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## RESEARCH ARTICLE

# Control of luminescence from pygmy shark (*Squaliolus aliae*) photophores

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### SUMMARY

**The smalleye pygmy shark (*Squaliolus aliae*) is a dwarf pelagic shark from the Dalatiidae family that harbours thousands of tiny photophores. In this work, we studied the organisation and physiological control of these photogenic organs. Results show that they are mainly situated on the ventral side of the shark, forming a homogeneous ventral photogenic area that appears well suited for counterillumination, a well-known camouflage technique of pelagic organisms. Isolated ventral skin patches containing photophores did not respond to classical neurotransmitters and nitric oxide but produced light after melatonin (MT) application. Prolactin and α-melanocyte-stimulating hormone inhibited this hormonally induced luminescence as well as the spontaneous luminescence from the photogenic tissue. The action of MT seems to be mediated by binding to the $MT_2$ receptor subtype, as the $MT_2$ receptor agonist 4P-PDOT inhibited the luminescence induced by this hormone. Binding to this receptor probably decreases the intracellular cAMP concentration because forskolin inhibited spontaneous and MT-induced luminescence. In addition, a GABA inhibitory tonus seems to be present in the photogenic tissue as well, as GABA inhibited MT-induced luminescence and the application of bicuculline provoked luminescence from *S. aliae* photophores. Similarly to what has been found in Etmopteridae, the other luminous shark family, the main target of the luminescence control appears to be the melanophores covering the photocytes. Results suggest that bioluminescence first appeared in Dalatiidae when they adopted a pelagic style at the Cretaceous/Tertiary boundary, and was modified by Etmopteridae when they started to colonize deep-water niches and rely on this light for intraspecific behaviours.**

Key words: α-MSH, bioluminescence, Chondrichthyes, Dalatiidae, *Squaliolus aliae*, melatonin, prolactin.

### INTRODUCTION

Except *Dalatias licha*, a benthopelagic shark that can attain almost 2 m in total length (TL), all sharks of the family Dalatiidae are small (≤50 cm TL) pelagic species that spent most of their time in the mesopelagic zone of the world's oceans (except the polar seas) (Compagno et al., 2004). Among the 10 currently described species encompassed by this family are some of the rarest sharks, known by extremely few specimens (*Mollisquama parini* and *Isistius labialis* – each known by one specimen; *Euprotomicroides zantedeschia* – known by two specimens; *Heteroscymnoides marleyi* – known by six specimens; and *Isistius plutodus* – known by 10 specimens), and others are localised or relatively inaccessible, which probably explains why most remain poorly known to science (Compagno et al., 2004; Zidowitz et al., 2004). At least nine of these sharks are endowed with thousands of tiny photogenic organs called photophores that allow them to produce a visible light as a result of a chemical reaction (Hubbs et al., 1967; Seigel, 1978; Reif, 1985; Stehmann and Kreft, 1988; Compagno et al., 2004). The presence of photophores in only *M. parini* remains to be determined. However, this shark is thought to possess specialised glands that produce a luminous fluid at the base of the pectoral fins (Dolganov, 1984). Although this has never been tested, the ventral position of dalatiid photophores strongly suggests that they are used to replace the residual downwelling light absorbed by the body of these sharks, hiding their silhouettes from below, a camouflage technique know as 'counterillumination' (Young et al., 1980; Reif, 1985). Although this camouflage is generally used to avoid predation, a predatory use of this behaviour has been suggested for the cookiecutter shark *Isistius brasiliensis*, a well-known dalatiid species: this shark cloaks its entire ventral side except a dark zone lacking photophores situated in the head region (the 'dog collar') to appear smaller and attract fast-swimming predators such as big fishes and cetaceans, on which it feeds by kleptoparasitism (Jones, 1971; Widder, 1993).

The smalleye pygmy shark *Squaliolus aliae* (Teng, 1959) is the smallest species of the Dalatiidae and maybe the smallest of all currently described shark species, attaining its sexual maturity at approximately 15 cm TL and a maximum size of 22 cm TL (Compagno et al., 2004). It occurs in coastal waters of the southeastern Indian and West Pacific Oceans (Compagno et al., 2004), where it can be locally abundant (Tanakamaru et al., 1999) and migrate from deep waters (down to 2000 m) during the day to the epipelagic zone at night, probably following small crustaceans, cephalopods and fishes, on which it preys (Seigel, 1978). Contrasting with photophores of the Etmopteridae or 'lantern sharks', the other luminous shark family, photophores of this shark are smaller and harbour a simplified structure that is composed of a single photogenic cell called a photocyte sheathed in a pigmented cup and covered by one or several lens cells (Seigel, 1978).

If pygmy shark photophores are involved in counterillumination, they have to be well controlled as this luminous behaviour is particularly demanding: to be efficient, the luminescence produced by the shark must demonstrate the same physical characteristics

(intensity, angular distribution and wavelength) as the residual downwelling light present in its environment (Clarke, 1963; Denton et al., 1972; Denton et al., 1985; Harper and Case, 1999). In addition, the shark should also be able to switch off its luminescence when not needed or when it could attract the attention of a predator, e.g. at depths situated below 1000 m where no more solar light penetrates (Warrant and Locket, 2004).

Until now, the physiological control of shark photophores has only been extensively studied in Etmopteridae (for a review, see Claes and Mallefet, 2011). Indeed, the only investigation of luminescence control in dalatiid photophores was performed by Herring and Morin, who tried without success to induce light in *I. brasiliensis* with peritoneal injections of adrenaline and acetylcholine (Herring and Morin, 1978). The two lantern sharks investigated so far, *Etmopterus spinax* and *E. splendidus*, demonstrate a complex control of their photogenic organs, which involves hormonal [melatonin (MT) and prolactin (PRL), stimulatory effect; and α-melanocyte stimulating hormone (α-MSH), inhibitory effect] and neural inputs [GABA, inhibitory effect; and nitric oxide (NO), modulatory effect] that provoke pigment expansion/retraction in melanophores covering the photogenic cells to regulate the amount of the light produced on the outside (Claes and Mallefet, 2011; Claes et al., 2011). A direct action of these substances on the photogenic cells themselves has not, however, been excluded (Claes and Mallefet, 2011). Interestingly, the control mechanism of etmopterid photophores gives clues to evolutionary pathway of luminescence in sharks, as it involves the same hormones as those acting in the physiological colour change used by elasmobranchs to remain cryptic against variable backgrounds [PRL and MT provoke skin lightning *via* pigment retraction in melanophores whereas α-MSH provokes skin darkening *via* pigment expansion in these cells (Visconti et al., 1999; Gelsleichter, 2004)]. It is therefore suggested that lantern sharks modified their original shallow-water crypsis mechanism when they started to colonize deep-water niches during the late Cretaceous (Adnet and Capetta, 2001; Straube et al., 2010) and develop their ability to produce light (Claes and Mallefet, 2010a).

