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**Function and control of luminescence from
lanternshark (*Etmopterus spinax*) photophores**

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Thesis presented for the degree of Doctor of Philosophy (Sciences)

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Fiat Lux.

Genesis 1:3

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Summary

In the permanent darkness of the deep-sea, a vast majority of organisms including molluscs, crustaceans and fishes developed photophores (= photogenic organs) to produce their own light and assume numerous functions including predation avoidance, predation help and intraspecific communication. This amazing capability evolved in two shark families, the Etmopteridae (= lantern sharks) and the Dalatiidae, which totalised more than 50 different luminescent sharks i.e. about 10% of currently described shark species. As a matter of fact, however, this amazing phenomenon has been poorly studied in these cartilaginous fishes, in comparison with bony fishes. By using a model species (the velvet belly lantern shark, *Etmopterus spinax*), the present work aims to provide the first experimental data on the adaptive benefit and the physiological control of photophore luminescence in sharks. Results were obtained by different techniques including fluorescent microscopy, microphotospectrometry, pharmacology (and luminometry), and immunohistochemistry.

E. spinax's photophores are able to produce light before birth and appear as a multi-use tool that could help the shark in different behaviours including intraspecific signalling, camouflage by counterillumination, aposematism, and prey illumination. The physiological control of their luminescence is complex and unique, involving hormonal [melatonin (induction), prolactin (induction) and α -MSH (inhibition)] but also nervous [GABA (inhibition), nitric oxide (modulation)] inputs. One of the main targets of these substances appears to be a melanophore iris, which regulates the amount of light emitted to the outside by pigment dispersion/retraction. This result suggests that the lantern sharks' luminescence capability evolved from the Elasmobranch (sharks and rays) skin colour change mechanism, inefficient in deepwater niches.

List of abbreviations

AC	adenylyl cyclase
Act	acetylated tubulin
Adr	adrenalin
α -MSH	α -melanocyte stimulating hormone
ANOVA	one-way analysis of variance
ATP	adenosine triphosphate
cAMP	3',5'-cyclic adenosine monophosphate
cGMP	3',5'-cyclic guanosine monophosphate
db-cAMP	N ⁶ ,2'-o-dibutyryl-adenosine 3',5'-cyclic monophosphate
$\Delta L_{\max}$	$L_{\max}$ (control preparations) - $L_{\max}$ (treated preparations)
DMSO	dimethylsulfoxide
$E_{z\ 500}$	downwelling irradiance
5-HT	5-hydroxytryptamine, serotonin
4P-PDOT	4-phenyl-2-propionamidotetralin
FSK	forskolin
GABA	γ -aminobutyryl acid
GC	guanylyl cyclase
8BrcGMP	8-Bromo-cyclic guanosine mono phosphate
Ig	immunoglobulin
ILS	iris-like structure
IR	immunoreactivity
JAK2	janus kinase 2
JAKi II	JAK2 inhibitor II
$K_{d\ 500}$	attenuation of downwelling irradiance at 500 nm
$K_{i\ 500\ Z}$	integrated attenuation between the surface and the depth z
$L_{\max}$	maximum intensity of light emission
L-NAME	N ^G -nitro-L-arginine methyl ester
L_T	total length
L_{tot}	total light emission

LZ	luminous zone
MAPK	mitogen-activated protein kinase
MDL-12,330A	cis-N-(2-phenylcyclopententyl)-azacyclotridec-1-en-2-amine monohydrochloride
$\text{Mq s}^{-1} \text{cm}^{-2}$	megaquanta per second per square centimeter (of skin)
MT	melatonin
MT1	melatonin receptor 1
MT2	melatonin receptor 2
MT3	melatonin receptor 3
Nadr	noradrenalin
NO	nitric oxide
NOS	nitric oxyde synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
P_D	photophore diameter
P_{De} (ou PD)	photophore density
PRL	prolactin
PRLR	prolactin receptor
q s^{-1}	quanta per second
r.d.u.	relative density units
r.l.u. (ou RLU)	relative light units
SNK test	Student-Newman-Keuls test
SQ22,536	9-(Tetrahydro-2-furanyl-9H-purin-6-amine)
Stat	signal transducers and activators of transcription
TL	total length
$TL_{\max}$	time between stimulation and maximum intensity light emission
$\text{Tq h}^{-1} \text{cm}^{-2}$	teraquanta per hour per square centimeter (of skin)
uNOS	universal NOS
V_{LZ}	ventral surface area occupied by luminous zones
V_P	ventral surface area occupied by photophores
V_S	ventral surface area of a luminous zone
VSP	ventral skin patch

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I. INTRODUCTION



Velvet belly lantern shark (*Etmopterus spinax*) swimming in a Norwegian fjord. Picture: Seapics.

1 Sharks' odyssey to the deep

Sharks appeared on Earth before the dinosaurs, during the Devonian period (more than 400 myrs ago) and their basic anatomy underwent relatively few changes from that time: they possess a cartilaginous skeleton, 5 to 7 apparent gill arches, and as the other cartilaginous fish groups (Batoids and Holocephalans) lack a gaseous swim bladder but instead harbour a large squalene-filled liver that allow them to maintain an almost neutral buoyancy (Corner et al., 1969).

During the course of their evolution, sharks survived several mass extinctions to progressively become apex predators in many aquatic environments including the world's oceans but also numerous rivers and several lakes (Thorson, 1971; Cortes, 1999; Motta and Wilga, 2001).

One of the hardest environments to colonize was certainly the deep-sea, since living in this environment, which is the largest on Earth, imposes to resist to difficult constraints including a high hydrostatic pressure, a cold water temperature, a low food availability, and, mainly, a permanent low light level which can be problematic for accomplishing their major biological roles: find prey, escape predators and reproduce (Bailey et al., 2002; Priede et al., 2006). Numerous species of invertebrates and vertebrates (including fishes but also mammals) successfully conquered the deepwater environments by developping striking anatomical, physiological and behavioural adaptations.

One of the most widespread and successful adaptations of living organisms to deep-sea life is certainly the possession of photogenic organs, which may solve the problems inherent to a life in an environment with little light or no light at all (at depths superior to 1000 m).

Two squalid shark families developed the ability to produce light from tiny photophores: the Etmopteridae (called the lantern sharks) and the Dalatiidae (Hubbs et al., 1967). These two families totalised more than 50 different species, and new species are increasingly found (Last et al., 2002; Compagno et al., 2004) probably due to the recent human interest for deep habitat exploration, which means that more than 10 % of currently known shark species are actually able to produce light. In comparison to luminous organisms including bony fishes, however, luminous sharks have been poorly studied and no experimental data are available concerning their bioluminescence.

This thesis consists in a first experimental investigation of the physiological control and the adaptive benefit of the photogenic organs in a common luminescent

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shark, the velvet belly lantern shark (*Etmopterus spinax*). By using a model species, this thesis aims to provide the first basis for further studies on shark luminescence.

The following introduction will give (i) a review on general concepts of bioluminescence with a special emphasis on its functions and control mechanisms of this phenomenon, (ii) a review on shark biology with a special focus on the cellular communication mechanisms and luminescence characteristics of these fishes, and finally (iii) biological information on *E. spinax*.

2 Bioluminescence

2.1 Distribution

Bioluminescence is the light produced through chemical reaction within an organism. Although rare in terms of species number, this phenomenon has a wide phylogenetic distribution, occurring in more than 700 genera representing 13 phyla and all the kingdoms of living things except archaeobacteria and plants (see the bioluminescence tree of life in **annexe 1**), and have probably appeared more than 40 times independently during the course of evolution (Hastings, 1983; 1995; Wilson and Hastings, 1998; Haddock et al., 2010) even if a lack of fossil record makes this only a rough estimate (Johnsen et al., 1999). This suggests that luminescence is of great ecological importance for luminescent organisms but also that the acquisition of the ability to emit light is a relatively easy and quick process (Haddock et al., 2010).

Luminescent species occupy a variety of habitats but are more represented in marine environments. In particular, it is in the deep-sea that luminescence attains its highest biodiversity since many deep-sea organisms, from bacteria to fishes, rely on this light emission to assume numerous biological functions in the permanent darkness of this habitat (**Fig. 1**; Harvey, 1952; Buck, 1978; Johnsen, 2005; Herring, 2007).

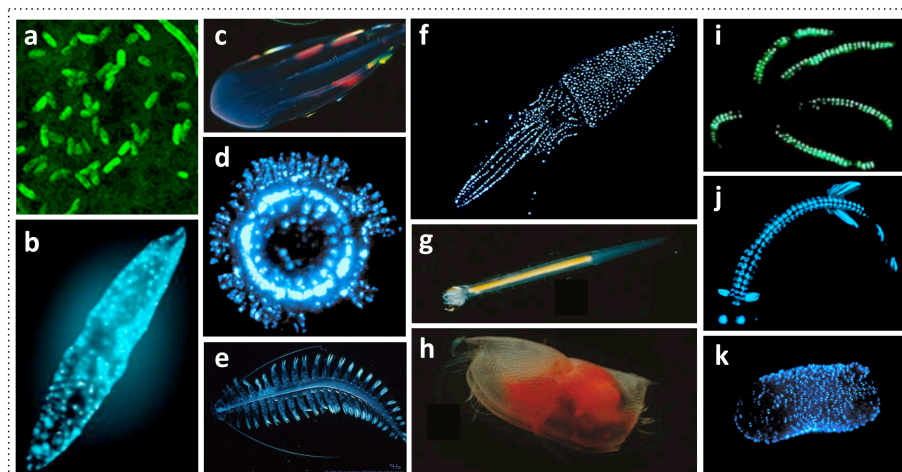


Figure 1 Examples of luminous marine organisms. **a**, Bacteria (*Vibrio fischeri*). **b**, Dinoflagellata (*Pyrocystis fusiformis*). **c**, Ctenophora (*Beroe forskalii*). **d**, Cnidaria (*Atolla wyvillei*). **e**, Annelida (*Tomopteris helgolandica*). **f**, Mollusca (*Abralia veranyi*). **g**, Chaetognatha (*Caecosagitta macrocephala*). **h**, Crustacea (*Conchoecia* sp.). **i**, Echinodermata (*Amphipholis squamata*). **j**, Teleostei (*Melanostomias bartobeani*). **k**, Tunicata (*Pyrosoma atlanticum*). Pictures: E. Nelson and L. Sycuro (**a**). E. Widder (**b,d,f,j,k**). S. Haddock (**c,e,g,h**). J. Mallefet (**i**). Organisms in **a,b,d,f,i-k** were photographed by their own light. Organisms in **c,e,g,h** are daylight photographs.

2.2 Chemical principles

Contrary to fluorescence and phosphorescence whose light emission requires a prior light absorption, luminescence is the results of an exergonic chemical reaction whose energy is emitted in the form of light (Hastings and Morin, 1991).

Two major luminous systems exist in luminous organisms: (i) luciferine/luciferase, and (ii) photoprotein systems (**Fig. 2**).

In the first system (**Fig. 2a**), an organic substrate (luciferin) is oxidised by an enzyme (luciferase) in the presence of oxygen (Wilson and Hastings, 1998). While luciferases are always species-specific (Boden and Kampa, 1974), luciferins are often common to different organisms, sometimes phylogenetically remote (for more details, see the **annexe 2**; Shimomura, 2006; Haddock et al., 2010). The current hypothesis for the evolution of luciferins and luciferases consists in the idea that luminous organisms acquired their luciferin through their food, and developed their luciferase later, probably from antioxydant molecules that emit light as a by-product of their reactive oxygen scavenging chemical activity (Warner et Case, 1980; Rees et al., 1998; Haddock et al., 2001; 2010). This hypothesis could partly explain the widespread occurrence of bioluminescence in organisms, and is supported by the presence of light-emitting luciferins in luminous but also non-luminous organisms (Shimomura, 1987). Coelenterazine-making organisms have not been discovered yet (Haddock et al., 2001).

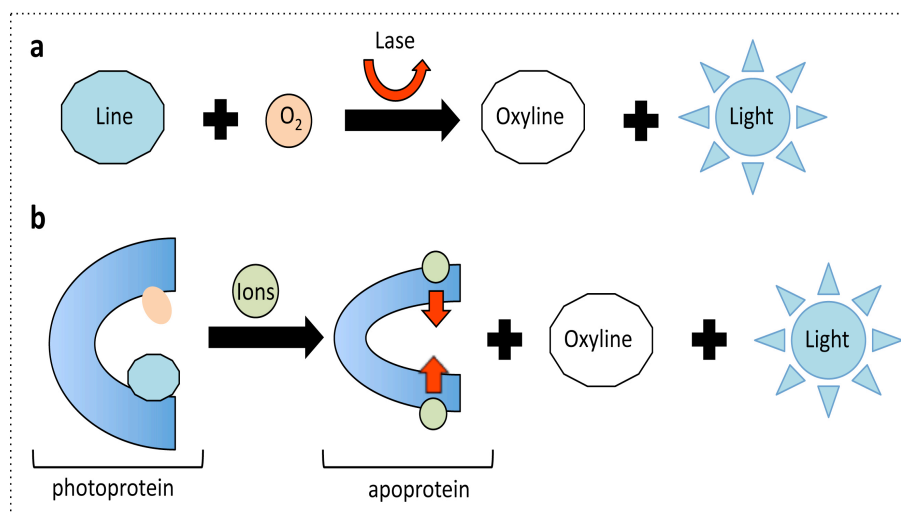


Figure 2 1 Luminous systems of luminescent organisms. a, luciferine/luciferase system. **b,** photoprotein system. Lase, luciferase; Line, luciferine.

In the second system (**Fig. 2b**), both the substrate and the enzyme are present in a single molecule that needs a cofactor (ions, oxygen, H_2O_2 , ATP) and, sometimes, specialized enzymes (catalase, peroxidase) to start to produce light (Shimomura, 1985; 1986; 2006). Although less frequent than the luciferine/luciferase system, it has been found in various organisms including cnidarians, ctenophores, annelids, mollusks and echinoderms and its presence was also suggested in fishes (Shimomura, 1985; Campbell, 1989; Shimomura, 2006).

2.3 Photogenic structures

Luminescent organisms produce light from a variety of different structures. The simplest structural unit for light emission is a single luminous cell (= photocyte) acting as an effective point source (**Fig. 3a**). This structural unit is of course found in luminescent unicellular organisms but also in luminescent pluricellular organisms, even if it is rare in crustaceans, cephalopods and fishes, which generally harbour more complex photogenic structures (Herring, 1985a). The photogenesis may be restricted to certain parts of the cell, in specialised organelles which are generically called “microsources” but bear different names according to the group of organisms in which they are present (dinoflagellates: “scintillons”, coelenterates: “lumisomes”, polychaetes: “photosomes”, insects: “photocyte granules”, crustaceans: “membrane network”, echinoderms (brittlestars): “vesicles”, fish: “microvesicles”) (DeSa et al., 1963; Anderson and Cormier, 1973, Bassot and Bilbaut, 1977; Herring, 1978; Deheyn et al., 2000).

Numerous luminescent pluricellular organisms harbour photogenic organs called photophores. These photophores may contain photocytes (= intrinsic photophores; **Fig. 3b**; in molluscs, crustaceans, and fishes), symbiotic luminous bacteria (= symbiotic or bacterial photophores; **Fig. 3c**; in tunicates, mollusc and fishes) or even a luminous secretion (= secretory photophores; **Fig. 3d**; in annelids, molluscs, crustaceans and fishes), which either stay in the organs (intra-glandular luminescence) or are propelled externally on demand (extra-glandular or secretory luminescence) (Herring, 1978; Leisman et al., 1980; Herring, 1985a; Haygood, 1993). In some cases, an organism can bear different types of photogenic organs (e.g. anglerfishes genus *Linophryne* which possess both intrinsic and bacterial photogenic organs) (Hansen and Herring, 1977).

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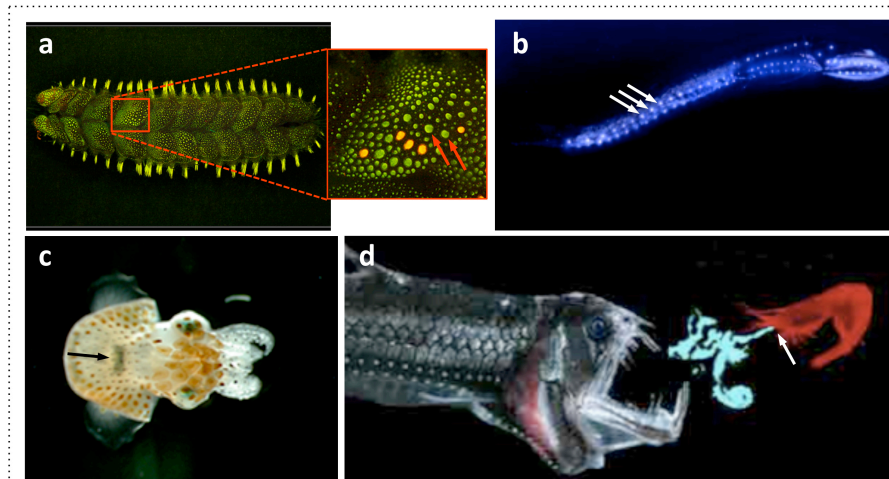


Figure 3.1 Photogenic structures in luminescent organisms. **a**, photocyte (green fluorescent dots, arrows) on a scale (insert) of a polynoid worm (*Lepidonotus squamatus*). **b**, intrinsic photophores (arrows) from a fish (*Cyclothone braueri*) whose glow conceals its silhouette from below. **c**, symbiotic photophore (arrow) from a squid (*Euprymna scolopes*) containing bacterial symbionts (*Vibrio fischeri*). **d**, secretory photophore in a shrimp (*Acantheephyra purpurea*) emitting a luminous fluid (arrow) towards a predatory fish (*Chauliodus sloani*). Pictures: A. Semenov (**a**). Deepsea photography (deepseaphotography.com) (**b**). M. McFall-Ngai (**c**). E. Widder (**d**).

In addition, accessory optical structures of several types (including pigmented tissues reflectors, lenses, filters, and light guides) are often present to modulate the physical characteristics (light intensity, wavelength and angular distribution) of the light emitted (**Fig. 4a**; Denton et al., 1972; 1985; Herring, 1985a), and other structures may be associated with a physical control of the light emission (occlusive mechanisms which serve to switch off the light from continuously glowing symbiotic photophores; **Fig. 4b-d**; Haneda, 1963; Young, 1977; Howland et al., 1992; Haygood, 1993) or with a specialised function (e.g. illicium of anglerfishes which allow photophore movement for prey/mate attraction; **Fig. 4e**; Munk, 1999) may also be present (Herring, 1978; 1985a).

In some particular cases, photogenic organs retain structural traits from the tissue from which they evolve, offering a glimpse into their evolutionary history. This is the case of the internal symbiotic photogenic organs of numerous fishes which are actually derived from the intestinal tract (Haygood, 1993), the internal photogenic symbiotic organs of some photophores which appears to be derived from the ink sac (Herring, 1978; Tong et al., 2009), and the ventral photogenic organ of the deep sea fish *Benthalbella infans* (Johnson and Herring, 1985) which is clearly derived from muscle tissue and of course the light-emitting suckers of the octopus *Cirrothauma murrayi* (Johnsen et al., 1999).

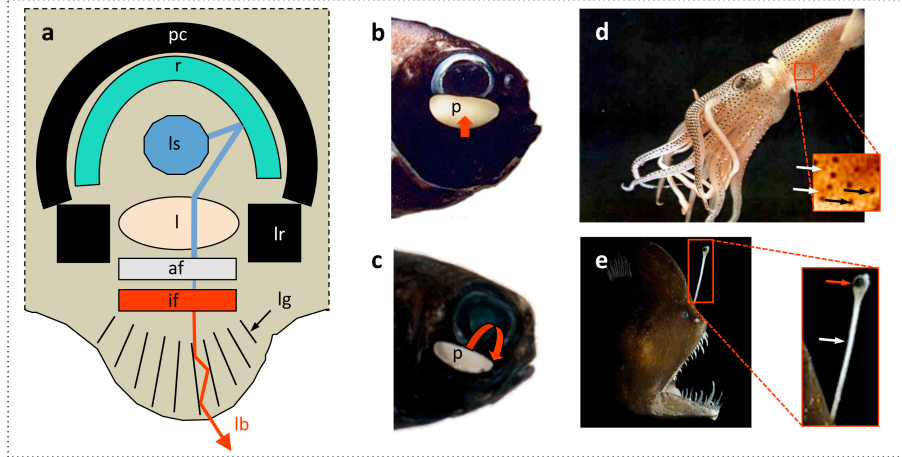


Figure 4 | Accessory structures of photophogenic organs in luminescent organism. **a**, schematic drawing of a photophore indicating the various dioptric structures that can change the physical characteristics of the initial light source (modified from Herring, 1985a). A light beam from a light source (ls) is reflected by a reflector (r), collimated by a lens (l), partially altered by an absorption (af) and interference filter (if) which respectively decreased its intensity and changed its spectral distribution, and finally guided to the outside by light guides (lg), which are sometimes called light pipes when they conduct light over a great distance (especially in animals containing internal bacterial glands). Pigmented cup (pc) and lamellar pigmented ring (lr) are used to intercept the light which otherwise would be emitted off-axis. **b**, enlarged view of the flashlight fish *Photoblepharon palpebratus* showing the symbiotic ocular photophore (p) whose light can be switched off by movement of an inverse pigmented eyelid (arrow). **c**, enlarged view of the head of the flashlight fish *Anomalops katoptron* showing the symbiotic ocular photophore (p) whose rotation into a dark pigmented pocket prevent its light to be emitted outside the fish's body. **d**, some squid species (including *Calliteuthis* sp. presented here) are thought to switch off their photophore (black arrows) luminescence by the mean of expanding chromatophores (small black dots, white arrows, insert) in the light path. **e**, enlarged view of the head of the anglerfish *Melanocetus murrayi* showing the fishing pole composed of the illicium (white arrow, insert) supporting the symbiotic photophore called esca (red arrow, insert) for movement and positioning. Pictures: Seapics (**b**, **e**). Fishbase (**c**). Il mondo degli animali, RCS Li (**d**).

The position of photogenic structures varies from one species to another and is generally related to their ecological functions in luminous organisms, for example, marine organisms which camouflage their silhouette with a ventral light emission generally harbour only ventral photogenic organs (McAllister, 1967; Young et al., 1979; Herring, 1985a).

The intensity of the luminescence point sources varies from 10^7 to 10^{14} photon s^{-1} (Warrant and Locket, 2004) and the light spectra (of a specific photogenic source) is monochromatic but extremely variable [from 395 nm (in the cnidarian *Thourella hilgendorfi*) to 705 nm (in the fish *Malacosteus niger*)] from one species to another (**Fig. 5**) or sometimes between photogenic structure of a same organism (e.g. *M. niger* which have blue-emitting ventral photophores and red-emitting ocular photophores; **Fig. 5d**) (Herring, 1983; Widder et al., 1983; Latz et al., 1988; De

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Cock, 2004). In general, the luminescence maximum of emission depends on the environment of the emitting species: terrestrial luminescent species are mostly yellow-green emitters, coastal (and benthic) species are mostly green emitters and pelagic species are mostly blue emitters (Herring, 1983), but exceptions exist. The angular distribution of light depends on the structure, the position and the function of the photogenic organs (since the efficiency of a luminous signal is dependent upon its perception by its target organism; Herring, 1985a). Single point emitting sources emit light in all directions while accessory structures of complex photophores generally restrict light in a particular direction (Denton et al., 1972). Epidermal photogenic organs usually emit only light to the outside while organs positioned on appendages such as esca of anglerfishes emit light in all directions. Ventral photophores of counterilluminating animals generally share an elliptical luminescence pattern, with most of the light emitted downward, while photophores involved in other biological functions harbour other angular patterns (Denton et al., 1972; Buck, 1978; Herring, 1985a).

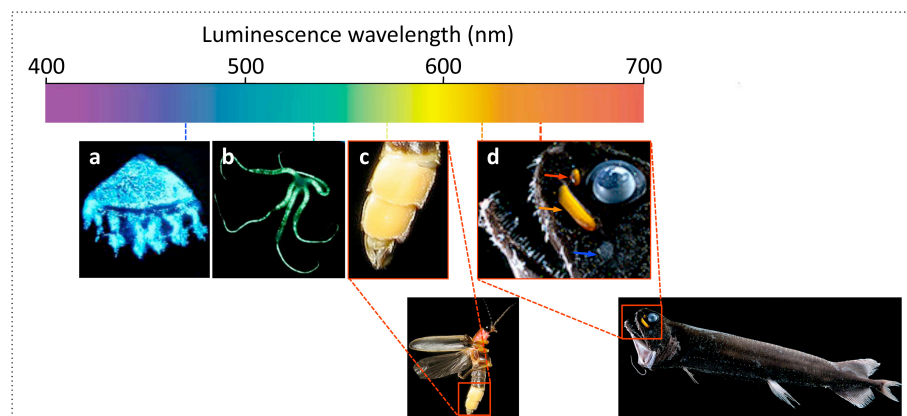


Figure 5 | Spectral characteristics of different luminous organisms. **a**, the jellyfish *Periphylla periphylla* emitting a blue light, typical of pelagic marine luminescent organisms ($\lambda = 470$ nm; Herring, 1983). **b**, the brittle star *Ophiopsila aranea* emitting a green light, typical of coastal (and benthic) marine luminescent organisms. **c**, the firefly *Photinus pyralis*, whose abdomen emit a yellow light (insert), typical of terrestrial luminescent organisms ($\lambda = 570$ nm; Seliger et al., 1964). **d**, the fish *Pachystomias microdon*, which is particular in the way that (i) it produces red light in a pelagic marine environment, and (ii) its photogenic organs are thought to emit luminescence of variable wavelength. The fluorescence patterns of the head's photogenic organs suggest that its preorbital organ (red arrow, insert) emit, as its relatives, *Malacosteus niger* and *Aristostomias sp.*, a red light (fluorescence peaks: $\lambda = 615, 640$ and 680 nm; mean = 645 nm), its suborbital organ (orange arrow) an orange light (fluorescence peaks: $\lambda = 590$ and 650 nm; mean = 620 nm) and its postorbital organs, as its ventral photophores, a blue light (fluorescence peak: $\lambda = 500$ nm) (Herring and Cope, 2005). Pictures: J. Mallefet (**a**, **b**). A. Farmer (**c**). S. Haddock (**d**).

Luminescent displays can be separated in two categories according to the timing pattern of their light emission: flashes (duration < 2s) and glows (duration ≥ 2 s). In

the general sense, glows are thought to be attractant signals and sudden flashes are supposed to be repellents (Haddock et al., 2010). Continuous light emission is not found in other organisms than bacteria and fungi, most luminescent signals being transitory events (Hastings, 1978; Herring, 1985a).

2.4 Control mechanisms

Luminescent organisms (except bacteria and fungi which are continuous light emitters; Case and Strause, 1978; Hastings, 1978; Wassink, 1978) display a variety of luminescence control mechanisms allowing them not only to turn their photogenic structures on and off, but also to modulate the physical characteristics (intensity, colour and angular distribution) of their light emission (Haddock et al., 2010).

A distinction is made between intrinsic control mechanisms, which correspond to controls at the intracellular level, and extrinsic control mechanisms, which occur at the extracellular level. The first mechanisms involved the control of the photogenic chemical reaction or intracellular control events while the second group includes physical and nervous control mechanisms.

Physical luminescence control mechanisms include (i) optical filtering and reflection (**Fig. 4a**); (ii) mechanical shuttering [via dark shutters (**Fig. 4b**), pigmented pocket hiding a mobile photogenic organ (**Fig. 4c**), retraction/extension of chromatophores covering the photogenic organ (**Fig. 4d**)]; (iii) control of the circulatory supply [mainly O₂, e.g. in the intrinsic serotonin (5-HT)-controlled photophore of the northern krill *Meganyctiphanes norvegica* (Krönström et al., 2009), in the luminous bacterial esca (see **Fig. 4e**) of anglerfishes (Bertelsen, 1951; Munk, 1999) and bacterial organs of leiognathids (Haygood, 1993)]; (iv) external mixing of reactants involved in the photogenic reaction, sometimes produced by separated secretory photophores, or ejection of a secretory product into the water (**Fig. 3d**) (Case and Strause, 1978; Herring and Morin, 1978; Christophe, 1984; Herring, 1985a). Physical control mechanisms of luminescence occur mostly in animals harbouring symbiotic photogenic organs whose bacteria are permanently glowing (Haygood, 1993; Haddock et al., 2010) as well as in some organisms possessing intrinsic organs (Buck, 1978; Young, 1973; 1977; Krönström et al., 2007).

Nervous substances involved in the control of luminescence emission are diverse and belong to the three groups of neurotransmitters (Nichols, 1994): (i) type I,

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including simple amino acid (glutamate and gamma-amino-butyric acid (GABA); (ii) type II, including “classical” neurotransmitters [(nor)adrenaline, acetylcholine, serotonin, octopamine and purines]; and (iii) type III, including a wide variety of neuropeptides generally used at very low concentration and acting more as neuromodulators rather than as primary neurotransmitters (they do not have specific effects alone but rather modulate light emission induced by a primary neurotransmitter; SALMFamide neuropeptides S1 and S2). In addition, the nitric oxide (NO), a particular membrane-permeable gaseous neurotransmitter with a very short half-life (about 5 seconds) has been found to be involved in the luminescence control mechanism of some animals. Nervous control mechanisms of luminescence only occur in organisms with intrinsic photogenic structures (Buck, 1978).

The **Table 1** synthesises the current knowledge of nervous mediators involved in bioluminescence control mechanisms.

Although luminescent organisms harbour a high diversity of luminescence control mediators, and although closely related species sometimes do not share common nervous control mechanisms (Dewael and Mallefet, 2002), some general patterns can be observed: (i) the main neurotransmitter of Insecta is thought to be octopamine; (ii) the main neurotransmitter of Ophiuroidea (brittle stars) appears to be acetylcholine; (iii) the main neurotransmitters of Osteichthya (bony fishes) is adrenaline; and (iv) the main neuromodulator of neural-induced luminescence is certainly NO, which modulate serotonin-induced luminescence of the krill *M. norvegica* and bony fishes including the hatchetfish *Argyrops leucurus*, the pearlfish *Maurolicus muelleri* and the midshipman fish *Porichthys notatus* and probably many others (since other bony fishes including *Hygophum benoiti*, *Myctophum punctatum*, *Cyclothone braueri*, *Electrona risso* and *Vinciguerria attenuata* show nitric oxide synthase immunoreactivity in their photogenic organs; Krönström and Mallefet, 2009).

Nerves can either act directly on the photocytes or on other elements of the photophores (lens, chromatophores, etc.), or even on structures not included in the photophore (e.g. muscles controlling the blood supply to the photogenic organs and maybe also the emission of the secretory luminous substance).

Table 1 Bioluminescence neural control mechanisms

<i>Taxon</i>	<i>Mediator [effect(s)]</i>
Protoctista	
Dinoflagellata	
<i>Gonyaulax polyedra</i>	¹ Adr [+]
Cnidaria	
Anthozoa	
<i>Renilla köllikeri</i>	² Adr [+]
Ctenophora	
<i>Mnemiopsis leidyi</i>	³ Adr [+], ³ Ach [+]
Annelida	
Polychaeta	
<i>Chaetopterus variopedatus</i>	⁴ 5-HT [+], ⁴ GABA [-]
<i>Harmothoe lunulata</i>	⁵ Ach [+]
Arthropoda	
Insecta	
<i>Photuris versicolor</i> ; other fireflies	⁶ Octo [+], ⁷ NO [+]
Crustacea	
<i>Meganocytiphanes norvegica</i>	⁸ 5-HT [+], ⁹ NO [M+/-]
Echinodermata	
Ophiuroidea	
<i>Amphipholis squamata</i>	¹⁰ Ach [+], ¹¹ Ado[+], ¹¹ Glu [+], ¹¹ ATP [+], ¹⁴ Adr [+], ^{12,13} GABA, Gly, cat., 51,2, pur.
<i>Amphiura arcystata</i>	¹⁵ Ach [+]
<i>Amphiura filiformis</i>	¹⁶ Ach [+], ¹⁷ Tryp [+], ¹⁷ Octo [+]
<i>Ophionereis fasciata</i>	¹⁸ Ach [+], ¹⁸ GABA [+]
<i>Ophionereis schayeri</i>	¹⁸ Ach [+], ¹⁸ GABA [+]
<i>Ophiopsila californica</i>	¹⁷ Tryp [+]
Chordata	
Osteichthya	
<i>Argyrolepiscus hemigymnus</i>	¹⁹ Adr [+], ¹⁹ Nadr [+], ²⁰ NO [+/-]
<i>Argyrolepiscus olfersi</i>	²¹ Adr [+]
<i>Chauliodus sloani</i>	¹⁵ Adr [+]
<i>Cyclothone braueri</i>	²² Adr [+]
<i>Dibranchius atlanticus</i>	²³ Adr [+]
<i>Echiostoma barbatum</i>	²⁴ Adr [+]
<i>Gonostoma spp.</i>	²⁵ Adr [+]
<i>Mauroliscus muelleri</i>	²⁶ Adr [+], ²⁶ Nadr [+]
<i>Mauroliscus pennanti</i>	²⁰ Adr [+]
<i>Porichthys notatus</i> , <i>P. myriaster</i>	²⁷ Adr [+], ²⁷ Nadr [+], ²⁸ 5-HT [-], ²⁹ NO [M+/-]
<i>Stomias boa</i>	²⁷ Adr [+]

¹Hardeland, 1980; ²Anctil et al., 1984; ³Anctil, 1985; ⁴Anctil, 1981; ⁵Nicolas et al., 1978; ⁶Nathanson, 1979; ⁷Trimmer et al., 2001; ⁸Kay, 1965; ⁹Krönström et al., 2007; ¹⁰De Breaeaker et al., 1996; ¹¹De Breaeaker, 1999; ¹²De Breaeaker et al., 1999; ¹³De Breaeaker, 2000a; ¹⁴Dupont et al., 2004; ¹⁵Mallefet, unpublished data; ¹⁶Dewael and Mallefet, 2002a; ¹⁷Vanderlinden and Mallefet, 2004; ¹⁸Mallefet et al., 2004; ¹⁹Baguet and Marechal, 1978; ²⁰Krönström et al., 2005; ²¹Bertelsen and Grontveld, 1949; ²²Mallefet and Dupont, unpublished data; ²³Crane, 1968; ²⁴Harvey, 1931; ²⁵Herring and Morin, 1978; ²⁶Baguet and Christophe, 1983; ²⁷Baguet, 1975; ²⁸Garipey and Anctil, 1983; ²⁹Krönström, 2009.
Ach, acetylcholine; Ado, adenosine; Adr, adrenaline; ATP, adenosine triphosphate; cat., catecholamines; GABA, gamma-aminobutyric acid; Nadr, noradrenaline; Gly, glycine; NO, nitric oxide; Octo, octopamine; Tryp, tryptamine; 5-HT, 5-hydroxytryptamine (= serotonin); +, stimulatory effect; -, inhibitory effect; M, modulatory effect; pur., purines

2.4.1 Ecological functions

Numerous functions have been suggested to explain the wide occurrence of the bioluminescence phenomenon (Harvey, 1952; Buck, 1978; Herring, 1985a; Haddock et al., 2010). In most cases, however, the suggested function often reflect the ingenuity of researchers rather than the fruit of strong experimental work (Buck, 1978), mostly due to the difficulty of performing behavioural experiments on bioluminescent animals, since most of them inhabit the deep-sea realm. General functions of bioluminescence fall in three groups: (i) predation avoidance; (ii) predation help; (iii) intraspecific communication (Buck, 1978; Widder, 1998; Haddock et al., 2010). In some cases, luminescent displays of an organism may have more than one function, and the function of an animal's luminescence may also change according to the environment and over ontogeny.

2.4.2 Predation avoidance

2.4.2.1 Camouflage

The bioluminescent anti-predatory defence that has been the most documented and tested in laboratory is camouflage by counterillumination (Haddock, 2010). This type of camouflage mainly evolved in the twilight zone (= mesopelagic zone, depths between 200 to 1000 m in the oceans) along with other crypsis mechanisms including transparency, cryptic coloration and mirroring (McFall-Ngai, 1990; Johnsen, 2001; 2003), but is also present in some epipelagic organisms (McFall-Ngai and Morin, 1991; Harper and Case, 1999). It occurs in many bony fishes (Denton et al., 1972; Case et al., 1977; McFall-Ngai and Morin, 1991; Harper and Case, 1999), squids (Young and Mencher, 1980; Young et al., 1980; 1977; Jones and Nishiguchi, 2004), and crustaceans (Warner et al., 1978) with either intrinsic or extrinsic photophores.

The function of counterillumination corresponds to the use of ventral (lower) photophores to emit a luminescence whose physical characteristics (angular distribution, intensity and wavelength) are similar to those of the light coming from the surface, thus obliterating the potential shadow (Clarke, 1963).

Mesopelagic counterillumination involved stereotypical patterns reflecting the homogeneous physical characteristics of this environment (Denton et al., 1972; 1985; Young, 1983): (i) elliptical angular distribution with most of the light being directed downward (**Fig. 6a**); (ii) intensity (**Fig. 6b**); and (iii) wavelength (**Fig. 6c**) (Harper and Case, 1999). Epipelagic counterillumination is generally more a

“disruptive illumination”, which allows organisms to disrupt their silhouette (rather than making it invisible) in a more heterogeneous and changing environment (Endler, 1978; McFall-Ngai and Morin, 1991).

In addition to match the physical characteristics of the environmental down-welling light, counter-illuminating organisms have to be able to emit light for long period of time (counterillumination luminescent displays typically do not include flashes) and cope with temporal variations of their light environment (Warner et al., 1978). Different strategies allow counter-illuminators to continuously match their changing light environment by modulating each characteristics of their luminescence: (i) angular distribution modulation is achieved by orientating photophores or reflectors to emit the light in the appropriate direction even when the orientation of the body change (**Fig. 6d**; Young, 1977; Latz and Case, 1982); (ii) adaptations to changes of ambient light intensity consist in either changes of luminescence intensity up to until nine orders of magnitude in some “luminescence intensity modulators” (**Fig. 6b,e**; Warner et al., 1978; Young et al., 1980; McFall-Ngai and Morin, 1991; Harper and Case, 1999; Jones and Nishiguchi, 2004), or simply vertical migrations in the water column in “isolume followers” which are not or less able to change the intensity of their luminescence (**Fig. 6b**); and (iii) adaptation to environmental spectral has only been shown in some squid species (*Abrolia veranyi*, *A. trigonura* and *Abrialopsis* sp.) which are able to change the colour of their luminescence by using different photophore types or/and by intra-photophore modulations (**Fig. 6c**; Young and Mencher, 1980; Herring et al., 1992). Luminescence modulation involves feedback control mechanisms. Numerous bony fishes, shrimps and squids bear ocular photophores whose light emission is directed toward the eye and are thought to allow comparison with down-welling daylight (**Fig. 6f**; Young, 1973; Young et al., 1979). More recently, it has been demonstrated that the bacterial photogenic organs of the bobtail squid *Euprymna scolopes* are coupled to photoreceptors which are though to directly monitor luminescence from their bacterial symbionts, independently from eye vision, offering a new way for luminescence feedback control mechanism (Tong et al., 2009). In some fish species, the pineal gland is also known to give information on ambient light intensity (e.g. in the lanternfish *Triphoturus mexicanus*; McNulty and Nafpaktitis 1976).

Finally, some organisms, including fishes (e.g. fishes genus *Scopelarchus* and *Argyropelecus*; Herring, 2002) and few squids genus *Histioteuthis* (Douglas and Marshall, 1999), evolved a yellow lens in their eyes that allow them to selectively filter short wavelengths and increase the contrast between luminescence emission and the background (which generally contains longer wavelengths than surrounding

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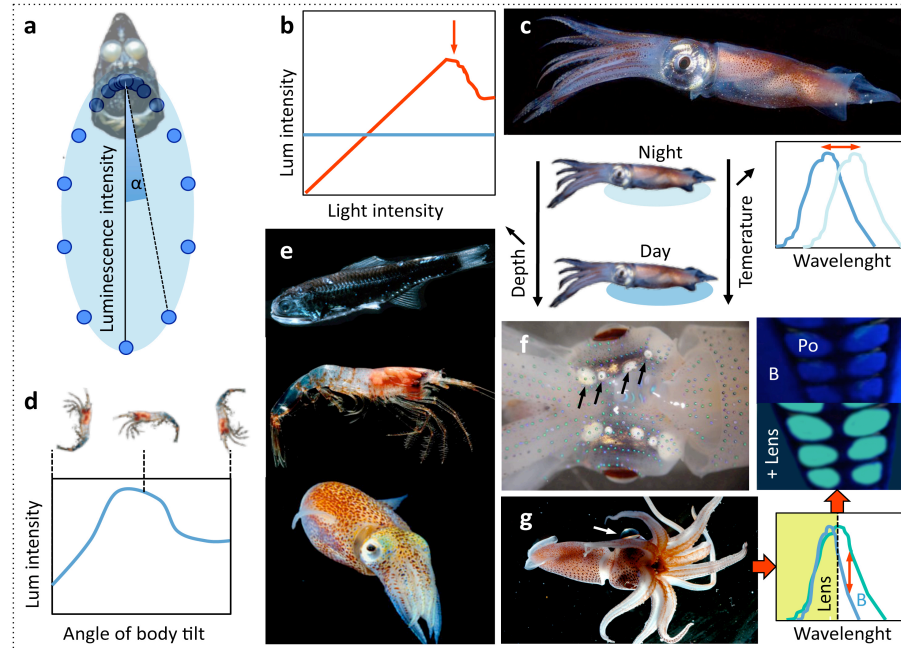


Figure 6 | Luminous camouflage in marine organisms. **a**, angular distribution (blue dots) of the hatchetfish *Argyrops hemigymnus* luminescence and residual down-welling solar light (blue area) according to the angle from the vertical (α), showing a nearly perfect matching (modified from Denton et al., 1972). **b**, relationship between residual light intensity and luminescence intensity of isolume followers (blue line) and modulators (red line; modified from Young et al., 1980) which increase their luminescence intensity until their camouflage fails (red arrow). **c**, the squid *Abralia veranyi* modify the colour of its luminescence from blue (peak: $\lambda = 400$ nm) at depth during the day to green (peak: $\lambda = 560$ nm) at surface at night. **d**, the shrimp *Sergestes similis* adjust the orientation of its photophores in order to maximize downward luminescence (modified from Latz and Case, 1982). **e**, examples of luminescence intensity modulators (from up to down): *Myctophum punctatum*, *S. similis*, *Euprymna scolopes*. **f**, ocular photophores (arrows) of *A. veranyi*. **g**, *Histioteuthis* sp. can break counterillumination camouflage by a yellow lens in its large tubular eye (arrow) that increases the contrast between photophore luminescence (P) and background (B). Pictures: M. Vecchione (c, f). S. Haddock (e, up). D. Fenolio (e, middle, g). T. Kleindinst (e, down). L. Madin, BBC (h).

daylight) (**Fig. 6g**), breaking the camouflage (Munk, 1980; Douglas and Thorpe, 1992; Herring, 2002; Warrant and Locket, 2004). Another technique for breaking counter-illuminating camouflage has also been proposed for cephalopods and crustaceans: having a polarised vision they could theoretically make the contrast between luminescence polarised by iridophores (when a reflector is present in the photogenic organ) and unpolarised down-welling daylight (Hanlon and Shashar, 2003; Warrant and Locket, 2004). The wide occurrence of mesopelagic animals relying on counterillumination suggest, however, that this camouflage remains an efficient way to escape from the sight of many upward-looking predators in a world

with no place to hide and where below 100 m predation is the main way to feed (Land, 2000; Warrant and Lockett, 2004; Haddock et al., 2010).

2.4.2.2 Lure

Luring predators is another way to use luminescence to avoid predation. Numerous luminescent displays may enter in this category. In general the prey can confuse its predator either (i) on its position or (ii) on its nature.

The ability to turn its luminescence off is certainly the first behaviour that allows organisms to escape from predator's sight (**Fig. 7a**; Buck, 1978). Other organisms such as ostracods and flashlight fishes perform movements with rapid on/off switches reinforcing the difficulty for a predator to catch them (**Fig. 7b**; Tsuji et al., 1970; Morin et al., 1975). Some organisms including crustaceans, molluscs and fishes also produced a luminous smoke screen to hide them when they are changing direction (**Fig. 7c**; Angel, 1968; Buck, 1978; Herring and Barnes, 1978; Herring, 1985a; Robison et al., 2003). In some cases, luminous part of the animal may be autotomized (= sacrificial lures; **Fig. 7d**; e.g. scales in polynoid worms, “self-luminous bombs” of swimming worms, arms in ophiuroids and the deep-sea squid *Octopoteuthis deletron*, or photocytes in the searsiid fishes), which distract the attention of the predators; the tissue is then generally regenerated by the organisms (Herring, 1978; Herring, 1985a; Bush et al., 2009; Osborn et al., 2009; Haddock et al., 2010). Adaptive sacrificial luminescence has also been postulated for the “sternchaser” photophores of the male of the myctophid fishes genus *Tarletonbeania*, which decoy away predator from a mate (Bolin, 1961; Buck, 1978).

In some cases, organism use display to try to confuse predator on their nature, either by intimidating the predatory organism (aggressive luminescent display, startle effect; **Fig. 7e**) or by looking like something of poor interest (mimicry; **Fig. 7f**) (Buck, 1978; Haddock et al., 2010). Startle effect has been demonstrated in dinoflagellates to avoid copepod grazing (Esaias and Curl, 1972). Mimicry may occur in the midshipman fishes *Porichthys sp.*, whose photophore pattern is believed to look like the ctenophore *Mnemiopsis sp.* (McAllister, 1967) or a small swarm of euphausiids (Tsuji et al., 1971), or in the luminous pelvic organ of the fishes *Kasidoron sp.*, which mimic a siphonophore (Buck, 1978; McAllister, 1967).

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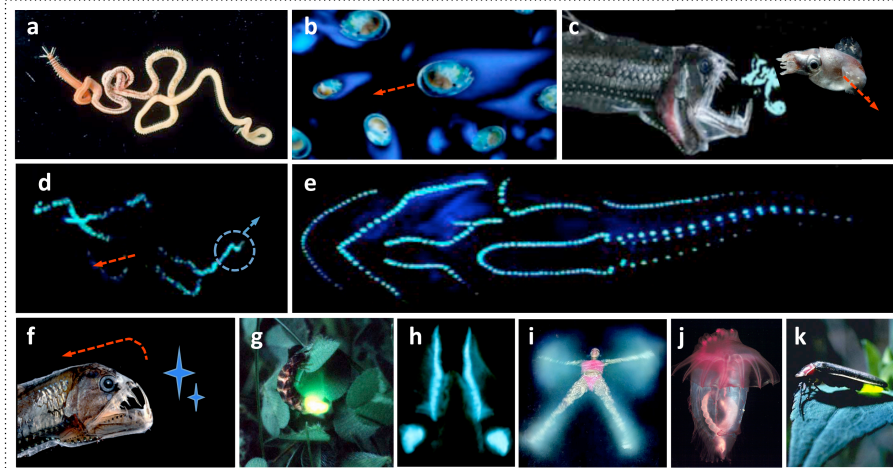


Figure 7 | Luminescent anti-predatory behaviours (other than camouflage). **a**, the continuous light emission of the palolo worm (*Palola siciliensis*) is known to attract predatory fishes. **b**, the ostracod *Vargula hilgendorfi* performed quick movements when emitting light preventing predator to know its exact position. **c**, the squid *Heteroteuthis dispar* which emit a luminous smoke screen when it is disturbed. **d**, the brittle star *Amphiura filiformis* is able to autotomize its self-luminous arms (dashed blue circle) to distract the attention of a predator when it is hiding. **e**, the photophore pattern of the midshipman fish *Porichthys notatus* look like a swarm of euphausiids or of ctenophores genus *Mnemiopsis*. **f**, startle flashes of a dinoflagellates towards a potential predator. **g**, the larvae of *Lampyris noctiluca* show an aposematic luminous signal to warn of their unpalatability. **h**, the luminous secretions of the bivalve *Pholas dactylus* are believed to deter potential settler to occupy the same space. **i**, the luminescence of the dinoflagellate *Pyrodinium bahamense* is believed to act as a burglar alarm, illuminating organisms that disturb them. **j**, when ingested, parts of the pelagic sea cucumber *Eupniastes eximia* continue to glow for hours, acting as a sacrificial tag that could attract the attention of secondary predators. **k**, fireflies genus *Photuris* are thought to use their light to see and avoid spider webs. The red arrows in **a-e** represent relative movement of the light emitter. Pictures: Seapics (**a**). Y. Ohmiya. (**b**). D. Shale (**c**). E. Widder (**c,f**). J. Mallefet (**d,e**). R. Scagell (**g**). KSL (**h**). F. Franky (**i**). L. Madin (**j**). E. Degginger (**k**).

2.4.2.3 Warning

Warning luminescent signals include (i) aposematism and (ii) settler deterrence.

Luminescent aposematism corresponds to the use by an organism of a luminescent signal to advertise a potential predator of its toxicity or unpalatability (**Fig. 7g**). It has been demonstrated in larvae of the firefly *Lampyris noctiluca* (De Cock and Matthysen, 1999; 2003) and suggested in other organism such as the fishes genus *Porichthys* which could use light to warn potential predators of its poisonous opercular spines (Nicol, 1967), the brittle star *Ophiopsila riisei* which is believed to have a bad taste (Grober, 1988), scyphozoan jellyfishes which are fragile yet potentially dangerous (Herring and Widder, 2004) and scale worm *Harmothoe*

lunalata (Bassot and Nicolas, 1995). It has to be noted that, contrary to classical aposematic signals which involve bright coloration, luminescent aposematism may be temporary, switched off when not needed.

Settler deterrence occurs when the luminescent displays of an organism discourage other organisms to occupy the same space (**Fig. 7h**). This behaviour has been suggested in some species including the worm *Chaetopterus variopedatus* and the bivalve *Pholas dactylus* whose luminescent secretions might discourage shadow-loving intruders (Nicol, 1962; Buck, 1978). It has also been suggested that dinoflagellates use luminescence to highlight their predators in order to refrain grazing on them (Nicol, 1962).

2.4.2.4 Burglar alarm

Burglar alarm is a secondary anti-predatory behaviour where the prey makes its predators vulnerable to the attack of higher order predators (Haddock et al., 2010). Two principal luminescent burglar alarm behaviours can be distinguished: (i) burglar alarm *stricto sensu* and (ii) sacrificial tag.

The luminescent burglar alarm *stricto sensu* correspond to behaviours where the prey illuminates its predators to make it visible to higher order predator (**Fig. 7i**; Burkenroad, 1943). Laboratory experiments demonstrate the efficiency of this behaviour in dinoflagellates using mysid shrimps as primary predators and the midshipman fish *P. notatus* (Mensinger and Case, 1992) or cephalopods (Fleisher and Case, 1995) as secondary predators.

Sacrificial tags are part of luminous organisms that can be lost during a predatory encounter and which continue to glow inside or on the body of the predator and may attract the attention of a higher order predator (**Fig. 7j**; Haddock et al., 2010). Since transparency is widespread in the deep-sea, it is likely that numerous luminous organisms may benefit from this stratagem, including fragile animals such as sea cucumbers (Robison, 1992) and scyphozoan jellyfishes (Herring and Widder, 2004). This may explain the great occurrence of black (and red) pigments in the gut of otherwise transparent animals (Johnsen, 2005). Sacrificial tag may therefore be a secondary function of the sacrificial lure and may occur in all organisms harbouring self-glowing body parts.

2.4.2.5 Illumination

Fireflies genus *Photuris* are known to emit flashes with a high frequency when landing, suggesting a role of luminescence in avoiding hazards such as water or spider's web, forming a particular case of luminescent anti-predatory behaviour (**Fig. 7k**; Lloyd, 1968; Buck, 1978).

