

Ultrastructure and biochemistry of the light-emitting
system of lantern shark (*Etmopterus spinax*)
photophores

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Summary

Bioluminescence, i.e. the ability for living organisms to emit light, is a widespread phenomenon in deep-ocean, due to valuable ecological advantages it provides to organisms endowed with this capability. Six percent of deep-sea cartilaginous fishes (sharks, rays, skates and chimaeras) are luminous. Among them, the lantern shark *Etmopterus spinax* Linnaeus, 1758 is one of the most commons and can be easily caught. For this reason, it has recently become a model species for studying bioluminescence in shark. Although various aspects (functions and control) of this complex phenomenon are now better understood in this species, the photocyte (i.e. the light-emitting cell) mechanism of light emission remains unsolved. This is the subject addressed in this work.

In the first chapter, photophore ultrastructure is explored since it is a key step in investigating an unknown bioluminescent mechanism. By using transmission electron microscopy (TEM), we were able to describe photophore fine organization (Paper 1): a new structure, a reflector, which was believed to be absent in shark photophores, was highlighted. Photocytes were found to be divided into three distinct areas, of which the apical one, oriented toward the photophore center and containing numerous granular inclusions, was proved to be the light-emitting area (Paper 2). By investigating photocyte ultrastructural changes during the course of a light-emission event, we proved granular inclusion to represent shark microsource (i.e. the intracellular light-emitting site, where the chemiluminescent reaction takes place), and we proposed the term “*glowon*” to characterize this novel particle (Paper 2). In the second chapter, we addressed *E. spinax* feeding habits in order to identify putative luminous preys and analyze whether the shark could obtain a known luciferin (i.e. the luminous substrate) through its diet and use it for its own light production. The results we obtained (Paper 3) suggested *E. spinax* does not use a known luciferin but might rather use a novel molecule as substrate or an unknown storage or active form of a known luciferin. The isolation and purification of putative new luminous molecules (luciferin and luciferase –i.e. the reaction enzyme) are initiated in the third chapter of which the purpose is to provide basis experiments and reflection for the future complete characterization of this system. Finally, all results were integrated and discussed together with regard to previous findings on bioluminescence functions and control in this shark species.

Résumé

La bioluminescence, la capacité d'un organisme à émettre sa propre lumière, est un phénomène largement répandu dans les profondeurs de l'océan de par les avantages écologiques qu'il apporte à ces organismes. Six pour cent des poissons cartilagineux (requins, raies, chimères) sont lumineux. Parmi eux, le requin lanterne *Etmopterus spinax* Linnaeus, 1758 est l'un des plus communs et peut être facilement collecté. Pour cette raison, il est récemment devenu l'espèce modèle pour l'étude de la bioluminescence chez les requins. Bien que plusieurs aspects de ce phénomène complexe soient maintenant mieux compris chez cette espèce (contrôle et fonctions), le mécanisme d'émission de lumière des photocytes (les cellules émettrices) reste incompris. C'est le sujet abordé dans ce travail.

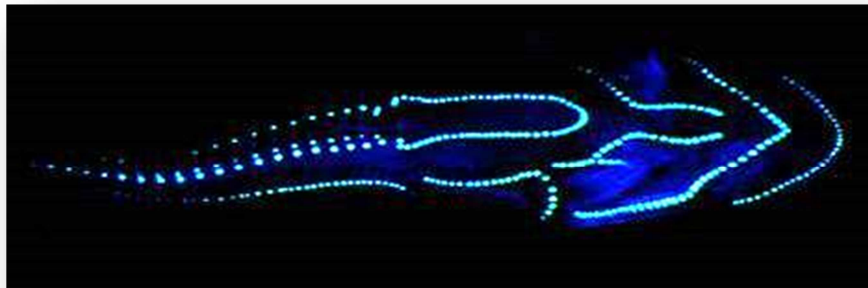
Dans le premier chapitre, l'ultrastructure des photophores est analysée car il s'agit d'une étape clé dans l'étude d'un système lumineux encore inconnu. Par l'utilisation d'un microscope électronique à transmission (TEM), nous avons pu décrire l'organisation précise du photophore (Article 1) : un réflecteur, une structure que l'on croyait absente dans les photophores des requins, a été découvert. Le photocyte a été subdivisé en trois régions cytoplasmiques distinctes. La zone d'émission de lumière est la partie apicale, orientée vers le centre du photophore et remplie d'inclusions granulaires. En analysant les changements ultrastructuraux à l'intérieur du photocyte, nous avons montré que les inclusions granulaires représentent les microsources (c'est-à-dire les sites intracellulaires où la réaction de chimiluminescence a lieu) et avons proposé le terme « glowon » pour qualifier ces nouvelles structures (Article 2). Dans le second chapitre, le régime alimentaire d'*E. spinax* est analysé afin d'identifier ses proies lumineuses et de savoir s'il pourrait acquérir sa luciférine (le substrat de la réaction) via son alimentation. Les résultats obtenus (Article 3) suggèrent qu'*E. spinax* n'utilise aucune luciférine connue mais plutôt un substrat encore inconnu, ou une nouvelle forme active ou de stabilisation spécifique d'une luciférine connue. L'isolation et la purification de ces nouvelles molécules (luciférine et luciférase, l'enzyme de la réaction) sont initiées dans le chapitre 3. Le but de ce chapitre est de fournir les expériences de base et la réflexion nécessaire à la future caractérisation complète de ce système. Finalement, les résultats ont été intégrés et discutés ensemble, en fonction des résultats obtenus précédemment quant au contrôle et aux fonctions de la bioluminescence chez les requins lumineux.

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INTRODUCTION



Porichthys notatus in vivo fluorescence (J. Mallefet)

INTRODUCTION

In the first part of this introduction, bioluminescence will be addressed in order to allow readers to better understand its various aspects (functions, mechanisms, morphologies and control). Because the model species of this work is a shark, the second part of the introduction will be devoted to basic knowledge on these animals (evolution and biology). Finally, both topics will be gathered to provide an overview of luminous sharks at the end of the introduction.

GENERALITIES ON BIOLUMINESCENCE

Origin and diversity of bioluminescence

The phenomenon of bioluminescence has been observed for many years and referred several times in civilizations ancient literature and myths. Aristotle described it as “cold light” in his book “*De Anima*”. Thereafter, while explorers travelled around the world, bioluminescence was regularly observed (Christopher Columbus observed mysterious light in water and later, Charles Darwin witnessed a milky sea during his travel on the *Beagle*) and began to be studied. In 1667, Robert Boyle demonstrated the oxygen requirement for luminescence and in 1887, Raphael Dubois made great advance in bioluminescence chemistry by extracting the two main compounds of the chemiluminescent reaction, the *luciferin* and the *luciferase* (Harvey, 1957; Hasting, 1983; Poisson, 2010). Nowadays, this phenomenon is well understood and is defined as “light produced by living organisms” or “living light”. In the first part of the 20th century, E. Newton Harvey undertook the first comprehensive synthesis on bioluminescence knowledge which he published it in his reference book from 1952 (Harvey, 1952).

