

## Hormonal control of luminescence from lantern shark (*Etmopterus spinax*) photophores

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### SUMMARY

The velvet belly lantern shark (*Etmopterus spinax*) emits a blue luminescence from thousands of tiny photophores. In this work, we performed a pharmacological study to determine the physiological control of luminescence from these luminous organs. Isolated photophore-filled skin patches produced light under melatonin (MT) and prolactin (PRL) stimulation in a dose-dependent manner but did not react to classical neurotransmitters. The  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) had an inhibitory effect on hormonal-induced luminescence. Because luzindole and 4P-PDOT inhibited MT-induced luminescence, the action of this hormone is likely to be mediated through binding to the MT2 receptor subtype, which probably decreases the intracellular concentration of cyclic AMP (cAMP) because forskolin (a cAMP donor) strongly inhibits the light response to MT. However, PRL seems to achieve its effects *via* janus kinase 2 (JAK2) after binding to its receptor because a specific JAK2 inhibitor inhibits PRL-induced luminescence. The two stimulating hormones showed different kinetics as well as a seasonal variation of light intensity, which was higher in summer (April) than in winter (December and February). All of these results strongly suggest that, contrary to self-luminescent bony fishes, which harbour a nervous control mechanism of their photophore luminescence, the light emission is under hormonal control in the cartilaginous *E. spinax*. This clearly highlights the diversity of fish luminescence and confirms its multiple independent apparitions during the course of evolution.

Key words:  $\alpha$ -MSH, bioluminescence, Chondrichthyes, *Etmopterus spinax*, melatonin, prolactin.

### INTRODUCTION

Lantern sharks (Etmopteridae) are deep-water Chondrichthyes, which received their name from their ability to produce a visible light through a chemical reaction. Due to the relative rarity of most of these sharks and the limited accessibility of their environment, the function of this bioluminescence has never been experimentally tested. Recent works strongly support the implication of this phenomenon in camouflage by counter-illumination as well as in intraspecific behaviours such as mating and schooling (Claes and Mallefet, 2008; Claes and Mallefet, 2009).

The velvet belly lantern shark (*Etmopterus spinax*) emits a blue luminescence from thousands of tiny (*ca.* 150  $\mu$ m) photophores organised into nine different luminous zones forming a complex luminous pattern (Claes and Mallefet, 2008; Claes and Mallefet, 2009). These epidermic organs are relatively simple in structure, lacking reflectors or specialised filters (Hubbs et al., 1967). The light from the photocytes (i.e. the photogenic cells) passes through the lens cell(s) before being emitted to the outside. A pigmented iris-like structure (ILS) is present between the photocytes and lens cells, and a pigmented sheath protects the underlying tissues; this sheath is penetrated by large sinuses bringing blood inside the photophore's lumen (Ohshima, 1911).

To be efficiently used by the shark in its natural environment, this light emission must be precisely controlled. Counter-illumination, for example, is only efficient if the light produced has the same characteristics (intensity, wavelength and angular distribution) as found in the luminous background (Clarke, 1963; Denton et al., 1972; Denton et al., 1985; Harper and Case, 1999). Moreover, the shark should be able to switch off its luminescence when not needed.

Control mechanisms of shark luminescence are poorly understood and rarely investigated (Case and Strause, 1978). The slow onset of luminescence (Ohshima, 1911) and the absence of photophore innervation (Johann, 1899) in some investigated species, led Harvey to hypothesise a hormonal control of luminescence in sharks (Harvey, 1952). This control could, for example, target the chromatophores of the ILS whose action has already been suggested to allow a regulation of the amount of light emitted outside (Ohshima, 1911; Iwai, 1960). This could explain why Herring failed to induce luminescence in the luminescent cookie-cutter shark, *Isistius brasiliensis*, using acetylcholine as well as adrenaline (Herring and Morin, 1978), which is currently considered the main neurotransmitter triggering luminescence in Osteichthyes (Baguet, 1975; Baguet and Marechal, 1978; Baguet and Christophe, 1983) (J.M., unpublished data).

Assuming the hypothesis of a hormonal control of *E. spinax*'s luminescence through the ILS, three different hormones, implied in the regulation of the physiological colour change in Elasmobranchs (sharks and rays), are good candidates: prolactin (PRL),  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) (which stimulate melanosome dispersion and therefore induce skin darkening after visual stimulation) and melatonin (MT) [which is thought to regulate (through changes in  $\alpha$ -MSH release) skin colouration resulting from a non-visual perception of light level] (Visconti et al., 1999; Gelsleichter, 2004).

In this work, the control mechanism of luminescence in the velvet belly lantern shark was pharmacologically investigated. Using isolated ventral skin patches of *E. spinax*, we performed a screening of test substances for neurotransmitters and hormones to identify

Table 1. Specimen collection period, number, size and ventral photophore density

Sampling period	N	Total length (cm)	Ventral photophore density (unit cm <sup>-2</sup> )
February 2008	7 (0)	46.4±1.2	1596±67
April 2008	8 (3)	42.6±1.3	1727±80
December 2008	22 (2)	44.6±1.1	2493±88
February 2009	20 (9)	43.3±0.9	2710±126

N, number of specimens used in the analysis (number of males used per period of time is included in parentheses).

those eliciting luminescence (extrinsic control) and then explored the intrinsic control pathways of these drugs.

## MATERIALS AND METHODS

### Experimental fish

Fifty-seven adult velvet belly lantern sharks, *Etmopterus spinax* Linnaeus [30–52 cm total length (TL)] were captured during four field collections (February 2008, April 2008, December 2008 and February 2009) by long lines lowered in a deep area (depth ≥ 200 m) of the Raunefjord, Norway (Table 1). Living sharks were brought to the High Technological Center of Bergen ('Hoyteknologisenteret i Bergen', HiB) or to the Espeland Marine Station and were housed in 1 m × 1 m × 1 m tanks placed in dark, cold (6°C) rooms. Following the rules of HiB for experimental fish care, all sharks were killed by a blow on their heads before experimentation took place.

