



Biochemical characterization of diverse deep-sea anthozoan bioluminescence systems

Manabu Bessho-Uehara^{1,2,3} · Warren R. Francis⁴ · Steven H. D. Haddock¹

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Abstract

Bioluminescence, light produced by living organisms, is a common trait in the ocean. In benthic ecosystems in the deep-sea, octocorals are some of the most abundant luminous animals. Among luminous sessile organisms, the shallow-water sea pansy *Renilla* has been well studied for its chemistry and molecular biology. Aside from *Renilla*, however, little is known about the bioluminescent mechanisms of other anthozoans, especially deep-sea corals. In this study, we investigated the characteristics of bioluminescence in deep-sea anthozoans. The bioluminescent capabilities of *Heteropolypus*, *Kophobelemnon*, *Paragorgia*, and a hormathiid anemone are newly described. Coelenterazine, a substrate for bioluminescent reactions, was detected in extracts from octocorals. Coelenterazine-dependent luciferase activity was found in all the anthozoans. Moreover, immunoreactivity against *Renilla* luciferase was detected in protein extracts from the families Isididae, Alcyoniidae, Umbellulidae, Funiculinidae, Kophobelemnidae and Protoptilidae, suggesting that all luminous octocorals may share a common biochemical mechanism, which utilizes coelenterazine and *Renilla*-type luciferase. Our results support the hypothesis that the last common ancestor of all the octocorals was bioluminescent, and that bioluminescence evolved a minimum of six times in Cnidaria. Future studies with robust phylogenies, wide taxon sampling, and comparative transcriptome analyses could reveal the patterns of evolution of bioluminescence in octocorals and anthozoans. Our study provides fundamental observations of deep-sea corals and experimental evidence of their coelenterazine-dependent luciferase systems.

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✉ Steven H. D. Haddock
haddock@mbari.org

- ¹ Monterey Bay Aquarium Research Institute, Moss Landing, CA, USA
- ² Institute for Advanced Research, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan
- ³ Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan
- ⁴ Department of Biology, University of Southern Denmark, Odense, Denmark

Introduction

Class Anthozoa is a diverse group of cnidarians containing 7500 species, including sea anemones and corals. They are found throughout marine environments, and provide structure that harbors biodiversity (Bellwood and Hughes 2001; Daly et al. 2007). Optical and structural variability of coral reefs provides ecological niches resulting in the evolution of morphologically diversified animals as represented in the Great Barrier Reef. In the deep sea, octocorals (predominantly pennatulaceans and gorgonians such as bamboo and primnoid corals) and stony corals (Scleractinia), also contribute to the diversity in their environments (Roberts et al. 2006; Watling et al. 2011).

Bioluminescence, light emission from living organisms, was noted in sea pens in an early published account by Boussuet (1558) (as cited in Williams 1999), subsequently by Pratje (1923) and Harvey (1952), and more recently documented in Anthozoa by Herring (1987), Williams (2001, 2003), and Johnson et al. (2012). The luminous anthozoan species are taxonomically sparsely reported among two subclasses, four orders, and 17 families

according to the classification of WoRMS (Cordeiro et al. 2019). Order Actiniaria and Zoantharia in subclass Hexacorallia include four luminous genera: *Epizoanthus*, *Savalia* (formerly *Gerardia*), *Parazoanthus*, and an unidentified Hormathiidae sea anemone. Order Alcyonacea in subclass Octocorallia includes ten luminous genera; *Acanthogorgia*, *Anthomastus*, *Eleutherobia*, *Chrysogorgia*, *Iridogorgia*, *Isidella*, *Keratoisis*, *Lepidisis*, *Primnoisis*, and *Thouarella*. Order Pennatulacea in subclass Octocorallia includes 16 luminous genera: *Funiculina*, *Halipteris*, *Pennatula*, *Pteroeides*, *Ptilosarcus*, *Distichoptilum*, *Protoptilum*, *Renilla*, *Stachyptilum*, *Umbellula*, *Cavernularia*, *Veretillum*, *Actinoptilum*, *Acanthoptilum*, *Stylatula*, and *Virgularia*. In the time since Herring (1987) listed luminous Alcyonacea, some species have been reclassified to different genera. For example, *Anthomastus ritteri* has been reclassified as *Heteropolypus ritteri* (Molodtsova 2013), and it remains unclear which species of the parent family might be bioluminescent. Recent phylogenetic analyses suggest polyphyly of Alcyonacea (Quattrini et al. 2018), but we still do not have a reliable phylogeny including a rich enough set of luminous species to fully evaluate the origins of bioluminescence in this lineage. Hence, in this study, we follow the classification presently accepted by WoRMS (marinespecies.org).

A typical chemical reaction producing bioluminescence involves an enzyme (generally called a luciferase), a substrate (luciferin), and oxygen with or without cofactors (Haddock et al. 2010). The shallow-water sea pansy *Renilla reniformis* (Pennatulacea: Renillidae) is the most biochemically studied anthozoan. The *Renilla* luciferase acts upon coelenterazine, a luciferin which is used among a variety of luminous marine animals (Inoue et al. 1977), in the presence of dissolved oxygen to emit blue light (maximum wavelength = 480 nm) without other cofactors (Matthews et al. 1977; Lorenz et al. 1991). Other associated molecules such as a green fluorescent protein (GFP), a coelenterazine binding protein (CBP), and coelenterazine sulfate have been identified and characterized in *Renilla* (Inoue et al. 1977; Ward and Cormier 1979; Kumar et al. 1990; Inouye 2007). The homologous genes encoding the luciferase, GFP, and CBP have also been also identified from the shallow-water sea cactus *Cavernularia obesa* (Vertillidae) (Ogoh 2012; Ogoh et al. 2013). Coelenterazine-dependent luciferase activity was also reported in other Pennatulacea (Cormier et al. 1973). However, little is known about the biochemistry of bioluminescence in the other anthozoans, especially deep-sea species.

In this study, we examined the bioluminescence of deep-sea anthozoans and found one hexacoral and three octocorals to be bioluminescent for the first time. All the anthozoans tested were positive for possessing coelenterazine and coelenterazine-dependent luciferases. Moreover,

immunoreactivity against *Renilla* luciferase was detected in Alcyonacea corals. These results suggest that extant Octocorallia species share a homologous bioluminescent mechanism.

Materials and methods

Samples

Animals were collected along the coast of California using the remotely operated vehicle (ROV) Doc Ricketts operated from RV Western Flyer from the Monterey Bay Aquarium Research Institute (MBARI) between 2018 and 2019 under California Fish and Wildlife Permit SC-4029 and Specific Use Permit S-191140006-19114-001 to SHDH. *Lepidisis* sp. (Fig. 1a, b), *Isidella tentaculum* (Fig. 2a–c), *Heteropolypus ritteri* (Fig. 3a, b), *Kophobelemnion* sp. (Fig. 4a, b), *Paragorgia arborea* (Fig. 5a), *Umbellula* sp. (Fig. 6a, b), *Distichoptilum gracile* (Fig. 7a), *Funiculina* sp. (Fig. 8a), *Pennatula* sp. (Fig. 9a), and a Hormathiid (Fig. 10a) were sampled by the ROV. For some samples, specimens were held by the robotic arm of the ROV and agitated in situ during collection to stimulate bioluminescence, which could be recorded by a low-light sensitive color camera. Once aboard the ship, collected specimens were kept in the dark at 4 °C until in vivo bioluminescent spectral measurements and photography, followed by being frozen in liquid nitrogen. Specimens were morphologically identified by Steven Haddock, Kyra Schlining, or Lonny Lundsten, and subsamples were frozen for DNA barcoding. The specific depths of the animals collected are described in Table 1.

Luciferase extraction and assay

Luciferase activity was measured using coelenterazine (Prolume Ltd., Pinetop, AZ, USA) in a Tecan Infinite 200 plater reader (TECAN, Männedorf, Switzerland) running i-control software. To extract luciferase, frozen tissues (100–400 mg) were homogenized in 2 mL of 200-mM Tris–HCl, 50-mM EDTA; pH 7.6. The homogenate was centrifuged at 15,000 × g at 4 °C for 10 min. The buffer in the supernatant was replaced by 20-mM Tris–HCl, 5-mM EDTA; pH 7.6 using PD-10 desalting columns (GE Healthcare, Chicago, IL, USA). The 50 µL of protein extracts were placed in the wells of 96-well plate and the light-emitting reaction was assayed by injection of 50 µL of 1-µM coelenterazine. As non-luciferase protein control, 107 µg/mL of bovine serum albumin (BSA) was used.

To characterize luciferase activity, we tested two pH conditions and three common cation species. The crude luciferase extracts were assayed with sodium chloride, calcium chloride, or magnesium chloride at pH 7.5 or 8.6. Fifty

