Morphology and control of photogenic structures in a rare dwarf pelagic lantern shark (*Etmopterus splendidus*)

Julien M. Claes [a,*], Keiichi Sato [b], Jérôme Mallefet [a]

[a] *Laboratoire de Biologie Marine, Earth and Life Institute, Université catholique de Louvain, 3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium*
[b] *Okinawa Churaumi Aquarium, 424 Ishikawa, Motobu-cho, Okinawa prefecture, 905-0206, Japan*

ABSTRACT

The shark genus *Etmopterus* encompasses numerous deep-sea species that are widely distributed throughout the world's oceans and share the capability to emit light thanks to numerous tiny epidermal photogenic organs called photophores. Despite the potential wide ecological interest of this light emission, it is still a poorly studied aspect of shark biology, mostly due to the challenges inherent to the study of uncommon deep-sea animals. During a collection trip in waters around Okinawa Island, we had the opportunity to collect, maintain and study specimens of *Etmopterus splendidus*, a small pelagic lantern shark that was not previously known from this area. Analyses show that (i) the photophore density of this species varies according to the different parts of the body, which led to a heterogeneous photogenic pattern; (ii) photophore harbour the classical structure found in other etmopterid sharks, i.e. a cluster of photocytes enclosed in a pigmented sheath and surmounted by pigmented and lens cells; (iii) the physiological control of these photophores appears similar to what was found in the distantly related *Etmopterus spinax*, i.e. including hormonal and neural inputs as well as the action of pigmented cells overlying the photocytes. These results indicate that *E. splendidus* luminescence is probably used for more than one purpose, and support the idea that the physiological control of lantern shark photophores was selected early in the evolution of these sharks.

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1. Introduction

The genus *Etmopterus* is certainly the most diverse of the shark family Etmopteridae, since it currently encompasses 33 described species that occupy the depths of the world's oceans, where they use small epidermal organs called photophores to emit a visible light (Compagno et al., 2004; Schaaf da Silva and Ebert, 2006). Furthermore, a recent molecular work showed that these species can be grouped in several clades that are supported by morphological differences in the organization of photophores present on their flanks (Straube et al., 2010). This supports the luminescence of these sharks to be involved in species recognition, and therefore to be a driver of their evolutionary success (Claes, 2010; Straube et al., 2010).

Despite its potentially great ecological implication, bioluminescence is still one of the least investigated aspects of shark biology, with only one species, *Etmopterus spinax*, for which experimental data are available in this field (Claes, 2010). The main reason for that is certainly the logistical challenge inherent to the study of deep-sea species that are, for some of them at least, relatively rare or localised (Claes and Mallefet, 2008).

The splendid lantern shark *Etmopterus splendidus* Yano, 1988, is a poorly known dwarf lantern shark with a cylindrical body (Fig. 1A), probably adapted to a pelagic life. Very localised, its presence has only been confirmed in East China Sea, off Taiwan and southern Japan (Compagno et al., 2004). Although no molecular data are currently available for this species, its general morphology and the organization of its flank photophores (Fig. 1A) strongly suggest that it belongs to the "*Etmopterus pusillus* clade" (Straube et al., 2010).

Recently, we were able to collect and maintain in captivity three specimens of *E. splendidus.* This represented a unique opportunity to document the luminescence of this uncommon species. The present paper aims to provide information on (i) the organization, (ii) the structure of the photophores of this shark and (iii) the physiological control of the light emitted by these organs.

2. Materials and methods

Three adult male splendid lantern sharks, *E. splendidus* [20.3–23.5 cm total length (TL)] were collected by hook-and-line in December 2010, during two fishing sessions, near a seamount located in East China Sea off Okinawa Island (26° 28.946′N, 27°41.207′E), at a depth comprised between 470 and 485 m.