Because the separation of Dalatiidae and Etmopteridae certainly occurred before the radiation of lantern sharks in the deep sea (Straube et al., 2010) and because dalatiid and etmopterid photophores harbour structural differences, it has been suggested that the luminescence capability observed in members of these two shark families is the result of convergent evolution (Hubbs et al., 1967; Claes and Mallefet, 2009a; Straube et al., 2010). In this context, the study of the physiology of luminescence control in a dalatiid species such as *S. aliae* is particularly interesting because it contributes to our understanding of the evolutionary history of luminescence in sharks; although a similar control mechanism would be in favour of a single acquisition of luminescence in sharks, a distinct mechanism would suggest multiple independent acquisitions of the phenomenon in these fishes.

In this work, we analysed the organisation and physiological control of smalleye pygmy shark photophores. Using isolated ventral skin patches (VSPs) of *S. aliae*, we performed: (1) a screening of test substances for hormones, nitric oxide (NO) and classical neurotransmitters to identify those involved in the extrinsic luminescence control, (2) morphological analysis of photophore change during pharmacologically induced luminescence and (3) combined experiments to explore the intrinsic control pathway of stimulatory drugs and their modulation. The results obtained are discussed with the ecology of the studied dalatiid species and are compared with results obtained from luminous shark species from the Etmopteridae family.

## MATERIALS AND METHODS

### Experimental fish

Twenty-seven adult smalleye pygmy sharks, *S. aliae* (eight male specimens 17–20.5 cm TL; 19 female specimens 14.3–22.3 cm TL) were captured in inshore waters off Donggang harbour in southwest Taiwan (22°26′N, 120°23′E) by several midwater trawls (seven collection days, 50–150 m depth) operating at dawn in July 2011. Living sharks were brought to the National Museum of Marine Biology and Aquarium (NMMBA; Pingtung, southern Taiwan) and were housed in two 0.8×0.6×0.4 m aquaria placed in the dark at 18–19°C. Following the rules of NMMBA for experimental fish care, all sharks were killed by decapitation before experimentation took place.

### Morphology

To determine the organisation of photogenic structures in *S. aliae*, skin patches from the caudal, pectoral, pelvic, rostral and belly regions were excised from six specimens (14.3–22.3 cm TL; two males and four females) and photographed under a microscope (Leitz Diaplan, Oberkochen, Germany). In addition, skin patches forming a dorsoventral continuum were dissected for each shark and included in the analysis. Digital pictures were analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to determine, for each skin patch, photophore density ($P_D$), mean photophore diameter ($P_{Di}$) and total photogenic cover ($P_C$; i.e. for a specific skin area, the number of photophores multiplied by the mean apparent surface of a photophore in the skin axis).

### Pharmacology

#### Luminometry

Physiological control of pygmy shark photophores was investigated following the method of Claes and Mallefet (Claes and Mallefet, 2009b). VSPs were dissected out from freshly dead specimens of *S. aliae* (Fig. 1A–C) using a metal cap driller (diameter=0.55 cm). They were then placed in small Perspex chambers containing 200 µl of shark saline (Bernal et al., 2005) with their photogenic surface area placed towards the photo-detector area of a luminometer (Berthold FB12, Pforzeim, Germany) calibrated with a standard 470 nm light source (Beta light, Saunders Technology, Hayes, UK). Finally, a pharmacological drug was applied onto the patch and light emission was recorded during either 20 min (for neural drugs) or 60 min [for hormonal drugs and the $GABA_A$ antagonist bicuculline (BICU)]. The slow kinetics of *S. aliae* luminescence allowed us to perform several experiments simultaneously using the Berthold multiple kinetic mode (Sirius protocol manager v1.4, Pforzeim, Germany). Data were collected every minute on a laptop computer to build original luminescence curves. These curves were then characterised using different parameters (Fig. 1D): the maximum intensity of light emission ($L_{max}$; $Mq\,s^{-1}$), the total quantity of light emitted during the experiment ($L_{tot}$; Gq) and the time to reach $L_{max}$ from stimulation time ($TL_{max}$; min). Light parameters were standardised by skin surface area ($cm^{-2}$).

#### Chemicals

The chemical agents used in this study were: (1) classical neurotransmitters or their stable agonists [(nor)adrenaline, serotonin (5-HT), GABA and carbachol; Sigma Chemical Co., St Louis, MO, USA; all these substances were applied at $10^{-3}\,mol\,l^{-1}$]; (2) the NO donor sodium nitroprusside (SNP) (Sigma Chemical Co.; applied at $10^{-3}\,mol\,l^{-1}$); (3) the hormones melatonin (MT), prolactin (PRL) and α-melanocyte stimulating hormone (α-MSH) (Sigma Chemical Co.; all applied at $10^{-6}\,mol\,l^{-1}$ but MT was also applied at $10^{-7}$ and

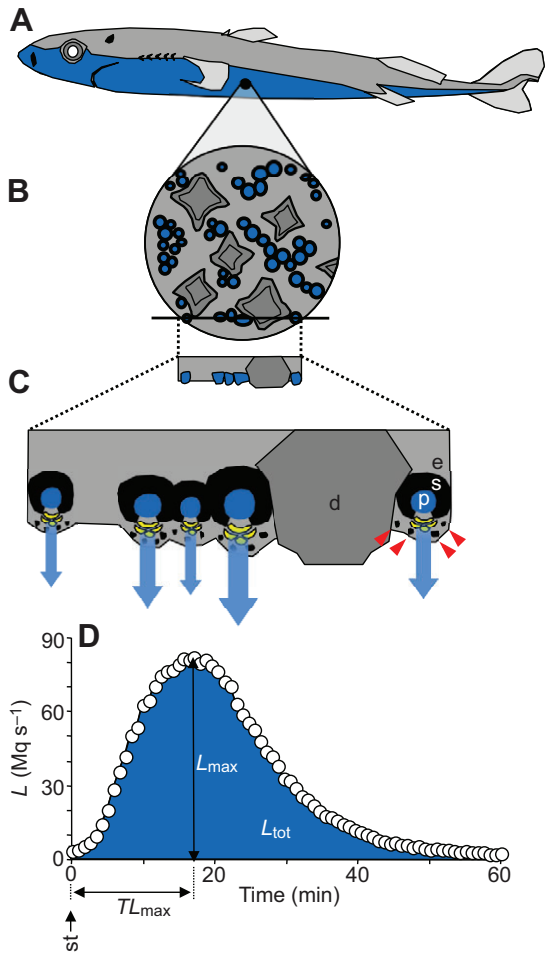


Fig. 1. Study of photophores and luminescence in *Squaliolus aliae*. (A) Lateral view of the shark with its luminous pattern (blue colour). (B) Ventral skin patch, showing dermal denticles and associated photophores (blue colour). (C) Structure of glowing photophores [adapted from Seigel (Seigel, 1978)]. The light (blue arrows) is produced in the photocyte (p) of each photophore, passes through the lens (yellow colour) and is finally emitted to the outside. A pigmented sheath (s) prevents the light from being emitted towards the underlying tissues. We make the assumption that the numerous pigmented cells (red arrows) present in the epidermis (e) play a role in the control of this shark's light emission by obliterating the photocytes. (D) Typical luminescence emission curve with associated parameters after a stimulation (st). d, dermal denticle; *L*, light emission; $L_{max}$, maximum intensity of light emission; $L_{tot}$, total quantity of light emitted during the experiment; $TL_{max}$, time from stimulation to $L_{max}$.