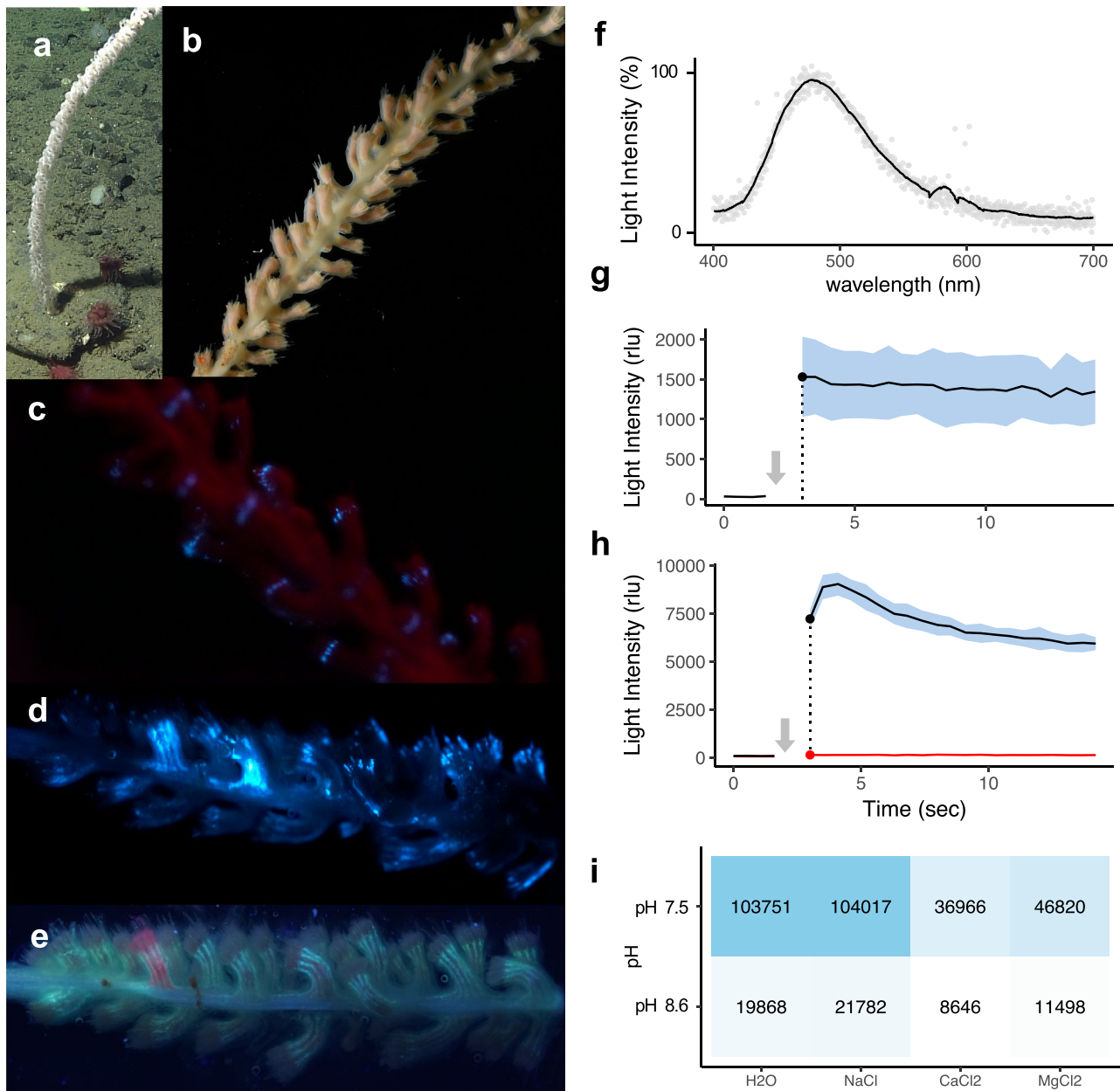


Fig. 1 *Lepidisis* sp. **a** Frame grab of ROV dive at 1288 m. **b** The specimen in the lab under white light. **c** Bioluminescence stimulated mechanically under red light. ISO 51200, F/2.8, exposure time 1/10 s. **d** Bioluminescence stimulated with KCl. ISO 16000, F/2.8, exposure time 0.625 s. **e** Fluorescence under UV light. **f** Bioluminescence spectrum smoothed by Savitzky–Golay method. **g** Coelenterazine

assay. The luciferin extract was mixed with 1 ng/μL *Renilla* luciferase at $t=2$ s (arrow). Standard deviation (\pm SD) is shown as a ribbon graph. **h** Luciferase assay. The protein extract was mixed with 1 μM coelenterazine at $t=2$ s (arrow). Coelenterazine added to BSA (red) is used as negative control. **i** Characterization of luciferase. Values are measured in relative light unit in the luminometer (rlu)

microliters of protein extracts were mixed with 10 μL of 0.5-M salt in 0.5-M Tris–HCl buffer at pH 7.5 or 8.6 in the well of 96-well plate, and the reaction was initiated by injection of 40 μL of 1-μM coelenterazine.

Luciferin extraction and assay

The coelenterazine content in the animal was quantified by comparison with native coelenterazine using commercially available recombinant *Renilla* luciferase (Prolume Ltd) in the luminometer. The frozen tissues (40 mg–150 mg) were homogenized in 200 μL of 99.5% EtOH. The homogenate

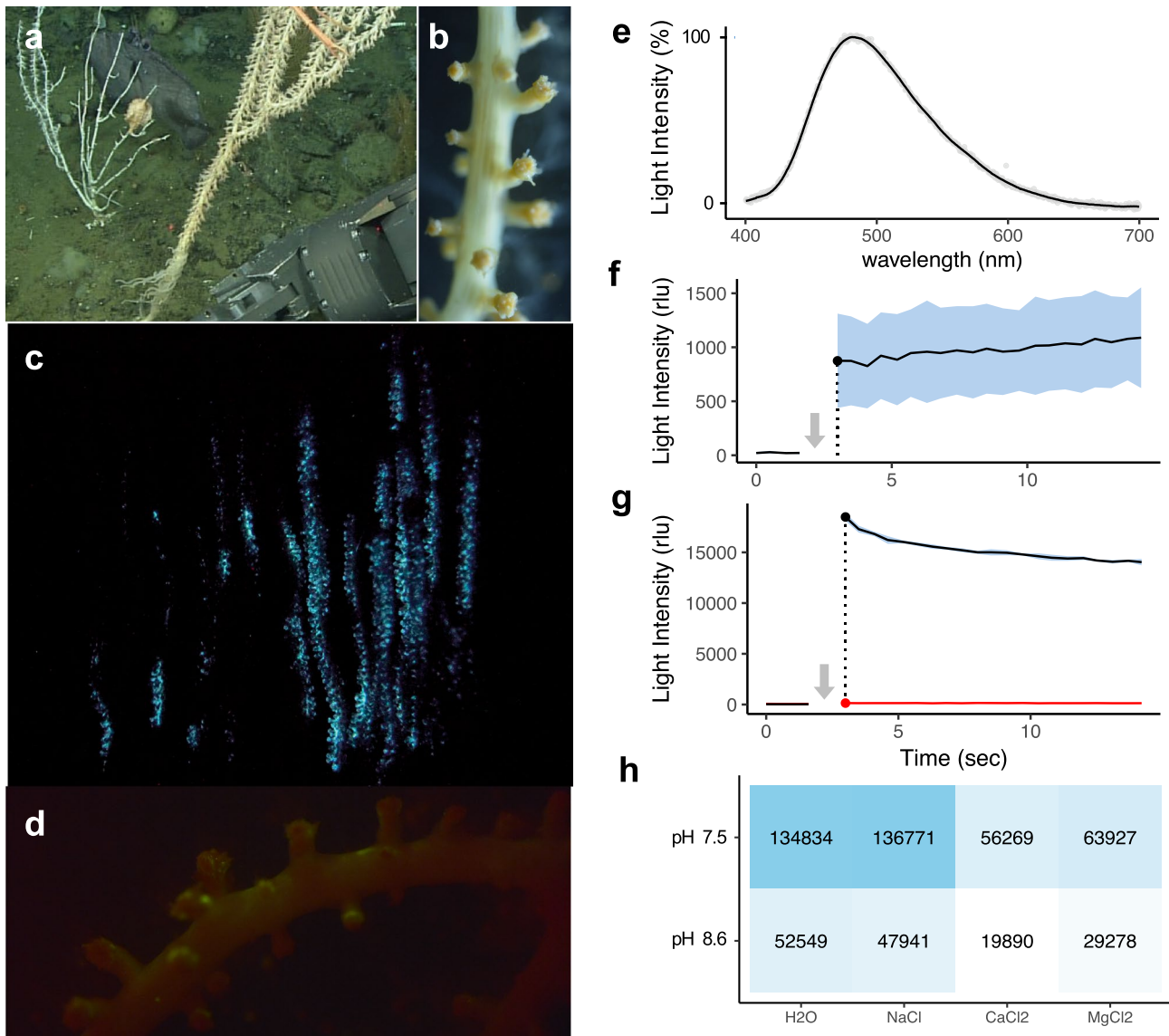


Fig. 2 *Isidella tentaculum*. **a** Frame grab of ROV dive at 1224 m. **b** The specimen in the lab under white light. **c** Bioluminescence stimulated mechanically. Frame grab of low-light camera on the ROV. **d**

Fluorescence under blue light through the yellow filter. **e** Bioluminescence spectrum. **f** Coelenterazine assay. **g** Luciferase assay. **h** The effects of pH and ions

was centrifuged at $15,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min. The supernatant was filtered using a $0.45\text{-}\mu\text{m}$ membrane filter (MilliporeSigma, Burlington, MA, USA). Ten microliters of the filtered luciferin extract were diluted with $90\text{ }\mu\text{L}$ of water and placed in a well of a 96-well plate. The luminescent reaction was initiated by injecting $100\text{ }\mu\text{L}$ of $100\text{-pg}/\mu\text{L}$ *Renilla* luciferase in the 20-mM Tris–HCl, 5-mM EDTA, pH 7.6.

Western blotting

Immunoreactivity was tested using anti-*Renilla* luciferase antibody to understand if the specimen possesses a luciferase homologous to *Renilla* luciferase. Protein samples

were reduced with SDS sample buffer containing dithiothreitol (62.5-mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 w/v % bromophenol blue, 100-mM dithiothreitol at the final concentration) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Novex 10–20% polyacrylamide gradient gel (Thermo Scientific, Waltham, MA). The separated protein in the gel was blotted onto a PVDF membrane using XCell II blot Module (Thermo Scientific) at 42 V for 90 min. The blotted membrane was treated with Pierce Western Blot Signal Enhancer (Thermo Scientific) followed by blocking with 5% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature and subsequently incubated