### Luminometry

The photophore preparations used in this work were skin patches of *E. spinax* specimens dissected out from their ventral luminous zone using a metal cap driller (diameter = 0.55 cm); this preparation will be called 'ventral skin patches' (Fig. 1A–C). After dissection, these ventral skin patches were placed in a shark saline [292 mmol l<sup>-1</sup> NaCl, 3.2 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 0.6 MgSO<sub>4</sub>, 1.6 mmol l<sup>-1</sup> Na<sub>2</sub>SO<sub>4</sub>, 300 mmol l<sup>-1</sup> urea, 150 mmol l<sup>-1</sup> trimethylamine N-oxide, 10 mmol l<sup>-1</sup> glucose, 6 mmol l<sup>-1</sup> NaHCO<sub>3</sub>; total osmolarity: 1.080 mosmol; pH 7.7 (Bernal et al., 2005)] following the method of Claes and Mallefet (Claes and Mallefet, 2008). The number of photophores present in a ventral skin patch of each animal was counted under binocular microscope (Leitz Diaplan, Oberkochen, Germany).

For screening of neural control, ventral skin patches were transferred to small Perspex chambers filled with 200 µl of the shark saline; the light-emitting surface areas of the patches were orientated to the photo-detector of a luminometer (Berthold FB12; Pforzheim, Germany) calibrated using a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK). Light emissions were recorded for 10 min using Berthold single kinetic mode (Sirius protocol manager v1.4). Data were collected every 0.2 s on a laptop computer to build original curves.

The usual slow kinetics of hormonal drugs permitted to conduct several long-lasting (about one hour) experiments simultaneously. For this purpose, preparations were placed in 100 µl saline-filled holes of one multiplate luminometer (Berthold MPL2/Orion; Pforzheim, Germany) calibrated with the same standard light source. According to the number of experiments, Berthold Simplicity software parameters were adjusted in order to obtain at least one point every minute. The luminescent responses were characterised using different parameters (Fig. 1D): the maximum intensity of light emission [ $L_{\max}$ , in megaquanta per second (Mqs<sup>-1</sup>)], the total quantity of light emitted during the experiment [ $L_{\text{tot}}$ , in teraquanta per hour (Tq h<sup>-1</sup>)], and the time to reach  $L_{\max}$  from the stimulation time [ $TL_{\max}$ , in seconds (s)]. Light parameters were standardised by skin surface area (in cm<sup>-2</sup>).

### Pharmacology

As a first step to trigger light emission, test substances (Table 2) were injected on ventral skin patches to reach the desired working dilution (10<sup>-4</sup> and 10<sup>-6</sup> for neural and hormonal testing, respectively; Table 2). In addition, potassium chloride (KCl, 200 mmol l<sup>-1</sup>) was also injected in order to induce luminescence by nerve depolarisation. Ventral skin patches were injected with

Table 2. Pharmacological drugs used in this study

Drugs	Action	Working dilution (mol l <sup>-1</sup> )	Source
<b>Extrinsic control</b>			
Adrenaline	Neurotransmitter	10 <sup>-3</sup>	Sigma Chemical Co. (St Louis, MO, USA)
Noradrenaline	Neurotransmitter	10 <sup>-3</sup>	Fluka (Buchs, Switzerland)
Carbachol	Neurotransmitter	10 <sup>-3</sup>	Sigma
5-HT	Neurotransmitter	10 <sup>-3</sup>	Sigma
GABA	Neurotransmitter	10 <sup>-3</sup>	Sigma
PRL	Hormone	10 <sup>-5</sup> –10 <sup>-8</sup>	Sigma
MT	Hormone	10 <sup>-4</sup> –10 <sup>-7</sup>	Acros Organics (Pittsburgh, PA, USA)
α-MSH	Hormone	10 <sup>-6</sup> –10 <sup>-7</sup>	Sigma
Luzindole	Antagonist MT1/MT2	10 <sup>-4</sup>	Sigma
4P-PDOT	Antagonist MT2	2 × 10 <sup>-4</sup> –2 × 10 <sup>-7</sup>	Tocris Bioscience (Ellisville, MO, USA)
<b>Intrinsic control</b>			
dibutyryl-cAMP	cAMP analogue (cAMP↓)	10 <sup>-4</sup>	Sigma
Forskolin	Adenylyl cyclase activator (cAMP↑)	10 <sup>-4</sup>	Sigma
SQ22,536	Adenylyl cyclase inhibitor (cAMP↑)	10 <sup>-5</sup>	Sigma
MDL-12,330A	Adenylyl cyclase inhibitor (cAMP↑)	10 <sup>-5</sup>	Sigma
1,2,3,4,5,6-Hexabromocyclohexane	JAK2 inhibitor (JAK2↓)	10 <sup>-4</sup>	Calbiochem Merck (Darmstadt, Germany)

PRL, prolactin; MT, melatonin; α-MSH, α-melanocyte-stimulating hormone; 5-HT, serotonin.

shark saline as control. For each drug, we compared the  $L_{\text{tot}}$  values obtained for treated and control skin patches from different adult sharks. After this screening, only drugs having significantly produced more (or less) light than the control were considered as