Although bioluminescence is rare in terms of species number, it is phylogenetically largely distributed since approximately 700 genera encompassing all kingdoms, except archaeas and plants, possess members endowed with this astonishing capability (Hastings, 1995; Widder, 2010). From them, 80% inhabit marine environments where bioluminescence is represented across major taxonomic groups

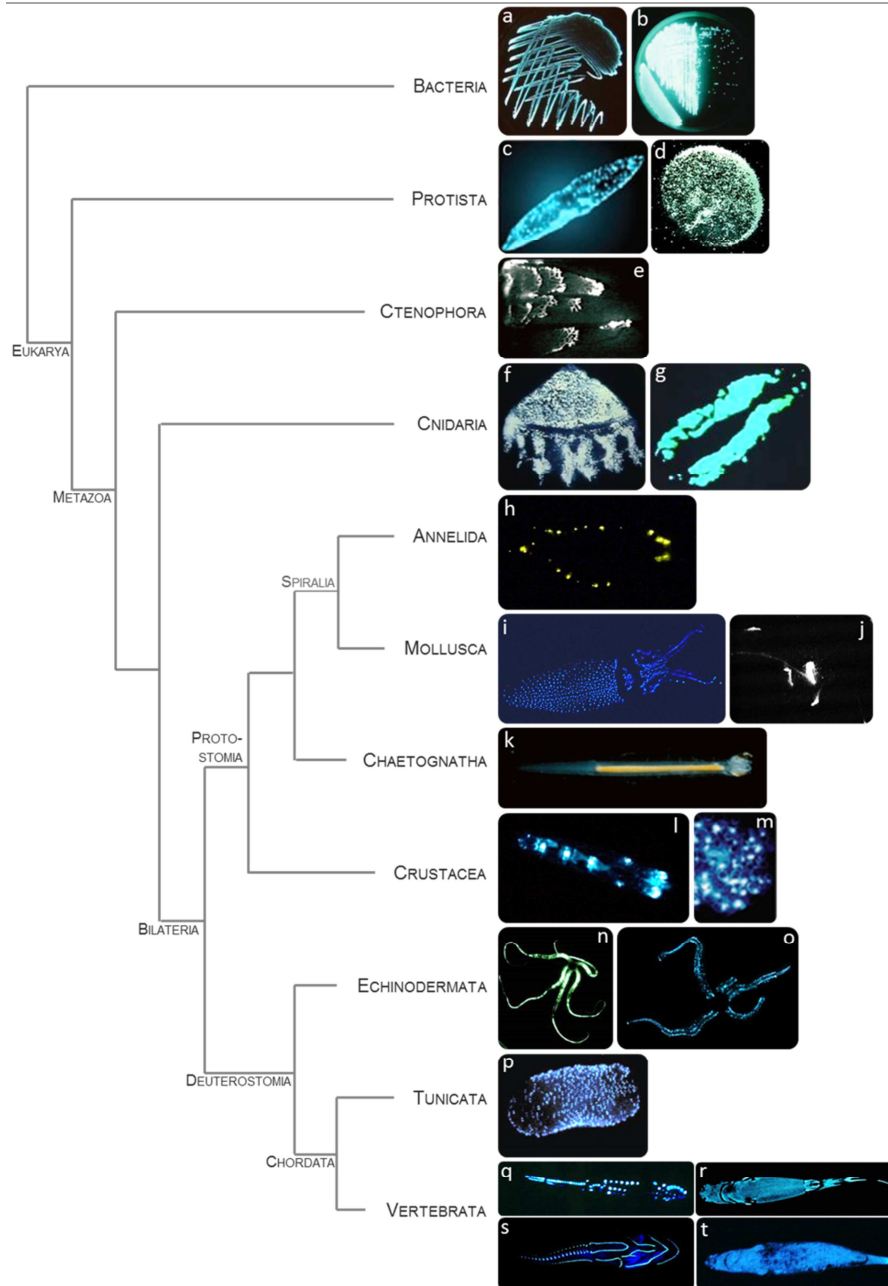


Fig. 1 Simplified marine bioluminescence tree of life with representative luminous species as examples. **a** *Photobacterium phosphoreum*. **b** *Vibrio fischeri*. **c** *Pyrocystis fusiformis*. **d** *Noctiluca scintillans*. **e** *Beroe forskalii*. **f** *Periphylla periphilla*. **g**

Pennatula rubra. **h** *Tomopteris helgolandica*. **i** *Watasenia scintillans*. **j** *Vampyroteuthis infernalis*. **k** *Caecosagitta macrocephala*. **l** *Meganyctiphanes norvegica*. **m** *Vargula hilgendorffii*. **n** *Ophiopsila aranea*. **o** *Amphipholis squamata*. **p** *Pyrosoma atlanticum*. **q** *Maurollicus muelleri*. **r** *Etmopterus spinax*. **s** *Porichthys notatus*. **t** *Squaliolus aliae*. Pictures: J. Mallefet (**a,d,f,g,l,n,o,q,r,s,t**); A. Gouveneaux (**h**); Widder, 2010 (**c,m,p**); Dunlap & Tsukamoto, 2006 (**b**); Haddock *et al.*, 2010 (**e,k**); Robison *et al.*, 2003 (**j**); Arkive (<http://www.arkive.org/>) (**i**). All pictures show animal's *in vivo* bioluminescence or fluorescence except **k** which is a white light picture. Modified from Haddock *et al.*, 2010.

(including bacteria, protists, ctenophores, cnidarians, annelids, mollusks, chaetognaths, crustaceans, echinoderms, tunicates and fishes - **Fig 1**) and occurs from coastal shallow waters to deep abyss (Morin, 1983; Haddock *et al.*, 2010; Widder, 2010). Dimness waters have the highest luminous organisms density and it has been estimated that 70% of deep-sea Osteichthyes were luminous (Herring, 1978). On the opposite, only few luminous organisms are found in fresh water environments (e.g. the limpet *Latia neritoides*) and terrestrial bioluminescence is mainly known through fireflies and fungi, although some earthworms, beetles or insect larvae also are luminous. Both fresh water and terrestrial bioluminescence will not be addressed in this study. Why seas and oceans account for major part of bioluminescent organisms is not fully understood yet but a hypothesis proposes the oceanic conditions (deep ocean is a stable environment with the greatest dim area and clear optical properties) to have favored bioluminescence evolution (Haddock *et al.*, 2010).

