Evidence for *de novo* Biosynthesis of the Luminous Substrate Coelenterazine in Ctenophores

Coelenterazine biosynthesis in an emerging model animal

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HIGHLIGHTS

The pathway for biosynthesis of the luciferin shared by over nine phyla is unknown

We found that luminous comb jellies can produce the luciferin coelenterazine

After 15 generations on a luciferin-free diet, ctenophores were still luminous

Culturable ctenophores enable us to study the biosynthesis of coelenterazine

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Evidence for de novo Biosynthesis of the Luminous Substrate Coelenterazine in Ctenophores

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SUMMARY

Coelenterazine is a key substrate involved in marine bioluminescence which is used for light-production by at least nine phyla. Some luminous animals, such as the hydromedusa Aequorea, lack the ability to produce coelenterazine endogenously and instead depend on dietary sources. Little is known about the source organisms or the metabolic process of coelenterazine biosynthesis. Here, we present evidence that ctenophores are both producers and suppliers of coelenterazine in marine ecosystems. Using biochemical assays and mass spectrometry analyses, we detected coelenterazine from cultured ctenophores fed with a non-luminous coelenterazine-free diet. We propose that ctenophores are an emerging model organism to study coelenterazine biosynthesis and the origins of bioluminescence.

INTRODUCTION

Bioluminescence is one of the most ubiquitous biological activities in the ocean. Although the proportion of luminous species in shallow neritic and benthic environments is small (1-2%) (Morin, 1983), luminous creatures in off-shore habitats are abundant from the surface down to the abyssal seafloor: 76% of macroscopic individuals in the water column and roughly 20-40% of organisms visible on the seafloor are capable of light emission (Johnsen et al., 2012; Martini and Haddock, 2017; Martini et al., 2019). In dim or dark environments, bioluminescence plays roles intraspecifically (communication) and interspecifically (offensively or defensively) (Haddock et al., 2010), which drove the independent evolution of bioluminescence at least 84 times across a wide range of taxa (Haddock et al., 2010; Davis et al., 2016; Verdes and Gruber, 2017; Lau and Oakley, 2020).

Despite multiple evolutionary origins of bioluminescence across phyla, the biochemical mechanisms responsible for light emission are partially shared among some groups. In general, a luminous substrate, luciferin, is catalyzed by an enzyme, luciferase, to produce light. Luciferase sequences are unique to the lineages in which they are found. This likely arose because the ancestral non-luminous protein-coding gene accumulated mutations to develop luminescent activity during the course of evolution, albeit with exceptions like kleptoproteins (Bessho-Uehara et al., 2020a). In fact, numerous non-homologous families of known luciferases have been identified and characterized (Markova and Vysotski, 2015).

In contrast to the diverse array of luciferases, luciferins are less diverse. Many marine organisms representing at least 9 phyla utilize an identical luciferin, coelenterazine: polycystine radiolaria in Retaria; Phaeodaria in Ctenophora; Hexacorallia, Octocorallia, Scyphozoa, and Medusozoa in Cnidaria; Vampyroteuthid cephalopods and Pholida clams in Cephalopoda; Ophiura in Echinodermata; Chaetognatha; Appendicularia, Myctophiformes, and Stomiformes in Chordata (Campbell and Herring, 1990; Haddock et al., 2010; Widder, 2010; Markova and Vysotski, 2015; Bessho-Uehara et al., 2020b). Some species modify coelenterazine before using it as luciferin: the firefly squid Watasenia scintillans uses coelenterazine disulfate (Inoue et al., 1976); the flying squid Sthenoteuthis oualaniensis uses a dehydrocoelenterazine as luciferin (Isobe et al., 2002). Other taxa modify luciferins for storage: the sea pansy Renilla reniformis possess luciferin enol-sulfate (Inoue et al., 1977a, 1977b); lanternfish
Myctophidae possess an enol-ether form of coelenterazine bound with glucopyranosiduronic acid (Inoue et al., 1987). The widespread occurrence of coelenterazine in marine animals is partially explained by the food web. The jellies *Aequorea victoria*, *Eutonina indicans*, and the lophogastrid shrimp *Gnathophausia ingens* are unable to produce coelenterazine and so depend on a dietary supply (Haddock et al., 2001; Frank et al., 1984). Despite the widespread occurrence of coelenterazine (Shimomura et al., 1980; Shimomura, 1987; Thomson et al., 1997; Duchatelet et al., 2019), only two animals have been shown to produce coelenterazine, the decapod shrimp *Systellaspis debilis*, and the copepod *Metridia pacifica* (Thomson et al., 1995; Oba et al., 2009a). Oba et al. (2009a) demonstrated that one molecule of phenylalanine and two molecules of tyrosine are the biosynthetic precursors to a coelenterazine molecule in *Metridia*. However, the detailed biosynthetic pathway and genes involved remain unknown, partially due to the inconvenience of those animals as experimental models.