$10^{-8}$ mol l$^{-1}$); (4) the $MT_2$ receptor antagonist 4P-PDOT (Tocris Bioscience, Ellisville, MO, USA; applied at $10^{-4}$ mol l$^{-1}$) and the $GABA_A$ receptor antagonist BICU (Sigma Chemical Co.; applied at $10^{-3}$ mol l$^{-1}$); and (5) an enzyme activator, adenylyl cyclase activator forskolin (FSK) (Sigma Chemical Co.; applied at $10^{-4}$ mol l$^{-1}$). All chemicals were dissolved 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 mol 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 and 6 mmol l$^{-1}$ $NHCO_3$; total osmolarity 1.080 mosmol; pH 7.7 (Bernal et al., 2005).

### Method

As a first step to trigger light emission from pygmy shark photophores, test substances were applied on VSPs. They consisted of classical neurotransmitters, NO donor SNP and hormones. Simultaneously, other VSPs from the same specimens received a shark saline application as control. For each test substance, we compared the $L_{tot}$ values obtained for control and treated patches from the different sharks. After this screening, only substances that produced significantly more (or less) light than the control were considered as good candidates for a more detailed pharmacological analysis. This analysis consisted of: (1) dose–light response curves to determine the sensitivity of the tissue to a test substance, (2) the use of antagonists to signal the presence of a specific receptor in the tissue investigated and (3) the use of enzyme activators to determine the intrinsic control of photophores.

Because of the large individual variability in luminescent responses of pygmy shark photophores, each dose–light response curve is given in relative units and effects of antagonists or enzyme activators on light emission parameters are expressed as a percentage of the control, i.e. the luminescence induced by the receptor agonist alone (without the antagonist or the enzyme activator). In addition, some VSPs were also observed under microscope after the application of a light-inducing substance in order to macroscopically observe the morphological variations occurring in the photophores of *S. aliae* during light emission. Digital pictures were taken at different times following a pharmacological light induction and ImageJ was used to measure the apparent photogenic area (i.e. the 'transparency index') of six different photophores as well as the total photogenic area of these photophores (both of these values were finally expressed in relative units).

All the experiments were performed at room temperature (22°C).

### Statistical analysis

All analyses (one-way ANOVA, Kruskal–Wallis ANOVA on ranks, one- and two-tailed Student's *t*-test and linear regression) were performed using the software JMP v.9 (SAS Institute Inc., Cary, NC, USA) and considered to be significant at the 0.05 level. Each analysis was performed after removal of significant outliers (>2σ), and after an arcsine transformation in the case of relative data (except when a relative value >1 was obtained with a test substance where a log-transformation was applied instead). Normality and equality of variance were tested by a Shapiro–Wilk test and a Levene's test, respectively. When these parametric assumptions could not be met, we used a non-parametric Kruskal–Wallis ANOVA rather than a one-way ANOVA to statistically analyse the difference between more than two groups. When a difference was detected by ANOVA or Kruskal–Wallis ANOVA, we tested all pairwise comparisons using a *post hoc* Student's *t*-test or a *post hoc* Dunn's test, respectively. Values are expressed as means ± s.e.m., and *N* equals the number of VSPs used for a specific treatment corresponding to the number of the shark tested.

## RESULTS

### Morphology

Fifty-four different skin samples were analysed for the morphological analysis of *S. aliae* luminous pattern. Among these samples, some were excised from the ventral side (*N*=30) of the sharks and others came from the lateral and dorsal sides of these animals (*N*=24).

Kruskal–Wallis ANOVA did not detect any difference ($\chi^2_4$=8.68, *P*=0.0637) between the $P_D$ of the different photogenic areas situated on the ventral side of the smalleye pygmy shark (Fig. 2A), which gave a mean $P_D$ of 8323.74±458.42 photophores cm$^{-2}$ for the whole ventral photogenic area. The $P_{Di}$ also appears to be similar across the different ventral photogenic areas of *S. aliae* (one-way ANOVA,