2.4.3 Predation help

2.4.3.1 Attracting lure

Since many animals are attracted to light, numerous organisms developed luminescent lures to attract their prey and therefore increase their predatory rate. They can be separated in two categories: (i) “intrinsic lure” and (ii) “extrinsic lure”.

Predators use intrinsic luminescent lure when they use their own light to produce attractant signals for their preys (i.e. simulating potential food or mate; **Fig. 8a**). A diversity of intrinsic luminescent lures exists in the wild. The best examples are probably (i) the esca and the barbel of anglerfishes which attract preys at close vicinity of the jaws of these fishes (Kenaley and Hartel, 2005; Pietsch, 2009); (ii) the modified luminescent tentacles of cephalopods genus *Chiroteuthis* and *Stauroteuthis* (Johnsen et al., 1999); (iii) the siphonophores genus *Erenna* which possess red-fluorescent lures believed to attract long wave-sensitive organisms (Haddock et al., 2005); (iv) imitation of firefly courtship behaviour by some insects (Buck, 1978); and (v) attraction of predators by the black collar of the cookie-cutter shark *Isistius brasiliensis* which could appear as a small fish, the rest of the body being camouflaged by counterillumination (Widder, 1998).

Predators use extrinsic luminescent lures when they use an exterior light source to attract their prey (“extrinsic lure”; **Fig. 8b**). This behaviour is particular because it generally involves non-luminous and large organisms, such as suggested for the megamouth shark *Megachasma pelagios* whose white pigmented jaws may reflect down-welling light and attract plankton (Takahashi, 2001), and the sperm whale *Physeter macrocephalus* which would voluntarily stimulate bioluminescence to induce a burglar alarm response from predatory squids, drawing them to its mouth (Fristrup and Harbison, 2002).

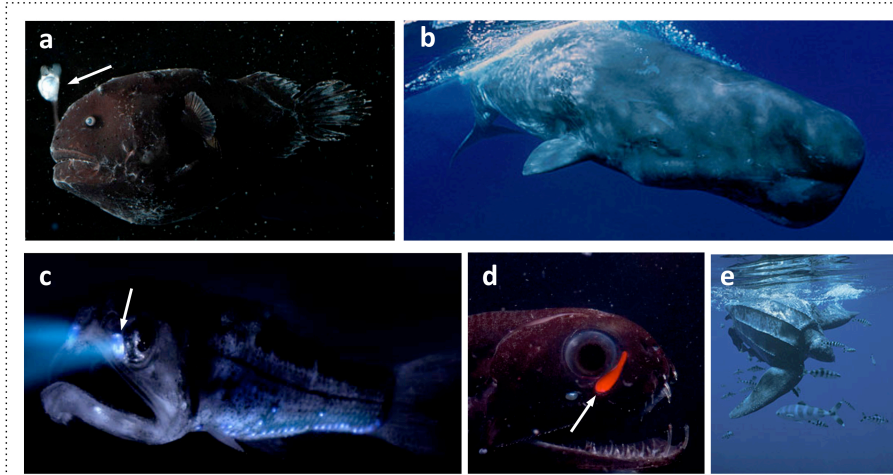


Figure 8 I Luminescent predatory behaviours. **a**, the deep-sea anglerfish *Chaenophryne longiceps* harbours a symbiotic luminescent esca (arrow) which is known to attract prey but also bacterial symbionts. **b**, the sperm whale *Physeter macrocephalus* is believed to use the bioluminescence of exterior sources to attract its squid preys. **c**, headlamp (arrow) of myctophids are thought to stun/confuse potential prey. **d**, the fish *Aristotomias erostoma* have two preorbital red light-emitting photophores (arrow) that are supposed to illuminate and see its prey without being detected. **e**, the leatherback turtle *Dermochelys coriacea* is thought to use the luminescence of their prey (jellyfishes) to increase their visual capabilities during their deep dives. Pictures: S. Haddock (a). D. Perrine (b,e). Deepseaphotography (c). D. Fenolio (d).

2.4.3.2 Illumination

Luminescence emitted towards the prey may help a predator (i) to stun, confuse its prey and (ii) to make it more visible for capture.

Stunning behaviours involve quick flashes directed towards the prey (**Fig. 8c**). It probably occurs in the squids genus *Taninga* which has been shown to produce flashes from its tentacles when attacking baits (Kubodera et al., 2007) and in the myctophids genus *Diaphus* which possess bright forward pointing “headlamp” photophores (Haddock et al., 2010). It has also been suggested that the lantern shark *Etmopterus spinax* could stun its prey by sudden flashes of its ventral pattern (Hickling, 1928).

Prey illumination as an aid for visual searching corresponds to behaviours where organisms use light to localise their prey (**Fig. 8d**), and generally involve forward pointing orbital photophores. It is thought to occur in flashlight fishes, in some myctophids and of course in dragonfishes genus *Aristostomias*, *Pachystomias*, *Malacosteus*, and *Tactostoma* whose red-emitting preorbital photophores appear

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well adapted to illuminate and see their prey without being detected since they also have red-sensitive visual pigments (Morin et al., 1975; McCosker, 1977; Herring and Cope, 2005; Haddock et al., 2010). In some cases predators including leatherback turtle *Dermochelys coriacea* and elephant seals genus *Mirounga* could use luminescence from other organism for visual searching of their preys (extrinsic illumination; **Fig. 8e**; Houghton et al., 2008; Haddock et al., 2010).

2.4.4 Intra-specific communication

2.4.4.1 Sexual signalling

Luminescent sexual signalling occurs when male and female of one species use luminescence to communicate (**Fig. 9a,b**). Although the use of luminescence for courtship has been well documented in terrestrial organisms such as fireflies that rely on extremely complex species-specific sexual displays, its ecological significance is still poorly understood in marine environment (Haddock et al., 2010). Recently, however, it has been shown that ostracods may also produce courtship displays whose complexity is similar to that of fireflies (Rivers and Morin, 2008). Sexual signalling probably also occurs in ponyfishes, which often demonstrate a sexual dimorphism in the size of their photogenic organ (those of males being larger) used in synchronised group displays (Sasaki et al., 2003; Herring, 2007). Luminescence from other fishes such as the flashlight fish *Photoblepharon palpebratus* and the midshipman fish *Porichthys notatus* is believed to be involved in sexual signalling as well (Crane, 1965; Lane, 1967; Morin et al., 1975).

Numerous other organisms (for which sexual luminescent communication has never been observed) harbour a sexual dimorphism in the size, the position, or the form of their photogenic organs, suggesting a possible role in sexual signalling and mate recognition (**Fig. 9c-e**; Herring, 2007). Caudal photophores of myctophids, especially, show a pronounced sexual dimorphism providing strong circumstantial evidence for their involvement in sexual signalling (Nafpaktitis, 1978; Herring, 2007). In some cases such as in many anglerfishes and some cephalopods, only one sex (female in these cases) is luminous (Robison and Young, 1981; Pietsch, 2005; Herring, 2007). Light emission or visual sensitivity at unusually long or short wavelengths (red emitters in deep-sea for example), also gives some evidence for a private light communication channel (Haddock et al., 2010).

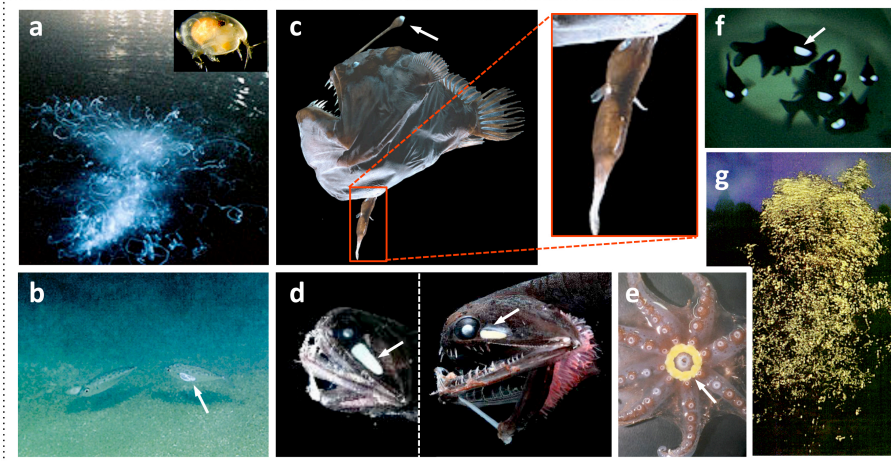


Figure 9 | Intraspecific luminescent communication. **a**, luminescent courtship display of the ostracod *Vargula hilgendorfi* (insert). **b**, luminescent signalling (arrow) in the pony fish *Leiognathus elongatus*. **c**, luminescent sexual dimorphism in the anglerfish *Melanocetus niger*: female possess a luminous esca (arrow) which is not present in the dwarf male (insert). **d**, luminescent sexual dimorphism the fish *Aristotomias erostoma*: male (left) has a larger postorbital photophore (arrow) than female (right). **e**, sexual luminescent dimorphism in the octopus *Jepetella diaphana*: female has a circumoral yellow photophore (arrow) which is not present in male. **f**, the fish *Photoblepharon palpebratus* is believed to use its flashling ocular photogenic organ (arrow) for group swimming/hunting. **g**, the firefly *Pteroptyx tener* form very dense, synchronously flashing, aggregations (here in a tree). Pictures: J.Morin (**a**). A. Sasaki (**b**). T. Pietsche (**c**). E. Widder (**d**). M. Vecchione (**e**). J. Hemdal (**f**). A. Mauroy (**g**).

2.4.5 Aggregation

Some luminescent organisms are known to form dense aggregations with synchronous light displays (**Fig. 9f,g**). Such **aggregations** are believed to provide numerous benefits for the light emitters including mutual promotion of feeding, defence, territoriality, mating, etc. (Buck, 1978). Some examples include luminescent quorum-sensing in bacterial colonies which allow the induction of host colonisation-related genes (Lupp et al., 2003) or attraction of animals for the recycling of biological material (dead animals, fecal pellets; Hastings and Nealson, 1977; Robison et al., 1977; Haddock et al., 2010), dense congregations of oriental fireflies associated with mating (Buck and Buck, 1976; 1978), schooling of flashlight fishes for cohesive hunting and defence (Morin et al., 1975, McCosker, 1977), mating associated-shoaling of the krill *Meganctiphanes norvegica* (Mauchline, 1960), and cohesive vertical migrations of various fishes (Colman, 1960).

3 Sharks

3.1 Evolution and zoogeography

Chondrichthyes (cartilaginous fishes) are believed to be monophyletic characterised by a peripheral tesserate mode of mineralising endoskeletal tissue and the presence of claspers (sperm conducting structures formed by the extension of the pelvic fins) in male individuals (Grogan and Lund, 2004; Lebrun, 2004).

Their first discovered remnants (spines and scales) were found during the Silurian (443.7-416 M.Y. ago) and cladodont sharks from the upper Devonian (400 M.Y. ago) already possessed the general aspect of current shark species (Grogan and Lund, 2004; **Fig. 10a**). Chondrichthyes radiated rapidly and expansively in all aquatic environments including marine and fresh waters (Rodina and Ivanov, 2002; Lund, 1976).

Current cartilaginous fishes include Elasmobranchs [(sharks and batoids (rays and skates))], and holocephalans (chimaeras). The divergence time of Holocephalans and Elasmobranchs is still in debate but probably occurred quite early in the evolution of cartilaginous fishes since chimaeroids underwent their major phylogenetic radiation during the Carboniferous period (Pradel et al., 2009). The common ancestor of all living forms of sharks, rays and skates plus its descendants are called neoselachians (Carvalho, 1996). The phylogenetic relationships among elasmobranchs are not totally resolved, but a recent publication brought new insights on the general organisation of current elasmobranch phylogeny, by comparing morphological and molecular data with stratigraphic distribution of fossil taxa (Kriwet et al., 2009; **Fig. 10a**). Following this paper, molecular phylogeny appears to give the most probable framework since it considerably reduces the length of ghost lineages. Molecular phylogeny establishes three major current elasmobranch groups: squalan sharks (Squalea), galean sharks (Galea) and batoids (Batoidea) (**Fig. 10a**). However, since other phylogenies exist, the debate is still open.

Height different orders of current sharks have been identified: Heterodontiformes, Orectolobiformes, Lamniformes, Carcharhiniformes, Hexanchiformes, Squaliformes, Squatiniformes and Pristiophoriformes. These orders encompassed currently around 500 described species (Kriwet et al., 2009) that harbour an amazing diversity of appearances, sizes, behaviours, and environments (**Fig. 10b-k**; Compagno et al., 2004). Sharks are found between the surface and abyssopelagic depths, in coastal or oceanic waters, and present different living modes including benthic (bottom living), pelagic (living in the water column) or benthopelagic habits (**Fig. 10b**; Compagno et al., 2004; Priede et al., 2006).

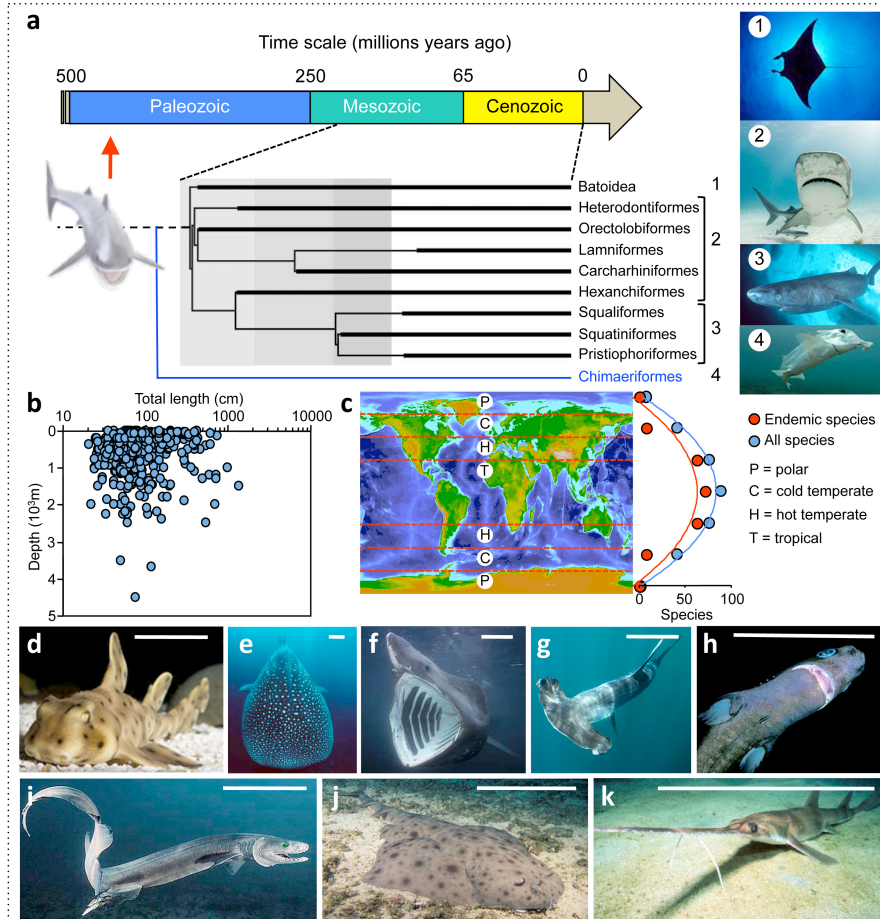


Figure 10 | Evolution, phylogeny and diversity of sharks. **a**, apparition of the first cartilaginous fishes during the Silurian (arrow; 443.7-416 M.Y. ago) and molecular phylogeny of cartilaginous fishes (blue colour = modification from Kriwet et al., 2009). Thick bars represent confirmed stratigraphic distribution of taxa and thin bars are ghost lineages (gaps in fossil records). Shaded areas represent the different parts of the Jurassic: lower, middle, upper. 1, Batoidea (rays); 2, Galeomorphii (sharks); 3, Squalia (sharks); 4, Holocephalii (chimaeras). **b**, relationship between maximum recorded size and maximum recorded depth for 446 current shark species (information from Compagno et al., 2004). **c**, relationship between climatic zones (latitude) and number of shark species (modified from Musick et al., 2004). **d-k**, species from the height current shark orders: **d**, Heterodontiformes (*Heterodontus francisci*). **e**, Orectolobiformes (*Rhynchodon typus*). **f**, Lamniformes (*Cethorhinus maximus*). **g**, Carcharhiniformes (*Sphyrna lewini*). **h**, Squaliformes (*Euprotomicrus bispinatus*). **i**, Hexanchiformes (*Chlamydoselachus anguineus*). **j**, Squatiniformes (*Squatina tergocellata*). **k**, pristiophoriformes (*Pristiphorus cirratus*). Informative scale bar: 10 cm in **h**, 50 cm in others. Pictures: Seapics (**a,d-h,j,k**). K. Aitken (**i**).

They present a wide aquatic distribution (in marine and freshwater environments), but are more represented in equatorial and tropical zones than in

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temperate waters and polar regions (**Fig. 10c**) and larger species have generally a larger distribution than smaller ones (Musick et al., 2004).

3.2 Anatomical features

Some particular features of sharks (and other cartilaginous fishes) distinct them from other fishes. Their endoskeleton (see **Fig. 11a**) is composed of chondrocytes in an extracellular matrix surrounded by a fibrous perichondrium. The extracellular matrix may harbour three calcification types (areolar, globular and prismatic). Areolar calcification form vertebral cartilage while the two other types form the tessellated cartilage, which is present in appendicular, axial (and cephalic) skeleton (Dean and Summers, 2006). The endoskeleton's hybrid nature of these fishes make this tissue particularly stiff and tough (Dean et al., in press).

In addition to their endoskeleton, sharks also possess other structures that form a dermal skeleton that covers the body surface and the mouth cavity (Lebrun, 2001). They consist in finspines (in Heterodontiformes, Squaliformes and some Echinorhiniformes), placoid scales and teeth. Finspines are present in front of the dorsal fin (**Fig. 11b**) and certainly serve as defensive tool but may also aid manoeuvrability in swimming and turning (Maisey, 1979). Contrary to placoid scales and teeth, finspines are not continuously replaced during growth, but instead increase in size by successive deposits that form specific annual bands sometimes used for ageing studies (Clarke and Irvine, 2006; Irvine et al., 2006). Placoid scales (**Fig. 11c**) are structures covering the entire surface of the sharks' body which harbour a very variable morphology according to the species, the age, the sex and even the position on the body. Their roles are multiple including protection from predators (and ectoparasites) and abrasion, hydrodynamism and accommodation of sensory and bioluminescent organs (Raschi and Tabit, 1992).

Teeth also demonstrate an important morphological diversity with variations according to species, age, sex and position in the jaw but also season (Kajiura and Tricas, 1996; Lebrun, 2001; Motta, 2004). This morphological diversity mainly reflects a high functional diversity since they are involved in numerous feeding modes but also in mating events (males stabilize themselves by biting the female during mating) (Wourms, 1977). There is strong correlation between the general form of a tooth and the main prey of its owner (**Fig. 11d**): planktonic sharks have small (non-functional) teeth, durophagous sharks have flattened teeth, piscivorous species have narrow teeth, sometimes with additional secondary crowns, and megacarnivorous sharks harbour large blade-like teeth, often with serrated edges

(Motta, 2004). Teeth are the link between the force produced by the jaw and the prey. To increase the efficiency of their dental battery, species can either show a combination of these functional types in their jaw or can change the orientation of their teeth during capture according to the prey (Ramsay and Wilga, 2007).

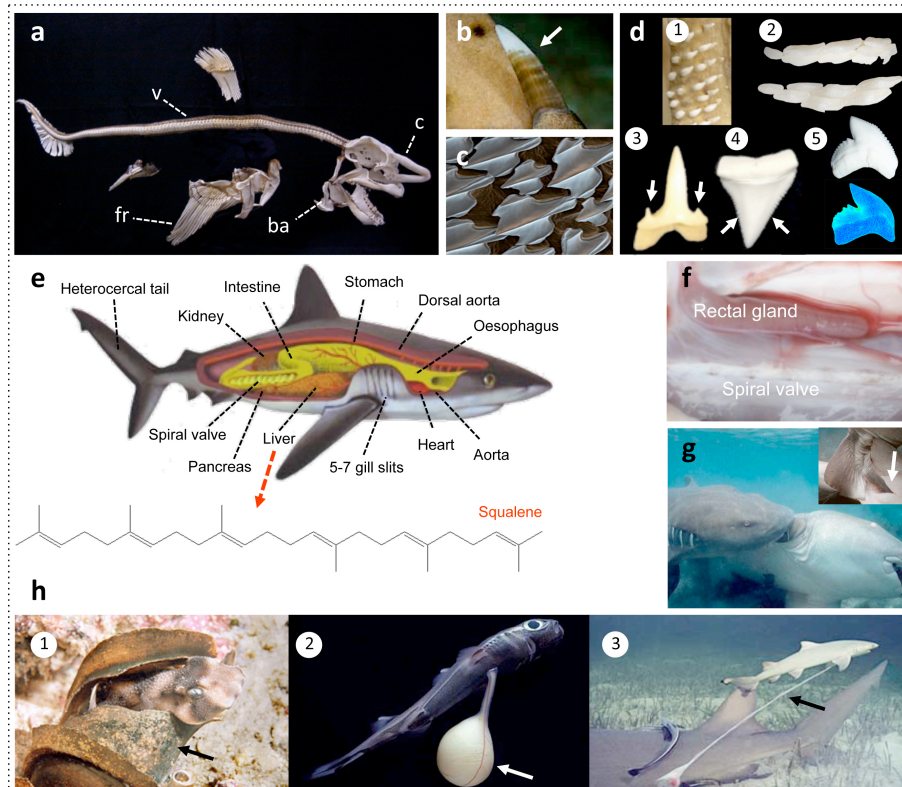


Figure 11 | Particularities of sharks. **a**, shark cartilaginous endoskeleton: chondrocrane (c), branchial arches (ba), vertebrae (v), and fin rays (fr). **b-d**, ossified structures of sharks. **b**, dorsal fin spine (*Heterodontus francisci*). **c**, dermal denticles. **d**, teeth: 1, *Cethorhinus maximus* (planktonic prey); 2, *H. francisci* (durophagous prey); 3, *Lamna nasus* (gliding preys); 4, *Carcharodon carcharias* (large preys); *Galeocerdo cuvier* (everything). Arrows indicate lateral denticles in (3), denticulated edge in (4). Bottom part of (5) represents stress underwent by the teeth when biting: blue colour, small stress; red colour, high stress. **e**, internal shark anatomy and structure of squalene. **f**, picture of a rectal gland from *Squalus acanthias*. **g**, mating pair of *Gynglimostoma cirratum*: the male bites female's pectoral fin and inserts one of its pterygopods in the cloaca of the female (arrow; insert). **h**, reproductive modes of sharks: 1, oviparity (*H. francisci*); 2, ovoviviparity (*Etmopterus spinax*); 3, viviparity (*Negaprion brevirostris*). Pictures: Canadian Shark Research Lab (**a**). Seapics (**b,g,h**). Eye of science (**c**). Reefquest Center for Shark Research (**d1,4**). R. Robertson (**d2**). L. Whitenack (**d3,5**). M. de Gruy (**e**). N. Hellman (**f**).

Other species show “universal teeth” whose morphology is adapted towards a broad range of organisms (the best example being teeth of the tiger shark, *Galeocerdo cuvier*; see **Fig. 11d**). The general internal anatomy of sharks is

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illustrated in **Fig. 11e**. Some interesting shark characteristics can be highlighted here. Sharks' stomach is capable of eversion out of the buccal cavity in order to get rid of indigestible food particles, parasites and mucus (Brunnschweiler et al., 2005). The liver of sharks is enormous and filled with squalene, a hydrocarbon that helps them to attain neutral buoyancy although lacking the swimbladder present in bony fishes (Corner et al., 1969). The intestine of these fishes, called spiral valve intestine, is also particular, increasing the surface area for digestion and absorption of food but also conserving space in the body cavity for liver and embryos (Moss, 1984). Finally, sharks harbour a rectal gland near the caudal region of the intestinal tract (**Fig. 11f**), which has been shown to be a site of net salt secretion in Elasmobranchs (in addition to kidneys and gills) for osmoregulation (Burger, 1965; Evans et al., 2004). In addition, kidneys and gills retain trimethylamine oxide (TMAO) to protect their enzymatic systems from the destabilizing action of urea (which is also retained by sharks as an osmolyte) (Seibel and Walsh, 2002).

Contrary to bony fishes, sharks have an internal fertilisation, male sharks inserting one of their claspers in the cloak of the female during mating (**Fig. 11g**; Carrier et al., 2004). At this time the male bite the head or the pectoral fin of the female to stabilise itself. Different reproductive modes exist in sharks (**Fig. 11h**; Wourms, 1977): oviparity, ovo-viviparity [with the embryos either depending only on yolk reserve (yolk dependency), or being egg (embryo)-eating (= oophagy), or being nourished through placental analogues] and viviparity. At this time, the ancestral condition is still in debate (Carrier et al., 2004).

3.3 Cellular communication

3.3.1 Endocrinology

Although the knowledge of hormonal regulation in sharks (and other Elasmobranchs) made some progress in the recent years, sharks and their relatives stay one of the least investigated vertebrate groups in the field of endocrinology. For what is known at present, the hormonal regulatory mechanisms of sharks appear strikingly similar to those of other vertebrates, indicating that these mechanisms have been well conserved during the course of evolution (Gelsleichter, 2004; **Table 2**). In this section we will therefore concentrate on a particular hormonally regulated mechanism which is only present in cold-blooded animals: the physiological colour change by the action of melanocytes or melanophores, which is different from the vertebrates' morphological colour change [involved in plumage/pelage color change

during the breeding season and in suntanning (**Fig. 12b**) by the fact that its action is more rapid (Bentley, 1982).

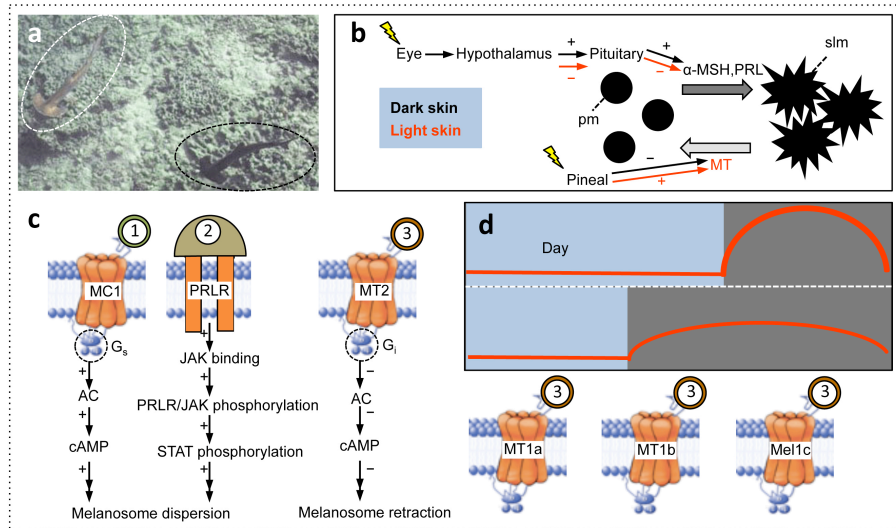


Figure 12 | Hormonally controlled colour change in sharks. **a**, suntanning in *Sphyrna lewini* (compare light form on the left and dark form on the right) illustrating how sharks can dramatically alter the colour of their skin by melanosome dispersion in melanophores. **b**, proposed mechanism for physiological regulation of skin coloration in Elasmobranchs (adapted from Gelsleichter, 2004): pm, punctate melanophore; slm, star-like melanophores. **c**, proposed (most probable) pathways of hormones involved in Elasmobranch colour change: 1, α -melanocyte stimulating hormone (α -MSH); 2, prolactin (PRL); 3, melatonin (MT). We chose the JAK/STAT pathway for the PRL because it is the most widespread but other pathways have also been demonstrated (see Bole-Feysot et al., 1998). Fishes were demonstrated to have four distinctive melatonin receptors (Ikegami et al., 2009), however, only the intrinsic pathway of the MT2 has been demonstrated (Falcon et al., 2007). **d**, profile of melatonin plasmatic content (y-axis) in fishes during summer time (up) and winter time (bottom), according to the time of the day (x-axis; modified from Falcon et al., 2007). AC, adenylyl cyclase; G_i , inhibitory G-protein; G_s , stimulatory G-protein; JAK, Janus kinase; STAT, signal transducers and activators of transcription. Pictures: G. Goodman-Lowe (**b**). NIAA (**c**).

The physiological colour change of sharks (and relatives) involves the action of three different hormones: (i) the α -melanocyte stimulating hormone (α -MSH) and (ii) the prolactin (PRL) which are both produced by the pituitary gland (α -MSH from the *pars intermedia* and PRL from the *pars distalis*; Gelsleichter, 2004), and (iii) the melatonin (MT) which is produced by the pineal gland and the retina in fishes (Falcon et al., 2007). Pituitary release of PRL and α -MSH provokes expansion of dermal melanophores and skin darkening (Wilson and Dodd, 1973; Visconti et al., 1999). Under conditions that favour skin pallor, the release of α -MSH (and PRL) is suppressed by neural signals from the hypothalamus (**Fig. 12c**).

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Table 2 Example of known hormonal regulations in sharks*

Gland (functional action ^o) - Hormone	Effects
S(D) - Gastrine ¹	Digestive acids secretion
S(D) - Somatostatin ²	Digestive process inhibition (gastrine inhibition)
I(D) - Secretine ³	Pancreatic bicarbonate-rich juices secretion
I(D) - Cholecystokinin-like ⁴	Bile and pancreatic enzymes supply regulation
P(D) - Insulin ⁵	Energy substrate uptake, conversion and storage
dt(D) - PYY/GRP/VIP/Tachykinins ^{6,7,8}	Gut motility and/or blood flow regulation
B(D) - Leptin, NPY, galanin ^{9,10,11}	Satiety regulation
Pi(G) - Growth hormone ¹²	Growth regulation
Ht(G) - GHRH ¹³	GH release
Ht(G) - IGF-I ¹⁴	Cell hypertrophy in fat, skeletal muscle, cartilage
K(S) - Catecholamines (Adr, Nadr) ¹⁵	Stress management
P(S) - ACTH ¹⁶	Stress management
Ir(S) - 1 α -OHB ¹⁷	Stress management
Ht(O) - CRF ¹⁴	ACTH release
H(O) - CNP ¹⁸	Osmoregulation
L(O) - Angiotensine II ¹⁹	Osmoregulation
Pi(P) - α -MSH ²⁰	Skin darkening
Pi(P) - PRL ²¹	Skin darkening
Pin(P) - MT ²²	Skin lightening
Pi(R) - GTH ²³	Reproduction (steroid release)
F/Ht(R) - GnRH ²³	Reproduction (GTH release)
G(R) - Steroids (E ₂ , P ₄ , T, DHT) ²⁴	Reproduction (sex diff., puberty, mating)
G(R) - Relaxin ^{11,25}	Reproduction (gametogenesis, parturition)
T(R) -T ₃ , T ₄ ^{11,26}	Reproduction (viviparous sharks; migration)
U(R) - Calcitonin ²⁷	Reproduction (viviparous sharks)

*This table summarises the information contained in Gelsleichter, 2004.

^oGlands were grouped according to their functional action: D, digestion and energy metabolism; G, growth; O, osmoregulation; P, physiological colour change; R, reproduction; S, stress.

References: ¹Vigna, 1983; ²Holmgren and Nilsson, 1983; ³Bayliss and Starling, 1903; ⁴Vigna, 1979; ⁵Bajaj et al., 1983; ⁶Holmgren et al., 1992a; ⁷Holmgren et al., 1992b; ⁸Kagström et al., 1996; ⁹Vallarino et al., 1991; ¹⁰Chiba et al., 2002; ¹¹Gelsleichter, 2004; ¹²Yamaguchi et al., 1989; ¹³Plesch et al., 2000; ¹⁴Gelsleichter and Musick, 1999; ¹⁵Butler et al., 1986; ¹⁶Lowry et al., 1974; ¹⁷Idler and Truscott, 1966; ¹⁸Takei, 2000; ¹⁹Anderson et al., 2001; ²⁰Sumpster et al., 1984; ²¹Visconti et al., 1999; ²²Wilson and Dodd, 1973; ²³Forlano et al., 2000; ²⁴Hayes, 1998; ²⁵Bullsbach et al., 1986; ²⁶Gash, 2000; ²⁷Wendelaar Bonga and Pang, 1991.

Gland (or production site) abbreviations: B, brain; dt, digestive tract; F, forebrain; G, gonads; H, heart; Ht, hypothalamus; I, intestine; In, interrenal; K, kidney; L, liver; P, pancreas; Pi, pituitary gland; Pin, pineal gland; S, stomach; T, thyroid; U, urobranchial.

Hormone abbreviations: 1 α -OHB, 1 α -hydroxycorticosterone; α -MSH, α -melanocyte stimulating hormone; ACTH, adrenocorticotrophic hormone; CNP, C-type natriuretic peptide; CRF, corticotrophin-releasing factor; DHT, dihydrotestosterone; E₂, 17 β -estradiol; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; GRP, bombesin/gastrin-releasing peptide; GTH, gonadotropins; IGF-I, insulin-like growth factor; MT, melatonin; NPY, neuropeptide Y; P₄, progesterone; PRL, prolactin; PYY, peptide YY; T, testosterone; T₃, triiodothyronine; T₄, thyroxine; VIP, vasoactive intestinal peptide.

The pineal gland is believed to regulate changes in skin coloration resulting from non-visual perception (Gelsleichter, 2004). The release of MT by this gland is supposed to induce skin lightening during nocturnal periods, as it appears to do in other vertebrates (it could act on the α -MSH release since it is unable to influence the melanosome dispersion in the freshwater ray *in vivo*; Visconti and Castrucci, 1993).

These three hormones induce their action after binding to their specific receptors, which are all metabotropic receptors i.e. receptors coupled to a G-protein (see next section, **Fig. 13d**).

Different melanocortin (MC) receptors have been found in sharks (Haitina et al., 2007), but it is believed that effects of α -MSH on fish physiological colour change are mediated by binding to the MC1 receptor (Logan et al., 2006; Selz et al., 2007), which has been suggested for sharks as well (Gelsleichter, 2004), which has been shown to be positively coupled to cyclic AMP (cAMP) (Im et al., 1998; Hill et al., 2006).

PRL binds to the PRLR, which shows structural differences from one fish species to another (Manzon, 2002), and whose homodimerisation initiates different downstream cascades including the JAK/Stat (Janus kinas/signal transducers and activators of transcription) and MAPK (mitogen-activated protein kinase) pathways (Bole-Feysot et al., 1998; Freeman et al., 2000).

Four different melatonin receptors (MT1a, MT1b, MT2 and Mel1c) have been found in fishes (Ikegami et al., 2009). Although MT receptors have been shown to be coupled to different intracellular transduction pathways in mammals, only one transduction cascade has been discovered in fishes: the pathway of MT2 receptor which involves a negative coupling to cAMP (Gaildrat and Falcon, 1999; Falcon et al., 2007).

The interaction between the three hormones is not well documented but α -MSH stimulates PRL release from the pituitary (Mountjoy et al., 1992), and melatonin is known to affect PRL (and GH) release in the trout (Falcon et al., 2003). The plasmatic concentration of melatonin is higher by night than during the day and the pattern of oscillation varies also seasonally (**Fig. 12e**), allowing fishes (and other vertebrates) to anticipate changes in their environment to which they have to synchronize their biological rhythms (Falcon et al., 2007).

3.3.2 Neurobiology

Sharks are cartilaginous fishes and as a matter of fact vertebrates. Hence in this section we will first describe the common features of vertebrates' nervous system (which stayed remarkably similar during the course of evolution; Gilles, 2006), and in a second time we will focus on the specificities of the sensory biology of sharks.

3.3.2.1 Nervous system

The nervous system encompasses a central nervous system made of the brain and the spinal cord (**Fig. 13a**) and a peripheral nervous system made of peripheral nerves innervating the different parts of the organism.

The basal element of nervous tissues is a cellular type called neuron, whose structure allows information transfer and processing. The neuron is composed of a cell body (called soma or perykaryon, which is the site of protein synthesis) that contains a nucleus and forms extensions called dendrites, which receive the information from other neurons, and axon, which transmit the information to other neurons or to the final target and is protected by a myelin sheath (**Fig. 13b**).

The information relayed by the neurons is electrical, based on the excitable nature of cell membranes. Cell membranes are composed of a bilayer of phospholipids acting as a barrier preventing the movement of ions and other substances. This, in addition to other cellular mechanisms including ATPase pumps and ion exchangers, induces differences in the ionic concentration present on both sides of the cell membrane (**Fig. 13b**, upper insert). The electrical characteristics [resistance, capacitance and transmembrane potential (-70 mV for resting potential)] of cell membrane result from this difference in ionic composition (Nichols et al., 2001; Gilles, 2006).

Nervous communication is based on the ability of neuron to respond to upstream information (after a summation of inhibitory, excitatory and spontaneous inputs) by a rapid depolarisation/repolarisation of their membrane (= action potential) that is propagated downstream through the axon and transmitted to another neuron. If the upstream information is not sufficient to trigger a membrane depolarisation, there is no propagation of an action potential (Hille, 2001).

The action potential is a phenomenon has been extensively studied and that can be summarised in three different steps (**Fig. 13b**, lower insert; Nicholls, 1994; Sperelakis, 1995; Gilles, 2006): (i) upstream stimuli provoke a depolarisation of the membrane of a neuron (to -55/-40 mV); (ii) the depolarisation of the membrane

induces the opening of voltage-activated Na^+ channels, which increase the Na^+ conductance and the depolarisation, and inactivates spontaneously after 1-2 ms, (iii) the delayed opening of voltage-activated K^+ channels which increase the K^+ conductance and repolarise the membrane and inactivates after 5-10 ms. The speed of axonal communication is increased by the myelin sheath, since its interruptions (nodes of Ranvier) provoke a salutatory conduction from one node to another (**Fig. 13b**; Sperelakis, 1995).

The electric information passes from one cell to another at the level of synapses, which can be either electrical synapses or chemical synapses. In electrical synapses, the cytoplasms of communicating cells are connected by the mean of special junctions, the gap junctions, which allow direct electric transmission. These junctions are actually composed of numerous channels, each formed by two oligomers (connexons) made of six proteins (connexin), which allow direct transmission of ions for electrical transmission (but also small metabolites and second messengers) (**Fig. 13c**; Sperelakis, 1995). Contrary to chemical synapses, they do not show any polarity in the information transmission, since the action potential can be propagated in more than one direction (Gilles, 2006). In chemical synapses, which are by far the most widespread, the information is transmitted from one cell to another thanks to a chemical signal, called neurotransmitter. This neurotransmitter is released by a presynaptic cell (when the action potential provokes the opening of voltage-activated Ca^{2+} channels) into a synaptic cleft and binds to receptors present on the membrane of a postsynaptic cell, or on the presynaptic cell itself for feedback information, is destructed by specialised enzymes and recycled by the presynaptic cell (**Fig. 13d**).

Neurotransmitters are numerous but are divided in four main categories (see **Table 3**; Nichols, 1994): (i) simple amino acids; (ii) “classical” neurotransmitters; (iii) neuropeptides; (iv) nitric oxide (NO), which is a particular gaseous neurotransmitter which freely diffuse through cell membranes and does not involve a specific receptor to exert its action (**Fig. 13d**, insert).

Receptors of neurotransmitters are either ionotropic or metabotropic, according to the nature of their downstream cascade (Gilles, 2006). Ionotropic receptors contain integrated ion channels that open themselves after neurotransmitter binding, increasing the conductance of cations or anions, decreasing (inhibitory) or increasing (excitatory) the likelihood of an electrical signal to be transmitted (**Fig. 13e**). Metabotropic receptors are coupled to a G-protein, which stimulates (after neurotransmitter binding) the intracellular production of a second messenger.

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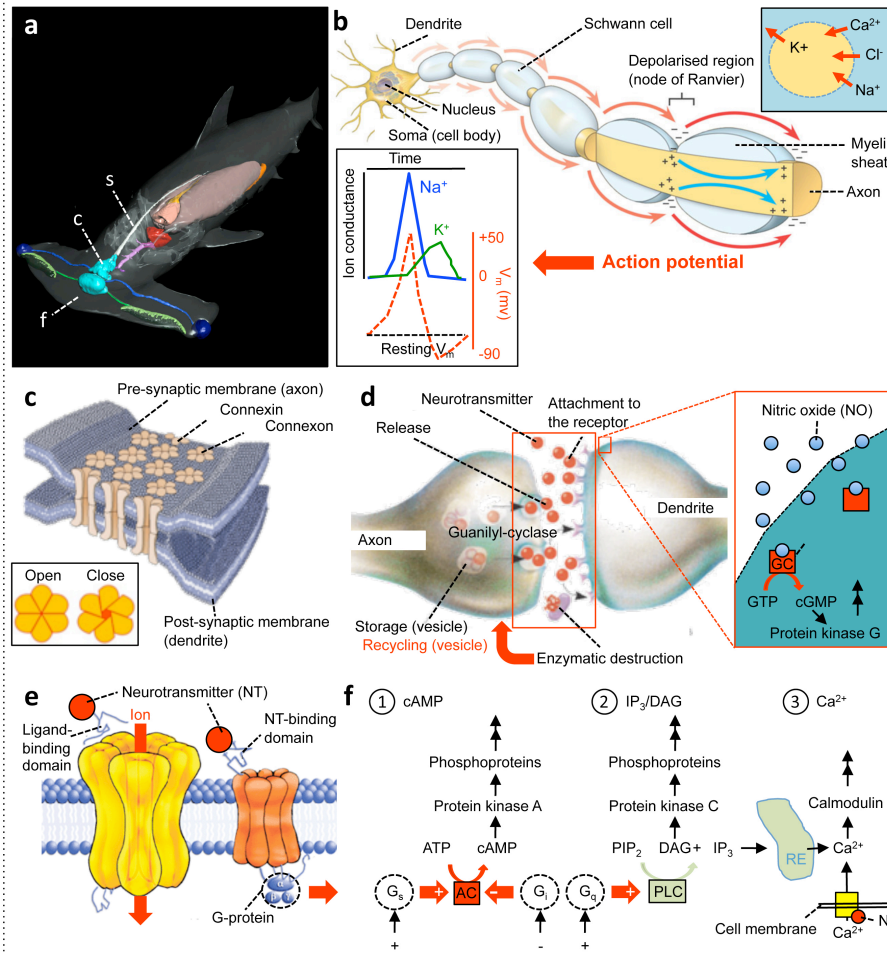


Figure 13 I Nervous system of sharks (vertebrates). **a**, 3D segmentation of the smooth hammerhead shark (*Sphyrna zygaena*) showing the forebrain (f), cerebellum (c) and spinal cord (s). **b**, organisation and action potential of a nerve. Upper insert represents the major ions contributing to the membrane potential (V_m) between interior (yellow) and exterior (blue) of the nerve cell (arrows show the direction of the ions' electrochemical gradients). Lower insert shows the variation of the ion conductance and V_m during an action potential. **c**, gap junction of an electrical synapse and its components. Insert shows a connexon in its open and close state. **d**, different components of the chemical synapse (square). Insert represents NO cascade (without receptor). **e**, structure and action mechanism of ionotropic (left) and metabotropic (right) receptors after ligand binding. **f**, schematic representation of second messengers cascades (cGMP cascade is described in **c**, insert). AC, adenylyl cyclase; ATP, adenosine 3 phosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAG, diacyl glycerol; GC, guanyl cyclase; G_i , inhibitory G-protein; G_q , PLC activating-stimulatory G-protein; G_s , stimulatory G-protein; IP₃, inositol 3 phosphate; PIP₂, phosphoinositol biphosphate; PLC, phospholipase C. Pictures: K. Gledhill (**a**). Encyclopedia Britannica, inc. (**b**). G. Johnson (**c**). Sinauer Associates (**d**). NIAA (**e**).

Table 3 Examples of neurotransmitters present in vertebrates

Neurotransmitter	Receptor	Agonist ^o	Antagonist ^o
<i>1. Amino acids</i>			
Glutamate	NMDA (i)	NMDA	Ketamine
	Kainate (i)	Kainate	DNQX1
	AMPA (i)	AMPA	DNQX
	mGluR ₁₋₈ (m)	BPI A (mGluR _{2,3})	EGLU (mGluR _{2,3})
Glycine	GlyR (i)	Alanine	Picrotoxin
γ -aminobutyric acid	GABA _A (i)	Muscimol	Biccuculin
	[GABA _C (i)]	[Muscimol]	[Biccuculin]
	GABA _B (m)	Baclofen	Phaclofen
<i>2. "Classical" nt</i>			
Acetylcholine	nAChR (i)	Nicotine (Carbachol)	Tubocurarine
	mAChR (m)	Muscarine (Carbachol)	Atropine
(Nor)Adrenaline	α_1 (m)	Phenylephrine	Prazosin
	α_2 (m)	Clonidine	Yohimbine
	β_1 (m)	Isoprenaline	Propanolol
	β_2 (m)	Levalbuterol	Butoxamine
	β_3 (m)	Amibegron	SR 59230A
	β_3 (m)	Azapride	SR 59230A
Dopamine	D ₁₋₅ (m)	Methysergide (5-HT ₁)	Alizapride
Serotonin	5-HT _{1,2,4-7} (m)	Quipazine	Methiotepine (5-HT ₁)
	5-HT ₃ (i)	Betahistine	Ondansetron
Histamine	H ₁ (m)	Betazole	Diphenhydramine
	H ₂ (m)	Immethridine	Cimetidine
	H ₃ (m)	4-Methylhistamine	Ciproxifan
	H ₄ (m)		Thioperamide
<i>3. Neuropeptides</i>			
Substance P	NK ₁ (m)	Neurokinin B	
VIP	VPAC ₁ (m)	VIP28 (VPAC ₁)	CP-96345
			[4Cl-D-Phe ₆ ,Leu ₁₇]VIP
Opioids	OP ₁₋₄ (m)	Etorphine (OP ₃)	(VPAC ₁)
Angiotensine II	AT ₁₋₄ (m)	Benzamide (AT ₂)	Cyprodime (OP ₃)
			Olmesartan (AT ₁)
<i>4. Nitric oxide</i>			
	/	/	/

^oThese lists of agonists and antagonists are of course not exhaustive.

i, ionotropic receptor; m, metabotropic receptor; nt, neurotransmitters; BPI A, biphenylindanone A.

Agonist = chemical that binds to a receptor of a cell and triggers a response by the cell.

Antagonist = type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Second messengers allow external information to be transmitted inside the cells. Four main second messenger pathways have been discovered: (i) 3',5'-cyclic adenosine monophosphate (cAMP) and (ii) 3',5'-cyclic guanosine monophosphate (cGMP), (iii) (1,4,5)-inositol triphosphate (IP₃)/diacylglycerol (DAG), and (iv) calcium (Ca²⁺). All these second messenger have their own cascades, which, however often interact together (**Fig. 13d**, insert; **13e**).

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The production of cAMP by the adenylyl cyclase (AC) is initiated by stimulatory G-protein (from the $G\alpha_s$ class) and inhibited by inhibitory G-protein (from the $G\alpha_i$ class). The cAMP activates a cAMP-dependent protein kinase (PKA) that catalyzes the phosphorylation of other proteins (**Fig. 13e**).

The cGMP is produced by the guanylyl cyclase (GC), present in different forms inside the cells including a membrane-associated and a soluble form, that can be activated/inhibited by NO binding (**Fig. 13d**, insert). The cGMP activates a cGMP-dependent protein kinase (PKG) that catalyzes the phosphorylation of other proteins. The IP_3 /DAG cascade is initiated by G-proteins ($G\alpha_q$ and $G\beta\gamma_q$ class) with active the membrane-associated phospholipase C (PLC) which cleaves the phospholipid phosphoinositol biphosphate (PIP_2) en DAG, which activates a DAG-dependent protein kinase (PKC) that phosphorylates other proteins, and IP_3 , which increases the intracellular Ca^{2+} concentration by provoking the opening of Ca^{2+} channels present in the membrane of the endoplasmic reticulum (**Fig. 13e**; Gilles, 2006).

The numerous functions of Ca^{2+} in the nervous system result from the activation of numerous calcium-binding proteins (such as calmodulin) that activate protein kinases that in turn regulates numerous other proteins (**Figure 13e**; Kennedy, 1994; Sperelakis, 1995).

3.3.2.2 Sensory biology

The sensory abilities of sharks have become practically legendary. Although sometimes exaggerated, senses of sharks are indeed acute, capturing numerous information from their environment, their prey and predators as well as from their conspecifics, which is in turn process by a large brain, sometimes even comparable to those of birds and mammals in brain-to-body ration (Northcutt, 1978; Hueter et al., 2004). This section aims to provid basic information on shark sense abilities.

Sensory systems of sharks can be divided in four main groups: (i) photoreceptors; (ii) mechanoreceptors; (iii) chemoreceptors; and (iv) electroreceptors.

The range at which each receptor type operates depends on the capabilities of the receptor, the strength of the source, and the physico-chemical characteristics of the environment (Hueter et al., 2004). In typical situation, however, hearing and olfaction are used at high distance, vision, electric sense and lateral line system are efficient at closer range, and mechanoreceptors not associated with the lateral line system as well as taste buds need direct contact.

3.3.2.2.1 Photoreceptors

Photoreceptors consist in (i) the eyes (**Fig. 14a,b**) and (ii) the pineal gland (**Fig. 14b**). Sharks have lateral eyes, which are however situated more dorsally in benthic species (Hueter et al., 2004). The visual field of these eyes attains nearly 360° in species which utilise a laterally sinusoidal swimming pattern, like hammerhead sharks for example (Hueter et al., 2004; McComb et al., 2009).

Ocular adnexa are more developed in sharks than in bony fishes, although eyelids of sharks do not cover the entire eyeball (Gilbert, 1963). These adnexa are more mobile in benthic sharks, which use them as a protection against abrasion. Some sharks (carcharhinids and sphyrnids) developed a third eyelid, the nictitating membrane, which can protect the entire eye (**Fig. 14b**). Other sharks (such as lamnids and the whale shark) do not possess a nictitating membrane, but instead developed the ability to rotate the entire organ back into the orbit to protect it when they are feeding, for example (Tricas and McCosker, Hueter et al., 2004). The structure of a typical shark's eye is presented in **Fig. 14a**. Sharks have a thin cornea and, unlike bony fishes, harbour a dynamic iris, which can increase/decrease the size of the pupil according to the light environment (deep-sea sharks, however, have less mobile pupils since they live in a near permanent darkness). The shape of the pupil is however very different from species to species (**Fig. 14b**). Sharks' crystalline lens provides the total refractive index of the eye (since the refractive indexes of the cornea and seawater are almost equal) (Hueter, 1991). Some carcharhinids and sphyrnids harbour lenses containing U.V.-filtering yellowish pigments which decrease chromatic aberrations, light scatter and glare, enhance contrast sensitivity, and provide a U.V. protection (Zigman, 1991; Nelson et al., 2003; Hueter et al., 2004). Sharks accommodate by moving the lens toward/away from the retina, and are hyperopic or emmetropic at the resting state (**Fig. 14a**; Sivak, 1978; 1991; Hueter et al., 2004). Benthic sharks appear to have greater accommodative range than pelagic sharks (Sivak, 1978). The back part of a shark's eye contains the retina, the sclera, and the choroid, which is the eye's only vascularised tissue and, in many species, contains a reflective *tapetum lucidum* (**Fig. 14b**; Denton and Nicol, 1964). This *tapetum*, made of guanine crystal, increases the eye's light sensitivity, and, in some (shallow water) species, can be occluded by dark pigment migration under light-adapted conditions (Nicol, 1964; Heath, 1991).

