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Nitric oxide in the control of luminescence from lantern shark (*Etmopterus spinax*) photophores

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SUMMARY

Photophores (photogenic organs) of the lantern shark *Etmopterus spinax* are under hormonal control, with prolactin (PRL) and melatonin (MT) triggering the light emission. Differential sensitivity to these hormones in adult individuals suggests, however, that the luminescence of this shark is controlled by an additional mechanism. In this study, different techniques were used to investigate a potential modulator of *E. spinax* luminescence – nitric oxide (NO). NO synthase (NOS)-like immunoreactivity (IR) was found in the photocytes (photogenic cells) of the photophores. In addition, acetylated tubulin IR also supported the presence of nerves running through the photogenic tissue and innervating different structural elements of the photophores: photocytes, pigmented cells from the iris-like structure and lens cells. Pharmacological experiments confirmed a modulatory action of NO on the hormonally induced luminescence: NO donors sodium nitroprusside (SNP) and hydroxylamine decreased the time to reach the maximum amplitude (TL_{max}) of MT-induced luminescence while these substances decreased the maximum amplitude of PRL-induced luminescence (and also the TL_{max} in the case of SNP). The small impact of the NOS inhibitor L-NAME on hormonally induced luminescence suggests that NO is only produced on demand. The cGMP analogue 8BrcGMP mimicked the effects of NO donors suggesting that the effects of NO are mediated by cGMP.

Key words: acetylated tubulin, bioluminescence, Chondrichthyes, melatonin, nitric oxide synthase, prolactin.

INTRODUCTION

The velvet belly lantern shark (*Etmopterus spinax*) is a common deepwater shark of the East Atlantic and the Mediterranean Sea (Coehlo and Erzini, 2008). Like other members of the Etmopteridae family, it is able to emit a visible chemical light from thousands of tiny epidermal organs called photophores (Compagno et al., 2005; Claes and Mallefet, 2008). These organs are organised in nine different luminous zones forming a complex luminous pattern (Claes and Mallefet, 2008). Although few studies have focused on the function of this bioluminescence, it is probably used for various purposes including camouflage by counter-illumination and intraspecific behaviours such as schooling and sexual signalling (Claes and Mallefet, 2009a).

Recently, photophores of this shark, unlike those of any other luminescent animal, have been shown to be under hormonal control: (i) application of classical neurotransmitters [(nor)adrenaline, GABA, serotonin and acetylcholine] on *E. spinax* photophore preparations does not induce a light reaction (Claes and Mallefet, 2009b), which contrasts with the neural luminescence control mechanism (mostly adrenergic) of teleost fish intrinsic (non-bacterial) photophores (Baguet, 1975; Baguet and Marechal, 1978; Baguet and Christophe, 1983), and (ii) melatonin (MT) and prolactin (PRL) respectively trigger a slow and a fast light emission, which are both inhibited by a prior application of α-melanocyte stimulating hormone (α-MSH) (Claes and Mallefet, 2009b). Hormonal luminescent responses, however, vary greatly between investigated shark specimens (Claes and Mallefet, 2009b) and a recent study highlighted a differential sensitivity to both hormones between the different luminous zones of the pattern and, in the case of PRL, between sexes for several luminous zones probably involved in mating (Claes and Mallefet, 2010). These results suggest therefore that an additional control mechanism might be present to modulate the hormonally induced luminescence in *E. spinax*.

Nitric oxide (NO) is a small gaseous signalling molecule that plays a key role in invertebrate as well as in vertebrate biological functions (Jacklet, 1997). Its effects are mediated either by the production of cyclic guanosine monophosphate (cGMP) or by the inhibition of mitochondrial respiration in the target cells (Brown, 1995; Jacklet, 1997). Since several recent studies demonstrated the involvement of NO as a neuromodulator of neurally induced luminescence from distantly related marine organisms, such as the northern krill *Meganyctiphanes norvegica* (Krönström et al., 2007) and teleost fish species including the hatchetfish *Argyropelecus hemigymnus* (Krönström et al., 2005) and the midshipman fish *Porichthys notatus* (Krönström, 2009; Krönström and Mallefet, 2009), we investigated the possible role of NO in the modulation of hormonally induced luminescence in the cartilaginous fish *E. spinax*.

In this study, therefore, this hypothesis was investigated by a combination of immunohistochemical and pharmacological methods in order to (i) detect the presence of nitric oxide synthase (NOS) in photophore structures, (ii) highlight photophore innervations, if any, (iii) investigate the putative role of NO in the luminescence control mechanism of *E. spinax* and, if any, (iv) determine the intracellular pathway used by this substance.

MATERIALS AND METHODS

Fish collection

Twenty-eight adult velvet belly lantern sharks *Etmopterus spinax* (Linnaeus 1758) (30–51.5 cm total length, TL) were caught during three field sessions (in February 2009, June 2009 and December 2009) by longlines lowered in a deep area (depth ≥200 m) of the

Raunefjord, Norway. Living specimens were transferred to two large (1 m^3) tanks in a dark cold (6°C) room at Espeland Marine Station (Espegrend, Norway) where they were kept until use. Captive animals were finally killed, before experimentation, by a blow to the head, following the local rules for experimental fish care.