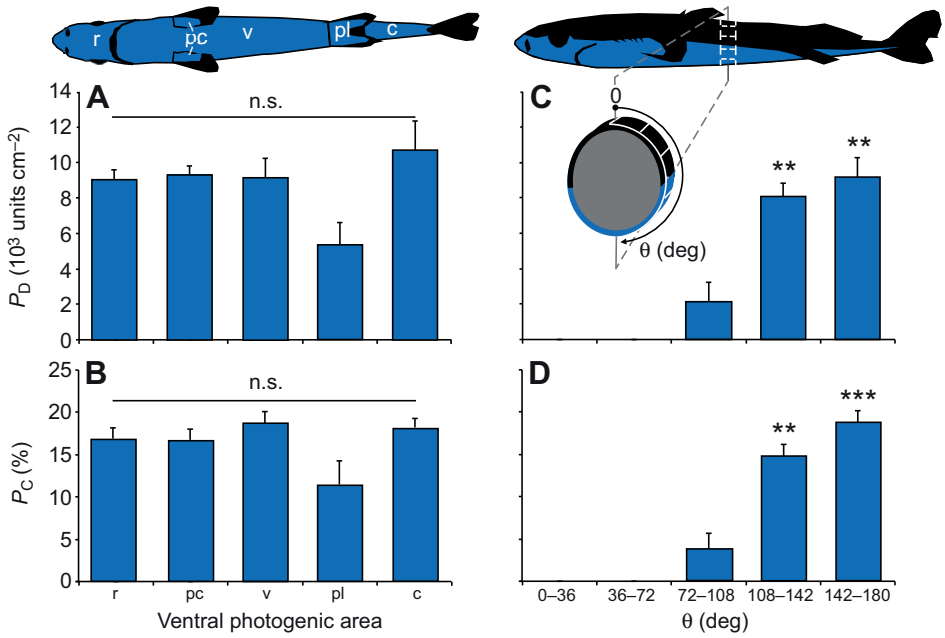


Fig. 2. Photophore organisation of *Squaliolus aliae*. (A) Photophore density ($P_D$) and (B) photogenic cover ($P_C$) of different areas composing the whole ventral photogenic surface area of the shark ($N$=6 for each ventral area). Kruskal–Wallis ANOVA failed to detect any difference in $P_D$ and $P_C$ among the different photogenic zones. n.s., Not significant. Top left: ventral view of the shark, showing the position of the different photogenic zones (blue colour). c, caudal; pc, pectoral; pl, pelvic; r, rostral; v, ventral. Black colour indicates non-luminous areas. (C) $P_D$ and (D) $P_C$ for different skin portions of *S. aliae* forming a continuum from dorsal to ventral ($N$=6 for each portion). Asterisks indicate significant differences between dorsal skin patches [angle from vertical (θ)=0–36 deg] and other skin patches (**$P$<0.01, ***$P$<0.001) detected by *post hoc* Dunn's tests. Top right: lateral view of the shark, showing the photogenic pattern (blue colour) as well as the different skin portions forming the dorsoventral continuum. Please note that the ventral photogenic area (v) in A and B corresponds to the skin portion situated between 144 and 180 deg from vertical in C and D.

$F_{4,25}$=1.34, $P$=0.2844), which gave a mean $P_{Di}$ of 50.42±0.81 µm for all measured ventral photophores of the shark ($N$=4058). As a consequence, $P_C$ followed the same tendency as $P_D$: no difference in $P_C$ was detected by Kruskal–Wallis ANOVA between the different zones forming the ventral photogenic area ($\chi^2_4$=7.44, $P$=0.1145; Fig. 2B); which gave a mean $P_C$ of 16.34±0.84% for the whole ventral photogenic area.

Contrasting with the homogeneity observed in the ventral photogenic areas, a significant difference in $P_D$ was detected among the skin patches forming the dorsoventral continuum (dorsolateral skin patches + six skin patches from the belly photogenic area): $P_D$ progressively increased from the dorsal side, where no photophores are present, to ventral side, where $P_D$ reached 9166.34±1096.08 photophores $cm^{-2}$ (Kruskal–Wallis ANOVA, $\chi^2_4$=24.50, $P$<0.0001; Fig. 2C). The first photophores appeared in the patch situated 72–108 deg from the vertical line passing at the mid-body in the transversal plane (Fig. 2C). However, it was only at an angular position of 108 deg that skin patches started to be statistically different from skin patches situated at the dorsal-most position ($Z$=3.22, $P$=0.0051). Similarly to what was found for the whole ventral photogenic area, the $P_{Di}$ was constant (47.91±11.39 µm, $N$=1030) in the different skin patches forming the dorsoventral continuum (Kruskal–Wallis ANOVA, $\chi^2_2$=2.1, $P$=0.9499). As a consequence, $P_D$ and $P_C$ again followed the same tendency: $P_C$ progressively increased from the dorsal side, where it was equal to 0%, to the ventral side, where it reached 18.74±1.40% (Kruskal–Wallis ANOVA, $\chi^2_4$=25.4895, $P$<0.0001; Fig. 2D) and skin patches with an angle between 108 and 144 deg were the first to be statistically different from the dorsal-most skin patches ($Z$=2.98, $P$=0.0116).

## Pharmacology

### Drug screening

The pharmacological screening performed on VSPs of adult pygmy sharks encompassed classical neurotransmitters, NO and hormones. Classical neurotransmitters and NO evoked a minor light emission whose $L_{tot}$ was, on average, not significantly different ($P$>0.05) from the $L_{tot}$ of a control injection of shark saline (Table 1). Injection of MT always induced a light response whose $L_{tot}$ was significantly ($P$<0.05) higher than those of the saline injection (Table 1). VSP treated with PRL and α-MSH, however, produced significantly ($P$<0.05) less light than the saline control (Table 1).

### Hormonal control of luminescence

Quickly after injection, VSPs stimulated with MT produced a slowly increasing glow ($N$=16, $L_{tot}$=191.24±42.71 Gq $cm^{-2}$, $L_{max}$=187.48±41.49 Mq $s^{-1}$ $cm^{-2}$, $TL_{max}$=27.82±6.61 min) that lasted at least 60 min, as these photogenic skin patches were still producing light at the end of the experiments (Fig. 3A). This light emission showed a highly ($N$=15, $F$=156.94, $P$<0.0001) significant relationship between $L_{tot}$ and $L_{max}$ (Fig. 3B); therefore, only the $L_{tot}$ will be considered in the following analyses. The dose–light response curve of MT showed that the photogenic tissue was more sensitive (i.e. produced more light) when this hormone was applied at $10^{-6}$ mol $l^{-1}$ than when applied at lower concentrations such as $10^{-7}$ and $10^{-8}$ mol $l^{-1}$ (Fig. 3C). This effect of concentration on $L_{tot}$ was detected by Kruskal–Wallis ANOVA ($\chi^2_2$=12.17, $P$=0.0023). However, the *post hoc* Dunn's test failed to detect any difference between $10^{-6}$ and $10^{-8}$ mol $l^{-1}$ ($Z$=0.73, $P$=0.9355); this test only detected a very significant difference between $10^{-7}$ and $10^{-6}$ mol $l^{-1}$ ($Z$=3.30, $P$=0.0019). No significant difference in $TL_{max}$ was found among the different concentrations (Kruskal–Wallis ANOVA, $\chi^2_2$=0.93, $P$=0.6289; Fig. 3C).

The $L_{tot}$ of MT-induced (at $10^{-6}$ mol $l^{-1}$) luminescence was very significantly decreased by the $MT_2$ antagonist 4P-PDOT applied at $10^{-4}$ mol $l^{-1}$ ($t_5$=5.87, $P$=0.0020; Fig. 3D). In addition, $10^{-3}$ mol $l^{-1}$ FSK application highly significantly decreased the $L_{tot}$ of this hormonally induced light emission ($t_7$=23.87, $P$<0.0001; Fig. 3D). Moreover, when applied on spontaneously luminous VSPs, $10^{-3}$ mol $l^{-1}$ FSK quickly switched them off in less than 20 min (Fig. 3E).

