



Embryonic expression of encephalopsin supports bioluminescence perception in lanternshark photophores

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Abstract

Counterilluminating animals produce a ventral light to hide their silhouette in the water column. This midwater camouflage technique requires a fine and dynamic control of the wavelength, angular distribution, and intensity of their luminescence, which needs to continuously match ambient downwelling light. Recently, extraocular opsins have been suggested to play a role in the bioluminescence control of several organisms, such as squids, comb jellies, or brittle stars, providing a way for photogenic structures to perceive their own light output. By analysing a growing embryonic series of the velvet belly lanternshark, *Etmopterus spinax*, we show that the development of lanternshark luminescence competence is associated with the expression of encephalopsin within epidermal cells and in the light-regulating structure of the photogenic organs. Such an intra-uterine expression of encephalopsin strongly supports this blue-sensitive extraocular opsin to allow bioluminescence perception in lanternshark photophores and suggests a clear physiological interaction between photoemission and photoperception.

Introduction

Counterillumination is an active camouflage method by which a midwater animal produces a ventral light to match the wavelength, intensity, and angular distribution of the background downwelling light to cloak its silhouette from both predators and prey passing below (Clarke 1963; Warner et al. 1979; Denton et al. 1985). Widespread in non-transparent mesopelagic organisms such as crustaceans, mollusks (cephalopods), and fishes, this fascinating camouflage strategy requires a fine and dynamic bioluminescence tuning to be effective, since any deviation from background light may attract the attention of upward-looking predators (Harper and Case 1999; Johnsen et al. 2004; Haddock et al. 2010). This control is performed via sophisticated optical structures [e.g., reflectors, wavelength-specific filters, lenses, etc. (Denton et al. 1972, 1985; Jones and Nishiguchi

2004)] and/or complex physiological control mechanisms, i.e., the use of neuromodulators, vasodilation, or regulation of oxygen supply (Nealson and Hastings 1979; Young and Mencher 1980; Latz 1995; Krönström et al. 2005, 2007, 2009). Counterillumination typically requires simultaneous perception of bioluminescence and ambient light via visual (e.g., the eyes) or non-visual organs (e.g., pineal organs in bony fishes, photoreceptive vesicles in cephalopods) closely associated with light organs (photophores) (Young et al. 1979). In some cases, light perception may occur within the photophore/photocyte itself via an extraocular opsin (Tong et al. 2009; Schnitzler et al. 2012; Delroisse et al. 2014). The recent discovery in ventral photophores of adult *Etmopterus spinax* specimens, of an extraocular opsin, encephalopsin (opsin 3), that is primarily controlled by hormones produced by the pineal gland and the retina (Claes and Mallefet 2009), suggested that bioluminescence and auto-perception mechanisms may concomitantly occur within photogenic tissues in a single species (Delroisse et al. 2018). Interestingly, *E. spinax* is a bioluminescent ovoviparous species, whose developing embryos are known to produce light ventrally, laterally, and dorsally in utero (Claes et al. 2010a, 2015; Claes and Mallefet 2008, 2014). *E. spinax* emits a blue–green glow [486 nm; Claes et al. (2010a)] from lanternshark-specific photophores, distributed mainly within the ventral epidermis. These structures are composed

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of multilayer cup-shaped pigmented cells that form a dark granular sheath enclosing the emitting cells, the photocytes, which are capped by one or several lens cells. A cell zone called the iris-like structure (ILS) is present between the lens cells and photocytes (Claes and Mallefet 2009; Renwart et al. 2014). As mentioned previously, shark light emission is under a hormonal control. Melatonin and prolactin trigger light emission, while alpha melanocyte stimulating hormone inhibits it (Claes and Mallefet 2009). Modulation of bioluminescence also involves GABA and NO in lanternshark (Claes et al. 2010b, 2011). Currently, the biochemical compound underlying the bioluminescent reaction occurring within photocytes of lanternsharks is not known (Renwart and Mallefet 2013; Oba et al. 2016).

This study set out to characterize the development of bioluminescence and encephalopsin photoreception during the morphogenesis of photophores from lanternshark *E. spinax*. We have assumed a close interaction between photoemission (i.e., bioluminescence) and photoperception (i.e., through encephalopsin) in photophores based on observations from adult *E. spinax* (Delroisse et al. 2018). We used fluorescence microscopy and immuno-histochemistry to first demonstrate that light perception is present when light emission appeared during the embryogenesis of *E. spinax*.