Specimens were then transferred to oxygen saturated plastic bags filled with cool (13 °C) seawater and brought in a refrigerated box to the Okinawa Churaumi Aquarium where they were housed in a cold
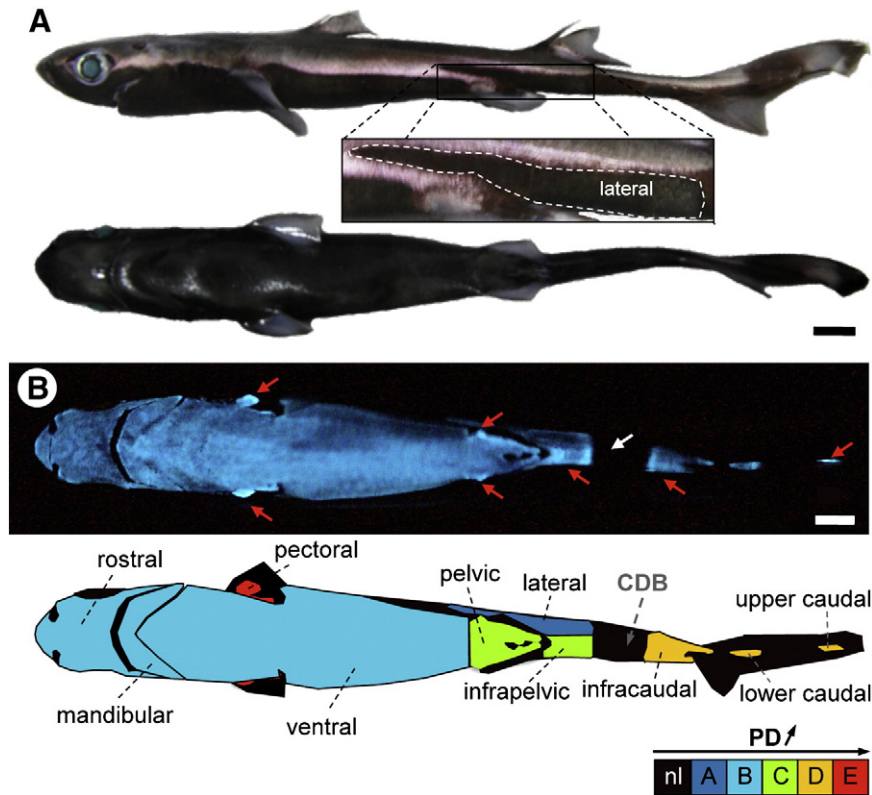
Fig. 1. Luminous pattern of *Etmopterus splendidus*. (A) Lateral (top) and ventral (bottom) view of an adult male specimen showing a typical pelagic habitus with a cylindrical morphology and the lateral luminous markings, which support its affiliation to "*Etmopterus pusillus*" clade (Straube et al., 2010). (B) Spontaneous luminescence of a freshly caught specimen (top) and description of the different photogenic zones composing the luminous pattern (bottom). Red and white arrows on top indicate bright luminescence and non-luminous caudal dark band (CDB), respectively. Photogenic zones shown in same colour have similar photophore density (PD). nl, not luminous. Scale bars: 2 cm.

(13 °C) seawater tank (0.75×1×2 m) maintained in the dark until experimentation took place.

2.1. Organization and structure of photophores

Ventral and lateral sides of the sharks were photographed with a digital camera (Canon 7D) in order to determine the organization of the luminous pattern using spontaneous luminescence. We then named the different photogenic zones using the same nomenclature used for the velvet belly lantern shark *E. spinax* (Fig. 1B). For each specimen, absolute surface area (in cm^2) of the different luminous zones was then calculated via Image J© (National Institute of Health, Bethesda, MD, USA).

Sharks were sacrificed by head decapitation, and pieces of skin containing photophores from different areas were dissected out in order to estimate their photophore density (PD) under binocular microscope.

Finally, in order to analyse the structure of *E. splendidus* photophores, skin patches from the ventral photogenic area were dissected out and fixed in seawater containing 4% formaldehyde for one week, stored in 70% ethanol, and then transferred to phosphate-buffered saline (PBS) with 0.5% sodium azide (NaN$_3$). Skin patches were then decalcified in ascorbic acid (2%) during 48 h, progressively dehydrated (50, 70, and 2×90% ethanol, one hour each), placed in 100% butanol for one hour, and left overnight in 100% butanol at 60 °C. Preparations were then submerged by paraffin wax for three different periods (12 h, 1 h and 3 h) at melting temperature (58 °C), cut with a classical microtome (section width=7 μm), and observed either (i) under UV stimulation using an epifluorescence microscope, or (ii) coloured with the Masson's trichrome and observed using a light microscope (Leitz Diaplan).

2.2. Luminescence control

The physiological control of *E. splendidus* photophores was investigated screening with test substances for neurotransmitters, nitric oxide (NO), one neurotransmitter antagonist (the GABA$_A$ antagonist bicuculline) and hormones to identify those inducing light emission (extrinsic control). All these substances were obtained from Sigma Chemical Co. (St Louis, MO, USA). In addition, the melatonin 2 (MT$_2$) receptor antagonist 4P-PDOT (Tocris Bioscience, Ellisville, MO, USA) in combination with melatonin was also used.