How organisms are able to produce their own light is one of the major questions arose by scientists. Briefly (this question will be later developed), bioluminescence occurs in particular cells where a chemical reaction takes place, involving oxidation of a light-emitting molecule (the *luciferin*) by an enzyme (the *luciferase*) and the subsequent emission of a photon (Henry & Michelson, 1978; Wilson & Hastings, 1998). Bioluminescence evolutionary origin is not completely understood and two hypotheses are currently proposed (Haddock *et al.*, 2010; Widder, 2010). The first one suggests a substrate-based origin: most luciferins, such as *coelenterazine*, have antioxidant properties and might have originally served in defense pathways against oxidative stress. When animals colonized the deep ocean, and encountered a reduced oxidative stress, these molecules might have been turned into other purposes, i.e.

light production (Rees *et al.*, 1998). On the opposite, the second hypothesis, based on enzymes evolution, proposes luciferase mutations to have switched their role in the cell from their original oxygenases function toward the light production catalyse. Anyhow, the current diversity of bioluminescent mechanisms suggests bioluminescence to be one of the most spectacular examples of convergent evolution. By summing the number of distinct light-producing systems across monophyletic lineages, Haddock *et al.* (2010) came to a conclusion that bioluminescence have independently appeared more than 40 times in the course of evolution!

Ecological functions of bioluminescence

All organisms emit light with the aim to reach the photodetection system of another organism in order for this latter to perform a specific behavior in return. Following the identity of both organisms (conspecifics, prey and predator) and the purpose of the light emission, bioluminescence serves many functions that are classically classified into three groups: defense, help in predation and intraspecific communication (Morin, 1983; Campbell, 1989; Hastings, 1995; Haddock, 2010).

Defense includes many mechanisms helping prey to escape their predators and is the prevalent bioluminescence function. Prey can emit a bright flash that will scare off the predator and make it hesitate (a technique called “startle effect”), or secrete a luminous fluid that will make itself difficult to be tracked by the predator (Morin, 1983; Robison *et al.*, 2003; Haddock *et al.*, 2010). More complex mechanisms have also been described, such as sacrificial tag, burglar alarm, aposematism or counterillumination.

Sacrificial tag: animals using this technique are able to autotomize a part of their body that continues to glow while the prey itself stays non luminous. As a result, the predator is attracted by the luminous tag and the prey can escape and regenerate its lost body part. This function has been particularly documented in ophiuroids (some species can autotomize an arm part - Deheyn *et al.*, 1998, Jones & Mallefet,

2013) and scale worms (which are known to autotomize luminous scales - Herrera, 1979; Morin, 1983) but also in squids (Robison *et al.*, 2003).

Burglar alarm: some species emit light with the purpose of attracting a secondary predator to make their primary predator vulnerable to attacks. This original defense mechanisms has been demonstrated by laboratory experiments on dinoflagellates: when the luminous micro-organisms are in presence of mysids (their primary predator), the glow they produce was proved to attract a secondary predator (the teleost *Porichthys notatus* or the cephalopods *Sepia officinalis* and *Euprymna scolopes*), which feed on mysids and consequently reduce predation pressure on dinoflagellates (Mesinger & Case, 1992; Fleisher & Case, 1995).

Aposematism is used by species that want to signal their unprofitability for predators. In the case of bioluminescence, the light is perceived by predators as a “don’t eat me” advertise. This function has been documented mainly in ophiuroid species (Grober, 1988; Jones & Mallefet, 2010) and recently in one shark species, *Etmopterus spinax* (Claes *et al.*, 2013).

Counterillumination is a form of camouflage used by mesopelagic species to disrupt or completely hide their shadow and become invisible to underneath predators (Clarke, 1963), which usually possess upwardly directed eyes and detect their prey from their silhouette (Haddock *et al.*, 2010). To do so, these luminous organisms (mainly crustaceans [Latz, 1995], cephalopods [Jones & Nishiguchi, 2004] and fishes [McFall-Ngai & Morin, 1991; Harper & Case, 1999; Claes *et al.*, 2010a]) bear ventral *photophores* (the light-emitting organs) matching the properties of residual downwelling light (intensity, wavelength and angular distribution).

Bioluminescence can also be used in offence strategies in order to help in predation. The best known example is the lure of anglerfish which they use to attract prey (Munk, 1999), a technique also documented in squids (Robison *et al.*, 2003). The “stun effect” is the predatory equivalent of the startle effect since some predators produce bright flashes that make the prey confuse and unable to escape (Morin, 1983). Some fishes, such as the anomalopids *Anomalops* and

Photoblepharon possess large light organs underneath their eyes and use them as flashlights in order to see potential prey. To avoid predator attraction with such continuous illumination, *Photoblepharon* for instance has developed complex escaping behavior by coupling light flashes with a rapid swimming mode (Morin *et al.*, 1975; Herring, 1982).

Thirdly, bioluminescence can be used for intraspecific communication, to allow conspecific recognition or for reproductive purposes such as localization of potential mates. Sexual recognition is allowed when both male and female display different luminous patterns (this has been documented in euphausiids, cephalopods and a lot of fishes including sharks [Herring, 2007; Claes & Mallefet, 2009a]), or when mates use specific “private channel” to communicate during courtship, such as fireflies (Copeland & Moiseff, 1995) and ostracods (Morin & Cohen, 2010). Finally, specific bioluminescent patterns can aid in schooling in order to facilitate shoal cohesion (Morin *et al.*, 1975) or to hunt in groups (Claes & Mallefet, 2009a; Renwart & Mallefet, 2013).