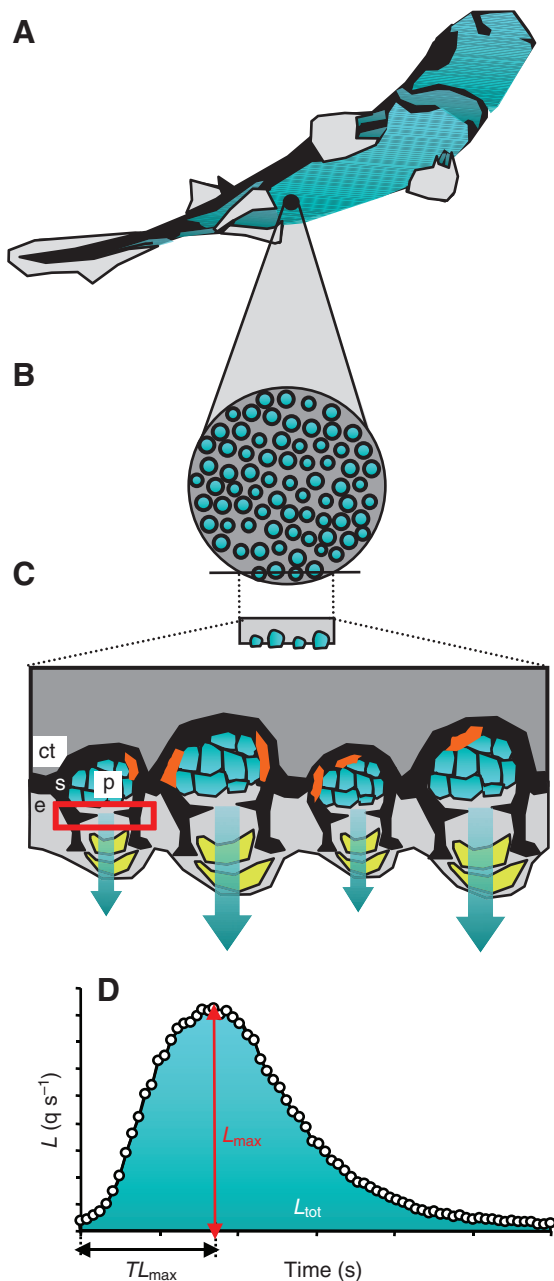


Fig. 1. Photophores and luminescence of *Etmopterus spinax*. (A) Ventral view of the shark with its luminous pattern (blue colour). (B) Ventral skin patch. (C) Structure of glowing photophores. The light (blue arrows) is produced in photocytes, passes through the lens (in yellow) and is finally emitted to the outside. It has been suggested that the iris-like structure (ILS, red rectangle) can regulate the amount of light emitted to the outside. Blood sinuses passing through the pigmented sheath are presented in orange colour. (D) Typical luminescence emission curve with associated kinetic parameters. ct, conjunctive tissue; e, epidermis;  $L$ , light emission ( $\text{q s}^{-1}$ );  $L_{\text{max}}$ , maximum intensity of light emission;  $L_{\text{tot}}$ , total quantity of light emitted during the experiment; p, photocyte; s, pigmented sheath;  $TL_{\text{max}}$ , time from stimulation to  $L_{\text{max}}$ .

good candidates for a more detailed analysis. Pharmacological analysis consisted in (i) dose–light response curves to determine the sensitivity of the tissue, (ii) use of antagonists (Table 2) to signal the presence of a specific receptor in the tissue investigated, and (iii) use of second messenger analogues and enzyme activators and inhibitors (Table 2) to determine the intrinsic control of the photophores in *E. spinax*. When inhibitors or antagonists were used, the ventral skin patches were first immersed for 20 min [except for the janus kinase 2 (JAK2) inhibitor, which necessitated a longer pre-treatment (Sandberg et al., 2005)] in a saline containing the inhibitor (or antagonist) at the desired final concentration and then received an injection of saline containing both the inhibitor (or antagonist) at the desired final concentration and the drug at a concentration that allows the final concentration to be the best working dilution. Due to large individual variability in the amplitude of light responses in *E. spinax*, dose–light response curves were given in relative units, and effect of antagonists, inhibitors and activators in percentage of control, i.e. the luminescence induced by the tested drug injected alone. All the experiments were conducted at room temperature ( $18^{\circ}\text{C}$ ).

#### Statistical analysis

All analyses [Student's  $t$ -tests and linear regressions] were performed with the software SAS/STAT (STAT Institute Inc., 1990, Cary, NC, USA). Two regression slopes were only considered to be significant if there was no overlapping between their 95% slope intervals. Each value was expressed with its standard error (means  $\pm$  s.e.m.), and 'N' equals the number of ventral skin patches used for a specific treatment (which actually corresponds to the number of individual sharks used).

## RESULTS

### Extrinsic control

#### Drug screening

The effect of classical neurotransmitters, KCl and hormonal drugs on ventral skin patches was investigated in adult specimens of *E. spinax*. Saline injection was used as control. Classical neurotransmitters and KCl evoked a minor light emission whose  $L_{\text{tot}}$  was, on average, not significantly ( $P > 0.05$ ) different from the  $L_{\text{tot}}$  obtained with a saline injection (Table 3). Injection of MT and PRL always induced light responses, which were significantly ( $P < 0.05$ ) higher than those of their control (Table 3). These light emissions typically lasted at least 30 min. Ventral skin patches pre-treated with  $\alpha$ -MSH, however, produced significantly ( $P < 0.05$ ) less light than the saline control (Table 3).

#### Kinetics of hormone-induced luminescence

Ventral skin patches stimulated by MT and PRL produced a glow quickly after injection but the time course was very different according to the hormone considered (Fig. 2A). The two hormones showed a highly significant ( $P < 0.001$ ) but different relationship between  $L_{\text{tot}}$  and  $L_{\text{max}}$  (Fig. 2B). The dose–light response curve of both hormones showed a similar pattern. The  $L_{\text{max}}$  progressively increased and attained its highest value at  $10^{-6} \text{ mol l}^{-1}$  to stay more or less constant at higher concentrations while the  $TL_{\text{max}}$  decreased when concentration increased (Fig. 3A,B).  $L_{\text{max}}$  in response to PRL and MT was also found to vary according to the time of year. In April, both hormonal drugs showed higher  $L_{\text{max}}$  than during the winter months and especially in February (Fig. 4A). However, the  $TL_{\text{max}}$  stayed constant over the year and corresponded to  $40.68 \pm 2.95 \text{ min}$  ( $N=44$ ) for MT and  $18.36 \pm 0.73 \text{ min}$  ( $N=49$ ) for PRL (Fig. 4B).