Circular standard (diameter=0.55 cm) skin patches were dissected from the ventral photogenic area of the sharks using a metal cap driller, following the method of Claes and Mallefet (2009b). Circular skin patches were then placed in small Perspex chambers containing 200 μl of a physiological shark saline (pH=7.7; see Bernal et al., 2005 for exact composition), with their photogenic area facing the photo-detector of a luminometer FB12 (Berthold; Pforzeim, Germany) calibrated with a standard light source (470 nm; Beta light; Saunders Technology, Hayes, UK). A test substance was finally applied onto the circular skin patches and light was recorded on a laptop computer for either 20 min (neurotransmitters) or 1 h (hormones), using the Berthold multiple kinetic mode (Sirius protocol manager v 1.4). Data were collected every minute to build original curves, and these curves were characterized using classical parameters: the maximum intensity of light emission [L_{max}, in megaquanta per second (Mq s^{-1})], the total quantity of light emitted during the experiment [L_{tot}, in gigaquanta (Gq)], and the time to reach L_{max} from the stimulation time [TL_{max}, in second (s)]. These parameters were standardised by skin surface area (in cm^{-2}). Saline applications were used as negative controls.

In some cases, skin patches that provided important light response were taken out of the luminometer and observed under binocular

microscope, to macroscopically observe morphological variations occurring in the photophores during light emission.

2.3. Statistical analysis

In order to test if PD was homogeneous across the different photogenic zones composing the luminous pattern of *E. splendidus*, we performed a Kruskal–Wallis analysis of variance on ranks. This non-parametric analysis of variance was used due to a lack of equality of variance between these data as determined by the Levene test ($F = 3.5021$, $P = 0.0250$). A post hoc Student's *t*-test was then performed to compare the PD of all the different photogenic zones two by two.

In order to test the effect of a pharmacological substance, we compared the light response obtained after application of this substance with the light response obtained after the application of saline (negative control) by a Student's *t*-test. Since basal luminescence (intensity of light emitted by the patch before stimulation) varied from one ventral skin patch to another, the light parameter L_{max} was preferred to L_{tot} to investigate the effect of a test substance. Furthermore, due to the small sample size ($N = 3$) and the variability of luminous response between individuals, we decided to use the parameter L_{max} in relative units in the statistics (i.e. R $L_{max, for}$ "relative L_{max}"), by dividing the L_{max} of the test substance and the saline application by the L_{max} value obtained with the test substance.

A Student's *t*-test was equally performed between the L_{max} of the melatonin (MT) application and the L_{max} of the MT + 4P-PDOT application, expressed as a percentage of the melatonin L_{max} value.

Kruskal–Wallis analysis of variance on ranks, Levene test and Student's *t*-tests were performed using the Jump software (JMP v. 9; SAS Institute Inc., 2011, Cary, NC, USA) and considered significant at the 0.05 level. Each mean value is expressed with its standard error (mean ± S.E.M.) and "*N*" represents the number of skin patches used for a specific treatment corresponding to the number of sharks tested.

3. Results

3.1. Photogenic structures

Directly after capture, one *E. splendidus* specimen exhibited a blue spontaneous luminescence from ventral and lateral sides of its body (Fig. 1B). The pattern of this light emission appeared relatively homogeneous on the rostrum and the belly part of the shark. However, small luminous areas located on the pectoral fins, on the border of the pelvic fins, and on the tail fin appear brighter (Fig. 1B). One striking observation was the presence of a dark, non-luminous zone between infrapelvic and infracaudal zone (Fig. 1B) referred to as "caudal dark band" (CDB).

The luminous pattern was divided into nine different luminous zones (Fig. 1B), which cover 90.74 ± 0.05% of non-translucent ventral surface area (i.e. total ventral surface area except eyes and pectoral fins). Kruskal–Wallis ANOVA showed significant difference in PD across the different luminous zones ($\chi^2_6 = 13.75$, $P = 0.0326$), while post hoc Student's *t*-test demonstrated the presence of different groups of luminous zones in which there was no statistical difference ($P > 0.05$) in photophore density (Table 1, Fig. 1B). PD ranged from 3940 ± 70 photophores cm^{-2} in the lateral luminous zones to 8670 ± 1760 photophores cm^{-2} in the pectoral luminous zones, and luminous zones displaying higher PD also appeared brighter. For each luminous zone, the total number of photophores was calculated by multiplying the surface area of the zone by its PD, giving an average total photophore number of $135,900 \pm 13,600$ for *E. splendidus* ($N = 3$).