Immunohistochemistry

Ventral skin patches containing photophores were taken from 8 different individuals (6 adults and 2 embryos from a pregnant female) and used for immunohistochemistry. Each tissue was fixed for 48 h in 3% formaldehyde (in saline) then transferred to phosphate-buffered saline (PBS) with sodium azide (NaN_3), and stored overnight in PBS with 30% sucrose before embedding. The tissue was embedded in OCT (Sakura, Zoeterwude, The Netherlands) and quickly frozen in isopentane chilled with liquid nitrogen.

A cryostat microtome (Zeiss Microm International GmbH, Walldorf, Germany) was used to cut 10 µm sections collected on chrome alum gelatine-coated slides and left overnight to dry.

Fluorescence histochemistry

Sections were preincubated with normal donkey serum (10%) for 30 min to prevent non-specific staining. Sections were incubated for 48 h after the application of primary antibodies (Table 1). They were rinsed (3×5 min in PBS with 2% NaCl) and incubated for 60 min after the application of the secondary antibodies (Table 1). All incubations occurred in a humid chamber at room temperature (20°C). Sections were rinsed anew with the same procedure and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Sections were examined using a Nikon eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DMX1200 digital camera.

In order to confirm the specificity of the secondary antibody, several control preparations were performed with omission of the primary antibody. These control experiments also allowed us to highlight potential autofluorescence from the tissue.

Avidin–biotin histochemistry

After preincubation with normal donkey serum, sections were first incubated with the primary and the secondary antibodies (Table 1) following the procedure described above (endogenous peroxidase activity was blocked by H_2O_2 preincubation; 0.3% or 3% for 5–10 min). Sections were then incubated with the avidin-biotinylated peroxidase complex (ABC Elite PK 6100 standard, Vector Laboratories) for 30 min and developed in Vector Nova red substrate kit (SK 4800, Vector Laboratories) for 3 min. Sections were then mounted in 50% glycerol and 50% carbonate buffer (pH 8.5) and examined as described above.

In order to confirm the specificity of the secondary antibody, several control preparations were performed with omission of the primary antibody.

NADPH-diaphorase activity

In addition to the different control experiments mentioned above, we also performed control experiments testing the NADPH-diaphorase activity in the photogenic tissue of *E. spinax*, in order to confirm the staining obtained with antibodies raised against universal NOS (uNOS).

Ventral skin patches containing photophores were taken from 6 different adult individuals, fixed in formaldehyde (3.7% in phosphate buffer) for 2 h, and rinsed for at least 1 h in PBS. The preparations were embedded, frozen and sectioned. They were then preincubated in a solution containing 0.2% β-NADPH (Sigma Chemical Company, St Louis, MO, USA), 0.025% nitro-blue tertrazolium (Sigma), 0.5% methanol, 0.5% dimethylformamide, 0.01% DMSO and 0.3% Triton X in PBS for 1 h at 37°C. The sections were rinsed for 4×5 min in PBS and mounted in carbonate-buffered glycerol (50% glycerol and 50% carbonate buffer, pH 8.5).

In order to confirm the specificity of the staining, we included several control skin patches that received a similar solution but without the β-NADPH.

Pharmacology

Luminometry

Photophore preparations used in this work consisted of circular ventral skin patches (0.25 cm^2) containing photophores dissected out from adult shark specimens with a metal cap driller and placed in a shark saline (Bernal et al., 2005) following the method of Claes and Mallefet (Claes and Mallefet, 2009b). These preparations will hereafter be called 'ventral skin patches' (VSP).

VSP were transferred to a 96-wells plate, each well containing 100 µl saline, which was inserted in a luminometer (Berthold Orion, Pforzheim, Germany) calibrated with a standard 470 nm light source (Beta light, Saunders Technology, Hayes, UK). The luminescent responses were recorded for 1 h after the stimulation and were characterised using different light parameters (Fig. 1): 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 teraquanta per hour, Tq h^{-1}), and the time to reach L_{max} from the stimulation time (TL_{max}, in seconds). Light parameters were standardised by skin surface area (cm^{-2}).

Chemicals

Chemical agents used in this study were the followings: 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt

Table 1. Antibodies and peptides used for immunohistochemistry

Antibody	Host	Working dilution	Antigen (peptide sequence)	Source
Primary				
uNOS PA1-039	Rabbit	1:100	Murine iNOS and nNOS C-terminal (1113–1122)	Transduction Laboratories[1]
Acetylated tubulin T-6793	Mouse	1:1000	Sea urchin acetylated tubulin	Sigma[2]
Secondary				
DaM-FITC 715-095-150	Donkey	1:100		Jackson ImmunoResearch Laboratories[3]
DaR-biotin 711-065-152	Donkey	1:400		Jackson ImmunoResearch Laboratories
DaR-Cy3 711-165-152	Donkey	1:800		Jackson ImmunoResearch Laboratories

Antibodies against nitric oxide synthase (NOS) are polyclonals while those against acetylated tubulin are monoclonal.
DaM, donkey anti-mouse; DaR, donkey anti-rabbit; uNOS, universal NOS, i.e. antibody reacting to all NOS isoforms.
iNOS, inducible NOS; nNOS, neuronal NOS.
[1]San Jose, CA, USA; [2]St Louis, MO, USA; [3]West Grove, PA, USA.