Chemistry and morphology of bioluminescent systems

In bioluminescent organisms, light is produced by a spontaneous exergonic chemiluminescent reaction involving the oxidation of a luciferin thanks to a luciferase catalyzing action. The existence of these components was first demonstrated by Raphael Dubois in 1885 as he was studying luminescence in the beetle *Pyrophorus*. In his experiments, Dubois managed to separate both components by making two aqueous extracts. In the first one, a cold extract, luminescence was preserved and gradually decreased with time. In the second one, which he heated, luminescence was straight quenched. By mixing both non-luminous cooled extracts, bioluminescence was triggered again and Dubois therefore concluded that the cold extract should contain a heat labile enzyme, which he named *luciferase*, while the hot extract should contain a heat stable molecule, which he named *luciferin* (Shimomura, 2006). As it was previously demonstrated by Boyle in 1667, bioluminescence also requires oxygen (Hasting, 1983). Later work on luciferin/luciferase system permitted to better understand bioluminescence chemistry, and it is now admitted that light is produced from luciferin oxidation, what

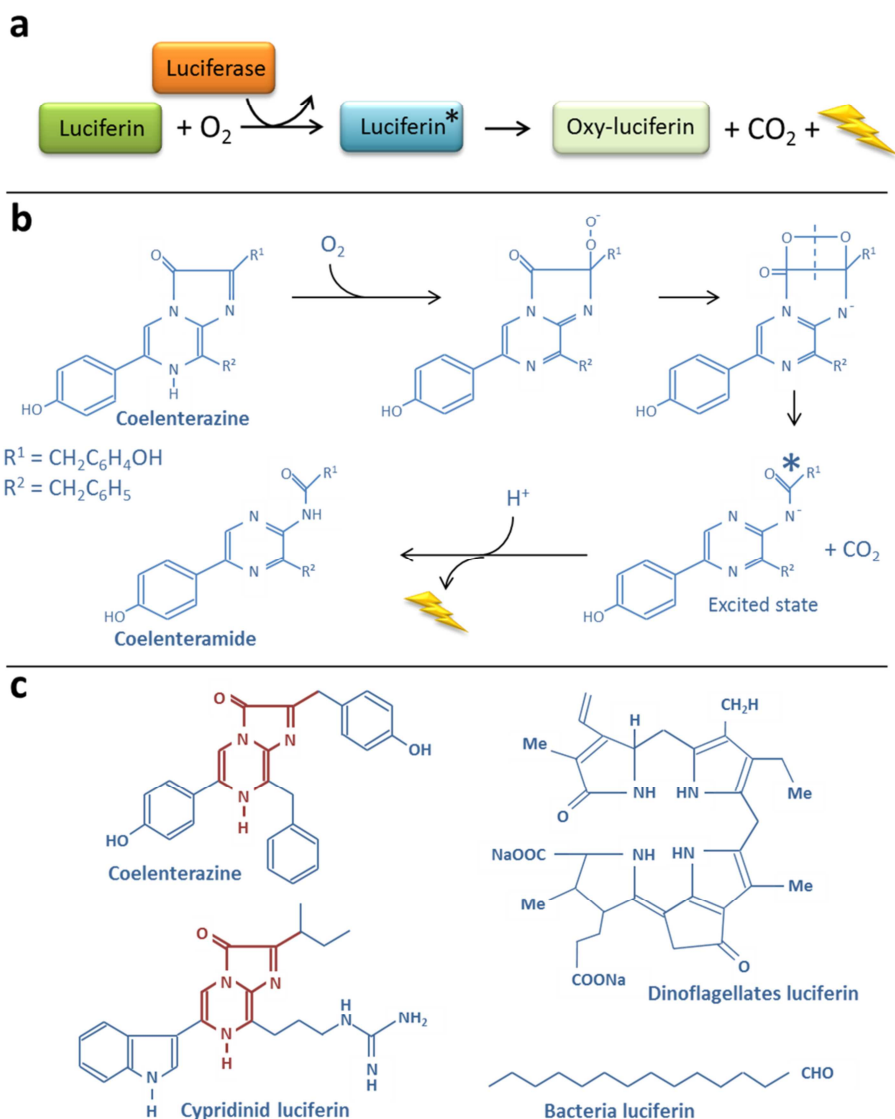


Fig. 2 Chemical mechanisms of light emission. **a** Diagram of a classical luciferin/luciferase chemiluminescent reaction. **b** Coelenterazine oxidation in chemiluminescent reaction (modified from Shimomura, 2006). **c** Chemical structure of the four best-known luciferins, with imidazopyrazine core shown in red bold.

produces a transitory excited state of this molecule that finally relaxes by emitting a photon and releasing a carbon dioxide molecule (**Fig. 2a**) (Hasting, 1983; Wilson & Hastings, 1998). All steps leading to the excited

state are not completely understood yet, but luciferin oxidation is believed to first create a peroxide, as the O_2 binds to the luciferin. This peroxide transitory stage then forms a dioxetanone that rapidly decomposes and generates the luciferin excited state. **Figure 2b** illustrates such a reaction with coelenterazine, one of the most studied luciferin (Shimomura, 2006). As new bioluminescent systems were progressively isolated and new luciferin molecular structures determined, scientists discovered with surprise that the same luciferin was sometimes used by various luminescent organisms from non-sister taxa. The most widespread luciferin is coelenterazine that has been found in not less than 9 phyla (Haddock *et al.*, 2010; Widder, 2010). The cypridinid luciferin, isolated from the ostracod *Vargula* (previously *Cypridina*) and also found in the teleost *Porichthys*, is structurally very close to coelenterazine since both molecules have the same imidazopyrazinone core (Kishi *et al.*, 1966; Cormier *et al.*, 1967; Tsuji *et al.*, 1972). The third most widespread marine luciferin is the dinoflagellates tetrapyrrole which is also found in euphausiids with two additional oxygen atoms (Dunlap *et al.*, 1980; Nakamura *et al.*, 1987, 1989). These three luciferins, together with the bacteria luciferin (a long-chain aldehyde) are the currently four best-known luciferins from marine environment and are illustrated in **figure 2c** (Widder, 2010).

Until the year 1961, all luminous systems were believed to be based on the luciferin/luciferase mechanism, but in that year, Shimomura *et al.* (1962) discovered a still unknown bioluminescent system in the jellyfish *Aequorea*. In this system, light was produced from a single protein, upon the addition of calcium ions and regardless of oxygen presence. The term *photoprotein* was introduced to name this new protein type and *aequorin* to name the *Aequorea* specific photoprotein (Shimomura, 2005). Since, photoproteins have been discovered in other luminous organisms (**Table 1**): in the coelenterates *Aequorea*, *Obelia* and *Mnemiopsis*, photoprotein is triggered by Ca^{2+} ; in the millipede *Luminodesmus*, photoprotein activation needs ATP, while a peroxide is required to trigger the light in the scaleworms *Harmothoe* and *Chaetopterus* as well as in the squid *Symplectoteuthis* and the ophiuroid *Ophiopsila* (Shimomura, 2006). It is interesting that various photoproteins require a reactive oxygen species (ROS) to emit light, as well as the need of a peroxidase or catalase activity, such as the bivalve *Pholas* or

Table 1 Discovered photoproteins together with their source organism, molecular weight and the cofactors required for light emission. Modified from Shimomura, 2006.