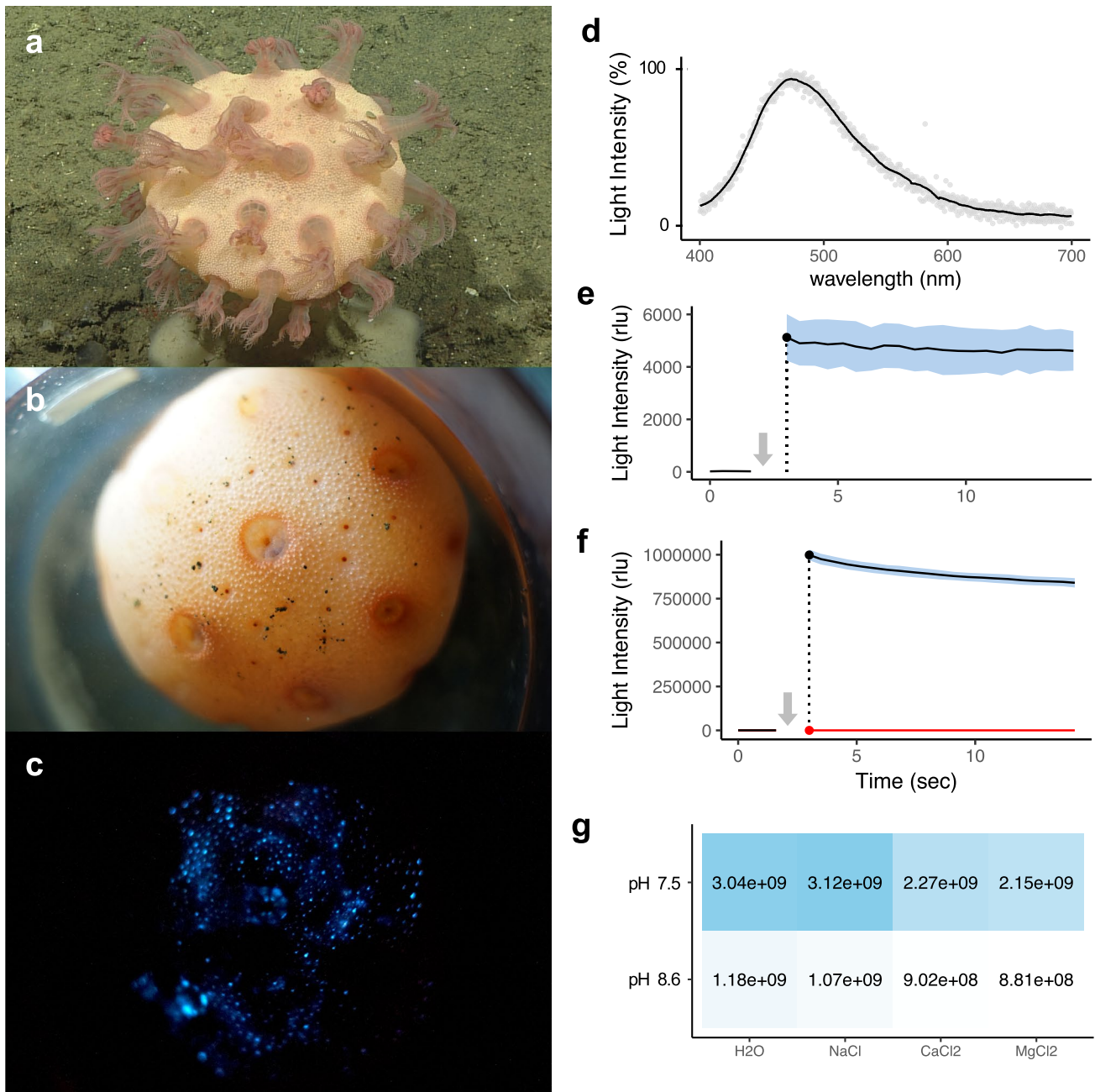


Fig. 3 *Heteropolypus ritteri*. **a** Frame grab of ROV dive at 1031 m. **b** The specimen in the lab under white light. **c** The bioluminescence stimulated with KCl in the same frame of **b**. ISO 25600, F/2.8, expo-

sure time 10 s. **d** In vivo bioluminescence spectra. **e** Coelenterazine assay. **f** Luciferase assay. **g** The effects of pH and ions

with 1.14 $\mu\text{g/mL}$ of anti-*Renilla* luciferase rabbit antibody (Invitrogen, catalog number PA5-32,210) for 16 h at 4 °C. The blot was washed three times with TBS-T for 10 min each at room temperature. Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Promega) was incubated with the blot for 1 h at room temperature, and the blot was washed three times with TBS-T. Immunoreactivity was visualized by chemiluminescence using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo

Scientific). SuperSignal Molecular Weight Protein Ladder (Thermo Scientific) was used as protein standard ladder.

Spectra measurement

In vivo bioluminescent spectra were measured using an Ocean Optics QE65000 spectrometer (CCD detector, 300–800 nm range at ~ 0.4 nm/pixel) (Ocean Optics, Largo, FL) with attached quartz fiber optic. The

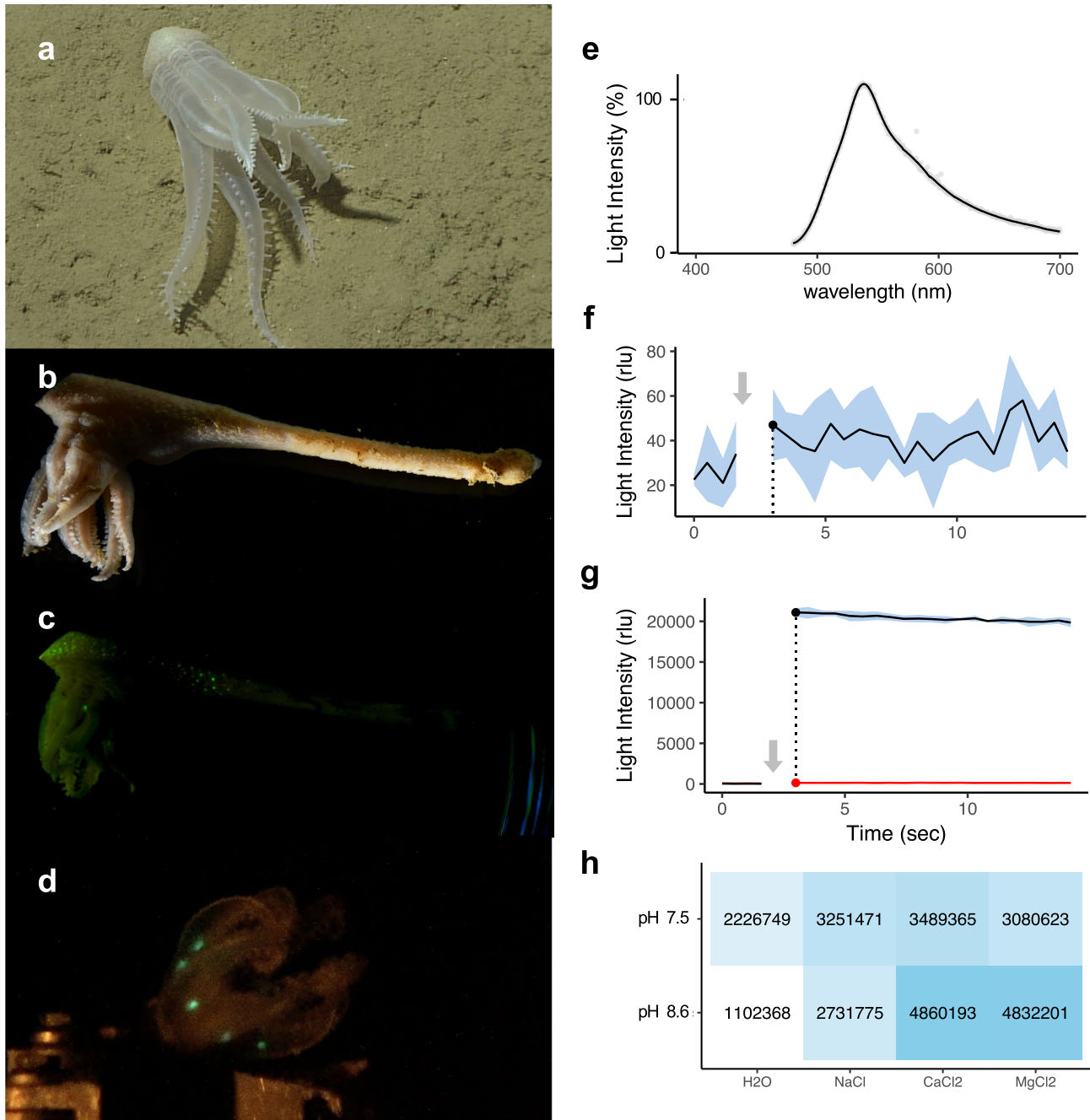


Fig. 4 *Kophobelemnion* sp. **a** frame grab of ROV dive at 3980 m. **b** The specimen in the lab under white light. **c** The fluorescence under blue light with yellow filter. **d** *In situ* bioluminescence taken by the camera on ROV. The green light is emitted from the spots between

tentacles. **e** Fluorescence spectra. **f** Coelenterazine assay. **g** Luciferase assay. **h** The effect of pH and ions. Note the more basic condition has higher emission only in the presence of divalent cations

specimens were stimulated mechanically (gently touched with a pipet) or chemically (KCl or CaCl₂) for bioluminescence. To collect the spectra data, we used the program brizzy (<https://github.com/conchoecia/brizzy>) modified from the associated Ocean Optics SeaBreeze API. The *in vitro* bioluminescent spectra were measured

from 500 μL of protein extract mixed with 1 μL of 1-mM coelenterazine. *In vivo* fluorescent spectra were measured under blue excitation through a yellow blue-blocking filter. The obtained spectra were smoothed in R software using a Savitzky–Golay filter with parameters: filter order = 3, filter length = 61.

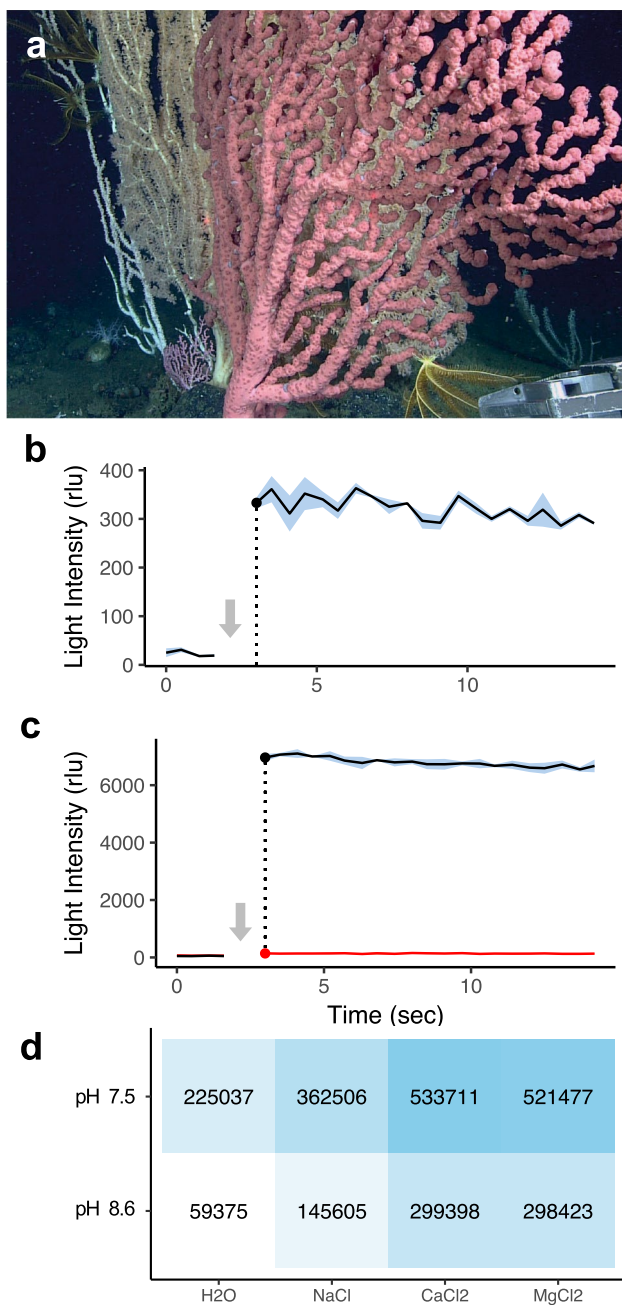


Fig. 5 *Paragorgia arborea*. **a** Frame grab of ROV dive at 1171 m. **b** Coelenterazine assay. **c** Luciferase assay. **d** The effects of pH and ions

DNA extraction and sequencing

The nucleotide sequences of the specimens were sequenced and deposited in GenBank (Table 1) as DNA reference barcodes. DNA was extracted from 40 to 60 mg of frozen sample using QIAamp blood and tissue extraction kit (QIAGEN, Hilden, Germany). The nuclear gene ribosomal RNA 28S for octocorals was amplified by polymerase chain reaction (PCR) with forward primer, LSUD1F 5'-ACCCGCTGA

ATTTAAGCATA-3'; and reverse primer, D3AR 5'-ACG AACGATTTGCACGTCAG-3' (Scholin et al. 1994). The mitochondrial gene cytochrome oxidase subunit III (COIII) for hormathiid anemone was amplified by PCR with forward primer, COIIIF 5'-CATTTAGTTGATCCTAGGCCCTTGACC-3'; and reverse primer, COIIIR 5'-CAAACCACATCTACAAAATGCCAATATC-3' (Geller and Walton 2001). The isolated gene fragments were sequenced by capillary sequencer 3500xL Genetic Analyzer Sanger (Applied Biosystems, Foster City, CA, USA). The sequences were deposited in NCBI and the BOLD System. The accession numbers for GenBank are listed in Table 1.