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Figure 14 | Sensory biology of sharks. **a**, structure of a shark's eye. **b**, Photoreceptors of sharks: 1-8, eyes of different sharks species. 4, nictitating membrane *Carcharhinus perezi*; 7, retractable eye of *Lamna nasus*; 8, tapetum of *Etmopterus spinax*; 9, pineal window (arrow) of *Scyliorhinus canicula*. **c**, position of lateral line canals (red) and free neuromasts (black dots) on *Carcharodon carcharias*. Insert, section of a pore canal with water flow (arrows): c, cupula; hc, hair cell; k, kinetocilia; n, nerve; p, pore; ps, placoid scale; s, stereocilia; sc, sustentacular cell. **d**, 3D head reconstruction of *C. carcharias* showing the position of the spiracular organ (arrow). **e**, inner ear: s, saccule; sc, semicircular canal; u, utricle. **f**, sharks have numerous taste buds in their mouth (arrows) and on their skin. **f**, head of *Galeocerdo cuvier* showing pores of Lorenzini's ampulla (arrow, insert). **g**, schematic section of a shark's skin showing the organisation of Lorenzini's ampullae: a, ampulla; c, canal; n, nerve; ps, placoid scale. **h**, *Sphyrna lewini* biting at a dipole electric field (the voltage equipotentials are shown in red). **j**, olfaction in sharks: 1, head of *S. lewini* with water entry (arrow); 2, dissected olfactory rosette with water entry (arrow); 3, individual lamella with water flows (arrows). Pictures: Seapics (**b,c,e**). S. Wroe (**d**). A. Kok (**f**). Getty Images (**g**). S. Kajiura (**h**). D. Doubilet (**i**). Shark foundation (**j**).

Nearly all sharks (and relatives) have duplex retina, with both rod (used for scotopic vision) and cone (used for photopic and color vision) photoreceptors (Gruber and Cohen, 1978). These photoreceptors contain pigment formed by a

protein (opsin) and a chromophore prosthetic group related either to vitamin A₁ (rhodopsins and chrysopsins) or A₂ (porphyropsins), which changes its conformation when exposed to light, inducing a cascade that finally transmits the visual information to the brain (Cohen, 1991). According to their environment, sharks demonstrate different combinations of these pigments: most species (and mainly epipelagic species) possess rhodopsins (sensitive to blue green light), mainly deepwater sharks have chrysopsins (sensitive to deep blue light), and porphyropsins (sensitive to yellow-red light) are found in some freshwater species (Denton and Shaw, 1963; Hueter et al., 2004). The density and spatial distribution of these photoreceptors and their associated retinal interneurons (bipolar, amacrine, horizontal and ganglion cells), which transmit the information to the central nervous system, determine the visual acuity and sensitivity of a species (Hueter, 1991; Collin, 1999; Bozzano and Collin, 2000; Hueter et al., 2004).

3.3.2.2 Mechanoreceptors

Mechanoreceptors of sharks include (i) lateral line system composed of superficial free neuromasts (= pit organs), pored/non-pored lateral line canals (**Fig. 14c**) and spiracular organs (**Fig. 14d**), and (ii) ears (**Fig. 14e**) (Hueter et al., 2004). Their functions are respectively: (i) the detection of waterflows (function in schooling behaviour, social communication, hydrodynamic imaging, rheotaxis, prey detection and predator avoidance) and jaw deformation (jaw proprioceptor), and (ii) sounds, angular acceleration and atmospheric pressure (Barry and Bennet, 1989; Heupel et al., 2003; Hueter et al., 2004).

The functional unit of the lateral line system is the neuromast, which is composed of sensory hair cells surrounded by support (= sustentacular) cells and surmounted by gelatinous cupula. These neuromasts are either freely positioned (with their cupulae exposed to the external environment) on different portions of the shark's body and fins, associated to modified placoid scales (or raised papillae), or in groups, associated to a pored or a non-pored canals, localised on the lateral portions of the shark's body and on dorsal portion of the head (Maruska, 2001; Hueter et al., 2004). Movement of the cupula due to viscous forces induces movements of the stereocilia towards the kinocilium and induce hair cell depolarisation, modulating the information transmitted by the neurons to the brain.

Other mechanoreceptors not associated to the lateral line system are also present in the shark's skin, bringing information on skin deformation (in relation to temperature, salinity, contact with a prey) (Nier et al., 1976; Lebrun, 2001).

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Ears of sharks are composed of a pair of membranous labyrinths made of three semicircular canals (orientated in three different planes) and four sensory areas each: the saccule (in connection to the exterior by the endolymphatic pore), the lagena and the utricle, forming the macula acustica and containing sensory hair cells on an epithelium overlain by an otoconial mass (made of CaCO_3 granules and otoliths embedded in a mucopolysaccharide matrix), and an additional macula neglecta not overlain by otoconia to which is connected a tympanic connection (absent in bony fishes), the fenestra ovalis (situated in the base of the skull's parietal fossa) which enhance audition (Howes, 1883; Maisey, 2001; Hueter et al., 2004). The semicircular canals contain similar hair cells embedded in a gelatinous fluid (= endolymph) whose movements induce hair cell depolarisation, transmitting acceleration information to the brain. A similar mechanism occurs in the hair cells of the macula acustica: sounds from the exterior provoke the vibration of the matrix, depolarisation of hair cells and transmission of low frequency (10-80 Hz) sound and pressure (hence atmospheric pressure and depth) information to the brain (Lebrun, 2001; Frazer and Shelmerline, Hueter et al., 2004). The macula neglecta has been shown to be sensitive to higher frequency sounds (100-600 Hz; Kalmijn, 1988).

3.3.2.2.3 Electoreceptors

The electoreceptors of sharks, called Lorenzini's ampullae, derived from the lateral line system, also contain ciliated cells, which are positioned in association with support cells in a chamber, the ampulla, at the end of a tubular canal filled with a mucopolysaccharide fluid (about 1 mm wide), which opens to the exterior (**Fig. 14f,g**). These ampullae are situated on different part of sharks' cephalic area, and form different groups: mandibular, buccal (extern and intern), supraorbital and hyoid (Ewart, 1891).

These electoreceptors are sensitive to low-frequency electrical stimuli from different sources (Lebrun, 2001; **Fig. 14h**): (i) electrical field produced by electrochemical disparity between the shark and its environment ($1\text{-}1000\text{ }\mu\text{V cm}^{-1}$; Kalmijn, 1974); (ii) the kinetic electric field produced by water (= conductor medium) is displaced by Earth's magnetic field (to $0.5\text{ }\mu\text{V cm}^{-1}$); (iii) the bioelectric field from electric organs, and (iv) the electromagnetic field from a moving organism (to 5 nV cm^{-1} ; Kalmijn, 1982).

The mechanism of information transmission is not well understood yet (Lebrun, 2001) but the basal mechanism has been highlighted. The receptors of the ampulla's sensory cells detect difference between the shark's internal potential and the

potential present at the surface of the pore. The low resistance (25-31 Ω -cm) gel filling the high resistance (6 M Ω -cm) canal transmits the electrical stimuli to the ampulla's sensory cells which fire and relay the information to the brain via the same nerve than the lateral line system (Norris, 1929; Bennet, 1971; Hueter et al., 2004). Ampullae with longer canals are more sensitive (Broun et al., 1979). Freshwater sharks have ampullae with shorter canals (due to higher resistivity of freshwater), and distributed individually rather than in clusters (Hueter et al., 2004).

The electric sense of sharks (and their relatives) is believed to be involved in prey and predator detection (Kalmijn, 1971, Peters and Evers, 1985), conspecific detection (Tricas et al., 1995), orientation and navigation (Kalmijn, 1982).

3.3.2.2.4 Chemoreceptors

In sharks, chemoreceptors include (i) taste buds from the oral-pharyngeal cavity (**Fig. 14i**) and exterior papillae (in *Cirrhigaleus barbifer*) and (ii) an olfactory epithelium present in two bag-like cartilaginous capsules situated in front of the mouth (to which they can be connected; Bell, 1993; Lebrun, 2001; **Fig.14j**). The functions of these receptors are, respectively, (i) to quantify the edibility of a potential prey (Hueter et al., 2004), and (ii) to detect odours of preys (Strong et al., 1992), predators (Rasmussen and Schmidt, 1992) and pheromones of conspecifics (Johnson and Nelson, 1978). The sense of olfaction is certainly one of the most acute senses of sharks, working up to hundreds of meters.

Taste buds of sharks (and their relatives) are similar than those of other vertebrates, composed of a small papillae covered with a multilayer epithelium that has a central cluster of elongate sensory receptor cells (Tester, 1963). The fixation of chemical substances to the receptors of these cells provokes them to depolarise and to send the gustatory information to the brain.

Sharks' olfactory epithelium is also structurally similar to those of most vertebrates excepted that bipolar receptor cells are not ciliated but rather have a dendritic knob from which extend a tuft of microvilli (Zeiske et al., 1987; Hueter et al., 2004). Again, the chemical stimulations of specific receptors from bipolar cells provoke them to depolarize and to send the olfactory information to a part of the brain, the olfactory bulb (Smeets, 1998).

3.4 Deep-sea adaptations

A great part of current shark species (about 60 %, i.e. about 300 species, which come from all orders of sharks except Heterodontiformes) live in the deep-sea (i.e. below 200 m, the lower limit of the epipelagic zone), or frequently undertakes incursions in this particular environment (**Figure 15a**; Martin and Treberg, 2002; Compagno et al., 2004), which is oligotrophic (low quantity of available food) and characterised by relatively constant physical variables including a high pressure, a cold temperature (around 4°C) and a permanent darkness.

Due to the inherent logistical challenges of studying deep-sea animals, the adaptations of deep-sea sharks have received little attention. The aim of this section is to summarise the main known adaptations/characteristics of sharks that could help them to survive in the conditions offered by the deep-sea environment, which is also one of the planet's largest environments (Priede et al., 2006).

The ability to feed on carcasses i.e. scavenging is a useful feeding particularity in the oligotrophic environment of the deep-sea. Contrary to scavenging bony fishes, whose size increases with depth (Collins et al., 2005), sharks, which are almost all potential scavengers, do not show any size-depth trend, deep-sea species encompassing a large range of sizes, which, on overage, are similar to those of shallow-water species (**Fig. 15b,c**; Compagno et al., 2004; Priede et al., 2006). The sharks' reproductive modes with no larval stages can also be considered as an important advantage in a low food environment since it allows embryonic sharks to develop slowly and to be born with fat reserves at a relatively less vulnerable size (**Fig. 15d**). Finally, one study, which compared the muscle enzyme activities of a shallow water squalid (*Squalus acanthias*) and a deep-sea equivalent (*Centroscyllium fabricii*), showed an overall lower metabolism in this later (Treberg et al., 2003), which implies a lower fuel utilisation which likely represent an adaptation to a low food availability (Collins et al., 1999). Deep-sea bony fishes have usually also lower concentration of metabolism-related enzyme than their shallow water counterparts (Childress and Somero, 1979; Bailey et al., 2002). On the other hand, the floatability system of sharks, which necessitates a lipid-rich liver, has been hypothesized to be the main reason explaining the poor representation of sharks (and their relative) in the abyss (below 3000 m; **Fig. 15a**; Priede et al., 2006).

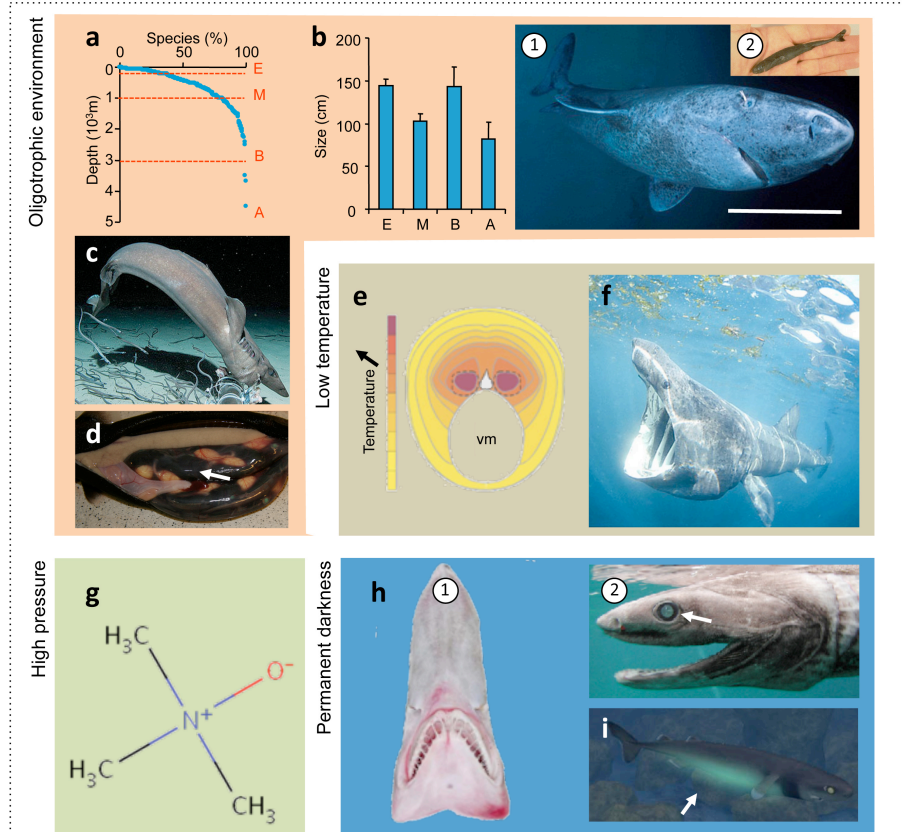


Figure 15 | Sharks' adaptations to the deep-sea. **a**, cumulative curve of 446 current shark species according to the maximal recorded depth (information from Compagno et al., 2004): E, epipelagic zone; M, mesopelagic zone; B, bathypelagic zone; A, abyssopelagic zone. **b**, mean size of shark species according to the different depth zones (same as in **a**): 1, *Somniosus microcephalus*, a large deep-sea shark (scale bar, 1 m); 2, *Etmopterus perryi*, probably the smallest deep-sea shark species. **c**, *Deania calcea* scavenging at a carcass. **d**, ovoviviparity (arrow) is common in deep-sea sharks contrary to bony fishes which generally harbour larval stages. **e**, some lamnid species, such as *Lamna ditropis*, are able to increase their body temperature thanks to a specialised counter-current heat exchanger, which help them to maintain a high activity in cold water. vm, visceral mass. **f**, large sharks such as *Cetorhinus maximus* trap heat in their enormous body, a phenomenon called "gigantothermy". **g**, the TMAO used by sharks to stay isotonic to seawater, help also them to support the high pressure present in the deep-sea. **h**, in the permanent darkness of the deep-sea, many sharks increased the acuity of their senses: 1, *Mitsukurina owstoni* harbours a long snout with numerous sensorial captors; 2, *Chlamydoselachus anguineus* has sensitive eyes with tapetum (arrow). **i**, some deepwater sharks such as *Isistius brasiliensis* evolved the ability to produce light (arrow) from photogenic organs. Pictures: Seapics (**b**). N. King (**c**). J. Mallefet (**d**). Bernal et al., 2005 (**e**). G. Parsons (**f**). G. Burgess (**h1**). Awashima marine park (**h2**). A. Nonnis (**i**).

In addition, some active species that undertake deep-sea incursions have morphological specialisation that allow them to keep a high metabolism in the cold of deep waters. Lamnids, which are active shark species known to often swim at

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great depths (> 200 m) evolved a rete mirabilis (= wonderful net) which allow the venous blood coming from the muscle to warm the artery blood, making these sharks' body core and other body regions such as the eyes up to 20°C warmer than the surrounding environment, a phenomenon called “heterothermy” (**Fig. 15e**; Bonfil et al., 2005; Bernal et al., 2005). Large species such as the basking shark (*Cetorhinus maximus*) and to a lesser extent (due to its warmer habitat) the whale shark (*Rhincodon typus*) can trap the heat in their enormous body, a phenomenon called “gigantothermy” also efficient during deep-sea journeys (**Fig. 15f**; Rowat and Gore, 2007; Skomal et al., 2009).

Deep-sea animals have generally higher concentration levels of TMAO (**Fig. 15g**), which offers a protection against a high hydrostatic pressure since, in addition to be an osmolyte, this molecule is known to act as a universal stabiliser of protein structure (Yancey et al., 2002). The high concentration of TMAO found in sharks reveal that they are pre-adapted to life at high pressure (Priede et al., 2006).

Finally, many sharks developed anatomical and physiological adaptations that help them to overcome the difficulty to perform their biological functions (predation, defence and intraspecific communication) in a world with a permanent darkness between 200 and 1000 m (mesopelagic zone) and no light at all below. Adaptations can be grouped in two axes: (i) increase sensory perception (**Fig. 15h**) and (ii) production of light i.e. bioluminescence (**Fig. 15i**). Sensory perception can be improved by increasing the surface available for sensory receptors (by the mean of expanded cephalic lobes e.g. the long rostrum of the goblin shark *Mitsukurina owstoni* and numerous squalids or lateral expansion of hammerhead sharks) or by changing the nature of the receptor themselves (*tapetum lucidum*, chrysopsin pigment, etc.). The capacity to produce light is known to be a preferential way for deep-sea organisms to accomplish the functions imposed by their biological cycle (Haddock et al., 2010), and numerous deep-sea sharks are known to possess photogenic organs.

3.5 Bioluminescence¹

While the majority of deep-sea Osteichthyes (70 %) are luminous, only a small fraction of deep-sea Chondrichthyes (6%) is endowed with this capability (Bone and

¹ This section is a part of the chapter « Bioluminescence of sharks : first synthesis » from the book « Bioluminescence in focus : a collection of illuminating essays » (Claes and Mallefet, 2009; annexe 3).

Marshall, 1982; Compagno et al., 2004). This led some authors to consider this amazing property like a burden in these fishes (Reif, 1985). Even though it is absent in Holocephali (chimaeras), the luminescence competence is nevertheless appeared at least three times independently in cartilaginous fishes, one time in basal Batoidea (rays) and two times in Squalan sharks, which totalize currently 51 luminescent sharks, which led to the fact that one deep-sea shark on five can produce light (Alcock 1902; Harvey, 1952; Hubbs et al., 1967; Reif, 1985).

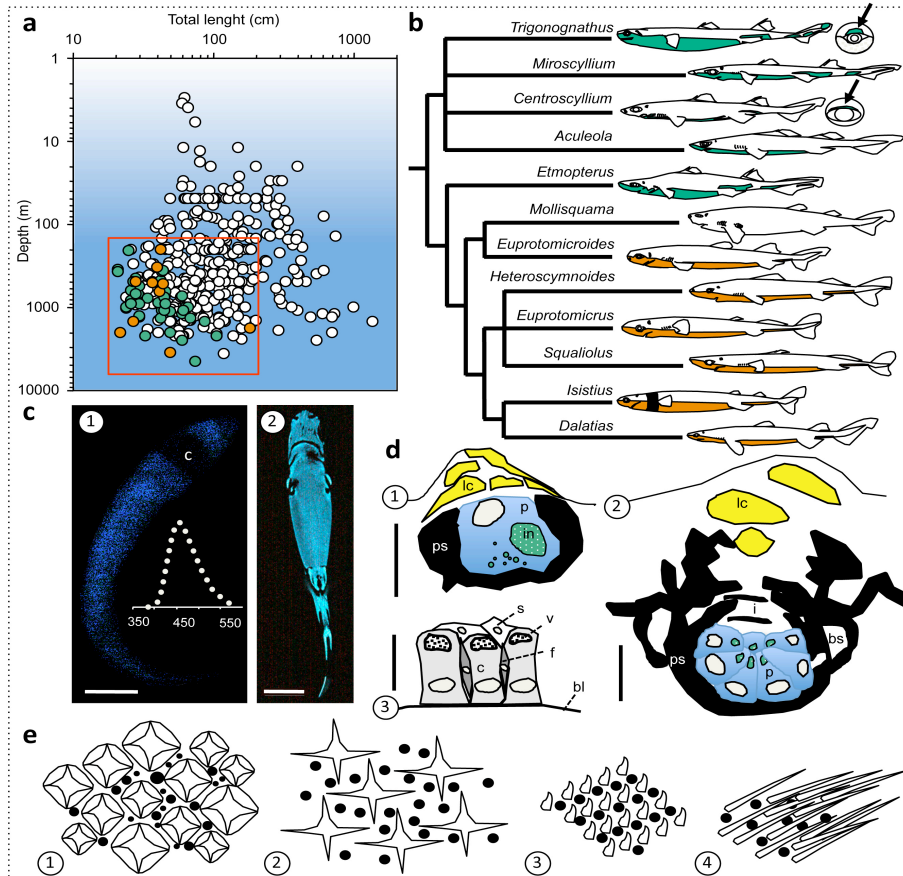
Due to the relative inaccessibility of their environment, the logistical difficulties linked to their maintenance in experimental conditions and their rarity (some are only known from one of several specimens), luminescent sharks have been poorly investigated (Reif, 1985; Wetherbee, 2000). This explains why only extremely limited and fragmented information is available in the literature on shark bioluminescence. This section aims to provide a first synthesis on what is currently known on the phenomenon of visible light emission in these cartilaginous fishes.

3.5.1 Luminescent sharks

3.5.1.1 Symbiotic luminescence

In symbiotic luminescence the light production is only present through the association with another organism, which is luminous. This kind of extrinsic luminescence is frequently observed in deep-sea squids and Osteichthyes harboring bacterial photophores (Hansen and Herring, 1977; Herring and Morin, 1978; Neilson and Hastings, 1979). Forms of symbiotic luminescence have been once suggested for some shark species: (i) the mouth of the megamouth shark, *Megachasma pelagios*, was considered to be a bacteria-filled luminous lure allowing krill attraction (Diamond, 1985), and (ii) ocular parasite copepods of sleeper sharks (*Somniosus* species) were thought to attract preys to the mouth of the shark (Berland, 1961). Now, since any evidence to find luminescent tissues in the mouth of *M. pelagios* and since tested specimens of sleeper shark copepods were not luminous, symbiotic luminescence seems to be very unlikely in sharks (Nakaya et al., 1967; Herring, 1985b).

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3.5.1.2 Intrinsic luminescence

Intrinsic bioluminescence appeared in two shark families: the Etmopteridae ("lantern sharks") and in the Dalatiidae ("dwarf mesopelagic sharks"), which encompass small species which can be found everywhere in the water column but generally at great (> 200 m) depths (only one dalatiid species, *Dalatias licha* exceeds 1 m in total length; **Fig. 16a**) (Hubbs et al., 1967; Musick et al., 2004; Compagno et al., 2004).

The Etmopteridae radiated 90 millions years ago when they start to leave shallow water environments to occupy the empty deep water niches during the marine life turnover which followed the mass extinction provoked by the global Cenomanian-Turonian anoxic event ("Bonarelli event") (Adnet and Capetta, 2001). This shark family encompasses five different genera (*Aculeola*, *Centroscyllium*, *Etmopterus*, *Miroscyllium* and *Trigonognathus*), which are all luminous (**Fig. 16b**; Compagno et al., 2005).

The Dalatiidae, on the other hand, evolved later when they replaced extinct large fishes and marine reptiles in the epipelagic fauna of the Paleocene after the Cretaceous/Paleocene mass extinction (65 millions years ago) (Adnet and Capetta, 2001). Seven genera are present in this family (*Dalatias*, *Euprotomicroides*, *Euprotomiscrus*, *Heteroscymnoides*, *Isistius*, *Mollisquama* and *Squaliolus*) among which at least six are known to contain luminescent species (the genus *Mollisquama* contains the species *M. parinii* which is only known from one individual, and luminescence has to be confirmed for this species) (**Fig. 16b**; Van Grevelinghe et al., 1999; Compagno et al., 2004).

While Etmopteridae are demersal species, which generally live in groups, all Dalatiidae are essentially solitary pelagic (excepted *D. licha* which is benthopelagic) predators, which undergo daily vertical migrations (Compagno, 1990; Musick et al., 2004).

3.5.2 Photogenic structures

3.5.2.1 Luminous patterns

Etmopteridae and Dalatiidae show two different types of photophore organizations (= luminous patterns) (**Fig 16c**; Reif, 1985).

In dalatiid species, photophores are arranged following a density gradient from dorsal to ventral region, where their number may attain 60 units per square millimeter (Hubbs et al., 1967; Reif, 1985; Zidowitz et al., 2004). Among the

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species possessing this pattern, a genus (*Isistius*) harbours a dark collar lacking photophores in front of the pectoral fins. Luminous patterns of Dalatiid species are presented in **Fig. 16b**. The luminous pattern of the genus *Mollisquama* is not represented because it has never been described and as already mentioned, the luminous status of *M. parinii* remains uncertain.

Etmopterid sharks possess considerably more complex luminous patterns than the Dalatiidae, as they show aggregations of photophores on the ventral side, but also on the flanks, the tail and even (in *Centroscyllium* and *Trigonognatus*) on the upper eyelid (**Fig. 16b**) (Iwai, 1960; Reif, 1985; Yano et al., 2003). In some species of lantern sharks, the photophores can also be inconspicuous because they are not arranged in specific markings (Compagno et al., 2005). As the luminous pattern of lantern sharks is supposed to be species-specific and to stay constant between sexes and over ontogeny, it has often been used for species determination (Reif, 1985; Springer and Burgess, 1985; Schofield and Burgess, 1997; Last et al., 2002).

3.5.2.2 Photogenic organs

3.5.2.2.1 Photophores

Photophores of sharks are extremely small (100 µm and 150 µm on average, for the Dalatiidae and Etmopteridae respectively) and numerous (several thousands); the sharks are probably the animals that have the highest number of photophores (Hubbs et al., 1967; Herring and Morin, 1978). In comparison with photophores of Osteichthyes, their structure is simpler, only composed of few elements (Hansen and Herring, 1977; Cavallaro et al., 2004). Structural differences exist between the two families of luminescent sharks (Hubbs et al., 1967).

Dalatiid sharks harbour photophores constituted of a single photogenic cell (= photocyte), a pigmented sheath and a lens made up of a group of small cells overlying the apical surface of the photogenic cells (**Fig. 16d**). Photocytes contain granules supposed to be photogenic and a large nucleus generally placed at the apical side. The lens cells contain a homogeneous cytoplasm, presumably transparent in life. The photophores are present in the stratified squamous epidermis of the skin and lack reflectors or any kind of specialized structure (Hubbs et al., 1967; Seigel, 1978).

Photophores of Etmopteridae are more complex than those of Dalatiidae. They are composed of a black circular pigmented sheath encompassing a group of 6-13 photogenic cells in general, a lens formed by 2 to 3 lens cells, and an iris-like

structure formed by chromatophores (sometimes divided into 3 groups: internal, horizontal, and external process following their distance to the photocytes; **Fig. 16d**). It has to be note that etmopterid circular photophores sometimes fusion together to produce larger linear photophores (300 μm in length by 100 μm broad) with a higher number of lens cell. The photophores also lack reflectors in this group (Ohshima, 1911; Harvey, 1952; Iwai, 1960; Hubbs et al., 1967).

3.5.2.2.2 Secretory luminous tissues

In *Euproctomicroides zantedeschia* a putatively luminous tissue of another type has been found (Munk and Jorgensen, 1988). This tissue is a probably stratified epithelium, present into the abdominal pouch of this species, with a thickness of 50-75 μm (**Fig. 16d**). It is composed of different cell types: (i) columnar cells possessing a big yellow apical inclusion (30 x 25 μm), basal nucleus; (ii) flattened cells with basal nucleus extending from the basal lamina towards the free surface of the epithelium, separating the lateral cell membranes of tall columnar cells; (iii) superficial cells, with a nucleus close to the surface of the epithelium, forming a very thin cover over the dome-shaped distal surfaces of columnar cells. A tissue of this kind may also exist in the pectoral gland of *M. parinii* (Compagno et al., 2004).

3.5.2.2.3 Specialised squamation

Placoid scales of sharks evolved to assume many functions in sharks including protection against parasites and predators, protection against substrate abrasion, hydrodynamism and finally accommodation of sensory and bioluminescent organs (Reif, 1985; Rschi and Tabit, 1992). Luminescent sharks had indeed to elaborate special squamation, which allows coexistence of epidermic photophores and placoid scales. Four major special squamation patterns are found in luminescent sharks and are presented in **Fig. 16e**. The bioluminescent squamation patterns of luminescent sharks are so specific that they could be used as a tool to determine bioluminescence in current but also in extinct sharks (Reif, 1985).

3.5.3 Bioluminescence

3.5.3.1 Physical and chemical characteristics

Most of shark luminescence knowledge is almost exclusively based on simple visual observations sometimes contradictory and doubtful (Bennet, 1840; Johann, 1899; Ohshima, 1911; Hickling, 1928; Dickens and Marshal, 1956; Parin, 1966). The only species for which spontaneous bioluminescence has been observed are *Euprotomiscrus bispinatus*, *Isistius brasiliensis*, *Etmopterus spinax* and *Etmopterus pusillus*. Only the bioluminescence spectrum of *I. brasiliensis* has been precisely measured with a spectrophotometer (Herring, 1983). This shark emits a bluish glow with a peak of emission at 455 nm with a half bandwidth of 73 nm (427-500 nm, **Fig. 16c**). *E. spinax* also shows a bluish glow with a peak of emission which seems however at higher wavelength even though it has never been measured (**Fig. 16c**) (Johann, 1899; Hickling, 1928). From literature, *E. bispinatus* emit a greenish glow (Dickens and Marshal, 1956; Parin, 1966) while *E. pusillus* emit a "whitish" glow (Ohshima, 1911). These assertions have however to be considered with the highest care until spectral measurements will be available.

Due to a lack of biochemical work on shark bioluminescence, the luminous system of sharks for light production is still totally unknown (Harvey, 1952; Herring, 1978; Reif, 1985).

3.5.3.2 Control

Physiological studies of luminous control mechanism in sharks have never been systematically carried out (Harvey, 1952; Reif, 1985). The impossibility to find innervations in photophores of the few species investigated, the slow onset of luminescence in *E. pusillus* and the absence of response to classical neurotransmitters such as adrenaline or acetylcholine in *I. brasiliensis* (only hydrogen peroxide stimulate light production in this species), led to the hypothesis that bioluminescence is hormonally controlled in sharks (Johann, 1899; Ohshima, 1911; Harvey, 1952; Herring, 1978). A control via the iris-like structure of the photophores through the action of chromatophores in Etmopteridae has been sometimes suggested but never tested (Ohshima, 1911; Iwai, 1960; Hubbs et al., 1967; Herring, 1978).

3.5.3.3 Functions

Due to the absence of experimental data on bioluminescent behaviors, the role of bioluminescence in sharks has never been demonstrated, as it is often the case for luminous deep-sea organisms (Reif, 1985). Functions of light emission in sharks can only be deduced from life trait history such as global ecology, arrangement of photogenic organs and bioluminescence spectrum. The best current functional hypotheses were presented here.

Dalatiidae are small pelagic sharks which are particularly vulnerable to predation in the water column, assuming they are mainly found in the mesopelagic zone (**Fig. 16a**), it is likely that their ventral pattern are used for camouflage by counterillumination (Reif, 1985). The ventral emission of *I. brasiliensis* produces however considerably shorter wavelength (455 nm) than what would be expected for a function of camouflage, as pelagic environments have peak of ambient light situated around 470-480 nm. Even though this could suggest that its photophores are not involved in camouflage, or at least not well adapted, one cannot exclude that this shark might also take advantage from the peculiar vision of deep-sea predators for which such differences in wavelength seems to have little effect (Herring, 1983). The presence of a dark collar in *I. brasiliensis* has led authors to suggest a secondary predatory use of its bioluminescence. This collar could indeed allow this species to use its light emission as a lure for big pelagic animals on which this shark feeds by ectoparasitism. Indeed, if bioluminescence matches the luminous background, the dark ventral collar would be the only part of the shark visible from below (**Fig. 16c**). This collar would therefore looks like a smaller fish very attracting for upward looking pelagic predators which would then be attacked by the shark at close distance (Jones, 1971; Widder, 1998).

Luminous patterns of Etmopteridae are more complex, suggesting that either their bioluminescence is not involved in camouflage or it is used for more than one purpose (Reif, 1985). Moreover, since they are benthopelagic species, counterillumination would be less useful in these fishes. As their patterns appear to be species-specific the best current hypothesis is that they use their luminescence for species recognition and schooling (Reif, 1985). Function of ocular photophores of *Centroscyllium* and *Trigonognatus* is relatively mysterious but may be involved in prey attraction or vision improvement (Iwai, 1960; Yano et al., 2003). One cannot exclude these ocular photophores to play a reference function in countershading.

4 The velvet belly lantern shark, *Etmopterus spinax*

The velvet belly lantern shark, *Etmopterus spinax* (Linnaeus, 1758) received its common name from the velvet-like aspect of its belly and its capability to produce light. In addition to these particular features, it harbours two dorsal finspines (**Fig. 17a**) that are believed to be used as a defence against predation and hydrodynamism (observations of freshly caught specimens clearly supports a defensive role for these spines and particularly for the second one, which is longer and can provoke serious damages to the fish handler; personal observation).

This shark is a deepwater (up to 2490 m; Priede et al., 2007) benthopelagic shark which is commonly found (between 200 and 500 m) in the East Atlantic, from Norway to South Africa, and in the Mediterranean Sea, where it lives mainly in the outer continental and insular shelves and upper slopes, and is frequently caught as bycatch (**Fig. 17b**; Compagno et al., 2004; Coelho and Erzini, 2008).

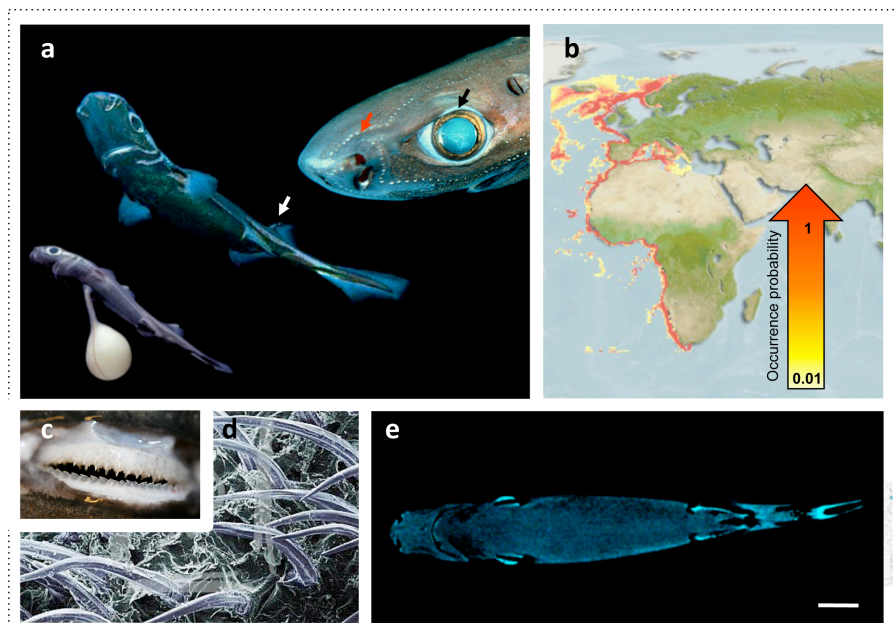


Figure 17 | Biology of the velvet belly lantern shark, *Etmopterus spinax*. **a**, Picture of an adult specimen (center), an embryo with its yolk sac (left), and a close up of the head (right). White arrow indicates second finspine; red arrow, a row of Lorenzini's ampullae; black arrow, the tapetum, which increases the visual sensitivity of this shark. **b**, map showing the probability of occurrence of this shark. **c**, bristle-like dermal denticles of this species. **d**, Mouth and teeth of this species showing a strong digenathic heterodonty since upper teeth are narrow and lower teeth are wider. **e**, luminous pattern of *E. spinax*. Scale bar, 5 cm. Pictures: Seapics (**a**). Modified from FishBase (**b**). J. Mallefet (**c,e**). Fotobank (**d**).

It reaches a maximum size of 60 cm in total length (TL) and attains its sexual maturity around 30 cm TL (25 cm TL for males and 30 cm for females; Coelho and Erzini, 2008) (Compagno et al., 2004). Different works used vertebral and fin spine bands to estimate the longevity of this species with different results: a maximum longevity comprised between 8 and 18 years for males and 11 and 22 years for females (Gennari and Scacco, 2007; Coelho and Erzini, 2008). While this species is known to segregate by size in the water column in the Mediterranean Sea (Coelho, 2007), it is known to vertically migrate in the water column of some Norwegian fjords, attaining very shallow depths (up to 15 m) at night (Moen and Svensen, 2004; Heidler, 2006). This species is ovo-viviparous (**Fig. 17a**) and is believed to reproduce only every three years in the Mediterranean Sea, which makes it particularly vulnerable to overfishing (Coelho and Erzini, 2008).

This species undergoes two main ontogenetic diet shifts: the first at around 19 cm when it quits a crustacean-based alimentation to become a fish eater (it then also starts to harbour some fish parasites), and the second at around 30 cm, when it starts to include new preys in its diet such as cephalopods and bigger fishes as well to use scavenging as a mode of food acquisition (Klimpel et al., 2003; Neiva et al., 2006). In Norway, its two main preys are the krill *Meganyctiphanes norvegica* and the pearlfish *Maurolicus muelleri*, which are small vertically migrating animals sometimes found in high number in certain fjords (Kaartvedt et al., 1988; Klimpel et al., 2003). The feeding mode of this shark is not known, but since it demonstrates small teeth (**Fig. 17c**), a high jaw protrusion (personal observation) and a low bite ability (Huber et al., 2009), and since numerous of its preys are often found intact in its stomach (personal observation), it is likely that it use ram or suction feeding (rather than bite feeding). Since some of its preys appear too big to have been hunt and killed by a single shark, and since it is known to school, some authors consider that this species (like other lantern sharks) may hunt in groups (Springer, 1967; McPherson, 1980; Reif, 1985). This species is infected by numerous parasites and is probably the preys of numerous bigger animals, including big fishes and cetaceans, even if its main predators in the Norwegian deep have not been discovered yet (Klimpel et al., 2003).

This species, finally, is well adapted to deep-sea life, harbouring a slow growth, tapeta-equipped eyes sensitive to low light level (**Fig. 17a**), a long snout with numerous sensory receptors, and numerous tiny photogenic organs (Hubbs et al., 1967), surrounded by bristle-like dermal denticles (**Fig. 17d**), which, in addition to protect the skin, let the light to be emitted to the outside (**Fig. 17e**). This luminescence is believed, like in other lantern sharks, to be involved in camouflage by counterillumination and schooling (Reif, 1985).

5 Aim of the study

The diversity of expression of bioluminescence mainly reflects the phylogenetic diversity of this phenomenon (Haddock et al., 2010). Among fishes, luminescent representatives are found in teleost and cartilaginous fishes, which both consist in the sole luminous vertebrates. Although the expression of bioluminescence has been extensively studied in teleost fishes (notably in the laboratory of Marine Biology of the Université catholique de Louvain), very few studies focus on the light emitted by cartilaginous fishes. This appears at first sight surprising since many sharks (> 50 species), including some very common and accessible species, are known to be luminous (Compagno et al., 2004).

By focusing on a model species, the velvet belly lantern shark *Etmopterus spinax*, this work aims to perform the first extensive experimental study on shark luminescence. In this context, the main targets were to identify (a) the potential function(s) and (b) the control mechanism of the luminescence from the photophores of this species. In addition to increase the understanding of the adaptive benefit of the luminescence of *E. spinax*, this work will also provide a basis for further studies on other luminescent sharks.

The fonction(s) of *E. spinax*'s light emission was(were) investigated (i) by determining the timing of the luminous competence acquisition (**Paper 1**), (ii) by investigating the evolution of photogenic structures throughout ontogeny (**Paper 2**) and (iii) by measuring the physical characteristics of the light emission (**Paper 3**).

The extrinsic and intrinsic control mechanism of light emission of *E. spinax* was first determined by applying different pharmacological substances on standard isolated photophore-containing skin patches; the extrinsic control proved to be hormonal (**Paper 4**). The action of the light-triggering hormones on the structural elements of the photophores was then investigated by a macroscopic examination under binocular microscope (**Paper 5**). These hormones were then applied on different areas of the photophore pattern and the luminescence pattern was proved to vary according to the area considered, suggesting that an additional control mechanism was present (**Paper 6**). We therefore tested the effect of different potential neuromodulators by pharmacology, immunohistochemistry and macroscopic observations under binocular (**Papers 7 & 8**).

The different results were integrated in order to propose (a) different potential functions of the light emitted by the shark throughout its life and (b) a model for the control of luminescence from this shark's photophores.

6 Experimental strategy

6.1 Collection field

Velvet belly lantern shark (*Etmopterus spinax*) specimens were collected by longlines and bottom trawling, principally in a deep area of the Raunefjord, a fjord situated close to Bergen and housing the Espeland marine research station, which provided the convenient facilities for the research : two research vessels, lab space, cold rooms with water tanks (Fig. 18).

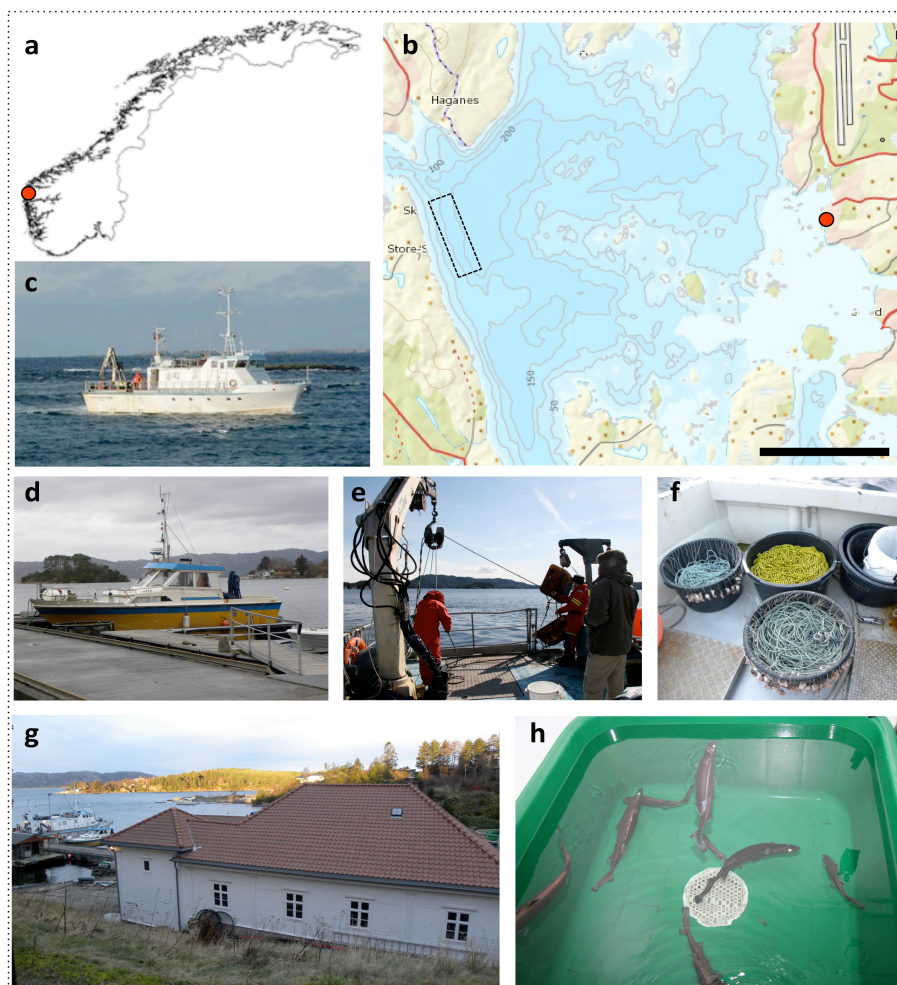


Figure 18 | Study site, fishing methods and marine station. **a**, Norway map indicating position of the Raunefjord (red dot). **b**, Raunefjord map indicating the position of the fishing site (dotted rectangle) and the marine station (red dot). **c**, research vessel (R. V.) Hans Brattström. **d**, R. V. Aurelia. **e**, bottom trawling. **f**, longlines. **g**, Espeland Marine Station. **h**, lantern sharks swimming in a tank placed in cold room. Scale bar in **(b)** indicates 1 NM.

I. Introduction

Velvet belly lantern sharks are known to be present in large numbers in Norwegian fjords, and especially in the Raunefjord, where they are found at relatively shallower depths [from 250 m (during the day) to 15 m (at night)], and can be easily caught and maintained in captivity for several days in good physiological conditions (Kryvi and Eide, 1977; Moen and Svensen, 2004).

6.2 Adaptive benefit

In this section we first tried to determine when *E. spinax* photophores start to become functional i.e. able to emit light. For that purpose, we followed the evolution of photogenic structures in an artificial embryogenic series formed by embryos from six different litters. We described (i) the successive apparition of (nine) different luminous zones i.e. zones containing photophores (we provided a specific nomenclature for them) by sight and (ii) the organogenesis of photophores (in the different luminous zones) by fluorescence and light microscopy. In addition, the density, the diameter and the ventral coverage of the photophores were measured on digital pictures using an specialised software (Image J). The luminescence competence was analysed by peroxide (H_2O_2) application on photophore-containing skin patches. In a second time, the evolution of the luminous zones' surface areas, photophore densities and peroxide-induced luminescence intensities was investigated throughout an ontogenetic series (~50 specimens, from 14 to 52.5 cm in total length), for each sex separately. Results were then analysed in relation with the main ecological transitions occurring throughout the life cycle of the shark (diet shifts, maturity, etc.).

The physical characteristics of *E. spinax* luminescence were analysed in order to demonstrate if they were convenient for camouflage by counterillumination, the most probable proposed function for this light emission. The luminescence spectrum was measured using a microspectrometer (Hamamatsu photonics K. K. TM-VIS/NIR: C10083CA, Hamamatsu-City, Japan; précision = 6 nm) and the luminescence intensity was measured using an optic fiber coupled to a luminometer (Berthold FB12, Pforzeim, Germany) (**Fig. 19a**) placed just below the photogenic surface area (= absolute luminescence intensity) or every 10° , at 10 cm from the center of the shark in a transversal plane, to determine the angular distribution of the shark's light emission (**Fig. 19a**). The intensity of luminescence from sharks placed in plastic water-filled boxes (**Fig. 19b**) was measured after different overhead light stimulations. The physical characteristics of *E. spinax* luminescence were then compared with the physical characteristics of the light present in the environment (Norwegian fjords) where this species is found, in order to test if camouflage might

occur. We also measured the angular distribution was measured at different position along the shark's body and performed several transverse histological sections to investigate (by light microscopy) the transversal morphology of the photophores.

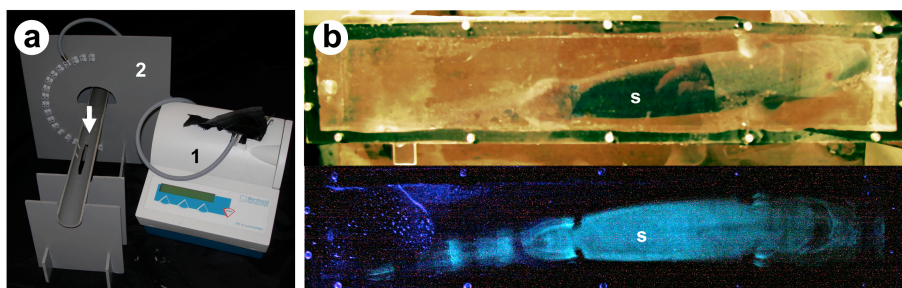


Figure 19 I Luminescence intensity measurement. **a**, Experimental PVC set-up (1) to measure the angular distribution of *E. spinax* luminescence. Sharks were positioned (belly-up) on a plastic channel (arrow) and luminescence was recorded on a circle around the animal (in a perpendicular plane) by means of clips placed every 10° that were able to hold the optic fibre coupled to the luminometer (2). **b**, plastic box containing a spontaneously luminous (living) shark (s) in daylight (up) and in the dark (below).

6.3 Physiological control

The physiological control of luminescence from *E. spinax* photophores were investigated by pharmacology, immunohistochemistry and macroscopic examination (under binocular microscope) of standard circular skin patches containing photophores (**Fig. 20**).

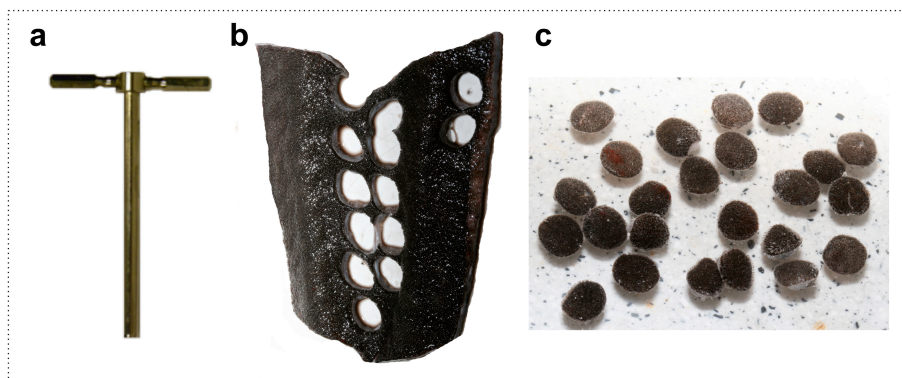


Figure 20 I Dissection technique for pharmacological experiments. **a**, metal cap driller. **b**, ventral photogenic tissue with several holes performed with the metal cap driller. **c**, skin patches containing photophores placed in the shark saline.

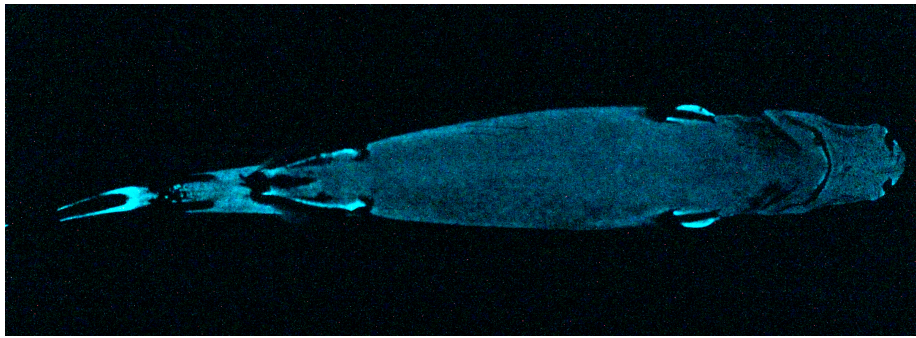
I. Introduction

Extrinsic control were determined by application of selective agonists/antagonists of the receptors while enzyme inhibitors and second messenger analogues were used to investigate the intrinsic (= intracellular) control ; all the drugs were dissolved in a specialised shark saline. Skin patches were placed in a luminometer and their light emission was measured after drug application. The effects of the different drugs tested were determined by comparison with control skin patches. In order to complete the pharmacological results using a visual approach, immunohistochemical staining using antibodies raised against different elements as well as dynamic observations of photophores under binocular microscope after application of pharmacological drugs were performed.

6.4 Integration

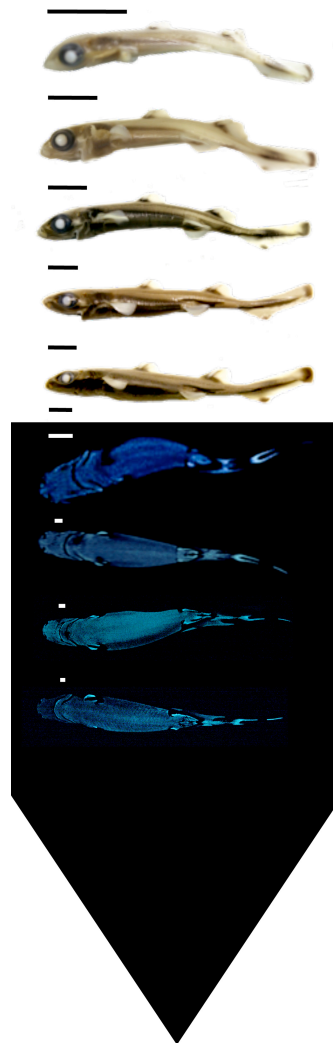
Results from the study were integrated and confronted with the biological information available on *E. spinax* to provide : (i) a dynamic view of the potential functions of bioluminescence throughout the life cycle of this shark and (ii) a first model of the luminescence control mechanism of this species.