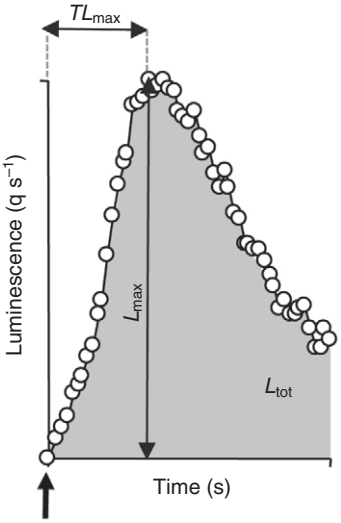

Fig. 1. Typical luminescence emission (in quanta per second, q s^{-1}) curve from photophores of *Etmopterus spinax* showing the different light parameters. L_{max}, maximum intensity of light emission; L_{tot} total quantity of light emitted during the experiment (shaded area); TL_{max}, time from stimulation (arrow) to maximum intensity of light emission (L_{max}).

monohydrate (8BrcGMP; Sigma), hydroxylamine hydrochloride (Sigma), melatonin (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; Tocris Cookson Inc., Ellisville, MS, USA), prolactin (Sigma), and sodium nitroprusside (SNP; Sigma). All chemicals were dissolved just before use and diluted to the proper concentration in a shark saline of the following composition: 292 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 5 mmol l^{-1} $CaCl_2$, 0.6 mmol l^{-1} $MgSO_4$, 1.6 mmol l^{-1} Na_2SO_4, 300 mmol l^{-1} urea, 150 mmol l^{-1} trimethylamine *N*-oxide, 10 mmol l^{-1} glucose, 6 mmol l^{-1} $NaHCO_3$, adjusted to pH 7.7 (Bernal et al., 2005).

Methods

This section aimed to investigate a potential modulatory action of NO on hormonally induced luminescence from *E. spinax* photophores, and, if this role was confirmed, the intracellular pathway used by this substance to mediate its effects. For this purpose, we first checked whether nitrergic drugs (NO donors, NOS inhibitor, cGMP analogue and soluble guanylyl cyclase inhibitor) were able to trigger light *per se*, and then tested the effects of a 20 min pre-treatment with nitrergic drugs on the light parameters of the luminescence induced by MT or PRL (both also applied alone as a control). The experimental procedure was performed at room temperature.

Statistics

Statistical analyses (Student's *t*-test and linear regressions) were performed with the software SAS/STAT (SAS Institute Inc., 1990, Cary, NC, USA). Two regression slopes were only considered to be significantly different if there was no overlap between their 95% slope intervals. Since luminescence responses showed a high variability in amplitude and kinetics between individuals, we expressed the light parameters of the luminescence from treated patches in relative units, i.e. as a percentage (%) of the control response of the same animal. Each mean value was expressed with its standard error (mean ± s.e.m.) and *N* is the number of skin patches used for a specific treatment (which actually corresponds to the number of shark specimens tested, as one skin patch from each individual was used in each treatment).

RESULTS

Immunohistochemistry

Etmopterus spinax photophores are relatively simple in structure: they consist of a pigmented cup containing 6–10 photogenic cells (photocytes) covered by one or several lens cell(s) (Fig. 2A, inset). Blood sinuses are present in the pigmented cup and probably provide a route for photophore luminescence-controlling hormones to reach their target, which could be the iris-like structure (ILS) present between photogenic and lens cells, since this structure is thought to regulate the amount of light emitted by photocytes (Ohshima, 1911; Claes and Mallefet, 2009b). Control experiments (with omission of the primary antibody) showed that endogeneous fluorescence is only found in *E. spinax*'s photocytes (Fig. 2A); this has already been demonstrated for embryos of this species (Claes and Mallefet, 2008).

Antibodies raised against acetylated tubulin supported the presence of nerve fibres in the connective tissues underlying *E. spinax*'s photophores, in the epidermis and also inside the photophores (Fig. 2B,C). Nerve-like bundles entered the pigmented cup of the photophores at different locations, reaching the photocytes directly as well as other functional cell types including pigmented cells of the ILS and lens cells (Fig. 2B,C). Nerve-like structures were also observed to link different photophores suggesting the presence of a nervous network (Fig. 2C).

NOS-like immunoreactivity (IR) was observed in the cytoplasm of photocytes by fluorescence histochemistry (Fig. 2B,C) as well as by avidin–biotin histochemistry (Fig. 2D,E). The specificity of this staining was confirmed by the strong NADPH-diaphorase activity also present in the cytoplasm of the photocytes as shown by the intense blue staining of these cells. Furthermore, control preparations lacking the primary antibody (immunohistochemistry; Fig. 2D) or β-NADPH (NADPH-diaphorase activity; Fig. 2F) did not show any unspecific straining.

Pharmacology

Since an identical relationship was found between the light parameters L_{tot} and L_{max} (Table 2) after injections of MT or PRL, with or without pre-treatment with a nitrergic drug, only the light parameters L_{max} and TL_{max} were considered in the following analyses.

