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Etmopterus spinax, the velvet belly lanternshark, does not use bacterial luminescence

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ABSTRACT

Marine organisms are able to produce light using either their own luminous system, called intrinsic bioluminescence, or symbiotic luminous bacteria, called extrinsic bioluminescence. Among bioluminescent vertebrates, Osteichthyes are known to harbor both types of bioluminescence, while no study has so far addressed the potential use of intrinsic/extrinsic luminescence in elasmobranchs. In sharks, two families are known to emit light: *Etmopteridae* and *Dalatiidae*. The deep-sea bioluminescent *Etmopteridae*, *Etmopterus spinax*, has received a particular interest over the past fifteen years and its bioluminescence control was investigated in depth. However, the nature of the shark luminous system still remains enigmatic. The present work was undertaken to assess whether the light of this shark species originates from a bioluminescent bacterial symbiosis. Using fluorescent *in situ* hybridization (FISH) and transmission electron microscopy (TEM) image analyses, this study supports the conclusion that the bioluminescence in the deep-sea lanternshark, *Etmopterus spinax*, is not of bacterial origin.

1. Introduction

Bioluminescence is a widespread phenomenon, ranging from bacteria to vertebrates (Haddock et al., 2010). The emission of visible light through biochemical reaction is referred to as (*i*) intrinsic bioluminescence, when the animal is able to produce its own light, and to (*ii*) extrinsic bioluminescence, when the light is produced by symbiotic bacteria (Haneda and Tsuji, 1971; Haddock et al., 2010; Widder, 2010). Some fish families possess the ability to emit their own light like the *Myctophidae* or *Stomiidae* (Barnes and Case, 1974; Case et al., 1977; Mallefet et al., 2019), while others display extrinsic bioluminescence as for the *Anomalopidae*, *Monocentridae* or *Ceratiidae* (Nealson and Hastings, 1979). Approximately 460 fish species are known to establish a symbiosis with bioluminescent bacteria (Dunlap, 2014), mainly with *Aliivibrio* (*Vibrio*) *fischeri*, *Photobacterium kishitanii, Photobacterium leiognathi, Photobacterium mandapamemsis, Candidatus* Enterovibrio luxaltus, *Candidatus* Enterovibrio escacola, *Candidatus* Photodesmus katoptron or *Candidatus* Photodesmus blepharon (Boettcher and Ruby, 1990; Ast et al., 2007; Dunlap et al., 2007; Kaeding et al., 2007; Hendry et al., 2014, 2018). Within a luminous symbiosis, the host likely controls the bacterial population within a luminous organ, called photophore. It provides the bacterial population with nutrients and oxygen they need to grow and emit light. In return, the host uses the light produced by the symbiotic bacteria (Haygood, 1993; Claes and Dunlap, 2000). In most extrinsic bioluminescent fish, the bacteria are extracellular and inhabit tubules of the fish photophore (Herring, 1982). In addition, these luminous organs are mainly associated with either the gastro-intestinal tract, or a subocular (*Anomalopidae*) or mandibular (*Monocentridae*) pocket or a modified dorsal fin ray (*Ceratiidae*) (Pietsch, 2009; Dunlap and Urbanczyk, 2013). In some cases, the fish photophore is connected to the environment through a small duct allowing the release of bacterial surplus due to the bacterial multiplication within the fish organ (Dunlap, 1984).

Nowadays, about 1064 fish species are able to produce their own light in photophores (Herring, 1982; Paitio et al., 2016) that resemble the following general structure : (i) a deep cup-shape pigmented sheet sometimes internally covered by (ii) a reflecting layer of guanine cristals that direct light produced by (iii) photocytes (luminous cells) toward a (iv) lens equipped or not with (v) filter; bioluminescence being directed outward (Herring, 1985). Although evolutionary convergent, the intrinsic photophore lack tubules, ducts or any pores such as those found in symbiotic light organs. Intrinsic photophores are mainly under

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cathecholamines nervous control with numerous modulations (Krönström et al., 2005; Zaccone et al., 2011; Mallefet et al., 2019) except in sharks where a hormonal control has been described (Claes and Mallefet, 2009a,c, 2010; Claes et al., 2010b, 2012).