Shortly after $10^{-6}$ mol $l^{-1}$ MT application, pigment retraction was observed in pigmented cells topping the photophores to reveal the underlying glowing photocytes (Fig. 4A). However, this mechanism was reversible: after approximately 20 min, pigment expansion occurred in the pigmented cells, re-obliterating the photocytes (Fig. 4A). Although pre-stimulating photophores appeared as dark

Table 1. Effects of test substances on luminescence from *Squaliolus aliae* photophores

| | | | | Student's *t*-test | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *P* | | | |
| | [PS] (mol l$^{-1}$) | Duration (min) | $L_{tot}$ (Gq) | d.f. | *t* | >\|*t*\| | >*t* | <*t* | Effect |
| Neural testing | | | | | | | | | |
| Neurotransmitters | | | | | | | | | |
| Adrenaline | $10^{-3}$ | 20 | 111.90±43.21 | 7.87 | –0.5 | 0.634 | 0.683 | 0.317 | 0 |
| Noradrenaline | $10^{-3}$ | 20 | 42.82±12.99 | 7.65 | 1.62 | 0.146 | 0.073 | 0.927 | 0 |
| 5-HT | $10^{-3}$ | 20 | 103.25±16.75 | 8.88 | –0.54 | 0.6032 | 0.6984 | 0.3016 | 0 |
| GABA | $10^{-3}$ | 20 | 153.18±79.36 | 5.93 | –0.79 | 0.4583 | 0.7709 | 0.2291 | 0 |
| Carbachol[a] | $10^{-3}$ | 20 | 76.77±32.47 | 9.26 | 0.26 | 0.7996 | 0.3998 | 0.6002 | 0 |
| NO | $10^{-3}$ | 20 | 286.19±155.47 | 5.24 | –1.26 | 0.2596 | 0.8702 | 0.1298 | 0 |
| Hormonal testing | | | | | | | | | |
| MT | $10^{-6}$ | 60 | 287.11±81.61 | 6.77 | –1.92 | 0.0977 | 0.9512 | **0.0488** | + |
| PRL | $10^{-6}$ | 60 | 7.31±3.71 | 5.11 | 3.11 | **0.0257** | **0.0128** | 0.9872 | – |
| α-MSH | $10^{-6}$ | 60 | 21.75±6.66 | 5.36 | 2.67 | **0.0414** | **0.0207** | 0.9793 | – |

[a]Cholinergic agonist.

*N*=6 for all pharmacological substances (PS).

Bold values indicate significant differences revealed by Student's *t*-tests. Control (saline) $L_{tot}$ values for neural and hormonal testing were 87.36±4.27 and 116.65±34.93 Gq, respectively.

$L_{tot}$, total light emitted during the experiment; 0, no effect; +, activation; –, inhibition; 5-HT, serotonin; GABA; γ-aminobutyric acid; NO, nitric oxide; MT, melatonin; PRL, prolactin; α-MSH; α-melanocyte stimulating hormone.

blotches (Fig. 4B), they were black rings surrounding luminescent bluish photocytes at maximum opening (Fig. 4C).

The $L_{tot}$ of MT-induced ($10^{-6}$ mol l$^{-1}$) luminescence was very significantly decreased by PRL ($t_5$=4.14, $P$=0.0090) and highly significantly decreased by α-MSH applied at $10^{-6}$ mol l$^{-1}$ ($t_7$=17.18, $P$<0.0001; Fig. 5A). In addition, the $TL_{max}$ of this hormonally induced luminescence was significantly reduced by these two hormones (PRL, $t_5$=3.71, $P$=0.0139; α-MSH, $t_7$=3.16, $P$=0.0159; Fig. 5B).

### Neural control of luminescence

GABA significantly decreased the $L_{tot}$ ($t_7$=3.49, $P$=0.0101; Fig. 6A) and highly significantly decreased the $TL_{max}$ ($t_7$=6.24, $P$=0.0004; Fig. 6B) of MT-induced ($10^{-6}$ mol l$^{-1}$) luminescence but had no effect on the $L_{max}$ ($t_7$=0.75, $P$=0.4765) of this light emission. Moreover, application of the $GABA_A$ antagonist BICU always provoked an important light emission whose $L_{tot}$ was significantly higher than those of the saline injection ($t_5$=2.90, $P$=0.0299; Fig. 6B). This light emission was a slowly increasing glow ($N$=7, $L_{tot}$=474.93±145.91 Gq cm$^{-2}$, $L_{max}$=253.79±65.09 Mq s$^{-1}$ cm$^{-2}$, $TL_{max}$=12.49±6.68 min) that lasted at least 60 min, as these photogenic skin patches were still producing light at the end of the experiments (Fig. 6C). This light emission showed a highly significant ($N$=7, $F$=40.59, $P$=0.0007) relationship between $L_{tot}$ and $L_{max}$ (Fig. 6D).