Control responses

Addition of PRL or MT at a final concentration of 10^{-6} mol l^{-1} always evoked a slow and sustained luminescence from photophores of control VSP. In these experiments the mean L_{max} induced by MT on 14 VSP (15.89±2.58 Mq s^{-1} cm^{-2}) was attained in 42.88±4.71 min (TL_{max}) while 20 control preparations stimulated by PRL gave a higher L_{max} (23.14±5.16 Mq s^{-1} cm^{-2}) that was reached earlier, in 20.46±0.96 min (TL_{max}).

NO donors

SNP

The effects of the NO donor SNP were tested. Application of SNP (10^{-6} to 10^{-3} mol l^{-1}; *N*=8) for 1 h on VSP did not induce light emission *per se*. SNP (10^{-6} to 10^{-3} mol l^{-1}) had no effect on the amplitude (L_{max}) of luminescence induced by MT but reduced the TL_{max} of this light emission (Fig. 3A). SNP (10^{-5} to 10^{-3} mol l^{-1}) decreased the amplitude (L_{max}) of PRL-induced luminescence, and also affected the TL_{max} of this light emission (Fig. 3B). Similar to what was found in the hatchetfish *A. hemigymnus* (Krönström et al., 2005), the inhibitory effect of SNP on the L_{max} of PRL-induced luminescence was not constant but instead directly dependent on the L_{max} of the control photophores, i.e. on the capacity of the photophore to luminesce in response to PRL (Fig. 4).