A majority of the ctenophores are bioluminescent (Figure 1A) except for members of the family Pleurobrachiidae: *Hormiphora* and *Pleurobrachia* (Haddock and Case, 1995). Ctenophores have been proposed as candidate coelenterazine producers based on reports of bioluminescence at early developmental stages (Freeman and Reynolds, 1973). However, the possibility of maternal loading is not excluded from those studies. In this study, we provide evidence that luminous ctenophores are coelenterazine producers. We detected coelenterazine in cultured specimens of *Bolinopsis infundibulum* and *Mnemiopsis leidyi* but not in their food items.

Figure 1. Coelenterazine in the Wild-Caught Ctenophores
(A) *Mnemiopsis leidyi* under white light (top) and its bioluminescence (bottom).
(B) Coelenterazine content normalized by body mass in the whole body of wild-caught specimens. The amount of coelenterazine was determined by luciferase assay (blue) and mass spectrometry (gray). The inset of panel B shows the same data rescaled to show lower values.
(C) Extracted ion chromatogram. (upper panels) Authentic coelenterazine (calculated mass, m/z 424.16557), coelenteramide (calculated mass, m/z 412,1657), and coelenteramine (calculated mass, m/z 278.12879). (lower panels) Representative chromatograms of *M. leidyi* extract. Observed mass values are indicated by the peaks. Photos in A by W.E.B. and M. B-U.
RESULTS AND DISCUSSION

Coelenterazine Was Detected in Luminous Ctenophores

The presence of coelenterazine was tested among wild-caught specimens by a luciferin-luciferase reaction using Renilla luciferase and by mass spectrometry. Using the luciferase assay, coelenterazine was detected in methanol extracts of the luminous ctenophores Beroe cucumis (on average 16.7 pmol/wet g), Mnniopsis leidyi (4.80 pmol/wet g), Bolinopsis infundibulum (0.129 pmol/wet g), and trace amounts in a non-luminous ctenophore Pleurobrachia bachei (0.0165 pmol/wet g) (Figure 1B). The trace amount of coelenterazine detected in wild-caught P. bachei was probably due to the presence of coelenterazine in the natural diet of P. bachei, as has been seen in other non-luminous taxa (Shimomura, 1987). Free coelenterazine in M. leidyi has been reported by luciferase assay (Anctil and Shimomura 1984). Although the enzymatic detection method has high substrate specificity, there is a possibility that luciferase reacts with compounds similar to coelenterazine, such as Cypridina luciferin (vargulin) or furimazine (Coutant and Janin, 2015). In addition to enzymatic detection, we used high-resolution mass spectrometry to verify the presence of coelenterazine by accurate mass-to-charge ratio (m/z) (Figure 1C). Using mass spectrometry, we detected coelenterazine in B. cucumis (on average 7.82 pmol/wet g), M. leidyi (1.04 pmol/wet g), B. infundibulum (0.256 pmol/wet g), and P. bachei (0.001 pmol/wet g, but in only one specimen out of nine biological replicates). We also detected coelenteramide and coelenteramine with exact mass values (Figure 1C).

Cultured Mnemiopsis and Bolinopsis Are Capable of Biosynthesizing Coelenterazine

To test the hypothesis that luminous ctenophores are capable of biosynthesizing coelenterazine, we cultured ctenophores while feeding them only non-luminous prey items (Figure 2A). Both cultured M. leidyi and B. infundibulum were bioluminescent and can emit light upon stimulation in the dark. By luciferase assay and mass spectrometry using selected ion monitoring, coelenterazine was detected only in the extracts of B. infundibulum and M. leidyi but not in the extracts of non-luminous Hormiphora californensis nor of prey items (algae, rotifer, copepod, mysid, zebrafish, and moon jelly) (Figures 2B and 2C). Selected fragmented ions of coelenterazine, coelenteramide, and coelenteramine were detected from the extract of cultured B. infundibulum at the identical retention time to those of the standards, but those compounds were not detected in H. californensis (Figure 2C). The possibility that the detected coelenterazine was derived from maternal provisioning by the wild-caught parent is extremely low because the specimens used in this study were the 15th generation for M. leidyi and the third generation for B. infundibulum. This result suggests that luminous ctenophores are capable of coelenterazine biosynthesis.

Evolution of Coelenterazine-Dependent Bioluminescence

Our study provides evidence that ctenophores are coelenterazine producers, joining the decapod shrimp S. debilis, and the copepod M. pacifica (Thomson et al., 1995; Oba et al., 2009a). It is assumed that coelenterazine is distributed through the food web to luminous and non-luminous animals. Since bioluminescence is a common trait in the ocean, we reveal the new ecological importance of ctenophores. Thus, the cnidian jelly, one of the major components of marine animals, may depend partly on ctenophores for their bioluminescent behavior. For example, bioluminescent narcomedusae are major predators of ctenophores (Choy et al., 2017).