Photophores were totally absent in the CDB, which instead contained numerous densely packed pigmented cells.

Photogenic structures consist in bell-shaped organs (circa 100 μm in diameter) composed of several photocytes enclosed in a pigmented

Table 1
Surface area, photophore density and number of photophores present in the different photogenic areas of *Etmopterus splendidus*.

	N	Surface area (cm²)	PD* (10^3 U c^{-2})	PN (10^4U)
Luminous zone				
Rostral	3	3.27 ± 0.05	4.46 ± 0.43[B,C]	1.46 ± 0.14
Ventral	3	17.12 ± 0.44	4.62 ± 0.36[B,C]	7.79 ± 0.76
Lower caudal	3	0.33 ± 0.04	7.83 ± 1.34[A,B]	0.27 ± 0.07
Upper caudal	3	0.07 ± 0.02	7.83 ± 1.34[A,B]	0.05 ± 0.02
Infracaudal	3	0.88 ± 0.05	7.83 ± 1.34[A,B]	0.69 ± 0.10
Mandibular	3	1.55 ± 0.07	4.62 ± 361[B,C]	0.71 ± 0.03
Pectoral	3	0.38 ± 0.12	8.67 ± 1.76[A]	0.34 ± 0.12
Pelvic	3	1.40 ± 0.10	6.98 ± 2.08[A,B,C]	0.96 ± 0.26
Lateral	3	2.79 ± 0.29	3.94 ± 0.07[C]	1.09 ± 0.10
Infrapelvic	3	0.42 ± 0.02	5.49 ± 0.85[A,B,C]	0.22 ± 0.02
Whole photogenic area	3	28.21 ± 3.13	/	13.59 ± 1.36

* Upper case letters indicate groups in which no statistical difference (P>0.05) has been found by post hoc Student's *t*-test.
PD, photophore density; PN, photophore number.

sheath and surmounted by one lens cell (Fig. 2A). Autofluorescence was found in the photocytes (Fig. 2B).

3.2. Physiological control of luminescence

Classical neurotransmitters and NO application on ventral skin patches only induced minor light responses that were not significantly different from the saline application control ($P > 0.05$, Table 2). However, application of the GABA$_A$ antagonist bicuculline (BICU) elicited a light response that was significantly higher than the control ($P < 0.01$). The saline application control produced nearly four times as much light as the GABA application (Table 2).

Application of MT and prolactin (PRL) elicited significantly more light than the control ($P < 0.01$, Table 2), while no significant difference was obtained for α-melanocyte stimulating hormone (α-MSH) application ($P > 0.05$, Table 2), although this hormone produced nearly two times less light than the saline application (Table 2).

The application of MT$_2$ antagonist 4P-PDOT decreased the amount of light elicited by MT in two of the three investigated specimens (inhibition = 57.21 ± 15.71%), however, Student's *t*-test did not detect any significant difference between application of MT alone and with 4P-PDOT ($P > 0.05$).

MT, PRL and BICU induced glows i.e. long lasting (>2 s) light emissions quickly after application, that showed different time courses (Fig. 3A). While PRL-induced luminescence was characterized by a nearly parabolic light emission that reached its L_{max} (81.78 ± 40.13 Mq s^{-1} cm^{-2}) quickly after application (10.60 ± 4.73 min), MT and BICU demonstrated similar slowly increasing curves that reached smaller L_{max} values (20.34 ± 2.85 and 19.01 ± 10.65 Mq s^{-1} cm^{-2}, respectively) in longer times (41.5 ± 9.37 and 44.33 ± 3.18 min, respectively). The L_{tot} of PRL-induced luminescence (167.34 ± 78.63 Gq cm^{-2}) was also an order of magnitude higher than L_{tot} of MT and BICU (33.96 ± 6.53 and 32.94 ± 10.65 Gq cm^{-2}).

Finally, as in *Etmopterus spinax* (Claes and Mallefet, 2010), non-luminous photophores of *E. splendidus* appeared as black dots while emitting ones adopted an iris-like morphology (Fig. 3B).

4. Discussion

This work represents the first record of *E. splendidus* from the waters of Okinawa Islands, and provides the first morphological and experimental data on the luminescence of this uncommon pelagic shark.