Among Chondrichthyes, two families are known to contain species able to emit light: the *Etmopteridae* and the *Dalatiidae* (Claes and Mallefet, 2009a; Straube et al., 2015). Over the last 15 years, the deep-sea bioluminescent *Etmopteridae*, *Etmopterus spinax* has received some interest for its particular bioluminescence. This deep-sea shark is indeed able to emit blue-green light at 486 nm thanks to organs, called photophores, spread mainly into the ventral epidermis (Claes and Mallefet, 2009a,b; Renwart et al., 2014). These photogenic organs (150 µm diameter on average for *E. spinax*), are composed of emitting cells, the photocytes, embedded in a cup-shaped pigmented cell sheath, covered by a reflective layer containing guanine crystals, and capped by one or several lens cells (Renwart et al., 2014). A multilayer cell zone, called the iris-like structure (ILS), is present between the lens cells and the photocytes, and is used as shutter of the light organ (Claes and Mallefet, 2009a; Renwart et al., 2015). Multiple bioluminescence functions have been highlighted for these species as counter illumination, aposematism and intraspecific communication (Claes and Mallefet, 2008, 2009a; Claes et al., 2010a, 2013; Duchatelet et al., 2019b). To achieve their biological functions, *Etmopteridae* exhibit a hormonal control of the light output: melatonin and prolactin triggering the light emission while inhibition occurs thanks to α-melanocyte stimulating hormone (Claes and Mallefet, 2009c). Nitric oxide and the γ-aminobutyric acid are also able to modulate the *E. spinax* light emission (Claes et al., 2010b, 2011). Despite the increasing collection of data about the luminous control and the ultra-structure description of the photophore (Claes and Mallefet, 2009c; Renwart et al., 2014), no evidence has been put forward to clearly discriminate the extrinsic *vs.* intrinsic light emission in luminous sharks (Renwart and Mallefet, 2013) even though some authors have suggested bacterial luminescence for the shark *Megachiasma pelagos* (Taylor et al., 1983; Herring, 1985; Nakaya, 2010).

In this paper, using transmission electron microscopy (TEM) observation of the light organ ultrastructure, and fluorescent *in situ* hybridization (FISH) technique with bioluminescent bacteria probes, the existence of extrinsic bioluminescence in *E. spinax* was ruled out.

2. Material and methods

2.1. Specimen collection

Adult *E. spinax* were captured during field collections in August 2014 by longlines lowered at 250 m depth in the Raunefjord, Norway (60°15′54″ N; 05°07′46″ E) (Claes and Mallefet, 2009c; Renwart et al., 2014, 2015). Six living specimens were kept in a 1 m^3 tank filled with running fresh cold seawater (6 °C) and placed in a dark cold room at Bergen University Marine Station (Espegrend, Norway). At the same location, three specimens of *Galeus melastomus* were captured and kept in the same condition in order to provide non-bioluminescent shark control. Sharks were sexed, measured, weighed then euthanized following the local rules for experimental vertebrate care. Animals were treated according to the European regulation for animal research handling. Skin patches of 3 cm^2 were dissected from the ventral luminous zone, bathed in 4% paraformaldehyde phosphate buffer saline (PBS) for 12 h at 4 °C, rinsed and stocked in PBS at 4 °C until use. Controls were performed on two species of luminous fish known to host symbiotic bacteria (Dunlap et al., 2014; Yasaki, 1928; Ruby and Nealson, 1976; Tebo et al., 1979). Three specimens of *Coelorinchus kishinouyei* (Gadiformes: *Macrouridae*) and two *Monocentris japonica* (Beryciformes: *Monocentridae*), obtained as bycatch in Taiwan, were processed as for *E. spinax*.