The distribution of coelenterazine through the food web contributes to the convergent evolution of bioluminescence in the sea (Haddock et al., 2001; Markova and Vysotski, 2015; Davis et al., 2016). To obtain bioluminescent capability, it is essential to gain both luciferin and luciferase. It is possible that de novo evolution of luciferase is easier than de novo evolution of luciferin biosynthesis. For example, beetle luciferase can gain its light-producing ability with a few amino acid mutations from a non-luciferase protein (Oba et al., 2009b) although no comparable evolutionary pathway has been found for the many coelenterazine-dependent luciferases. Any protein with a ‘solvent-accessible cavity’, such as albumin, has the potential to convergently evolve luciferase activity (Vassel et al., 2012). In contrast, the occurrence of coelenterazine biosynthesis requires the evolutionary assembly of multi-domain biosynthetic machinery, such as a non-ribosomal peptide synthetase, or a multi-enzyme metabolic pathway (Francis et al., 2015). Dietary supplementation of coelenterazine potentially accelerates the evolution of bioluminescence by circumventing the steps required to evolve a coelenterazine biosynthesis pathway.

Coelenterazine biosynthesis appears to have evolved independently in at least three lineages; decapoda (Thomson et al., 1995), copepoda (Oba et al., 2009a), and ctenophores. The convergence of chemical
structure benefits generalist predators that lack biosynthetic ability by allowing them to obtain luciferin from multiple food sources, perhaps helping the evolution of coelenterazine-type bioluminescent systems in multiple lineages. However, it is unclear how natural selection resulted in an imidazopyrazinone luciferin from Phe-Tyr-Tyr (F-Y-Y) amino acids, and why this may have occurred multiple times. An imidazopyrazinone skeleton is essential to form the dioxetanone reaction to excite an electron. In another luminescent chemistry, the amino acid side chains are different but the backbone remains the same: Shallow water luminescent ostracods produce and utilize a luciferin, vargulin, that is composed of Arg-Ile-Trp (R-I-W), with the same imidazopyrazinone core (Oba et al., 2002). The discovery of a biosynthetic pathway for coelenterazine in ctenophores or other animals may address the question of how this luciferin arose independently.

Among ctenophores, the occurrence of coelenterazine from this study is consistent with known bioluminescent capabilities (Haddock and Case, 1995) and with the presence of a putative pathway involving isopenicillin-N-synthase homolog with a tripeptide Phe-Tyr-Tyr at the C-terminus (FYY gene) (Francis et al., 2015). The absence of coelenterazine in *H. californensis* and a trace amount in wild-caught *P. bachei* are consistent with bioinformatics analyses. Transcripts encoding isopenicillin-N-synthase homolog were found in the transcriptomes of 24 luminous ctenophore species but were not detected in the transcriptomes or genomes of two non-luminous species (Francis, et al., 2015). Although the involvement of the FYY gene in coelenterazine biosynthesis lacks experimental evidence, the cultured system of ctenophores may enable future interrogation by using gene knockout techniques (Presnell and Browne, 2019).
To date, *M. leidyi* may be the best model to study the biosynthesis of coelenterazine because (1) the genome and transcriptome are available (Ryan et al., 2013), (2) specimens can be cultured in the lab (Patry et al., 2020), (3) eggs are accessible for genetic experiments (Pang and Martindale, 2008; Davidson et al., 2017), and (4) *H. californensis* can also be cultured, to serve as a control lineage that is not able to produce coelenterazine. A recent study performed knock down of a ctenophore gene using morpholino (Yamada et al., 2010; Jokura et al., 2019). Using ctenophores to study coelenterazine biosynthesis has these several advantages, which are not applicable to other known coelenterazine producers, such as *S. debilis* nor *M. pacifica*.

Thus far, the biosynthetic pathway of luciferin has been revealed only from bacteria (Meighen and Dunlap, 1993) and fungus (Kotlobay et al., 2018). Revealing the coelenterazine biosynthetic pathway would provide alternative tools for imaging life and cellular processes.

**Limitations of the Study**

In this study, the detection limits of the luciferase assay for coelenterazine were 7 nM in the extract, and 1 nM per wet weight for mass spectrometry calculated using a standard curve. The luciferase assays and mass spectrometry could only detect free coelenterazine molecules, not bound to photoprotein or in storage form. Thus if animals used in this study possess undiscovered coelenterazine analogs, we would fail to estimate the ‘effective’ coelenterazine amount. However, in that case, those animals would potentially also possess coelenterazine, coelenteramide, or coelenteramine, which we did not detect in non-luminous prey items nor non-luminous ctenophores.