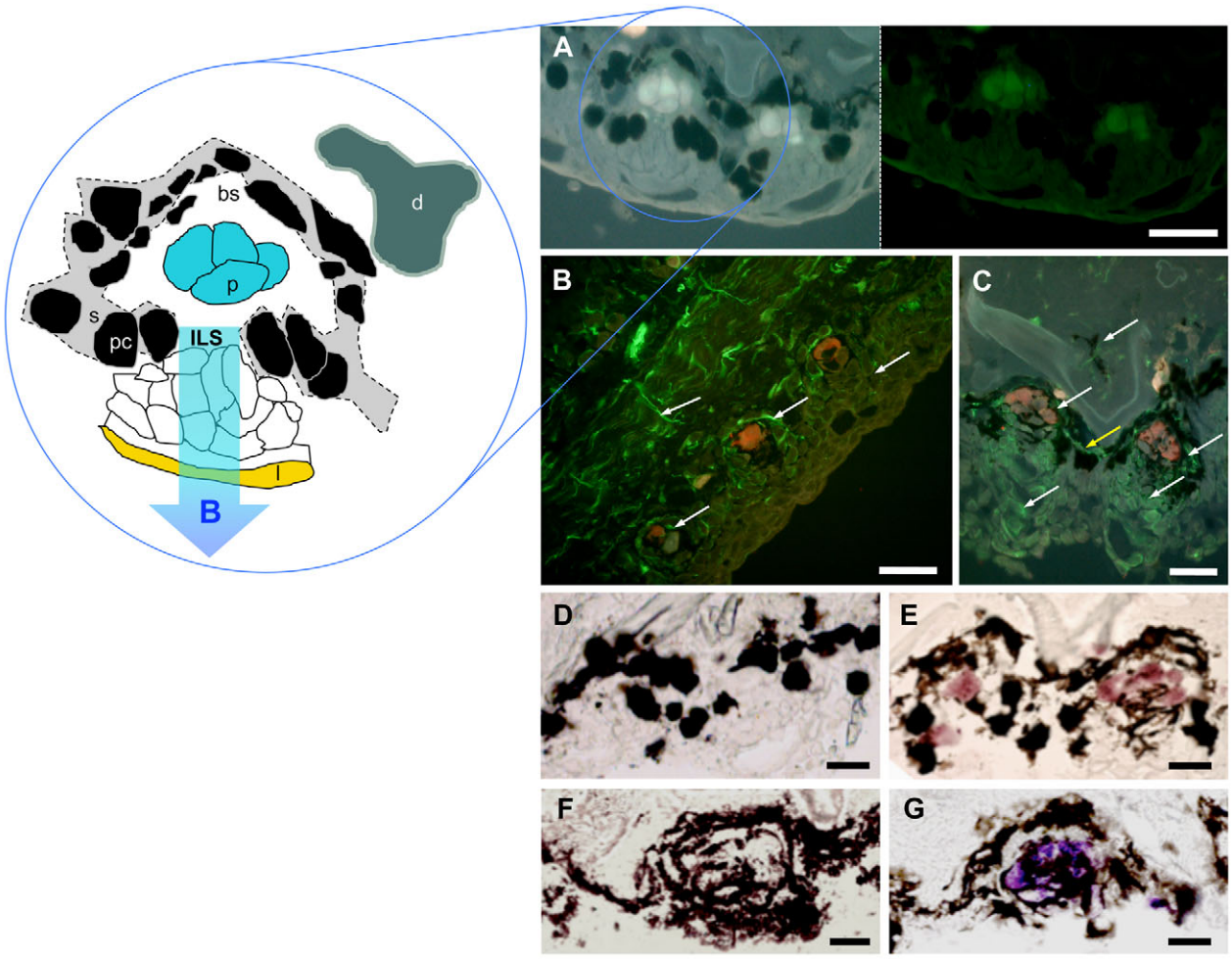

Fig. 2. Sections through ventral photophores from embryo (B) and adult specimens (A,C–G) of *E. spinax*. (A) Control photophore (with omission of the primary antibody) without (left) and with (right) UV stimulation (for fluorescence histochemistry). Right picture shows green autofluorescence inside the photocytes. Inset, schematic representation of the different structural elements present in a photophore. Blue B, bioluminescence; bs, blood sinus; d, dermal denticle (placoid scale); ILS, iris-like structure; l, lens cell; p, photogenic cell; pc, pigmented cell; s, pigmented sheath. (B,C) Acetylated tubulin-like immunoreactivity (AcT-like IR; green fluorescence, fluorescence histochemistry) and nitric oxide synthase-like immunoreactivity (NOS-like IR, uNOS PA1-039; red fluorescence, fluorescence histochemistry). AcT-like IR is found in nerve fibres (white arrows) that innervate the photophores and their surrounding tissue. Yellow arrow in C indicates a nerve fibre connecting two photophores. NOS-like IR is found in the cytoplasm of photocytes. The embryo's photophores contain a unique photocyte. (D) Control photophore (with omission of the primary antibody) for avidin–biotin histochemistry. (E) NOS-like IR (uNOS PA1 039; reddish precipitate, avidin–biotin histochemistry). NOS-like IR is found in the cytoplasm of photocytes. (F) Control photophore (with omission of β-NADPH) for NADPH-diaphorase activity testing. (G) NADPH-diaphorase activity (blue-mauve colour) was present inside the photocytes. Scale bars, 50 μm.

Hydroxylamine

Hydroxylamine is converted into NO in organs having a catalase activity (DeMaster et al., 1989; Krönström et al., 2005). Application of hydroxylamine (10^{-3} mol l^{-1}; N=8) for 1 h on VSP did not induce light emission *per se*. Hydroxylamine (10^{-3} mol l^{-1}) decreased the TL_{max} of MT-induced luminescence but had no effect on its L_{max} (Fig. 5A,B). Conversely, hydroxylamine (10^{-3} mol l^{-1}) decreased the L_{max} of PRL-induced luminescence but had no effect on its TL_{max} (Fig. 5A,B).

NOS inhibitors

In order to investigate the level of endogenous NO production in *E. spinax* photophores, we studied the effect of the NOS inhibitor L-NAME. Application of L-NAME (10^{-6} to 10^{-3} mol l^{-1}; N=6) for 1 h on VSP did not induce luminescence *per se*. L-NAME (10^{-6} to 10^{-3} mol l^{-1}) had no effects on the light parameters (L_{max} or TL_{max}) of the MT-induced luminescence (Fig. 6A). On the other hand, L-NAME treatment unexpectedly decreased the TL_{max} of PRL-induced luminescence at 10^{-4} and 10^{-3} mol l^{-1} (Fig. 6B).

Effects on the cGMP pathway

In order to determine whether the effect of NO on hormonally induced luminescence of lantern shark photophores was mediated through the production of cGMP, we tested the effects of a membrane-permeable cGMP analogue (8BrcGMP) and a selective inhibitor of NO-sensitive guanylyl cyclase (ODQ) on *E. spinax* light emission. None of these substances induced light emission *per se* in *E. spinax* when applied on VSP for 1 h (at 10^{-3} and 10^{-5} mol l^{-1} for 8BrcGMP and ODQ, respectively).

8BrcGMP (10^{-4} mol l^{-1}) decreased the TL_{max} of the MT-induced luminescence but had no effects on its L_{max} (Fig. 7A,B). No significant effect of 8BrcGMP was detected on PRL-induced luminescence (Fig. 7A,B). However, there was a strong tendency towards a decrease in L_{max} after 8BrcGMP application (P=0.06).

Table 2. Relationship between L_{max} and L_{tot} for control photophore preparations and nitrergic drugs*

	N	Slope	R^2	*P*
Melatonin				
Control (10^{-6} mol l^{-1})	13	0.38±0.03	0.99	<0.0001
+SNP (10^{-3} mol l^{-1})	8	0.39±0.02	1.00	<0.0001
+Hydroxylamine (10^{-3} mol l^{-1})	8	0.37±0.03	0.99	<0.0001
+8BrcGMP (10^{-4} mol l^{-1})	8	0.38±0.02	1.00	<0.0001
+L-NAME (10^{-3} mol l^{-1})	6	0.35±0.04	0.99	<0.0001
Prolactin				
Control (10^{-6} mol l^{-1})	14	0.61±0.05	0.98	<0.0001
+SNP (10^{-3} mol l^{-1})	8	0.63±0.05	0.99	<0.0001
+Hydroxylamine (10^{-3} mol l^{-1})	8	0.68±0.08	0.98	<0.0001
+8BrcGMP (10^{-4} mol l^{-1})	8	0.64±0.12	0.96	<0.0001
+L-NAME (10^{-3} mol l^{-1})	6	0.81±0.25	0.93	0.0004

*L_{max} (maximum intensity of light emission) is in Mq s^{-1} cm^{-2}, L_{tot} (total quantity of light emitted) is in Tq h^{-1} cm^{-2} (where q is quanta).
L-NAME, N^G-nitro-L-arginine methyl ester (NO inhibitor); SNP, sodium nitroprusside (NO donor).