Materials and methods

Specimen collection

Gravid females of *E. spinax* were collected during field session in January 2017 by longlines lowered at 200 m depth in the Raunefjord, Norway (60°15'54"N; 05°07'46"E). Living specimens were brought to Espeland marine station (Espeland, Norway), kept in a tank filled with 6 °C sea water and placed in a dark cold room. Sharks were weighed and sized before to be euthanized following techniques outlines in Claes and Mallefet (2009). Female uteri were excised to access the embryos. Embryos from each uterus were counted and measured to the nearest millimeter before being euthanized by a quick incision in the spinal cord. Embryo measurements were used to assign life stages. A total of ten embryos (50–127 mm TL) from four different litters [two embryos from litter 1; two embryos from litter 2 (both were unpigmented embryos and lacking photogenic organs); three embryos from litter 3; and three embryos from litter 4 (both were pigmented embryos with photogenic organs)] forming a growing embryonic series as well as six adult females (stage V) were analysed for this study (Fig. 1). The yolk sac of each embryo was removed before daylight as well as bioluminescence photographs captured thanks to a Sony alpha 7S II camera (Sony Corporation, Japan) (Fig. 1a, b). Ventral skin patches (1 cm²) between the pectoral and pelvic

fins were dissected from each embryo and females with a metal cap drill (0.6 cm diameter) following the method described in Claes and Mallefet (2009), fixed in phosphate buffer saline (PBS) with 4% paraformaldehyde for 12 h, and stored in PBS prior to experimentation.

Encephalopsin localization

Preserved ventral shark skin patches were immersed in a series of PBS baths with increasing concentrations of sucrose (10% for 1 h, 20% for 1 h and 30% overnight) for cryoprotection. Each tissue was then embedded in optimal cutting temperature compound (OCT compound, Tissue-Tek, The Netherlands) and rapidly frozen at −80 °C. Cryostat microtome (CM3050 S, Leica, Solms, Germany) was used to perform 10 µm sections that were laid on coated Superfrost slides (Thermo Scientific) and left overnight to dry. To visualize the encephalopsin expression and localization within the photophore, an immunofluorescence technique was applied on *E. spinax* embryo and female ventral skin sections. Slides were rinsed 15 min with Tris buffer saline 1% Tween [TTBS: Trizma base (Sigma) 20 mM, NaCl 150 mM, pH 7.5 + 1% Tween 20 (Sigma)], then blocked with TTBS containing 10% bovine albumin serum (BSA, Amresco). Following Delroisse et al. (2018), slides were incubated overnight with the anti-encephalopsin primary antibody (anti-encephalopsin Pab in Homo sapiens, Genetex, GTX 70609, lot number 821 400 929) at a dilution of 1/500 in TTBS 5% BSA. Slides were bathed again in TTBS during 30 min before to be incubated in the dark with the secondary antibody Alexa Fluor[®] 594 Goat Anti-Rabbit IgG (Goat Anti-Rabbit, Alexa Fluor[®] 594, Life Technologies Limited) at a dilution of 1/200 in TTBS 5% BSA and rinsed 15 min in TTBS. Finally, slides were subjected to 15 min DAPI (DAPI nucleic acid stain, Invitrogen) nucleus staining, rinsed 10 min in TTBS, and mounted with Mowiol (Mowiol[®] 4-88, Sigma). Sections were observed under Polvar SC epifluorescence microscope (Leica Reichter Jung) equipped with a Nikon DS-UI digital camera coupled with NIS elements FW software. Control sections (i.e., omission of the primary antibody as well as immunodetection in dorsal skin and retina; immunoblot) were made following Delroisse et al. (2018). Immunodetection experiments were performed on each embryo and applied on at least ten sections for each embryo.

