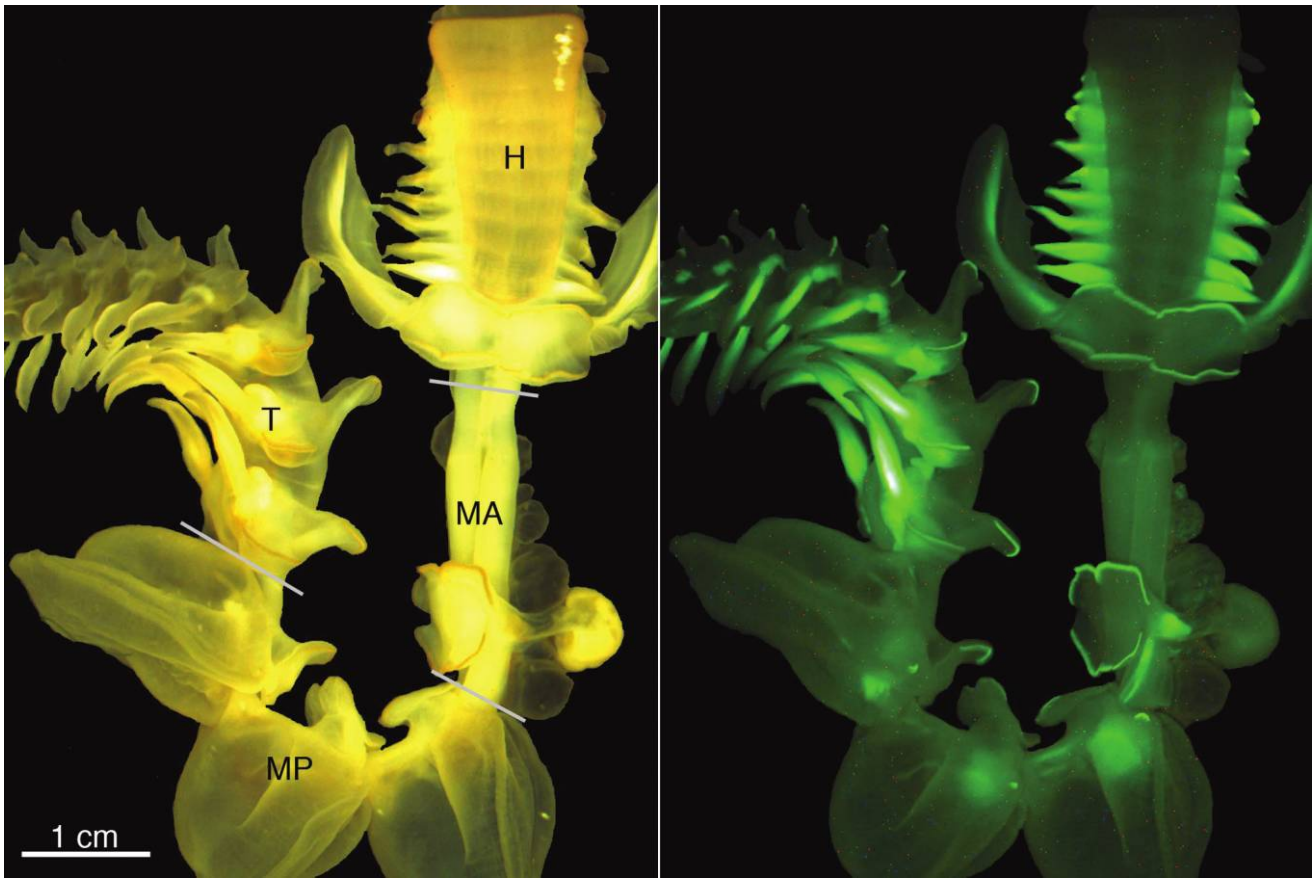

Figure 1. Full-body imaging of live *Chaetopterus* sp. marine worm in bright field (*left*) and fluorescence (*right*), with indication of the studied body parts. H, head; MA, middle anterior; MP, middle posterior; T, tail.

section (spontaneous light production induced by worm dissection) and for an additional 90 s after KCl addition (light production triggered by tissue depolarization that induces mucus secretion). The kinetics of light production were also recorded for 180 s from mucus only, once collected from the various body parts (mucus was thus collected after secretion from the body parts upon KCl exposure and separated from the body part). Mucus that was processed for light production was also analyzed for protein content concentration by colorimetry, following the protocol for a Bradford protein assay (QuickStart, BioRad).

Bioluminescence Recording

Bioluminescence was measured from 50-μL subsamples of the mucus preparation, and the light production was recorded using a Sirius dual internal injector integrated luminometer (Berthold Detection Systems, Germany). Records varied in duration depending on the experiment, but the acquisition rate was always kept at 5 acquisitions per second. Data were acquired from light spontaneously produced by mucus once secreted but also after the addition of ammonium persulfate (APS; $(NH_4)_2S_2O_8$, v : v, 20 mM; Sigma-Aldrich) as well as hydrogen peroxide (H_2O_2, 30% American Chemical Society grade; Fisher Scientific) in various concentrations (v : v). Both APS and H_2O_2 were injected consecutively into the mucus but also in the reverse order to ensure that there was no treatment effect from the order in which solutions were injected. All solutions were prepared freshly before each experiment.

Spectral Analysis of Light Production

The spectrum of bioluminescence was measured from one live organism at a time directly from the body parts showing light as well as from the secreted mucus collected and placed in a spectrophotometric cuvette. Measurements started immediately after the addition of KCl to the body part, which stimulates secretion of the luminous mucus, or when the luminous mucus was first transferred into a separate cuvette for mucus-only analysis. Light collection was performed every 30 s for as long as the bioluminescence was detectable (usually not more than 10 min). The spectral collection was then switched to the fluorescence mode, with excitation light set to 380 nm (Ocean Optics, Light Source model LS-450). A Low Light Echelle SE200 Digital Spectrograph (Catalina Scientific) was used for instantaneous (no scanning involved) acquisition of a full spectrum

using a short integration time (0.1–1 s), which involved light collection through a 1-μm fiber-optic cable. The Echelle SE200 was used for all spectra characterization (live and fluorescence, from worm and/or mucus only) but also for absorbance spectra (mucus only); this was done by analyzing the spectral change of a tungsten halogen white reference light beam (Ocean Optics, Light Source LS-1) passing through the mucus placed in a cuvette. The fiber optic was then connected to a dark microchamber made for light measurements in cuvettes (the emitting fiber was opposite and aligned with the collecting fiber for absorbance, while the fibers were in the same plane but perpendicular for fluorescence), while the collecting fiber was attached to a micromanipulator (M-3333, Japan) for application on certain areas of the worm body (for live and fluorescence measurements directly from the worm). In this case, the worm was placed under a Nikon SMZ1500 stereomicroscope coupled to a Retiga 2000R color digital camera for imaging (QImaging, Canada). The microscope setup was also equipped with a 100-W mercury lamp (X-Cite EXFO 120) and filter cube for epifluorescence, using a Nikon filter cube with excitation at 380–400 nm and longpass emission barrier >435 nm (Nikon Blue GFP/DAPI cube, Melville, NY).