Control and ODQ (10^{-5} mol l^{-1})-treated photophores show similar hormonally induced luminescence curves, with values of light parameters L_{max} and TL_{max} that are not significantly different (Fig. 8A,B).

DISCUSSION

This study shows for the first time the presence of nerve fibres and NOS-like IR associated with *E. spinax* photophores, suggesting a role for NO and nerves in the control of luminescence in this shark. Pharmacological results confirm the involvement of NO in the control of *E. spinax*'s luminescence. However, rather than having a direct effect on the luminescence control mechanism, NO appears to act more as a modulator of the effects of MT and PRL: NO donors applied onto photophore preparations failed to trigger luminescence *per se* but modulated the parameters of luminescence induced by MT or PRL.

Control of hormone-induced luminescence

Contrary to earlier views (Johann, 1899), this study suggests that photophores of the lantern shark *E. spinax* are innervated. Various attempts to trigger luminescence in this species using classical neurotransmitters [acetylcholine, (nor)adrenaline, serotonin and GABA] have failed (Claes and Mallefet, 2009b), which suggests that either these nerves do not contain classical neurotransmitters or they do not have a stimulatory role in the light production/emission in this shark. However, since the nerves reach the photophores through the underlying connective tissue and enter these photogenic organs to reach several structural components (photocytes, ILS, lens cells), they must be involved somehow in the control mechanism of *E. spinax* luminescence. Moreover, since they sometimes branch into different photophores, they may also serve to coordinate the luminescence activity of these organs. However, further investigations are needed to determine whether the nerves are spinal autonomic or some other type of neuron (Nilsson, 1983).

In the hatchetfish (*A. hemigymnus*), the NO modulation of adrenaline-induced luminescence appears to occur *via* nerves reaching the photocytes, and other luminescent bony fish harbour NO-containing nerves inside their photogenic organs (Krönström et al., 2005; Krönström and Mallefet, 2009). No clear evidence of such NO-containing nerves was found in the photophores of the cartilaginous *E. spinax*. We found, however, strong NOS-like IR and NADPH-diaphorase activity in the photocytes of this species, suggesting a role for NO in the luminescence control mechanism of *E. spinax*.

Pharmacological results clearly supported the involvement of NO in the modulation of luminescence induced by MT as well as PRL. However, the effects of NO on these hormonal light responses were not similar: NO only had an effect on the kinetics of MT-induced luminescence but affected the kinetics and the amplitude of PRL-induced luminescence. This result is not surprising: MT and PRL have different intracellular transmission pathways (Claes and Mallefet, 2009b), and one can therefore assume that NO will act at

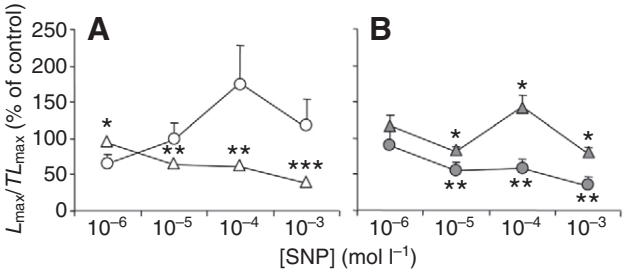

Fig. 3. Effect of the NO donor sodium nitroprusside (SNP) on hormonally induced luminescence from *E. spinax* photophores. (A) Dose-dependent effect of SNP on melatonin (MT; 10^{-6} mol l^{-1})-induced luminescence. SNP had no effect on L_{max} (circles) but significantly decreased TL_{max} (triangles) of this light emission. (B) Dose-dependent effect of SNP on prolactin (PRL; 10^{-6} mol l^{-1})-induced luminescence. SNP decreased L_{max} (circles) but also had an effect on TL_{max} (triangles) of this light emission. Values are expressed as a percentage of values obtained in control photophore preparations, i.e. the ventral skin patches (VSP) stimulated with MT or PRL alone. *N*=8 for each experiment. Asterisks indicate significant differences between control and treated ventral skin patches: **P*<0.05, ***P*<0.01, ****P*<0.001.

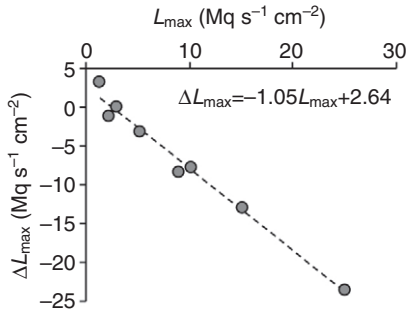

Fig. 4. Relationship between L_{max} of control photophores and ΔL_{max} (difference between L_{max} values of SNP-treated and control VSP), for PRL-induced luminescence (*P*<0.001, R^2=0.98, *N*=8). The inhibitory effect of SNP is more important in VSP with a high luminescence capability, i.e. those harbouring high L_{max} values in control VSP.