The photogenic structures of *E. splendidus* share remarkable similarities with those of *E. spinax* (Claes and Mallefet, 2008, 2009a), both species showing a complex heterogeneous luminous pattern with distinct photogenic areas composed of thousands of tiny epidermal
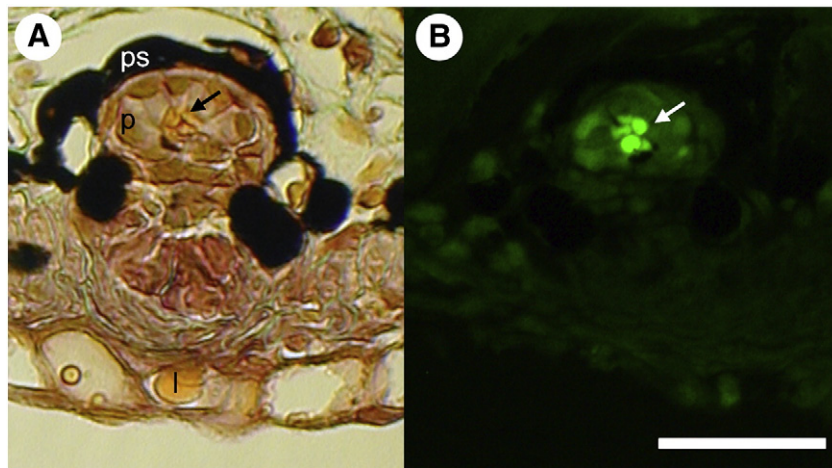
Fig. 2. Transversal section of a ventral photophore of *Etmopterus splendidus*, with (A) and without (B) UV stimulation, showing the different structural elements of the organ. Photocytes (p) emit a green fluorescence under UV stimulation from intracellular vesicles (arrows). l, lens cell; ps, pigmented sheath. Scale bar: 100 μm.

photophores harbouring the same basic structure (several photocytes in a black sheath covered by a lens cell). Knowing that *E. spinax* luminescence is believed to be involved in camouflage by counter-illumination (i.e. when light emission cloaks the silhouette of the luminous animal from below; Claes et al., 2010) and intraspecific functions including sexual signalling and schooling (Claes and Mallefet, 2009a), we could therefore suggest similar functions for *E. splendidus* luminescence. Following this idea, the bright luminescence observed from pelvic areas of this shark is in agreement with sexual signalling (see Claes and Mallefet, 2009a), while the counterillumination hypothesis is suggested by the presence of a long lasting blue luminescence that covers a large portion (>90%) of the ventral surface area in freshly caught *E. splendidus* specimens. Moreover, such a "cloak of invisibility" would be particularly convenient in such a dwarf pelagic shark, which can be an easy prey for upward-looking predators of the mesopelagic zone. Interestingly, the observable differences in the luminous pattern of *E. splendidus* and *E. spinax* (the morphology of the lateral luminous zones and the separation of caudal luminous zones in two parts) concern only bright photogenic areas that are believed to be used in schooling behaviours (Claes and Mallefet, 2009a). This supports the idea that these areas allow species discrimination during cohesive

swimming behaviours. This would be particularly useful for *E. splendidus* which live in sympatry with other similar lantern sharks such as *Etmopterus molleri* and *Etmopterus brachyurus*, as indicated by our catch during the collecting trip. The presence of CDB is intriguing. A similar non-luminous pigmented zone is well known in the bioluminescent dalatiid shark *Isistius brasiliensis* (the "dark collar") that it is supposed to act as a lure for big pelagic predators when the shark counterilluminates (Widder, 1998). In *E. splendidus*, this CDB disrupts the luminous pattern of the tail, probably to increase the contrast with the adjacent photogenic area, improving the visibility of these latest during intraspecific behaviours.

The pharmacological screening demonstrates that *E. splendidus* photophores, like those of *E. spinax* (for a review, see Claes and Mallefet, 2011) are under a complex physiological control that includes hormonal and neural inputs: (i) light-inducing hormones (MT and PRL), (ii) light-inhibiting hormone (α-MSH), and (iii) light-inhibiting classical neurotransmitter (GABA), which is certainly produced permanently since the GABA$_A$ antagonist BICU can elicit light from the photophores (inhibitory tonus). Furthermore, the control of luminescence from *E. splendidus* photophores seems to involve movement of pigments in pigmented cells overlying the

Table 2
Results of the pharmacological screening.