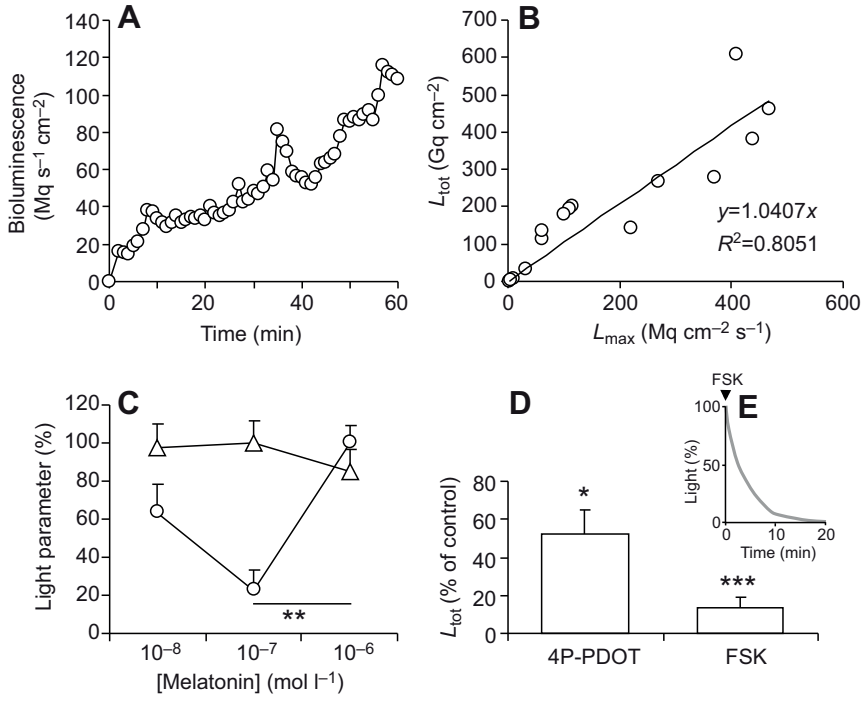


Fig. 3. Melatonin (MT)-induced luminescence from the photophores of *Squaliolus aliae*. (A) Original recording of luminescence induced by MT ($10^{-6}$ mol l$^{-1}$) from ventral skin patches (VSPs) of a 14.8 cm total length female specimen of *S. aliae*. (B) Relationship ($P$<0.001) between maximum intensity of light emission ($L_{max}$) and total quantity of light emitted during the experiment ($L_{tot}$) for MT-induced ($10^{-6}$ mol l$^{-1}$) luminescence ($N$=15). (C) Dose–light response curve of MT from VSPs of *S. aliae*. Values of $L_{tot}$ (circles) and time from stimulation to $L_{max}$ ($TL_{max}$) (triangles) are expressed as a percentage of the highest value obtained ($N$=9 for each concentration). A *post hoc* Dunn's test only detected a significant difference in $L_{tot}$ between concentrations of $10^{-7}$ and $10^{-6}$ mol l$^{-1}$ (**$P$<0.01). (D) Effect of the $MT_2$ antagonist 4P-PDOT ($10^{-4}$ mol l$^{-1}$; $N$=6) and the adenylyl cyclase activator forskolin (FSK; $10^{-4}$ mol l$^{-1}$; $N$=8) on the $L_{tot}$ of MT-induced ($10^{-6}$ mol l$^{-1}$) luminescence from VSPs of *S. aliae* (control $L_{tot}$ value for 4P-PDOT=287.11±81.61 Gq; control $L_{tot}$ value for FSK=240.07±64.40 Gq). Values are expressed as a percentage of the control, i.e. VSPs stimulated by MT alone. Asterisks indicate significant differences between control and treated VSPs (*$P$<0.05, ***$P$<0.001). (E) Average time course of spontaneous luminescence after application of $10^{-4}$ mol l$^{-1}$ FSK (arrow; $N$=5); FSK quickly decreased the intensity of spontaneous luminescence from *S. aliae* photophores, which became totally extinct within 20 min.

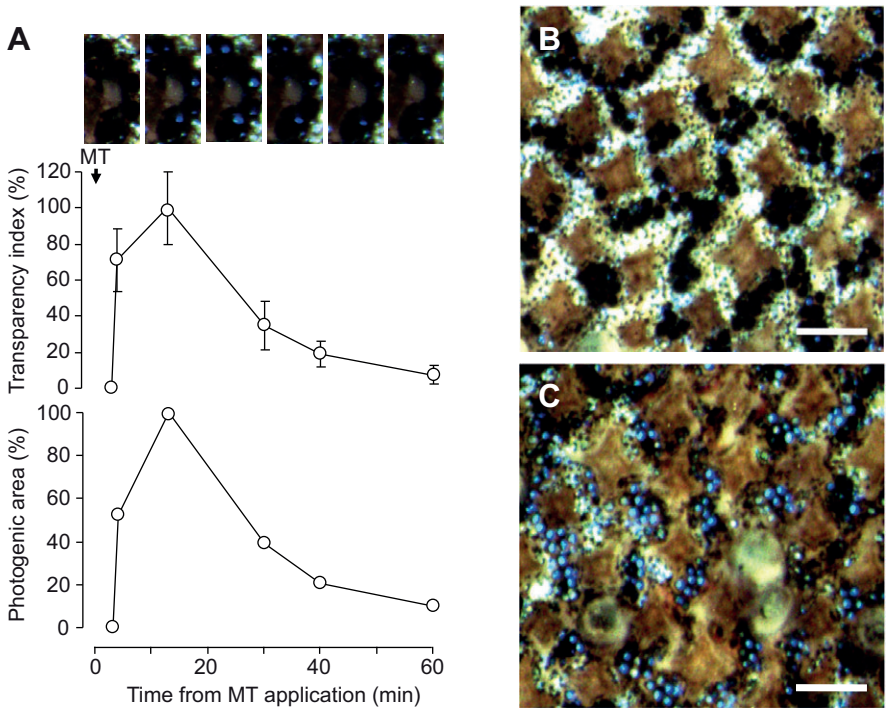


Fig. 4. Iris-like movement of pigmented cells underlying the photophores of *Squaliolus aliae*. (A) Evolution of the 'transparency index' and photogenic area of photophores after melatonin (MT) application (arrow). Serial pictures (top) illustrate the group of photophores ($N$=6) from which the data points were calculated. (B) Photophore-containing skin patch before MT stimulation (i.e. not producing light). (C) Photophore-containing skin patch producing light after MT application. Scale bars, 250 μm.

NO had no effect on any of the MT-induced ($10^{-6}$ mol l$^{-1}$) luminescence parameters ($L_{tot}$, $t_{10}$=1.77, $P$=0.1071; $L_{max}$, $t_{10}$=2.15, $P$=0.0575, $TL_{max}$, $t_{10}$=0.92; $P$=0.3818).

## DISCUSSION

This work represents the first detailed study on the physiological control of the photophore of a dalatiid shark, the smalleye pygmy shark (*S. aliae*), at both the extrinsic and intrinsic levels. The results obtained differ from those found for luminous etmopterid sharks investigated so far and provide new insights into the evolutionary history of luminescence in Chondrichthyes. It also confirms the use of isolated, physiologically active VSPs containing photophores as a valid technique to study the control of luminescence in sharks, whose photophores can be extremely small in comparison with those of members of the Osteichthyes (Claes and Mallefet, 2009b).

### Control of pygmy shark luminescence

Contrary to NO donor SNP and the different classical neurotransmitters used in this study, MT shows a stimulatory effect on pygmy shark photophores, which start to produce a long-lasting (>1 h; J.M.C., unpublished data), slowly increasing light response just after the application of this hormone. As in the lantern shark *E. spinax*, whose luminescence is also triggered by MT (Claes and Mallefet, 2009b), the sensitivity of *S. aliae* photophores appears to be higher at $10^{-6}$ mol l$^{-1}$, as revealed by the dose–light response curve. The dynamic analysis of photophore morphological changes occurring after MT application shows that this hormone, in addition to triggering light, also induces pigment retraction in melanophores covering the photocytes. In addition to this hormonal stimulating effect, an inhibitory effect of PRL and α-MSH on spontaneous and MT-induced luminescence was also shown. These different results suggest strongly that, as in luminous Etmopteridae (Claes and Mallefet, 2009b; Claes et al., 2011), pygmy shark luminescence is under the control of different hormones, and the photocyte-covering melanophores are one target of this hormonal control. In etmopterids, hormones are believed to use the large blood sinuses that go through the pigmented sheath of the photophores to act on specific targets in these photogenic organs (Ohshima, 1911; Claes and Mallefet, 2009b). Such blood sinuses are lacking in *S. aliae* photophores (Seigel, 1978), probably because the smaller size and simpler structure (they contain only a single photocyte) of these photogenic organs offer a more permeable barrier to hormonal signals.

Effects of MT are mediated by four different receptors in vertebrates, including one cytosolic enzyme involved in detoxification processes (low affinity receptor $MT_3$) and three transmembrane receptors [high affinity receptors $MT_1$ (=$Mel_{1a}$), $MT_2$ (=$Mel_{1b}$) and $Mel_{1c}$] (Vanecek, 1998; Barrett et al., 2003; Boutin et al., 2005). High affinity receptors can be coupled with several intrinsic pathways, including adenylyl/guanylyl cyclase inhibition (which induces a decrease in intracellular cAMP/cGMP) and phospholipase C activation, which modulates the intracellular concentration of $Ca^{2+}$ (Vanecek, 1998; Barrett et al., 2003).