II. RESULTS



Velvet belly lantern shark (*Etmopterus spinax*) emitting a spontaneous luminescence after capture. Picture: Jérôme Mallefet.

Chapter 1: *Development*



Chapter 1: Development

This first section aims to describe the evolution of *E. spinax*'s photogenic structures during its life cycle. It is composed of two papers, which cover the embryology (**Paper I**) and the ontogeny (**Paper II**) of this ovo-viviparous species.

In **Paper I**, a combination of light and epifluorescent microscopy as well as luminometry was used to follow the development of the photogenic organs of this shark across a complete embryonic series formed by seven different litters. Results indicate that photophores are grouped in nine different types of “luminous zones” that appear sequentially during embryogenesis (they received a specific nomenclature), harbour different photophore densities and start to be functional (i.e. to be able to emit light) before birth. In addition we show that this luminescence competence was linked to the presence of green fluorescent vesicles present at the apex of photogenic cells (photocytes). This early acquisition of bioluminescence competence strongly suggests camouflage by counter-illumination in newborn individuals.

Paper II aims to investigate the scaling of the surface area and photophore density of the different luminous zones composing the luminous pattern as well as the ecological consequence of an ontogenetic variation in bioluminescence efficiency. Results indicate that the different zones do not form an homogeneous pool but rather can be placed in two different groups: (i) “coverage zones”, probably used for counter-illumination whose density dramatically decrease over ontogeny, and (ii) “isolated” zones, whose densities show only a feeble ontogenetic decrease. The discrepancy of photophore insertion rate between these two groups, leads to an increase in the heterogeneity of the luminous pattern over ontogeny, the density of a zone appearing to directly influence its luminescence intensity. We suggest this heterogeneity to reflect the functional trade-off existing between the continuously useful function of camouflage (performed by coverage zones) and periodic intraspecific functions such as cohesive swimming/hunting and sexual signalling (performed by isolated zones). We indeed show in this paper that the sexually dimorphic sexual organs of large sharks emit a light brighter than the adjacent luminous tissues.

PAPER I

Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark, *Etmopterus spinax* (Squaloidea: Etmopteridae).

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**Early development of bioluminescence suggests
camouflage by counter-illumination in the velvet
belly lantern shark *Etmopterus spinax*
(Squaloidea: Etmopteridae)**

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The development of luminous structures and the acquisition of luminescence competence during the ontogeny of the velvet belly lantern shark *Etmopterus spinax*, a deep-sea squalid species, were investigated. The sequential appearance of nine different luminous zones during shark embryogenesis were established, and a new terminology for them given. These zones form the complex luminous pattern observed in free-swimming animals. The organogenesis of photophores (photogenic organs) from the different luminous zones was followed, and photophore maturation was marked by the appearance of green fluorescent vesicles inside the photocytes (photogenic cells). Peroxide-induced light emissions as well as spontaneous luminescence analysis indicated that the ability of *E. spinax* to produce light was linked to the presence of these fluorescent vesicles and occurred prior to birth. The size of photogenic organs, as well as the percentage of ventral body surface area occupied by the luminous pattern and covered by photophores increased sharply during embryogenesis but remained relatively stable in free-swimming animals. All these results strongly suggest camouflage by counter-illumination in juvenile *E. spinax*.

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Key words: bioluminescence; counter-illumination; development; *Etmopterus spinax*; photophore; shark.

INTRODUCTION

Bioluminescence is the production of visible light by an organism due to a chemical reaction. In sharks, this capability occurred at least twice independently in two squaloid subfamilies: the Dalatiidae, which are known as ‘dwarf pelagic sharks’ and the Etmopteridae, commonly called ‘lantern sharks’ (Hubbs *et al.*, 1967). Probably due to logistical limitations, *i.e.* the deep-sea habitat and the relative rarity of luminous shark species, this phenomenon has been poorly investigated in these fishes and therefore remains in great part unknown.

Until now, only the structure of the photogenic organs (photophores) has drawn the attention of some scientists. Those of the Dalatiidae are made of a small pigmented cup covering a single photocyte (photogenic cell), which is

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surrounded by one or several lens cells (Hubbs *et al.*, 1967; Seigel, 1978), while the light organs of the Etmopteridae are composed of a large pigmented sheath containing several photocytes, a pigmented iris and generally several lens cells (Oshima, 1911; Hickling, 1928; Iwai, 1960; Hubbs *et al.*, 1967; Seigel, 1978). In both subfamilies, granules are present in the photocytes and are supposed to contain luminescent material (Iwai, 1960; Hubbs *et al.*, 1967; Seigel, 1978; Munk & Jorgensen, 1988). The control and the biochemistry of light production in sharks remain until this day totally unknown (Reif, 1985).

Observing the relatively simple ventral photophore arrangement (luminous pattern) of the Dalatiidae, it has been suggested that they would use their luminescence for counter-illumination (Hubbs *et al.*, 1967; Reif, 1985; Widder, 1998). The luminous pattern of the Etmopteridae, on the other hand, is more complex and species specific, hence often used for species determination (Springer & Burgess, 1985; Schofield & Burgess, 1997; Last *et al.*, 2002). It has been suggested that it might be an aid for schooling and counter-illumination (Reif, 1985).

The velvet belly lantern shark *Etmopterus spinax* (L.) is a common ovo-viviparous deep-sea species frequently caught as by-catch in fisheries (Neiva *et al.*, 2006). In spite of this, very limited information is available in the literature concerning its bioluminescence (Johann, 1899; Oshima, 1911; Hickling, 1928) and practically nothing is known about the acquisition of this competence even if embryos of this species already seem to possess photophores (Johann, 1899).

The aim of this work is to follow the development of photogenic structures during the ontogeny of *E. spinax* and particularly to detect when the photogenic organs of this fish start to produce light. Knowing how and when an animal becomes luminous during its life history is helpful to determine the mechanism and the function of its bioluminescence (Anctil, 1977; Mensinger & Case, 1991; Foster *et al.*, 2002). The main goals were: (1) to characterize the establishment of the luminous pattern of *E. spinax*, (2) to follow the development of photophores during its embryogenesis, (3) to establish when these photophores become functionally operational, *i.e.* are able to emit light and (4) to make comparison between luminous structures of embryos and free-swimming individuals of *E. spinax*.

MATERIALS AND METHODS

SPECIMEN COLLECTION

Specimens of *E. spinax* were collected during February 2007 and December 2007 by longlines lowered in the deep waters of the Raunefjord near Bergen, Norway (60°16' N; 05°08' E). The living fish were brought to the HiB (Hoyteknologisenteret, Bergen, Norway) where they were kept in a 1 m³ tank filled with cold (6° C) running sea water pumped from the depths of the adjacent fjord. The tank was placed in a dark room to keep animals under good physiological conditions.

All the fish were sexed and total length (L_T) was measured. Six pregnant females were sacrificed by a blow on their head and their uteri were excised to free the embryos. In each uterus, embryos were counted and L_T measured. Embryos were sacrificed by quick decapitation and the volume of their yolk sac was digitally estimated *via* Image J[®], an image analysis software (<http://rsb.info.nih.gov/ij/>).

MORPHOLOGY OF LUMINOUS STRUCTURES

Luminous pattern

Digital picture of embryos from the six different litters were taken using a digital camera (Canon D20 with 100 mm macro lens; Canon, Shimomaru, Japan) and analysed in order to determine the progressive establishment of the luminous pattern. Considering that the size of embryos reflect their relative age (larger embryos being older), the formation of the pattern was formed by the juxtaposition of different pigmented luminous zones. The order of appearance of the different luminous zones was then determined. When more than 1 zone was discovered at one embryonic stage, the darkest zone was considered as the one that had first appeared.

The proportion of ventral surface area of the whole body occupied by luminous zones (V_{LZ}) was then estimated for embryos (three per litter) and free-swimming fish using Image J. In this calculation, translucent surfaces (*i.e.* eyes and fins) were not considered to be part of the total ventral surface area.

Photophores

To follow the organogenesis of the photophores of *E. spinax*, formalin-fixed patches of luminous zones from embryos of each litter were progressively dehydrated (50, 70, 90 and 100% ethanol, 60 min each) and stored overnight in 100% butanol. Preparations were submerged overnight in paraffin wax (Paraplast Plus, tissue embedding medium; Tyco Healthcare, St Louis, MO, U.S.A.) at melting temperature (58° C) under vacuum. After replacement of the paraffin wax, the tissues were put under vacuum for a second shorter period (6 h) before embedding. Fifteen different sections (7 µm) were obtained for each zone (of each litter) using a Biocut 2030 microtome (Reichert-Jung, Heidelberg, Germany). Sections were collected on 0.1% poly-L-lysine-coated slides before partial heat dewaxing in order to make observations under UV excitation using an epifluorescence microscope (Leitz Diaplan, Wild Leitz, Germany). Sections were then completely dewaxed, xylene treated and rehydrated using the standard procedure (100, 90, 70 and 50% ethanol), coloured with the Masson's trichrome, dehydrated, and finally mounted with a classical medium (Sub X TM mounting medium; Surgipath, Richmond, IL, U.S.A.). Sections were observed using a light microscope (Leitz Diaplan).

To determine the size and density of light organs in the different luminous zones, pieces of skin containing photophores were placed in a small transparent recipient containing a solution of 50% ethanol and observed under a light microscope. Pictures were taken with a digital camera (Nikon Coolpix 950; Nikon, Tokyo, Japan) mounted on the microscope and analysed with Image J. For each zone, the theoretical maximal photophore diameter (P_D) was estimated by taking the mean diameter value of the 10 largest photophores found in six 1 mm² skin patches per zone. Photophores density (P_{De}) present in the luminous zones was estimated by taking the mean density value of six 1 mm² skin patches per luminous zone. A mean P_D was calculated for all the zones of each embryos used in this analysis (*i.e.* three embryos per litter), while in free-swimming animals, only the P_D of the ventral zone was estimated and considered as representative of the P_D of all luminous zones.

To estimate the ventral surface area occupied by photophores (V_p) in a luminous zone, the following expression was used: $V_p = P_{De} [\pi (0.5 P_D)^2] V_s$, where V_s is the ventral surface area of the zone. For embryos (three per litter) and all the free-swimming animals, the proportion of ventral surface area occupied by photophores was then calculated.

LUMINESCENCE COMPETENCE DETECTION

To determine the timing at which photophores become able to produce light, six embryos of the litter that harbour luminous zones containing photophores in different maturation states were used. A metal cap drill was used to excise six 23.8 mm²

circular skin patches from each embryo: two with fluorescent photophores, one with non-fluorescent photophores and three from non-luminous zones (zones with no photophores) for the control.

Excised skin patches were then placed in small perspex chambers with 200 μ l of an isotonic shark physiological solution (saline) of the following composition: 292 mM NaCl, 3.2 mM KCl, 5 mM CaCl_2 , 0.6 mM MgSO_4 , 1.6 mM Na_2SO_4 , 300 mM urea, 150 mM trimethylamine N-oxide, 10 mM glucose, 6 mM NaHCO_3 ; total osmolarity: 1.080 mosM; pH 7.7 (Bernal *et al.*, 2005). Stock solution of hydrogen peroxide (H_2O_2) was prepared in shark saline every morning and stored at 4° C. Skin patches were stimulated by injection into the perspex chambers of H_2O_2 to a final concentration of 3.7% using a Hamilton syringe (200 μ l). The perspex chambers were placed inside a luminometer (Berthold FB 12, Pforzheim, Germany) with the skin patches surface area facing the photo-detector. The luminometer was calibrated using a standard beta-light source (Saunders Roe Ltd, Hayes, England) placed at the same position than the skin patches. The light production was recorded on a laptop computer during 5 min after the stimulation. The luminescence of skin patches was characterized using the total amount of light emitted (L_{tot}) during a period of 5 min, in megaquanta (Mq). Since the photophores were extremely numerous and densely packed, the luminescence was expressed by the surface area, *i.e.* in Mq cm^{-2} .

STATISTICAL ANALYSIS

In order to determine how the luminous structures evolve during the entire ontogeny of *E. spinax*, P_D , V_{LZ} and V_P were linearly regressed against L_T , in embryos and in free-swimming fish. Differences between slopes of embryos and free-swimming animals were only considered to be significant if none of the values present in the 95% slope interval of the first was encompassed by the 95% CI of the second. P_{De} and P_D were $\log_{10}$ transformed and a regression analysis was performed between $\log_{10}(P_{De})$ and $\log_{10}(P_D)$ as theoretically the density is expected to decrease with P_D at the power of two following geometric similarity. To test differences in L_{tot} between the different skin patches, data were tested for normality and equality of variance, and, as variances were unequal, a one-way ANOVA was done on $\log_{10}$ transformed values, as indicated by Sokal & Rohlf (1995). A Student–Newman–Keuls *post hoc* test (SNK test) was then performed in order to test all pair-wise comparisons. Statistical analyses were performed using SAS/STAT (SAS Institute, Cary, NC, U.S.A.) and considered significant at the 0.05 level. All results are given as mean $\pm$ S.E.

RESULTS

A total of 28 free-swimming *E. spinax* (146–520 mm L_T) were collected, and among them, six females were pregnant and provided 64 embryos (31–122 mm L_T) for analysis. As the pregnant females were of similar size (460–475 mm L_T), it was assumed that the different embryo mean L_T measured for each litter corresponded to different developmental levels; the litters were ranked and named (L1 to L6) according to the mean size of their embryos (see Fig. 1 and Table I)

EVOLUTION OF THE LUMINOUS STRUCTURES DURING ONTOGENY

Luminous pattern establishment

In this study, six different litters containing embryos of growing size, showing a progressive reduction of the yolk sac and a succession of appearance of new luminous zones (Fig. 1 and Table I) were used. As a result, the smallest

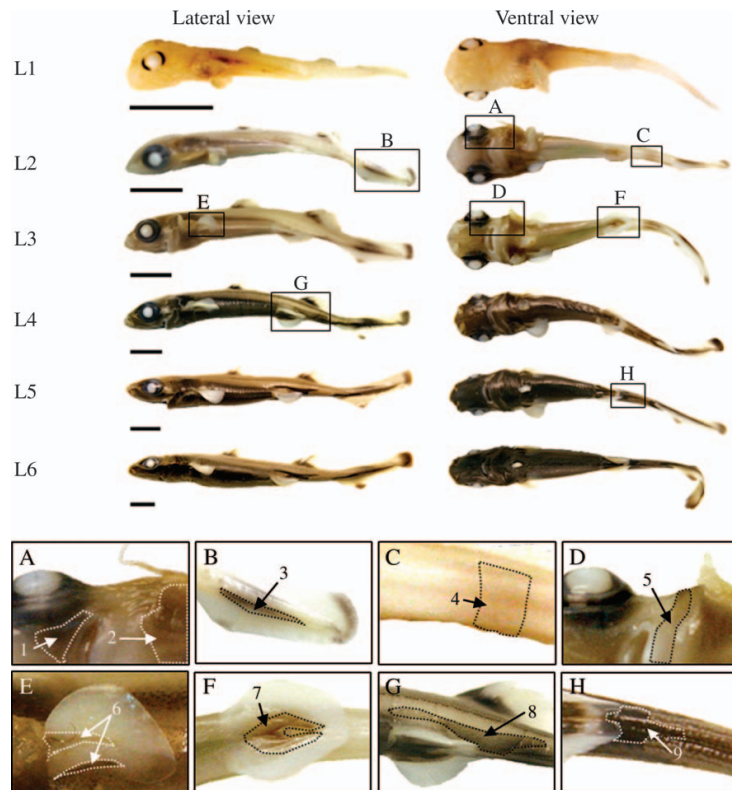


FIG. 1. Growing embryonic series of *Etmopterus spinax* constituted six different litters (L1–L6). Insets contain enlarged skin portions of the sharks. Black arrows indicate luminous zones at their first appearance delimited by a hatched line. The numbers indicate sequential appearance order of the luminous zones. For practical purposes yolk sac are excised. Scale bars = 10 mm.

embryos lacked any luminous zone and had the largest yolk sacs, while the largest embryos had a mean L_T of 117 mm and represented fish ready to hatch since their yolk sacs were almost totally reduced (Table I).

Nine different luminous zones appeared following a precise order (Fig. 1). The appearance of the first luminous zone occurred in litter 2, and the final luminous pattern was already observed in 95 mm L_T embryos. The density of photophores was homogeneous in one luminous zone, and the separation between two distinct adjacent luminous zones was easily visible due to a clear change in photophores density. The photophores pattern was therefore relatively heterogeneous, being more a composite of different individual zones than a homogeneous area.

As it has been done for other luminous fishes (Greene, 1899; Ahlstrom *et al.*, 1984), the first nomenclature for *E. spinax* luminous zones followed their

TABLE I. Characteristics of the different litters of *Etmopterus spinax* used in this study

Litter	Female L_T (mm)	Number of embryo*	Mean $\pm$ S.E. embryo L_T (mm)	Mean $\pm$ S.E. yolk-sac volume (cm ³) [†]	Number of luminous zones present
L1	470	8/6	34 $\pm$ 1	22.2 $\pm$ 1.7	0
L2	475	6/5	55 $\pm$ 1	10.7 $\pm$ 0.4	4
L3	460	4/3	70 $\pm$ 1	9.6 $\pm$ 1.0	7
L4	470	5/5	91 $\pm$ 1	5.3 $\pm$ 0.4	8
L5	460	7/6	95 $\pm$ 1	1.9 $\pm$ 0.3	9
L6	470	5/4	117 $\pm$ 1	0.5 $\pm$ 0.2	9

L_T , total length.

*For each uterus separately.

[†]Estimation based on digital imaging analysis.

position along the body of the fish and according to their sequential appearance during embryogenesis (Fig. 2). The following zones are proposed: (1) rostral, (2) ventral, (3) caudal, (4) infra-caudal, (5) mandibular, (6) pectoral, (7) pelvic, (8) lateral and (9) infra-pelvic.

Photophore development

Three different developmental steps in the photophore building were observed during the histological study of the various luminous zones from the different litters of *E. spinax*. The first step corresponded to the appearance of a layer of pigmented cells between epidermis and dermal connective tissue [Fig. 3(a), (d) (1)]. At this moment, the photophore was not yet formed. The second step consisted of a complete photophore with a pigmented sheath, an iris (created by the juxtaposition of pigmented cells), and lens cells, as well as non-fluorescent photocytes; these cells should be called protophotocytes

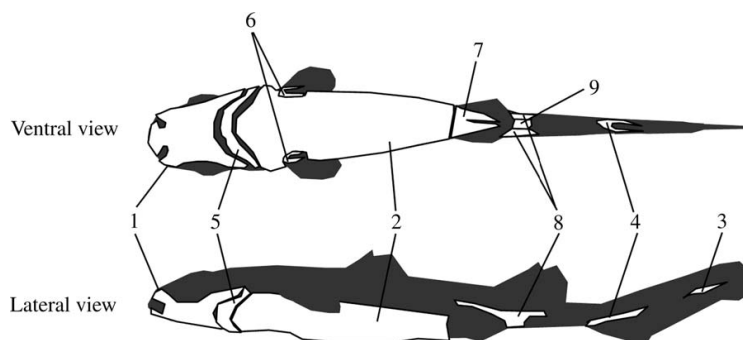


FIG. 2. Ventral and lateral view of the complete luminous pattern of *Etmopterus spinax* in addition to the proposed nomenclature for the different luminous zones: 1, rostral; 2, ventral; 3, caudal; 4, infra-caudal; 5, mandibular; 6, pectoral; 7, pelvic; 8, lateral; 9, infra-pelvic. Numbers correspond to the order of their sequential appearance.

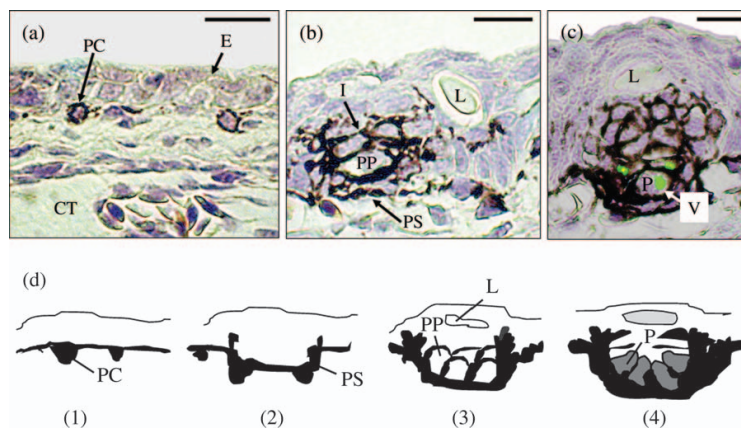


FIG. 3. Development of *Etmopterus spinax* photophores. (a)–(c) Histological sections of photophores at different developmental stages, Masson's trichrome. (a) Stage A photophore (mandibular zone) from a 71 mm total length (L_T) embryo. (b) Stage B photophore (lateral zone) from a 91 mm L_T embryo. (c) Combined image (light and fluorescence microscopy) of a fully developed photophore (stage C, lateral zone) of a 96 mm L_T embryo. (d) Schematic representation of photophore development: (1) appearance of pigmented cells (stage A); (2) and (3) formation of pigmented sheath, cluster of protophotocytes and appearance of lens cells (stage B); (4) maturation of the photophore, fluorescence is present inside the photocytes (stage C). CT, connective tissue; E, epidermis; I, iris; L, lens cell; P, photocyte; PC, pigmented cell; PP, protophotocyte; PS, pigmented sheath; V, fluorescent vesicle. Scale bars = 50 μ m.

following the terminology of Anctil (1977) for *Porichthys notatus* Girard [Fig. 3 (b), (d) (2), (3)]. The third step was observed when green fluorescent vesicles appeared inside the photocytes that Anctil (1977) called the secretory state, *i.e.* early fluorescent photocytes [Fig. 3(c), (d) (4)].

Light microscopy revealed an asynchronous development of photophores at different times according to the luminous zone (Table II). The fluorescence was already present in the yolk sac of litter 1 (L1) embryos but only appeared in luminous zones 1–4 (rostral, ventral, caudal and infra-caudal) in litter 4 (mean embryo L_T = 91 mm). In L5 (mean embryo L_T = 95 mm), all the luminous zones contain secretory photocytes. It is also in this litter that the first dermal denticles were observed, which indicated that these denticles started to develop after the photophores.

A highly significant relationship was found between P_D and P_{De} ($P < 0.001$), revealing a decrease in photophore density when photophore diameter increased [Fig. 4(a)]. The slope of the regression was, however, significantly different from the theoretical negative slope of 2 predicted by geometric similarity ($P < 0.05$). A strong relationship was also found between P_D and L_T during embryogenesis ($P < 0.001$), while no significant relationship ($P > 0.05$) was found between P_D and L_T in free-swimming animals [Fig. 4(b)]. The P_D of embryos from litter 6 ($119.4 \pm 1.6 \mu\text{m}$) attained *c.* 75% of the size of P_D in free-swimming fish ($164.9 \pm 4.3 \mu\text{m}$). The P_D of male ($161.8 \pm 9.2 \mu\text{m}$) and female ($167.6 \pm 4.5 \mu\text{m}$) free-swimming fish was very similar.

TABLE II. Photophore building through the growing embryonic series of *Etmopterus spinax*

Luminous zone	Litter				
	L2	L3	L4	L5	L6
Rostral	PC	PP	P	P	P
Ventral	PC	PP	P	P	P
Caudal	PC	PP	P	P	P
Infra-caudal	PC	PC	P	P	P
Mandibular	/	PC	PP	P	P
Pectoral	/	PC	PP	P	P
Pelvic	/	PC	PP	P	P
Lateral	/	/	PP	P	P
Infra-pelvic	/	/	/	P	P

P, (secretory) photocyte; PC, pigmented cells; PP, protophotocytes; /, nothing observed.

Coverage of ventral surface area

A highly significant relationship ($P < 0.001$) was found between the L_T of embryos and V_{LZ} , which increased during embryogenesis (Fig. 5). In free-swimming fish, V_{LZ} still increased relative to L_T but the slope obtained was less significant ($P < 0.05$) than the slope found for embryos (Fig. 5). A highly significant relationship ($P < 0.001$) was also found between the L_T of embryos and V_P , which also increased during embryogenesis (Fig. 5). No significant relationship was found between V_P and L_T in free-swimming fish ($P > 0.05$). The V_{LZ} of embryos from L6 ($81.9 \pm 1.2\%$) attained *c.* 95% of the size of P_D in free-swimming fish (mean $\pm$ s.e. $87.1 \pm 0.5 \mu\text{m}$). The V_P of embryos from L6 ($38.1 \pm 1.4\%$) reached $>80\%$ of the size of V_P in free-swimming sharks ($46.0 \pm 1.6\%$). The V_P of male ($46.8 \pm 2.4\%$) and female ($44.9 \pm 2.1\%$) free-swimming fish was very similar.

LUMINESCENCE COMPETENCE

The original recording of hydrogen peroxide-induced luminescence of an excised patch of the ventral zone of a 91 mm L_T embryo is presented in Fig. 6. The light emission was a monophasic glow (duration >2 s) characterized by a peak, occurring usually after 10 s. There was a highly significant difference ($P < 0.001$) for L_{tot} between luminous zones with secretory photocytes in their photophores and patches lacking secretory photocytes, *i.e.* non-luminous tissues and luminous tissue containing only protophotocytes (Fig. 7). Finally, spontaneous blue luminescence was observed in embryos (Fig. 8), showing that the luminous system was operational prior to birth.

DISCUSSION

The present work follows the development of photogenic structures and the acquisition of bioluminescence competence during the ontogeny of *E. spinax*.

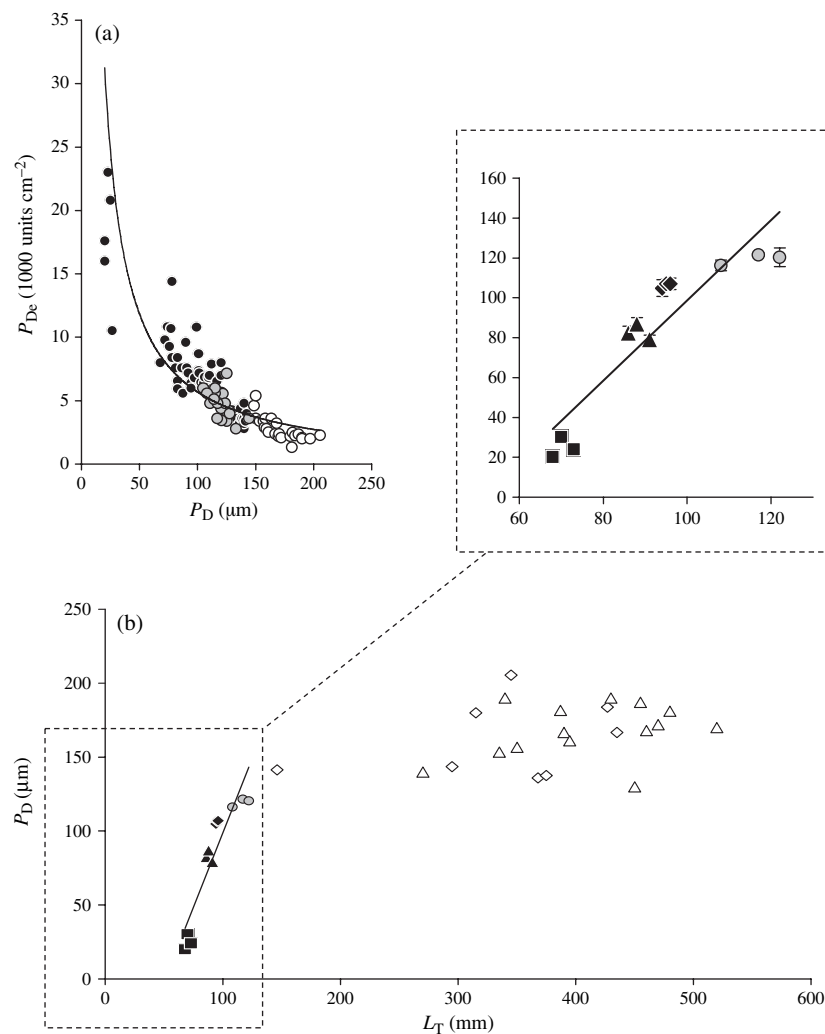


FIG. 4. Changes in the photophore diameter (P_D) in relation to (a) photophore density (P_{De}) and (b) total length (L_T) in *Etmopterus spinax*. (a) Relationship for the different luminous zones of young embryos (●) and ready-to-hatch embryos (○), as well as for the ventral luminous zone of free-swimming animals (○). Data are presented with calculated least-square regression of P_{De} and P_D according to the equation: $\log_{10} y = -1.058 \log_{10} x + 5.87$ ($r^2 = 0.73$, $n = 105$). (b) Inset, shows the relationship found between P_D and L_T in embryos [litters: L3 (■), L4 (▲), L5 (◆) and L6 (●)]. Values are means $\pm$ s.e. and data are presented with calculated least-square regressions according to the equation: $y = -102.79 + 20.16x$ ($r^2 = 0.84$, $n = 12$). No significant relationship was found between P_D and L_T for free-swimming fish ($r^2 = 0.09$, $n = 22$) (Δ , males and $\diamond$, females).

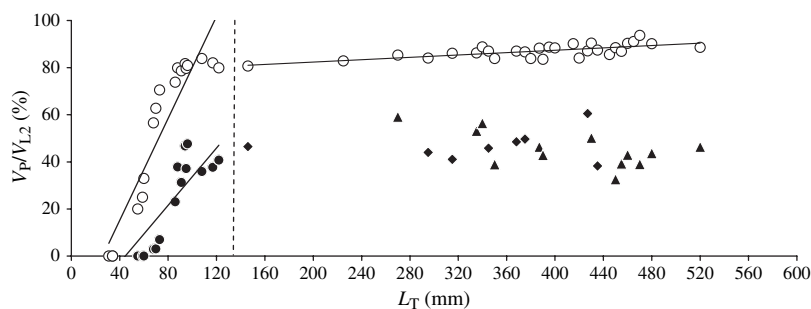


FIG. 5. Percentage of ventral surface covered by luminous zones (V_{LZ}) (○) and photophores (V_P) [embryos (●), free-swimming males (◆) and free-swimming females (▲)] in relation to total length (L_T). Dashed line separates embryos from free-swimming *Etmopterus spinax*. The curves were fitted by: V_{LZ} , $y = -28.07 + 1.08x$ ($r^2 = 0.86$, $n = 18$) for embryos and $y = 77.43 + 0.025x$ ($r^2 = 0.50$, $n = 28$) for free-swimming sharks; V_P , $y = -27.12 + 0.61x$ ($r^2 = 0.76$, $n = 18$) for embryos; no significant relationship was found between V_P and L_T for free-swimming sharks ($r^2 = 0.09$, $n = 21$).

The study gives for the first time strong clues concerning the function of luminescence in a species of shark.

ELABORATION AND ORGANIZATION OF PHOTOGENIC STRUCTURES

The luminous pattern of *E. spinax* is formed by the juxtaposition of at least nine different zones, which appear sequentially during the embryogenesis of this fish. A sequential appearance of different groups of photophores has also been described for other luminescent fish species (Moser & Ahlstrom, 1970;

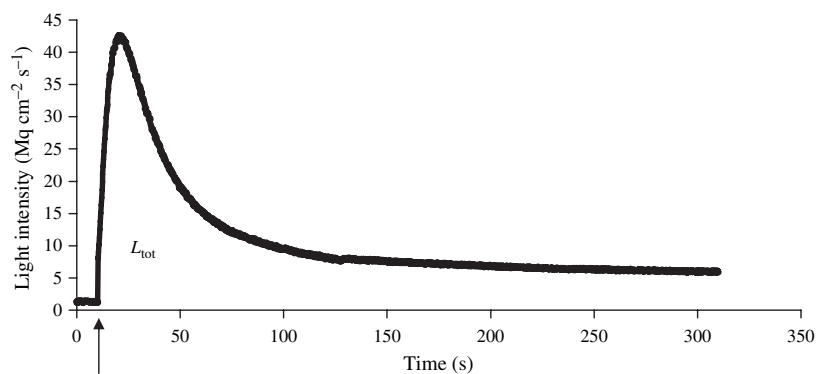


FIG. 6. Original recordings of light production in relation to the time from the rostral luminous zone of a 90 mm total length (L_T) embryo induced by hydrogen peroxide application (↑). L_{tot} , total amount of light emitted after the application.

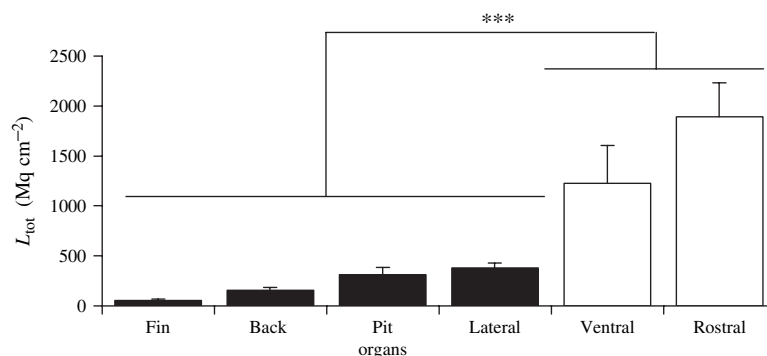


FIG. 7. Total amount of light (L_{tot}) emitted by different skin patches stimulated by hydrogen peroxide (H_2O_2) (■, non-fluorescent tissues (lacking the fluorescent vesicles); □, fluorescent tissues). Non-fluorescent tissues are either in non-luminous tissues, *i.e.* tissues lacking photophores (a fin skin patch, a skin patch from the back part of the fish and a skin patch containing pit organs from the lateral part of the fish) or luminous tissue (the lateral luminous zone) containing only protophotocytes (including fluorescent vesicles). Fluorescent tissues emitted highly significantly more light than non-fluorescent tissues (ANOVA, $n = 36$, ***, $P < 0.001$). Values are means $\pm$ s.e.

Ancil, 1977; Ozawa & Katayama, 2003). In contrast to what was observed with the teleost *P. notatus* (Ancil, 1977), there is no antero-posterior gradient of appearance for the luminous zones in *E. spinax*. As a consequence of sequential appearance, however, luminous zones are not similar in terms of their photophore building state.

The development of light organs in this fish is a well-controlled process and is relatively similar to what occurs in *P. notatus* (Ancil, 1977). The first noticeable step is the disposition of a layer of pigmented cells between the epidermis and the dermis. The second step is the progressive encapsulation of protophotocytes by the pigmented cells, which form the pigmented sheath and the iris of photophores as well as the differentiation of lens cells. The differentiation of protophotocytes into secretory photocytes consists in the next observable building event of photophores. At this stage, the light organs adopt a glandular-like organization, but until now no sign of exocytosis is observed.

In a comparative study of free-swimming luminous shark placoid scales, it was assumed that the density of photophores remains constant during the

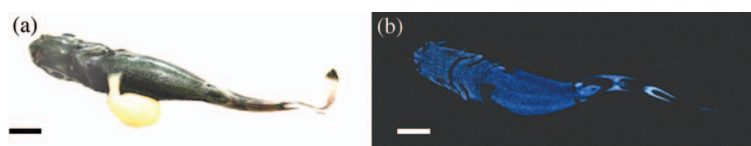


FIG. 8. Spontaneous luminescence of a 115 mm total length embryo. (a) Digital picture of the embryo when the light was turned on. (b) Digital picture of the same embryo when the light was turned off. Scale bars = 1 cm.

ontogeny of lantern sharks such as *E. spinax*, which implies a permanent insertion of new light-emitting organs during their growth (Reif, 1985). The present results do not support this hypothesis since a density decrease with a diameter increase of the photophores for the different luminous zones was shown, at least during embryogenesis. The fact that photophore diameter seems more stable in free-swimming animals suggest that there is a size limit (probably around 200 μm) for *E. spinax*'s photophores. This value is far below those attained in *P. notatus* and other luminous fishes (e.g. *Argyropelecus*, *Maurollicus*, lantern fishes) whose light organs can reach a diameter of at least 1 mm (Harper & Case, 1999; Cavallaro *et al.*, 2004; pers. obs.).

ACQUISITION OF LUMINESCENCE COMPETENCE

For the first time, light emission from a shark was analysed. Luminous capabilities of *E. spinax* are strongly linked to the presence of photocytes' green fluorescent vesicles; a similar phenomenon has been demonstrated for *P. notatus* where luminescence acquisition was correlated to the differentiation of protophotocyte (non-fluorescent) into fluorescent photocytes (Anctil, 1977).

The weak light production from zones with non-fluorescent photophores, *i.e.* with protophotocytes, or even from non-luminous skin zones might be related to a wide distribution of the luminous substrate in the entire embryo's body as it was demonstrated in other luminous mesopelagic fishes (Mallefet & Shimomura, 1995). Fluorescence is present in the yolk sac before photophore development in embryos. This observation supports the hypothesis of a maternal transfer of the luminous compounds to the embryo as already suggested in other luminescent fishes (Rees *et al.*, 1990, 1992; Mensinger & Case, 1991). The dietary acquisition of luminescent material has been demonstrated for a wide range of organisms (Rees *et al.*, 1990, 1992; Mensinger & Case, 1991; Mallefet & Shimomura, 1995; Haddock *et al.*, 2001) and it is possible that *E. spinax* could indeed acquire its luminous compounds through alimentation, as it is known that free-swimming individuals of *E. spinax* regularly prey on luminous organisms (Klimpel *et al.*, 2003; Neiva *et al.*, 2006).

FUNCTION OF LUMINESCENCE

The results obtained in this study are strongly in favour of a counter-illuminating use of luminescence in juvenile *E. spinax*. Indeed, the ventral photogenic organs develop quickly during embryogenesis, and ready-to-hatch embryos are already able to emit a downward blue light. Moreover, the proportion of ventral surface occupied by photophores and luminous tissues also increases quickly during *in utero* development to allow ready-to-hatch fish to have >80% of their ventral surface area covered by the luminous zones forming the luminous pattern. Although the percentage of ventral surface area covered by photophores is somewhat smaller (*c.* 40%), it is still far above the value obtained for juvenile *P. notatus* (<10%), which are considered to use luminescence for camouflage by counter-shading (Harper & Case, 1999). Natural predators of *E. spinax* are not known but probably consist of cetaceans and large fishes (Reif, 1985).

If counter-illumination is probably the function of luminous zones present on the ventral side of this fish, the function of luminous zones from the lateral zones is less clear. Indeed, the lateral zones represent a small portion of the fish side hence a camouflage function by counter-illumination for these zones seems unlikely. It cannot be ruled that the fish may camouflage itself by a disruptive silhouette. Nevertheless, the heterogeneity in the luminescence observed among the different luminous zones of the adult (pers. obs.) contribute to the idea that luminescence in *E. spinax* is not restricted to only one purpose (as in *Dalatidae*) but may be more versatile and complex.

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PAPER II

Ontogeny of photophore pattern in the velvet belly lantern shark,
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Ontogeny of photophore pattern in the velvet belly lantern shark, *Etmopterus spinax*

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Abstract

Bioluminescence is known to be of great ecological importance to a luminous organism but extremely few studies investigate the ontogeny of luminous capabilities. The photogenic pattern of the velvet belly lantern shark *Etmopterus spinax* was investigated over ontogeny (14.0–52.5 cm total length) to determine the scaling of the surface area and the photophore density of different luminous zones as well as the ecological consequences of ontogenetic variations in bioluminescence efficiency. According to the luminous zone considered, different scaling patterns were found for the surface areas while the photophore densities of all zones scale with negative allometry, even though photophore insertion occurs. No sexual differences in these relationships were found. Luminous zones can be placed in two morphologically different groups: the “coverage” and the “isolated” zones. While counter-illumination is certainly the function of the former, the latter are probably involved in intraspecific behaviours. Due to the discrepancy between luminous capabilities of these two luminous zone categories, there is an ontogenetic increase in the luminescence heterogeneity of the luminous pattern as it was shown by luminescence modelling and confirmed by direct observations of spontaneous luminescence in living sharks. This heterogeneity certainly represents a trade-off between an efficient ventral camouflage and a strong identification tool for intraspecific behaviours such as coordinate hunting, which would be particularly useful when *E. spinax* become fish eaters (>19 cm total length), and for sexual recognition in mature individuals.

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Keywords: Elasmobranch; Etmopteridae; Bioluminescence; Luminous zones; Counter-illumination

Introduction

Bioluminescence is a widespread phenomenon in the deep-sea environment where many species from bacteria to fishes emit light for numerous purposes including predation, defence and reproduction (Buck, 1978;

Wilson and Hastings, 1998; Herring, 2007). While the majority of deep-sea Osteichthyes (circa 70%) are luminous, only a small fraction (circa 6%) of deep-sea Chondrichthyes is endowed with this capability (Harvey, 1952; Herring and Morin, 1978; Compagno et al., 2004). This led some authors to consider this amazing property a burden on these fishes (Reif, 1985). Even though it is absent in Holocephali (chimaeras), the luminescence competence appeared at least three times

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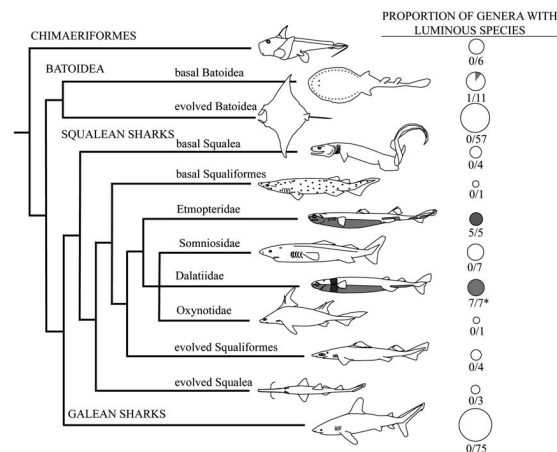


Fig. 1. Cladogram indicating the occurrence of bioluminescence in the cartilaginous fishes (grey shade represents photogenic tissues). Circles to the right indicate the proportion of luminous genera within a given taxonomic grouping (shaded, luminous; white, non-luminous; Harvey, 1952; Hubbs et al., 1967; Compagno et al., 2004). Circle size is proportional to the total number of genera in a given taxonomic grouping. The typology of the cladogram is based on Shirai (1996) and was improved following Naylor et al. (2005). One dalatid genus (*Mollisquama*) shows pectoral glands supposed to be luminous but more information is needed to confirm the luminous status of this very rare species (only known from one individual; Compagno et al., 2004).

independently in cartilaginous fishes, since three different phylogenetic groups harbour luminous species (Harvey, 1952; Hubbs et al., 1967; Fig. 1).

Luminous sharks are from two families, the Etmopteridae and the Dalatiidae, with distinct differences in the structure and the arrangement of their photophores (i.e. photogenic organs) (Hubbs et al., 1967; Reif, 1985; Fig. 1). While the Dalatiidae have an exclusively ventral pattern of photophores probably used for counter-illumination, the Etmopteridae show complex, species-specific patterns with photophore concentrations on the ventral part, but also on the flanks, tail, and sometimes on the upper eyelid (Iwai, 1960; Reif, 1985; Last et al., 2002; Yano et al., 2003; Fig. 1). The function of these patterns is still unknown but it is thought that they could play multiple roles, e.g. in schooling and counter-illumination (Reif, 1985; Claes and Mallefet, 2008).

If we assume that the function of the etmopterid bioluminescence is profoundly linked to the arrangement of their light organs, and knowing that these organs attain their maximum size at birth (Claes and Mallefet, 2008), the question arises as to how these sharks manage to maintain the integrity of their luminous pattern during growth.

The velvet belly lantern shark, *Etmopterus spinax*, Linnaeus 1758, is a good model to investigate the effect of growth on bioluminescence capabilities in a shark, as

it is abundant in all size classes in the East Atlantic and the Mediterranean Sea and can be easily caught and maintained in captivity for long periods of time (>1 week; Compagno et al., 2004; Neiva et al., 2006; Claes and Mallefet, 2008). The luminous pattern of this shark is composed of nine luminous zones, which appear sequentially during embryogenesis and are already functional before birth (Claes and Mallefet, 2008).

The objectives of this study were (i) to investigate how the luminous capabilities of *E. spinax* are maintained during growth, (ii) to test for a potential sexual dimorphism in bioluminescence, (iii) to model the luminous pattern of this shark, and (iv) to discuss the results in relation to the ecology of this species.

Materials and methods

Experimental animals

Free-swimming specimens of *E. spinax* of 14.0–52.5 cm total length (TL) were obtained during three field trips (February 2007, December 2007, and April 2008) in the Raunefjord (longlines and bottom trawling) as well as in the Lysefjord (bottom trawling) in Norway, and brought alive to the Hoyteknologiseret in Bergen

where they were kept in a tank placed in a dark cold room (4 °C).

Maturity of individuals was determined macroscopically following the maturity scale of Coelho and Erzini (2007) and rating all those able to reproduce or that had already reproduced in the past as mature (Conrath, 2004; Coelho and Erzini, 2008a). Immature individuals were divided into two categories according to their feeding preference: planktivorous ≤ 19 cm TL and piscivorous > 19 cm TL (Klimpel et al., 2003). Mature individuals were divided according to sex. All animals were sacrificed by a blow on the head, according to the experimental protocol of Hoyteknologisenteret in Bergen.

Morphological analysis

Digital pictures of the ventral and lateral sides of the sharks were taken with a digital camera (Canon D20; Canon Inc., Tokyo, Japan). The surface areas of the nine luminous zones of each individual were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). Photophore densities were estimated by counting photophores present on standard (0.25 cm²) skin patches placed under a binocular microscope in an isotonic shark saline following the method of Claes and Mallefet (2008). Since the small size of the caudal zone prevents precise measurement of photophore density, we considered the density in this zone the same as in the infra-caudal zone, of which it is an extension. The total number of photophores present in a shark was obtained by multiplication of the surface area of each zone by its specific density.

Luminometry

Excised luminous skin patches were placed in small perspex chambers with 200 μ l of isotonic shark saline. Skin patches were stimulated by injecting hydrogen peroxide into the chambers to a final concentration of 0.1 M using a micropipette (200 μ l). The chambers were placed inside a luminometer (Berthold FB 12; Berthold,

Pforzheim, Germany) with the skin patches' exterior surface facing the photo-detector. The luminescence was recorded on a laptop computer for 5 min after stimulation, and characterised using the maximum intensity of light emission ($L_{\max}$, in megaquanta per second (Mq s⁻¹)) standardised by surface area (cm²).

Statistical analysis

To highlight the scaling relationship in the different variables investigated (luminous zone surface area, photophore density, photophore number), they were log₁₀ transformed and linearly regressed against log₁₀-transformed TL. Slopes obtained for these scaling relationships were compared with theoretical isometrical slopes (−2 for photophore density, 0 for photophore number, and 2 for luminous surface area). For luminous zone surface areas and photophore densities, slopes obtained independently for the two sexes were compared and if no difference was detected, the analysis was performed on all animals without sexual distinction. Differences between slopes were only considered significant if none of the values present in the 95% confidence interval of the first was encompassed by the 95% confidence interval of the second slope.

Linear regressions between mean $L_{\max}$ values and mean photophore densities from the different luminous zones were performed in each shark category to identify the maximum intensity of light emission from a single photophore (i.e. the slope of the regression). These analyses were performed after removal of significant outliers ($> 2\sigma$). Statistical analyses were performed using the software SAS (SAS Institute Inc., Cary, NC, USA).

Results

In total, fifty free-swimming sharks were collected and used in this study. Immature specimens were mainly caught by trawl at shallower depths than adult individuals, which were only obtained with longline (Table 1).

Table 1. Summary of data for specimens of *Etmopterus spinax* collected for this study.

	<i>n</i>	TL			Collection depth (m)	Collection method	
		Minimum (cm)	Maximum (cm)	Mean (cm)		Trawl	Longline
Immatures							
Planktivorous	10	14.0	18.5	16.4 ± 0.5	60–180	10	0
Piscivorous	12	20.5	34.0	25.2 ± 1.4	60–250	12	3
Matures							
Males	10	31.5	43.5	38.0 ± 1.3	180–250	0	10
Females	18	38.7	52.5	44.8 ± 1.0	180–250	0	18

Table 2. Results of scaling analyses on $\log_{10}$ -transformed luminous zone surface areas of *E. spinax*.

Luminous zone	n^a	Regression equation ($\log LZ_S = a \log TL + b$)		r^2	p -value
		Slope (a) ^b [a_M/a_F]	Intercept (b)		
Rostral	45	1.34 [$1.28 \pm 0.17/1.38 \pm 0.10$]	−1.17	0.96	<0.0001
Ventral	45	2.42 [$2.31 \pm 0.17/2.15 \pm 0.12$]	−2.02	0.98	<0.0001
Caudal	44	1.43 [$1.52 \pm 0.34/1.39 \pm 0.22$]	−2.91	0.87	<0.0001
Infra-caudal	45	1.92 [$2.06 \pm 0.24/1.84 \pm 0.14$]	−2.83	0.96	<0.0001
Mandibular	45	1.75 [$1.85 \pm 0.16/1.71 \pm 0.10$]	−2.22	0.98	<0.0001
Pectoral	45	1.93 [$1.99 \pm 0.28/1.82 \pm 0.18$]	−3.17	0.93	<0.0001
Pelvic	45	2.36 [$2.36 \pm 0.22/2.37 \pm 0.17$]	−2.99	0.97	<0.0001
Lateral	44	2.08 [$2.18 \pm 0.23/2.08 \pm 0.16$]	−3.85	0.96	<0.0001
Infra-pelvic	42	2.52 [$2.18 \pm 0.23/2.42 \pm 0.26$]	−2.73	0.94	<0.0001

a_M , slope obtained for males (mean $\pm$ S.E.M.); a_F , slope obtained for females (mean $\pm$ S.E.M.); LZ_S , luminous zone surface area.

^aNumber of sharks used, i.e. 20 males and 25 females except for caudal, lateral, and infra-pelvic zones for which some female values were not available for calculation.

^bWhen no differences between a_M/a_F were found, analysis was done on all data giving one slope value. Bold values indicate slopes deviating significantly from isometry.

The luminous zone surface areas of male and female specimens of *E. spinax* showed similar scaling patterns (Table 2). The surface area of some zones (rostral, caudal, and mandibular) showed negative allometry, while others (ventral, pelvic, and infra-pelvic) showed a positive allometry over ontogeny (Table 2, Fig. 2). The surface area of the infra-caudal, pelvic, and lateral zones showed isometric growth (Table 2, Fig. 2).

The luminous zone photophore densities showed similar scaling patterns in both sexes (Table 3). In all zones, photophore density scaled with positive allometry relative to the total length of *E. spinax*, which means that new photophores are continuously produced during growth (Table 3, Fig. 3A). However, the production rate is too low to keep a constant photophore density throughout ontogeny (i.e. to have a slope equal to 0).

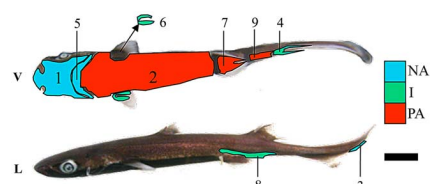


Fig. 2. Ventral (V) and lateral (L) views of a specimen of *E. spinax* (22 cm TL) showing how the surface area of the different luminous zones scales over ontogeny. I, isometry; NA, negative allometry; PA, positive allometry. 1 = rostral zone; 2 = ventral zone; 3 = caudal zone; 4 = infra-caudal zone; 5 = mandibular zone; 6 = pectoral zone; 7 = pelvic zone; 8 = lateral zone; 9 = infra-caudal zone. Scale bar = 2 cm. Results of scaling analyses of luminous zone surface areas are available in Table 2.