Effect of Temperature on Light Production

Samples of mucus were exposed to temperatures of −20°, 4°, 20°, 40°, 60° and 80°C for up to 30 min in the dark. Mucus secreted from three worms (following KCl stimulation) was combined and homogenized to make a total of 2.6 mL (i.e., the stock mucus for all temperature treatments). A total of 42 aliquots of 60 μL of mucus were split into separate 0.5-mL Eppendorf tubes (six temperatures × seven time points × 60 μL) that were sealed (snap cap and parafilm) and exposed to the different temperature treatments. At the specific time points investigated, one of the aliquots (thus one of the tubes) was retrieved from each of the temperature treatments and let sit on the bench for 2 min (allowing it to come back to room temperature), and 50 μL were transferred to a luminometer tube and then analyzed for light production. Light production was measured with the Sirius luminometer, and each measurement included three 10-s consecutive readings of light production from the same sample. The first measurement represented spontaneous light production from temperature-treated mucus, the second represented temperature-treated mucus + APS, and the third represented temperature-treated mucus + APS + H_2O_2. In a subset of experiments, spontaneous light production as well as fluorescence (excitation, 380 nm) of temperature-treated mucus was measured for 120 min, using the $20/20^n$ Turner luminometer equipped with the ultraviolet excitation (365–395 nm) fluorescence module (Turner Biosystems). This setup allowed consecutive measurements of bioluminescence quickly followed by analysis of fluorescence from the same sample without involving any movement of the cuvette, thus allowing only a very short time between the bioluminescence and the fluorescence analyses (<5 s). The detection of fluorescence without bioluminescence (see "Results") was indicative of a light-producing compound that was not broken down by heat treatment, which is an optimal condition for performing a hot/cold experiment.

Hot/Cold Experiments

Mucus samples were placed either into a 90°C water bath (hot treatment) or on the laboratory bench (cold treatment) after bioluminescence was measured for 1 min (time 0). In a traditional luciferin-luciferase reaction, placing a mucus sample into a cold treatment will oxidize the luciferin (substrate) and preserve the luciferase (enzyme). Alternatively, placing a mucus sample in a hot treatment will denature the luciferase and preserve the luciferin (in other systems, the luciferin is usually thermostable, and its oxidation is low without apoprotein; Shimomura 2006*a*). If, after the temperature treatment, mixing the hot and cold extracts (now both at room temperature) does not lead to restoring some of the light production, it can be inferred that the bioluminescent system does not involve a luciferin-luciferase reaction but rather a photoprotein (Shimomura 2006*b*).

Cold treatment samples were left on the laboratory bench for up to 90 min to allow the substrate to be fully oxidized. Enzyme denaturing in hot samples took 20 min (checks of spontaneous light production were made regularly using the luminometer), after which time samples were placed on ice until ready for use and rechecked for bioluminescence before starting the cross-reactivity experiment. Samples were combined as follows: cold + ASW + APS, hot + ASW + APS, cold + hot + APS, cold + cold + APS, and hot + hot + APS. The experiments were always conducted in darkness (main lights of the laboratory off) to avoid the possibility of photobleaching and/or photooxidation. Bioluminescence was measured for 60 s for each treatment, with an initial 30 s to measure any spontaneous light.

Effect of Oxygen Depletion on Light Production

Mucus samples (300 μL) from worms were bubbled in luminometer tubes with helium, nitrogen, or argon for 20 min at room temperature to deplete oxygen within the samples. Control samples consisted of mucus that was bubbled with air or not bubbled at all and left sitting on the laboratory bench. Following bubbling, the tubes were immediately corked with rubber stoppers, sealed with parafilm, and measured for spontaneous bioluminescence for 1 min. APS (300 μL) was injected manually through the rubber stopper using a syringe and needle, with light production measured for 1 min thereafter. The process was repeated with injection of H_2O_2 (600 μL) on the same sample then containing 300 μL of mucus + 300 μL of APS. APS and H_2O_2 solutions were subjected to the same experimental parameters used on the mucus samples in order to respect the various hypoxic treatments (yet the solutions were kept in glass tubes with rubber screw caps to maintain anaerobic conditions).

Effect of Iron and Hydrogen Peroxide on Light Production

Dose response experiments with hydrogen peroxide were completed by injection (v : v) into mucus, using 30%, 20%, 10%, 5%, or 1% H_2O_2 solution in ASW (initial concentration). Mucus samples (50 μL) were first measured for spontaneous light production for 30 s; hydrogen peroxide was then injected, and the light produced was recorded for an additional 100 s. In addition to the H_2O_2 dose response, analyses were completed from dual consecutive injections in the mucus of H_2O_2 (30%) and ferrous chloride ($FeCl_2$; 20 mM). Injections (v : v) were performed at 30 and 60 s (H_2O_2 then $FeCl_2$, but also $FeCl_2$ then H_2O_2), with the entire record lasting 130 s. All these experiments were typically done using 50 μL of samples, yet 100 and 300 μL were also shown to lead to similar results.

Rheological Analysis

Mechanical testing of secreted mucus was performed using a stress-controlled rheometer (Anton Parr GmbH MCR-301). The tool geometry was a peltier temperature-controlled, 50-mm-diameter plate-plate configuration. Oscillatory measurements were performed to determine the viscoelastic modulus as a function of strain amplitude and oscillation frequency. To determine the linear modulus, the oscillation frequency was fixed at $\omega = 6.2$ rad s^{-1}, while the strain amplitude was increased logarithmically from $\gamma = 0.1\%$ to 100%. The frequency response was determined by fixing the strain amplitude at $\gamma = 1.0\%$ and logarithmically varying the oscillation frequency from $\omega = 0.32$ to 62.8 rad s^{-1}. Flow curves (viscosity vs. shear rate) were measured with the same tool geometry through a logarithmic variation in the applied strain rate from 0.01 to 100 s^{-1}.

Results

Bioluminescence and Protein Concentration in Mucus from Different Body Parts

All body parts were always able to produce luminous mucus, and accordingly, all showed internal sources producing fluorescence (fig. 1). Kinetics of the light production was variable from one organism to another and among body parts; overall, the head and then tail and middle posterior sections produced the most light, with the middle anterior—which is the smallest and most pigmented body part—producing the least (fig. 2*A*). Mucus from each of the corresponding body parts showed no color or a faint yellowish tint and produced light in the form of a long-lasting glow, with mucus from the head usually the most intense and mucus from the middle anterior consistently the least (fig. 2*B*). Mucus from the main body section (which includes every part except the tail) showed characteristics similar to the head alone. Whichever the body part and the initial level of light intensity, the glow usually remained relatively constant over at least 180 s yet sometimes showed a slow decay in intensity (fig. 2*B*, head and anterior curves). Protein content results showed that mucus from the tail contained about 10–50 times more protein than the other body parts (fig. 2*C*), which sometimes could be seen by the opaque whitish color due to the release of gametes. Mucus from the head and middle parts therefore appeared more appropriate to study the mucus from its light production standpoint. In what follows, the data will always refer to mucus that was collected from only the main body section.