2.2. Section preparation

Photogenic shark skin patches preserved in PBS were bathed in sterilized PBS with increasing concentrations of sucrose (10% for 1 h, 20% for 1 h and 30% overnight). The tissues were then embedded in optimal cutting temperature compound (O.C.T. compound, Tissue-Tek, The Netherlands) and rapidly frozen at −80 °C. Cryostat microtome (CM3050 S, Leica, Solms, Germany) was used to obtain 10 µm sections that were laid on coated Superfrost slides (Thermo Scientific) and left overnight to dry under sterile conditions.

2.3. FISH bacterial detection

E. spinax slides were immersed in successive ethanol solutions (50, 80 and 100%, 3 min each) to permeabilized cells and left to dry. 10 µL of the hybridization buffer (900 mM sodium chloride, 200 mM Tris/HCl, 40% Formamide and 0.01% SDS in ultrapure water) containing the RNA probes at a final concentration of 5 ng μl^{-1} were applied per slides in the dark at 4 °C. An equimolar mix of three RNA probes coupled with Cy3 fluorochrome (EUB 338 I-cy3; EUB 338 II-cy3 and EUB 338 III-cy3), complementary to a 16S rRNA region highly conserved among bacteria, was tested in order to cover a highest range of bacteria domain and highlight the potential presence of bacteria within photophores (Table 1). NON EUB-Texas red probe, an oligonucleotide complementary to the EUB 338 probe, was use as negative control for nonspecific binding. After the probe application, the slides were incubated in wet conditions for 2 h at 46 °C, rinsed and incubated with a washing solution (46 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA and 0.01% SDS in ultrapure water) for 25 min at 50 °C. Slides were let to dry, coated with Cityfluor (AF1) allowing the limitation of photobleaching and cover with coverslips. A first positive control was realized using samples incubated with luminous bacteria. In brief, before cryo-sections and after PBS-sucrose baths, skin samples were placed in fresh *V. fischeri* suspension (ATCC 7744; American Type Culture Collection, PO Box 1549 Manassas, VA 20108 USA) for 4 h at 25 °C in a marine medium with constant stirring. The *V. fischeri* suspensions were prepared by inoculating 100 ml of marine medium followed by incubation for 24 h at 25 °C. The second series of controls was performed on *G. melastomus* (non-bioluminescent shark), *C. kishinouyei* and *M. japonica* (two extrinsic luminous fish). *C. kishinouyei* is known to host *P.*

Table 1
DNA probes used in the FISH experiments.

Probes	Targets	Sequences (5' – 3')	References
EUB 338 I	90% of bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. (1990), Loy et al. (2003)
EUB 338 II	69% of Planctomycetales	GCA GCC ACC CGT AGG TGT	Daims et al. (1999), Loy et al. (2003)
EUB 338 III	93% of Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	Daims et al. (1999), Loy et al. (2003)
NON EUB	Complementary to EUB 338	ACT CCT ACG GGA GGC AGC	Wallner et al. (1993), Loy et al. (2003)

kishitanii to produce light (Dunlap et al., 2014), while *M. japonica* displays a symbiotic association with *V. fischeri* (Ruby and Nealson, 1976).

All sections were observed with an inverted microscope DMI 6000B (Leica) equipped with a DFC 365 FX camera coupled with LAS AF 3.1.0 software (Leica).