Not all zones decreased in density to the same degree; for example, there was a large decrease in the ventral zone, while the pectoral zone showed only a slight decrease (Table 3, Fig. 3A).

Similar relationships between total length and total number of photophores were found for males (slope = 1.2 ± 0.46) and females (slope = 1.12 ± 0.15), and the slope obtained for the regression on all specimens was significantly higher than 0, showing a significant ontogenetic increase in the total number of photophores (Fig. 3B). The total number of photophores ranged from 67,105 units (in a male of 15.5 cm TL) to 439,227 units (in a female of 52 cm TL).

In all categories, a significant relationship was found between mean peroxide-induced L_{max} and mean photophore densities (Table 4; Fig. 4).

A theoretical relative bioluminescence model for the luminous pattern of each category of *E. spinax* at different sizes (planktivorous immatures, piscivorous immatures, mature males, and mature females) was realised (Fig. 5A). This model assumes that the luminescence of a luminous zone is directly dependent on its photophore density. Photophore density of each zone was determined using the mean size of sharks from each category as input in the regression equations presented in Table 3. Zonal density was normalised to the photophore density of the ventral luminous zone and expressed relative to L_{max} . In this model, the heterogeneity of luminescence capabilities between luminous zones increases throughout ontogeny (Fig. 5A). Direct observation of long-lasting (> one hour) spontaneous luminescence from *E. spinax* confirmed the results obtained with the theoretical model (Fig. 5B). The two sexes showed dimorphism in the shape of their luminous pelvic zone (Fig. 5C).

Table 3. Results of scaling analyses on log₁₀-transformed luminous zone photophore densities of *E. spinax*.

Luminous zone	<i>n</i> ^a	Regression equation (log PD = <i>a</i> log TL + <i>b</i>)		<i>r</i> ²	<i>p</i> -value
		Slope (<i>a</i>) ^b [<i>a</i> _M / <i>a</i> _F]	Intercept (<i>b</i>)		
Rostral	50	−0.73 [−0.74 ± 0.31/−0.71 ± 0.25]	4.73	0.59	<0.0001
Ventral	50	−1.57 [−1.31 ± 0.51/−1.74 ± 0.33]	5.79	0.75	<0.0001
Infra-caudal (and caudal)	50	−0.69 [−0.44 ± 0.37/−0.82 ± 0.30]	4.89	0.45	<0.0001
Mandibular	50	−1.14 [−1.07 ± 0.23/−1.14 ± 0.20]	5.26	0.84	<0.0001
Pectoral	46	−0.25 [−0.05 ± 0.27/−0.39 ± 0.32]	4.27	0.12	0.0189
Pelvic	50	−0.60 [−0.47 ± 0.35/−0.57 ± 0.29]	4.60	0.40	<0.0001
Lateral	50	−0.93 [−0.99 ± 0.32/−0.92 ± 0.26]	5.00	0.69	<0.0001
Infra-pelvic	49	−0.66 [−0.22 ± 0.32/−0.81 ± 0.32]	4.75	0.38	<0.0001

*a*_M, slope obtained for males (mean ± S.E.M.); *a*_F, slope obtained for females (mean ± S.E.M.); PD, photophore density.

^aNumber of sharks used, i.e. 20 males and 30 females except for pectoral and infra-pelvic zones for which some female values were not available for calculation.

^bWhen no differences between *a*_M/*a*_F were found, analysis was done on all data giving one slope value. Bold values indicate slope deviating significantly from isometry.

Discussion

The size range of the velvet belly lantern shark specimens used in this study (14.0–52.5 cm TL) encompasses more than 75% of the absolute size range observed in wild individuals (9.0–60.0 cm TL); our sample is therefore suitable for an ontogenetic study of photogenic structures in this luminous shark species (Borges, 2002; Compagno et al., 2004; Gennari and Scacco, 2007). Our fishing results confirm those of a study on Mediterranean individuals which shows that small specimens are mainly captured by trawl fishing while bigger specimens are principally caught with baited longline (Coelho and Erzini, 2008b). The total absence of mature individuals in trawl catches, which were realised at shallower depths (≤180 m) than longline catches (≥200 m), may indicate size-related depth stratification in fjord-inhabiting populations of Norwegian velvet belly lantern shark, as it is the case in the Mediterranean Sea, with immature individuals principally occurring in the upper part of the water column and mature individuals living in greater depths (Coelho and Erzini, 2007).

The bioluminescence modelling presented in this study is a first attempt to determine the functional morphology of the complex luminous pattern of a lantern shark. The validity of our approach is demonstrated by the fact that luminous pattern models obtained for the different shark categories are similar to the luminescent patterns observed in spontaneously glowing specimens of *E. spinax*.

Ontogeny of photogenic structures

The complex luminous patterns observed in Etmopteridae are often used for species determination as they

are assumed to be species-specific and to stay identical over ontogeny (Reif, 1985; Springer and Burgess, 1985; Schofield and Burgess, 1997; Last et al., 2002). Recently, Claes and Mallefet (2008) showed variations in the luminous structures of *E. spinax* during embryogenesis. Our work demonstrates for the first time that the luminous pattern of *E. spinax* is not constant during growth in free-swimming specimens.

In *E. spinax*, as in other etmopterid sharks, the luminous pattern is formed by different luminous zones, which are aggregations of numerous tiny (<200 μm) photophores (Hubbs et al., 1967; Compagno et al., 2004; Claes and Mallefet, 2008). This explains why, in a lantern shark species, luminous pattern variations can occur at different levels: (i) the relative surface area covered by each luminous zone, (ii) the photophore density of the luminous zones, and (iii) the size of the photophores present in the luminous zones. Even if no ontogenetic differences occur at the third level (Claes and Mallefet, 2008), we demonstrate that growth-linked variations do occur at the two first levels, as different zones (rostral, ventral, caudal, mandibular, pelvic, and infra-pelvic zones) show an allometric growth of their surface area and all zones show a decrease in photophore density, in spite of the continuous insertion of new photophores during growth. This ontogenetic decrease in photophore density is probably due to a space conflict between growing placoid scales and photophores (which are epidermal organs; Reif, 1985; Raschi and Tabit, 1992).

Two morphologically distinct types of zones can be distinguished: (i) coverage zones (rostral, ventral, and mandibular), which represent circa 75% of the ventral surface of the shark and in which the photophore insertion rate is low; and (ii) isolated zones (caudal, infra-caudal, pectoral, pelvic, lateral, and infra-pelvic), which are well-defined zones located either ventrally,

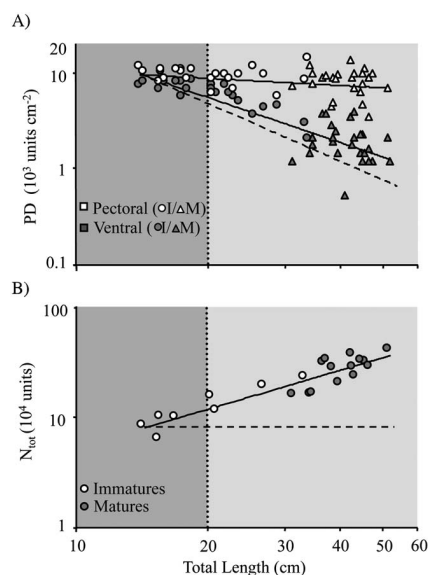


Fig. 3. (A) Photophore density of the ventral and the pectoral zones of *E. spinax* plotted versus total length on logarithmic axes. The dashed line indicates an isometric slope of -2 , while the solid lines are regression lines of data (see Table 3 for details). Results of scaling analyses of luminous zone surface densities are available in Table 2. (B) Total number (N_{tot}) of photophores of *E. spinax* plotted versus total length on logarithmic axes. The solid line is the regression line for all specimens ($\log N_{\text{tot}} = 1.19 \log TL + 3.54$; $n = 22$; $r^2 = 0.85$; $P < 0.0001$), while the dashed line indicates an isometric slope of 0. Shading indicates the feeding transition occurring around 20 cm TL in this species; dark shading = planktivorous; light shading = piscivorous.

showing higher photophore insertion rates during ontogeny, or laterally. The observed morphological differences indicate different luminous capabilities of these two groups of zones, and hence different uses of luminescence.

Bioluminescence ecology

Numerous adaptative benefits have been suggested for bioluminescence, highlighting the perceived importance of this phenomenon in the ecology of luminous organisms (Harvey, 1952; Buck, 1978; Hastings, 1983). In teleost fishes, bioluminescence is thought to be used for different purposes: (i) interspecific ones such as camouflage by counter-illumination (and disruptive illumination), predator jamming, decoying (to approach prey), prey attraction (luminous lure), prey illumination and (ii) intraspecific ones such as sexual signalling and schooling (Clarke, 1963; Young and Roper, 1976; Young, 1977; Buck, 1978; McFall Ngai and Morin, 1991; Herring, 1985, 2000, 2007). Though often quite plausible, these hypotheses have never been tested due to the difficulty of working with luminous fishes (Buck, 1978; Wetherbee, 2000). Only camouflage by counter-illumination has been relatively well documented by behavioural experiments in the myctophid *Myctophum obtusirostrum* and in the juveniles of the midshipman fish *Porichthys notatus* (Young et al., 1980; Harper and Case, 1999).

Our work allows us to establish strong hypotheses about the ecology of bioluminescence in *E. spinax* and to propose that morphologically divergent luminous zones play different roles. At first sight, the coverage zones are designed for camouflage by counter-illumination. Indeed, having similar photophore densities, these zones form a large homogeneous luminescent surface emitting a long-lasting blue glow that is a good match for down-welling light, so the fish disappears from the sight of its predators (Harper and Case, 1999; Claes and

Table 4. Results of regression of photophore density against L_{max} for different *E. spinax* categories.

Shark group	n^a	Regression equation ($L_{\text{max}} = a \text{ PD} + b$)		r^2	p -value
		Slope (a)	Intercept (b)		
Immatures					
Planktivorous	10	62.39 ± 51.70	-365.56	0.59	0.0255
Piscivorous ^b	12	65.54 ± 27.04	-290.96	0.89	0.0016
Matures					
Males	10	19.84 ± 9.86	-37.64	0.80	0.0026
Females	17	15.97 ± 6.05	-26.52	0.87	0.0007

PD, photophore density in $1000 \text{ units cm}^{-2}$.

^aNumber of sharks used to determine the PD and the L_{max} of each luminous zone.

^bIn this group, the regression was performed without the infra-caudal zone which gave a very weak luminescence.

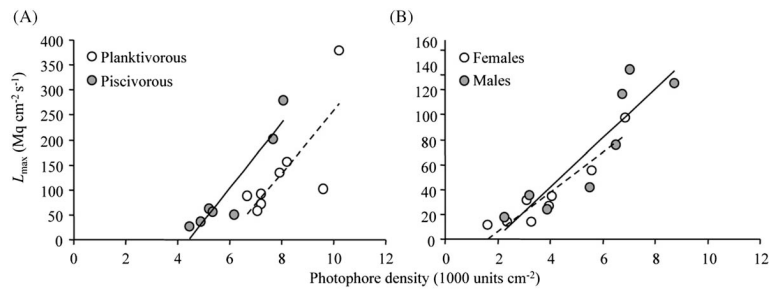


Fig. 4. $L_{\max}$ plotted versus photophore densities of the different luminous zones of (A) immature and (B) mature specimens of *E. spinax*. Solid and dashed lines represent regression lines of the data (dashed lines = open circles; solid lines = filled circles). Results of the regressions are available in Table 4.

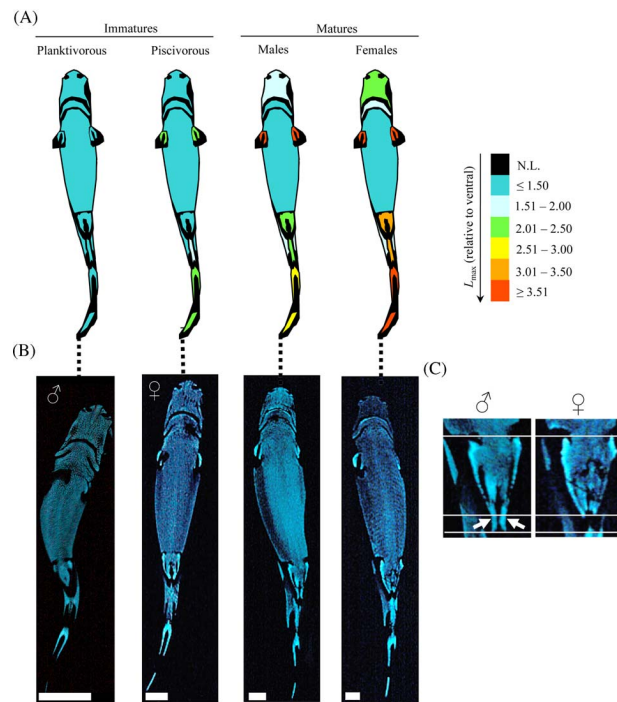


Fig. 5. (A) Luminous pattern modelling of the different *E. spinax* categories investigated in this study. In each shark category, the luminescence of the luminous zones is expressed in relation to the luminescence of the ventral zone. N.L. = non-luminous tissue. (B) Spontaneous luminescence of different specimens. Scale bars = 2 cm. Dashed lines link the pictures to the luminous pattern models to which they are supposed to correspond. (C) Close-up of pelvic areas of an adult male and an adult female specimen of *E. spinax*. Presence of a high photophore density in this body region allows the two sexes to be distinguished in the dark: only male sharks exhibit luminous pterygopods (white arrows), which are used for mating.

Mallefet, 2008). The positions of the isolated zones as well as their luminous characteristics render these zones more visible in adults, which favours intraspecific signalling. It is known that numerous non-luminous sharks probably live and reproduce in deep waters with the help of pheromonal signals (Wourms, 1977; Johnson and Nelson, 1978; Pratt and Carrier, 2001; Compagno et al., 2004). One cannot exclude, however, that the presence of brighter luminescence in the sexually dimorphic pelvic areas of mature specimens of *E. spinax* might facilitate sexual recognition, comparable to lantern fishes (Osteichthyes: Myctophidae) where sexual dimorphism of caudal photophores provides circumstantial evidence for sexual signalling (Herring, 2007). The high luminous capabilities of isolated zones could also serve as visual clues helping to coordinate swimming and/or hunting since it is known that teleost fishes and other shark species frequently use visual information for intraspecific behaviours (Myrberg, 1990; Grégoire and Chaté, 2004). Springer (1967) already suggested that luminescence in the green lantern shark (*Etmopterus virens*) is a schooling aid and that this species predares in groups. He based his hypothesis on the fact that the prey consumed by this shark is too big to be hunted and killed by a single shark. Based on the same observations, Macpherson (1980), and more recently Neiva et al. (2006), formulated the same hypothesis for *E. spinax*. Bright luminous markings on flanks, tail, and pectoral fins would give helpful visual clues for such coordinated movements in the darkness of the deep sea.

Shift in photophore pattern organisation

Our results show that the luminous capabilities of a zone are dependent on its photophore density. The low photophore insertion rates of coverage zones, and the constant size of photophores in free-swimming specimens of *E. spinax* (Claes and Mallefet, 2008) strongly suggest that small sharks of this species can counter-illuminate at shallower depths than bigger ones. This assumption is totally in accordance with recent findings of Coelho (2007), who showed that depth stratification occurs in Mediterranean *E. spinax*, with larger individuals occurring predominantly in deep waters, and females migrating vertically to give birth at shallower depths. A similar trend was observed in this study, with no mature specimens caught above a depth of 180 m and only few (13%) immature sharks found below this depth. Isolated zones, on the other hand, face a pressure to have their luminescence capabilities maintained over ontogeny. This is conclusive if we consider that these zones help sharks to dominate large prey such as large fishes and squids which only start to be part of the alimentation when the shark grows over 19 cm TL

(Klimpel et al., 2003; Neiva et al., 2006), the size at which coverage and isolated luminous zones start to diverge in terms of luminous capabilities. The luminous pattern heterogeneity observed in fish-eating sharks would thus represent the answer to the trade-off faced by this shark that tries to keep an efficient ventral luminescent camouflage when faced with the need for a visual tool for intraspecific behaviours. Krill-eating sharks (≤ 19 cm TL) do not need to increase the visibility of their isolated zone and have a homogeneous luminous pattern (with only slight differences in photophore density between luminous zones), which covers a very large part of the ventral surface area, thus giving a nearly perfect camouflage. This confirms the adaptive advantage of ventral counter-shading in young specimens of this species, as is the case for *Porichthys notatus*, a luminous teleost (Mensingher and Case, 1991; Claes and Mallefet, 2008).

Acknowledgements

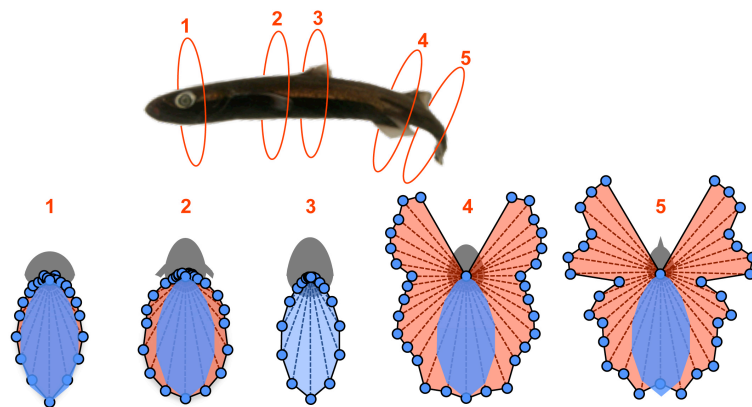
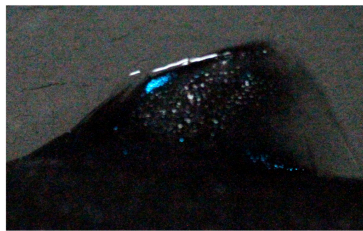
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Chapter 2: *Luminescent behaviours*



Chapter 2: Luminescent behaviours

This second section aspires to investigate the putative function(s) of the luminescent pattern of *E. spinax*. It is composed of one paper (**Paper III**), that aims to test the function of counterillumination in this species, and preliminary data that suggest other potential functions for this shark's luminescence.

Results from previous studies (Claes and Mallefet, 2008; 2009a) strongly suggested the use of ventral photophores as a way for the shark *E. spinax* to illuminate its ventral surface and efficiently cloak its silhouette from its prey and predator's sight, a process known as counterillumination, which is common in midwater animals including crustaceans, molluscs (squids) and bony fishes (Young, 1980; Warner et al., 1978; Harper and Case, 1999). This tricky camouflage depends, however, of the physical characteristics of the luminescence, which have to be similar to those present in the environment at the depth at which the animal want to counterilluminate (Denton et al., 1985). In addition the light must be emitted during extended period of time and cope with temporal variations in their light environment (Warner et al., 1978). In **Paper III**, the physical characteristics (wavelength, intensity and angular distribution) of *E. spinax*'s spontaneous luminescence were measured and compared with those present in its environment (Norwegian fjords), modelled using a CTD probe coupled to a chlorophyll sensor and taking data from the literature. Results show that the luminescence of *E. spinax* is well suited for counter-illumination, being emitted for long periods of time and possessing the convenient physical characteristics. Besides, we also show that the species was apparently not able to modulate the intensity of its luminescence in response to changes of overhead light intensity, which suggest that it migrates vertically in the water column to remain cryptic and could explain the nocturnal observations of this shark at shallow depths in the fjords.

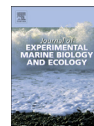
The preliminary data aim to show that in this shark the luminescence is not restricted to the sole purpose of camouflage, but rather is involved in intraspecific behaviours such as cohesive swimming/hunting and sexual signalling (as it was already suggested see Claes and Mallefet, 2009a), in aposematism, and in prey illumination.



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Phantom hunter of the fjords: Camouflage by counterillumination in a shark (*Etmopterus spinax*)

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ABSTRACT

Many midwater animals emit ventral light to hide their silhouette in the water column. This phenomenon known as counterillumination typically requires fine control over light emission since it needs a luminescence that closely matches the properties of downwelling light (intensity, angular distribution and wavelength). Here we provide evidence that, although lacking complex structures of counterilluminating animals, the deepwater luminescent shark *Etmopterus spinax* could, in Norwegian fjords, efficiently cloak its silhouette from downwelling ambient light to remain hidden from predator and prey. This represents the first experimentally tested function of luminescence in a shark and illustrates how evolution can take different routes to converge on identical complex behavior.

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1. Introduction

Luminescent organisms are able to produce visible light via a chemical reaction. Luminescence's functions in animals are very diverse but mainly fall into intraspecific communication, predation help and predation avoidance (Buck, 1978). Many deepwater sharks (>50 species in 12 genera of two squalid families) harbor thousands of tiny (≈ 0.15 mm in diameter) epidermal photogenic organs called photophores (Compagno et al., 2004). Hypotheses regarding the function(s) of these organs have been proposed by different authors (Hubbs et al., 1967; Reif, 1985; Widder, 1998; Claes and Mallefet, 2008) but have never been tested due to the inherent difficulties of working with deepwater animals in good physiological condition.

The dense ventral photophore coverage and long-lasting glow of luminescent sharks has led to the idea that these fishes produce light to obliterate their silhouettes seen against the downwelling ambient light (Reif, 1985; Widder, 1998; Claes and Mallefet, 2008, 2009a). This camouflage mechanism, known as counterillumination, is documented for midwater organisms including crustaceans, mollusks (squids) and bony fishes that used it to virtually disappear in the water column (Clarke, 1963; Warner et al., 1979; Young et al., 1980). To achieve this illusion, the luminescence should however match the physical characteristics of the downwelling ambient light, i.e. intensity,

angular distribution, and, if the viewer has a color vision, wavelength (Denton et al., 1985), which vary according to the radiating source (sun, moon, or stars) and the depth (Johnsen, 2003). Some counter-illuminating animals have been shown to modulate the physical characteristics of their glow in response to these changes to remain cryptic in the water column (Warner et al., 1979; Young et al., 1980; Young and Mencher, 1980; Latz and Case, 1982; McFall-Ngai and Morin, 1991; Harper and Case, 1999; Jones and Nishiguchi, 2004). Light from the animal needs also to be emitted for extended period of time to make the camouflage efficient; flashes of light are not well adapted for counterillumination (Jones and Nishiguchi, 2004).

Shark photophores are intrinsic, i.e. they do not contain symbiotic luminous bacteria but instead an intrinsic luminous system. They are tiny and simple in structure, lacking the specialized reflectors and filters generally found in photophores of counterilluminating teleosts that allow them to control the angular distribution and wavelength of their luminescence (Hubbs et al., 1967; Denton et al., 1972, 1985). In addition, the luminescence spectrum of the cookie-cutter shark *Isistius brasiliensis* (the only available spectrum from the luminescence of a shark), peaks at a considerably shorter wavelength (455 nm) than that of the sunlight found in the oceanic water where it lives (470–480 nm), suggesting that either the photophores of this shark are not involved in camouflage by counterillumination or not well tuned for this purpose unless this camouflage is directed towards deep-sea organisms lacking specialized color vision (Herring, 1983). However, the recent discovery of a hormonal luminescence control in the velvet belly lantern shark, *Etmopterus spinax* (Linnaeus 1758), fits in with counterillumination

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since it allows a long-lasting light production, which will be operating in a slowly changing or unchanging ambient light (Claes and Mallefet, 2009b).

Here, the lantern shark *E. spinax* was used in order to experimentally test the counterillumination hypothesis in a shark. We investigated the luminescence characteristics of this shark to determine if they comply with the required conditions to match the light characteristics of its environment (the Norwegian fjords in this case). In addition, we tested if this species was able to modulate the intensity of its glow in response to ambient light variations in intensity.

2. Materials and methods

2.1. Lantern shark luminescence

E. spinax specimens were collected at noon in winter by long lines lowered in a deep area (180–250 m) of the Raunefjord and brought to Espeland Marine Station where they were maintained in water tanks placed in a dark cold (6 °C) room where experiments were also performed. All the experiments were done in accordance with the Norwegian law for experimental fish care (fish handling approval #1664).

The physical characteristics of lantern shark luminescence were determined by analyzing the spontaneous glow observed in some sharks immediately after the catch (Fig. 1a).

The spectrum of the glow of three *E. spinax* specimens was measured with the optical fiber (model A976201, core diameter = 0.6 mm, length = 1.5 m with SMA905D connectors at both ends) of a mini-spectrometer (Hamamatsu photonics K. K. TM-VIS/NIR: C10083CA, Hamamatsu-City, Japan; precision = 6 nm) positioned perpendicularly to the photogenic tissue at a distance of 1 mm. As it is generally done, we used the function “Gaussian smoothing” of the spectrometer to present the data with more clarity.

In order to be able to record the intensity of the ventral luminescence of the sharks, we coupled an optical fiber (model FFO3914, International Light Technologies, Peabody, MA, USA; core diameter = 3 mm, length = 0.9 m) to a luminometer (Berthold FB12, Pforzeim, Germany), by placing one extremity into the entrance slit of the luminometer in front of its photomultiplier tube (effective spectral range 300–600 nm), and the other extremity placed towards the surface area of the photogenic tissue (Fig. 1b). The transmission power of the optical fiber was calculated using a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK), which was measured once with the optical fiber, coupled to the luminometer, as well as with the luminometer alone. The ratio between the two measures [130 000 vs. 960 000 relative light units (RLU)] gave us the transmission power of the fiber (14%). RLU were converted into absolute values using our calibrated Beta-light source (emission of 29 megaquanta (Mq) s^{-1} in December 2008).

The luminescence intensity of the shark was measured in different experimental conditions: (i) immediately after catch, to measure the intensity and angular distribution of spontaneous glows, and (ii) after an overhead light stimulation, some days after catch, to test the response of the sharks to light stimuli.

The glow intensity from twenty-one sharks (9 adult males, 9 adult females and 3 sharks born in our tank), was measured by placing the optical fiber perpendicularly to the surface area of the photogenic tissue at a distance of 1 mm. In some animals, the intensity of the glow was monitored for a long period of time (up to 1 h) to test its stability over time.

This optical fiber was then used to generate a polar diagram of the angular distribution of the luminescence in three sharks. The optical fiber was positioned at different positions around the shark (every 10°, Fig. 1b) and the light intensity was measured for 5 s at each position at a distance of 10 cm. For each shark, the values were standardized by the intensity value of the luminescence taken vertically [angle from vertical (θ) = 0°] below the shark (perpendicular to the photogenic tissue)

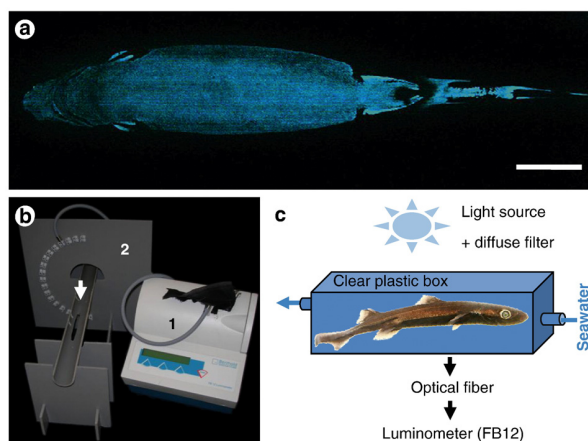


Fig. 1. (a) Spontaneous ventral luminescence in an adult specimen of *E. spinax*. (b) Experimental PVC set-up (1) to measure the angular distribution of *E. spinax* luminescence. Sharks were positioned (belly-up) on a plastic channel (arrow) and luminescence was recorded on a circle around the animal (in a perpendicular plane) by means of clips placed every 10° that were able to hold the optical fiber coupled to the luminometer (2). (c) Experimental set-up to measure the intensity of the luminescence of the sharks in response to light stimuli (overhead illumination of variable intensity). The sharks were positioned in clear water-filled plastic boxes with running seawater and surmounted by a diffuse light source. A 480 nm filter allowed us to restrict the wavelengths of the source in the range of the wavelengths found in the environment of the sharks, while the intensity of the source was modulated by neutral density filters. The luminescence was measured by the optical fiber coupled to the luminometer.

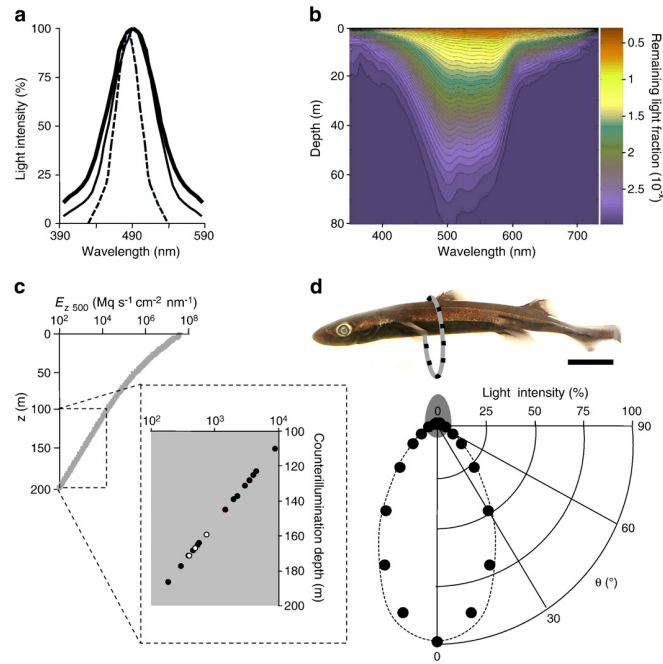


Fig. 2. (a) Luminescence spectrum (Gaussian smoothing) of 3 adult specimens of *E. spinax* (dashed, thin and thick line). (b) Light spectrum according to the depth in the Masfjord (unpublished data given with the courtesy from Stein Kaartvedt and Thor Klejver). Below 80 m wavelengths around 500 nm dominate. (c) Irradiance at 500 nm according to the depth (z) ($E_{z, 500}$) calculated for the Raunefjord. Insert, ventral luminescence intensities of 21 different sharks plotted against the depth at which these intensities are found in the Raunefjord. (d) Polar diagram representing the angular distribution of luminescence just in front of the first dorsal fin. Dots are mean values from 3 adult *E. spinax* specimens (due to low variation between individuals s.e.m. are smaller than dots' size). The dotted line represents the near-asymptotic angular distribution of the light at 66.1 m depth in the Lake Pend Oreille (modified from Tyler, 1960). The light intensity decreases as the angle from vertical (θ) increases. Scale bar, 5 cm.

which always gave the highest luminescence intensity following the method of Harper and Case (1999).

The effect of a light stimulation on shark luminescence was tested after two days of captivity in constant darkness in order to let the sharks recover from the stress from the catch and to obtain behaviors as natural as possible in laboratory conditions. Seven sharks (6 adult sharks and one newborn) were placed in water-filled clear plastic boxes that were approximately of their size and width in order to restrict their movements (Fig. 1c). A diffuse light source (with a filter restricting the wavelength around 480 nm and neutral density filters for intensity modulation) was used and projected on a portion of the room's white ceiling situated about 50 cm above the shark and intensity was recorded with the optical fiber coupled to the luminometer. To quantify downwelling light intensities, the optical fiber was placed directly below the plastic box without the shark present. To measure the luminescence intensity, the optical fiber was placed just beneath the ventral photogenic area of the shark when the overhead light was switched off. This experimental device allowed us (i) to test if an overhead illumination would induce luminescence in the sharks, and (ii) if the sharks would be able to modulate the intensity of their glow in response to variation of overhead light intensity. The overhead light intensities were in the range of spontaneous glow intensities of the

sharks, except for one intensity that was ten times higher than the most intense recording. Each overhead light stimulation (when the light was suddenly switched on) lasted at least 1 h in order to allow the sharks to acclimate themselves to the light regime, and the luminescence measurement was done just after the end of the light stimulation, when the light was suddenly switched off.

2.2. Environmental light

To estimate the physical characteristics of the light environment encountered by the sharks, we used data from three Norwegian fjords situated close to each other: the Masfjord, the Raunefjord and the Samnangerfjord.

The wavelength distribution of the downwelling irradiance at 80 m depth was measured using a hyperspectral radiometer (RAMSES ACC, Trios-optical sensors, Oldenburg, Germany) in the Masfjord (see Aksnes et al., 2009 for method).

The amount of downwelling irradiance in the Raunefjord was calculated by a proxy method. Aksnes et al. (2009, their Eqs. 2 and 3) report the observed relationship between the attenuation of downwelling irradiance at 500 nm [$K_d 500$, in m⁻¹], salinity (S), chlorophyll concentration (C , in mg Chl a m⁻³), and dissolved oxygen concentration

for several western Norwegian fjords in winter time when chlorophyll *a* (Chl*a*) concentrations were low ($<0.6 \text{ mg Chl } a \text{ m}^{-3}$):

$$K_{d500} = 0.62 - 0.017S + 0.16C$$

(for a dissolved oxygen concentration of 5 ml l^{-1})

This relationship was used to obtain an estimate of the attenuation of downwelling irradiance (E_z , in $\text{quanta m}^{-2} \text{ s}^{-1} \text{ nm}^{-1}$) as a function of depth (z) from the salinity and chlorophyll concentrations obtained from *in situ* fluorescence measured using a fluorometer (Seapoint chlorophyll fluorometer, Seapoint Sensors, Inc., Exeter, NH, USA) coupled to a mini CTD (model SD204, SAI/AS, Bergen, Norway) in the Raunefjord on 11th of December 2007 (at the same time and location of the shark catch) according to:

$$E_z = E_0 \exp[-K_{i500}z]$$

where $K_{i500}z$ is the integrated attenuation between the surface and the depth z (ranging from 0 to 200 m). We assumed a surface irradiance (E_0) corresponding to $5 \cdot 10^7 \text{ Mq s}^{-1} \text{ cm}^{-2} \text{ nm}^{-1}$ reflecting the low solar irradiance at wintertime in Raunefjord. This value corresponds to a clear sky noon irradiance as measured by the Geophysical Institute, University of Bergen, Bergen, Norway.

The near asymptotic angular distribution of light in these fjords was considered to be similar to the distribution obtained by Tyler (1960) for the Lake Pend Oreille, since this lake shows a similar ratio of scattering to absorption which is similar to the ratio found in the Samnangerfjord (Hamre et al., 2003). The angular structure of the underwater light field approaches an asymptotic state with increasing depth, and below 1% light depth there are only small changes in the angular distribution (Kirk, 1994). The actual shape of the asymptotic distribution is determined by the ratio of scattering to absorption. At a high ratio the shape of the angular distribution (plotted as a polar diagram such as in Fig. 2d) tends towards a circle, while at a low ratio the polar diagram takes on the form of a narrow pencil (Kirk, 1994). Hamre et al. (2003) measured seasonal variations in absorption and scattering down to 50 m depth in the Samnangerfjord. Their results (their Fig. 1) indicate a transition to absorption ratio close to 2 in the deepest part (40–50 m), which is near the value (2.4) reported for Lake Pend Oreille by Tyler (1960) that is discussed by Kirk (1994, his Figs. 5.11 and 6.12).

2.3. Statistical analysis

The statistical analyses (Student's *t*-test, linear regression) were performed using the software SAS/STAT (STAT Institute Inc., 1990, Cary, NC, USA) and considered to be significant when *P*-value was lower than 0.05.

3. Results

Directly after catch, the vast majority of specimens produced a spontaneous long-lasting (sometimes more than 1 h) luminescence. The physical characteristics of this glow were very similar to those of the ambient light environment encountered by the sharks.

The luminescence spectrum showed a peak at $486 \pm 1 \text{ nm}$ (Fig. 2a), which falls in the small range of wavelengths (around 500 nm) detected at 80 m in the Masfjord (Fig. 2b). The luminescence intensities ($295\text{--}8620 \text{ Mq s}^{-1} \text{ cm}^{-2}$) of tested sharks were stable over time (variations of less than one order of magnitude) and corresponded to E_z between 110 and 186 m in the Raunefjord at time of the catch (Fig. 2c). Adult male and female sharks did not show any significant difference in the intensity of their glow (*t*-test, $P=0.22$), or in the depth at which the intensity of their glow was theoretically capable of matching the ambient light intensity present in the fjord (*t*-test, $P=0.33$).

The angular distribution of *E. spinax* luminescence was also very close to that present in its environment, with a polar diagram showing a near-asymptotic angular distribution slightly narrower than the one found in the lake Pend Oreille, with the majority of the light emitted downward (Fig. 2d).

After two days of captivity, spontaneous luminescence was only observed in two animals and in a transitory manner. Two sharks (an adult and a newborn) responded to an overhead illumination by switching on their luminescence at an intensity level falling into the range of the spontaneous glows recorded the day of the catch (Fig. 3). The five other tested sharks did not luminesce even after different trials with variable light intensities. However, the adult shark that started to produce light was not able to modulate the intensity of its glow in response to an increase in the overhead light intensity (Fig. 3), it continued to emit light at a relatively constant intensity (from 74 to $160 \text{ Mq s}^{-1} \text{ cm}^{-2}$) during the entire experiment (which lasted more than 5 h). No correlation was therefore found between the intensities of shark luminescence and overhead light (linear regression, $r^2=0.07$, $P=0.67$).

4. Discussion

Here we show that in the southern Norwegian fjords velvet belly lantern sharks (*E. spinax*) emit a ventral luminescence whose physical characteristics are very close to those of the light present in their environment. This is the first evidence that sharks could use camouflage by counterillumination.

E. spinax photophores produce a luminescence whose spectrum has a narrow peak which is closer to the wavelength range found in the deep waters of the fjords than the spectral luminescence of the dalatiid shark *I. brasiliensis* (Herring, 1983). The origin of such a difference in the luminescence spectrum of these two shark species is unknown but may be linked to a difference in the luminous substrate of the chemiluminescent reaction as well as to the presence of pigments in the photophore lens that alter the spectral characteristics of luminescence (Denton et al., 1985).

The angular distribution of *E. spinax* luminescence is remarkably similar to the near-asymptotic distribution found in its environment as well as in other counterilluminating animals (Denton et al., 1972; Harper and Case, 1999). This shows that a diffuse pattern of photophores differentially organized around the axis of the animal is another way to achieve an appropriate angular distribution of the light field. A gradual transverse change in the orientation of the photophore axis was already observed by Hickling (1928) in this species.

Luminescence intensities for 500 nm light measured in *E. spinax* fall in the range of downwelling irradiance at 110–186 m deep in its environment. Counterilluminating animals are generally able to

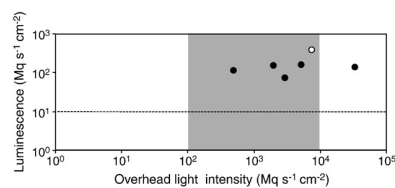


Fig. 3. Relationship between overhead light intensity and *E. spinax* luminescence. The dashed area represents the range of intensities of spontaneous glows measured the day of the catch. The dotted line represents the background level of luminescence found in the sharks two days after the catch. Two sharks switched on their luminescence in response to overhead light. Open symbol, shark 1 (newborn); filled symbols, shark 2 (adult). Shark 2 maintained its luminescence intensity during all the experiment at a relatively constant level, even when the overhead light intensity changed.

modulate the intensity of their luminescence in response to ambient light changes by comparing visually their luminescence output and the ambient light intensity (Warner et al., 1979; Young et al., 1979, 1980; Jones and Nishiguchi, 2004). The temporal stability of *E. spinax* glow intensity after the capture and in response to different light intensities suggests that this animal maintains a diel vertical migration pattern in the Norwegian fjords to stay in a constant light level at a wavelength of 500 nm. This idea is supported by observations of *E. spinax* at very shallow depth (up to 15 m) at night in some Norwegian fjords (Moen and Svensen, 2004; Heidler, 2006). The poor ability of this shark to rapidly adjust its luminescence on a wide range of intensities is probably a consequence of the hormonal control of luminescence present in this species (Claes and Mallefet, 2009b). Indeed, the intensity of prolactin and melatonin-induced luminescence from *E. spinax* photophores does not vary much even if a wide range of concentrations is applied on the tissue (Claes and Mallefet, 2009b). In this study, however, some sharks showed small variations in their luminescence intensity (always less than one order of magnitude). One cannot exclude the possibility that these variations represent temporary adjustments of luminescence intensity that would allow the shark to move in the water column to stay in an isolume. It is likely that this shark uses both the eyes and pineal gland to monitor information on downwelling light during their vertical migrations as it has been already suggested in lantern sharks (Clark and Kristof, 1990) and in other animals including fishes and cephalopods that use camouflage by counterillumination (Young et al., 1979; Jones and Nishiguchi, 2004). In this case the information would be directly transmitted to the photophores by hormonal inputs.

Counterilluminating animals generally use their luminescence to disappear from their predator's sight, and this is probably also the case in *E. spinax*, especially at small size when it is more vulnerable. The ability of newborn individuals to match intensities found in their environment, to switch on their luminescence in response to overhead light illumination, and the high photophore coverage of their ventral surface area (Claes and Mallefet, 2008, 2009a) supports this hypothesis. However, as with almost all sharks, this animal captures its prey with a ventral mouth. A ventral camouflage by counterillumination may therefore double the adaptive advantage in this species since it would make the shark invisible not only to its predator but also to its prey. The main Norwegian preys of *E. spinax* are krill and pearlfish (Klimpel et al., 2003), which are also luminous and undergo diel vertical migrations in Norwegian fjords (Kaartvedt et al., 1988). This also supports the idea that, in Norwegian fjords, this shark is an isolume follower that performs diel migrations in the water column to remain hidden from predator and prey. It remains to be determined, however, if this capability to disappear in a world with no place to hide might also be present in other lantern sharks with ventral photophores or even in other populations of *E. spinax*, such as those present in the Mediterranean Sea, for example, whose biotic (predator and prey) and abiotic (light level, depth, temperature) environments are different.

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PAPER III (complementary results)

Paper III (complementary results)

INTRODUCTION

The velvet belly lantern shark (*Etmopterus spinax*) was shown to possess the requirements for camouflage by counterillumination (**Paper III**). On the other hand, the heterogeneity in photophore density hence luminescence intensity found across different luminous zones, at least in adult specimens, strongly suggest that the luminous pattern of this shark may be more versatile than just restricted to the function of camouflage.

MATERIALS AND METHODS

Sharks were collected by longlines in the Raunefjord (Norway) and brought to Espeland Marine Station where they were housed in seawater tanks placed in a dark cold (6°C) room until use for experimentation. Immediately after capture, some of these sharks were seen to emit a spontaneous long lasting glow. One shark was used to map the angular distribution of luminescence across five different body positions using an optical fiber (model FFO3914, International Light Technologies, Peabody, MA, USA; core diameter=3 mm, length=0.9 m) connected to a luminometer (Berthold FB 12, Pforzeim, Germany) and a PVC device (to restrain the shark and stabilise the fibre optic at a specific angular position) following the method of Claes et al. (**Paper III**). The luminescence intensity from five different luminous zones was also measured using the optical fiber (placed at a distance of 1 mm from the photogenic tissue) in seven different adult individuals and plotted against their photophore density. Since important luminescence intensity variations were observed among the different sharks, luminescence intensity and photophore density were expressed in relative value (value obtained from ventral zone=1). A linear regression was then performed using the software SAS/STAT (STAT Institute Inc, 1990, Cary, NC, USA) and considered significant at the 0.05 level.

In one shark (killed by a blow to the head following local rules for experimental fish care), we dissected out a skin strip (2 cm wide) encompassing the half part (in a plane perpendicular to the fish axis) of the ventral luminous zone, in front of the first dorsal fin. The strip was fixed in formaldehyde and sectioned (10 µm) using a cryo-microtome (in a plane perpendicular to the fish axis). The different sections were then analysed using the image analysing software Image J (National Institutes of Health, Bethesda, MD, USA): (i) the angular position (α) of each photophore encountered was estimated by

measuring its position in the skin strip, whose length was considered to represent 90°; (ii) the photophore axis was determined as the axis passing through the medium of XY, the 2 junction points of the photophore pigmented sheath (s) and the melanophore layer (m), and the intersection of the lines coming from X and Y and encompassing the first lens cell (l); and (iii) the orientation of the photophore axis (β) was determined as the angle between the photophore axis and the line passing through the points Y and Y.

Observations were then performed on captive animals. Luminescence from the dorsal finspines was observed in numerous animals and digitally photographed (Canon D07, Canon inc., Tokyo, Japan).

RESULTS AND DISCUSSION

Although the anterior part of *E. spinax* harbours a spontaneous luminescence whose angular distribution approaching the one found in the Norwegian fjord, this was not the case for the posterior part (tail region) of this shark (**Fig. 1a**). Instead, the luminescence in this region is also emitted laterally and dorsally, certainly due to the presence of the lateral luminous zone in this part of the animal (**Paper II**).

In addition, results confirm the previous observation that the intensity (I) of the luminescence from this shark is directly proportional to the photophore density (PD) of the emitted zone (linear regression: $I=0.724 \text{ PD}$, $R^2=0.711$, $P<0.05$) (**Fig. 1b**).

All these results strongly suggest that luminous zones of the tail part of the shark are not involved in counterillumination, but rather are involved in intra-specific behaviours such as sexual signalling and group swimming/hunting, as it was already suggested (**Paper II**).

The relationship between the angular position of a photophore and the orientation of its axis shows that, in front of the dorsal fin, the morphology of the photophores are gradually changing to maximise downward photic emission, as it was suggested by Hickling (1928). This morphological change allows sharks to emit the angular distribution necessary for counterillumination and other behaviours such as those suggested above without the help of specialised light reflectors in their photophores.

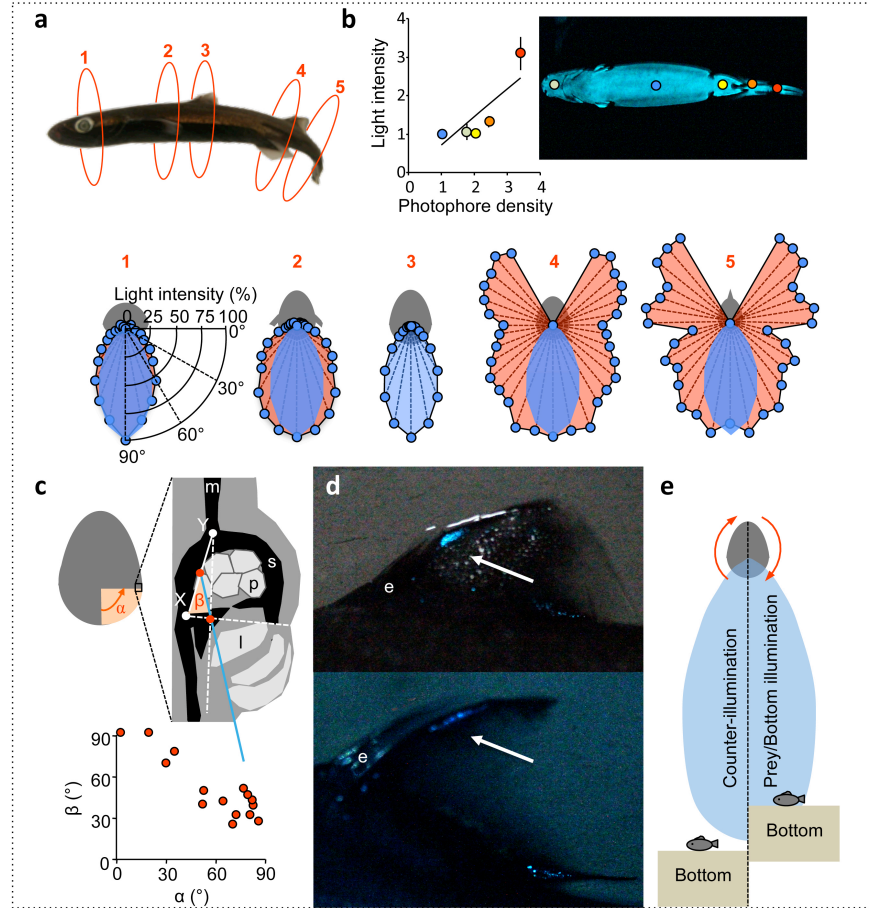


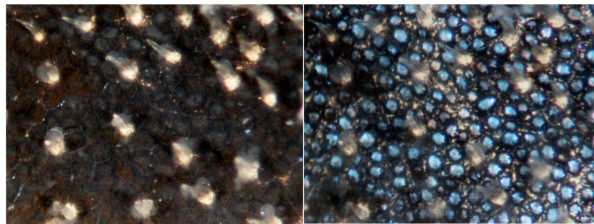
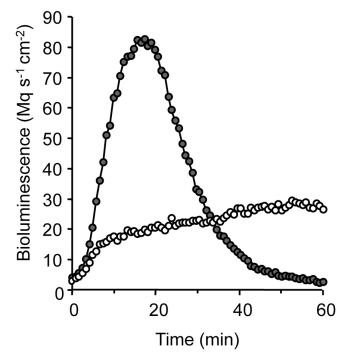
Figure 1 | Versatile luminous pattern of *Etmopterus spinax*. **a**, angular distribution of spontaneous luminescence at different transversal positions across the shark's body (blue shade, angular distribution required for counterillumination; grey shade, transversal section of the shark; red shade, deviation from that distribution). The angular distribution differs strongly from the requirement for counterillumination in the tail region of the shark. **b**, relationship between photophore density and the spontaneous luminescence intensity for five luminous zones of the pattern (each value is a mean obtained on seven different sharks). Coloured dots indicate from which zone were made the measurement (see the picture of the spontaneously luminescent adult). **c**, relationship between the axis orientation (β) the angular position (α) of 15 different photophores across the shark body at position 3 in (a). Upper drawing represents sections of the shark's body (grey shade) and photophore: l, lens cell; m, melanophore layer; p, photocyte; s, pigmented sheath; XY, the 2 junction points between s and m. **d**, Photophores present behind the first (upper picture) and the second (lower picture) dorsal fin spine. **e**, Schematic drawing explaining how the shark could manage to use its luminous pattern in counterillumination and bottom (prey) lightening at once (blue colour, counterillumination pattern, grey colour, transversal section of the shark). The red arrows indicate the transversal movement made by numerous sharks when swimming.

Observation of luminescence just beneath the dorsal finspines of numerous specimens (**Fig. 1d**) strongly suggests that photophore of this region serves to illuminate the spines and warn the potential predators of *E. spinax* from the danger to catch it. Luminous aposematism has been demonstrated in invertebrates (Grober, 1988; De Cock and Matthysen, 2000; 2003), but never in vertebrates. To confirm this hypothesis, we need to demonstrate that dorsal fin photophores fill the requirements for aposematism (Grober, 1989): (i) the shark is not profitable for a food (this first condition is likely achieved by dorsal finspines); (ii) potential predators should show an initial reluctance to attack *E. spinax* (the long lasting glow of the photophore could in a way be a clue that the shark tries to signal permanently the danger of its spines); (iii) potential predators should show rapid avoidance learning of *E. spinax*; (iv) the shark is less eat than a similar non-luminous species; (v) the shark should be able to survive to a predator attack to favour the evolution of the aposematic signal although the aposematic signal can be maintained at the population level.

Direct observations of captive sharks' swimming behaviour also bring the idea that luminescence in *E. spinax* is not restricted to the purpose of camouflage. Sharks were observed to luminesce spontaneously when swimming at the bottom of the tank in the absolute darkness of the cold room. Although it is always very dangerous to speculate natural behaviours from captive animals, this might represent a way for the shark to illuminate the bottom hence its potential prey (benthic animals or carcasses) (**Fig. 1e**; Neiva et al., 2006). Finally, numerous spontaneously luminescent sharks swam with an oscillatory movement (**Fig. 1e**), which may indicate a way for them (due to tail's light angular distribution) to turn their long-lasting glow into serial flashes, performing a cryptic intraspecific luminous signal without being located by their predators.

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Chapter 3: *Hormonal control*



Chapter 3: Hormonal control

This third section focuses on the physiological control of the luminescence from *E. spinax*'s photophores. It is composed of three different papers (**Papers IV-VI**).