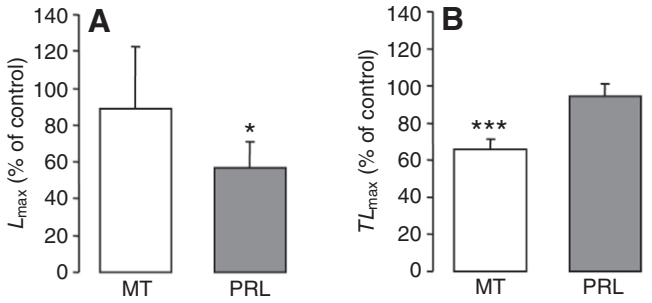

Fig. 5. Effects of the NO donor hydroxylamine (10^{-3} mol l^{-1}) on (A) L_{max} and (B) TL_{max} of hormonally (10^{-6} mol l^{-1}) induced luminescence from *E. spinax* photophores. Values are expressed as a percentage of values obtained in control photophore preparations, i.e. VSP stimulated with MT or PRL alone. N=8 for each experiment. Asterisks indicate significant differences between control and treated VSP: *P<0.05, ***P<0.001.

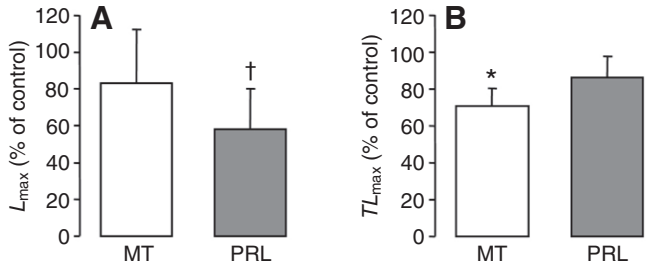

Fig. 7. Effects of the cGMP analogue 8BrcGMP (10^{-4} mol l^{-1}) on (A) L_{max} and (B) TL_{max} of the hormonally (10^{-6} mol l^{-1}) induced luminescence from *E. spinax* photophores. Values are expressed as a percentage of values obtained in control photophore preparations, i.e. VSP stimulated with MT or PRL alone. N=8 for each experiment. Asterisks indicate significant differences between control and treated VSP: *P<0.05, †P=0.06.

different levels when modulating the action of these two hormones on *E. spinax* photophores.

The effect of the NO donors used in this study (SNP and hydroxylamine) on PRL-induced luminescence in *E. spinax* can be compared with the effect of NO on the hatchetfish and the northern krill (*M. norvegica*), which is predominantly inhibitory (Krönström et al., 2005; Krönström et al., 2007). Similar to what was found in hatchetfish, the inhibitory effect of NO on the amplitude of the luminescent response was not constant but directly depends on the luminous capability of the photophore preparation: the inhibition was maximal in VSP that produced a high PRL-induced luminescence and minimal in VSP that exhibited a low response to this hormone.

On the other hand, both NO donors speeded up the luminous response from *E. spinax* photophores after MT application. This suggests that NO affects a mechanism controlling the rate of light emission in *E. spinax* photophores. A similar modulation of the luminescence time course by NO has already been observed in other organisms including the hatchetfish, the northern krill and the midshipman fish (*P. notatus*) (Krönström et al., 2005; Krönström et al., 2007; Krönström, 2009).

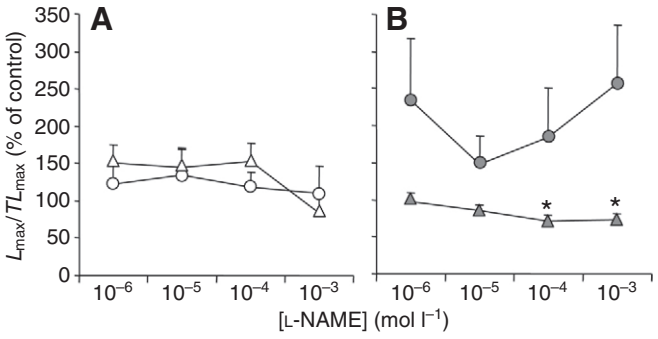

Fig. 6. Effect of the NOS inhibitor L-NAME on the hormonally induced luminescence from *E. spinax* photophores. (A) Dose-dependent effect of L-NAME on MT (10^{-6} mol l^{-1})-induced luminescence. L-NAME had no effect on the light parameters (L_{max}, circles; TL_{max}, triangles) of this light emission. (B) Dose-dependent effect of L-NAME on PRL (10^{-6} mol l^{-1})-induced luminescence. L-NAME had no effect on L_{max} (circles) of the PRL (10^{-6} mol l^{-1})-induced luminescence but significantly reduced TL_{max} (triangles) of this light emission at 10^{-4} and 10^{-3} mol l^{-1}. Values are expressed as a percentage of values obtained in control photophore preparations, i.e. VSP stimulated with MT or PRL alone. N=6 for each experiment. Asterisks indicate significant differences between control and treated VSP: *P<0.05.

Hydroxylamine is a precursor of NO in biological systems that possess a catalase activity (DeMaster et al., 1989). Since the effects of hydroxylamine on hormonally induced luminescence of *E. spinax* are similar to the effect of a NO donor, this suggests the presence of endogenous catalase activity in this shark's photophores, as previously demonstrated in the photophores of the hatchetfish (Krönström et al., 2005).

The NOS inhibitor L-NAME only had a small impact on the timing of PRL-induced luminescence and did not affect the light parameters of MT-induced luminescence from *E. spinax* photophores. This supports the idea that NO is generated only at certain times, when changes occurring in the environment require the shark to quickly modulate the expression of its luminescence. The effects of this molecule on hormonally induced luminescence are therefore mainly transitory rather than permanent, similarly to what has been suggested for hatchetfish (Krönström et al., 2005).