Spectral Analysis

Bioluminescence and fluorescence of *Chaetopterus* sp. followed a similar spectral profile, both with a peak at 455 nm (fig. 3). Fluorescence, however, showed an additional peak at 670 nm, with a shoulder at 725 nm. Mucus transmittance was not homogeneous throughout the entire spectrum, showing a sharp decrease between 400 and 550 nm, with the lowest transmittance (i.e., maximum absorbance) at 475 nm (fig. 3). These profiles were similar whether collected from the organism or the mucus and were independent of the body part tested.

Effect of Temperature on Light Production

The intensity of light produced by mucus changed with temperature, particularly on warming. The amount of spontaneous light produced by mucus treated at 60° and 80°C decreased by 100–400 times within 5 min (fig. 4*A*) and remained low until the end of the analysis, despite the addition of APS (fig. 4*B*) and then H_2O_2 (fig. 4*C*), thus indicating degradation/denaturation of the source of light at these temperatures. In comparison, the −20° and 4°C treatments induced approximately a 10× decrease of spontaneous light production within 5 min, which was also observed for the ambient reference treatment (20°C), thus indicating a slowing of the chemical reaction (or decrease in rate of photons emitted per second) as a result of inherent factors of the mucus rather than temperature. In all cases, the subsequent treatments with APS did not trigger any large changes in the amount of light produced; however, a 2–5× increase was occasionally noted in the lower temperature treatments (−20° to 40°C; compare fig. 4*A* and fig. 4*B*).

In contrast, injection of H_2O_2 always induced a 10–50× decrease in light produced that was proportional to the spontaneous and/or APS amount over the various time points, which resulted in a similar change in the rate of decay, in particular for the lower temperature treatments (−20° to 40°C; fig. 4*C*). Surprisingly, the higher temperatures (60° and 80°C) actually showed a slight but steady increase of light production on exposure to H_2O_2 (fig. 4*C*); for the 80°C temperature in particular, the light intensity increased 2–7× (for all times, except for time 0) relative to spontaneous/APS-treated mucus after addition of H_2O_2 (compare the bioluminescence intensity for the 80°C data in fig. 4*B* and fig. 4*D*). This suggests that at higher temperatures, a compound (chromophore) is likely released and available for oxidation with H_2O_2, when such accessibility to oxidizing the chromophore is not possible at lower temperatures. In fact, at such nondenaturing temperatures, H_2O_2 appears to have an inhibitory effect rather than stimulatory (see "Dose Response with Hydrogen Peroxide").

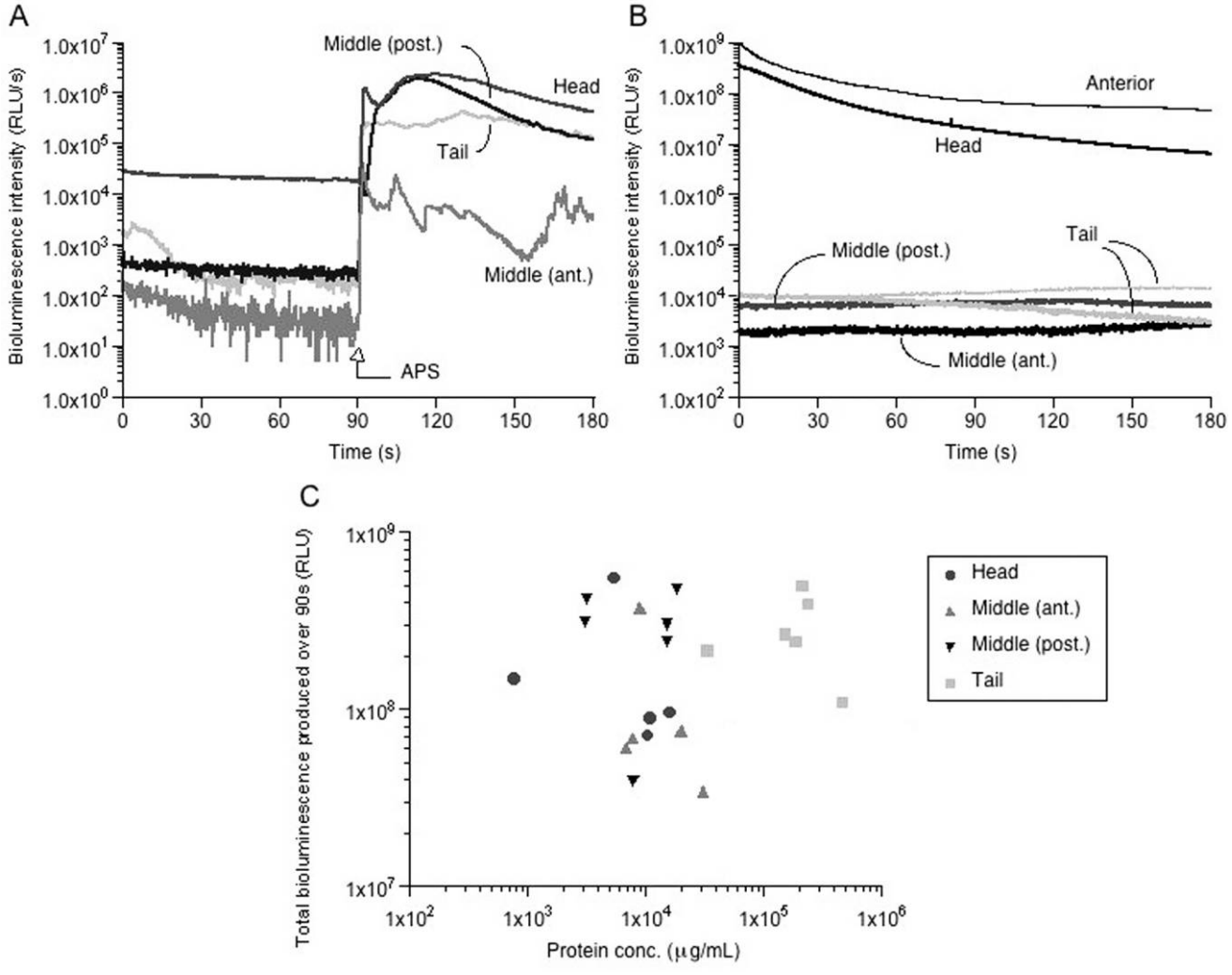

Figure 2. Representative kinetics records (from $N = 12$ replicates) of KCl-induced light production from the various body parts of one *Chaetopterus* sp. individual, followed by stimulation with ammonium persulfate (APS; *A*) and spontaneous light production from the mucus secreted by the various body parts of one individual and by the anterior and tail body sections of another individual (*B*). *C*, Total amount of light produced spontaneously by mucus of different individuals as a function of protein concentration in mucus. RLU, relative light unit.