2.4. TEM photophores analysis

Fresh ventral skin patches with photophores from *E. spinax* were fixed at 4 °C in 3% glutaraldehyde in cacodylate buffer (100 mM sodium cacodylate, 270 mM sodium chloride, pH 7,8) during 3 h, rinsed in the same buffer, and immersed at room temperature in a decalcifying solution (OsteoRAL R fast decalcifier, RAL diagnostics) during at least 10 days. Sections were then post-fixed in an osmium tetroxide solution (1% osmium tetroxide, 100 mM sodium cacodylate, 270 mM sodium chloride, pH 7.8) for 45 min, and dehydrated gradually in ethanol. Tissues were embedded in Spurr's resin, thin section of 100 nm were made *via* ultramicrotome and placed on copper grids following Renwart et al. (2014). Uranyl acetate (18 mM uranyl acetate solution: ethanol (2:1) for 45 min) and lead citrate solution (80 mM lead citrate, 120 mM sodium citrate, 160 mM sodium hydroxide for 4 min) were used for contrasting the slides. Sections were let to dry and observed in a transmission electron microscope Zeiss Leo 906E.

3. Results and discussion

3.1. Intrinsic luminescence

The general features of photophore could be observed on the lanternshark cryosections and TEM images, *i.e.* autofluorescent photocytes embedded in a pigmented sheath, capped by a multilayer cell zone with pigmented cells (ILS) and topped by one or two lens cells (Fig. 1A–C). Specific tubular structures hosting bacteria classically observed in extrinsic light organs has never been observed in *E. spinax* photophore (Fig. 1A, C). Shark photophores are of epidermal origin; ontogenic development studies have never shown any association with either gastro-intestinal tract or subocular or mandibular pocket (Claes and Mallefet, 2008, 2009b; Duchatelet et al., 2019a)

Using TEM, we were unable to detect the presence of bacteria in the photophore structure, and around or within the light emitting cells. Moreover, no duct leading to the external environment was observed within photophores (Fig. 1C). Internal structure of photocytes, revealed three distinct zones: nuclear, granular and vesicular zones (Fig. 1D, E): the granular inclusions represent the intracellular luminescence source called "glowons" (Renwart et al., 2015). Also, the size of inclusions (± 400 nm) within the vesicular and granular zones of photocytes does not match with the one of luminous bacteria (1–3 µm) (Nealson and Hastings, 1979).

In term of FISH labeling, no bacterial signal was detected at the level of photophore, neither in the photocytes, nor in the multilayer cell zone (ILS) or in the lens (Fig. 1F–G), while a strong signal was observable in the placoid scales (Fig. 1F). As a negative control, the absence of any red fluorescence using NON EUB-Texas red probe ruled out the possibility of unspecific hybridization (Fig. 1H). Positive control, consisting of slides pre-incubated with *V. fischeri,* displays a very strong labeling at the level of the epidermis corresponding to bacteria agglutination at the skin surface and a weak signal in the connective tissue surrounding the light organ (Fig. 1I).

Sections of *C. kishinouyei* (Fig. 2A–D) and *M. japonica* (Fig. 2E–G) luminous organ displayed bacterial labeling at the level of specific