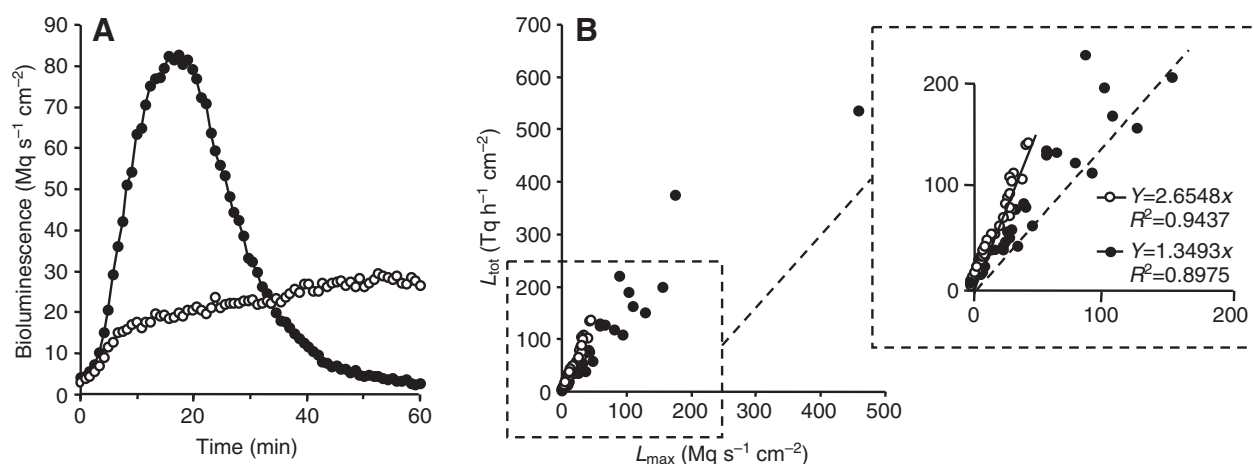


Fig. 2. (A) Original recordings of luminescence induced by melatonin (MT) ( $10^{-6}$  mol l<sup>-1</sup>; open circles) and prolactin (PRL) ( $10^{-6}$  mol l<sup>-1</sup>; closed circles) from ventral skin patches of a mature 44 cm total length female specimen of *Etmopterus spinax*. Data points were recorded every 49 s using a multiplate luminometer MPL2 (Berthold, Pforzheim, Germany). (B) Relationship ( $P < 0.001$ ) between maximum intensity of light emission ( $L_{\max}$ ) and total quantity of light emitted during a given period ( $L_{\text{tot}}$ ) for MT (open circles;  $N=36$ ) and PRL (closed circles;  $N=41$ ). For a same  $L_{\max}$  value, MT shows a higher  $L_{\text{tot}}$ . Insert, enlarged view showing the relationship  $L_{\max}$ - $L_{\text{tot}}$  for lower  $L_{\max}$  values of light production obtained with both hormones.

An additive effect for  $L_{\max}$  and  $L_{\text{tot}}$  was obtained with a simultaneous injection of  $10^{-6}$  mol l<sup>-1</sup> MT and  $10^{-6}$  mol l<sup>-1</sup> PRL (Table 4). The luminescence resulting from this injection, however, showed a  $TL_{\max}$  equal to the  $TL_{\max}$  of PRL and significantly ( $P < 0.01$ ) smaller than the  $TL_{\max}$  of MT (Table 4).

To estimate the inhibitory effect of  $\alpha$ -MSH, dose–light response curves were conducted with a simultaneous injection of either PRL or MT at their most effective stimulating concentration ( $10^{-6}$  mol l<sup>-1</sup>) and  $\alpha$ -MSH at different concentrations. The patterns of the dose–light response curves are given in Fig. 5A,B. Depending on the experiment, the control was either PRL or MT injected alone at  $10^{-6}$  mol l<sup>-1</sup>. At  $10^{-8}$  mol l<sup>-1</sup>, no significant effect of  $\alpha$ -MSH on the time course of luminescence was detected. At  $10^{-7}$  mol l<sup>-1</sup>, however,  $\alpha$ -MSH very significantly decreased the  $TL_{\max}$  of MT ( $P < 0.01$ ) and the  $L_{\max}$  of PRL ( $P < 0.001$ ) whereas all light parameters of both stimulating hormones were decreased by a  $10^{-6}$  mol l<sup>-1</sup> injection of  $\alpha$ -MSH (Fig. 5A,B). While the  $TL_{\max}$  and the  $L_{\max}$  of MT-induced light response as well as the  $L_{\max}$  of PRL-induced light response were significantly reduced ( $P < 0.001$ ) by  $\alpha$ -MSH, this hormone had a lesser effect ( $P < 0.05$ ) on the  $TL_{\max}$  of PRL-induced luminescence (Fig. 5A,B).

#### Effect of antagonists on hormonal-induced luminescence

The treatment of ventral skin patches with  $10^{-4}$  mol l<sup>-1</sup> MT1/MT2 receptor antagonist luzindole and  $2 \times 10^{-4}$  mol l<sup>-1</sup> MT2-specific receptor antagonist 4P-PDOT did not induce luminescence *per se*.

The MT ( $10^{-6}$  mol l<sup>-1</sup>)-induced luminescence was highly significantly ( $P < 0.001$ ) decreased by  $2 \times 10^{-4}$  mol l<sup>-1</sup> and  $2 \times 10^{-5}$  mol l<sup>-1</sup> 4P-PDOT as well as by  $2 \times 10^{-4}$  mol l<sup>-1</sup> luzindole (Fig. 6A,B). 4P-PDOT had, however, no effect on MT-induced luminescence ( $10^{-6}$  mol l<sup>-1</sup>) at lower concentrations (Fig. 6A).

#### Intrinsic control

##### MT and cAMP pathway

The treatment of ventral skin patches with  $10^{-4}$  mol l<sup>-1</sup> dibutyryl-cAMP (db-cAMP), a membrane-permeable analogue of adenosine 3',5'-cyclic monophosphate (cAMP),  $10^{-4}$  mol l<sup>-1</sup> forskolin (FSK) and  $10^{-5}$  mol l<sup>-1</sup> of the two adenylyl cyclase inhibitors (SQ22,536 and MDL-12,330A) did not induce luminescence *per se*.

Even though  $10^{-4}$  mol l<sup>-1</sup> FSK significantly ( $P < 0.001$ ) decreased the MT-induced luminescence ( $10^{-6}$  mol l<sup>-1</sup>) from ventral skin patches, this luminescence was not affected by  $10^{-4}$  mol l<sup>-1</sup> db-cAMP,  $10^{-5}$  mol l<sup>-1</sup> SQ22,536 and  $10^{-5}$  mol l<sup>-1</sup> MDL-12,330A (Fig. 7A).