Comparison of spontaneous light production and fluorescence (excitation, 390 nm) from mucus treated at 80°C showed that while the bioluminescence sharply declined within 5 min and remained low for 120 min, the fluorescence showed the opposite trend and increased over the time of analysis (fig. 4*D*). This suggests that the chromophore (or luciferin) is still functional after heat treatment, which is compelling evidence to perform a traditional hot/cold experiment.

Hot/Cold Experiments

Intensity of light production from untreated mucus was between 10^7 and 10^8 relative light unit (RLU) s^{-1}, which dropped to 10^4–10^5 RLU s^{-1} for cold extracts and to 10^2–10^3 RLU s^{-1} for hot extracts (fig. 5). Control recombination samples (cold + cold, hot + hot, cold + ASW, hot + ASW) did not result in any increase in light emission after addition of the second extract (fig. 5). Similarly, mixing a hot sample to a cold and vice versa did not result in any recovery of spontaneous light production (fig. 5).

Oxygen Depletion

Decreasing oxygen from the surrounding medium did not affect the level of spontaneous bioluminescence produced by the mucus (fig. 6). The trend of light production remained the same whether the mucus was bubbled with argon, helium, nitrogen, or air or not bubbled at all. The addition of APS increased light production in all treated samples in a similar fashion, and the subsequent addition of hydrogen peroxide immediately inhibited light production to as little as 10% of initial light levels, independent of the oxygen depletion treatment (fig. 6). These

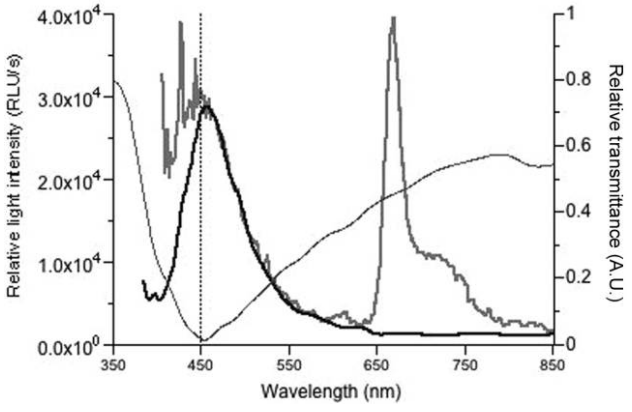

Figure 3. Emission spectra from mucus producing spontaneous light (thick black line) and fluorescence (thick gray line) superimposed with relative transmittance (thin black line). The fluorescence spectrum peaking at 670 and 730 nm indicates the presence of chaetopterin, a chlorophyll-derived compound long known in *Chaetopterus*. All emission spectra were very similar among $N = 10$ replicates tested. RLU, relative light unit; AU, absorbance unit.

effects were always found among treatments but also from one sample to the other, independent of the initial intensity of spontaneous light.

Dose Response with Hydrogen Peroxide

Adding H_2O_2 to mucus triggered rapid inhibition of the spontaneous light production (fig. 7*A*). The inhibition was dose dependent, showing gradual and slow inhibition at lower concentrations of hydrogen peroxide (1%, 5%, and 10%). For these lower concentrations, the light production was sometimes first stimulated very rapidly for just a couple of seconds before undergoing a 5–10 × inhibition by the end of the analysis (fig. 7*A*). This was not always observed among replicates and seemed affected by the initial amount of spontaneous light produced by the mucus (mucus with lower bioluminescence would be more likely to show this pattern). In contrast, the inhibition of mucus light production was immediate and faster for greater concentrations of hydrogen peroxide (20% and 30%). The bioluminescence was then reduced by about 100 × within 10 s following the H_2O_2 exposure and by about 1,000 × within 40 s following the H_2O_2 exposure (fig. 7*A*). Adding 20 mM APS to the 30% H_2O_2 treated mucus increased the light production by 2–10 ×, which was always only a small fraction of the initial level of bioluminescence (fig. 7*B*).

Effects of Ferrous Iron and Hydrogen Peroxide

The addition of ferrous iron (Fe^{2+}) induced a limited (1.2–3 ×) increase in mucus spontaneous bioluminescence, which was rapidly inhibited by addition of H_2O_2 (fig. 8). In contrast, the addition of Fe^{2+} subsequent to H_2O_2 inhibition first generated a greater (8–15 ×) increase of light production (fig. 8).

Rheological Analysis

When subjected to a low-amplitude oscillatory shear strain, unfiltered raw mucus exhibited a viscoelastic response with a storage modulus G' greater than the loss modulus G'', indicating that the mucus is an elastic gel. Under continuous shear conditions, the mucus flowed with a viscosity that decreased as the shear rate increased. The observed shear thinning behavior is indicative of a yield stress gel. We observed that the functional form of the mucus flow curves were well described by a power law of $\eta \sim (d\gamma/dt)^{-a}$, with $0.8 < a < 1.1$, independent of the additives or filtration history (fig. 9). The exception comes from the addition of H_2O_2 that produced a large (≈500 ×) increase in viscosity, uniform across all shear rates tested (fig. 9).

Discussion

This study provides a fundamental description of the optical and physicochemical properties of the luminous mucus secreted by the marine worm *Chaetopterus* sp. Even though collected in shallow coastal waters, the mucus produces a long-lasting glow of blue light, which is unique for this environment where bioluminescence is usually produced in the green, especially for benthic species (Harvey 1952; Haddock et al. 2010).

Light Production in Chaetopterus *sp.: Learning from Past Studies on Worm Tissues*

Biochemistry of the light production from *Chaetopterus* sp. has been subject to only a handful of studies several decades ago (Nicol 1952*a*, 1952*b*, 1954, 1957; Sie et al. 1958; Johnson 1959; Shimomura and Johnson 1966, 1968). Most of these studies were completed on purified light-producing compounds that were extracted from worm tissues of whole individuals and rarely from the mucus itself. The mucus, however, is where the reaction of light production takes place upon secretion of the mucus only. The earlier studies on purified proteins from worm tissue extracts indicated that light production involves a photoprotein that uses iron as cofactor while being strongly stimulated by H_2O_2 (Shimomura and Johnson 1966, 1968). The luminous system also seems to involve an inhibitor mechanism on the basis of the observation that adding seawater to the mucus triggers a rapid burst of light followed by an acceleration in the decay of light production (Johnson 1959), which might have also been seen here following some injections (see figs. 5, 7). A chemiluminescent protein was isolated from worm tissues extracts, yet no direct indication of its involvement with the light production process was found. Nevertheless, a putative photoprotein was crystallized (Shimomura and Johnson 1968), but elucidation of the full protein sequence and its tertiary structure is not available in the scientific literature, to the best of our knowledge. The conclusions of these studies, however, indicate that biochemistry of the light production in *Chaetopterus* sp. is different from the ones currently known for other luminous organisms, as summarized by Shimomura (2006*a*).