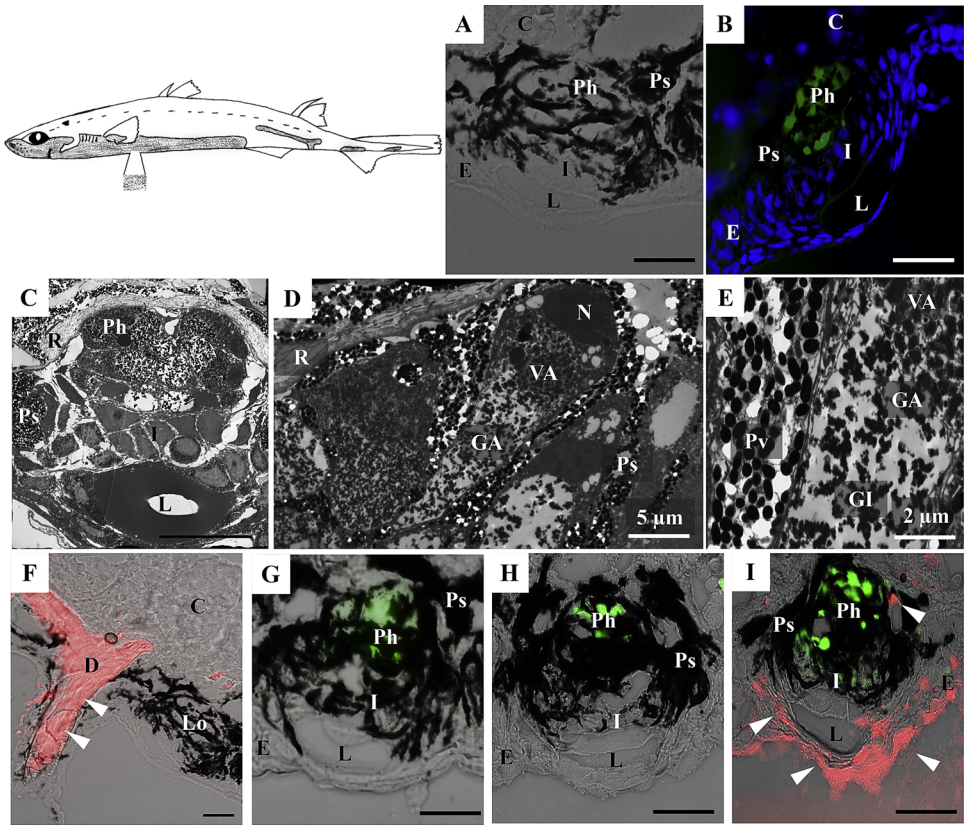

Fig. 1. *E. spinax* luminous organ histology, cytology and FISH labeling. *E. spinax* with grey shaded luminous zones and insert showing where ventral tissues were sampled. (**A**) Histological cryosection. (**B**) DAPI blue staining section showing green autofluorescence of photocytes. (**C–E**) Photophore transmission electron microscopy micrography. FISH labeling (**F, G**) with EUBI, II, III probes, (**H**) with NON EUB probe, (**I**) pre-incubated photophore with *vibrio fischeri*, with EUBI, II, III probes. Green color corresponds to autofluorescence of photocytes (G–I). White arrow head: bacterial red labelling. C: connective tissue; D: dermal denticule; E: epidermis; GA: granular area; GI: granular inclusions; I: iris-like structure cells; L: lens cells; Lo: light organ; N: nucleus; Ph: photocytes; Ps: pigmented sheath; Pv: pigmented vesicules; R: reflective guanine layer; VA: vesicular area. Scale bars: 50 µm (except D, E) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