Homology search for luciferase and phylogenetic analysis

Luciferase homologs were sought by BLAST searches against publicly available genome and transcriptome data. For all searches, *Renilla reniformis* luciferase was used as the query sequence (SwissProt accession: P27652). Genomic data of *Renilla muelleri* (Jiang et al. 2019) and *Dendronephthya gigantea* (Jeon et al. 2019) were downloaded from the websites indicated in their respective papers. Transcriptome data were used from *Gorgonia ventalina* (Burge et al. 2013), *Leptogorgia sarmentosa* (Romiguier et al. 2014), *Corallium rubrum* (Pratlong et al. 2015), *Heliopora coerulea* (Hongo et al. 2017), *Tubipora musica*, *Sinularia cruciata*, *Pinnigorgia flava* (Conci et al. 2019), *Anthomastus* sp., Cerianthid sp., Keratoisid sp., *Scleronephthya* sp. (Zapata et al. 2015), and *Pennatulula rubra* (Simion et al. 2017). Other homologs were taken from previous analyses of this protein family by Delroisse et al. (2017).

Sequences were aligned with MAFFT v7.313 (Katoh and Standley 2013), using the LINSI options (equivalent to—maxiterate 1000—localpair). Phylogenetic tree was generated with IQTREE v1.6.12 (Nguyen et al. 2015), with 1000 fast bootstrap replicates (-bb 1000) and protein model LG with 6 rate categories (LG + R6).

Results and discussion

Lepidisis sp.

The luminescence of *Lepidisis* sp. (Fig. 1a, b) was evoked by agitation using the ROV manipulator. The blue-light emission propagated as a wave (Electronic Supplementary Material Video). The light intensity was especially strong on the polyps, but the whole body including along the stem is capable of light emission. In the lab, when one of the polyps of the specimen was stimulated, blue flashes were emitted from the tips of polyps and the light was distributed as a wave, from the point disturbed to the

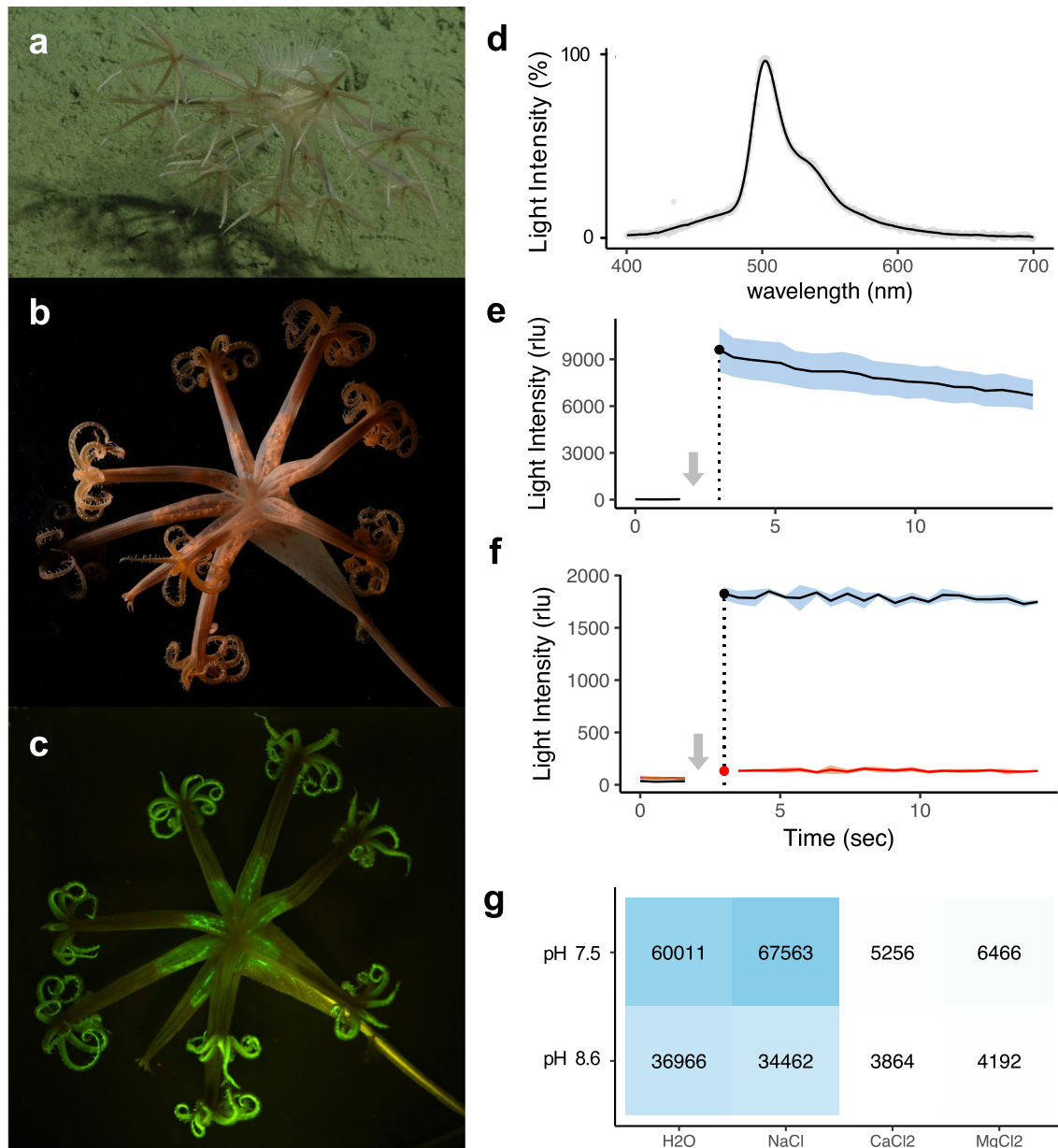


Fig. 6 *Umbellula* sp. **a** Frame grab of ROV dive at 3226 m. **b** The specimen in the lab under white light. **c** The fluorescence under blue light through a yellow filter. **d** Bioluminescence spectrum, with char-

acteristic GFP shape. **e** Coelenterazine assay. **f** Luciferase assay. **g** The effects of pH and ions

tip of the next polyp (Fig. 1c, Electronic Supplementary Material Video). This observation was consistent with that of *Lepidisis olapa* by Muzik (1978). Chemical stimulation using KCl made the whole specimen glow continuously for a few minutes without its natural kinetics (Fig. 1d). The specimen had green fluorescence localized along the stem and in polyps under UV excitation (wavelength 365 nm) (Fig. 1e). Interestingly, a few polyps emitted pink fluorescence. The peak wavelength of the bioluminescent spectrum was at 477 nm (Fig. 1f), corresponding to the light

of *Lepidisis* sp. collected from Bahamian Islands (Johnsen et al., 2012).

Ethanol extract was positive for coelenterazine when assayed with *Renilla* luciferase (Fig. 1g). Light was produced upon the injection of the coelenterazine-specific luciferase, indicating the presence of coelenterazine. The assay for luciferase activity in crude protein extract was positive upon injection with 1- μ M coelenterazine (Fig. 1h). Light was produced upon the injection of the coelenterazine, suggesting the presence of coelenterazine-dependent luciferase. Upon injection by coelenterazine, light intensity of the