Here, we studied the luminous mucus secreted from the worm and revised and compared its optical and physicochem-

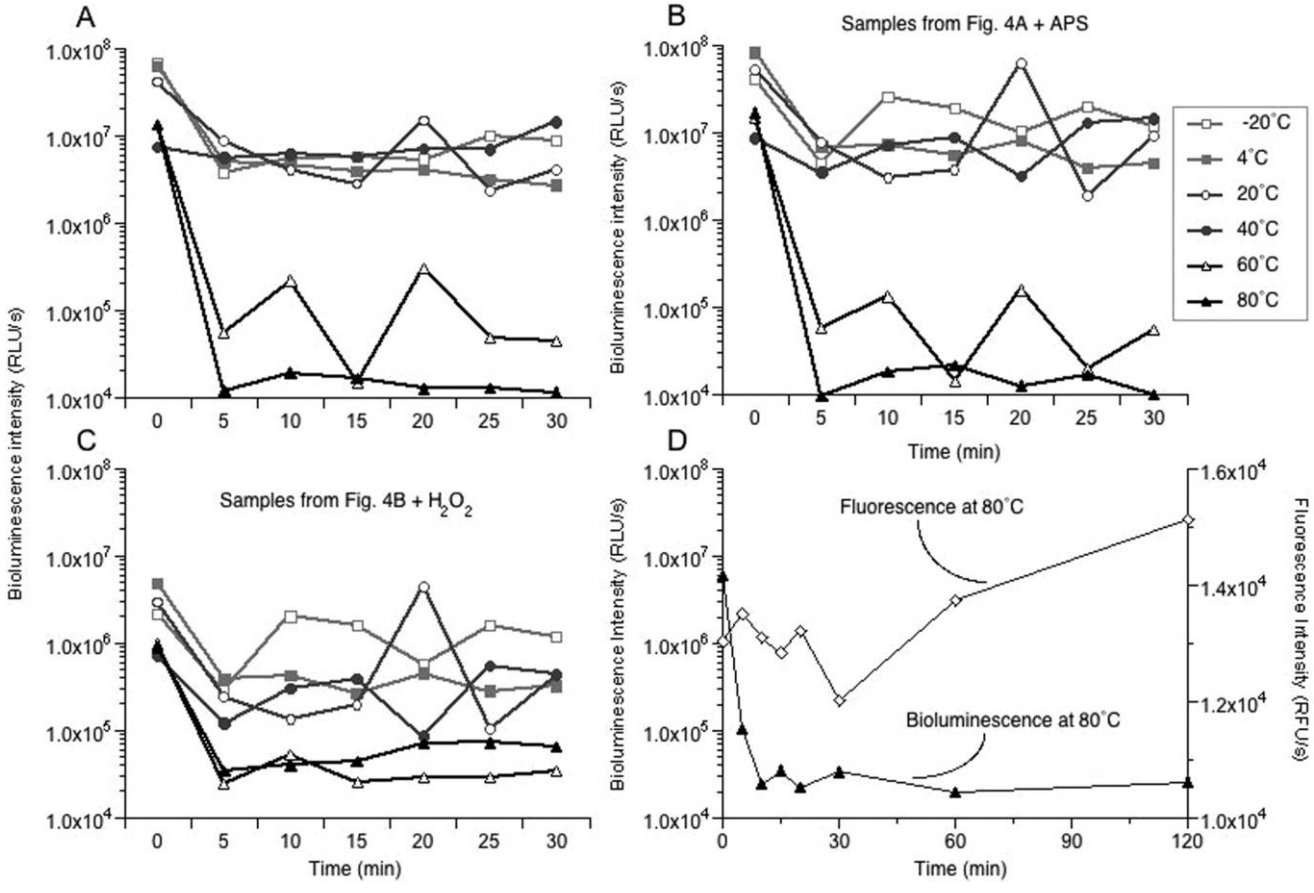

Figure 4. Effect of temperature over time on mucus spontaneous light production (*A*) subsequently followed by the addition of ammonium persulfate (APS; *B*) and hydrogen peroxide (*C*). *D*, Variation over time of spontaneous bioluminescence and fluorescence from 80°C-treated mucus. These effects are representatives from a total of 15 replicates, some completed with different time points. RLU, relative light unit; RFU, relative fluorescence unit.

ical properties to previous works that examined the bioluminescence of purified compounds from full worm tissues. Hence, we provide an alternative route of sample processing that may lead to complementary data for easier identification and purification of the photoprotein and other biochemical components involved in the control of the light production in *Chaetopterus* sp.

Light Production and Mucus Mechanical Response Are Governed by Distinct Compounds

Processes leading to the light production still remain poorly understood in *Chaetopterus* sp. Anatomical and morphological analysis of light-producing versus non-light-producing epidermis identified some cells as putative photocytes, also referred to as the eosinophilic light-producing cells (Nicol 1952*b*). The photocytes exude luminescent material in the external medium under nervous stimulation through a cascade of cellular mechanisms that remain unknown (Anctil 1979). Other types of cells surround the photocytes; these cells also appear able to secrete material, which is likely to be the sticky and thick mucus that surrounds the worm once mechanically stimulated. Mucus and bioluminescence, however, show an association. Indeed, when collecting mucus from different body areas, no mucus has been found that is not luminescent, and luminescence appears to always be associated with the production of mucus, on the basis of our experience and reference to the literature. In addition, the density of mucus appears to affect the level of light production (the denser the mucus, the less light produced), which was experimentally demonstrated by decreasing the mucus bioluminescence upon increasing pressure on the mucus (Sie et al. 1958) but also by triggering a burst of light production upon addition of seawater (Johnson 1959).

These experimental observations coincide well with anecdotal observations during collection of the tube worms in the field and from the tube worms maintained in the aquarium. In either case, luminous mucus was observed secreted at first as a dense and sticky material from the worm inside the tube, while its secreted bioluminescence seemed to increase in intensity when diluted into the surrounding seawater (to a certain point, after which it disappears), which also seems to coincide

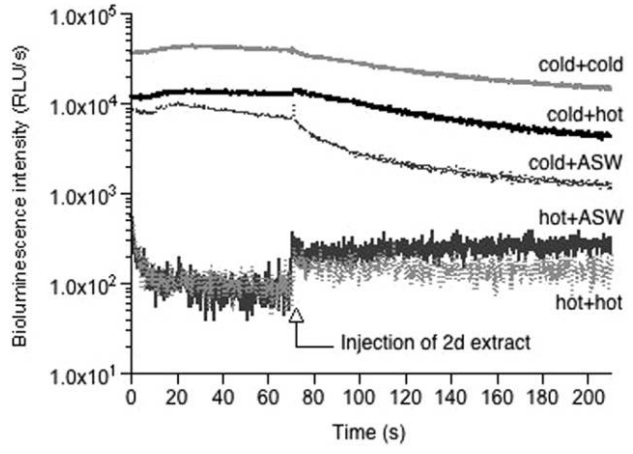

Figure 5. Representative results from the hot/cold mucus extracts recombination experiment, with artificial seawater (ASW) as a control (from $N = 6$ replicates). Note the difference among the cold samples, especially after addition of their respective treatment; the ASW control shows a dilution effect that is more pronounced than when the ASW contains material from mucus material. The hot extracts showed much less spontaneous light, since they have been denatured; hence, there was little difference between adding ASW or hot extract back to them. RLU, relative light unit.

with the loss of stickiness and density of the mucus. One could then consider that the light production of the mucus is associated with the breakdown of the mucus while expanding in the surrounding environment over time. However, it is still not clear at this stage whether the compounds involved in light production are also the ones constitutive of the mucus.