In this work, the $MT_2$-specific receptor antagonist 4P-PDOT ($10^{-4}$ mol l$^{-1}$) decreased the luminescence induced by application of MT ($10^{-6}$ mol l$^{-1}$). This suggests that, similarly to what was found in *E. spinax* (Claes and Mallefet, 2009b), melatoninergic $MT_2$ receptors are present in pygmy shark photophores. Moreover, the strong inhibitory effect of FSK ($10^{-3}$ mol l$^{-1}$) on spontaneous and MT-induced luminescence strongly suggests that the $MT_2$ receptor is negatively coupled to cAMP, as FSK is known to increase the intracellular cAMP concentration by stimulating adenylyl cyclase.

Similarly to what was found in etmopterids (Claes et al., 2010b; Claes et al., 2011), GABA has an inhibitory effect on MT-induced luminescence from *S. aliae* photophores and BICU has a stimulatory effect on these photogenic organs. These elements are strongly in favour of the presence of a GABA inhibitory tonus in pygmy shark photogenic tissue that would prevent, *via* binding to $GABA_A$ receptors, undesired light emission, probably by acting on melanophore pigments; this light inhibition is apparently partially inhibited by higher MT concentration levels.

Contrary to GABA, NO does not seem to act similarly in the two luminescent shark families. Indeed, although NO is known to

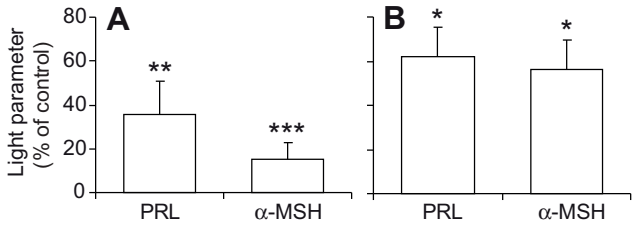


Fig. 5. Inhibitory effect of $10^{-6}$ mol $l^{-1}$ prolactin (PRL; $N$=6) and $10^{-6}$ mol $l^{-1}$ α-melanocyte-stimulating hormone (α-MSH; $N$=8) on (A) total quantity of light emitted during the experiment ($L_{tot}$; control $L_{tot}$ value for PRL=183.13±89.19 Gq; control $L_{tot}$ value for α-MSH=237.9.07±65.42 Gq) and (B) time from stimulation to $L_{max}$ ($TL_{max}$) of MT-induced ($10^{-6}$ mol $l^{-1}$) luminescence (control $TL_{max}$ value for PRL=183.13±89.19 min; control $TL_{max}$ value for α-MSH=32.50±7.80 min). Values are expressed as a percentage of the control, i.e. VSPs stimulated by MT alone. Asterisks indicate significant differences between control and treated VSPs (*$P$<0.05, **$P$<0.01, ***$P$<0.001).

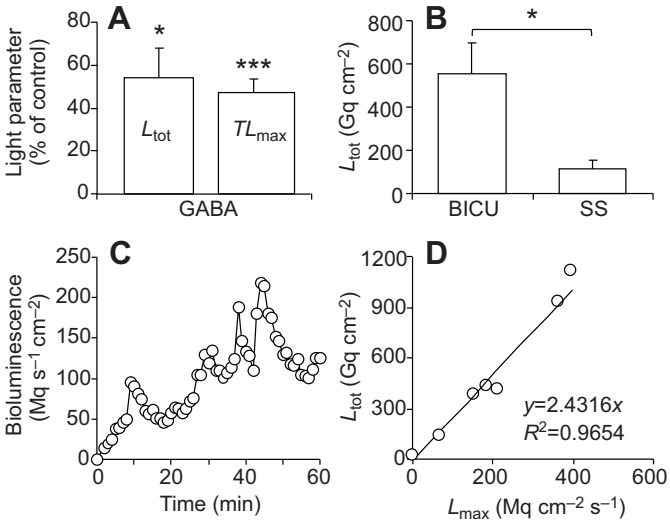


Fig. 6. (A) Effect of $10^{-3}$ mol $l^{-1}$ γ-aminobutyric acid (GABA; $N$=8) on total quantity of light emitted during the experiment ($L_{tot}$; control $L_{tot}$ value=209.57±74.53 Gq) and time from stimulation to maximum intensity of light emission ($TL_{max}$; control $TL_{max}$ value=36.50±7.40 min) of the MT-induced ($10^{-6}$ mol $l^{-1}$) luminescence. Values are expressed as a percentage of the control, i.e. VSPs stimulated by MT alone. Asterisks indicate significant differences between control and treated VSPs (*$P$<0.05, ***$P$<0.001). (B) Comparison ($N$=6) between the $L_{tot}$ of luminescence from *Squaliolus aliae* photophores after application of $10^{-3}$ mol $l^{-1}$ $GABA_A$ antagonist bicuculline (BICU) and shark saline (SS). Asterisk indicates significant differences between the two treatments (*$P$<0.05). (C) Original recording of BICU-induced ($10^{-3}$ mol $l^{-1}$) luminescence from *S. aliae* VSPs. (D) Relationship ($P$<0.001) between maximum intensity of light emission ($L_{max}$) and $L_{tot}$ for BICU-induced ($10^{-3}$ mol $l^{-1}$) luminescence ($N$=7).

modulate the hormonally induced luminescence in *E. spinax* (Claes et al., 2010c), it did not have any effect on MT-induced luminescence from *S. aliae* photophores. Effects of NO on the luminescence of marine organisms are, however, known to be versatile and complex (Krönström et al., 2005; Krönström et al., 2007; Claes et al., 2010c), and additional experiments are certainly needed to completely exclude NO from the pygmy shark luminescence control.

### Evolution of photophore control in sharks

Except bacteria and fungi, which are continuous light emitters, luminescent organisms demonstrate a wide array of intracellular and extracellular mechanisms to control the physical characteristics and the onset/termination of the light emitted by their photogenic organs (Case and Strause, 1978; Hastings, 1978; Herring and Morin, 1978; Wassink, 1978; Claes and Mallefet, 2009b; Haddock et al., 2010). These mechanisms are essential to ensure a proper use of luminescent behaviours and guarantee the survival of the emitter.

Until recently, fish photophores were classified into two categories: (1) symbiotic photophores containing luminescent bacteria whose light was controlled by accessory structures (dark shutters and chromatophores) or physiological suppression of the circulatory supply [e.g. $O_2$ (Bertelsen, 1951; Haygood, 1993; Munk, 1999)] and (2) intrinsic photophores containing an endogeneous system under an exclusive nervous control, mostly adrenergic (for a review, see Claes and Mallefet, 2009b). Etmopterid sharks, however, were found to be exceptions to this rule when it was shown that their intrinsic photophores were controlled by a dual action of hormonal and nervous inputs whose main target was an accessory melanophore iris (Claes and Mallefet, 2009b; Claes and Mallefet, 2010a; Claes et al., 2010b; Claes et al., 2010c; Claes and Mallefet, 2011; Claes et al., 2011). In this work, we show that the luminescence of the dalatiid shark *S. aliae* is also dually controlled by nervous (GABA) and hormonal (MT, PRL and α-MSH) substances that probably exert their action *via* the movement (retraction/expansion) of pigments inside the melanophores covering the photocytes.