Source organism	Name	Molecular weight (Da)	Required cofactor
Protozoa			
<i>Thalassicolla sp.</i>	Thalassicollin		Ca ⁺⁺
Cnidaria			
<i>Aequorea aequorea</i>	Aequorin	21,500	Ca ⁺⁺
<i>Halistaura cellullaria</i>	Halistaurin	21,800	Ca ⁺⁺
<i>Phialidium gregarium</i>	Phialidin	23,000	Ca ⁺⁺
<i>Obelia geniculata</i>	Obelin	21,000	Ca ⁺⁺
<i>Obelia longissima</i>	Obelin	22,200	Ca ⁺⁺
Ctenophora			
<i>Mnemiopsis sp.</i>	Mnemiopsin	24,000	Ca ⁺⁺
<i>Beroe ovata</i>	Berovin	25,000	Ca ⁺⁺
Annelida			
<i>Chaetopterus variopedatus</i>		120,000	Fe ⁺⁺ , hydroperoxide, O ₂
<i>Harmothoë lunulata</i>	Polynoidin	500,000	Fe ⁺⁺ , H ₂ O ₂ , O ₂
Mollusca			
<i>Pholas dactylus</i>	Pholasin	34,600	Peroxidase/Fe ⁺⁺ , O ₂
<i>Symplectoteuthis oualaniensis</i>	Symplectin	60,000	Alkaline pH? O ₂
<i>Symplectoteuthis luminosa</i>	Symplectin	50,000	Catalase, H ₂ O ₂ , O ₂
Diplopoda			
<i>Luminodesmus sequoiae</i>		60,000	ATP, Mg ⁺⁺ , O ₂
Echinodermata			
<i>Ophiopsila californica</i>		45,000	H ₂ O ₂

Symplectoteuthis. This observation supports the hypothesis of bioluminescence origin based on an ancient ROS detoxifying mechanism. Photoproteins are actually “pre-charged” compounds in

which luciferin, luciferase and oxygen are bound together in a more stable form than are dissociated components found in other organisms. Indeed, after reaction, apoaequorin (the “discharged” form of aequorin) has been found to be successfully “recharged” when mixed with a solution containing oxygen and coelenterazine (**Fig. 3**), what suggest oxygen to be required for luminescence and aequorin chromophore to be coelenterazine, as it was later proved by Shimomura and Johnson (Shimomura & Johnson, 1975, 1978; Shimomura *et al.*, 1993; Shimomura, 2005). It has to be noted that *in vivo* recycling of coelenterazine from coelenteramide (coelenterazine oxidized form) has never been demonstrated in a luminescent organism.

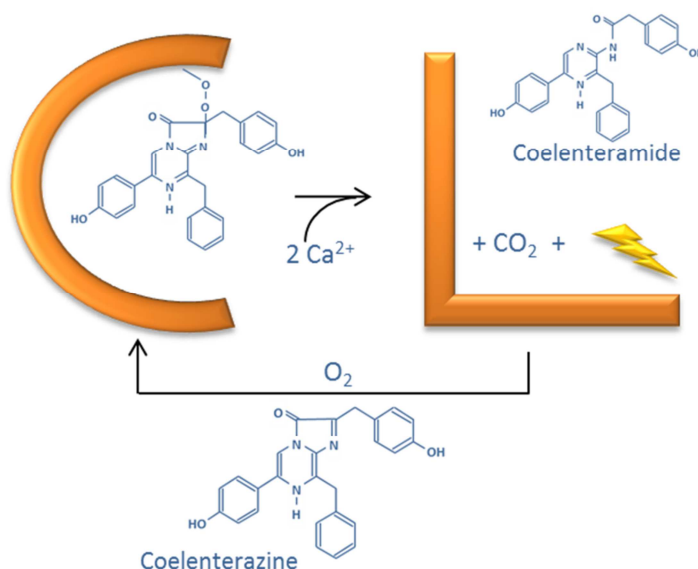


Fig. 3 Diagram of aequorin chemiluminescent reaction and regeneration (modified from Shimomura, 1985).

Chemiluminescent reactions, either from a luciferin/luciferase or from a photoprotein system, occurs in particular cells, named *photocytes*, and more precisely, in specific organelles or cytoplasmic structures of these cells, named *microsources* (Sweeney, 1980). In most luminous organisms, several photocytes are grouped together and sometimes enclosed in other structural elements in order to form a light-emitting organ called *photophore*. In luminous unicellular organisms (bacteria and dinoflagellates), the cell itself refers to photocyte, while luminous

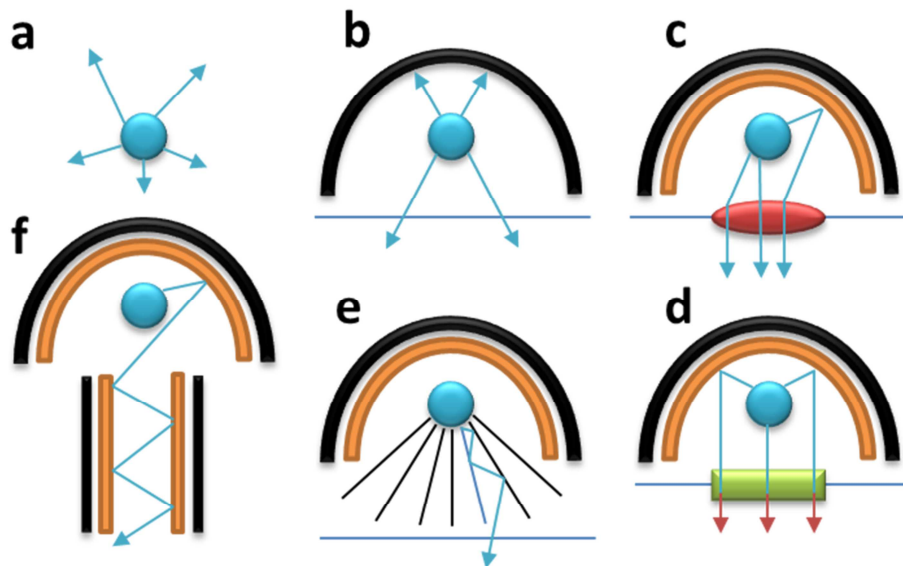


Fig. 4 Photophore morphologies showing various accessory structures aiming to increase light emission effectiveness. **a** Group of photocytes (light blue) emitting light (blue arrows) with no accessory structure. **b** A sheath of pigments (black) restricts light emission to one direction. **c** A reflector (orange) reflects light rays into the right direction and a lens (red) focusses the light. **d** A filter (green) changes light wavelength or intensity. A light guide diffuser (**e**) and a light pipe (**f**) collimate the light. Modified from Hastings & Morin, 1991.

metazoans can bear from several to hundreds of photophores. As illustrated in the **figure 4**, photophores can be very complex organs, displaying various structural elements in addition to photocytes, which aim to increase light emission effectiveness: pigmented cells protect underlying tissues from light undesirable effects; reflector reflects the light toward the right direction; lens focusses the light to make it easily detectable from the outside; filter has the property to tune the light emission wavelength (Hastings & Morin, 1991).