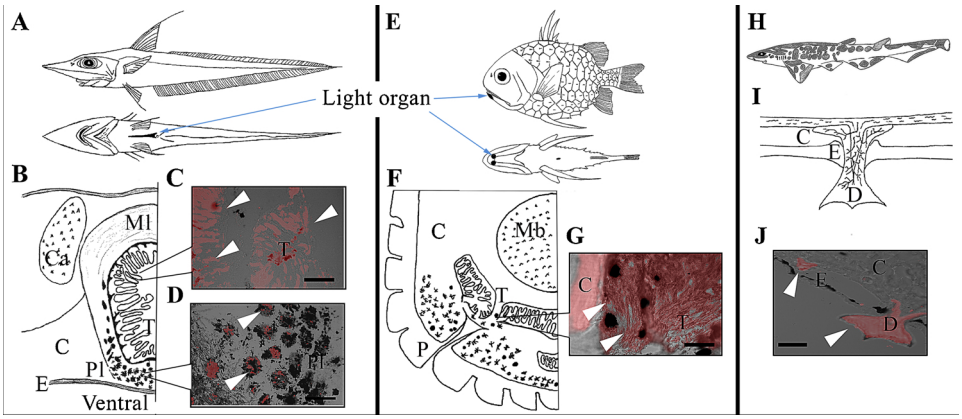

Fig. 2. Control experiments perform on (**A–D**) *Coelorinchus kishinouyei*, (**E–G**) *Monocentris japonica*, (**H–J**) *Galeus melastomus*. (A) Lateral and dorsal schematic view of *C. kishinouyei* (B) schematic transversal section of the gastro-intestinal linked ventral light organ. Control FISH experiment with EUBI, II, III probes on (C) internal bacterial tubules, (D) ventral pigmented layer. (E) Lateral and dorsal schematic view of *M. japonica*. (F) Schematic transversal section of the mandibular light organ. (G) Control FISH experiment with EUBI, II, III probes. (H) Schematic lateral view of *G. melastomus*. (I) schematic cross section of epidermis showing a placoid scale. (J) Control FISH experiment with EUBI, II, III probes. White arrow head: bacterial red labelling. C: connective tissue; Ca: cartilaginous tissue; D: placoid scale; E: epidermis; P: dermal papillae; Pl: pigmented layer; Mb: mandibular bone; Ml: muscular layer; T: bacteria-filled tubules. Scale bars: 100 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

tubules (Fig. 2C, D, G) with EUB probes, confirming their specificity. Finally, sections of *G. melastomus* (Fig. 2H–J) did not show any labeling in the epidermis or connective tissue except at the level of placoid scales (Fig. 2J) suggesting an unspecific placoid scale probe trapping as it has also been observed for *E. spinax* (Fig. 1F).

The number of luminous bacteria present in symbiotic light organs is typically high, reaching concentration of 10^{11} bacteria per mL (Dunlap, 1984). Observed FISH labeling for both extrinsic luminous fishes highlight a high density of bacteria within specific tubules (Fig. 2C, D, G), while the absence of labeling in *E. spinax* photophores indicate a very low bacterial density. These FISH results strongly suggest that light production of *E. spinax* is unlikely to be of bacterial origin.

A last intrinsic luminescence evidences come from ontogeny and development of juveniles. Most of juvenile bioluminescent fishes and cephalopods using bacterial symbiosis do not have light capacity at birth, but must obtain it by recruiting luminous bacteria from surrounding waters through duct or connection openings. The luminous bacteria colonize the light organ during the post-hatching juvenile stage of these organisms (Ruby and McFall-Ngai, 1992; Ruby and Asato, 1993; Wada et al., 1999; Fukui et al., 2010; Dunlap et al., 2014; Gould et al., 2016). Conversely, in *Etmopteridae*, embryonic development takes place in the uterus and embryos are already able to produce light *in utero* before birth (Claes and Mallefet, 2008; Duchatelet et al., 2019a).

4. Conclusion

Based on (*i*) ultrastructural and histological descriptions, (*ii*) FISH labeling, (*iii*) embryonic bioluminescence observations, it can be concluded that bacteria, or structures that can host symbiotic luminous bacteria, are absent from the photophores of *E. spinax*. Taken together, these data strongly support an intrinsic bioluminescence in the deep-sea lanternsharks and highlight the importance of studying the nature of this light emission system in these enigmatic organisms.

Ethics approval

The shark collection and experiments were performed under the "Experimental fish care PERMIT" number 12/14048. Following the local instructions for experimental fish care (Permit 12/14048), captive animals were euthanized by a blow to the head followed by a full incision of the spinal cord at the back of the head. Animal procedures were conducted in compliance with the Belgian national guidelines and in agreement with the European directive 2010/63/UE, under the approval of the Animal Ethics Committee of the Catholic University of Louvain in Louvain-la-Neuve.

Declarations of interest

None.

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Authors' contributions

L. Duchatelet: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing original draft. J. Delroisse: conceptualization, data curation, formal analysis, methodology, validation. P. Flammang: resources, methodology, project administration, supervision. J. Mahillon: resources, methodology, project administration, supervision. J. Mallefet: conceptualization, methodology, investigation, data handling, resources, project administration, supervision, funding acquisition. All authors reviewed the final version and agree to final article submission.

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