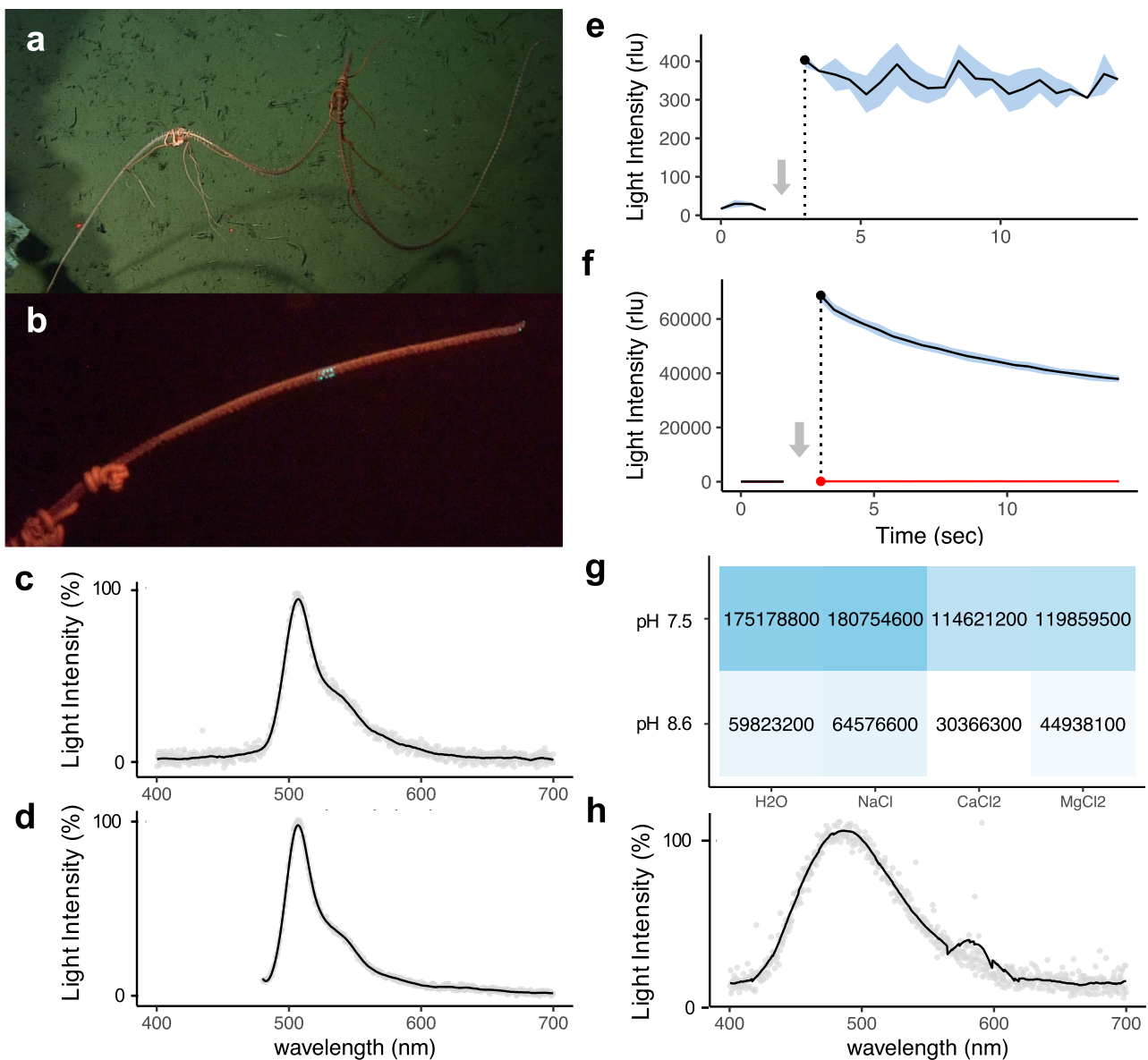


Fig. 7 *Distichoptilum gracile*. **a** Frame grab of ROV dive at 1945 m. **b** The specimen in the lab under white light. **c** In vivo bioluminescence spectrum. **d** The fluorescence under blue light through a yellow

filter. **e** Coelenterazine assay. **f** Luciferase assay. **g** The effects of pH and ions. **h** In vitro bioluminescent spectrum

luciferase sample rose significantly higher than the response of bovine serum albumin used as a negative control protein. The luciferase activity was higher at pH 7.5 than pH 8.6. The luciferase activity was sensitive to divalent cations of Ca^{2+} and Mg^{2+} at 50 mM (Fig. 1i). These results suggest that the bioluminescence of *Lepidisis* is produced by coelenterazine and a coelenterazine-dependent luciferase system.

Isidella tentaculum

The bamboo coral *Isidella tentaculum* (Fig. 2a, b) emitted bright blue light upon mechanical stimulation by the ROV

manipulator or by hand (Fig. 2c). The specimen secreted luminous mucus upon strong stimulation (Electronic Supplementary Material Video). Etnoyer reported that *I. tentaculum* emits green–blue light when the specimen was agitated by the ROV manipulator (Etnoyer 2008). Weak green fluorescence was observed under blue light through a yellow filter, though it did not clearly correspond to the color of bioluminescence (Fig. 2d). The peak wavelength of the bioluminescent spectrum was 483 nm (Fig. 1e), close to the 480 nm peak observed in the Atlantic *Isidella* sp. (Johnsen et al. 2012).

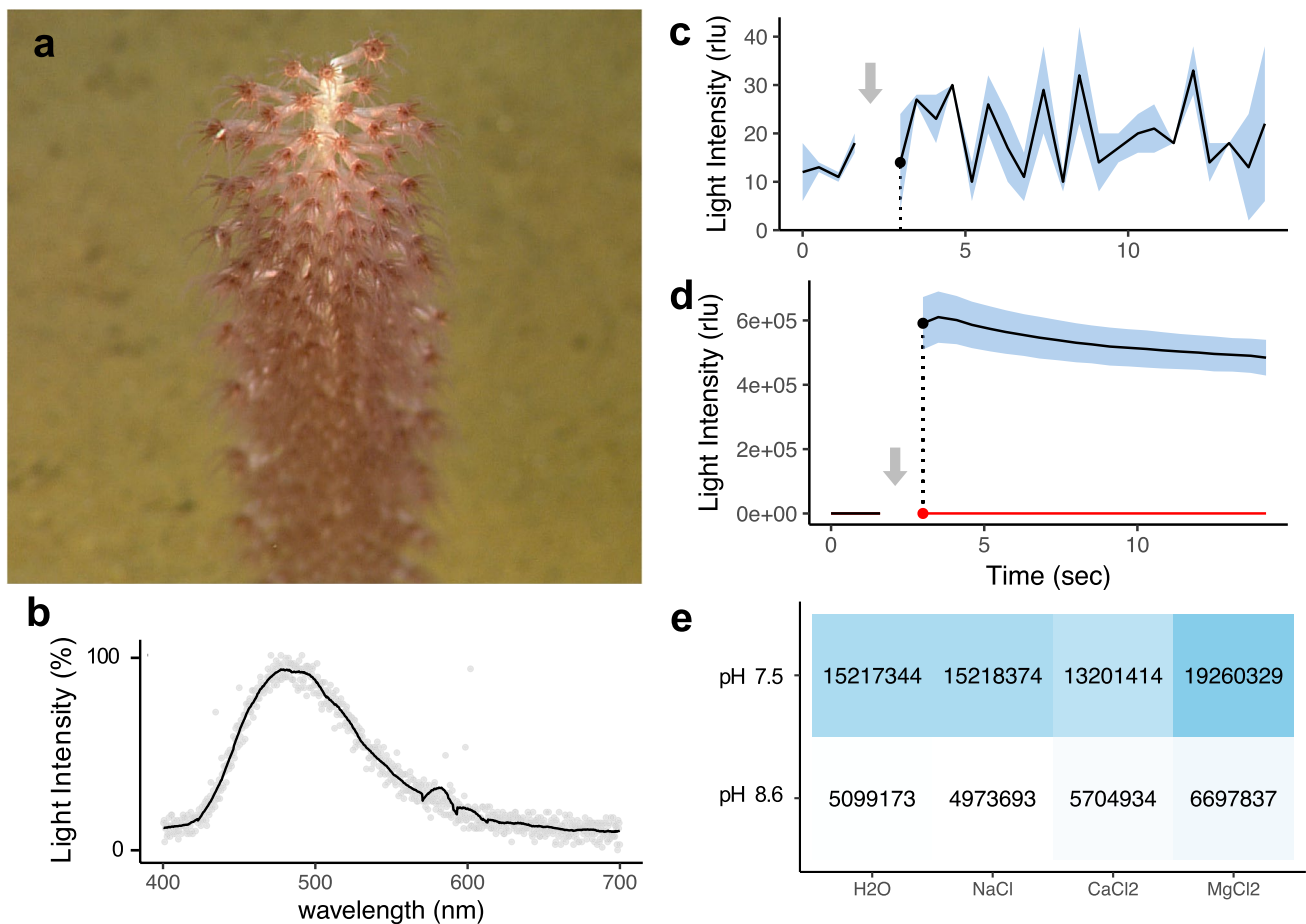


Fig. 8 *Funiculina* sp. **a** Frame grab of ROV dive at 879 m. **b** Bioluminescence spectrum. **c** Coelenterazine assay. **d** Luciferase assay. **e** The effects of pH and ions

Ethanol extract was positive for coelenterazine (Fig. 2g). The luciferase assay was positive, with the crude protein extract emitting light upon addition of coelenterazine (Fig. 2h). The luciferase activity was higher at pH 7.5 than pH 8.6. Luciferase activity was sensitive to Ca^{2+} and Mg^{2+} at 50 mM (Fig. 2i).

Heteropolypus ritteri

The mushroom coral *Heteropolypus ritteri* (Fig. 3a, b) extends polyps in situ but those polyps were retracted in the lab on the ship. Blue light was emitted from the autozooids and siphonozooids but not mesozooids when the specimen was stimulated with KCl (Fig. 3c). The light emission was slow and dim though nonetheless visible by eye. This is the first report of bioluminescence in genus *Heteropolypus* although this genus was recently reclassified from *Anthomastus* which is known to bioluminescence (Molodtsova 2013; Herring 1987). The specimen did not show fluorescence

under either UV or blue light. The peak wavelength of the bioluminescent spectrum was at 476 nm (Fig. 3d).

The coelenterazine assay (Fig. 3e) and the assay for luciferase activity (Fig. 3f) were both positive. The peak wavelength of the in vitro luminescent spectrum was 475 nm, almost identical to the bioluminescent spectrum. The luciferase activity was higher at pH 7.5 than pH 8.6 (Fig. 3g).

Kophobelemnon sp.

The specimen of *Kophobelemnon* sp. was collected at 3980 m depth (Fig. 4a), which is practically the deepest depth that ROV *Doc Ricketts* can access. Green fluorescence was expressed at the basis between tentacles and were distributed at the siphonozooid under blue light through a yellow filter (Fig. 4b, c). During in situ observations, the specimen emitted green light from the photophores located between tentacles (Fig. 4d) when the specimen was agitated by the manipulator of the ROV (Electronic Supplementary

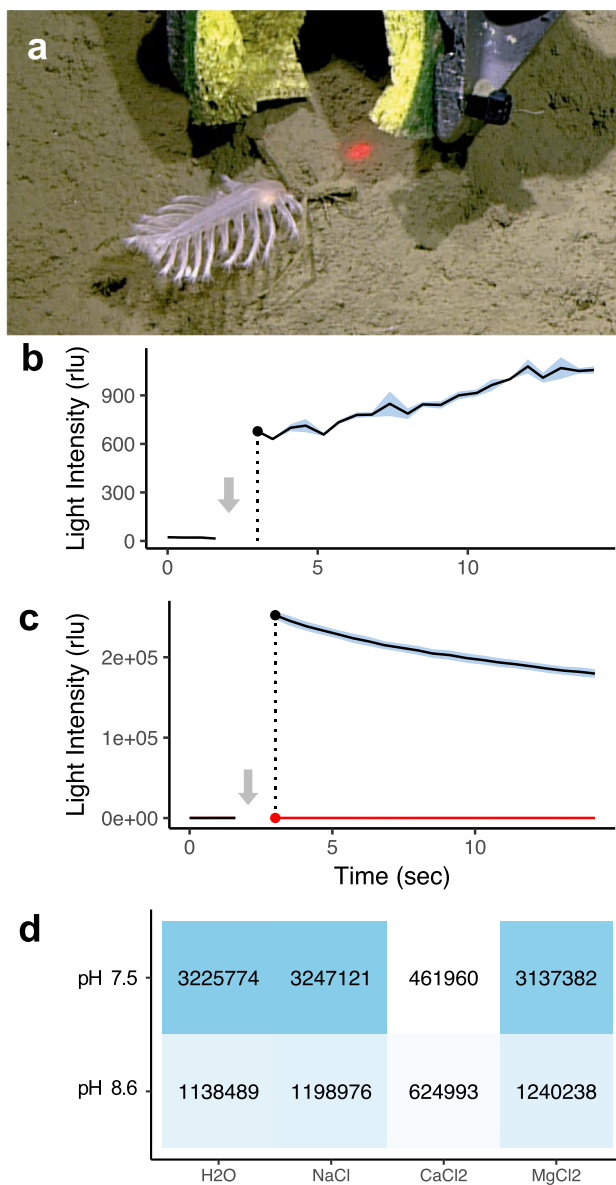


Fig. 9 *Pennatulid* sp. **a** Frame grab of ROV dive at 3457 m. **b** Bioluminescence spectrum. **c** Coelenterazine assay. **d** Luciferase assay. **e** Effect of pH and ions

Material Video). This is the first report of bioluminescence in genus *Kophobelemnon*. The peak wavelength of the fluorescent spectrum was 538 nm (Fig. 4e). If this green fluorescence is produced by the GFP family, as suggested by the shape of the spectrum, then *Kophobelemnon* sp. possesses the deepest GFP yet found in the ocean.