Photophores display a large variety of morphologies, each of them being associated with particular light emission chemistry, meaning that all luminous species possess a unique light emission mechanism. In order to overview this diversity, we aim to describe the chemistry and corresponding morphology of light-emitting systems in representative species from various taxa.

Bacteria

Luminous bacteria (**Fig. 5a, b**), some species of *Photobacterium*, *Shewanella* and *Vibrio* genus, are found as free-living populations in all marine waters and sediments. They also can colonize marine organisms (mainly fishes and squids) to make symbioses (see hereinafter). At the beginning of research on luminous bacteria, classical luciferin/luciferase reactions did not work. Indeed, in addition to the long chain aldehyde (the luciferin), the luciferase, and molecular oxygen, chemiluminescent reaction requires FMNH₂ as cofactor to trigger light production (**Fig. 5c**) (Meighen, 1991; Dunlap & Kita-Tsukamoto, 2006; Shimomura, 2006). Moreover, this reaction is only possible if bacteria have reached a sufficient concentration, e.g. in milky sea phenomenon (**Fig. 5d**), a mechanism called quorum sensing (Meighen, 1993; Fuqua *et al.*, 1994; Miller *et al.*, 2005).

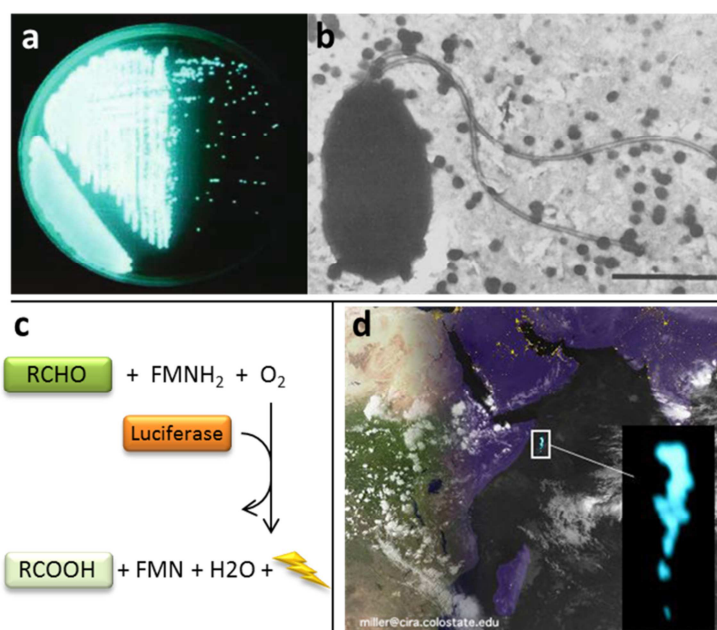


Fig. 5 Bacterial luminescence. **a** *In vivo* luminescence in *Vibrio fischeri* (from Dunlap & Kita-Tsukamoto, 2006). **b** Electron micrograph of free-living (non-symbiotic) *Vibrio fischeri* (from Rudy & Asoto, 1993). Scale bar = 1μm. **c** Diagram of bacterial chemiluminescent reaction. **d** A milky sea of about 300 km long, captured by a U.S. defense meteorological satellite above the Indian Ocean. Author: Dr Steve Miller, from the Naval Research Laboratory, Monterey. Picture: <http://biolum.eemb.ucsb.edu/organism/milkysea.html>.

Dinoflagellates

The model species considering dinoflagellates bioluminescence study is certainly *Lingulodinium polyedrum* since it was used for both chemistry and morphology investigations. Dinoflagellates luminescence is based on a luciferin/luciferase system, the former being a tetrapyrrole (**Fig. 2c**) (Nakamura *et al.*, 1989). Both components, together with a luciferin-binding protein, are found in particular organelles called *scintillons* (i.e. the microsources) (**Fig. 6a**) protruding in a low pH vacuole (Shimomura, 2006). Luciferin-binding protein aims to stabilize luciferin before reaction and should not be confused with photoprotein which one takes part to the reaction. To experimentally trigger the light, DeSa *et al.* (1963) lowered the pH of isolated scintillons solution to 5.7. Later, Fritz *et al.* (1990) proposed a model to explain *in vivo* light production in *L. polyedrum* (**Fig. 6b**): as an action potential spreads along the low pH vacuole membrane, protons are released and come into contact with scintillon where pH is therefore lowered, permitting the luciferin to be released from its binding protein and to encounter the luciferase. Indeed, luciferin-binding protein binds the luciferin at pH 8 but no more at pH 6 (Shimomura, 2006).

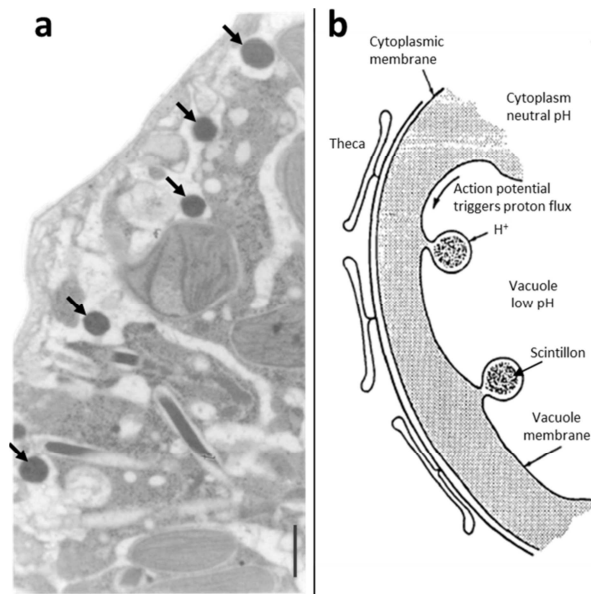


Fig. 6 Dinoflagellates luminescence (modified from Fritz *et al.*, 1990). **a** Electron micrograph of *Lingulodinium polyedrum* cell showing scintillons (arrowheads). Scale bar = 1 μ m. **b** Diagram of *L. polyedrum* light emission mechanism.

Coelenterates

Coelenterates encompass cnidarians and ctenophores. Bioluminescence in the latest has been mainly studied in *Mnemiopsis* and *Beroe* in which Ca^{2+} -activated photoprotein, respectively named *mnemiopsin* and *berovin*, have been reported (Ward & Seliger, 1974). The discharged photoprotein can be successfully recharged with coelenterazine, suggesting this luciferin to be photoprotein chromophore as in aequorin (Shimomura, 2006).