Although intrinsic photophores of teleost fishes are under nervous (mainly adrenergic) control (Baguet, 1975; Baguet and Marechal, 1978; Baguet and Christophe, 1983), these organs have been suggested to be hormonal control in sharks due to (i) the slow onset of their luminescence (Ohshima, 1911), (ii) their apparent lack of innervation (Johann, 1899) and (iii) their absence of response (in the cookie-cutter shark *Isistius brasiliensis*) to acetylcholine and adrenaline (Herring and Morin, 1978). In **Paper IV**, we tested this hypothesis by applying classical neurotransmitters (adrenaline, noradrenalin, acetylcholine, 5-HT and GABA) and hormones [melatonin (MT), prolactin (PRL) and α -MSH] on the shark's isolated ventral skin patches containing photophores. Results confirm that, in this species, the luminescence is under hormonal control: MT and PRL induced luminescence, while α -MSH inhibited this light emission. Using specialised antagonists we identified the receptor of the MT, which proved to be the MT₂, and determined, using enzyme inhibitor and second messenger analogues the extrinsic pathways of the two stimulating hormones: MT₂ appears negatively coupled to cAMP, and prolactin receptor (PRLR) appears coupled to Jak2.

It has been suggested that the pigmented iris-like structure present in *E. spinax*'s photophores between photocytes (photogenic cells) and lens cells could act as an iris to regulate the amount of photocyte light emitted to the outside (Ohshima, 1911; Iwai, 1960). This hypothesis was tested in **Paper V** by following photophores under binocular microscope after MT and PRL application. Results confirm the hypothesis and therefore suggest that the luminescence control mechanism of *E. spinax* actually derives from the (shallow water) Elasmobranch color change mechanism that involved the action of the same hormones on pigmented cells (Gelsleichter, 2004).

Since *E. spinax* photophores appear to be involved in more than one function (**Chapter 1 & 2**), the question arises whether functionally different luminous zones present different control mechanism. **Paper IV** showed the photophores from the ventral luminous zones of *E. spinax* to be under hormonal control. In **Paper VI**, MT and PRL were applied on skin patches isolated from other luminous zones of male and female specimens of this species to test (i) if all zones responded similarly to these hormones and (ii) if sexual differences are observed in some luminous zones. Results indicate that all tested zones respond to MT and PRL even if the intensity

II. Results

and the amount of hormonally induced light are directly dependent of the photophore density of the zone. Surprisingly, photophore density-independent differences in luminescence time course were observed among luminous zones and, in luminous zones of the pelvic region, between sexes (for PRL only). All these results suggest that the hormonally induced luminescence is controlled by other actors and support the idea that the luminescence of this shark is used in reproduction.

PAPER IV

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

Claes, J. M., Mallefet, J. (2009)

J. Exp. Biol. **212**, 3684-3692.

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 α -melanocyte-stimulating hormone (α -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: α -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 μ 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), α -melanocyte-stimulating hormone (α -MSH) (which stimulate melanosome dispersion and therefore induce skin darkening after visual stimulation) and melatonin (MT) [which is thought to regulate (through changes in α -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 ⁻²)
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⁻¹ NaCl, 3.2 mmol l⁻¹ KCl, 5 mmol l⁻¹ CaCl₂, 0.6 mmol l⁻¹ MgSO₄, 1.6 mmol l⁻¹ Na₂SO₄, 300 mmol l⁻¹ urea, 150 mmol l⁻¹ trimethylamine N-oxide, 10 mmol l⁻¹ glucose, 6 mmol l⁻¹ NaHCO₃; total osmolality: 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⁻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 (s)]. Light parameters were standardised by skin surface area (in cm²).

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 (10⁻⁴ and 10⁻⁶ for neural and hormonal testing, respectively; Table 2). In addition, potassium chloride (KCl, 200 mmol l⁻¹) 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 ⁻¹)	Source
Extrinsic control			
Adrenaline	Neurotransmitter	10 ⁻³	Sigma Chemical Co. (St Louis, MO, USA)
Noradrenaline	Neurotransmitter	10 ⁻³	Fluka (Buchs, Switzerland)
Carbachol	Neurotransmitter	10 ⁻³	Sigma
5-HT	Neurotransmitter	10 ⁻³	Sigma
GABA	Neurotransmitter	10 ⁻³	Sigma
PRL	Hormone	10 ⁻⁵ –10 ⁻⁸	Sigma
MT	Hormone	10 ⁻⁴ –10 ⁻⁷	Acros Organics (Pittsburgh, PA, USA)
α-MSH	Hormone	10 ⁻⁶ –10 ⁻⁷	Sigma
Luzindole	Antagonist MT1/MT2	10 ⁻⁴	Sigma
4P-PDOT	Antagonist MT2	2 × 10 ⁻⁴ –2 × 10 ⁻⁷	Toocris Bioscience (Ellisville, MO, USA)
Intrinsic control			
diButyryl-cAMP	cAMP analogue (cAMP1)	10 ⁻⁴	Sigma
Forskolin	Adenylyl cyclase activator (cAMP1)	10 ⁻⁴	Sigma
SQ22,536	Adenylyl cyclase inhibitor (cAMP1)	10 ⁻⁵	Sigma
MDL-12,330A	Adenylyl cyclase inhibitor (cAMP1)	10 ⁻⁵	Sigma
1,2,3,4,5,6-Hexabromocyclohexane	JAK2 inhibitor (JAK21)	10 ⁻⁴	Calbiochem Merck (Darmstadt, Germany)

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

shark saline as control. For each drug, we compared the L_{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

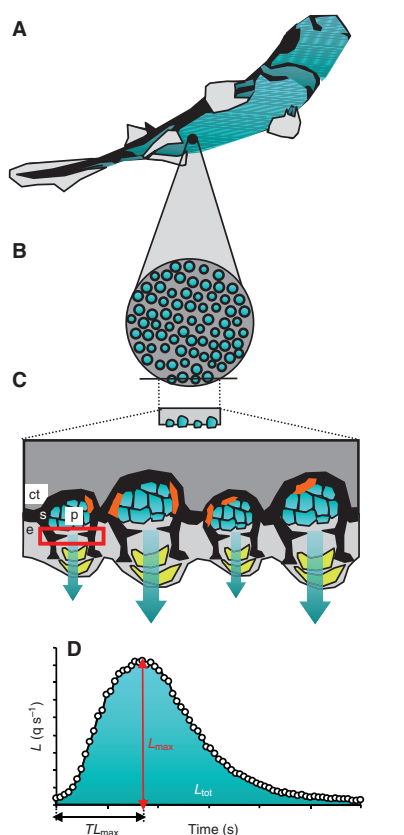


Fig. 1. Photophores and luminescence of *Etmopterus spinax*. (A) Ventral view of the shark with its luminous pattern (blue colour). (B) Ventral skin patch. (C) Structure of glowing photophores. The light (blue arrows) is produced in photocytes, passes through the lens (in yellow) and is finally emitted 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 (qs^{-1}); L_{max} , maximum intensity of light emission; L_{tot} , total quantity of light emitted during the experiment; p, photocyte; s, pigmented sheath; TL_{max} , time from stimulation to L_{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 ($18^{\circ}C$).

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_{tot} was, on average, not significantly ($P > 0.05$) different from the L_{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 α -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_{tot} and L_{max} (Fig. 2B). The dose–light response curve of both hormones showed a similar pattern. The L_{max} progressively increased and attained its highest value at 10^{-6} mol l^{-1} to stay more or less constant at higher concentrations while the TL_{max} decreased when concentration increased (Fig. 3A,B). L_{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_{max} than during the winter months and especially in February (Fig. 4A). However, the TL_{max} stayed constant over the year and corresponded to 40.68 ± 2.95 min ($N=44$) for MT and 18.36 ± 0.73 min ($N=49$) for PRL (Fig. 4B).

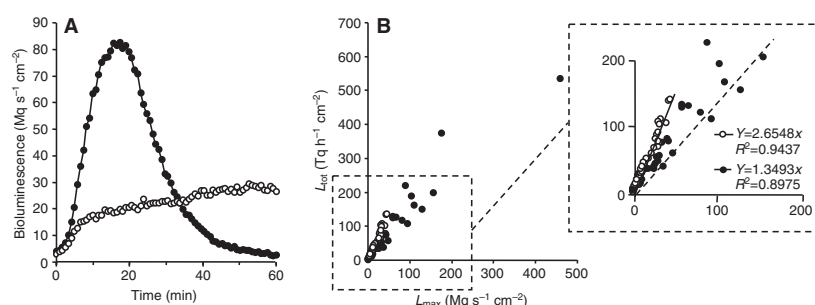


Fig. 2. (A) Original recordings of luminescence induced by melatonin (MT) (10^{-6} mol l⁻¹; open circles) and prolactin (PRL) (10^{-6} mol l⁻¹; closed circles) from ventral skin patches of a mature 44 cm total length female specimen of *Etmopterus spinax*. Data points were recorded every 49 s using a 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_{tot}) for MT (open circles; $N=36$) and PRL (closed circles; $N=41$). For a same $L_{\max}$ value, MT shows a higher L_{tot} . Insert, enlarged view showing the relationship $L_{\max}$ – L_{tot} for lower $L_{\max}$ values of light production obtained with both hormones.

An additive effect for $L_{\max}$ and L_{tot} was obtained with a simultaneous injection of 10^{-6} mol l⁻¹ MT and 10^{-6} mol l⁻¹ 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 α -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⁻¹) and α -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⁻¹. At 10^{-5} mol l⁻¹, no significant effect of α -MSH on the time course of luminescence was detected. At 10^{-7} mol l⁻¹, however, α -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⁻¹ injection of α -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 α -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⁻¹ MT1/MT2 receptor antagonist luzindole and 2×10^{-4} mol l⁻¹ MT2-specific receptor antagonist 4P-PDOT did not induce luminescence *per se*.

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

Intrinsic control

MT and cAMP pathway

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

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

Table 3. Results of the pharmacological screening

	[Drug] (mol l ⁻¹)	N	Duration (min)	L_{tot} saline (Tq h ⁻¹ cm ⁻²)	L_{tot} treatment (Tq h ⁻¹ cm ⁻²)	P-value	Effect
Neural testing							
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	2×10^{-1}	6	10	2.31±0.97	4.37±2.66	0.4922	0
Hormonal testing							
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	+
α -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; α -MSH, α -melatonin-stimulating hormone; L_{tot} , total light emission; Tq h⁻¹ cm⁻², teraquantas per hour per square centimetre.

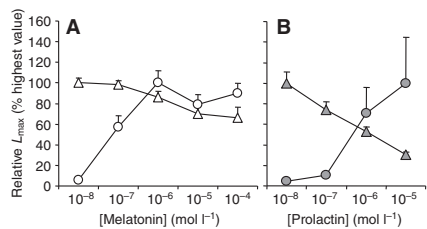


Fig. 3. Dose–light response curve of (A) melatonin and (B) prolactin from ventral skin patches of *Etmopterus spinax*. Values of maximum intensity of light emission ($L_{\max}$) (circles) and time from stimulation to $L_{\max}$ ($TL_{\max}$) (triangles) are expressed as a percentage of the highest value 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 $^{-1}$) 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

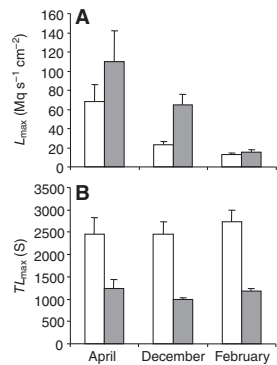


Fig. 4. Comparison of (A) the maximum rate of light emission ($L_{\max}$) produced by ventral skin patches of *Etmopterus spinax* as a response to melatonin (MT) and prolactin (PRL) stimulations as well as (B) the time between stimulation and $L_{\max}$ ($TL_{\max}$) between the different months when 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 $^{-1}$) and PRL (10^{-6} mol l $^{-1}$) 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 $^{-1}$ cm $^{-2}$)	1	1.51±0.52	0.98	0.3588
L_{tot} (Tq h $^{-1}$ cm $^{-2}$)	1	0.92±0.25	−0.31	0.7683
$TL_{\max}$ (min) [†]	1	1.00±0.08	0.04	0.9661
$TL_{\max}$ (min) [‡]	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.
†Student *t*-test performed using $TL_{\max}$ (time between stimulation and maximum intensity light emission) of PRL injected alone as control value.
‡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_{tot} , total light emission; Mq s $^{-1}$ cm $^{-2}$, megaquanta per second per square centimetre; Tq h $^{-1}$ cm $^{-2}$, 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 (α . 150 μ m) 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

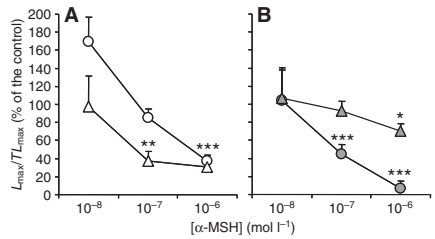


Fig. 5. Dose-dependent inhibitory effect of α -melanocyte-stimulating hormone (α -MSH) on maximal intensity of light emitted ($L_{\max}$; circles) and time between the start of light emission to $L_{\max}$ ($TL_{\max}$; triangles) of (A) melatonin (10^{-6} mol l $^{-1}$) and (B) prolactin (10^{-6} mol l $^{-1}$)-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 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

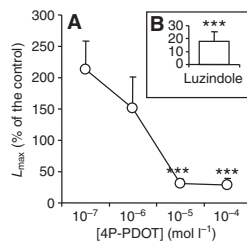


Fig. 6. Effect of antagonists on melatonin-induced luminescence. (A) Dose-dependent inhibitory effect of 4P-PDOT on maximal intensity of light emitted (L_{max} , open circles) from melatonin ($10^{-6} \text{ mol l}^{-1}$)-stimulated ventral skin patches of *Etmopterus spinax*. (B) Effect of luzindole at $10^{-6} \text{ mol l}^{-1}$ 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 α -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

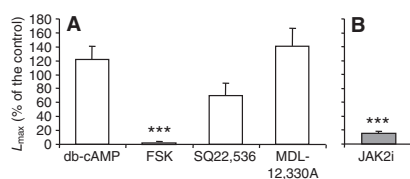


Fig. 7. Intrinsic pathway of melatonin and prolactin in the control of luminescence from ventral photophore of *Etmopterus spinax*. (A) Effect of $10^{-4} \text{ mol l}^{-1}$ dibutyryl-cAMP (db-cAMP), $10^{-4} \text{ mol l}^{-1}$ forskolin (FSK), $10^{-5} \text{ mol l}^{-1}$ SQ22,536 and $10^{-5} \text{ mol l}^{-1}$ 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 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 melatonergic 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
Batrachoididae		
<i>Porichthys myriaster</i> , <i>P. notatus</i>	Nervous (Adr, Nadr, 5-HT [†])	Baguet, 1975; Gariépy and Ancill, 1983
Myctophidae		
<i>Benthosema glaciale</i>	Nervous	Ancill, 1972
<i>Diaphus holli</i>	Nervous	Baguet, 1975; Baguet and Marechal, 1976
<i>Myctophum punctatum</i>	Nervous	Ancill, 1972; Christophe and Baguet, 1982
<i>Triphoturus mexicanus</i>	Nervous	Barnes and Case, 1974
Gonostomatidae		
<i>Gonostoma</i> spp.	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>Argyrolepeus hemigymnus</i>	Nervous (Adr, Nadr)/NO [‡]	Baguet and Marechal, 1978; Krönström et al., 2005
<i>Argyrolepeus offersi</i>	Nervous (Adr)	Bertelsen and Grontved, 1949
<i>Maurolucus muelleri</i>	Nervous (Adr, Nadr)	Baguet and Christophe, 1983
<i>Maurolucus pennanti</i>	Nervous (Adr)	Bertelsen and Grontved, 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, α -MSH [§])	Present study

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

[†]Inhibitory effect.

[‡]Modulatory effect.

Adr, adrenaline; α -MSH, α -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 *Argyrolepeus hemigymnus* (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 Lockett, 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 α -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., 2007) (Falcón et al., 2007), seasonal variation in PRL-induced luminescence may be directly dependent of seasonal variation in plasma MT content.

LIST OF ABBREVIATIONS

α-MSH	α-melanocyte-stimulating hormone
cAMP	cyclic adenosine monophosphate
db-cAMP	dibutylryl cyclic adenosine monophosphate
FSK	forskolin
ILS	iris-like structure
JAK2	janus kinase 2
L	light emission
L _{max}	maximum intensity of light emission
L _{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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PAPER V

The lanternshark's light switch: turning shallow water crypsis into midwater camouflage.

Claes, J. M., Mallefet, J. (in press)

Biol. Lett.

The lantern shark's light switch: turning shallow water crypsis into midwater camouflage

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Bioluminescence is a common feature in the permanent darkness of the deep-sea. In fishes, light is emitted by organs containing either photogenic cells (intrinsic photophores), which are under direct nervous control, or symbiotic luminous bacteria (symbiotic photophores), whose light is controlled by secondary means such as mechanical occlusion or physiological suppression. The intrinsic photophores of the lantern shark *Etmopterus spinax* were recently shown as an exception to this rule since they appear to be under hormonal control. Here, we show that hormones operate what amounts to a unique light switch, by acting on a chromatophore iris, which regulates light emission by pigment translocation. This result strongly suggests that this shark's luminescence control originates from the mechanism for physiological colour change found in shallow water sharks that also involves hormonally controlled chromatophores: the lantern shark would have turned the initial shallow water crypsis mechanism into a midwater luminous camouflage, more efficient in the deep-sea environment.

Keywords: bioluminescence; chromatophore; *Etmopterus spinax*; lantern shark; midwater fish; photophore

1. INTRODUCTION

In the permanent darkness of the deep ocean a surprising diversity of organisms, from bacteria to fishes, generate their own light to find food, communicate or avoid predation (Buck 1978; Haddock *et al.* 2010). In fishes, which are the only luminous vertebrates, this bioluminescence is either self-produced by intrinsic photophores or harnessed from glowing symbiotic bacteria (extrinsic photophores), light sources that have different 'on/off switches'. Whereas photophores are typically under nervous control, fishes regulate their bacterial light by secondary means, either physiologically by controlling the physico-chemical parameters of the bacterial chamber (Haygood 1993), or simply by hiding it with chromatophores, dark shutters or by rotation of the entire bacterial organ into a dark pocket (Herring & Morin 1978; Herring 1985).

Lantern sharks (Etmopteridae), one of the few luminous shark families, were recently demonstrated

as an exception to this rule (Claes & Mallefet 2009a). Although their simple light organs are intrinsic, made of tiny clusters of photogenic cells (photocytes) sheathed in a pigmented layer and topped by one or more lens cells (Hubbs *et al.* 1967), work on the velvet belly lantern shark (*Etmopterus spinax*) demonstrated that, unlike any other known system, their luminescence is under hormonal control (Claes & Mallefet 2009a): prolactin and melatonin trigger the light emission using specific extrinsic and intrinsic pathways while α -MSH inhibited these light emissions.

Since these hormones are also involved in the physiological control of colour changes in elasmobranchs (sharks, skates and rays) whose functional unit is a pigmented cell, the melanophore (Visconti *et al.* 1999; Gelsleichter 2004), it has been postulated that the hormones could act on an iris-like structure present between the photocytes and the lens cells, and therefore that the shark *E. spinax* would control its intrinsic luminescence mechanically (Claes & Mallefet 2009a).

2. MATERIAL AND METHODS

Adult lantern shark (*E. spinax*) specimens were collected by longlines lowered in a deep area (180–250 m) of the Raunefjord (Norway) and brought to Espeland Marine Station where they were maintained in water tanks placed in a dark cold (6°C) room until they were killed by a blow to the head before experimentation took place, following the local rules for experimental fish care.

Using the method of Claes & Mallefet (2009a), we excised photophore-containing skin patches from the sharks and placed them in Perspex chambers filled with a shark saline (292 mM NaCl, 3.2 mM KCl, 5 mM CaCl₂, 0.6 mM MgSO₄, 1.6 mM Na₂SO₄, 300 mM urea, 150 mM trimethylamine N-oxide (TMAO), 10 mM glucose, 6 mM NaHCO₃; total osmolarity: 1.080 mosM; pH 7.7; from Bernal *et al.* 2005).

The hormones prolactin (PRL; Sigma Chemical Co., St Louis, MO, USA) and melatonin (MT; Across Organics, Pittsburgh, PA, USA) were diluted in the saline and applied at a final concentration of 10^{−3} mM (which is known to be the concentration to which the tissue is the most sensitive; Claes & Mallefet 2009a) on the skin patches in separate trials ($n = 12$ for each hormone) to stimulate photophores to glow and a luminometer (Berthold FB12, Berthold Technologies, Pforzheim, Germany) was used to record the time course of the light emission during 45 min. Concomitantly, similar hormone-stimulated photophore preparations were digitally photographed (Canon D07, Canon, Tokyo, Japan) under a binocular microscope (Leitz Diaplan, Oberkochen, Germany) to investigate possible morphological changes of the chromatophores in the superficial layer of the skin; the apparent photogenic surface area i.e. the 'transparency index' of six different photophores was measured using the image-analysing software Image J (National Institutes of Health, Bethesda, MD, USA) over the course of each post-stimulation trial.

3. RESULTS

Immediately after application, both hormones always induced light emission from the photophores in the investigated lantern shark specimens. The two hormones, however, showed two different luminescence time courses (figure 1a), similar to those described by Claes & Mallefet (2009a).

Concomitantly, both hormones also provoked an iris-like structure of the pigmented layer that revealed the glowing underlying photocytes. The 'transparency index' of the photophores varied according to the hormone, showing general patterns of evolution similar to the time course patterns of hormonal-induced luminescence curves, although luminescence intensity peaked before transparency index (figure 1b). Whereas pre-stimulation photophores appeared as dark blotches,

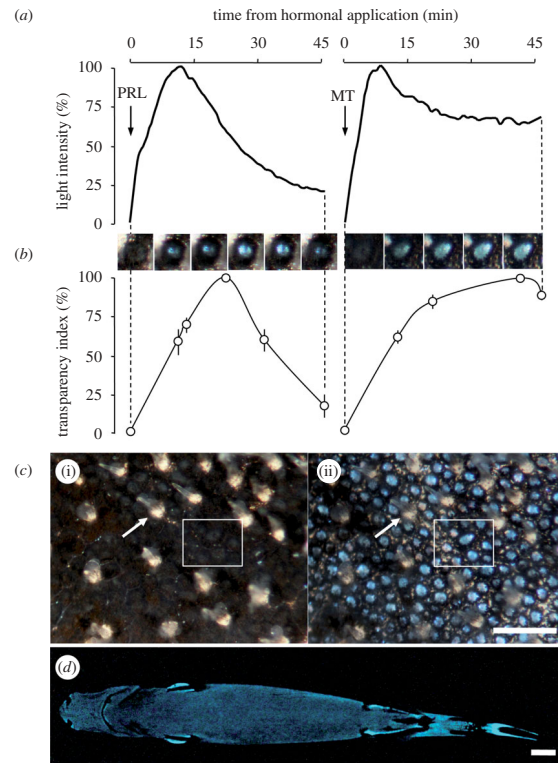


Figure 1. Mechanism for luminescence control in *Etmopterus spinax*. (a) Photophore luminescence recordings after melatonin (MT) and prolactin (PRL) application (arrows). (b) Evolution of the 'transparency index' of photophores (dots, mean data points calculated on six different photophores) after hormonal stimulation. Serial pictures (top) illustrate individual photophore evolution after MT and PRL stimulation. (c) Photophore-containing skin patch before hormonal stimulation (i) and at luminescence peak (ii). The same spiny dermal denticle (white arrow) and group of photophores (white box) are represented in both panels. Scale bar, 1 mm. (d) Ventral luminous pattern of an adult *E. spinax* specimen. Scale bar, 2 cm.

they were black rings surrounding luminescent bluish photocytes at maximum opening, due to the pigment translocation occurring in chromatophores (figure 1c). The photophores are small (about 150 μm wide) and yet because of their high density, formed homogeneous luminous areas at greater distance (figure 1d).

4. DISCUSSION

The 'irising' feature of lantern shark photophores is to our knowledge a unique mechanism of light regulation among luminescent animals and the only one known to be under hormonal control (Claes & Mallefet 2009a). The use of occlusive structures to control luminescence was believed to be restricted to fishes with continuously glowing bacterial organs (Herring 1985; Haygood 1993; Claes & Mallefet 2009a). In the case of *E. spinax*, however, the discrepancy between the highest values of luminescence intensity and

'transparency index' suggests that hormones also stimulate photocytes in addition to provoking pigment retraction in melanophores. The lantern shark mechanism illustrates the range of physiological and morphological means by which diverse taxa have separately arrived at bioluminescent capability.

Bioluminescence is a soft-tissue phenomenon that leaves no fossil track; we therefore rely on extant taxa for clues to evolutionary pathways (Johnsen *et al.* 1999). Our data provide the valuable suggestion that this lantern shark's luminescence is the result of a co-opting of existing skin structures. The hormonally controlled retraction/expansion capabilities of elasmobranch melanophores (Gelsleichter 2004) provided the basis for the control mechanism of lantern shark photophores.

Skin physiological colour change has been found in shallow water elasmobranch species, which use this adaptation to remain cryptic against variable backgrounds and defeat visual detection by prey and

predators (Gelsleichter 2004). On the other hand, *E. spinax*'s extended ventral pattern of photophores radiating a long lasting blue light suggests that this shark uses its luminescence to cloak its silhouette from below (Claes & Mallefet 2008, 2009b), a camouflage mechanism known as counter-illumination that is found in numerous other midwater organisms including crustaceans, molluscs (squids) and bony fishes (Clarke 1963; Warner *et al.* 1979; Young *et al.* 1980). We therefore suggest that this species turned the initial shallow water crypsis mechanism into a midwater bioluminescent camouflage, more efficient in the darkness of the deep-sea. This functional transition might have occurred during colonization of the deep-sea by lantern sharks during the late Cretaceous (Adnet & Capetta 2001).

Research was carried out under approval from the Norwegian Animal Research Authority (NARA) and under University of Bergen (UiB)/High Technology Centre in Bergen (HiB)/Espeland Marine Station ethics guidelines.

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PAPER VI

Functional physiology of lantern shark (*Etmopterus spinax*) luminescent pattern:
differential hormonal regulation of luminous zones.

Claes, J. M., Mallefet, J. (in press)

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Functional physiology of lantern shark (*Etmopterus spinax*) luminescent pattern: differential hormonal regulation of luminous zones

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SUMMARY

Lantern sharks are small deep-sea sharks that harbour complex species-specific luminescent photophore patterns. The luminescent pattern of one of these sharks, *Etmopterus spinax*, is made up of nine luminous zones. Previous experiments revealed that in the largest of these zones (ventral zone), photophores are under hormonal control, light being triggered by both melatonin (MT) and prolactin (PRL). In this study, we analysed the luminescent responses to MT and PRL in five other luminous zones from twelve female and eight male *E. spinax* specimens. The results showed that all luminous zones respond to both hormones, with each zone having its own kinetic parameters (maximum light intensity, $L_{\max}$; total light emitted, L_{tot} ; time from stimulation to $L_{\max}$, $TL_{\max}$), which confirms the multifunctional character of this shark's luminescence. L_{tot} and $L_{\max}$ were found to be directly dependent on the photophore density (PD) of the luminous zone, while $TL_{\max}$ varied independently from PD. In addition, we demonstrated a sexual dimorphism in the luminescent response to PRL, with male specimens having smaller L_{tot} and $TL_{\max}$ in the luminous zones from the pelvic region. As this region also harbours the sexual organs of this species, this strongly suggests a role for the luminescence from these zones in reproduction.

Key words: bioluminescence, Chondrichthyes, melatonin, photophores, prolactin, sexual dimorphism.

Running title: Lantern shark luminous pattern physiology

INTRODUCTION

A vast majority of deep-sea organisms rely on luminescence for numerous functions including predation avoidance, predation help, and intraspecific communication (Buck, 1978; Wilson and Hastings, 1998; Herring, 2007). The possession of photogenic organs (= photophores) is a common feature in many of them including crustaceans, molluscs and fishes (Haddock et al., 2010). These photophores are divided into two types: extrinsic photophores, which shelter symbiotic luminous

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bacteria, and intrinsic photophores, which are self-luminescent, containing an endogenous luminescent system (Herring, 1982). To bring an adaptive advantage to their owner, photophores should be precisely controlled since the efficiency of luminescent behaviours depends on the timing and the physical characteristics of luminescence (Warner et al., 1979; Denton et al., 1985; Branham and Greenfield, 1996; Harper and Case, 1999; Demary et al., 2006; Rivers and Morin, 2008).

A variety of luminescence control mechanisms exist in fishes (for a review see Herring and Morin, 1978; Herring, 1982; Claes and Mallefet, 2009a), which are the only luminous vertebrates. Photogenic organs with bacterial symbionts in bony fishes are controlled mechanically via mobile dark shutters, rotation into a dark pocket or chromatophores, and/or physiologically, by controlling the physicochemical characteristics (osmolarity, glucose, O₂ availability, etc.) of the bacterial growth medium (Bertelsen, 1951; Herring, 1978; Herring, 1982; Haygood, 1993; Munk, 1999; **Fig. 1A**). By contrast, intrinsic photophores are under neural control (mostly by adrenergic nerves) in bony fishes but appear to be under hormonal control in Chondrichthyes (Baguet, 1975; Baguet and Marechal, 1978; Baguet and Christophe, 1983; Claes and Mallefet, 2009a; **Fig. 1B,C**). In addition to these “luminescence switches”, accessory optical structures (including reflectors, light guides, optical filters and lenses) are often present and allow a physical modulation of the light emitted by photophores (Herring and Morin, 1978; Munk and Bertelsen, 1980; Herring, 1982; Denton et al., 1985).

The velvet belly lantern shark, *Etmopterus spinax* (Linnaeus, 1758), is a small deep-sea shark endowed with numerous photophores forming a complex luminous pattern organised in nine different types of luminous zones (Claes and Mallefet, 2008; **Fig. 2A,B**). Recent studies suggested that this pattern is involved in camouflage by counterillumination as well as in intraspecific behaviours such as cooperative swimming/hunting and even sexual signalling (Claes and Mallefet, 2008; Claes and Mallefet, 2009b).

Luminescence from the ventral luminous zone of *E. spinax* is controlled by hormones involved in the physiological control mechanism of Elasmobranch (sharks and rays) skin pigmentation: melatonin (MT) and prolactin (PRL), which trigger light emission, as well as α -melanocyte stimulating hormone (α -MSH) whose application prevent MT and PRL-induced luminescence (Claes and Mallefet, 2009a; in press). Since the *E. spinax* luminescent pattern is believed to be involved in more than one function (Claes and Mallefet, 2008, 2009b), the question arises whether functionally different luminous zones present different control mechanisms.

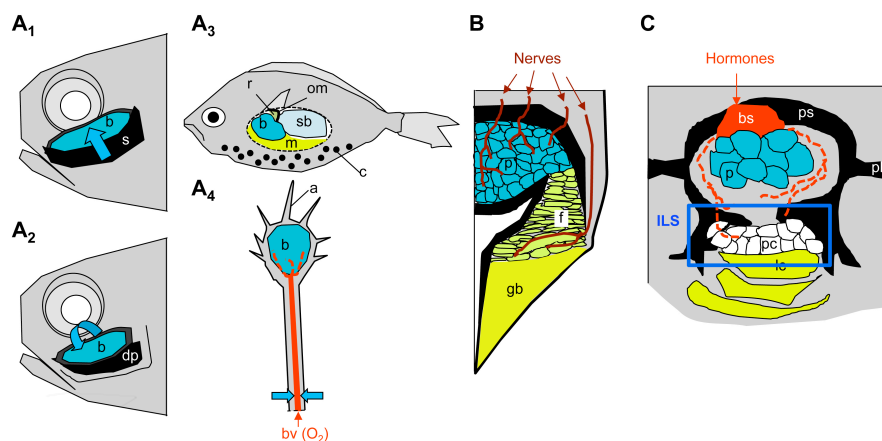


Figure 1 | Luminescence control mechanisms in fishes. (A) Symbiotic luminescence (bony fishes). (A₁) The luminescence from the bacterial photogenic organ (b) of *Photoblepharon palpebratus* is controlled by the movement (blue arrow) of a dark shutter (s), which acts as an inverse eyelid (modified from Howland et al., 1992). (A₂) The luminescence from the bacterial photogenic organ (b) of *Anomalops katoptron* is controlled by the movement (blue arrow) of the whole photogenic organ, which can rotate downward into a dark pocket (dp) (modified from Howland et al., 1992). (A₃) Light organ system of a leiognathid (modified from Haneda and Tsuji, 1976). The bacterial photogenic organ (b) opens laterally into the oesophagus and its light emission is transmitted to the ventral surface by a translucent muscle (m). Expansion of chromatophores (c) on the ventral surface prevents the light to be emitted to the outside. (A₄) A modulation (blue arrows) of the blood supply to the bacterial photogenic esca (section) of a ceratioid anglerfish could allow a control of its luminescence by varying the amount of oxygen (O₂) available for the chemiluminescent reaction (Bertelsen, 1951; Munk, 1999). (B) Photophore section of the hatchetfish *Argyropelecus hemigymnus* [Intrinsic luminescence (bony fishes)]. As other luminous bony fishes endowed with intrinsic photophores, *A. hemigymnus* exhibits a neurally controlled luminescence (modified from Krönström et al., 2005). (C) Photophore section of the lantern shark *Etmopterus spinax* [Intrinsic luminescence (cartilaginous fishes)]. The photophore luminescence from *E. spinax* is hormonally controlled (modified from Claes and Mallefet, 2009a), via movement of pigments inside the pigmented cells (pc) composing the iris-like structure (ILS, blue rectangle). Dashed red lines represent hypothetic movement of the blood inside the photogenic organ. The luminous tissue is represented in blue colour. a, appendage; bv, blood vessel; bs, blood sinus; f, filter; gb, gelatinous body; lc, lens cell; om, opaque membrane; p, photocyte; pl, pigmented layer; ps, pigmented sheath; r, guanine reflector; s, swimbladder.

Through MT and PRL application on different luminous zones composing the pattern of the velvet belly lantern shark, *E. spinax*, the specific goals of this study were (i) to determine if all the luminous zones respond to hormonal application, and if it is the case then (ii) to show if different hormonal luminescent responses are observable among the different luminous zones, (iii) to highlight a potential sexual

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dimorphism in the luminescent responses to hormones, which would be a clue for the existence of sexual signalling, (iv) to investigate the effect of photophore density on the luminescence kinetic parameters, and (v) to discuss the results in relation to the functional ecology of this shark species.

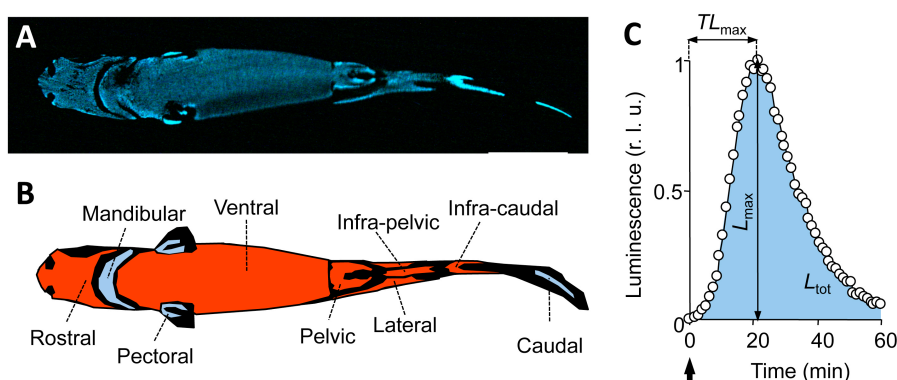


Figure 2 I (A) Ventral view of a spontaneously glowing specimen of the lantern shark *Etmopterus spinax* showing the heterogeneity of luminescence intensity present among the luminous zones composing the luminescent pattern. (B) Schematic drawing of the ventral part of *E. spinax* showing the different luminous zones composing the luminescent pattern of this shark with their associated nomenclature (Claes and Mallefet, 2008). Zones tested in this study are presented in red colour while others are presented in blue colour. (C) Typical luminescence emission curve [in relative units, (r. l. u.)] with associated light parameters: L_{max} , maximum intensity of light emission; L_{tot} , total quantity of light emitted during a given period; TL_{max} , time from stimulation to L_{max} . Scale bars=5 cm.

MATERIAL AND METHODS

Experimental animals

Twelve adult female and eight adult male living specimens of the velvet belly lantern shark *Etmopterus spinax* [30-51 cm total length (TL)] were caught by longlines lowered in a deep (≥ 200 m) area of the Raunefjord (Norway) during two field sessions (December 2008 and February 2009), and transferred to Espeland Marine Station (Espesrend, Norway) where they were kept in two 1 m x 1 m x 1 m tanks placed in a dark cold room (6°C) until use. Captive animals were sacrificed by a blow to the head before the start of experimentation, following the local rules for experimental fish care.

Photophore preparations

In this work, photophore preparations consisted of standard (diameter=0.55 cm) circular skin patches dissected out from *E. spinax* specimens [following the method of Claes and Mallefet (2009a)]. Since we wanted to test the effects of MT and PRL separately, we selected all the luminous zones that were large enough to give two separate but similar patches, which correspond to six out of the nine zones described initially (Claes and Mallefet, 2008; **Fig. 2B**).

After dissection, skin patches were placed in a shark saline [292 mM NaCl, 3.2 mM KCl, 5 mM CaCl₂, 0.6 MgSO₄, 1.6 mM Na₂SO₄, 300 mM urea, 150 mM trimethylamine N-oxide, 10 mM glucose, 6 mM NaHCO₃; total osmolarity: 1.080 mosM; pH 7.7 (Bernal et al., 2005)]. For each shark, photophore densities present in the six investigated luminous zones was estimated using a binocular microscope (Leitz Diaplan, Oberkochen, Germany) by counting the number of photophores present in one of the two patches and by dividing this number by its surface area (0.2375 cm²). Skin patches were then stored in saline at 6°C until use (for less than two hours; maximum survival time of skin patches = more than 12 hours).

Luminometry

The same procedure was applied for each luminous zone (LZ) of the 20 *E. spinax* specimens. Skin patches were transferred to a 96 wells microplate (each well containing 100 µl saline-filled holes) from a luminometer (Berthold Orion; Pforzheim, Germany) calibrated with a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK). These patches were stimulated by an application of either MT (Tocris Bioscience, Ellisville, MO, USA) or PRL (Sigma, St Louis, MO, USA) at a final concentration of 10⁻⁶ mol l⁻¹, which is the concentration eliciting the highest luminescent response in the ventral zone i.e. the concentration to which the luminous tissue is the most sensitive (Claes and Mallefet, 2009a). The experimental procedure was performed at room temperature (18°C).

The luminescent responses were recorded for 1 hour after the stimulation and were characterised using different kinetic parameters (**Fig. 2C**): 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 (in cm²). In order to compare the luminous response of six different luminous zones to the two hormones, a total of 12 skin patches were dissected out from each animal and stimulated simultaneously.

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Then, according to the number of experiments run at the same time in the luminometer, Berthold Simplicity software parameters were adjusted in order to obtain at least one point every minute.

Statistical analysis

Knowing that $L_{\max}$ and L_{tot} parameters of luminescent responses to MT and PRL vary according to the period of the year (Claes and Mallefet, 2009a), and since our specimens were performed at two different times (February and June), a standardisation was made in each specimen by dividing the values obtained for these two parameters in the different luminous zones by value of $L_{\max}$ and L_{tot} of the luminescent response from the ventral zone (which is the one with the smallest photophore density). In order to perform coherent comparisons, the photophore density of skin patches was also standardised using ventral density as reference. No such standardisation was applied for $TL_{\max}$, which was found to be constant (for both hormones) over the year (Claes and Mallefet, 2009a). Control Student t -tests were performed between sexes for (absolute) luminescence parameters and photophore density in the ventral luminous zone.

Two-way analyses of the variance (ANOVAs) were performed to determine if the effect of sex and the luminous zone on the three different luminescence parameters and the photophore density. If sexual difference was detected, each luminous zone was investigated for sexual differences by using Student t -test. In addition, a linear regression was performed to identify a potential link, across the different luminous zones, between the mean values of the light parameters of hormonal-induced luminescence and the mean photophore density.

Statistical analyses (ANOVAs, Student's t -tests and linear regressions) were performed with the software SAS/STAT (STAT Institute Inc, 1990, Cary, NC, USA). Each mean value is expressed with its standard error (mean $\pm$ S.E.M.) and " N " equals the number of skin patches used for a specific treatment (which corresponds to the number of shark specimens tested as one skin patch from each individual was used in each treatment).

RESULTS

Control Student t -test showed that the ventral luminous zone (LZ) of male and female *E. spinax* specimens used in this study are rather uniform in terms of their photophore density (PD) and their MT-induced luminescence kinetic parameters

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(**Table 1**). Sexual differences were, however, observed for the luminescence induced by PRL; females showed higher $L_{\max}$ and L_{tot} than males (**Table 1**). This difference might reflect the fact that males and females were not tested at the same period of the year (all females were tested in February while 6 out of the 8 males were tested in June) since seasonal variations in the $L_{\max}$ (and L_{tot}) of hormonally-induced luminescence has already been detected in this species (Claes and Mallefet, 2009a). This seasonal variation in $L_{\max}$ (and L_{tot}) confirms the need, for these two light parameters, to use the standardisation detailed above in the following analyses.

Table 1 Hormonal-induced luminescence parameters ($L_{\max}$, L_{tot} , and $TL_{\max}$) and photophore density for the ventral luminous zone of male and female specimens of *Etmopterus spinax*

	Males (N=8)	Females (N=12)	Student's <i>t</i> -test	
			<i>t</i> -value	<i>P</i> -value
Melatonin				
$L_{\max}$	15.28±3.44	22.74±3.05	1.24	0.2293
L_{tot}	40.32±6.96	58.16±12.41	1.02	0.3218
$TL_{\max}$	45.61±6.49	31.17±6.75	-1.47	0.1594
Prolactin				
$L_{\max}$	10.04±3.43	50.31±13.41	2.91	0.0126
L_{tot}	14.41±4.56	83.34±22.21	3.04	0.0103
$TL_{\max}$	14.01±1.61	20.15±3.29	1.68	0.1667
PD	3152±318	2614±147	-1.72	0.1034

Bold values indicate significant sexual differences.

Units for the different variables : min ($TL_{\max}$); $\text{Mq s}^{-1} \text{cm}^{-2}$ ($L_{\max}$); Tq h^{-1} (L_{tot}); units cm^{-2} (PD)

Two-way ANOVA revealed significant difference in PD among the different luminous zones but failed to show any effect of the sex on this variable (**Table 2**).

Melatonin

Application of MT always evoked light emission in all the skin patches from the different tested luminous zones. Two-way ANOVAs detected highly significant differences ($P < 0.001$) among the light parameters of luminescence from these luminous zones (**Table 2**). These ANOVAs failed, however, to detect any sexual effect on the MT-induced luminescence (**Table 2**).

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Table 2 Results of two ways-ANOVAs performed on the hormonally-induced luminescence parameters

	<i>df</i>	<i>F</i> -value	<i>P</i> -value
PD			
LZ	5	88.98	<0.0001
Sexe	1	1.25	0.2668
LZ*Sex	5	2.06	0.0756
Melatonin			
<i>L</i> _{max}			
LZ	5	7.29	<0.0001
Sexe	1	0.75	0.3900
LZ x Sex	5	0.31	0.9032
<i>L</i> _{tot}			
LZ	5	10.17	<0.0001
Sexe	1	0.10	0.7528
LZ x Sex	5	0.52	0.7584
<i>TL</i> _{max}			
LZ	5	5.66	0.0001
Sexe	1	3.14	0.0792
LZ x Sex	5	1.19	0.3193
Prolactin			
<i>L</i> _{max}			
LZ	5	2.36	0.0452
Sexe	1	0.12	0.7347
LZ x Sex	5	1.13	0.3500
<i>L</i> _{tot}			
LZ	5	3.38	0.0070
Sexe	1	23.06	<0.0001
LZ x Sex	5	2.34	0.0465
<i>TL</i> _{max}			
LZ	5	4.67	0.0007
Sexe	1	9.97	0.0021
LZ x Sex	5	0.79	0.5599

PD, photophore density; LZ, luminous zones. Bold values indicate significant relationships.

Linear regression analysis revealed a significant relationship between PD and the kinetic parameters *L*_{max} and *L*_{tot} of the MT-induced luminescence (**Fig. 3A, B; Table 3**). No relationship was found between PD and *TL*_{max} (**Fig. 3C; Table 3**).

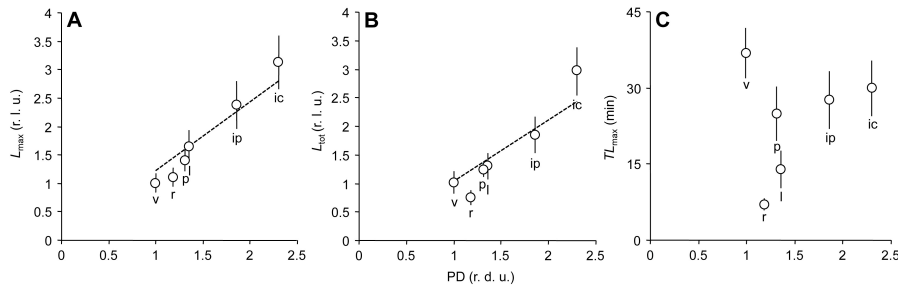


Figure 3 I Relationship between photophore density [PD, in relative density units (r. d. u.)] and light parameters of melatonin-induced luminescence from different luminous zones of *Etmopterus spinax* luminescent pattern: maximum intensity of luminescence (A), total light emitted during the experiment (B), time from stimulation to $L_{\max}$ (C). Each value is associated with its S.E.M. and $L_{\max}$ and L_{tot} values are presented in relative light units (r. l. u.). For the three light parameters, two-ways ANOVAs showed significant differences among the luminous zones but not among sexes (see **Table 2). Dashed lines represent regression lines of the data. Results of the regressions are available in Table 3. Number of replicates: $N=20$ per luminous zone. ic, infra-caudal; ip, infra-pelvic; l, lateral; p, pelvic; r, rostral, v, ventral.**

Table 3 Relationship between the hormonally-induced luminescence parameters ($L_{\max}$, L_{tot} and $TL_{\max}$) and photophore density among the different luminous zones of *Etmopterus spinax*

	<i>N</i>	Intercept	Slope	r^2	<i>P</i> -value
Melatonin					
$L_{\max}$	6	0*	1.22±0.17	0.9852	<0.0001
L_{tot}	6	0*	1.06±0.23	0.9650	<0.0001
$TL_{\max}$	6	15.34±17.19	5.35±30.44	0.0561	0.6514
Prolactin					
$L_{\max}$	6	0*	1.15±0.34	0.9378	0.0003
L_{tot}	9	0*	0.56±0.24	0.7733	0.0008
$TL_{\max}$	8	14.13±14.84	-2.78±9.80	0.0744	0.5133

*These curves do not have an intercept because no light can be emitted when no photophores (photophore density = 0) are present in the tissue.

Bold values indicate significant relationships.

Prolactin

Application of PRL always evoked light emission in all the skin patches from the different tested luminous zones. Two-ways ANOVAs detected significant differences ($P<0.05$) among the luminescence kinetic parameters of these luminous zones as well as sexual differences in L_{tot} and $TL_{\max}$ (**Table 2**).

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Student t -tests confirmed sexual differences in L_{tot} in lateral ($t_{12,3}=4.89$, $P=0.0003$), pelvic ($t_{11,3}=3.90$, $P<0.01$) and infra-pelvic ($t_{11,5}=3.39$, $P<0.01$) luminous zones, and sexual differences in TL_{max} in lateral ($t_{17,3}=3.31$, $P<0.01$) and pelvic ($t_{12,8}=2.82$, $P<0.05$) luminous zones. Linear regression analysis revealed a significant relationship between PD and the kinetic parameters L_{max} and L_{tot} of the PRL-induced luminescence (**Fig. 4A,B; Table 3**). No relationship was found between PD and TL_{max} (**Fig. 4C; Table 3**).

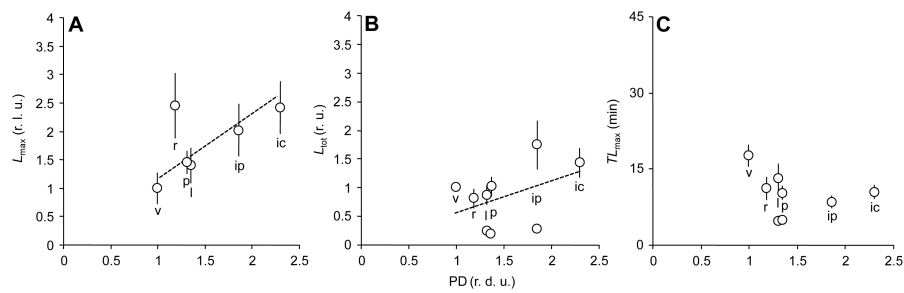


Figure 4 I Relationship between photophore density [PD, in relative density units (r. d. u.)] and light parameters of prolactin-induced luminescence from different luminous zones of *Etmopterus spinax* luminescent pattern. Categories, values, regression lines, source and abbreviations as in **Fig. 3**. Two-ways ANOVAs showed a significant difference among the luminous zones in the three light parameters but sexual differences were only found in L_{tot} and TL_{max} (see **Table 2**). Student t -test show that females have significantly higher L_{tot} for lateral and pelvic luminous zones ($P<0.05$) and significantly higher TL_{max} for lateral, pelvic and infra-pelvic luminous zones. Number of replicates: $N=20$ per luminous zone except in sexually dimorphic zones where $N=12$ for females and $N=8$ for males.

Standardised mean profiles of MT and PRL-induced luminescence time courses from the different luminous zones investigated in this study (**Fig. 5**) clearly demonstrate that luminous zones respond differently to prolactin and melatonin. While no difference was detected between male and female *E. spinax* specimens in response to MT, some zones (lateral, pelvic and infra-pelvic zones) showed sexual differences in response to PRL, with male sharks showing proportionally quicker light responses than females for these zones.

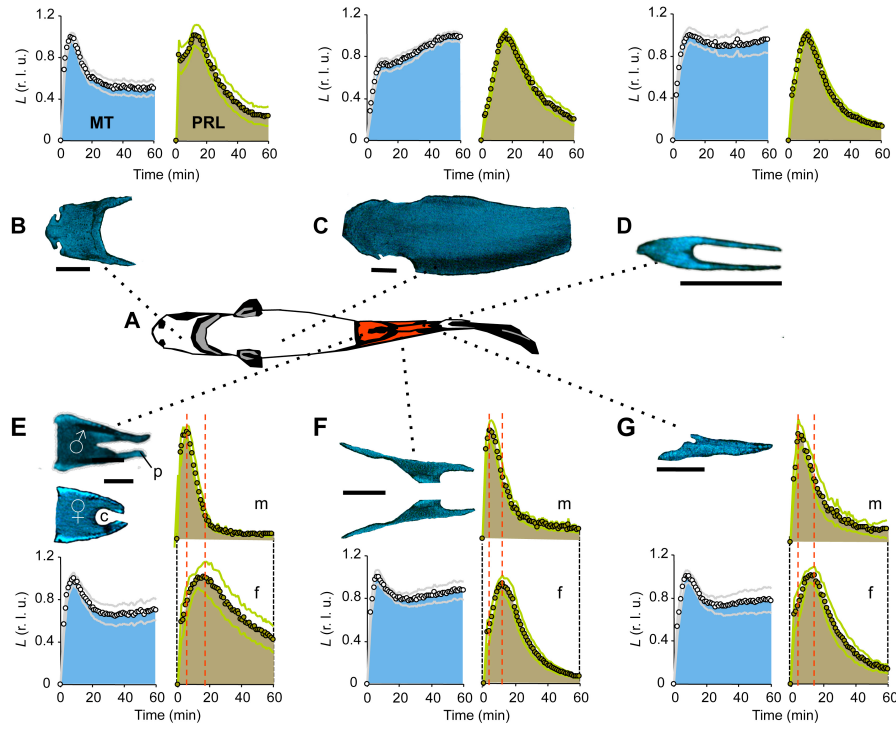


Figure 5 | Time course of luminescence (L) induced by MT and PRL for different luminous zones of *Etmopterus spinax*. Standardised luminescence curves [in relative units (r. l. u.), since luminescence curve of each single shark was standardised in a way that $L_{\max}$ value = 1] from luminescent pattern after application (time = 0) of MT (left (blue) panel of each pair) or PRL (right (brown) panel of each pair). Grey and green lines represent error limits. (A) Schematic drawing of the ventral part of *E. spinax* showing the different luminous zones of the luminescent pattern of this shark: black colour, non-luminous tissue; grey colour, zones that were not tested; red colour, zones that react differently according to the sex after application of PRL; white colour, zones that did not show such a dimorphism in luminescence response. (B) Rostral luminous zone. (C) Ventral luminous zone. (D) Infra-caudal luminous zone. (E) Pelvic luminous zone. (F) Lateral luminous zone. (G) Infra-pelvic luminous zone. Dashed vertical red lines in E-G allow to compare the sexual difference in $TL_{\max}$ observable in these zones in male (m) and female (f) specimens. Scale bars=2 cm.