The presence of coelenterazine was not supported by the coelenterazine assay (Fig. 4f). The luciferase assay was positive using crude protein extract (Fig. 4g). Luciferase activity was detected from rod tissue in which fluorescent siphonozooids are distributed but bioluminescence was not

observed from this tissue in situ or in vivo. The luciferase activity was high at pH 8.6 with 50-mM CaCl₂ or MgCl₂ (Fig. 4h). Interestingly, the luciferase activity in the pH 8.6 buffer was lower than that in pH 7.5 although the relationship of pH on the activity was opposite when the calcium or magnesium cations were present.

Paragorgia arborea

The bubblegum coral *Paragorgia arborea* (Fig. 5a) emitted blue light upon mechanical stimulation by the ROV manipulator (Fig. 5b). This is the first report of bioluminescence in suborder Scleraxonia.

The coelenterazine assay (Fig. 5c) and the assay for luciferase activity (Fig. 5d) were both positive. The luciferase activity was higher at pH 7.5 than pH 8.6 (Fig. 5e). Divalent cations such as calcium and magnesium cations positively affect luciferase activity.

Umbellula sp.

The *Umbellula* sp. (Fig. 6a, b) showed green fluorescence on the tentacles of polyps and basal of polyps on the stalk (Fig. 6c). Green bioluminescent light was also emitted from the polyps upon mechanical stimulation by the ROV manipulator. The peak wavelength of the bioluminescent spectrum was at 502 nm with a long-wavelength shoulder, characteristic of the presence of GFP (Fig. 6e), and corresponded closely with the measured fluorescence peak of 505 nm. These values are also close to the 501-nm light measured from the stalk in *Umbellula magniflora* (Widder et al. 1983).

The coelenterazine assay (Fig. 6e) and the assay for luciferase activity (Fig. 6f) were both positive. The luciferase activity was higher at pH 7.5 than pH 8.6 (Fig. 6g). The luciferase activity was sensitive to calcium and magnesium cations.

Distichoptilum gracile

Distichoptilum gracile (Fig. 7a) emitted green light waves upon mechanical stimulation by the ROV manipulator (Fig. 7b, Electronic Supplementary Material Video). In the laboratory, touching the polyps elicited bright green light, visible by eye. The addition of a few drops of KCl triggered bright sustained bioluminescence, lasting a few minutes. A luminous mucus was released, though a green glow was also visible at the sites of fluorescence on the stalk. The peak wavelength of the bioluminescent spectrum was at 507 nm with a long-wavelength shoulder (Fig. 7c), identical to the fluorescent spectrum (Fig. 7d), and again, strongly suggestive of the presence of a green fluorescent protein.

The coelenterazine assay was positive, although the activity was very weak (Fig. 6e). The luciferase assay was

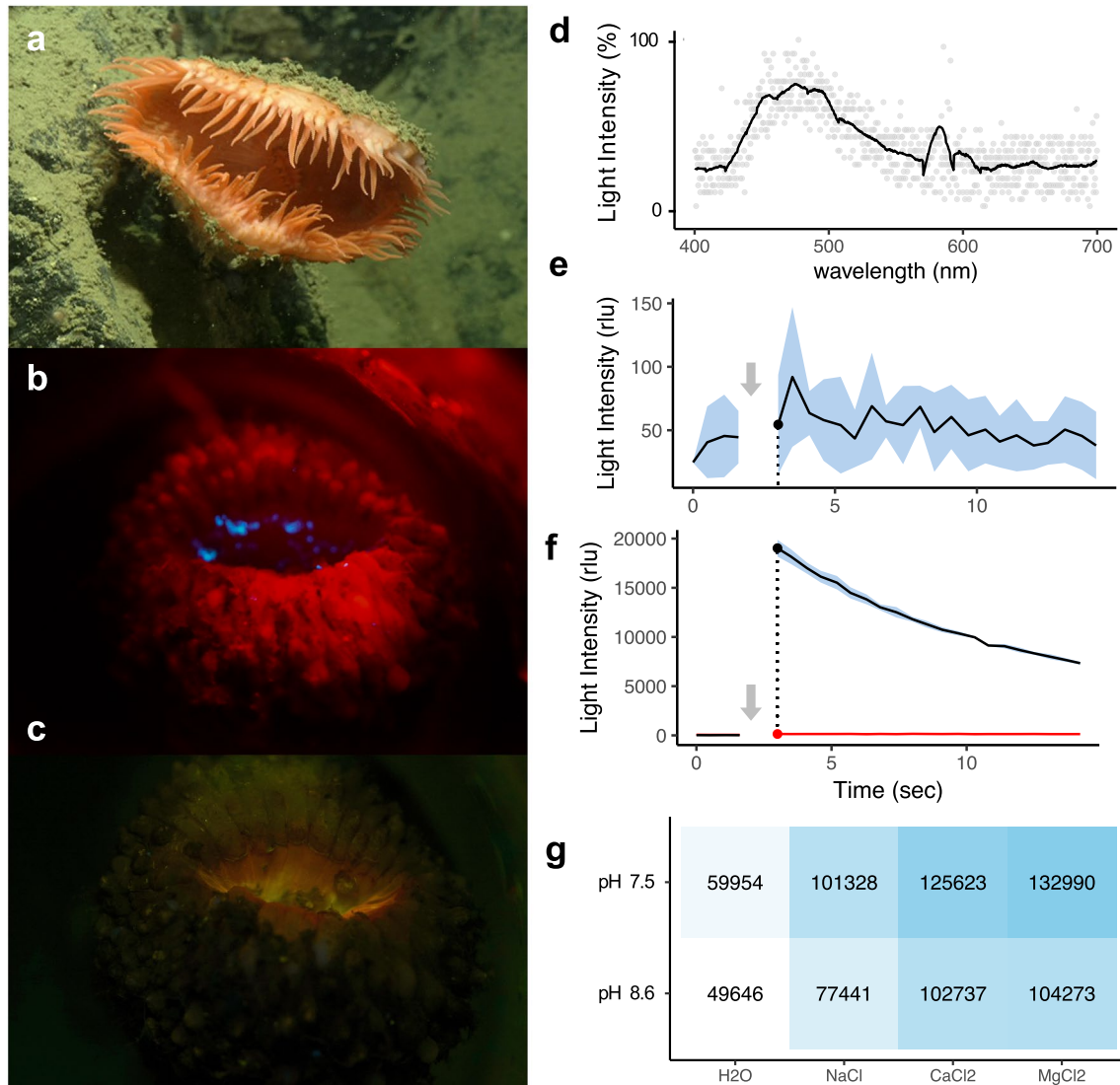


Fig. 10 Hormathiidae. **a** Frame grab of ROV dive at 1287 m. **b** Bioluminescence stimulated with KCl. ISO 25600, F/2.8, exposure time 2 s. **c** Fluorescence of oral disc excited with blue light. **d** In vivo bio-

luminescence spectra. **e** Coelenterazine assay. **f** Luciferase assay. **g** Effect of pH and ions

positive with the crude protein extract (Fig. 6f). The luciferase activity was higher at pH 7.5 than pH 8.6 (Fig. 6g). The luciferase activity was sensitive to calcium and magnesium cations. The luminescent spectrum of the in vitro luciferase assay was measured, and had a peak wavelength of 487 nm, without a shoulder (Fig. 7h). The different spectra between in vivo and in vitro luminescence is due to energy transfer (Ward and Cormier 1979). Bioluminescent resonant energy transfer (BRET) occurs when luciferase and GFP are closely co-localized, less than 100 Å apart. Under these conditions, excited energy resulting from the luciferin-luciferase reaction is transferred to excite the fluorophore in GFP, which is

well demonstrated in vitro using the aequorin system from *Aequorea* jellyfish (Morise et al. 1974). This connection is lost in in vitro assays, leading to shorter wavelength bioluminescence emission.

Funiculina sp.

Funiculina (Fig. 8a) emitted blue light upon mechanical stimulation of the stalk or polyps. Polyps rapidly contracted, but gentle squeezing could trigger light emission from the stalk. The peak wavelength of the bioluminescent spectrum was 485 nm (Fig. 8b). The addition of CaCl₂ and coelenterazine

Table 1 Taxonomy, habitat, bioluminescence spectra of coral specimens

Species	Family	Depth collected (m)	Major observation ^b (m)	Bioluminescent spectra (nm)	Accession no. ^c
<i>Lepidisis</i> sp.	Isididae	1288	1250–2440	477	MN955305
<i>Isidella tentaculum</i>	Isididae	1224	860–1230	Blue-green	MN955304
<i>Heteropolypus ritteri</i> ^a	Alcyoniidae	1037	520–1510	476	MN955303
<i>Kophobelemnion</i> sp. ^a	Kophobelemnidae	3980	2670–3970	Green	MN955300
<i>Paragorgia arborea</i> ^a	Paragorgodiidae	1243	950–1360	Blue-green	MN955306
<i>Umbellula</i> sp.	Umbellulidae	3266	3950–3970	502	MN955308
<i>Distichoptilum gracile</i>	Protoptilidae	1945	690–3070	507	MN955301
<i>Funiculina</i> sp.	Funiculinidae	879	830–1250	485	MN955302
<i>Pennatula</i> sp.	Pennatulidae	3457	3210–3210	Green	MN955307
Hormathiid	Hormathiidae	1287	1030–2710	472	MN954949

^aGenus newly described as bioluminescent in this study

^bMajor observation depth of 10 and 90 percentile frequencies of the animals found in Monterey Bay

^cNucleic 28S ribosomal gene for octocorals and mitochondrial COIII gene for the hormathiid anemone were deposited in GenBank

was sufficient to yield light from a 1-cm-long piece of the stalk.