In physiological processes, the effects of NO are mediated either by cGMP induced after guanylyl cyclase activation (Katsuki et al., 1977), or by a cGMP-independent pathway involving the inhibition of mitochondrial respiration (Brown, 1995). In our study, the cGMP analogue 8BrcGMP mimicked the effect of the NO donors SNP and hydroxylamine. This suggests that the NO–cGMP pathway is important in the luminescence control mechanism of *E. spinax*, contrary to what was found in other species in which NO appears

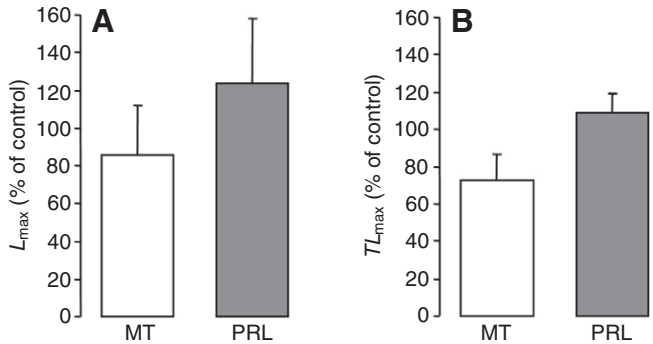

Fig. 8. Effects of the guanylyl cyclase inhibitor ODQ (10^{-5} mol l^{-1}) on (A) L_{max} and (B) TL_{max} of the hormonally (10^{-6} mol l^{-1}) induced luminescence from *E. spinax* photophores. ODQ had no effect on this light emission. Values are expressed as a percentage of values obtained in control photophore preparations, i.e. VSP stimulated with MT or PRL alone. N=8 for each experiment.

to regulate light emission using a cGMP-independent pathway (Trimmer et al., 2001; Krönström et al., 2005; Krönström et al., 2007). The absence of effect of the guanylyl cyclase inhibitor ODQ on MT-induced luminescence is consistent with our hypothesis that the effect of NO is transient.

Function of NO in luminescence control

Luminescent behaviours generally necessitate rapid, precise timing and coordination to be ecologically successful (Buck, 1978; Denton et al., 1985; Branham and Greenfield, 1996; Haddock et al., 2010).

The rapid action and short half-life of NO make it a perfect mediator for quick adjustments of luminescence properties. In a recent study, it was suggested that NO could be involved in the control of light production/emission of many luminescent teleost fish species (Krönström and Mallefet, 2009). Our results confirm this idea and show that cartilaginous luminescent fish also evolved a NO regulation system for their luminescence. It is not clear, however, at this time whether nerves reaching photocytes in the lumen of *E. spinax* photophores are NO containing or not. The presence of these nerves nevertheless confirms the idea that hormonal inputs need an additional mechanism(s) for the efficient tuning of *E. spinax* photophore luminescence.

Various adaptive advantages have been suggested for the luminescence produced by the velvet belly lantern shark, including camouflage through counterillumination and intra-specific communication (Claes and Mallefet, 2008; Claes and Mallefet, 2009a). These different functions appear to be achieved by different zones of the luminous pattern: the ventral zone (investigated in the present study) is believed to be restricted to camouflage by counterillumination while luminous zones of the pelvic area are probably used for sexual signalling (Claes and Mallefet, 2009a). With this in mind, NO could finely tune the intensity of the luminescence from glowing *E. spinax* photophores to allow this shark to counterilluminate efficiently in the mesopelagic zone, the light properties of which change with time and depth (Johnsen, 2003), similar to what was suggested in the hatchetfish (Krönström et al., 2005).

The early appearance of NOS inside the photocytes of embryonic shark photophores underlines the importance of NO modulation in *E. spinax*'s life, from the very beginning. The presence of NOS in the caudal organ of the myctophid *M. punctatum* (Krönström and Mallefet, 2009), which is probably used for sexual communication (Herring, 2007), supports the idea that the effects of NO on luminescence are not restricted to the counterilluminating behaviour in fishes. It is therefore possible that NO modulation also exists in luminous zones of *E. spinax* that are not involved in counterillumination, but this needs further analysis.

LIST OF ABBREVIATIONS

AcT	acetylated tubulin
8BrcGMP	8-bromo cyclic guanosine monophosphate
cGMP	cyclic guanosine monophosphate
5-HT	5-hydroxytryptamine (serotonin)
ILS	iris-like structure
IR	immunoreactivity
L_{max}	maximum intensity of light emission
ΔL_{max}	L_{max} (control preparations)–L_{max} (treated preparations)
L_{tot}	total light emission
$Mq\,s^{-1}\,cm^{-2}$	megaquanta per second per square centimetre (of skin)
α-MSH	α-melanocyte stimulating hormone
MT	melatonin
L-NAME	N^G-nitro-L-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PRL	prolactin
TL_{max}	time between stimulation and maximum intensity of light emission
$Tq\,h^{-1}\,cm^{-2}$	teraquanta per hour per square centimeter (of skin)
uNOS	universal nitric oxide synthase
VSP	ventral skin patch

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