Table 3. Results of the pharmacological screening

	[Drug] (mol l <sup>-1</sup> )	N	Duration (min)	$L_{\text{tot}}$ saline (Tq h <sup>-1</sup> cm <sup>-2</sup> )	$L_{\text{tot}}$ treatment (Tq h <sup>-1</sup> cm <sup>-2</sup> )	P-value	Effect
<b>Neural testing</b>							
Neurotransmitters							
Adrenaline	$10^{-3}$	6	10	1.64±0.92	0.55±0.38	0.2981	0
Noradrenaline	$10^{-3}$	6	10	1.50±0.82	0.73±0.20	0.3972	0
5-HT	$10^{-3}$	7	10	2.02±0.87	3.25±0.99	0.3697	0
GABA	$10^{-3}$	7	10	2.02±0.87	2.59±0.18	0.6101	0
Carbachol*	$10^{-3}$	6	10	1.64±0.92	0.58±0.16	0.3067	0
KCl	$2 \times 10^{-1}$	6	10	2.31±0.97	4.37±2.66	0.4922	0
<b>Hormonal testing</b>							
PRL	$10^{-6}$	8	60	3.74±0.90	193.82±60.80	0.0167	+
MT	$10^{-6}$	8	60	3.74±0.90	71.86±13.46	0.0014	+
$\alpha$ -MSH	$10^{-6}$	8	60	3.74±0.90	1.15±0.40	0.0265	-

\*Cholinergic agonist. -, inhibition; 0, no effect; +, potentiation. PRL, prolactin; MT, melatonin; 5-HT, serotonin;  $\alpha$ -MSH,  $\alpha$ -melatonin-stimulating hormone;  $L_{\text{tot}}$ , total light emission; Tq h<sup>-1</sup> cm<sup>-2</sup>, teraquanta per hour per square centimetre.



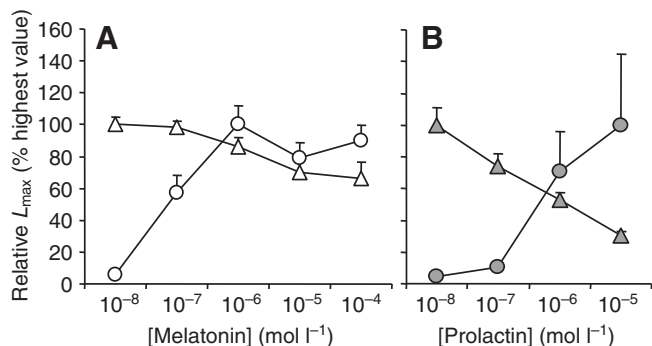


Fig. 3. Dose–light response curve of (A) melatonin and (B) prolactin from ventral skin patches of *Etmopterus spinax*. Values of maximum intensity of light emission ( $L_{\max}$ ) (circles) and time from stimulation to  $L_{\max}$  ( $TL_{\max}$ ) (triangles) are expressed as a percentage of the highest value obtained ( $N=16$  for each concentration).

#### PRL and JAK2 pathway

The JAK2 inhibitor did not induce luminescence from ventral skin patches *per se* but significantly ( $P<0.001$ ) decreased the PRL-induced luminescence ( $10^{-6}$  mol l<sup>-1</sup>) from ventral skin patches pre-treated during 5 h (Fig. 7B).

#### DISCUSSION

This work represents the first detailed study of control mechanisms of photophore luminescence in a cartilaginous fish, the velvet belly lantern shark (*E. spinax*), at both the extrinsic and intrinsic levels. It provides evidence for the first time of a hormonal control of luminescence in a fish. It also highlights the diversity of fish

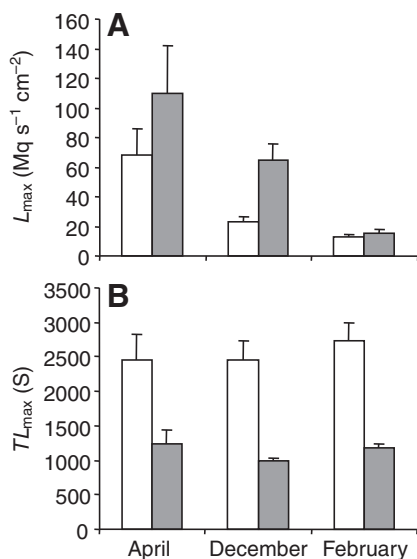


Fig. 4. Comparison of (A) the maximum rate of light emission ( $L_{\max}$ ) produced by ventral skin patches of *Etmopterus spinax* as a response to melatonin (MT) and prolactin (PRL) stimulations as well as (B) the time between stimulation and  $L_{\max}$  ( $TL_{\max}$ ) between the different months when experiments were performed (open bars=MT; closed bars=PRL). Specimens tested in April show higher  $L_{\max}$  than those tested during winter months.  $TL_{\max}$  values of luminescence provoked by both hormones do not show any significant variations over the year. Number of replicates: April,  $N=8$ ; December,  $N=22$ ; February,  $N=14$  (MT) and  $N=19$  (PRL).

Table 4. Effect of a simultaneous injection of MT ( $10^{-6}$  mol l<sup>-1</sup>) and PRL ( $10^{-6}$  mol l<sup>-1</sup>) on light emission

Light parameters	Control* ( $N=8$ )	MT+PRL ( $N=8$ )	Student's <i>t</i> -test	
			<i>t</i> -value	<i>P</i> -value
$L_{\max}$ (Mq s <sup>-1</sup> cm <sup>-2</sup> )	1	1.51±0.52	0.98	0.3588
$L_{\text{tot}}$ (Tq h <sup>-1</sup> cm <sup>-2</sup> )	1	0.92±0.25	-0.31	0.7683
$TL_{\max}$ (min) <sup>†</sup>	1	1.00±0.08	0.04	0.9661
$TL_{\max}$ (min) <sup>‡</sup>	1	0.54±0.11	-4.09	0.0046

\*Control is the sum of the values obtained for prolactin (PRL) and melatonin (MT) tested separately.