Here we showed that the mechanical properties of *Chaetopterus* sp. mucus are similar to those of most other forms of animal mucus, which show shear thinning behavior with slopes of log viscosity versus log shear rate close to −1 (Lai et al. 2009). The shear thinning in yield-stress elastic gels is typically associated with the breakup of mucin (and related proteins) fibers and other structures whose connections are entanglements or other reversible linkages.

From earlier studies and observations described in the literature from decades ago, one might consider that the light production of the mucus is associated with the breakdown of the mucus over time, as it dilutes off in the surrounding water. Interestingly, here we found that the rheological behavior of the *Chaetopterus* mucus was largely independent of the amount of light it was producing and unaffected by most of the perturbations employed to influence the physical entanglements. Indeed, whether the mucus was bright or dim to start with, or whether it was filtered, diluted, or used freshly or later upon secretion, it exhibited a similar viscoelastic response, thus independent of its ability to produce light at the time of testing for bioluminescence. Thus, our results are not consistent with the consideration that the light production of the mucus is associated with the breakdown of the mucus, and the compounds involved in the light production and making the mucus seem to be of two independent sets involving two relatively independent biochemical processes.

However, the addition of hydrogen peroxide to the mucus rapidly suppressed the light production, which is unusual for light-producing systems (as discussed further below). Moreover, hydrogen peroxide concomitantly increased the overall viscosity of the mucus, which is in contrast to the effect described for higher organisms for which hydrogen peroxide can be used to break down and liquefy mucus (Brownlee et al. 2007; Pillai et al. 2012). In *Chaetopterus*, one possible physical mechanism is that the hydrogen peroxide promotes chemical cross-linking of the mucus components, leading to an enhancement of the interchain interactions and thus increase of viscosity uniformly across a wide range of shear rate; this is also consistent with the behavior when the pressure is increased, which also leads to an inhibition of light production (Sie et al. 1958). It is clear that the mucus and the bioluminescence reaction are intimately linked, and one possibility is that the mucus acts as a structural and/or physical barrier that can trap the luminous compounds and prevent them from dissolving too fast into the surrounding medium.

Fluorescence Analysis Indicates That Chlorophyll Derivative Chaetopterin Compound Is Abundant in the Luminous Mucus

We showed that all body parts of *Chaetopterus* sp. produce a mucus that is bioluminescent in the blue range (455 nm), which accordingly corresponds to its maximum light absorbance. The anterior part of the worm, however, is the most interesting in the sense that it secretes mucus with intense and long-lasting light production while containing significantly less protein than the other body parts. Working only with mucus secreted from the main body section thus allowed us to specifically target our characterization to biochemical reactions mainly involved in the light production. The mucus also produces fluorescence peaking in the blue, similar to bioluminescence, yet also has two peaks in the red (670 and 725 nm), which is indicative of the chlorophyll-related compound called chaetopterin, usually found in the guts of the worm (Kennedy and Nicol 1959). Concentrated chaetopterin shows absorbance related to chlorophyll, with peaks at 410–450, 500–540, and 610–670 nm (French et al. 1956; Kennedy and Nicol 1959), which were not observed here likely because they are masked by the absorbance properties of other components of the mucus; yet chaetopterin shows intense fluorescence in the red with the same spectrum as chlorophyll (Lankester 1897), which was also observed here. However, the systematic occurrence of chaetopterin in the secreted luminous mucus of all body parts (as observed in this study in fluorescence) suggests that chaetopterin might be involved in the light production process directly or indirectly, possibly being a precursor or derivative of the chromophore involved in the bioluminescence process. Such a biochemical relationship remains to be clearly established.

The fluorescence of fresh and untreated mucus was always found here to peak at 455 nm (blue). However, recent inves-

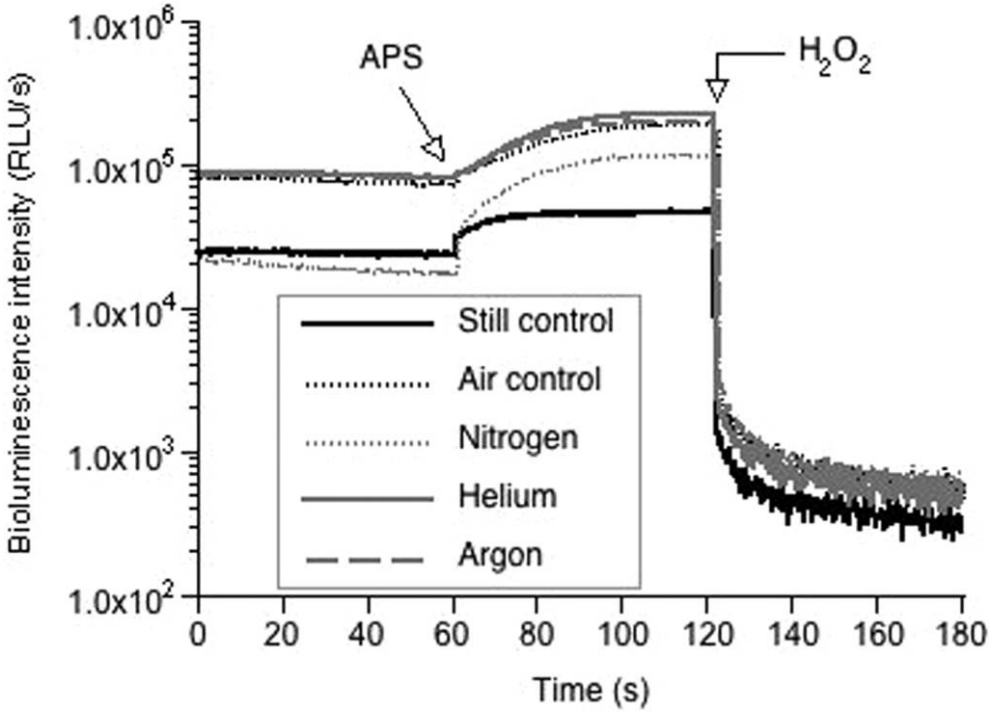

Figure 6. Effect of oxygen depletion by gas bubbling (with still and air bubbling controls) on bioluminescence produced spontaneously first and then following the addition of ammonium persulfate (APS) and hydrogen peroxide (H_2O_2). Representative records from $N = 4$ replicates. RLU, relative light unit.

tigation on chemically treated or spent mucus over time showed that the fluorescence can shift to greater wavelengths, with a peak emission at 525 nm (green; Branchini et al. 2013). Such color corresponds to the green observed in fluorescence from entire worms (see fig. 1), although the green color might also appear more pronounced than the blue because of the emission filter barrier cutoff. Nonetheless, the green fluorescence seems to be related to a riboflavin-like compound found in the mucus but also in various body parts of the worm (Branchini et al. 2013). This is to be expected, considering that riboflavin is ubiquitous and that riboflavin-related compounds can be involved in many biological processes, including bioluminescence in a diversity of luminous organisms (McCapra 1990; Uyakul et al. 1990; Bassot and Nicolas 1995; Mager and Tu 1995; Kanakubo et al. 2005). Hence, further research will focus on characterizing the fluorescence of riboflavin and chaetopterin and on identifying any possible respective and/or interactive role(s) these compounds might have with the biochemical reaction leading to blue bioluminescence in *Chaetopterus*.