Results

Figure 1 summarizes the different embryonic developmental stages (stages I–IV) leading to the adult (stage V) in *E. spinax* (Fig. 1a). Spontaneous luminescence (ventral, lateral, and dorsal) was observed only in stage IV embryos and in

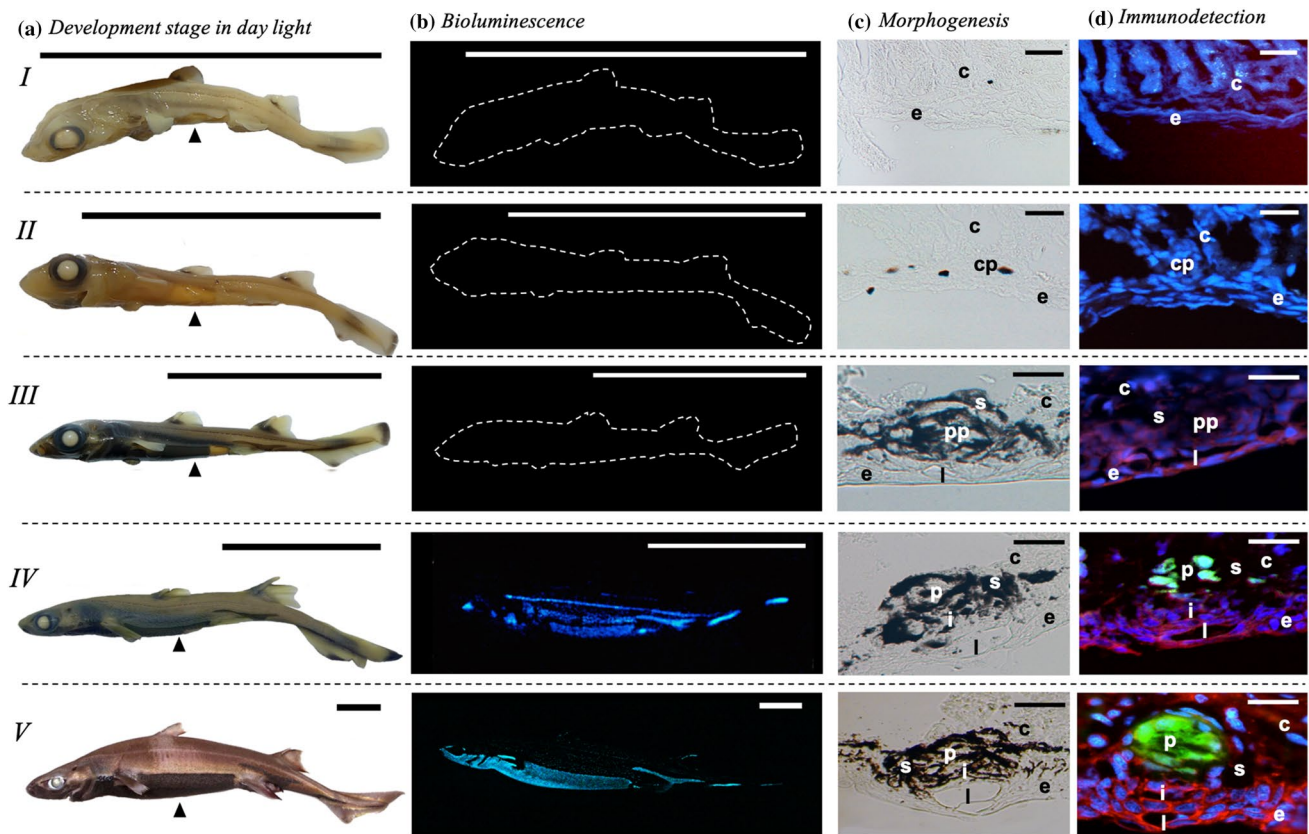


Fig. 1 Joint appearance of encephalopsin and photophores during *Etmopterus spinax* photophore embryogenesis within the ventral skin epidermis. **a** Developmental series of *E. spinax* in natural light (stages I–IV come from four distinct litters collectively constituting an embryonic series, while stage V is an adult female specimen). Arrowheads depict the ventral location sampled for histological and immunodetection experiments. **b** Images taken in the dark highlighting spontaneous luminescence, when present. Contour dot line depicted the non-luminous embryo from stage I to III. **c** Histological section of the ventral epidermis under bright light highlighting the

presence and the ontogeny of photophore structures, when present. **d** Histological section of the ventral epidermis under UV stimulation highlighting the presence of photophore structures, when present. The autofluorescence of the photocyte vesicles (green fluorescence, only present in mature photocytes) and encephalopsin immunodetection (red labelling) is shown. Dapi blue staining associated with the cell nucleus. *c* connective tissue, *cp* pigmented cells, *e* epidermis, *i* iris-like structure cells, *l* lens cells, *p* photocytes, *pp* protophotocytes, *s* pigmented cell layer. Scale bars represent 5 cm in **a** and **b**; 50 μm in **c** and **d**