Most studied luminous cnidarians (the hydrozoans *Obelia*, *Clytia*, *Earleria* and the scyphozoans *Aequorea* and *Pelagia*) also possess Ca^{2+} -activated photoprotein (Shimomura, 2006), except the sea pansy *Renilla* (anthozoan) and the scyphozoan *Periphylla periphylla* that have a luciferin/luciferase system based on coelenterazine. Interestingly, *P. periphylla* displays two luminous sites (ovaries and exumbrellar epithelium), both of them having their own luciferase (Shimomura & Flood, 1998). Coelenterates microsources are called *lumisomes* and refer to vacuoles in which photoproteins or luciferins/luciferases are stored (**Fig. 7a**). In *Renilla*, coelenterazine is found in a protein-bound form and, as Ca^{2+} enter the vacuole upon nervous stimulation, they release the luciferin which become available for the luciferase (Anderson & Cormier, 1973; Shimomura, 2006).

Crustaceans

Luminous crustaceans are found in copepods, ostracods, amphipods, euphausiids and decapods. Studied copepods (mainly *Gaussia* and *Metridia*) as well as the decapod *Oplophorus*, were found to possess a luciferin/luciferase system based on coelenterazine. On the opposite to most luminous animals, reaction does not occur inside photocytes but instead a luminous fluid is excreted in surrounding water (Shimomura, 2006). In 2009, Oba *et al.* demonstrated the capability of *Metridia pacifica* to *de novo* produce its own coelenterazine from three amino-acids, while most other coelenterazine users are believed to alimentary acquire the molecule.

The ostracod *Vargula hilgendorfii* was a model species in bioluminescence field during 20th century and therefore possesses one

of the most extensively studied luminous systems. Its luciferin (the cypridinid luciferin, **fig. 2c**) is the second most widespread luciferin after coelenterazine since it is also used as luminescent substrate by various fishes (see below). Such as copepods, *Vargula* has excretory luminescence and a luciferin/luciferase system. The gland responsible for molecules production is composed of two cellular types, one secreting luciferin and the other secreting luciferase. Both components are evacuated concomitantly and reaction occurs in water (Shimomura, 2006).

Euphausiids possess the same luciferin than dinoflagellates with two additional oxygen atoms (Nakamura *et al.*, 1987), what means that crustaceans possess all three most widespread luciferins in marine environment. Most euphausiids bear ten bright photophores where a luciferin/luciferase reaction takes place. Both components are very unstable, especially the luciferin which is very sensitive to oxidation upon pH, temperature and acidity variations (Herring & Locket, 1978; Shimomura, 1995).

Worms

Bioluminescence in marine annelids concerns polychaetes. Most of known bioluminescent members (polinoid scaleworms, *Chaetopterus* and *Tomopteris*) possess a photoprotein triggered by Fe^{2+} ions, H_2O_2 and other specific components, but one (*Odontosyllis*) has developed a luciferin/luciferase system requiring Mg^{2+} ions. Luciferin structure in *Odontosyllis* still remains to be characterized but seems to be different from other known luciferins (Shimomura, 2006). Photogenic tissue ultrastructure has been mainly investigated in scaleworms in which dorsal surface is covered with luminescent elytra containing numerous photocytes. Each of them is filled with paracrystalline networks of endoplasmic reticulum, considered to be the worms microsources and named *photosomes* in consequence (**Fig. 7b**) (Bassot & Bilbaut, 1977). When an action potential occurs along photosome membrane, required cofactors are released and trigger light production (Bassot & Nicolas, 1987).

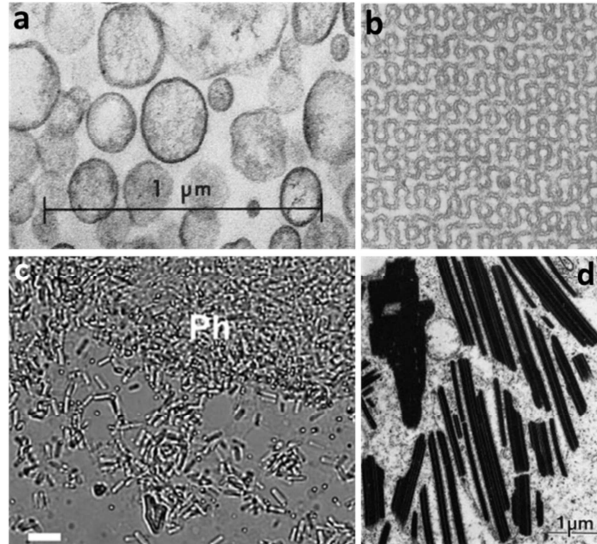


Fig. 7 Electron micrographs of various microsource structures (**a-c**). **a** *Renilla reniformis* (cnidarian) lumisomes (from Anderson & Cormier, 1973). Scale bar = 1 µm. **b** *Acholoe astericola* (scaleworm) photosomes (from Bassot & Nicolas, 1978). Magnification x 78,000. **c** *Watasenia scintillans* (cephalopods) rod-like crystals (from Hamanaka et al., 2011). Ph photogenic tissues; Scale bar = 10 µm. **d** Photocyte paracrystalline material in *Mesonychoteuthis hamiltoni* (cephalopods) (from Herring et al., 2002). Scale bar = 1 µm.

Mollusks

Bioluminescent mollusks are mostly cephalopods, whereas bioluminescence has also been studied in two gastropods (*Latia neritoides* and *Planaxis labiosus*) and one bivalve (*Pholas dactylus*) as well as observed in numerous nudibranchs in which no information is available on light emission chemistry. Various light-emitting mechanisms have been developed by cephalopods: some possess symbiotic organs, such as *Loligo*, *Sepiola* and *Euprymna* (described below), while others have a coelenterazine based system, either luciferin/luciferase (*Onychoteuthis*, *Watasenia*) or photoprotein (*Symplectoteuthis*) system. *Watasenia* luciferin is coelenterazine disulfate, similar to coelenterazine with two sulfate bonds in addition, and its *in vivo* oxidation requires ATP and Mg^{2+} (Shimomura, 2006; Hamanaka et al., 2011). Regarding photophores morphology, cephalopods possess a lot of complex and different organ types, with sometimes two or three types observed on the

same specimen, variously located on its body (e.g. eyes, arm tip, mantle or digestive gland) (Herring *et al.*, 2002). In *Watasenia*, arm photocytes contain many rod-like crystals which have been proved to be the light emission site (**Fig. 7c**) (Hamanaka *et al.*, 2011). Herring *et al.* (2002) also found variously shaped crystalloids in photocytes from species of Taoniinae family (**Fig. 7d**) but no information is available about their possible role in light emission.