A Photoprotein System with Light Production Affected by the Mucus

Our data showed that mucus secreted by *Chaetopterus* sp. is still able to produce spontaneous and chemically induced light in anoxic conditions, indicative that the biochemical system leading to light production is independent of the presence of molecular oxygen (Shimomura 1985, 2006*b*). This was confirmed by the fact that mixing hot and cold extracts of the mucus did not lead to an increase in light production, thus validating earlier statements in the literature (Nicol 1952*b*; Johnson 1959; Shimomura and Johnson 1966). In contrast to past studies indicating that ferrous iron and H_2O_2 are required stimulatory cofactors to light production in *Chaetopterus* (Shimomura and Johnson 1966), this work showed that adding ferrous iron has a limited positive effect on light production, while adding H_2O_2 always has a strong inhibitory effect that is proportional to the H_2O_2 concentration. This inhibitory effect is not destructive, since light production can be restored by the addition of iron and APS to the mucus and thus would rather indicate the presence of an H_2O_2-sensitive factor or process in the mucus that in turn inhibits the light production (which could be related to the strong effect of H_2O_2 on the rheology of the mucus, as discussed earlier). Such a scenario is also supported by the fact that at low concentrations (1%–10%), H_2O_2 seems to stimulate light production for a couple of seconds first (see fig. 7*A*) before being rapidly inhibited afterward by what would be an H_2O_2-sensitive inhibitor.

In any case, the discrepancy of our data with previous studies might be related to the fact that the work has been completed with different body parts (worm tissues vs. secreted mucus in this study) and different sample types (partially purified tissue homogenates vs. raw mucus in this study) and thus at different

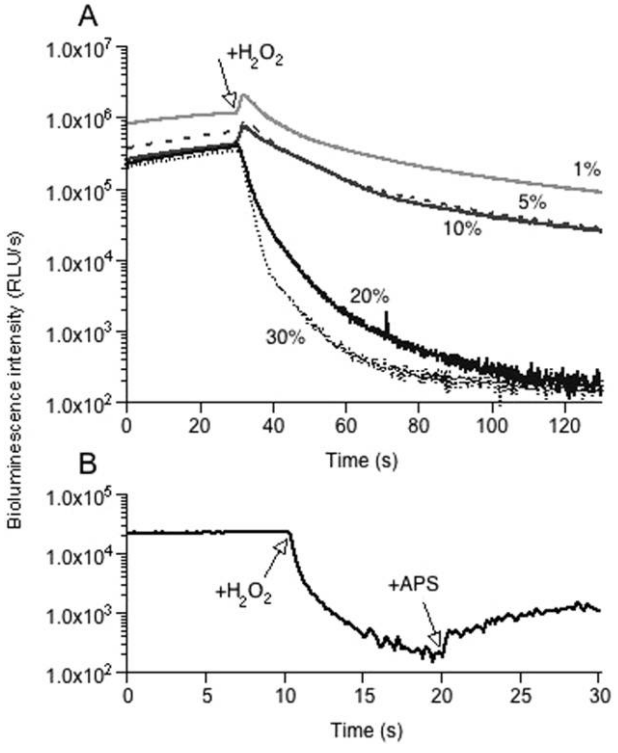

Figure 7. Representative records (from $N = 9$ replicates) showing the effect of increasing H_2O_2 concentration on the kinetics of spontaneous light production from mucus (*A*), which was followed by the addition of ammonium persulfate (APS; 20 mM) for the highest concentration of H_2O_2 only (*B*). Note that at low concentration (1%–10%), the H_2O_2 first triggers light momentarily before the H_2O_2-driven inhibitor factor starts to have an effect on the light production. RLU, relative light unit.

relative concentrations between the treatment solution and the bioluminescent system. Nonetheless, the inhibitory effect of H_2O_2 is very unusual for luminous organisms that are usually stimulated to produce light upon exposure to H_2O_2 and thus oxidation of the chromophore. There is the possibility that the H_2O_2 does not act directly on the photoprotein but rather acts on the mucus, which in turns inhibits the light production process through a physicochemical transformation. Indeed, adding ferrous iron to H_2O_2-treated mucus still induces some light production, indicating that the process by which light is produced is still functional under heavy H_2O_2 concentration. This effect thus seems to reflect the fact that the H_2O_2-related level of light in the mucus results from the balance of the rate of reaction between the light production compounds (in particular, the chromophore) and the inhibitory peroxide and/or the stimulatory cofactor, ferrous iron. Similarly, oxidation of the chromophore with other oxidizers (such as ammonium persulfate) was able to trigger some light production as well, even after H_2O_2 treatment. The inhibitory effect of H_2O_2 on the light production appears associated with rheological changes induced in the mucus. Testing this scenario of the mucus controlling the level of light produced would require having the ability to separate the bioluminescent component from the mucus. One would then expect the isolated bioluminescent component to be able to produce light upon oxidation with H_2O_2. However, such separation has not yet been observed from natural samples or achieved from experimental manipulation.

Chromophore of the Photoprotein Is Active after High-Temperature Treatment

We found that high temperatures (60°–80°C) negatively affected spontaneous and chemically induced light production from the mucus while lower temperatures (−20° to 40°C) did not, which is typical of photoprotein systems (Shimomura 1985, 2006*b*). The light production in *Chaetopterus* was reported fully degraded at 40°C in the past (Shimomura and Johnson 1966), and the discrepancy with this study might reflect differences in methodology, notably in using purified fraction from tissue homogenates (Shimomura and Johnson 1966) versus secreted mucus (this study). Hence, the temperature data presented here are similar to the ones of the luminous mucus secreted from another marine polychaete, *Odontosyllis phosphorea* (Deheyn and Latz 2009). For *Chaetopterus* sp., we showed here that H_2O_2 always rapidly induced a strong inhibition that remained persistent in the mucus for at least 2 h. The treatments at lower temperatures (−20° to 40°C) all showed similar extent of inhibition by H_2O_2 (compare light intensity of −20° to 40°C curves in fig. 4*B* and fig. 4*C*). The only difference was observed for higher temperatures, where the H_2O_2 treatment actually induced an increase in light production; this trend appeared greater at 80°C than at 60°C (compare 60° and 80°C curves in fig. 4*C*, especially after 15 min). Thus, under our scenario of interaction between the bioluminescence and the mucus ("A Photoprotein System with Light Production Affected by the Mucus"), these temperature data suggest that the thickening or cross-linking of the mucus triggered by H_2O_2 can be com-