stage V adult specimens (Fig. 1b). Ventral skin morphogenesis (Fig. 1c) shows pigmented cells dispersed within the epidermis layer (stages I, II), but lacks photogenic structures or encephalopsin immunoreactivity (Fig. 1d). Stage III embryos displayed protophotophores, with protophotocytes lacking fluorescent vesicles and hence without luminescent capabilities (Fig. 1c, d). Protophotocytes were embedded in an integrated layer of pigmented cells that was capped by an iris-like pigmented structure (ILS) and a lens cell (Fig. 1c, d). This, in turn, was surrounded by epidermis cells that labelled positively for encephalopsin (Fig. 1d). Stage IV embryos showed functional photophores (i.e., photophores containing photocytes with autofluorescent vesicles). A strong encephalopsin labelling was observed within ILS and photophore-topping epidermis cell membranes from stage IV embryos (Fig. 1d). Stage V encephalopsin labelling was similar to those observed in Delroisse et al. (2018) in the

ventral epidermis of *E. spinax* adult specimens (Fig. 1d). Immunodetections were not observed within photocytes, while weaker labelings were detected at the level of the epidermis surrounding the photophore for these two former stages (Fig. 1d). Moreover, observed immunolabelings were consistent through the repetition of the experimentation for each stage. All controls (data not shown) produced results consistent with those reported in Delroisse et al. (2018).

Discussion

The photophore development steps observed in this study are similar than those described in Claes and Mallefet (2008), which supports the idea that lanternshark photophore development follows a stereotypical trajectory during embryogenesis. The development of luminous competence

during embryogenesis is associated with the appearance of encephalopsin expression in the cell membranes of the light organ ILS. This structure is known to act as a diaphragm that regulates the amount of light emitted to the outside (Claes and Mallefet 2010; Claes et al. 2011). This and the embryonic luminescence, that can only be detected by encephalopsin, within the female's uteri strongly suggests a close association between the development of photoreception and bioluminescence in lanternshark photophores. The extraocular perception of bioluminescence could represent a feedback control mechanism analogous to the one suggested for squid *Euprymna scolopes* (Tong et al. 2009), which has to be present before the photophore starts to produce light or, at least, before embryos hatch and start to rely on counterillumination for predator evasion (Claes and Mallefet 2008). In addition, a recent research highlighted the blue light sensitivity and the regulatory role of melanocyte for the human homologous encephalopsin (Regazzetti et al. 2018), adding further evidence of a potential link between extraocular opsin and the blue–green light emitted, at 486 nm (Claes et al. 2010a, b), by this lanternshark. The link between photoreception and regulation of light by pigmentation might be mediated by encephalopsin (Delroisse et al. 2018) regulating melanocytes dispersion in ILS (Renwart et al. 2014, 2015).

The pattern of encephalopsin expression observed in this study is very similar to the immunoreactivity of GABA within the shark photogenic tissue (Claes et al. 2011). GABA is known to play an inhibitory role on the light emission of *E. spinax* (Claes et al. 2011) and hence could act jointly with different transduction pathways on the ILS cells to regulate the light output (Bertolesi et al. 2015). All together, hormones and neuromodulators controlling the light emission [melatonin, alpha melanocyte stimulating hormone and GABA (Claes and Mallefet 2009; Claes et al. 2010b, 2011), as well as extraocular opsin (i.e., encephalopsin)] could act in concert to regulate the ILS cells' pigmentation and refine the bioluminescence output. Simultaneous emission and perception of light by a single organ have only been suggested for Hawaiian bobtail squid *Euprymna scolopes*, warty comb jellies *Mnemiopsis leidyi*, and brittle stars *Amphiura filiformis* (Tong et al. 2009; Schnitzler et al. 2012; Delroisse et al. 2014). The presence of a similar dual capability in the photophores of a vertebrate supports the idea that light perception might be common in counterilluminating photophores. Further research is required to better understand the role of this extraocular opsin and confirm its implication in the control mechanism of lanternshark photophores.

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Author contributions LD performed, analysed, and interpreted the immunodetection and was a major contributor in writing and revising the manuscript. JC revised the manuscript. JM took live pictures, supervised the work, contributed to, and revised the manuscript. Both authors read and approved the final manuscript.

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Compliance with ethical standards

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

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. 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. This article does not contain any studies with human participants performed by any of the authors.

Data accessibility All data supporting the paper are presented in the main manuscript.

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