	[Drug] (mol l^{-1})	N	Duration (min)	R L_{max} (Mean ± SEM)	Student's t-test			Effect
					DF	t-value	P-value	
Neural testing								
Neurotransmitters								
Adrenaline	10^{-3}	3	20	1.05 ± 0.05	2	1.0000	0.4226	0
Noradrenaline	10^{-3}		20	1.66 ± 0.59	2	1.0000	0.4226	0
5-HT	10^{-3}	3	20	0.81 ± 0.19	2	1.0000	0.4226	0
GABA	10^{-3}	3	20	3.75 ± 2.54	2	1.0841	0.3916	(−)
Carbachol[†]	10^{-3}	3	20	1.83 ± 0.83	2	1.0000	0.4226	0
NO	10^{-3}	3	20	0.98 ± 0.13	2	0.1287	0.9094	0
BICU	10^{-3}	3	60	0.30 ± 0.05	2	15.5603	0.0041**	+
Hormonal testing								
PRL	10^{-6}	3	60	0.13 ± 0.08	2	11.2483	0.0078**	+
MT	10^{-6}	3	60	0.28 ± 0.07	2	10.7330	0.0086**	+
α-MSH	10^{-6}	3	60	1.82 ± 1.15	2	0.7130	0.5498	(−)

0, no effect of the test substance; +, when saline control elicited significantly less light than the test substance. "response curves"; (−), when saline control elicited more light than the test substance (when relative L_{max} ± SEM>1). *P<0.05, **P<0.01.

α-MSH, α-melanocyte stimulating hormone; 5-HT, 5-hydroxytryptamine (serotonin); BICU, bicuculline; GABA, γ-aminobutyric acid; MT, melatonin; α-MSH, α-melanocyte stimulating hormone; 5-HT, 5-hydroxytryptamine (serotonin); hydroxytryptamine (serotonin); L_{max}, total quantity of light emitted during the experiment; NO, nitric oxide; PRL, prolactin; R L_{max}, relative L_{max} i.e. L_{max} saline control/L_{max} test substance.
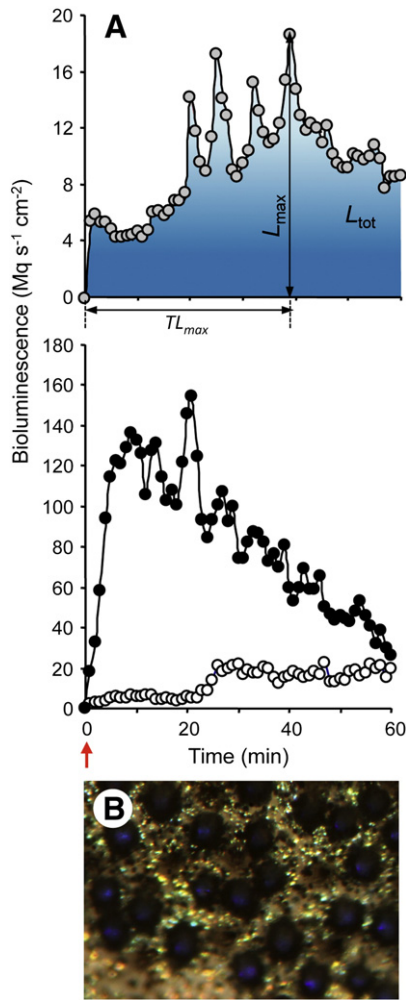
[†] Cholinergic agonist.

Fig. 3. (A) Original recordings of luminescence induced by bicuculline (BICU) (10^{-3} mol l^{-1}; grey circles), melatonin (10^{-6} mol l^{-1}; white circles) and prolactin (10^{-6} mol l^{-1}; black circles). For informative purpose, luminescence parameters are presented on BICU-induced luminescence recording: (i) the maximum light emission (L_{max}), (ii) the total quantity of light emitted during the experiment (L_{tot}) and the time to reach L_{max} from test substance application (red arrow) (TL_{max}). (B) Picture showing iris-like morphology of photophore when they are emitting light. Scale bar: 100 μm.

respective clades split up at least 31.55 MYA (Straube et al., 2010), is interesting since it supports the idea that this control was selected early in the evolution of lantern sharks, probably quickly after (or concomitantly with) their colonisation of deepwater niches (Claes and Mallefet, 2010).

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photocytes, similar to what was found in *E. spinax* (Claes and Mallefet, 2010). The similarity between the physiological control of photophore luminescence in these two distantly related lantern sharks, their