The potential sources for the discrepancy between the luciferase assay and LC/MS detection for coelenterazine content might be explained as follows. For luciferase assays, the crude methanol extract for the luciferin + luciferase assay possibly contains unidentified compounds that enhance or inhibit the luminescent reaction. On the other hand, the samples are separated by liquid chromatography on the C18 column before ionization for MS detection, so that the signal intensities are not affected by other compounds.

**Resource Availability**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Manabu Bessho-Uehara (bessho.manabu.lumi@gmail.com).

**Materials Availability**

The cultured ctenophore line of *Mnemiopsis leidyi* used in this study will be made available on request. There may be restrictions on the availability to have *Mnemiopsis leidyi* because this species is invasive species in some areas.

**Data Availability**

This study did not generate data sets and code.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101859.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.B.-U. and S.H.D.H.; Methodology, M.B.-U., and S.H.D.H.; Investigation, M.B.-U. and W.H.; Writing – Original Draft, M.B.-U.; Writing – Review and Editing, All authors; Funding Acquisition, S.H.D.H. and J.K.W.; Resources, W.L.P., W.E.B., and S.H.D.H.; Supervision, S.H.D.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

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Supplemental Information

Transparent Methods

**Specimen collection and maintenance**

The specimens of *B. infundibulum, B. cucumis, and P. bachei* were collected by blue-water SCUBA diving from the research vessel Western Flyer in March 2019. These specimens were kept in the dark in a plankton kreisel for five days to complete the digestion of food ingested in the wild. The specimen of *M. leidy* was caught from King’s Bay, Miami, FL, USA by ladle on April, 2018. In Monterey Bay Aquarium, specimens of *B. infundibulum* and *H. californensis* originally caught in Monterey Bay were cultured in Diffusion tubes (Patry et al., 2020) for three and five generations, respectively. The animals were fed non-luminous prey: the copepod *Parvocalanus crassirostris*, enriched for nutrients with algae *Isochrysis galbana* (provided by Reed Mariculture, Inc.), the non-luminous mysid *Mysidopsis bahia* (provided by Aquatic Indicators, Inc.), and the non-luminous moon jelly *Aurelia coerulea* raised upon those copepods. At the University of Miami, *M. leidy* was cultured in a pseudo-plankton kreisel for 15 generations. The animal was fed non-luminous prey: rotifer enriched nutrients with algae, and juvenile zebrafish *Danio rerio*.

**Coelenterazine extraction**

Live specimens were kept in the dark at least for 6 hours before flash freezing in liquid nitrogen. Lyophilized samples were suspended in a one-tenth volume of the wet weight of methanol on ice. For samples of less than 5 g (algae, copepod, rotifer, zebrafish, and *P. bachei*), 500 µL of methanol was used to extract coelenterazine. Homogenate was centrifuged at 15,000 g, for 10 min, at 4 ºC. The supernatant was filtered by 0.45 µm membrane filters (MilliporeSigma). Coelenterazine extract was immediately used for luciferase assay or kept in -80ºC until mass spectrometry.

**Luciferase assay**

Coelenterazine was quantified by an assay for reactivity with luciferase. Ten microliters of the extract was mixed with 100 µL of 100 ng/mL *Renilla* luciferase (ProtoLum) in 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0. The light was measured for 10 sec with 0.5 sec increments using an Infinite m200 platereader (Tecan). The coelenterazine concentration in the extract was determined using a standard curve made with synthesized coelenterazine (ProtoLum). The samples were diluted with methanol when the measurement was higher than the standard curve.
Mass spectrometry

High-resolution MS1/MS2 full scan of samples and standards were performed on LC/MS (Kinetex® 2.6 µm C18 100 Å LC Column 150 x 3 mm, Q Exactive Orbitrap Mass Spectrometers) with buffer A (water + 0.1% formic acid) and buffer B (acetonitrile + 0.1 formic acid). Both positive mode and negative mode were used in High-resolution MS1/MS2 full scan. For the quantification of coelenterazine, coelenteramide, and coelenteramine, the samples were analyzed using SRM method by LC/MS (Kinetex 2.6 µm C18 100 Å LC Column 50 x 3 mm, Thermo TSQ Quantum Access MAX Triple Quadrupole) at positive mode with buffer A (water + 0.1% formic acid) and buffer B (acetonitrile + 0.1 formic acid). The coelenterazine, coelenteramide, and coelenteramine in the methanol extract were identified by the single reaction monitoring (SRM) method. The SRM method was determined by running standards of coelenterazine, coelenteramide, and coelenteramine. For coelenterazine, the precursor and product ions are 424.100 m/z and 302.100 m/z respectively. For coelenteramide, the precursor and product ions are 412.100 m/z and 278.100 m/z, and for coelenteramine, the precursor and product ions are 278.100 m/z and 200.100 m/z. The quantification of each compound’s content was completed by comparing the peak area of each sample to the standard.