DISCUSSION

Physiology

The luminescent pattern of the lantern shark *E. spinax* is complex and appears to be involved in different functions (Reif, 1985; Claes and Mallefet 2008; 2009a;b). Since bioluminescent behaviours generally imply kinetic differences in light emission or even do not occur at the same time, we asked if the different functional areas of *E. spinax* share a similar or different luminescence control mechanisms? A recent study showed that luminescence from the ventral luminous zone of this shark may be triggered by MT and PRL (Claes and Mallefet, 2009a). If other luminous zones of this shark are controlled by hormones as well, another question arises: how does a signalling molecule that diffuses in the blood throughout all part of the organism efficiently control a multifunctional luminescent pattern such as the one of *E. spinax*?

In this study we showed that all luminous zones investigated so far respond to application of either MT or PRL by glows of variable time courses [all other zones of the luminous pattern also responded to both hormones but their small size prevent them from being tested quantitatively (Claes and Mallefet, unpublished data)]. The observed difference in the light parameters $L_{\max}$ and L_{tot} among the different luminous zones certainly reflects the difference in PD present in this zone. Not surprisingly zones with higher photophore densities emitting more light than others after hormonal application. This relationship is quite simple to understand: when MT- or PRL-containing blood reaches a specific luminous zone, all the photophores present in this zone are stimulated to glow. Direct observation of spontaneous luminescence from living specimens of *E. spinax* confirm that brighter zones are the zones displaying the highest photophore densities (Claes and Mallefet, 2009b). They found direct relationship between chemically induced luminescence intensity [via oxygen peroxide (H_2O_2)] and photophore density accross *E. spinax*'s luminous zones. In this case, however, the relationship reflected mainly that the amount of luminous substrate available for the chemiluminescent reaction in a given luminous zone is linked to its number of photophore in a given surface area (Claes and Mallefet, 2009b) suggesting that each photophore might contain a similar amount of luminous substrate. However, since the variation in $TL_{\max}$ between the different luminous zones of *E. spinax* does not appear to be linked to difference in PD, it provides evidence that the hormonal luminescence control present in this shark may act differently according to the stimulated part of the luminescent pattern. This heterogeneity in the timing of the light emission reflects a difference in the sensitivity of the luminous zones to both hormones, indicating that additional

control mechanisms might be present. The observed sexual differences in response to PRL, which also do not seem to be linked to a difference in PD, gives also another clue that the hormonal luminescence control mechanism of *E. spinax* is more complex than what was previously thought, and approaches in selectivity the neural luminescence control mechanism of bony fishes.

Ecology

The luminous zones of *E. spinax* do not appear at the same time during embryogenesis (Claes and Mallefet, 2008) and present different surface area and photophore density scaling patterns throughout ontogeny. These differences lead to the establishment of a complex heterogeneous luminescent pattern in subadult and mainly in adult individuals (Claes and Mallefet, 2009b). This luminous pattern heterogeneity appears to actually represent a trade-off between the continuously useful function of camouflage and periodic intraspecific functions such as cohesive swimming/hunting and sexual signalling which would be only useful in larger sharks (Reif, 1985; Claes and Mallefet, 2009b).

Results of this study (which focused on adult individuals of *E. spinax*) agree well with these hypotheses since they show that hormones controlling the luminescence from photophores of the *E. spinax* ventral luminous zone are not only able to trigger light in other luminous zones, but also that, applied at a same concentration, make them to luminesce proportionally to their PD, thus conserving the heterogeneity of the luminescent pattern. Moreover, this study adds a new step in the complexity of the luminescent pattern since it shows that the kinetics of the luminescent response to PRL and MT also varies independently from the PD of the zone. The rate of light emission is very different from one zone to another, thus reinforcing the idea of the multifunctionality of the luminous pattern. Similar versatility in the luminescence emission have been found in lanternfishes (myctophids) which feature a caudal organ that flashes rapidly and is believed to be used in intraspecific communication and slow glowing ventral photophores involved in camouflage (Young et al., 1980; Herring, 2007). In addition, the data presented here bring, for the first time, evidence of a clear dimorphism in the response to PRL between males and females of *E. spinax*. Interestingly, this dimorphism is only apparent in the pelvic region, where the sexually dimorphic organs of sharks, i.e. male's claspers and the cloaca of the female, are present (Wourms, 1977). This dimorphism strongly supports the involvement of luminescence from this region in reproduction, as already suggested (Claes and Mallefet, 2009b). For example, the differences in L_{tot} and TL_{max} found in this study might reveal the presence of a luminescent courtship display between

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male and female *E. spinax* specimens. Such luminous sexual signals have already been documented/suggested in other marine animal species including polychaetes (Tsuji and Hill, 1983), crustaceans (Rivers and Morin, 2008), squids (Herring, 1988) and bony fishes (Crane, 1965; Sasaki et al., 2003; Herring, 2007). Since this sexual dimorphism is only observed in response to PRL, it should be more pronounced when luminescence response to PRL are higher, which appears to be in April, at the winter-spring transition (Claes and Mallefet, 2009a). In Mediterranean, *E. spinax* is believed to mate in winter (when the proportion of active male is higher) with female storing the sperm until fertilisation which then occurs in summer (Coehlo and Erzini, 2008). It can therefore be expected that similar specific timing may exist in Norwegian velvet belly lantern sharks with mating occurring at a specific period of the year, and that possessing a more pronounced luminescent dimorphism at this period increases sexual signalling and reproductive success.

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LIST OF ABBREVIATIONS

α -MSH	α -melanocyte stimulating hormone
ILS	iris-like structure
$L_{\max}$	maximum intensity of light emission
L_{tot}	total light emission
LZ	luminous zone
Mol l^{-1}	mole per liter
$\text{Mq s}^{-1} \text{ cm}^{-2}$	megaquanta per second per square centimeter (of skin)
MT	melatonin
PD	photophore density
PRL	prolactin
q s^{-1}	quanta per second
SNK test	Student-Newman-Keuls test
TL	total length
$TL_{\max}$	time between stimulation and maximum intensity light emission
$\text{Tq h}^{-1} \text{ cm}^{-2}$	teraquanta per hour per square centimeter (of skin)

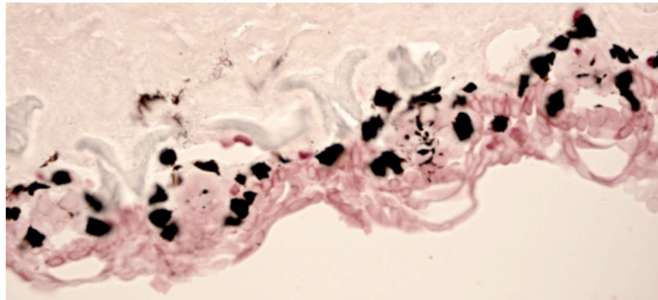
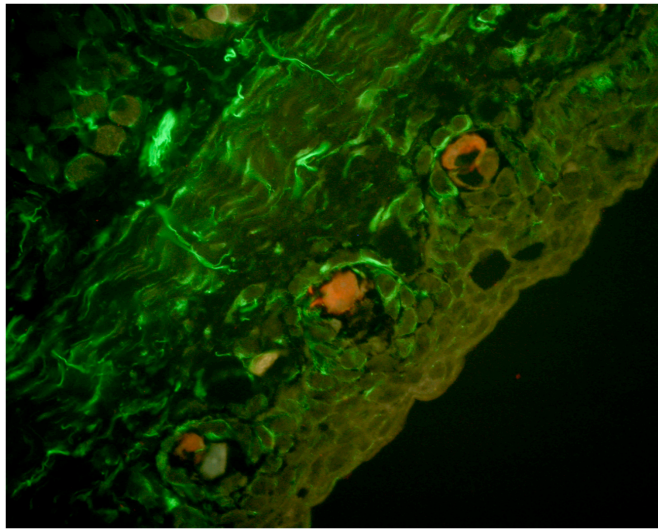
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Chapter 4: *Neuromodulation*



Chapter 4: Neuromodulation

This fourth and last section provides additional data on the physiological control of *E. spinax*'s photophores. It aims to analyse potential modulators of these organs's hormonally induced luminescence that could explain the sexual and zone-related variations in luminescent responses observed in **Paper VI** that could not be explained by a difference in photophore density. **Paper VII** focuses on the role of nitric oxide (NO) while **Paper VIII** investigates a potential neuromodulation of *E. spinax* luminescence by classical neurotransmitters.

The NO is a freely diffusible substance with a short half-life that is known to control firefly flashing (Trimmer et al., 2001), and was recently found to act as a neuromodulator in the luminescence control mechanisms of the krill and different fish species (Krönström et al., 2005; 2007; Krönström, 2009; Krönström and Mallefet, 2009). In **Paper VII**, we therefore used pharmacology and immunohistochemistry [with antibodies raised against NO-synthases (NOS) and acetylated tubulin (AcT, abundantly present in fish nerve fibers)] to investigate a potential role of this molecule in the modulation of *E. spinax* hormonally induced luminescence. Results are strongly in favour of a NO-modulation of the hormonally induced luminescence of this species since (i) the presence of NOS and AcT-like immunoreactivity were found in different parts of the photophores, and (ii) NO-donors (which had no effect *per se*) were able to modulate the hormonally induced luminescence patterns (probably via the production of cGMP). This NO-modulation could help the shark to finely tune the timing and intensity of its luminescence in response to environmental stimuli, as it has been already suggested for the hatchetfish (Krönström et al., 2005).

The presence of specific AcT-like immunoreactivity in photophores of *E. spinax* strongly suggests the presence of nerves in the photophores, and therefore a neuromodulation of this shark's luminescence. Since NOS were not found in these putative nerve fibers, they are probably not NO-containing nerves such as those found in the hatchetfish (Krönström et al., 2005) but instead containing more classical neurotransmitters. If this is the case, their action should be inhibitory or modulatory since classical neurotransmitters, however, failed to induce luminescence from *E. spinax* photophores (**Paper IV**). By using pharmacological and immunohistochemical methods as well as direct dynamic observations under binocular microscope, the **Paper VIII** aims to determine the nature of the nerves, as well as their role in the light control mechanism. Results suggest GABA as the putative candidate since (i) strong immunoreactivity to this substance was found

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around the photophore [in association with the iris-like structure (ILS)], (ii) the application of GABA modulates hormonally induced luminescence, and (iii) the application of the GABA_A antagonist bicucullin modulates hormonally induced luminescence but also triggers light from the photophores and was as potent as the hormones to “open” the photophores i.e. to provoke pigment retraction in pigmented cells of the ILS. This suggests that, contrary to NO, GABA is produce permanently, in order to “close” the photophores i.e. to provoke pigment expansion in the pigmented cells of the ILS. Since the light intensity of bicuculin-induced luminescence was only about 10% of values obtained with the hormones (MT and PRL), it also supports the idea that hormones have additional targets, the photocytes for example.

PAPER VII

Nitric oxide in control of luminescence from lanternshark (*Etmopterus spinax*)
photophores.

Claes, J. M., Krönström, J., Holmgren, S., Mallefet, J. (submitted)

J. Exp. Biol.

Nitric oxide in 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, prolactin (PRL) and melatonin (MT) triggering the light emission. Differential sensitivity to these hormones in adult individuals suggests, however, the luminescence of this shark to be controlled by an additional mechanism. In this study, different techniques were used to investigate a potential modulator of *E. spinax* luminescence, the 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 SNP and hydroxylamine decreased the time to reach the maximum amplitude (TL_{max}) of melatonin (MT)-induced luminescence while these substances decreased the maximum amplitude of prolactin (PRL)-induced luminescence (and also the TL_{max} in the case of SNP). The little impact of 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 effects of NO are mediated by cGMP.

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

Running title: NO control of lantern shark luminescence

INTRODUCTION

The velvet belly lantern shark (*Etmopterus spinax*) is a common deepwater shark of the East Atlantic and the Mediterranean Sea (Coelho 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

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zones forming a complex luminous pattern (Claes and Mallefet, 2008). Although few studies focused on the function of this bioluminescence, it is likely used for various purposes including camouflage by counter-illumination and intraspecific behaviours such as schooling and sexual signalling (Claes and Mallefet, 2009a).

Recently, unlike those of any other luminescent animal, photophores of this shark 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 to 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, in press). 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 either mediated 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 *Argyrolepelecus 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 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.

MATERIAL 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 (February 2009, December 2009) by longlines lowered in a deep area (depth ≥ 200 m) of the Raunefjord, Norway. Living specimens were transferred to two large (1 m³) tanks in a dark cold (6°C) room at Espeland Marine Station (Espesgrend, Norway) where they were kept until use. Captive animals were finally sacrificed, 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 48h in 3% formaldehyde (in saline) then transferred to PBS with sodium azide (NaN₃), and stored overnight in PBS with 30% sucrose before embedding. The tissue was embedded in OCT (Sakura, Zoeterwoude, 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 μ m 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 x 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.

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Table 1 Antibodies and peptides used for immunohistochemistry

Antibody (source)	Host	Working dilution	Antigen (peptide sequence)
<i>Primary</i>			
uNOS PA1-039 (1)	Rabbit	1:100	Murine i and nNOS c-terminal (1113-1122)
Acetylated tubulin T-6793 (2)	Mouse	1:1000	Sea urchin acetylated tubulin
<i>Secondary</i>			
DaM-FITC 715-095-150 (5)	Donkey	1:100	
DaR-biotin 711-065-152 (5)	Donkey	1:400	
DaR-Cy3 711-165-152 (5)	Donkey	1:800	

Antibodies against 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.

1, Transduction laboratories, San Joe, CA, USA ; 2, Sigma, St Louis, MO, USA. 3, Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

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 a preincubation with normal donkey serum, sections were preincubated with the primary and the secondary antibodies (**Table 1**) following the procedure described above [endogeneous peroxidase activity was blocked by H₂O₂ preincubation (0.3 or 3 % for 5 to 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 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 phosphate buffered saline. The preparations were embedded, frozen and sectioned. The preparations were preincubated in a solution containing 0.2% b-NADPH (Sigma Chemical Company, St Louis, MO, USA), 0.025% nitro blue tetrazolium (Sigma), 0.5% methanol, 0.5% dimethylformamide, 0.01% DMSO and 0.3% triton X in PBS for 1 h at 37 C. They were rinsed for 4 X 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 performed several control skin patches that receive a similar solution but without the b-NADPH.

Pharmacology

Luminometry

Photophore preparations used in this work consisted of circular ventral skin patches (0.25 cm²) 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 (2009b). These preparations will 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 during 1 hour 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 (in cm⁻²).

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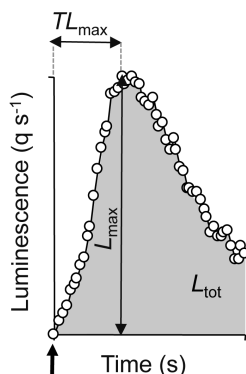


Figure 1 Typical luminescence emission curve from photophores of *Etmopterus spinax* showing the different light parameters. $L_{\max}$, maximum intensity of light emission; L_{tot} total quantity of light emitted during the experiment (shaded area); $TL_{\max}$, time from stimulation (arrow) to maximum intensity of light emission ($L_{\max}$).

Chemicals

Chemical agents used in this study were the followings: 8-Bromoguanosine 3',5'-cyclic monophosphate sodium salt monohydrate (8BrcGMP; Sigma, St Louis, MO, USA), hydroxylamine hydrochloride (Sigma), melatonin (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), 1H-[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 CaCl₂, 0.6 mmol l⁻¹ MgSO₄, 1.6 mmol l⁻¹ Na₂SO₄, 300 mmol l⁻¹ urea, 150 mmol l⁻¹ trimethylamine N-oxide, 10 mmol l⁻¹ glucose, 6 mmol l⁻¹ NaHCO₃, adjusted to a 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 is confirmed, the intracellular pathway used by this substance to mediate its effects. For that purpose, we first checked that nitrenergic drugs (NO donors, NOS inhibitor, cGMP analogue and soluble guanylyl cyclase inhibitor) were able or not to trigger light *per se*, and then tested the effects of a 20 min pre-treatment with nitrenergic

drugs on the light parameters of the luminescence induced by MT or PRL (both also applied alone as control). These experiments were performed at room temperature.

Statistics

Statistical analyses (Student's *t*-test 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 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. in percentage (%) of the control response of the same animal. Each mean value was expressed with its standard error (mean $\pm$ s.e.m.) and "*N*" equals 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

E. 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**, insert). Blood sinuses are present in the pigmented cup and likely 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 was already 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**).

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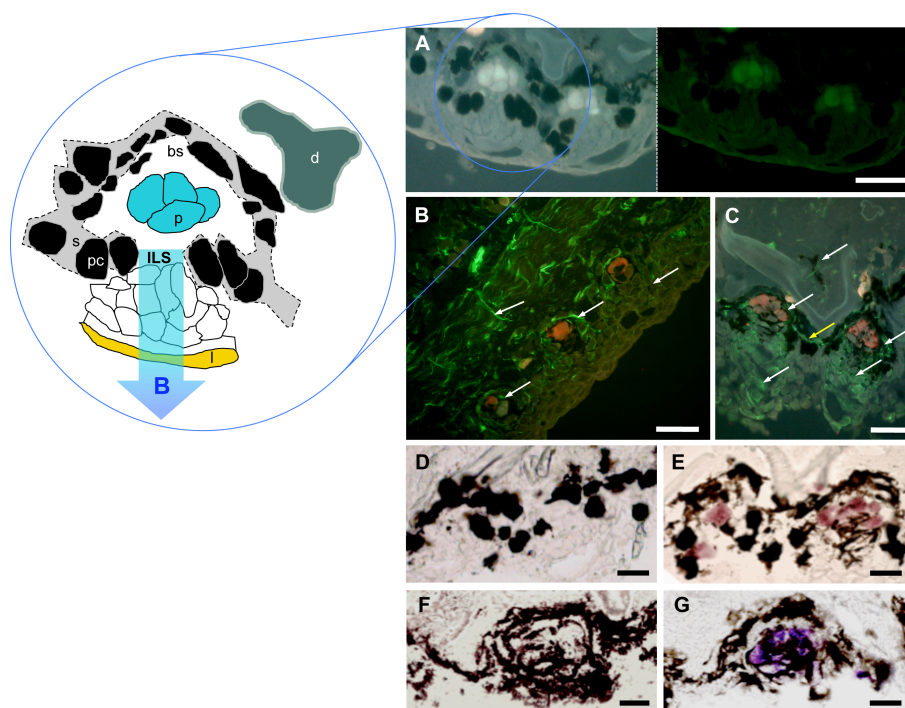


Figure 2 I Sections through ventral photophores from embryo (B) and adult specimens (A,C-G) of *Etmopterus spinax*. (A) Control photophore (with omission of the primary antibody) without (left) and with (right) U.V. stimulation (for fluorescence histochemistry). Right picture show green autofluorescence inside the photocytes. Insert, schematic representation of the different structural elements present in a photophore. (B,C) Acetylated tubulin-like (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 fibers (white arrows) innervate the photophores and their surrounding tissue. Yellow arrow in (B) indicates nerve fibre connecting two photophores. NOS-like IR is found in the cytoplasm of photocytes. 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 b-NADPH) for NADPH-diaphorase activity testing. (G) NADPH-diaphorase activity (blue-mauve colour) was present inside the photocytes. B, bioluminescence; bs, blood sinus; d, dermal denticle (placoid scale); ILS, iris-like structure; in, intermediate cell; l, lens cell; p, photogenic cell; pc, pigmented cell; s, pigmented sheath. Scale bars, 50 μ m.

Nitric oxide synthase (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 (immuno-histochemistry; **Fig. 2D**) or the b-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 a pre-treatment with a nitrenergic drug, only the light parameters L_{max} and TL_{max} were considered in the following analyses.

Table 2 Relation between L_{max} and L_{tot} for control photophore preparations and nitrenergic drugs*

	<i>N</i>	Slope	r^2	<i>P</i>
<i>Melatonin</i>				
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
<i>Prolactin</i>				
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} is in $\text{Mq s}^{-1} \text{cm}^{-2}$, L_{tot} is in $\text{Tq h}^{-1} \text{cm}^{-2}$.

L-NAME, N^G-nitro-L-arginine methyl ester (NO-inhibitor); SNP, sodium nitroprusside (NO-donor).

II. Results

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 maximal intensity of light emission ($L_{\max}$) 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

Sodium nitroprusside (SNP)

The effects of the NO donor SNP were tested. Application of SNP (10^{-6} to 10^{-3} mol l $^{-1}$; $N=8$) for one hour on VSP did not induce light emission *per se*. SNP (10^{-6} to 10^{-3} mol l $^{-1}$) had no effects 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**).

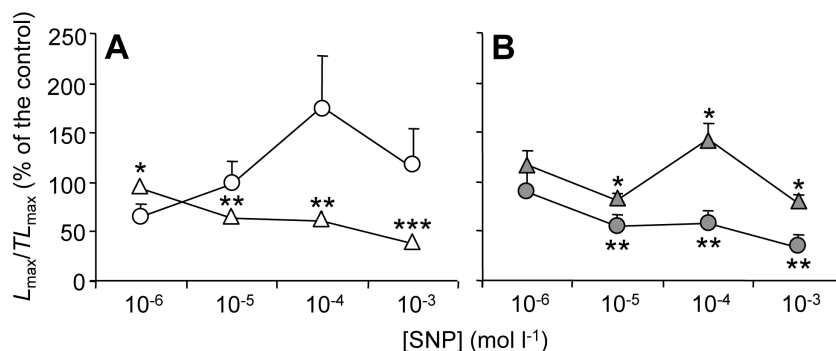


Figure 3 I Effect of the NO donor SNP on hormonally induced luminescence from *Etmopterus spinax* photophores. (A) Dose-dependent effect of SNP on melatonin (MT; 10^{-6} mol l $^{-1}$)-induced luminescence. SNP had no effect on the $L_{\max}$ (circles) but significantly decreased the $TL_{\max}$ (triangles)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

Similarly 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**).

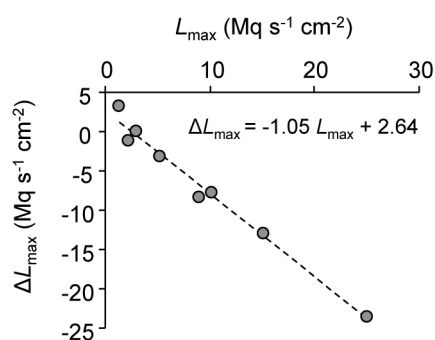


Figure 4 I Relationship between $L_{\max}$ of control photophores and $\Delta L_{\max}$ (difference between $L_{\max}$ values of SNP-treated and control VSP), for PRL-induced luminescence ($P < 0.001$, $R^2 = 0.98$, $N = 8$). The inhibitory effect of SNP is more important in VSP with a high luminescence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

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 one hour 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 one hour 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**).

II. Results

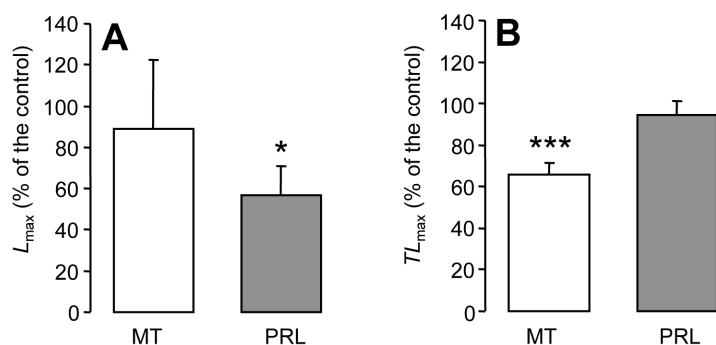


Figure 5 I Effects of the NO-donor hydroxylamine ($10^{-3} \text{ mol l}^{-1}$) on (A) the L_{max} and (B) the TL_{max} of hormonally ($10^{-6} \text{ mol l}^{-1}$)-induced luminescence from *Etmopterus spinax* photophores. Values are expressed as a percentage of values obtained in control photophore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

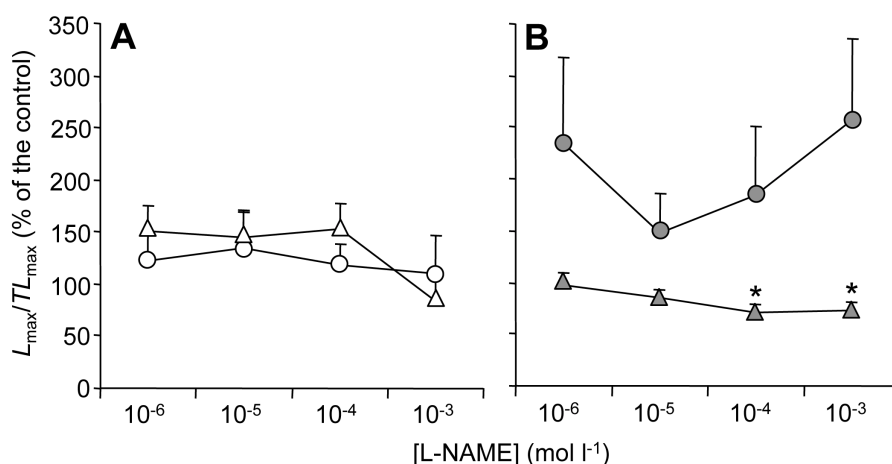


Figure 6 I Effect of the NOS inhibitor L-NAME on the hormonally induced luminescence from *Etmopterus spinax* photophores. (A) Dose-dependent effect of L-NAME on melatonin (MT; $10^{-6} \text{ mol l}^{-1}$)-induced luminescence. L-NAME had no effects on melatonin on the light parameters (L_{max} , circles;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

Effects on the cGMP pathway

In order to determine if 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) in *E. spinax* light emission. None of these substances induced light emission *per se* on the luminescence of *E. spinax* when applied on VSP for one hour (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 $L_{\max}$ of after 8BrcGMP application ($P=0.06$).

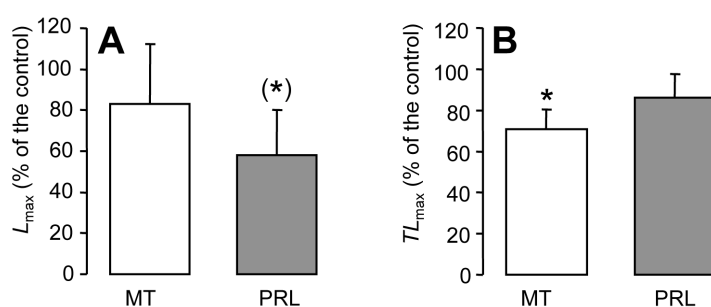


Fig. 7. Effects of the cGMP analogue 8BrcGMP (10^{-4} mol l $^{-1}$) on (A) the $L_{\max}$ and (B) the $TL_{\max}$ of the hormonally (10^{-6} mol l $^{-1}$) induced luminescence from *Etmopterus spinax* photophores. Values are expressed as a percentage of values obtained in control photophore preparations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

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

II. Results

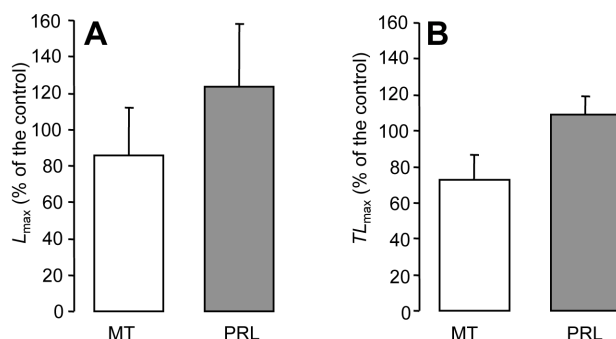


Figure 8 I Effects of the guanylyl cyclase inhibitor ODQ (10^{-5} mol l^{-1}) on (A) the L_{max} and (B) the TL_{max} of the hormonal (10^{-6} mol l^{-1})-induced luminescence from *Etmopterus spinax* photophores. ODQ had no effects on this light emission. Values are expressed as a percentage of values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

DISCUSSION

This study supports for the first time the presence of nerve fibres and NOS-like IR associated to *Etmopterus spinax* photophores, suggesting a role of NO and nerves in the luminescence control of 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 hormonal-induced luminescence

Contrary to previous conception (Johann, 1899), this study suggests that photophores of the lantern shark *E. spinax* are innervated. Since various attempts to trigger luminescence in this species using classical neurotransmitters [acetylcholine, (nor)adrenaline, serotonin, and GABA] have failed (Claes and Mallefet, 2009b), this 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 they 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 belong to some other type of neuron (Nilsson, 1983).

In the hatchetfish (*Argyroteleus 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 of 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 were not similar on these hormonal light responses: NO only had an effect on the kinetic of MT-induced luminescence but affected the kinetic and the amplitude of PRL-induced luminescence. This result is not surprising: since MT and PRL have different intracellular transmission pathways (Claes and Mallefet, 2009b), one can therefore suppose the NO to act at different levels when modulating the action of these two hormones on *E. spinax* photophores.

The effect of NO-donors used in this study (SNP and hydroxylamine) on PRL-induced luminescence in *E. spinax* can be compared to the effect of NO on the hatchetfish and the northern krill (*Meganyctiphanes norvegica*), which is predominantly inhibitory (Krönström et al., 2005; Krönström et al., 2007). Similarly to what was found in hatchetfish, the inhibitory effect of NO on the amplitude of the luminescent response is not constant but directly depends on the luminous capability of the photophore preparation: the inhibition is 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 fastened the luminous response of *E. spinax* photophores after MT application. This suggests that NO affect 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 (*Porichthys notatus*) (Krönström et al., 2005; Krönström et al., 2007; Krönström, 2009).

II. Results

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 NO-donor, this suggests the presence of endogenous catalase activity in this shark's photophores, as it was also demonstrated in the photophore of the hatchetfish (Krönström et al., 2005).

The NOS inhibitor L-NAME did not affect the light parameters of MT-induced luminescence from *E. spinax* photophores and had only a low impact on the timing of PRL-induced luminescence. This supports the idea that NO is generated on demand in this shark's photophores, and therefore that the effects of this molecule on hormonally induced luminescence are mainly transitory rather than permanent.

In physiological processes, 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 mitochondrial respiration inhibition (Brown, 1995). In this work, the cGMP analogue 8Br-cGMP mimicked the effect of 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 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 coherent if we keep the idea that NO is only involved in this system on temporary demand.

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, 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 has been 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 of their luminescence. It is not clear, however, at this time, if nerves reaching photocytes into the lumen of *E. spinax* photophores are NO-containing nerves or not. The presence of these nerves nevertheless confirms the

idea that hormonal inputs need additional mechanism for an 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, 2009a). These different functions appear to be achieved by different luminous 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 tunes the intensity of luminescence from glowing *E. spinax* photophores to allow this shark to counterilluminate efficiently in the mesopelagic zone, whose light properties change with time and depth (Johnsen, 2003), similarly 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 *Myctophum punctatum* (Krönström and Mallefet, 2009), which is likely used for sexual communication (Herring, 2007), supports the idea that the effects of NO on luminescence is not restricted to the counterilluminating behaviour in fishes. It is therefore possible that a NO modulation also exists in luminous zones of *E. spinax* that are not involved in counterillumination, but this needs further analysis.

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II. Results

LIST OF ABBREVIATIONS

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

GABA inhibition of luminescence from lanternshark (*Etmopterus spinax*)
photophores.

Claes, J. M., Krönström, J., Holmgren, S., Mallefet, J. (manuscript)

GABA inhibition of luminescence from lanternshark (*Etmopterus spinax*) photophores

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SUMMARY

Photogenic organs (photophores) of the velvet belly lanternshark (*Etmopterus spinax*) are under hormonal control, since melatonin (MT) and prolactin (PRL) trigger luminescence while α -MSH prevents this light to be emitted. A recent study supported, however, the presence of numerous nerve fibres in the photogenic tissue of this shark. In this work we used immunohistochemical and pharmacological methods to identify the nerve type(s) present in the photogenic tissue of this shark and to determine their role in its photogenesis control mechanism. Results indicate that GABA inhibits MT and PRL-induced luminescence, which are on the other hand increased by the GABA_A antagonist bicuculin. In addition we also demonstrated that bicuculin can induce light *per se* by provoking pigment retraction in the pigmented cells composing the iris-like structure of the photophore (as do MT and PRL). This study strongly supports that a GABA inhibitory tonus controls photophore aperture in the photogenic tissue of *E. spinax*.

Key words: bioluminescence, Chondrichthyes, GABA_A receptor, melatonin, neurotransmitter, prolactin.

Running title: GABA control of lanternshark luminescence

INTRODUCTION

The velvet belly lanternshark (*Etmopterus spinax*) is a small deepwater shark occurring in the East Atlantic, from Norway to South Africa, and in the Mediterranean Sea (Compagno et al., 2005; Coelho and Erzini, 2008). As other members of the lanternshark family (Etmopteridae), it is able to emit a chemically based visible light (= luminescence) from thousands of tiny photogenic organs called photophores (Compagno et al., 2004). These organs aggregate themselves during embryology to form, according to their position on the shark's body, nine distinctive luminous zones (Claes and Mallefet, 2008; **Fig. 1A**). Due to the discrepancy existing in their photophore densities, these markings form a complex luminous pattern harbouring a heterogeneous luminescence intensity (Claes and

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Mallefet, 2009a). This particularity suggests that luminescence brings different adaptive benefits to this shark such as an efficient camouflage and a powerful tool for intraspecific communication in the darkness of the deep-sea (Claes and Mallefet, 2009a).

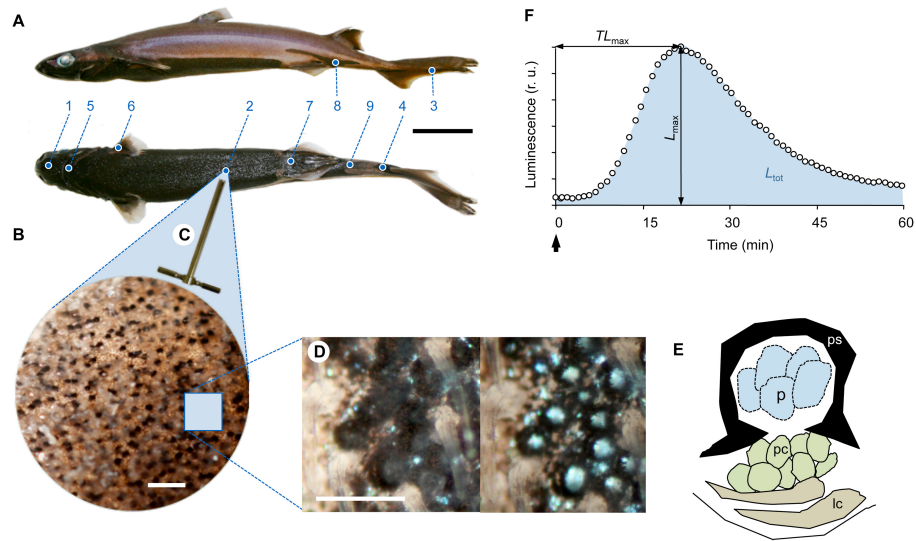


Figure 1 (A) Ventral (up) and lateral (down) view of a mature male specimen of *Etmopterus spinax* showing the position of the nine different luminous zones: 1, rostral; 2, ventral; 3, caudal; 4, infra-caudal; 5, mandibular; 6, pectoral; 7, pelvic; 8, lateral; 9, infra-pelvic. Scale bar, 5 cm. (B) Standard circular skin patch excised from the ventral zone. Scale bar, 1 mm. (C) Metal cap drill used to excised circular skin patches from *E. spinax* luminous zones. (D) Specific photophores before (left) and during (right) light emission. Scale bar, 0.5 mm. (E) Schematic drawing of a photophore showing the different structural elements present in this photogenic organ, including the pigmented iris-like structure (ILS), which regulates the amount of light emitted by the photocytes (p) to the outside. lc, lens cell; pc, pigmented cell of the ILS; ps, pigmented sheath. (F) Typical luminescence emission curve with associated light parameters: L_{max} , maximum intensity of light emission; L_{tot} , total quantity of light emitted during the experiment; TL_{max} , time from stimulation (arrow) to the peak of light emission (L_{max}).

Since they often directly influence the survival of an organism, luminescent behaviours are usually precisely controlled. Different control levels exist, targeting either the timing or the physical properties (intensity, wavelength and angular

distribution) of the light emitted, and in most cases both controls are present (Baguet and Case, 1971; Baguet and Marechal, 1978; Denton et al., 1985; Harper and Case, 1999; Demary et al., 2006; Rivers and Morin, 2008).

While the physical properties of an organism's luminescence are generally controlled in a passive way i.e. via accessory optical structures including optical lens, reflectors and specialized filters (Herring and Morin, 1978, Herring, 1982; Denton et al., 1985), timing control generally involves active on/off switches from the emitter in response to environmental stimuli (Morin et al., 1975; Case, 1980; Harper and Case, 1999; Chan et al., 2008).

While intrinsic (non-bacterial) photophores of bony fishes harbour a neural control of their luminescence, photophores of the cartilaginous *E. spinax* do not respond to the application of classical neurotransmitters (Baguet, 1975; Baguet and Marechal, 1978; Baguet and Christophe, 1983; Claes and Mallefet, 2009b) but instead appear hormonally controlled (Claes and Mallefet, 2009b). Three different hormones were discovered to be involved in this control: melatonin (MT) and prolactin (PRL) which trigger light emission and α -melanocyte-stimulating hormone (α -MSH) whose application prevent the shark's photogenic tissue to glow, which indicates a putative inhibitory effect of this hormone on the light emission/production. These hormones are also those controlling the skin pigmentation of Elasmobranchs (sharks and rays) via movements of chromatophore (Visconti et al., 1999; Gelsleichter, 2004) and MT and PRL were recently shown to target a pigmented iris-like structure [ILS, which appears to be made of aggregated chromatophores (Ohshima, 1911)] situated between the light-emitting cell(s) i.e. photocyte(s) and the lens cell(s) (Claes and Mallefet, in press 1; **Fig. D,E**).

Sexual and zone-related differences in hormonally induced luminescence suggested an additional control mechanism (Claes and Mallefet, in press 2). The presence of nitric oxide synthases (NOS) and putative nerve fibers in the photogenic organs of *E. spinax* was recently demonstrated, suggesting a role of NO and these nerves in the luminescence control mechanism of this shark (Claes et al., submitted). In the same study, pharmacological experiments confirmed a neuromodulatory effect of NO on the hormonal-induced *E. spinax* luminescence but did not focus on the role of the nerve-like structures in the light emission control mechanism of this shark. Since photophores of this lanternsharks did not luminesce after application of classical neurotransmitters, it can be postulated that these nerves are either modulator or inhibitor nerves such as those present in other luminescent organisms (Baguet, 1975; Ancil, 1981; Krönström et al., 2005).

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By combining immunohistochemical, pharmacological and morphological approaches, this paper aims (i) to identify the nerve type(s) present in the photogenic tissue of *E. spinax* and (ii) to determine the role of these nerves in *E. spinax* luminescence control mechanism.

MATERIALS AND METHODS

Experimental animals

Thirty-six adult velvet belly lanternsharks *Etmopterus spinax* (Linnaeus 1758) [29-52 cm total length (TL)] were caught during three field sessions (April, 2008, June 2009 and December 2009) by longlines lowered in a deep area (depth ≥ 200 m) of the Raunefjord, Norway. Living specimens were transferred to large (1 m³) tanks in a dark cold (6°C) room [at the High Technology Center of Bergen ('Hoyteknologisenteret i Bergen', HiB) or at Espeland Marine Station (Espegrend, Norway)], where they were kept until use. Following the local rules for experimental fish care, all the sharks were killed by a blow to the head before experimentation started.

Immunohistochemistry

Ventral skin patches containing photophores were taken from 6 different individuals and used for immunohistochemistry. Tissue were fixed for 48h in 3% formaldehyde, transferred to PBS with sodium azide (NaN₃) and stored overnight in PBS with 30% sucrose before embedding. The tissue were embedded in OCT (Sakura, Zoeterwude, The Netherlands) and quickly frozen in isopentane chilled with liquid nitrogen.

Histological sections (10 μ m) were performed using a cryostat microtome (Zeiss Microm International GmbH, Walldorf, Germany) collected on chrome alum gelatine-coated slides and left overnight to dry.

Fluorescence histochemistry

Sections were preincubated with normal donkey serum (NDS, 10%) for 30 min to prevent non-specific staining and then incubated for 48 h after the application of primary antibodies (**Table 1**). They were rinsed (3 x 5 min in PBS with 2% NaCl) and incubated for 60 min after the application of the secondary antibodies (**Table 1**).

Table 1 Antibodies and peptides used for immunohistochemistry

Antibody (source)	Host	Working dilution	Antigen (peptide sequence)
<i>Primary</i>			
Tyrosin hydroxylase (1)	Goat	1:400	Full lenght tyrosin OHase from rat
Choline acTase (2)	Goat	1:100	Choline acTase from rat brain
Serotonin (3)	Rabbit	1:4000	Serotonin-creatinine sulfate complex conjugated to BSA
GABA (3)	Rabbit	1:400	GABA conjugated to BSAX
<i>Secondary</i>			
DaG-DTAF 705-016-147 (4)	Donkey	1:100	Goat IgG
DaG-biotin 705-065-148* (4)	Donkey	1:400	Goat IgG
DaR-FITC 711-095-152 (4)	Donkey	1:100	Rabbit IgG
DaR-biotin 711-065-152* (4)	Donkey	1:400	Rabbit IgG

*For further processing of the sections stained with biotin secondaries we have used Vectastain ABC-kit and the vector Nova RED (brownish precipitate) or vector VIP (purple precipitate), Vector laboratories.

1,Abcam inc., Cambridge, MA, USA; 2, Chemicon International inc. (Millipore), Billerica, MA, USA; 3, Sigma, St Louis, MO, USA; 4, Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

actasse, acetyltransferase; DaG, donkey anti-goat ; DaR, donkey anti-rabbit;OHase, hydroxylase.

All incubations took place 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.

Avidin-biotin histochemistry

After a preincubation with NDS, sections were preincubated with the primary and the secondary antibodies (**Table 1**) following the procedure described above [endogeneous peroxidase activity was blocked by H₂O₂ preincubation (0.3 or 3 % for 5 to 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 finally mounted in 50 % glycerol and 50 % carbonate buffer (pH 8.5) and examined as described above.

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Luminometry

Photophore preparations used in this work consisted of circular skin patches (0.25 cm², **Fig. 1B**) containing photophores dissected out from luminous zones of adult shark specimens with a metal cap driller (**Fig. 1C**) and placed in a shark saline (Bernal et al., 2005) following the method of Claes and Mallefet (2009b).

Skin patches were transferred either to small Perspex chambers filled with 200 µl saline with their photogenic part orientated to the photo-detector of a luminometer FB12 (Berthold; Pforzheim, Germany) or to a plate containing 100 µl saline-filled holes inserted in a luminometer Orion (Berthold; Pforzheim, Germany). Both luminometer types were calibrated using a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK). The luminescent responses were recorded during 1 hour after the stimulation and were characterised using different parameters (**Fig. 1F**): 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)], the time to reach $L_{\max}$ from the stimulation time [$TL_{\max}$, in seconds], and the time from $L_{\max}$ to the half-extinction of light emission [$TL_{1/2}$ in seconds(s)]. Light parameters L_{tot} and $L_{\max}$ were standardised by skin surface area (in cm²).

Chemicals

Chemical agents used in this study were the followings: bicuculin (Sigma; StLouis, MO, USA); γ -amino-butyric acid (GABA, Sigma); melatonin (MT; Across Organics; Pittsburgh, PA, USA); prolactin (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 CaCl₂, 0.6 mmol l⁻¹ MgSO₄, 1.6 mmol l⁻¹ Na₂SO₄, 300 mmol l⁻¹ urea, 150 mmol l⁻¹ trimethylamine N-oxide, 10 mmol l⁻¹ glucose, 6 mmol l⁻¹ NaHCO₃, adjusted to pH 7.7 (Bernal et al., 2005).

Morphology

In order to macroscopically determine the effect of chemical agent(s) on *E. spinax* photophores, photophore-containing skin patches were digitally photographed under binocular after chemical application, and the transparency index i.e. the apparent

photogenic surface areas of six specific photophores was measured (for different times after the application) with Image J (National Institutes for Health, Bethesda, MD, USA), using the technique of Claes and Mallefet (in press 1). In order to make a correspondence between macroscopic changes and luminescence emission, binocular observations were made concomitantly to a luminescence recording with the luminometer on a similar patch from the same shark (as in Claes and Mallefet, in press 1).

Methods

Antibodies were first used to identify the nerve type present in the photogenic tissue of *E. spinax*. Then we started the pharmacological study. This study aimed (i) to identify the receptor used by the agonist via the use of antagonists, (ii) to determine the sensitivity of this receptor via a dose-light response curve, (iii) to check if this receptor was present in all *E. spinax* luminous zones and if its effects were photophore density dependant (which is a strong clue that it is closely involved in the photophore control mechanism), and finally (iv) to test the effect of agonist and antagonist application on hormonal-induced luminescence.

The experimental procedure was performed at room temperature. Statistical analyses (Student's *t*-test and linear regressions) were performed with the software SAS/STAT (STAT Institute Inc, 1990, Cary, NC, USA).

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. in percentage (%) of the control response of the same animal. Each mean value is expressed with its standard error (mean±S.E.M.) and "N" equals the number of skin patches used for a specific treatment (which corresponds to the number of shark specimens tested, as one skin patch from each individual was used in each treatment).

RESULTS

Immunohistochemistry

The photogenic organs of *E. spinax* appeared as small bell-shaped structure in the epidermis. Photocytes are enclosed in a pigmented sheath surrounded by pigmented cells of the iris-like pigmented structure as well as one or several lens cells (**Fig. 1E, 2A**).

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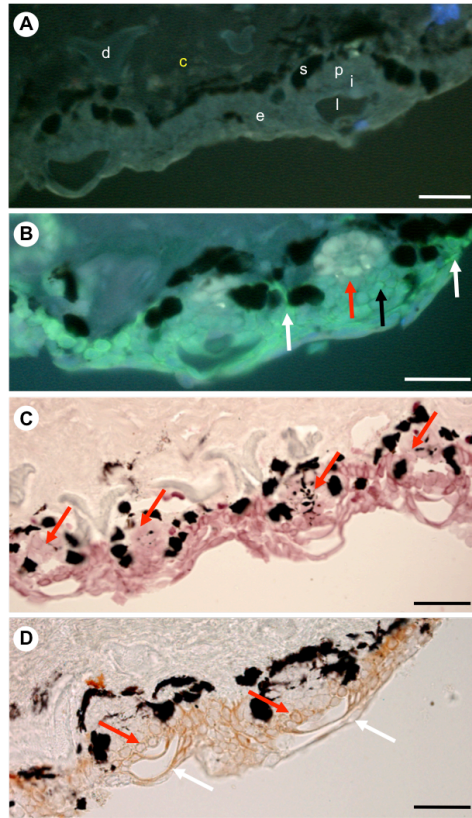


Figure 2 I Longitudinal sections of ventral photophore from *Etmopterus spinax*. (A) Control without antibody does not show any staining. (A-C) Strong GABA immunoreactivity (IR) in the epidermis of the shark, around lens and ILS-related pigmented cells as well as inside the photophore. (B) GABA-IR (green fluorescence) obtained with DaR-FITC 711-O95-152. Autofluorescence from photocytes (red arrows) appears different from fluorescence of the staining (black arrow). White arrows indicate nerve fibres. (C) GABA-IR (purple precipitate) obtained with DaR-biotin 711-065-152 and vector VIP. Arrows indicate presence of the staining inside the photophores. (D) GABA-IR (brownish precipitate) obtained with DaR-biotin 711-065-152 and vector Nova Red. Staining is abundant at the surface of intermediate cells (red arrows) and lens cells (white arrows). c, conjunctive tissue; d, dermal denticle; e, epidermis; i, ILS-related pigmented cell; l, lens cell; p, photocyte; s, pigmented sheath. Scale bar, 50 µm.

No immunoreactivity (IR) was obtained with antibodies raised against tyrosine hydroxylase (which is an enzyme involved in the synthesis of adrenalin and noradrenaline), choline acetyltransferase (which an enzyme involved in the synthesis of acetylcholine), and serotonin, suggesting the absence of adrenergic, noradrenergic, cholinergic and serotonergic nerves in the photogenic tissue of *E. spinax*.

On the other hand antibodies raised against GABA show strong immunoreactivity in the epidermal cells and nerve fibres (**Fig. 2B**), in the photophores (**Fig. 2C**), and at the surface of ILS-related pigmented and lens cells (**Fig. 2D**). Omission of the primary antibody (raised against GABA) did not show any non-specific binding of the secondary antibodies (**Fig. 2A**). These results strongly supported a role of GABA in the photogenesis control of *E. spinax*.

Pharmacology

Staining was only detected with antibodies raised against GABA, we therefore decided to focus the pharmacological analysis on this neurotransmitter and its receptors. Since GABA application does not induce light *per se* from *E. spinax* photophores (Claes and Mallefet, 2009b), we postulated that if this neurotransmitter is involved in photogenesis control, it has either a modulatory or an inhibitory effect on this shark's light emission. Hormonal applications (MT or PRL) were therefore used as positive controls and saline applications as negative controls.

GABA receptor

E. spinax photophores responded to application of GABA_A receptor antagonist bicuculin (10^{-3} mol l⁻¹) by a light emission whose L_{tot} was significantly ($P < 0.05$) different from saline application (**Table 2**). Application of the GABA_B antagonist phaclofen (10^{-3} mol l⁻¹) did not induce light emission (**Table 2**).

Table 2 Results of antagonist applications

Antagonist	[Drug] (mol l ⁻¹)	<i>n</i>	Duration (min)	L_{tot} saline (Tq h ⁻¹ cm ⁻²)	L_{tot} treatment (Tq h ⁻¹ cm ⁻²)	<i>P</i> -value (Student <i>t</i> -test).
Bicuculin	10^{-3}	6	60	13.39±4.01	0.81±0.14	0.0258
Phaclofen	10^{-3}	6	60	0.60±0.15	0.81±0.14	0.3238

Bold value indicates significant difference between saline control and treatment

The time course of the light emission induced by bicuculin application (**Fig. 3A**) was similar to the time course of MT-induced luminescence (Claes and Mallefet, 2009) and a highly significant ($P < 0.001$) relationship was found between L_{max} and L_{tot} light parameters (**Fig. 3B**). Dose light response curve of the bicuculin application indicated that highest luminescence responses are obtained with the highest concentration i.e. 10^{-3} mol l⁻¹ (**Fig. 3C**). At this concentration, bicuculin efficiently

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induced light emission in all the luminous zones of *E. spinax*; zones whose size was sufficient to provide at least one standard photophore-containing patch showed a highly significant ($P<0.001$) relationship between the mean $L_{\max}$ of their bicuculin-induced luminescence and their mean photophore density (**Fig. 3D**).