The coelenterazine assay was negative (Fig. 8c). The luciferase assay was positive with the crude protein extract (Fig. 8d). The luciferase activity was higher at pH 7.5 than at pH 8.6 (Fig. 8e).

***Pennatula* sp.**

The sea pen *Pennatula* (Fig. 9a) emitted green bioluminescence. However, we could not determine the maximum wavelength due to weak luminescence.

The coelenterazine assay (Fig. 9b) and the assay for luciferase activity (Fig. 9c) were both positive. The luciferase activity was higher at pH 7.5 than pH 8.6 (Fig. 9d). Calcium cations negatively affected luciferase activity.

Hormathiidae

The hormathiid anemone (Fig. 10a), when mechanically stimulated using a paintbrush on the ROV arm, secreted a bright blue cloud from the mouth (Electronic Supplementary Material Video). The specimen discharged luminous mucus in the lab upon mechanical stimulation or chemical stimulation using potassium chloride (Fig. 10b). The discharge of blue luminous slime upon the mechanical stimulus has been reported from *Hormathia alba* (Tur 1993). The mouth emitted greenish-yellow fluorescence at the center with a gradation to orange fluorescence at the edge of the mouth under blue light (Fig. 10c). The peak wavelength of the bioluminescent spectrum was 472 nm (Fig. 10d).

The coelenterazine assay was negative with the ethanol extract (Fig. 10e). The luciferase assay was positive with the crude protein extract (Fig. 10f). The luciferase activity

was higher at pH 7.5 than pH 8.6 (Fig. 10g). Ion strength increased luciferase activity.

Bioluminescent behavior

All of the deep-sea anthozoans tested in this study were bioluminescent (although some corals previously found not to be bioluminescent were not examined). *Lepidisis* sp., *Isidella tentaculum*, *Heteropolypus ritteri*, *Kophobelemnion* sp., *Paragorgia arborea*, *Umbellula* sp., *Distichoptilum gracile*, *Funiculina* sp., *Pennatula* sp., and a Hormathiid anemone emitted light in situ when they were agitated by the arm of ROV or in vivo when they were mechanically and/or chemically stimulated in the laboratory.

The bioluminescence signals of *Lepidisis* and *Isidella* consist of waves of light emitted from the point of stimulation as described previously (Figs. 1a–c, 2d, Electronic Supplementary Material Video) (Muzik 1978). *Distichoptilum* also showed a wave-like flash pattern (Electronic Supplementary Material Video). Such a pattern is often observed in marine bioluminescent animals such as the shallow-water sea cactus *Cavernularia*, the sea pansy *Renilla* (Buck, 1973), the brittle star *Amphipholis* (Deheyn et al. 2000), the ctenophore *Beroe forskalii*, and deep-sea sea cucumber *Pannychia* (Herring 1995). The ecological significance of this display may be misdirection for predators, aposematic signal, or burglar alarm (Haddock et al. 2010), although there is no experimental evidence. *Isidella*, *Distichoptilum*, and the hormathiid secreted luminous clouds which contain homogenous luminous fluid and brighter particles (Electronic Supplementary Material Video). The secretion of luminous exudate in octocorals is reported here for the first time. The mucus contained luciferase activity using

coelenterazine. The sticky, luminous mucus might serve as a deterrent to predators by marking the predator, which makes the predator vulnerable to attack from higher-order predators (Mensing and Case 1992). The secretion of luminous fluid with particles is also known in ctenophores, scyphozoans, and polychaetes (Haddock and Case 1999; Herring and Widder 2004; Francis et al. 2014).

Prevalence of blue or green light

Hastings (1996) had noted a bimodal distribution in peak wavelength for pelagic and deep-sea organisms, where many species produced blue light, but others from similar habitats emitted green light. Of the species we collected, colors appeared to be related by site: *Umbellula* sp. and *Pennatula* sp. both produced green light and were collected from the same 3200-m site. However, at a relatively shallower site around 1200 m, *Isidella*, *Lepidisis*, *Heteropolypus*, and the hormathiid all produced blue light (Table 1).

This suggests selective pressure on the wavelength. In coastal habitats, green is a more common color (Hastings 1996), for two reasons (Haddock and Case 1999). Firstly, shorter wavelength light is more easily scattered by particles, so environments with a high particle load may select for producing longer wavelengths. Secondly, shallow or coastal environments may broadly have higher primary productivity, and chlorophyll of phytoplankton is likely to absorb blue light, again favoring green over blue. For the deep sea, particles may be more abundant at some sites, and may favor longer wavelengths.

The bioluminescence spectra of *Distichoptilum* and *Umbellula* have a long-wavelength shoulder that is characteristic of GFP (Ward and Cormier 1979; Ogoh et al. 2013), suggesting that these two species possess GFP to modify the emitted light by resonant energy transfer. Of the many luminous cnidarians, three groups use GFP to modulate the color of bioluminescence: octocorals (as presented here), siphonophores, and many hydromedusae (e.g., *Aequorea*, *Olindias*, *Clytia*, *Obelia*), and other non-luminous anthozoans commonly possess GFP. Given the presence of GFP in cnidarians, but also in arthropods and chordates (Shagin et al. 2004; Deheyn et al. 2007), it is likely that GFP existed in the common ancestor of Cnidarians, and which could already produce fluorescence (Shagin et al. 2004; Francis et al. 2016). This would mean that some species kept GFP for bioluminescence, use in general pigmentation (as fluorescence or chromoproteins for many hexacorals), or lost it.

Occurrence of coelenterazine

The presence of coelenterazine was determined for most of the octocorals tested in this study except *Kophobelemnon*, *Funiculina*, and the hormathiid anemone (Figs. 1g, 2f, 3e, 4f, 5b, 6e, 7e, 8c, 9b, 10e). These specimens might keep undetected luciferin in storage forms such as coelenterazine sulfate, which is found in *Renilla* and converted into coelenterazine by luciferin sulfokinase (Cormier et al. 1970). Coelenterazine content was determined by a substrate-specific enzyme assay using *Renilla reniformis* luciferase. The results were summarized in Table 2. All the values in

Table 2 Calculated content of coelenterazine and luciferase

Specimen	CTZ assay		Luciferase assay				Immunoreactivity
	Light intensity (rlu)	CTZ in tissue (fmol/mg)	Light intensity (rlu)	Tissue (wt mg)	Protein Conc (µg/mL)	Specific activity (rlu/µg)	
<i>Lepidisis</i>	2.95×10^4	28	1.73×10^5	267	175	1.98×10^4	Positive
<i>Isidella</i>	2.05×10^4	11	3.19×10^5	124	226	2.82×10^4	Positive
<i>Heteropolypus</i>	9.94×10^4	210	1.88×10^9	188	280	1.34×10^8	Positive
<i>Kophobelemnon</i>	8.72×10^2	N.D	4.28×10^5	230	454	1.89×10^4	Positive
<i>Paragorgia</i>	6.76×10^3	16	1.39×10^5	202	366	7.58×10^3	
<i>Umbellula</i>	1.67×10^5	189	3.75×10^4	173	282	2.66×10^3	Positive
<i>Distichoptilum</i>	4.24×10^3	N.D	1.02×10^8	92	214	9.50×10^6	Positive
<i>Funiculina</i>	4.19×10^2	N.D	1.17×10^7	221	430	5.45×10^5	Positive
<i>Pennatula</i>	1.80×10^4	23	4.38×10^6	209	392	2.24×10^5	
Hormathiid	1.12×10^3	N.D	1.97×10^3	214	78	5.02×10^2	
10-nM Coelenterazine	2.19×10^4						
<i>Renilla</i> Luciferase			3.18×10^5		0.001	6.37×10^9	Positive
BSA			2.72×10^3		107	5.09×10^2	

N.D. not detected

Table 2 should be considered minimum values because of the consumption of substrate during collection, in situ bioluminescence tests, and spontaneous decomposition during storage and extraction. In our experimental conditions, the minimum detection limit of coelenterazine in the extract was 3 nM. The results for which we detected small amounts of coelenterazine suggest that anthozoans possess a storage form of coelenterazine, such as coelenterazine sulfate, which has been reported in *Renilla* (Inoue et al. 1977).

Coelenterazine-dependent luciferases

The activity of coelenterazine-dependent luciferase was tested using crude protein extracts. Assays for luciferase activity using coelenterazine resulted in light production from all the anthozoans (Fig. 11a, Table 2). The values of luciferase activity are significantly different among species. For example, the specific activity of *Heteropolypus* was 50,000 times higher than that of *Umbellula*. This variation is due to not only the differences in luciferase content of the tissues but also the differences in the optimum conditions for

luciferase. For example, *Kophobelemnon* luciferase showed higher activity at pH 8.6 although most of the others showed higher activity at pH 7.5. Thus, the values of specific activity in Table 2 do not represent an absolute value to compare luciferase activity between specimens but are nonetheless useful to identify bright systems for further study.

We biochemically investigated luciferase activities in the crude protein extract and found various responses under different conditions. Divalent cations (calcium and magnesium) negatively affected the luciferase from *Lepidisis*, *Isidella*, *Umbellula*, and *Distichoptilum*. *Penatula* luciferase was selectively sensitive to calcium ion (Fig. 8d). Future studies of gene cloning, protein expression, and comparison of biochemical characters will help to explain how the amino acid sequence determines those properties, which will lead further development of new life science tools. It should be noted that we used crude protein extract that potentially contains more than two luciferase isoforms and other proteins which may affect luciferase activity.