<sup>†</sup>Student *t*-test performed using  $TL_{\max}$  (time between stimulation and maximum intensity light emission) of PRL injected alone as control value.

<sup>‡</sup>Student *t*-test performed using  $TL_{\max}$  of MT injected alone as control value.

MT, melatonin; PRL, prolactin;  $L_{\max}$ , maximum intensity of light emission;  $L_{\text{tot}}$ , total light emission; Mq s<sup>-1</sup> cm<sup>-2</sup>, megaquanta per second per square centimetre; Tq h<sup>-1</sup> cm<sup>-2</sup>, teraquanta per hour per square centimetre.

luminescence physiology and confirms that this capability evolved independently multiple times (Hastings, 1983; Herring, 1987). Finally, by working on isolated ventral skin patches, it offers a new valid technique to investigate luminescence control mechanisms of sharks whose photophores are extremely tiny (*ca.* 150 μm) compared with those of bony fishes.

#### Hormonally controlled luminescence: pathways

The different classical neurotransmitters used in this study failed to induce luminescence from isolated photophores of *E. spinax*. This and the absence of a reaction to KCl strongly suggest that, contrary to luminescent bony fishes, shark's photophores are not under direct nervous control. This is in agreement with the observation of Johann (Johann, 1899), who was not able to find any innervation in the photophores of the velvet belly lantern shark.

MT and PRL, however, show a dose-dependent stimulatory effect on light emission from photophores of isolated ventral skin patches of *E. spinax*. The comparison of the dose–light response curves show

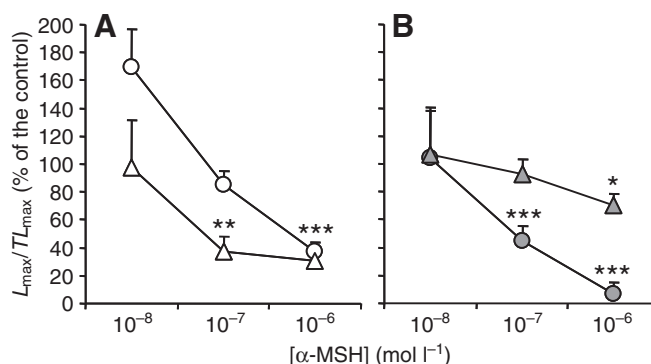


Fig. 5. Dose-dependent inhibitory effect of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) on maximal intensity of light emitted ( $L_{\max}$ ; circles) and time between the start of light emission to  $L_{\max}$  ( $TL_{\max}$ ; triangles) of (A) melatonin ( $10^{-6}$  mol l<sup>-1</sup>) and (B) prolactin ( $10^{-6}$  mol l<sup>-1</sup>)-induced light emission from ventral skin patches of *Etmopterus spinax*. Values are expressed as a percentage of values obtained in control ventral skin patches ( $N=10$  for each concentration). Asterisks indicate significant differences between control and treated ventral skin patches (\* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ).

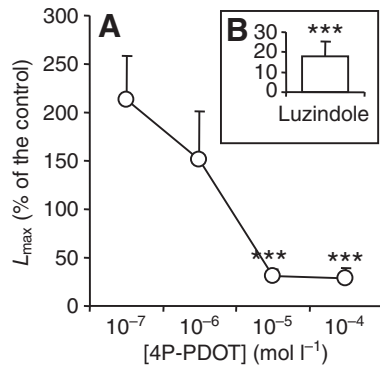


Fig. 6. Effect of antagonists on melatonin-induced luminescence. (A) Dose-dependent inhibitory effect of 4P-PDOT on maximal intensity of light emitted ( $L_{max}$ ; open circles) from melatonin (MT) ( $10^{-6}$   $\text{mol l}^{-1}$ )-stimulated ventral skin patches of *Etmopterus spinax*. (B) Effect of luzindole at  $10^{-4}$   $\text{mol l}^{-1}$  on maximal intensity of light emitted ( $L_{max}$ ; open bar) from MT ( $10^{-6}$   $\text{mol l}^{-1}$ )-stimulated ventral skin patches of *E. spinax*. Values are expressed as a percentage of those obtained in control ventral skin patches ( $N=8$  for each treatment). Asterisks indicate significant differences between control and treated ventral skin patches ( $***P<0.001$ ).

similar sensitivity of the photophores to both hormones between  $10^{-5}$   $\text{mol l}^{-1}$  and  $10^{-8}$   $\text{mol l}^{-1}$ . The time course of luminescence triggered by the two hormones is, however, different but reproducible: while response to MT injection is characterised by a slow increase of light intensity (which can last several hours; J.M.C., unpublished data), PRL induced a relatively quicker response, which lasts one hour at maximum. This, with the additive effect of a simultaneous injection of MT and PRL, suggests that these two hormones use a different pathway to induce light emission in *E. spinax*'s photophores. In addition to these hormonal stimulating effects, an inhibitory effect of the  $\alpha$ -MSH on photophore luminescence was also shown. These combined results strongly suggest that the light emission of lantern shark is controlled by different hormones. This would explain the slow onset of luminescence observed in this species (Ohshima, 1911). Assuming luminescence to be hormonally controlled, the hormones certainly use the large blood sinuses that go through the pigmented sheath

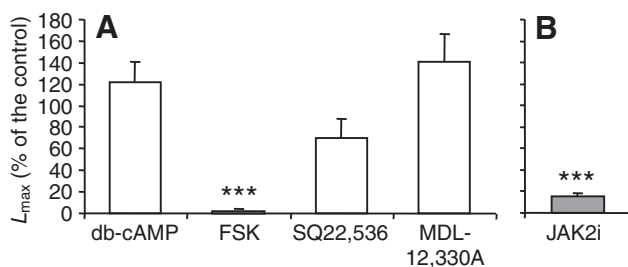


Fig. 7. Intrinsic pathway of melatonin and prolactin in the control of luminescence from ventral photophore of *Etmopterus spinax*. (A) Effect of  $10^{-4}$   $\text{mol l}^{-1}$  dibutyryl-cAMP (db-cAMP),  $10^{-4}$   $\text{mol l}^{-1}$  forskolin (FSK),  $10^{-5}$   $\text{mol l}^{-1}$  SQ22,536 and  $10^{-5}$   $\text{mol l}^{-1}$  MDL-12,330A on melatonin ( $10^{-6}$   $\text{mol l}^{-1}$ )-stimulated ventral skin patches from *E. spinax* (open bars;  $N=8$  for each treatment). (B) Effect of JAK2 inhibitor 1,2,3,4,5,6-Hexabromocyclohexane (JAK2i) on prolactin ( $10^{-6}$   $\text{mol l}^{-1}$ ) stimulated ventral skin patches from *E. spinax* (closed bar,  $N=8$ ). Asterisks indicate significant differences between control and treated ventral skin patches ( $***P<0.001$ ).

of shark's photophores to act on a specific target inside these luminous organs.