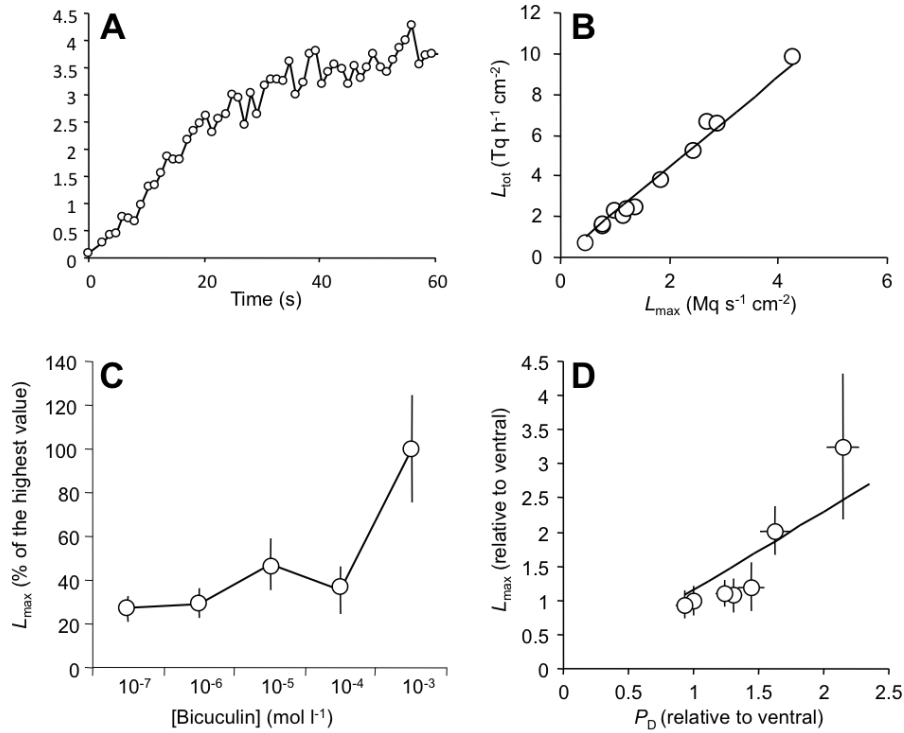


Figure 3 I (A) Original luminescence recording from a ventral skin patch of an adult specimen of *Etmopterus spinax* after bicuculin application (10^{-3} mol l $^{-1}$). (B) Relationship ($P<0.001$) between the maximum intensity of light emission ($L_{\max}$) and total amount of light emitted during the experiment (L_{tot}) for luminescence induced by 10^{-3} mol l $^{-1}$ bicuculin application ($N=12$): $L_{\text{tot}}=2.21 L_{\max}$, $r^2=0.99$. (C) Dose light response curve for bicuculin application. Values of the maximum intensity of the light emission ($L_{\max}$) are expressed as a percentage of the highest value [that was in all cases ($N=12$) obtained with the highest concentration, 10^{-3} mol l $^{-1}$]. (D) Relationship between relative (ventral zone=1) photophore density (P_D) and relative (ventral zone=1) maximal intensity of light emission ($L_{\max}$) after bicuculin application (10^{-3} mol l $^{-1}$) for *E. spinax* luminous zones whose surface area was sufficient to provide at least one standard skin patch ($N=7$): $L_{\max}=1.15 P_D$, $r^2=0.94$ ($N=12$ for each luminous zone). Other luminous zones ($N=2$) not included in this regression analysis also responded to bicuculin application, but their small size prevents them to be used quantitatively in this analysis.

Effects of bicuculin on hormonal application

The GABA_A antagonist bicuculin significantly ($P<0.05$) enhanced the $L_{\max}$ of MT and PRL (**Fig. 4A**). In addition bicuculin also very significantly ($P<0.01$) increased the L_{tot} of MT-induced luminescence and significantly ($P<0.05$) delayed this light emission (**Fig. 4B,C**).

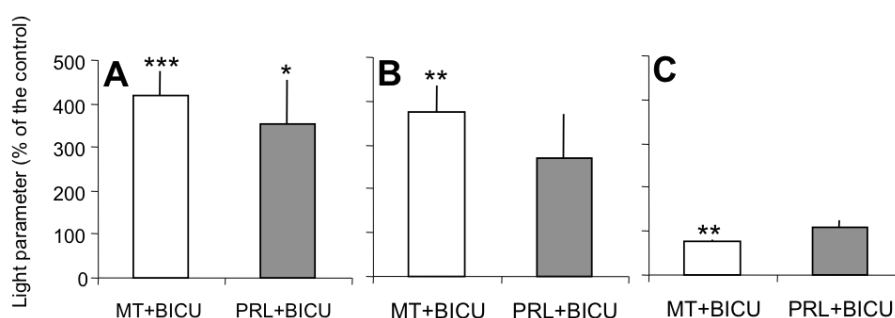


Figure 4 | Effect of bicuculin application (10^{-3} mol l⁻¹) on light parameters of MT (white bars) and PRL (grey bars)-induced luminescence. (A) Maximum intensity of light emission ($L_{\max}$). (B) Total quantity of light emitted during the experiment (L_{tot}). (C) Time from stimulation to the luminescence peak ($TL_{\max}$). Values are expressed as percentage of the control values [$N=11$ for each pairs of control (MT or PRL) and treated ventral skin patches]. Asterisks indicate significant differences between control and treated ventral skin patches (* $P<0.05$, ** $P<0.01$, * $P<0.001$).**

Effects of GABA on hormonal application

GABA decreased the $L_{\max}$ of PRL-induced luminescence at 10^{-3} mol l⁻¹ ($P<0.05$) but had no effect at smaller concentrations on this parameter (**Fig. 5A**). L_{tot} of both hormones are significantly ($P<0.05$) decreased by 10^{-3} mol l⁻¹ GABA application but no effect on L_{tot} was observed at smaller concentrations (**Fig. 5B**). Finally, 10^{-5} mol l⁻¹ GABA application significantly ($P<0.05$) decreased the $TL_{\max}$ of MT-induced luminescence, but no significant effect was obtained with other GABA concentrations (**Fig. 5C**).

In vivo effects of bicuculin

Application of bicuculin (10^{-3} mol l⁻¹) on photophore-containing *E. spinax* skin patches always provoked luminescence (**Fig. 6A**) and the “irising” of the photophores (**Fig. 6B**); the transparency index coincided almost perfectly with the

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luminescence time course. $L_{\max}$ of bicuculin-induced luminescence was however highly significantly ($P<0.001$) lower than $L_{\max}$ of hormonal (MT or PRL)-induced luminescence (**Fig. 6C**). $L_{\max}$ values of MT and PRL-induced luminescence are summarised in Table 3.

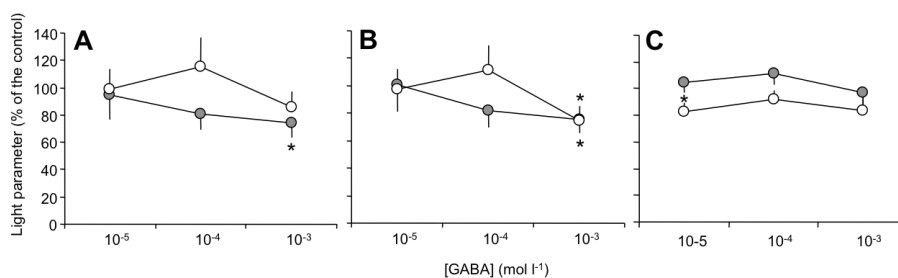


Figure 5 I Effect of different concentrations of GABA (10^{-5} to 10^{-3} mol l⁻¹) on light parameters of MT (white circles) and PRL (grey circles)-induced luminescence. (A) Maximum intensity of light emission ($L_{\max}$). (B) Total quantity of light emitted during the experiment (L_{tot}). (C) Time from stimulation to the luminescence peak ($TL_{\max}$). Values are expressed as percentage of the control values [$N=12$ for each pairs of control (MT or PRL) and treated ventral skin patches at each concentration]. Asterisks indicate significant differences between control and treated ventral skin patches ($*P<0.05$).

Table 3 Light parameters of MT and PRL-induced luminescence		
	June 2009	December 2009
Hormone*	$L_{\max}$	$L_{\max}$
	(Mq s ⁻¹ cm ⁻²)	(Mq s ⁻¹ cm ⁻²)
MT	9.66±3.10	12.60±1.47
PRL	1.81±0.59	29.24±8.41

*Number of experiments: MT, $n=7$ (June) and 21 (December); PRL, $n=6$ (June) and 21 (December).

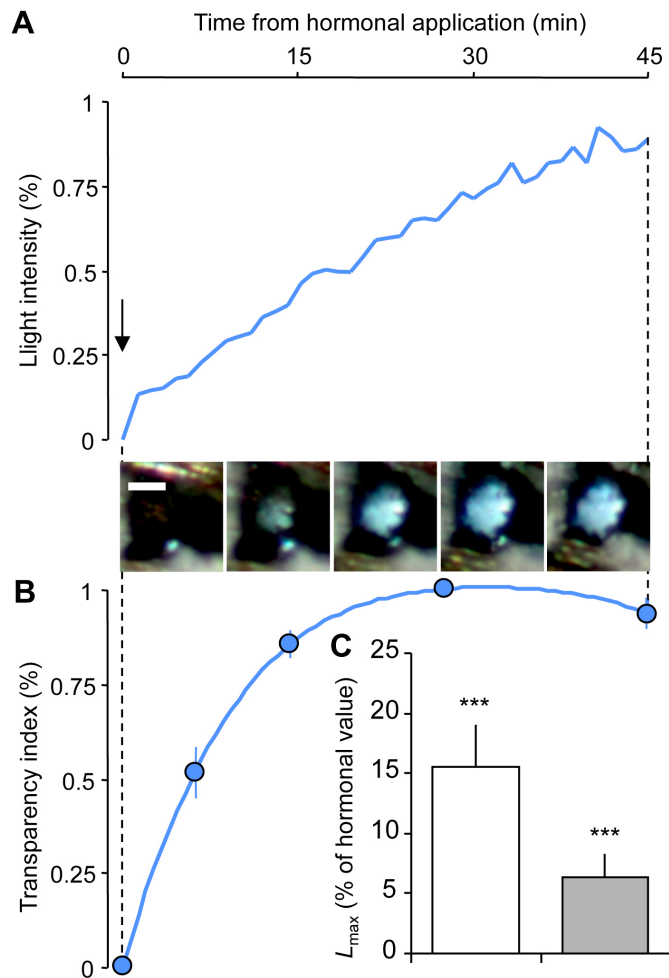


Figure 6 I In vivo effects of bicuculin on the photophores of *Etmopterus spinax*. (A) Relative luminescence recording ($N=8$) after bicuculin application (10^{-3} mol l^{-1} ; arrow). (B) Transparency index after the same bicuculin application ($N=6$ per period of time). Serial pictures at the top illustrate the evolution of a specific photophore after bicuculin application. Scale bar, 50 μm . As MT and PRL (Claes and Mallefet, in press 1), bicuculin application provoked photophore “irising”, which reveals glowing photocytes. (C) Ratio of the maximum intensity ($L_{\max}$) of luminescence induced by bicuculin and the $L_{\max}$ of MT and PRL-induced luminescence. Bicuculin values are expressed as a percentage of MT (white bar) and PRL (grey bar) $L_{\max}$ values ($N=12$ for each hormone). Asterisks indicate significant differences between $L_{\max}$ of bicuculin and $L_{\max}$ of each hormone (*** $P<0.001$).

DISCUSSION

Photophores of the velvet belly lanternshark *E. spinax* appear to be under hormonal control (Claes and Mallefet, 2009b). Nitric oxide (NO) probably allows quick adjustments of this luminescence in response to environmental stimuli (Claes et al., submitted). Here we present evidence that nervous inputs are likely also involved in the photogenesis control of this shark.

Immunohistochemistry supported the presence of GABA in different elements of *E. spinax* photogenic tissue including photocytes, intermediate cells and epidermal cells. In addition GABA-containing nerve-like structures were also found in this tissue. This suggests an involvement of GABA in luminescence control mechanism of this shark. Since application of GABA did not provoke light emission from photophores of this species (Claes and Mallefet, 2009b), this neurotransmitter should act as a luminescence inhibitor or modulator. GABA is the major inhibitory neurotransmitter of the vertebrate nervous system (Kwakowky et al., 2008) and is known to inhibit light emission in the worm *Chaetopterus variopedatus* (Ancil, 1981). However, this molecule is also known to trigger light emission in other animals such as the brittle star *Ophionereis fasciata* (Mallefet et al., 2004).

Results of our pharmacological experiments confirm the involvement of GABA in the control of *E. spinax* luminescence. Indeed, we showed that the GABA_A receptor antagonist bicuculin increases the intensity of the light induced by MT and PRL, and that the light emission induced by these two hormones are partially inhibited by GABA application. GABA seems therefore to act as an inhibitor agent of this shark's photogenesis. Interestingly, contrary to NO, GABA appears to be produced permanently since we demonstrated that the bicuculin injected alone was able, as MT and PRL, to elicit light from this shark's photophores by provoking retraction of the pigmented cells composing the ILS of the photophore i.e. by provoking photophore "irising". This supports the idea that there is an inhibitory GABA tonus in *E. spinax* photogenic tissue, but does not exclude GABA to be also produced on demand.

Light emission induced by a receptor antagonist supports the idea that photocytes are continuously emitting a background light. Permanent light emission is a rare phenomenon in other organisms than bacteria, bioluminescent outputs being generally transitory events (Hastings, 1978). Furthermore, squids and fishes endowed with bacterial photophores generally use mechanical ways (occlusive chromatophore, pigmented shutters, or mobile photophores that can be hidden pigmented pockets) to control their light emission and switch off the light when it is not needed (Herring, 1978; Herring, 1982; Haygood, 1993; Tong et al., 2009).

Photophores of the velvet belly lanternshark *E. spinax* harbour photogenic cells, which start to produce a fluorescent material thought to be photogenic during the embryology. Moreover, this shark is known to be able to produce light before birth (Claes and Mallefet, 2008). This strongly supports the idea that photophores of this species are able to produce light intrinsically, without the help of luminous bacterial symbionts. The spontaneous photocyte luminescence observed in this shark's photophore is therefore intriguing and the exact underlying mechanism needs therefore further examination. However, the intensity of bicuculin-induced luminescence appears is very low and a lot dimmer than the intensity of MT and PRL-induced luminescence. This result therefore supports the previous conception that these hormones also directly target the photocyte in addition to target the pigmented cells of the ILS (Claes and Mallefet, in press 1).

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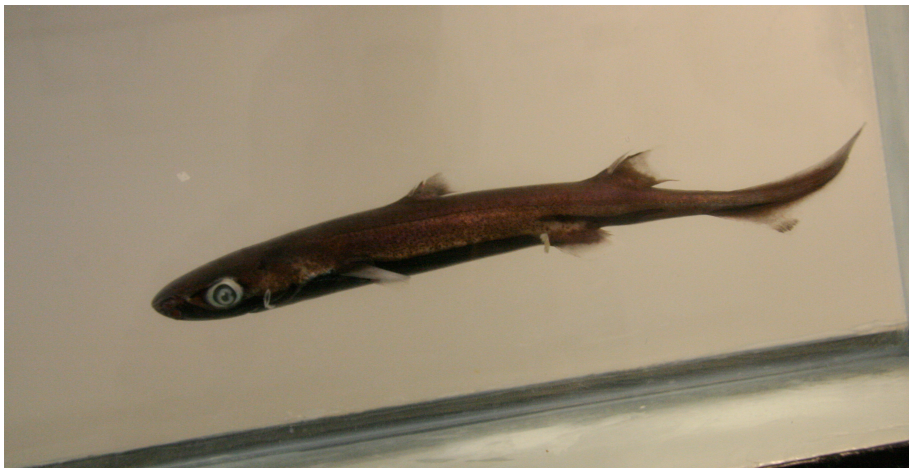
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III. GENERAL DISCUSSION and PERSPECTIVES



Captive velvet belly lantern shark (*Etmopterus spinax*) swimming in a small seawater aquarium. Picture: Jérôme Mallefet.

1 General discussion

The present thesis consists in the first experimental study on shark luminescence. As a consequence, this pioneer work brought numerous new findings, which represent at once the basis and the state-of-the art information available in the literature on shark luminescence function and control mechanism. The present section aims to discuss the choice of model species and methods used to experimentally investigate the ecological role and the physiological control of luminescence from a shark's photophores. In addition, by integrating all the information available from the different chapters, (i) the main putative functions of our model shark's luminescence will be presented in a dynamic view, throughout this shark's life cycle, and (ii) a first model of luminescence control of this species will be provided.

1.1 Methodology

At the beginning of this research, the rare information available on shark luminescence in the literature was descriptive [excepted the spectral measurement of the cookie cutter shark (*Isistius brasiliensis*) luminescence (Herring, 1983), and the mention that peritoneal injection of adrenalin failed to induce luminescence in this species (Herring, 1978)], mostly because this information was based on the observation of dead animals. In order to bring new experimental insights in this field, the first objective was to find a luminous shark species that was abundant, easy to catch, and mainly resistant to the capture and the captivity, in order to perform experiments on living specimen and photogenic tissues. These conditions were filled by the velvet belly lantern shark (*Etmopterus spinax*), a common by-catch etmopterid species from the East Atlantic and the Mediterranean Sea (Coelho and Erzini, 2008) whose Norwegian specimens are known to survive for some days in captivity (Kryvi and Eide, 1977). This species became our model luminous shark species and Norwegian fjords our favourite collection field. The fishing area was situated in the Raunefjord and its adjacent fjords, which were known to shelter an important *E. spinax* population (Kryvi and Eide, 1977) but also to be situated very close to a marine station (Espeland Marine Station) providing the ideal facilities for our research. This preferential location allowed us to rapidly collect (by longlines and bottom trawls) and transport numerous *E. spinax* specimens to the station where they were housed in seawater tanks placed in a dark cold room. During this process, the stress underwent by the sharks was reduced to the minimum since: (i) sharks, lacking swimbladder, are not sensitive to limited pressure changes, (ii) the surface water temperature were similar (especially in winter) than the temperature at depth,

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(iii) after the bringing to the surface sharks were directly placed in dark buckets topped by black plastic bag to avoid light stress, and (iv) the time between the capture and the placement in water tanks was generally inferior to 120 minutes. All these elements guaranteed to furnish animals in a good physiological state for the experiments.

To investigate particular behaviours in deep-sea animals is always challenging due to the limited accessibility of their environment for *in situ* observation and the inherent difficulties, in laboratory conditions, to reproduce conditions encountered by these animals in the wild (Haddock et al., 2010). Therefore, in order to determine the potential ecological benefit of luminescence from *E. spinax* photophores, I decided to mainly study, by a multidisciplinary analysis, the physical characteristics (wavelength, angular distribution and intensity) of this light emission [by adapting the method used in previous works for smaller luminescent species (Warner et al., 1978; Young et al., 1980; Harper and Case, 1999; Jones and Nishiguchi, 2004)] and the spatial organisation of photophores rather than to perform numerous behavioural experiments with the sharks. This approach had the advantages to reduce the misinterpretation of biased, captivity-related behaviour as natural behaviours and to directly exclude luminescent functions that cannot be achieved by the luminescence characteristics of this shark (e.g. a red, or dorsally-directed luminescence is *a priori* not suitable for counter-illumination). In addition, this approach is also less affected by the stress of the capture. Finally, a special effort was made to perform this analysis throughout the whole ontogeny of *E. spinax*, since different sized sharks may use their luminescence for different purposes, as it was shown in the teleost fish *Porichthys notatus* (Harper and Case, 1999).

Recent studies focusing on luminescence control mechanisms of animals generally involved pharmacological study of light response from isolated photogenic organs/cells i.e. photophores/photocytes respectively, sometimes coupled with histological description and/or immunolabelling of these structures (Vanderlinden et al., 2003; Vanderlinden and Mallefet, 2004; Trimmer et al., 2005; Krönström et al., 2005). In this study, the small size (<200 μm ; **Paper I**) of *E. spinax* photophores, prevented us to work on isolated photogenic organs. Instead, we therefore used skin patches containing photophores excised from luminous zones of the shark using a metal cap driller for our pharmacological experiments. The major disadvantage of this approach is the inability to control the exact target of the tested drugs (Vanderlinden, 2006). The structural simplicity of lantern shark photophores—around 12 photocytes enclosed in a pigmented sheath surmounted by an iris-like structure (ILS) and one of several lens cells (Hubbs et al., 1967)—, however, limited the number of available targets, and our approach, which combine pharmacological

results with dynamic microscopic observations as well as immunohistochemical studies, allowed us to identify the ILS as the main target of hormonal [melatonin (MT) and prolactin (PRL)] as well as nervous (GABA) drugs (**Papers V, VIII**) and strongly suggested an action of nitric oxide (NO) on photocytes (**Paper VII**). Another problem linked to the use of skin patches instead of isolated photophores is the fact that the exact number of photophores in each tested skin patch was not always counted at the end of the experiments. In this study, this problem was easily solved by working on relative values of light parameters, obtained by comparing control and tested patches from the same luminous zone (usually the ventral luminous zone) of the same animal since each luminous zone was shown to harbour a constant density of photophores of about the same size to form an homogeneous luminous area (**Papers I, II**). The viability of the skin patches was preserved by placing them into a special shark saline designed for physiological studies (Bernal et al., 2005). The maximum viability of the photogenic skin patches was superior to twelve hours. Since the vast majority of experiments were performed on the skin patches within two hours after their isolation, we can be confident that the tissues were still in good physiological conditions. Moreover, even if the composition of the saline may impact the absolute luminescence performance from the tissue, it would have no effect on the relative values used in the pharmacological analysis (see above), since the effect of saline on control and tested patches are supposed to be identical.

1.2 Ecological function of photophores

Functions of bioluminescence in luminous organisms are very diverse and fall into three main functional categories: predation avoidance, predation help and intraspecific communication (Haddock, 2010).

The analysis of the photophore pattern and the luminescence physical characteristics of *E. spinax* coupled to direct visual observations of spontaneously luminescent captive specimen of this shark lead to the conclusion that this species uses its luminescence for more than one purpose. The versatility of this shark's luminescence appears to increase ontogenetically, with young, immature sharks using mainly their ventral luminous pattern for the anti-predatory function of camouflage (by counterillumination), and large, mature sharks demonstrating a more complex pattern whose characteristics suggest a wide range of potential ecological benefits including anti-predatory functions, prey illumination and intraspecific signalling.

III. General discussion and perspectives

The development of functional photogenic structures before birth (**Paper I**) strongly suggests a great ecological need to be luminous for newborn sharks. Since, at this stage, sharks (i) have already an important (~80%) portion of their ventral surface area covered by photophores (**Paper I**), (ii) harbour luminous zones with similar photophores density hence forming a homogeneous luminescent ventral pattern (**Paper II**), (iii) are planktivorous, targeting mainly krill in the water column (pelagic stage) (Klimpel et al., 2003; Neiva et al., 2006), and (iv) are immature hence not yet involved in reproductive behaviours, they certainly use their luminescence mainly as a way to disappear from their predator's sight in the period of their life cycle during which they are more vulnerable due to their small size and pelagic habits.

The function of camouflage by counterillumination is believed to occur in molluscs (squids), crustaceans and bony fishes. This camouflage technique is particularly demanding, imposing the luminescence to be emitted for extended period of time and to possess the same physical characteristics (wavelength, intensity, angular distribution) than the surrounding light environment of the light-emitting organism (Denton et al., 1985). In a variable light environment, counterilluminating animals are also supposed either to modulate the physical characteristics of their luminescence or to move in the water column to stay cryptic (Warner et al., 1978; Young et al., 1980). As a consequence, an animal whose luminescence fills the particular requirements necessitated by this complex behaviour can be considered to use counterilluminating camouflage (Warner et al., 1978; Young et al., 1980; Harper and Case, 1999; Jones and Nishiguchi, 2004). In this thesis we showed that the ventral luminous zone of newborn and adult *E. spinax* produces a long-lasting light whose intensity is identical to the intensity found in the Raunefjord between 110 and 186 m, and that (at least in adult specimens) the wavelength and angular distribution of this light is similar to those present in the fjord at these depths (**Paper III**). In addition, it was shown that newborn and adult sharks started to produce light after an overhead light (480 nm) stimulation, but, however, were not able to modulate the intensity of their glow over a wide range in response to variation in overhead light intensity. Considering that *E. spinax* is known to perform diel vertical migrations in the water column, which is supported by nocturnal observations of this species at shallow water (Heidler, 2006; Moen and Svensen, 2004), hence that this shark stays in a specific isolume in which it does not need to modulate its glow intensity, this sharks fulfils all the conditions to use its luminescence to cloak its silhouette from prey and predators, in Norwegian fjords at least (**Paper III**). This result is particularly interesting since (i) it is the first time that counterillumination is shown in a cartilaginous fishes, and (ii) sharks does not

appear to possess the complex filters and reflectors of counterilluminating teleosts (Hubbs et al., 1967; Denton et al., 1972; 1985). Instead they appear to achieve the convenient angular distribution by a gradual transversal morphological change in the orientation of their photophores [to maximise downward photic emission; **Paper III (complementary results)**] and to use a luminous substrate whose spectral emission peak is close to the peak of their ventral glow i.e. 486 nm, such as the coelenterazine (whose spectral emission peaks around 480 nm; Shimomura, 2006) which has been found in other mesopelagic teleost fishes known to counterilluminate (Denton et al., 1972; Mallefet and Shimomura, 1995).

Although counterillumination is certainly the function of the ventral luminous zone (as well as the rostral and mandibular luminous zones) of these sharks, other experimental evidences demonstrated that other part of the luminous pattern i.e. other luminous zones produce a luminescence whose characteristics are not convenient for camouflage by counterillumination due to the density, position and orientation of their photophores [**Papers II, III (complementary results)**]. These zones are associated to the tail and fins (dorsal, pectoral, pelvic and caudal), and are designed to produced either dorsally or laterally directed light with a high intensity (due to their high photophore density), forming important visual clues in the permanent darkness of the deep-sea where *E. spinax* lives. In addition, observations of captive sharks rotating their body in a transversal plane when swimming strongly suggests that these sharks may transform their long-lasting glow in flashing signals [**Paper III (complementary results)**]. In other instance, sharks were seen to emit light when swimming with their belly upward, which in the wild would have the consequence to increase the contrast between the shark and the dark background (personal observation). These elements are strongly in favour of a function of intra-specific signalling for cohesive swimming/hunting, behaviours that certainly occur in lantern sharks (Springer, 1967; McPherson, 1980; Reif, 1985), especially when they become eaters of large prey such as fishes and squids i.e. around 20 cm in total length in the case of *E. spinax* (Klimpel et al., 2003; Neiva et al., 2006; **Paper II**). In addition, evidence for sexual signalling is given in this species by the presence of a high photophore density hence brighter luminescence in the sexually dimorphic pelvic areas of mature specimens, which also harbour a differential hormonal control between sexes (**Papers II, VI**). To be able to visually recognise a potential mate and also, to have luminous clues that enhance visual localisation during mating (which involves internal fertilisation in sharks; Wourms, 1977) is certainly a great advantage in the permanent darkness of the deep-sea. Furthermore numerous other fish species are believed to use sexual signalling including pony fishes and lantern fishes, whose luminescence has also been shown to serve as camouflage (Young et

III. General discussion and perspectives

al., 1980; McFall Ngai and Morin, 1991, Sasaki et al., 2003; Herring, 2007). Direct observations of natural behaviours (with R.O.V., for example) could confirm these hypotheses. Finally, the presence of photophores just behind the two dorsal finspines [on the margin of the fins; **Paper III (complementary results)**], are probably used to lighten the spines, which would allow (i) to signal the position of the spines to conspecifics, preventing them to get hurt in social behaviours (mating, schooling, etc.) but also (ii) to warn predators of the danger to attack and eat the shark, in a form of a particular luminous aposematism. In these cases, the translucent spines probably act as a focal lens. Luminous aposematism has been demonstrated in invertebrates (Grober, 1988; De Cock and Matthysen, 2000; 2003) but never in vertebrates, and in the case of *E. spinax*, would be difficult to confirm since it implies to test the different conditions required for aposematism in laboratory. *E. spinax*'s non-profitability can be assumed by the presence of the spine but advantage against a non luminous shark, predator learning and initial reluctance [**Paper III (complementary results)**], would impose in particular to work with quite large predators since this aposematism probably only occurs in large velvet bellies whose spines are big enough to provoke damages (personal observation).

The luminescence of *E. spinax* appears then as a versatile tool used in numerous functions throughout the life cycle of this shark (**Fig. 1**). These apparent great ecological advantage of the phenomenon in this shark leads to the following questions: (i) why aren't there more luminous deepwater shark species [only about 15% of them are luminous; **Review on shark luminescence** (see Introduction 3.5)], and (ii) how can other sharks manage to accomplish their life cycle without being luminous? These questions are difficult to answer but it can be stated that luminescence certainly brings numerous advantages to *E. spinax* (and likely to other luminous sharks) but is probably not essential for its survival. The ability to produce light likely allowed lantern sharks (Etmopteridae) to rapidly radiate and colonize the deep-sea environment at the end of the Cretaceous (Adnet and Capetta, 2001), and may explain the apparent success of this group which contain numerous species (more than 30 currently described species for the genus *Etmopterus*) which are present in virtually all the planet's oceans (Compagno et al., 2004). The incredible number of *E. spinax* found in the Raunefjord (this sharks generally composed 100% of our captures with longlines) illustrates the adaptive advantage of some species of lantern sharks. Other successful non-luminous deepwater sharks may simply have developed different deep-sea adaptations (e.g. an elongated snout filled with sensory receptors such in sharks of the prolific genus *Apristurus*; Compagno et al., 2004) and use pheromonal instead of luminescent signals (Johnson and Nelson, 1978; Pratt and Carrier, 2001).

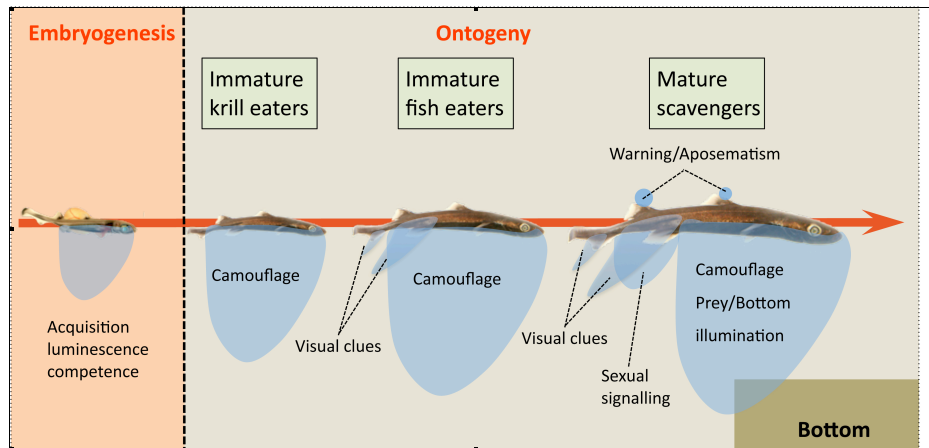


Figure 1 | Potential functions of lantern shark (*Etmopterus spinax*) photophores throughout its life cycle (red arrow). The luminescence is acquired early in the life of this shark (before birth), would mainly serve as camouflage by counterillumination in newborn sharks, and would be involved in other functions in larger individuals such as intraspecific signalling with visual clues for cohesive swimming/hunting and reproduction (in mature specimens), lightening of the two dorsal finspines to warn conspecifics and potential predators (aposematism), and bottom lightening which certainly could help large sharks to find their food including benthic species and remnants of dead animals (scavenging). This increasing number of potential functions is allowed by an ontogenetic increase in the luminous pattern heterogeneity.

1.3 Physiological control of photophores

Involved in numerous ecological functions, luminescence needs to be precisely controlled to advantage an animal during its life cycle (Buck, 1978; Warner et al., 1979; Young et al., 1980; Denton et al., 1985; Branham and Greenfield, 1996; Wilson and Hastings, 1998; Harper and Case, 1999; Demary et al., 2006; Rivers and Morin, 2008). While the physical characteristics (wavelength, intensity and angular distribution) of animal luminescence are generally controlled by accessory structures (including reflectors, absorption/interference filters, lenses) the time course of light production is under direct nervous control in animals possessing intrinsic photophores (Case and Strause, 1978; Vanderlinden, 2006; Krönström, 2009; **Paper IV**). Animals with bacterial photophores usually modulate the continuous light emission of their bacterial symbionts either by changing the physicochemical characteristics (osmolarity, glucose, O₂ availability, etc.) present in the bacterial growth medium in the photogenic organ, or by occluding this organ via mechanical

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ways including mobile dark shutters, chromatophores or rotation into a dark pocket (Bertelsen, 1951; Herring, 1978; Herring, 1982; Haygood, 1993; Munk, 1999).

In this work, we investigated the physiological control of luminescence from *E. spinax* photophores by different techniques including pharmacology/luminometry, immunohistochemistry and dynamic microscopic examination under binocular. Luminescence control mechanisms can be complex (Trimmer et al., 2001; Krönström et al., 2005; Vanderlinden, 2006; Krönström et al., 2007; 2009), and using different complementary methods to study them allows an integration of the experimental information to end up in a coherent model.

1.3.1 Extrinsic control

Photophores of the velvet belly lantern shark present photocytes that contain vesicles with a fluorescent photogenic material, and start to produce light before birth (**Paper I**), they are therefore considered to be of intrinsic origin. Since bony fishes with intrinsic photophores harbour a direct (mostly adrenergic) control of their light emission, we first tried to induce luminescence from *E. spinax* photophores by classical neurotransmitters [(nor)adrenaline, acetylcholine, 5-HT and GABA] application (**Paper IV**). This first neural screening failed to induce luminescence from this shark's photophores, we therefore tried a hormonal screening with hormones involved in Elasmobranch skin color change (MT, PRL and α -MSH; Gelsleichter, 2004). This second screening showed what is probably one of the most important results of the present work: luminescence from *E. spinax* photophores is hormonally controlled: MT and PRL are responsible of light inductions with different time courses, which are inhibited by a simultaneous application of α -MSH (**Paper I**).

Separate applications of MT and PRL on the different luminous zones forming the complex pattern of this shark show that (i) all zones started to luminesce after hormonal application, which indicate a common hormonal control for the whole luminous pattern, (ii) the maximum intensity (and quantity) of light produced was directly dependent of the photophore density of the zone, (iii) the time to reach the peak of light emission was different from one zone to another, independently from their photophore density, which indicates that an additional mechanism regulates the hormonal action on the photophores (**Paper VI**).

Potential candidates involved in a regulation of the primary hormonal control of luminescence from *E. spinax* photophores were investigated by

immunohistochemistry. This technique revealed the presence of numerous nitric oxide synthases (NOS) and nerve-like structures, including GABAergic nerves, in the photophores (**Papers VII, VIII**). We therefore decided to pharmacologically investigate the role of nitric oxide (NO) and GABA in the control of light production in *E. spinax*. Results confirmed the involvement of these substances in this control mechanism.

NO appeared to have a modulatory role, decreasing time to reach the light emission induced by both stimulatory hormones (MT and PRL) and the maximum intensity of light triggered by PRL, but having no effect *per se* on light production from *E. spinax* photophores (**Paper VII**). This suggests the NO to be produced on demand to modulate hormonal-induced luminescence. A similar transitory neuromodulatory role of NO has been found on the luminescence of the krill and in the fishes *Argyrops leucostomus* and *Porychthys notatus* (Krönström et al., 2005; 2007; Krönström, 2009). Results on *E. spinax* confirm the wide involvement of NO in the control of luminescence of fishes (Krönström and Mallefet, 2009). The rapid action, short half-life and transmembrane diffusion properties of NO (Jacklet, 1997) probably allowed this molecule to be selected many times during the course of evolution as a preferential modulatory substance for rapid fine tuning of luminescence.

GABA application did not induce luminescence from the shark's photophores (**Paper IV**). We therefore postulated an inhibitory (or modulatory) role for this substance in the luminescence control of *E. spinax*. Application of bicucullin (GABA_A antagonist) on the different luminous zones composing the luminous pattern always provoked light emission from the shark's photophores, and the intensity of this light emission was directly dependent on the photophore density of the luminous zone, which lead us to propose the presence of an inhibitory GABA tonus (**Paper VIII**). These results also suggest that photocytes of this shark's photophores are continuously glowing, which is a rare phenomenon in non-bacterial (intrinsic) photophores (Hastings, 1978).

The targets of hormones and neurotransmitters involved in the control of *E. spinax* photogenesis have been highlighted by immunohistochemistry and dynamic microscopic examination under binocular. MT and PRL have been shown to induce pigment retraction in melanophores present between photocytes and lens cells which forms an iris-like structure (ILS), triggering photophore "irising" and emission of luminescence (**Paper V**). In addition these two hormones probably also directly target the photocytes, since the hormonal luminescence peaks before maximum

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photophore “irising” (**Paper V**). Since α -MSH appeared to inhibit the effect of these two hormones, and induced pigment dispersion in melanophores of other Elasmobranch species (to induces skin darkening; Gelsleichter, 2004), we can make the valuable suggestion that this substance provokes pigment dispersion in the ILS, therefore occluding the light emitted by the photocytes. This mechanical luminescence control, which has no equivalent among intrinsic photophores of fishes (Herring and Morin, 1978; **Papers IV, V**), is certainly a consequence of (i) the apparent continuous light emission of photocytes (**Paper VIII**), and/or (ii) the use of hormones that were originally involved, by their action on melanophore pigments, in the elasmobranch skin coloration (Gelsleichter, 2004). The strong NOS immunoreactivity and NADPH diaphorase activity found in photocytes suggest these photogenic cells to be the main target of NO (**Paper VII**). Finally the strong GABA immunoreactivity in cells present between photocytes and lens cells (=pigmented cells of the ILS), and microscopic examination showing ILS’s pigment dispersion after bicucullin application suggest that the main target of GABA is also the ILS. Luminescence induced by bicucullin application is, however, a lot weaker than hormonal-induced luminescence, which supports the idea that photocytes are another potential target of MT and PRL (**Paper VIII**).

Effects of MT appear to be mediated by the binding to the MT2 receptor subtype since a prior application of luzindole (MT1/MT2 receptor antagonist) and 4P-PDOT (MT2 receptor antagonist) greatly inhibited the MT-induced luminescence from *E. spinax*’s photophores (**Paper IV**). The PRL receptor (PRLR) is likely, of course, to be present in the photogenic tissue but the current absence of commercial PRLR antagonist prevented us to perform pharmacological experiments to confirm it. Action α -MSH on melanophores probably involves binding to MC1 receptors (Mountjoy et al., 1992; Gelsleichter, 2004) but this was not tested. Mediation of NO effects does not involve receptor binding since this substance freely diffuses through cytoplasmic membranes (Jacklet, 1997). Finally the effects of GABA on photocyte luminescence are mediated through the binding to the GABA_A receptor since (i) application of bicucullin provoked photophore luminescence and (ii) application of phaclofen (GABA_B receptor antagonist) had no effects on photophore luminescence (**Paper VIII**).

1.3.2 Intrinsic control

Some of the intrinsic (=intracellular) pathways of the different substances involved in the luminous control of *E. spinax* have been identified during this study. The MT2 receptor appears to be negatively coupled to the cyclic AMP (cAMP) since the

application of forskolin (an activator of adenylyl cyclase) strongly inhibits MT-induced luminescence (**Paper IV**). The PRL receptor appears to be coupled to the Janus kinase 2 (JAK2, the most common intrinsic pathway of PRLR; Bole-Feysot et al., 1998), since an inhibitor of this enzyme (1,2,3,4,5,6-Hexabromocyclohexane) strongly inhibits PRL-induced luminescence (**Paper IV**). The intrinsic pathway of α -MSH has not been investigated in this study but the binding of to MC1 receptor involves activation of adenylyl cyclase (Gilles, 2006). The effects of NO are probably mediated by cyclic GMP (cGMP) since the effects of this second messenger on hormonal-induced luminescence are similar than those of NO (**Paper VII**). Finally the GABA_A receptor known to be an ionotropic receptor that classically provokes the entry of ions Cl⁻ inside the cell (Gilles, 2006), but this was not tested by experiments in the present study.

1.3.3 Integrated model

The different results obtained during the study allow to establish a first integrated model of the luminescence control mechanism of *E. spinax* photophores (**Fig. 2**), which is also the first described luminous control mechanism of a cartilaginous fish. This model ends up on a unique luminescence control mechanism among luminous organisms investigated so far. Indeed, this mechanism not only involves a dual control of neurotransmitters/neuromodulators and hormones, but also demonstrates a mechanical light switch in intrinsic photophores.

The duality of *E. spinax*'s photophore control is amazing and probably reflects in some way the evolutionary history of luminescence competence acquisition in lantern sharks. Etmopteridae are believed to have left shallow waters to colonize the deep-sea environment after the Bonarelli anoxic event, during the Cretaceous (Adnet and Capetta, 2001). The hormonal inputs involved in the luminescence control of the velvet belly lantern shark are the same than those involved in the physiological colour change of shallow water Elasmobranchs, both mechanisms involving the translocation of melanophore pigments (Gelsleichter, 2004; **Papers IV, V**). We can therefore make the valuable suggestion that this lantern shark's photogenesis control is actually the result of a co-opting of existing structures (including melanophores) involved in the ancestral concealment mechanism of its shallow water relatives. During their odyssey to the deep-sea, lantern sharks would have turn their initial crypsis mechanism, inefficient in the permanent darkness of this environment, into a deepwater luminescent camouflage (**Paper V**). At the same time or later, their luminescent patterns became more complex and started to be used in other intra and interspecific functions.

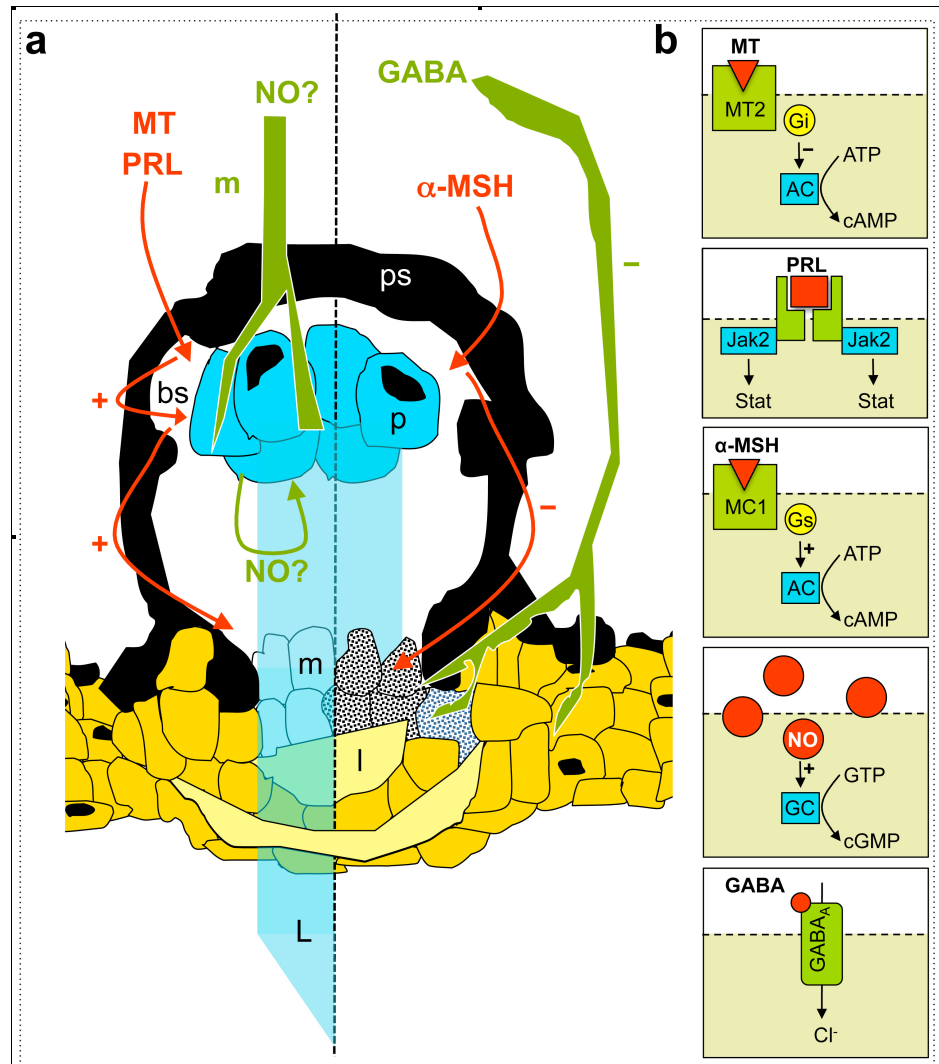


Figure 2 | Proposed model for luminescence (L) control mechanism of *E. spinax*. **a**, main actors and targets (extrinsic control pathways) involved in the photophore aperture (pigment retraction in melanophores; left) and photophore closing (pigment extension in melanophores; right). The photophores are controlled by hormonal (MT, PRL and α -MSH) and neural (NO, GABA) inputs. Nerves are presented in green and putative hormonal pathways are represented by red arrows. **b**, intrinsic control pathways). α -MSH, α -melanocyte stimulating hormone; AC, adenylyl cyclase; bs, blood sinus; GC, guanylyl cyclase; l, lens cell; m, melanophore; MT, melatonin; p, photocyte; PRL, prolactin; ps, pigmented sheath.

III. General discussion and perspectives

The complementary neural inputs involved in *E. spinax* photophore control mechanism (**Papers VII, VIII**) probably evolved in a second time, driven by the need for a precise fine-tuning of light emission in a dark environment where uncontrolled light displays rapidly attract the attention of predators. Their action, combined with the action of (i) PRL and α -MSH, whose release is dependant upon direct visual perception and (ii) MT which is mainly released by non-visual perception of light (by the pineal gland), and can be used as a an internal clock, provide a complex framework that supports the numerous suggested functions of *E. spinax* luminescence: counter-illumination (**Papers I, III**); aposematism, prey illumination [**Paper III (complementary results)**]; group swimming/hunting and sexual signalling [**Papers II, III (complementary results), VI**].

2 Perspectives

Contrary to what was once believed, luminescence in sharks is certainly not a burden (Reif, 1985). This study reveals that, in the velvet belly lantern shark (*E. spinax*), this phenomenon is certainly involved in numerous functions of great ecological interest through the life cycle, and is controlled by a unique mechanism that involves a dual control and a mechanical light switch of intrinsic photogenic organs.

Ecological functions of luminescence in *E. spinax* should be tested by (i) theoretical modelling, (ii) behavioural observations/experiments on captive specimens and (iii) direct observations of natural behaviours in the field, via the use of a R.O.V. coupled to a light-intensified camera for example. In particular, it will be interesting to determine the function of the photophores illuminating the finspines since they may provide an explanation for the apparent ecological success of this species, which appears to have few natural predators and to be quite abundant in the different parts of its distribution area.

Immunohistochemical studies with antibodies targeting the receptors of the different elements involved in the luminous control of this shark will help to confirm their targets. The slow kinetics of hormonal-induced luminescence in this species also gives the opportunity to analyse, by light and electron microscopy, the cellular and intracellular rearrangements occurring in the photophores during a light emission.

Since the blood concentration of circulating melatonin and prolactin are known to vary seasonally (Falcon et al., 2007), it would be interesting to determine if hormonally induced luminescence expression also follows a seasonal pattern. This can be achieved by completing data taken during the present study, which cover four different months (**Fig. 3**). Seasonal variations in hormonally induced luminescence can be related to different steps of the life cycle (mating season, birth, etc.). It would also be particularly judicious to investigate ontogenetic variations in hormonally induced luminescence. For that purpose, data on immature individuals are particularly needed since the present study focused mainly on mature sharks (**Fig. 4**). Investigating seasonal and ontogenetic variations on hormonally induced luminescence will help to improve our comprehension of luminescence importance throughout major transitions (diet shifts, maturity) encountered by *E. spinax* throughout its life cycle.

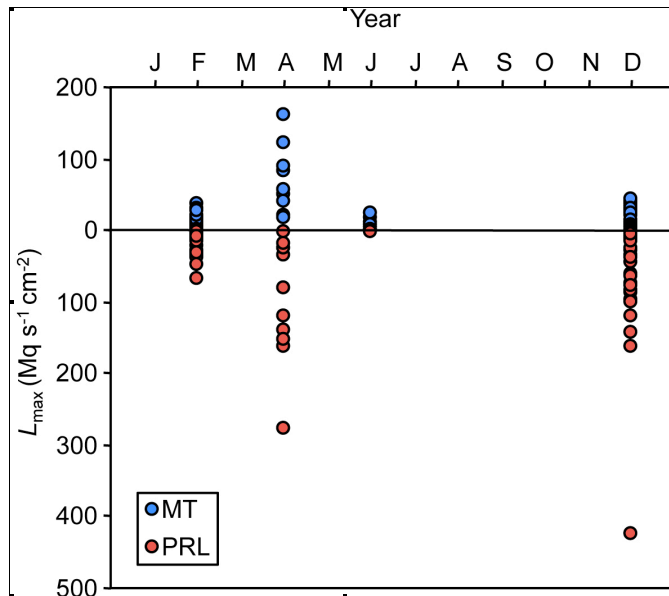


Figure 3 I Maximum intensity ($L_{\max}$) of hormonally induced luminescence from *Etmopterus spinax* photophores for the different collection months. MT, melatonin. PRL, prolactin. Number of sharks tested: February, MT = 17, PRL = 20; April, MT = 11, PRL = 10; June, MT = 7, PRL = 6; December, MT = 43, PRL = 43.

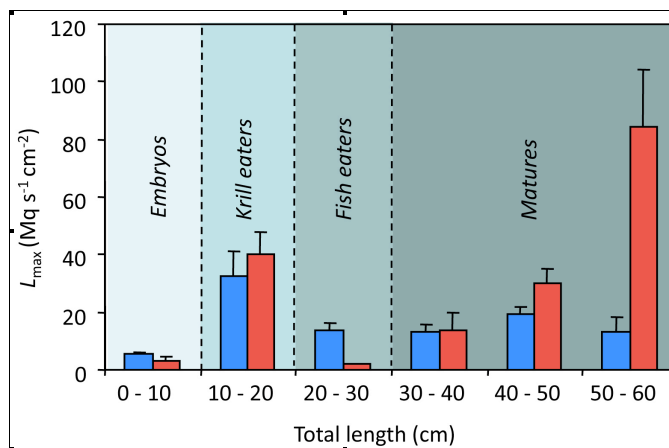


Figure 4 I Maximum intensity ($L_{\max}$) of hormonally induced luminescence from the photophores of different size classes of *Etmopterus spinax*. Melatonin (MT), blue bars ; prolactin (PRL), red bars. Number of sharks tested: 0 - 10 cm, MT = 4, PRL = 4; 10 - 20 cm, MT = 17, PRL = 18; 20 - 30 cm, MT = 2, PRL = 1; 30 - 40 cm, MT = 15, PRL = 15; 40 - 50 cm, MT = 55, PRL = 56; 50 - 60 cm, MT = 4, PRL = 5.

III. General discussion and perspectives

Finally, the results obtained on *E. spinax* are surprising and encouraging: they underline the potential interest to experimentally investigate the control and functions of luminescence in other luminous shark species such as other Etmopteridae but also Dalatiidae. A comparative study of shark luminescence would allow (i) to understand the biotic and abiotic factors that determine the expression of luminescence in sharks, and (ii) to establish the evolutionary history of this phenomenon in these fishes.

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Sunrise on the Raunefjord, Norway (December 2009). Picture: Julien M. Claes

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