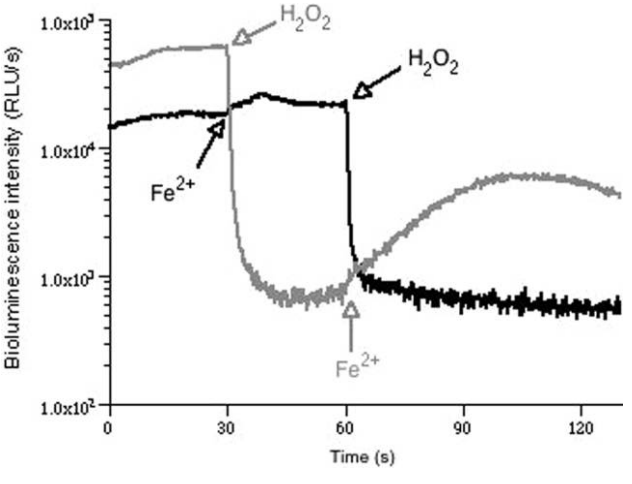

Figure 8. Representative data (from $N = 12$ replicates) showing the consecutive effect of iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) on spontaneous light production of mucus when injected in alternate order. RLU, relative light unit.

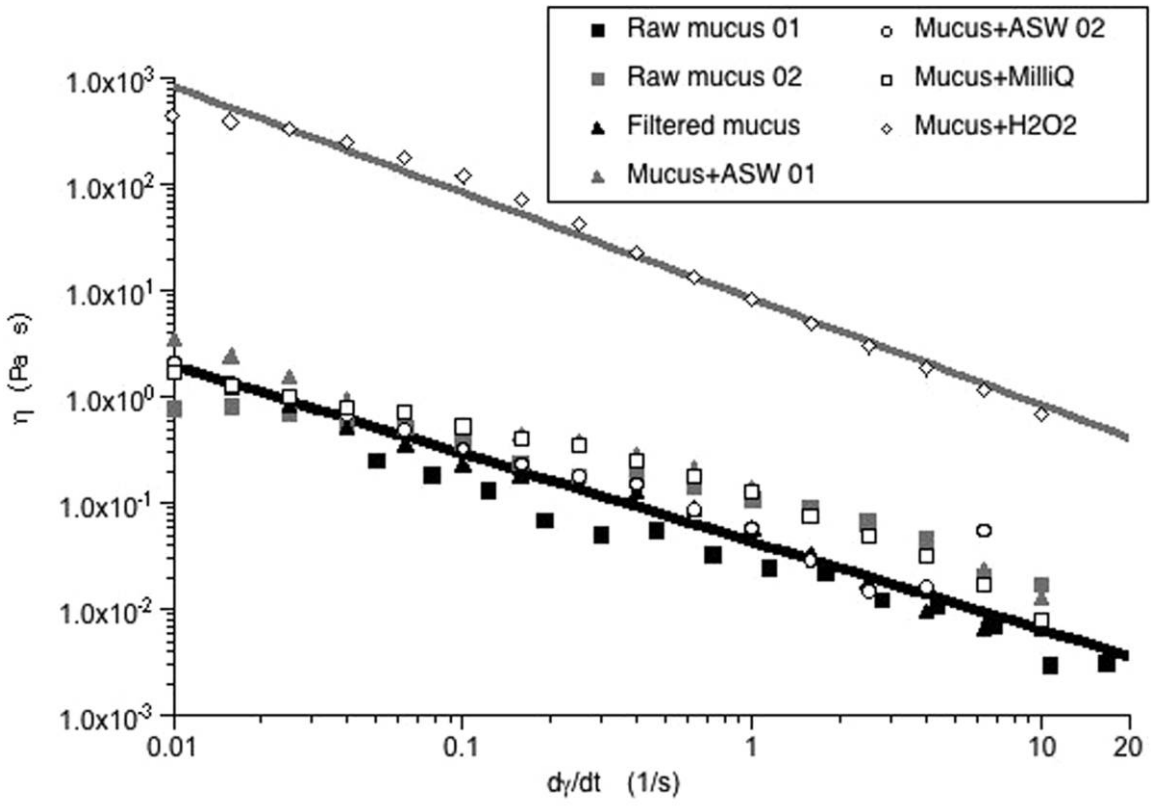

Figure 9. Change in viscosity with increasing shear rate in mucus when untreated (raw), filtered, diluted (v : v) with artificial seawater (ASW) or MilliQ water, and treated (v : v) with 30% hydrogen peroxide (H_2O_2). A total of $N = 3$ replicates were conducted per treatment yet sometimes using a different range of shear rates.

promised by high temperature. Accordingly, while the spontaneous luminescence still remains low for the high-temperature treatment over time, the fluorescence of the mucus increases, suggesting that denaturation of the apoprotein allows the biochromophore to be optically interactive and producing fluorescence under blue light (fig. 4*D*). These data suggest that high-temperature treatment of the mucus might help isolate the chromophore from the surrounding components of the mucus by using its preserved fluorescence as a screening tool.

Learning from the Natural Variability of the Light Production in Chaetopterus

There was variability from one individual worm to the other in terms of the amount of mucus produced, its viscosity, and its levels of luminescence intensity, which was probably dependent on the season the worms were collected from the field, the size and gender of the worm, and their stage in life history (as reported by earlier authors; Enders 1909; Nicol 1954; Johnson 1959; Shimomura and Johnson 1968). Each worm tested produced luminous mucus, but the research presented here did not consider the weaker ones ($<10^4$ RLU s^{-1}), only the ones with intensity between 10^5 and 10^7 RLU $^{-1}$. Within that group, data remained similar across individuals, yet despite showing the same trend in the effects induced by experimental conditions, the induced effects could have significant variation in their extent; for example, an inhibitory effect could range from 80% to 100% inhibition, relative to the initial values of spontaneous light. Such variability in the extent of an effect, although always remaining either inhibitory or stimulatory, might reflect the fact that the mucus is a complex mixture of various components (yet to be identified), with relative abundance affected by various natural factors (e.g., from life-history stages to diet). Further studies on the nature of the biochemical reactions occurring in the mucus and associated (or not) with the light production will help understand the factors controlling and affecting variability of the bioluminescence in this organism.

Conclusion

This study investigated the optical and physicochemical properties of the luminous mucus secreted by the marine worm *Chaetopterus* sp. Data show that the mucus contains a photoprotein emitting blue light. The light emission is associated with the rheological properties of the mucus, yet the compounds involved in the bioluminescence reaction do not appear to be the same ones giving the mucus its rheological properties.

Polymerization and thickening of the mucus upon exposure to H_2O_2 seem to be the only processes affecting and suppressing the light emission. High-temperature treatment appeared to have the potential to preserve the mucus capacity to produce fluorescence, thus offering an avenue to further isolate and characterize the chromophore in this organism.

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