In vertebrates, the effects of MT are mediated through high and low affinity receptors. While low affinity receptors (MT3) correspond to a cytosolic enzyme implicated in detoxification processes (the 'quinone reductase-2'), high affinity receptors are transmembrane receptors (Vanecek, 1998; Barrett et al., 2003; Boutin et al., 2005). In fishes, three different high affinity receptors have been found: MT1 (=Mel1a), MT2 (=Mel1b) and Mel1c (Barrett et al., 2003). These high affinity MT receptors can be coupled with several intrinsic pathways, including adenylyl cyclase inhibition (which induces a decrease in intracellular cAMP) or phospholipase C (PLC) activation, which finally modulate intracellular  $\text{Ca}^{2+}$ , or even *via* guanylyl cyclase inhibition (Vanecek, 1998; Barrett et al., 2003).

In this work, MT1/MT2 receptor antagonist luzindole ( $10^{-4}$   $\text{mol l}^{-1}$ ) and MT2-specific receptor antagonist 4P-PDOT ( $2 \times 10^{-4}$   $\text{mol l}^{-1}$ ) both inhibit the action of MT, with 4P-PDOT showing a dose-dependent (from  $2 \times 10^{-7}$   $\text{mol l}^{-1}$  to  $2 \times 10^{-4}$   $\text{mol l}^{-1}$ ) inhibitory effect on MT-induced luminescence. These results are strongly in favour of the presence of melatoninergic MT2 receptors in the ventral photophores of *E. spinax*. Moreover, the strong inhibitory effect of FSK ( $10^{-4}$   $\text{mol l}^{-1}$ ), which activates the adenylyl cyclase and increases intracellular cAMP concentration, on MT-induced luminescence indicates that the shark MT2 receptor is negatively coupled to the cAMP pathway. The absence of effects of adenylyl cyclase inhibitors SQ22,536 ( $10^{-5}$   $\text{mol l}^{-1}$ ) and MDL-12,330A ( $10^{-5}$   $\text{mol l}^{-1}$ ) on light emission triggered by MT are coherent if we consider that the intensity of light emitted after MT injection correspond to the maximum physiological response of the tissue. The contradictory absence of effects with the cAMP analogue db-cAMP ( $10^{-4}$   $\text{mol l}^{-1}$ ) might reveal the presence of a high phosphodiesterase activity inside the photophore that prevents cAMP to act. The lack of effect of db-cAMP has already been observed in other animal preparations and could also be explained by solubility or permeability problems of this substance in preparations (Vanderlinden et al., 2004).

Due to its numerous actions in many different biological mechanisms, PRL is considered as the most versatile hormone in vertebrates (Bole-Feysot et al., 1998; Manson, 2002). Effects of PRL are mediated by the PRL receptor (PRLR), a receptor of the class 1 cytokine receptor superfamily (Bole-Feysot et al., 1998). The PRLR is known to be present in several bony fish species but, to our knowledge, has never been reported in a cartilaginous species (Sandra et al., 1995; Tse et al., 2000; Le Rouzic et al., 2001; Santos et al., 2001; Higashimoto et al., 2001). The binding of PRL to the PRLR induces a homodimerisation of this receptor and activates a tyrosine kinase called JAK2. When activated, JAK2 phosphorylates different proteins and initiates different downstream cascades, including JAK/Stat (signal transducers and activators of transcription) and MAPK (mitogen-activated protein kinase) pathways (Bole-Feysot et al., 1998; Freeman et al., 2000). The strong inhibition of PRL luminescence observed after pre-treatment with the inhibitor of JAK2 suggests strongly that the luminescence triggered by PRL is mediated by the action of JAK2, which is also in favour of the presence of PRLR in the shark photogenic organs.

#### Comparative control of luminescence in fishes

The survival of a luminescent organism depends on its capability to control its light emission. In multicellular animals, this control occurs at two levels: (i) the intrinsic control level, which focuses

Table 5. Photophore control mechanism in luminous fish

	Photophore control*	References
Osteichthyes		
Ogcocephalidae		
<i>Dibranchius atlanticus</i>	Nervous (Adr)	Crane, 1968
Batrachoididea		
<i>Porichthys myriaster</i> , <i>P. notatus</i>	Nervous (Adr, Nadr, 5-HT <sup>†</sup> )	Baguet, 1975; Gariépy and Anctil, 1983
Myctophidae		
<i>Benthoosema glaciale</i>	Nervous	Anctil, 1972
<i>Diaphus holli</i>	Nervous	Baguet, 1975; Baguet and Marechal, 1976
<i>Myctophum punctatum</i>	Nervous	Anctil, 1972; Christophe and Baguet, 1982
<i>Triphoturus mexicanus</i>	Nervous	Barnes and Case, 1974
Gonostomatidae		
<i>Gonostoma spp.</i>	Nervous (Adr)	Herring and Morin, 1978; Herring, 1982
<i>Cyclothone braueri</i>	Nervous (Adr)	J.M. and S. Dupont, unpublished data
Phosichthyidae		
<i>Ichtyococcus ovatus</i>	Nervous	Baguet, 1975; Baguet and Marechal, 1976
Sternoptychidae		
<i>Argyrolepecus hemigygnus</i>	Nervous (Adr, Nadr)/NO <sup>‡</sup>	Baguet and Marechal, 1978; Krönström et al., 2005
<i>Argyrolepecus olfersi</i>	Nervous (Adr)	Bertelsen and Grontveld, 1949
<i>Maurolicus muelleri</i>	Nervous (Adr, Nadr)	Baguet and Christophe, 1983
<i>Maurolicus pennanti</i>	Nervous (Adr)	Bertelsen and Grontveld, 1949
Stomiidae		
<i>Chauliodus sloani</i>	Nervous (Adr, Nadr)	Denton et al., 1972; J.M., unpublished data
<i>Echiostoma barbatum</i>	Nervous (Adr)	Harvey, 1931
<i>Stomias boa</i>	Nervous (Adr)	Baguet, 1975
Chondrichthyes		
Etmopteridae		
<i>Etmopterus spinax</i>	Hormonal (PRL, MT, $\alpha$ -MSH <sup>†</sup> )	Present study