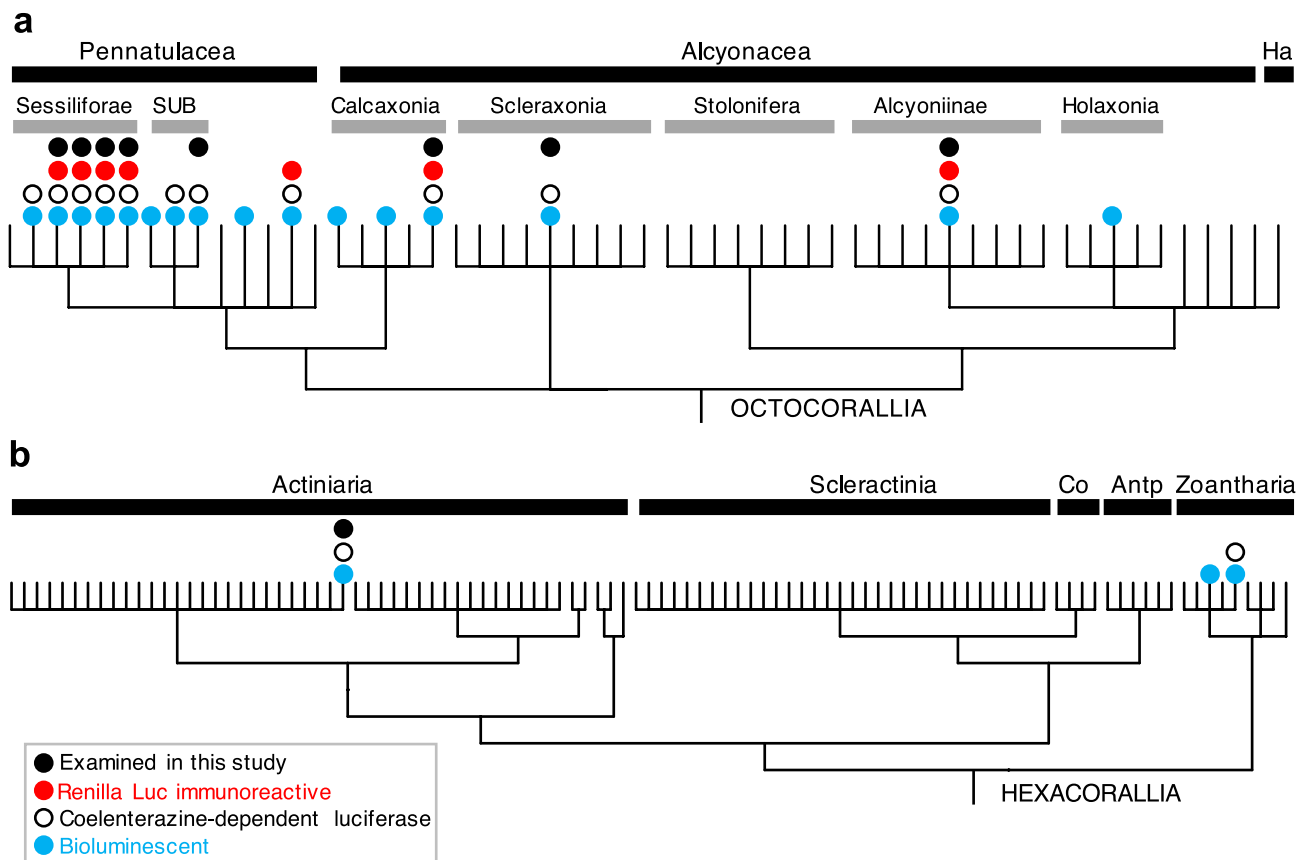


Fig. 11 Distribution of bioluminescence in **a** Octocorallia and **b** Hexacorallia. Families including bioluminescent taxa are indicated as blue filled circles. Taxonomy is assigned according to WoRMS. Phylogenetic relationship is derived from Quattrini et al. (2018). Black

bars and gray bars indicate the classification of order and suborder. *Antp* Antipatharia, *Co* Corallimorpharia, *Ha* Helioporacea, *SUB* Subsessilifora

Western blotting analysis detected positive signals against the anti-*Renilla* luciferase antibody in protein extracts from *Lepidisis*, *Heteropolypus*, *Kophobelemnon*, and *Distichoptilum* (Fig. 11a, Electronic Supplementary Material Fig. S1). The bands in the blot have similar molecular size (32–40 kDa) to those of *Renilla* luciferase (37 kDa). Those immunoreactive bands indicate the presence of a luciferase which is homologous to *Renilla* luciferase. This finding suggests that it would be feasible to identify new luciferase genes using homology searches within anthozoan transcriptomes.

Our results provide fundamental information for seeking out new reporter genes of potential use for luminescence imaging. For example, calcium-ion specific sensitivity found in *Pennatulula* luciferase could provide a calcium sensor, as alternative to aequorin, the Ca-triggered photoprotein. Cloning, protein expression, and precise characterization of pure luciferase will be needed to understand the property of each luciferase and to find new reporter genes to meet new imaging challenges.

Evolution of bioluminescence in Octocorallia

The presence of coelenterazine, coelenterazine-dependent luciferase activity, and immunoreactivity against anti-*Renilla* luciferase antibody in protein extracts from luminous octocorals including Alcyonacea (Fig. 11a) suggests that all luminous octocorals share a common biochemical mechanism for bioluminescence. It leads two major hypotheses for the evolution of bioluminescence in octocorals: (1) the common ancestor of all the luminous octocorals was bioluminescent and that bioluminescence was lost multiple times in the course of evolution, or (2) luciferase activity evolved independently multiple times from a common non-luciferase homolog through parallel evolution.

In the order Pennatulacea, luciferase activity using coelenterazine was reported from the sea pens *Renilla*, *Ptilosarcus*, *Stylatula*, and *Acanthoptilum* (Cormier et al. 1973) and the sea cactus *Cavernularia* (Ogoh 2012). The luciferase genes from *Renilla* and *Cavernularia* have been cloned, and the amino acid sequences show 57% identity (81% similarity in BLOSUM62 matrix), suggesting that the most recent common ancestor of those corals had a single origin of bioluminescence.

We sought luciferase genes in the *Renilla muelleri* genome (Jiang et al. 2019) and found two additional paralogs of luciferase. Those paralogs share 47–50% sequence identity and 72–78% similarity (BLOSUM62 matrix). The proposed catalytic triad (D120, E144, and H285 in *R. muelleri* luciferase; GenBank Accession number AAG54094) (Fanaei-Kahrani et al. 2018) was conserved in all three paralogs (Electronic Supplementary Material Fig. S2). However, two homologs were also found in the genome of

the non-luminous soft coral *Dendronephthya gigantea* (Jeon et al. 2019), and these also have the same conserved catalytic triad. Further BLAST searches against the publicly available octocoral genome and transcriptome data found genes similar to *Renilla* luciferase. Phylogenetic analysis of those genes recovered the relationship that a luciferase homolog from *Pennatulula rubra* is nested in the clade with validated luciferases of *Renilla* and *Cavernularia* (Electronic Supplementary Material Fig. S3).

Although several luciferase homologs were paraphyletic to the validated *Renilla* luciferase, experimental evidence is needed to determine whether those genes are functional as luciferases or not. Paralogous luciferase genes evolve differently regarding an enzymatic character and gene expression regulation, as shown in fireflies (Bessho-Uehara et al. 2017). There is still the possibility that the immunoreactive bands are false positives due to the presence of non-luminous homologs of *Renilla* luciferase, and that the true luciferases of Alcyonacea have a different origin than luciferases of Pennatulacea. Recent phylogenetic analyses pointed out that the order Alcyonacea is polyphyletic (McFadden et al. 2006; Quattrini et al. 2018). Keratoisidinae, including *Isidella* and *Lepidisis*, are suggested to form a clade with Pennatulacea; thus, it is considerably more plausible that those two groups share a common origin of bioluminescence.

Future studies on anthozoan phylogenetics will be needed to understand the evolution of bioluminescence within octocorals. Comparative transcriptome analysis with wide taxon sampling is desired to address those hypotheses, as we previously demonstrated in the case of independent origin of beetle bioluminescence (Fallon et al. 2018).

Luciferase in hexacorallia

Luciferase activity using coelenterazine was detected in the hormathiid anemone. The presence of coelenterazine-dependent luciferase in the hexacoral *Parazoanthus* (Fig. 11b) has previously been reported by Cormier et al. (1973). Immunoreactivity was not detected in protein extracts of the hormathiid specimen. BLAST searches against two publicly available transcriptomes from Hormathiidae anemones, *Bolocera tuediae* and *Hormathia digitata*, could not find any genes similar to *Renilla* luciferase. Another species, *Hormathia alba*, was the first reported luminous hormathiid (Tur 1993); so, *H. digitata* might be expected to be luminous as well. These results suggest that it is less likely that luciferases of those hexacorals share homology to *Renilla* luciferase, indicating an independent origin of bioluminescence origin in hexacorallia. Considering the phylogenetic distances between Hormathiidae and Parazoanthidae + Epizoanthidae (Macrocnemina: Zoantharia) (Fig. 11b), it is likely that the bioluminescence in hexacorallia evolved independently in each lineage.

There are two well-characterized bioluminescence systems in Cnidaria: *Renilla*-type among octocorals and photoproteins among Hydrozoa. There are four additional evolutionary origins of bioluminescence in other Cnidaria: two origins in Hexacorallia, the unidentified luciferase of coronate scyphozoans such as *Periphylla* (Shimomura and Flood 1998), an unidentified mechanism of *Pelagia noctiluca* (Semaestomeae: Scyphozoa), which was claimed by Morin and Hastings (1971) to be a photoprotein, though was never identified further. Thus, bioluminescence evolved at least six times in Cnidaria, but as many as 11 times, depending on the results from future studies. Surprisingly, all these animals use the identical molecule, coelenterazine, as the light-producing substrate, indicating that cnidarians provide an intriguing model group to study parallel evolution of bioluminescence. Detailed molecular characterization may be required to understand whether there are further novel mechanisms in those groups.

Conclusions

Bioluminescence in anthozoans is widely distributed, but biochemical aspects have been less studied, especially among deep-sea species. Here, we sampled deep-sea corals using a ROV down to 4000 m depth and observed bioluminescence in four genera not known to be luminous. A coelenterazine-dependent luciferase system was detected in all ten species of deep-sea octocorals and the hexacoral, and green-fluorescent protein was observed at nearly 4000 m. We report for the first time the presence of coelenterazine-dependent luciferases in Alcyonacea and Hormathiidae. Immunoreactivity against *Renilla* luciferase in Alcyonacea suggests that all luminous octocorals share the similar biochemical mechanisms. While GFP is found consistently across cnidarians, and plausibly existed in the last common ancestor of cnidarians, the different mechanisms of bioluminescence are evidence for convergent evolution of light production during the course of diversification. Future studies including robust phylogeny, functional assays of luciferases, transcriptomes and genome analyses are needed to understand the evolution of anthozoan bioluminescence.

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Data availability All data needed to evaluate the conclusions in the manuscript in the paper are present in the paper, the Electronic Supplementary Materials, and NCBI. DNA sequences are available in the NCBI GenBank repository, with accession numbers MN954949 and MN955300-MN955308. Additional data related to this paper may be requested from the authors.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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