However, a major difference appears in this work between etmopterid and dalatiid photophore luminescence control. Indeed, if the absence of effect of NO might be linked to the versatility of this particular neurotransmitter/modulator, PRL has an opposite action on etmopterid and dalatiid luminescence: although this hormones triggers light in *E. spinax* (Claes and Mallefet, 2009b) and *E. splendidus* (Claes et al., 2011), it inhibits spontaneous and MT-induced luminescence in *S. aliae*. This opposite effect of PRL suggests that dalatiid photophore control represents an intermediate between the control of elasmobranch physiological colour change, where PRL also provokes pigment expansion in skin melanophores (Visconti et al., 1999), and the etmopterid luminescence control, where this hormone triggers pigment retraction in melanophores covering the photocytes (Claes and Mallefet, 2010a). In an evolutionary context, this is particularly interesting because it is in favour of a unique apparition of luminescence in sharks, this capability first appearing in dalatiid sharks and evolving later in Etmopteridae.

The phylogenetic relationship existing between Dalatiidae and Etmopteridae remains unclear. Although a squaliform phylogeny based on dental characters previously suggested that Etmopteridae benefited from the Cenomanian–Turonian global anoxic event to colonize newly empty deepwater niches (approximately 90 million years ago) (Adnet and Capetta, 2001), a more recent molecular phylogeny places the origin of these sharks earlier (approximately 65 million years ago) at the Cretaceous/Tertiary (C/T) boundary (Straube et al., 2010). Both phylogenies (morphological and molecular) are, however, unanimous in placing the origin of the Dalatiidae at the C/T boundary. Dalatiid sharks would have taken advantage of the dramatic reduction of large marine pelagic predators that occurred at this period (Capetta, 1987; Bardet, 1995) to leave shallow waters and integrate with the pelagic predator fauna (Adnet and Capetta, 2001). Because camouflage by counterillumination is probably the main function of dalatiid luminescence (Hubbs et al., 1967; Seigel, 1978; Reif, 1985; Widder, 1993), we therefore propose that the luminescence first evolved in Dalatiidae, which

took advantage of this capability to protect themselves from the dangers of the pelagic environment. Etmopteridae would have subsequently split and colonized deepwater niches and adopt a more benthopelagic lifestyle. This environmental change would have been accompanied by a deep reorganisation of etmopterid photogenic structures. Indeed, although lantern shark luminescence can still be used in counterilluminating behaviours (Claes et al., 2010a), it started to be involved in intraspecific functions such as schooling, cooperative hunting and mating, favouring their rapid radiation in the darkness of the deep sea (Claes and Mallefet, 2009c; Straube et al., 2010). Photophores of Etmopteridae would first have become larger, encompassing more than one photogenic cell, and more regularly distributed in the skin, forming more homogeneous photogenic surface areas well adapted to their luminescent behaviours. This could have been facilitated by the desertion of pavement-like denticles [found in all dalatiid genera and few etmopterids such as *E. schultzi* and *E. pusillus* (Dolganov, 1984; Reif, 1985; Stehmann and Kreft, 1988; Shirai and Tachikawa, 1993; Stehmann et al., 1999)] for squamations more adapted to let photophore light pass through the epidermis, such as those found in the majority of Etmopteridae which are cross-, bristle- or hook-shaped (Reif, 1985). It also has to be noted that during their organogenesis, etmopterid photophores first contained a unique photocyte such as those found in Dalatiidae (Claes and Mallefet, 2008; Claes et al., 2010c), which may be a relic of this structural transition.

Finally, the differential use of luminescence by Dalatiidae and Etmopteridae probably explains the opposite action of PRL on the photophores of these two shark families. Indeed, in etmopterid sharks, MT is thought to mediate the light information perceived by the pineal gland, and therefore to be particularly important in counterillumination behaviour (Claes and Mallefet, 2009b; Claes and Mallefet, 2010b). PRL, in contrast, is thought to be produced in response to direct visual stimuli perceived by the eyes of the sharks and to be involved in intraspecific behaviours such as sexual signalling and group swimming/hunting, which necessitate quicker on/off light switching (Claes and Mallefet, 2009b; Claes and Mallefet, 2010b). This second assertion was strongly supported by the fact that, in *E. spinax*, luminous areas involved in these intraspecific behaviours (i.e. lateral, pelvic and infra-pelvic luminous areas) demonstrate a sexually dimorphic light response to PRL (Claes and Mallefet, 2010b). MT is sufficient to induce the light switch in smalleye pygmy shark photophores because the luminescence of these organs is probably only devoted to camouflage by counterillumination, as indicated by their ventral homogeneous repartition. The stimulatory action of PRL probably evolved later to fine tune the intraspecific luminous signals of etmopterid sharks, maybe as a result of a different PRL receptor, but this remains to be tested.

## CONCLUSIONS

The present work shows that the smalleye pygmy shark appear to be a good model species to study dalatiid luminescence because: (1) its luminescence can be investigated using isolated VSPs, (2) it can be locally abundant, which allows proper repetition of pharmacological experiments, and (3) it can be maintained in captivity for short period of time.

Results are in favour of an unique apparition of luminescence in sharks: dalatiid sharks would have turned the initial crypsis mechanism of shallow water elasmobranchs into a luminescent midwater camouflage well adapted to the pelagic lifestyle they acquired at the C/T boundary, and, shortly afterwards, Etmopteridae would have modified the physiological control and the organisation of initial photophores to allow them to fulfil additional functions, such as sexual signalling and schooling, when they started to adopt a benthopelagic lifestyle in the darkness of the deep sea.

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| α-MSH | α-melanocyte stimulating hormone |
| BICU | bicuculline |
| 5-HT | 5-hydroxytryptamin (serotonin) |
| FSK | forskolin |
| GABA | γ-aminobutyric acid |
| $GABA_A$ | GABA receptor A |
| $L_{max}$ | maximum intensity of light emission |
| $L_{tot}$ | total quantity of light emitted during one experiment |
| MT | melatonin |
| $MT_2$ | melatonin receptor 2 |
| NO | nitric oxide |
| $P_C$ | photogenic cover |
| $P_D$ | photophore density |
| $P_{Di}$ | photophore diameter |
| PRL | prolactin |
| TL | total length |
| $TL_{max}$ | time elapsed from stimulation to reach $L_{max}$ |
| VSP | ventral skin patch |

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