\*The effect of the drugs mentioned is stimulatory except other indication.

<sup>†</sup>Inhibitory effect.

<sup>‡</sup>Modulatory effect.

Adr, adrenaline;  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; MT, melatonin; Nadr, noradrenaline; NO, nitric oxide; PRL, prolactin.

on intracellular events and on the photogenic reaction, and (ii) the extrinsic control level whose action is extracellular. Luminescent bony fishes, especially, show a high diversity of extrinsic control mechanisms depending on the nature of their photogenic organs. While luminescence of fishes harbouring symbiotic bacteria in their photophores can be controlled either mechanically *via* dark shutters and chromatophores or physiologically by controlling the blood supply to these organs, luminescence of fishes with photophores containing endogenous systems are neurally controlled and often show accessory structures, including optical filters and reflectors (Case and Strause, 1978; Herring and Morin, 1978; Denton et al., 1985).

In bony fishes (Osteichthyes), luminescence is triggered by adrenaline (and sometimes noradrenaline) while serotonin (5-HT) is known to inhibit light emission in the midshipman fish *Porichthys notatus* (Table 5). In addition, a modulatory effect of nitric oxide (NO) on adrenaline-stimulated luminescence has been detected in the hatchetfish *Argyrolepecus hemigygnus* (Table 5). Direct neural control of luminescence generally allows precise and rapid on/off light switching, which is useful in predator avoidance and in intraspecific communication.

In the velvet belly lantern shark, photophore luminescence is controlled by hormones, which are also involved in the skin pigmentation control of Elasmobranchs *via* the action of melanophores (Visconti et al., 1999; Gelsleichter, 2004). This strongly suggests that, in this shark, light emission from photocytes is, at least partly, controlled *via* the action of the ILS (formed by pigmented cells) as it has been sometimes suggested in different lantern shark species (Ohshima, 1911; Iwai, 1960). This hormonal control system of *E. spinax*'s luminescence explains the slow

kinetic of light emission, especially in the case of MT luminescence. Far from being unsuited to this shark's ecology, this slow luminescence kinetic could be very convenient for vertical migrations during which the ambient light intensity may increase/decrease gradually. Lantern sharks like other deep-sea shark species possess a 'pineal window' and it has already been suggested that visual information collected by the pineal gland help these fishes to accomplish their vertical migrations (Clark and Kristof, 1990). Assuming that the visual information from the pineal gland is mediated by a release of MT in the blood (Underwood, 1989; Arendt, 1997) and because MT triggers light emission in a dose-dependent manner in *E. spinax*, this hormone could make a direct connection between ambient light and luminescence intensity in this shark. This could allow *E. spinax* to match the intensity of downwelling light with its luminescence, which is particularly convenient for camouflage by counter-illumination, a function already suggested for this shark's luminescence (Claes and Mallefet, 2008; Claes and Mallefet, 2009). Despite the lack of a rapid fine tuning of luminescence by neural control and the absence of ocular photophore, which are used as a luminescence reference standard by counter-illuminating midwater bony fishes (Nicol, 1962; Herring, 1977; Denton et al., 1985; Warrant and Locket, 2002), the hormonal long-lasting luminescence associated to pineal gland might therefore represent a new way to counter-illuminate efficiently. Nevertheless more experiments are needed in order to properly document counter-illumination in *E. spinax*. In Elasmobranchs, PRL and  $\alpha$ -MSH are thought to be released and induce skin darkening after a visual stimulation (Visconti et al., 1999; Gelsleichter, 2004). Similarly, the action of these hormones on shark luminescence may be dependent on visual perception and



therefore to be involved in behaviours necessitating quicker on/off light switching than responses to MT can support, such as in schooling, mating or predator escape.

In this study we show that PRL and MT trigger light emission in *E. spinax*. In addition we report a possible seasonal variation in the response to these hormones, with a higher response in April than in December and February. A similar seasonal variation in luminescence intensity has been suggested for the krill *Meganyctiphanes norvegica* and is thought to be linked to higher ambient light levels in the summer period (Krönström et al., 2007). Seasonal variation in MT-induced luminescence is not surprising because plasma MT content vary with day-length and the season. Using this molecule as a biological internal clock, fishes can therefore anticipate changes in their environment to which they have to synchronise their biological rhythms (Falcón et al., 2007). Because MT is known to affect PRL release in some fish species (Falcón et al., 2003) (Falcón et al., 2007), seasonal variation in PRL-induced luminescence may be directly dependent of seasonal variation in plasma MT content.

#### LIST OF ABBREVIATIONS

$\alpha$ -MSH	$\alpha$ -melanocyte-stimulating hormone
cAMP	cyclic adenosine monophosphate
db-cAMP	dibutylryl cyclic adenosine monophosphate
FSK	forskolin
ILS	iris-like structure
JAK2	janus kinase 2
<i>L</i>	light emission
$L_{\max}$	maximum intensity of light emission
$L_{\text{tot}}$	total light emission
MT	melatonin
MT1	melatonin receptor 1
MT2	melatonin receptor 2
MT3	melatonin receptor 3
PRL	prolactin
PRLR	prolactin receptor
TL	total length
$TL_{\max}$	time between stimulation and maximum intensity light emission

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