Fishes

Among fishes, bioluminescence has evolved in both osteichthyans and chondrichthyans. Initiating the study of light-emitting system morphology and chemistry in chondrichthyans is the aim of the present work. Most luminous osteichthyans have a luciferin/luciferase system based either on coelenterazine or cypridinid luciferin, but some of them have also developed symbiotic photophores (see below) (**Table 2**). So far, no photoprotein system has been discovered in fishes.

Table 2 Bioluminescent systems of teleosts with selected species as examples.

Family	Luminous species	System	Substrate
Anomalopidae	<i>Anomalops katoptron</i> ¹	Bacterial symb.	-
Apogonidae	<i>Siphamia tubifer</i> ²	Bacterial symb.	-
Linophryinae	<i>Linophryne arborifera</i> ³	Bacterial symb.	-
Monocentridae	<i>Monocentris japonica</i> ⁴	Bacterial symb.	-
Apogonidae	<i>Apogon truncatus</i> ⁵	Line/Lase	Vargula line
Batrachoididae	<i>Porichthys notatus</i> ⁶	Line/Lase	Vargula line
Myctophidae	<i>Diaphus elucens</i> ⁷	Line/Lase	Vargula line
Pempheridae	<i>Parapriacanthus ransonneti</i> ⁸	Line/Lase	Vargula line
Gonostomatidae	<i>Cyclothone braueri</i> ⁹	Line/Lase	Coelentera.
Myctophidae	<i>Myctophum punctatum</i> ⁹	Line/Lase	Coelentera.
Neoscopelidae	<i>Neoscopelus microchir</i> ¹⁰	?	Coelentera.
Phosichthyidae	<i>Vinciguerria attenuata</i> ¹¹	Line/Lase	Coelentera.
Sternoptychidae	<i>Argyropelecus hemigymnus</i> ¹²	Line/Lase	Coelentera.
Stomiidae	<i>Chauliodus sloani</i> ⁹	Line/Lase	Coelentera.

Line = luciferin; lase = luciferase; symb. = symbionts; coelentera. = coelenterazine. ¹Haneda & Tsuji, 1971; ²Fishelson *et al.*, 2005; ³Hansen & Herring, 1977; ⁴Ruby & Nealson, 1976; ⁵Haneda *et al.*, 1958; ⁶Tsuji *et al.*, 1972; ⁷Tsuji & Haneda, 1971; ⁸Johnson *et al.*, 1961; ⁹Mallefet & Shimomura, 1995; ¹⁰Inoue *et al.*, 1977; ¹¹Rees *et al.*, 1990; ¹²Rees *et al.*, 1992.

The most-studied luminous teleost is the midshipman fish *Porichthys notatus* that bears several photophores arranged in a particular and beautiful way (**Fig. 8a**). In 1967, Cormier *et al.* showed the luminous system of *P. notatus* to cross-react with the one of *Vargula*, suggesting both species to have the same luciferin, and later Barnes *et al.* (1973) experimentally proved a dietary acquisition of cypridinid luciferin (**Fig. 2c**) in this fish, as the latest was fed with *Vargula*. Photophores of *P. notatus* are composed of several photocytes, enclosed in a reflector and a pigmented sheath in their basal end and oriented toward the adjacent lens in their apical end. The pear-shaped lens, which focuses the light produced in photocytes, is composed of numerous cells (**Fig. 8b**) (Nicol, 1957). *P. notatus* photocytes contain many vesicles full of flocculent material (**Fig. 8c**), which are believed to be the microsources where luciferin/luciferase reaction takes place (Anctil, 1979b). In fishes, numerous luminous reactions still remain unknown, such as the one of *Gonostoma*, which possesses excretory luminescence, or the one of *Malacosteus*, which surprisingly bears two photophore types, one blue-emitter and one red-emitter.

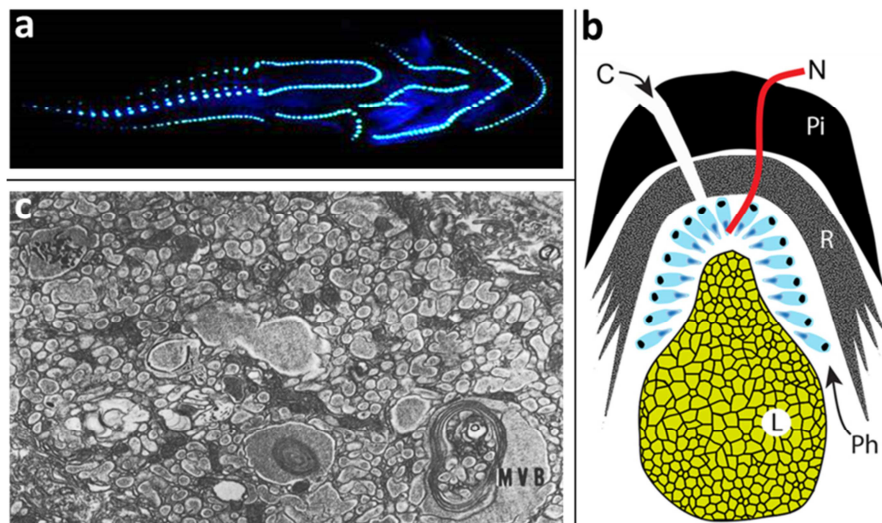


Fig. 8 *Porichthys notatus* luminescence. **a** *P. notatus* *in vivo* fluorescence (picture: J. Mallefet). **b** Diagram of *P. notatus* photophore. C capillary; N nerve; Pi pigments; R reflector; Ph photocytes; L lens (diagram: J.M. Claes). **c** Electron micrograph of photocyte vesicles in *P. notatus* (from Anctil & Case, 1976). Magnification x 9,000.

Symbiotic bioluminescence

Some fishes and squids do not possess an intrinsic bioluminescent system (i.e. based on chemicals within organisms intrinsic tissue – Haddosk *et al.*, 2010) but instead developed symbioses with luminous bacteria (genus *Photobacterium* and *Vibrio*) (**Fig. 9**), of which both host and symbionts can benefit. While the host gets advantage of emitting light from bacteria for many purposes, symbionts find an adequate environment in host photophores (including nutrients and oxygen) in order to multiply, as well as an enhanced dissemination potential as symbionts surplus is regularly released in sea water by the host (Dunlap & Kita-Tsukamoto, 2006; Haddock *et al.*, 2010). Once bacteria have colonized host photophore, they multiply to reach a suitable population and glow continuously.

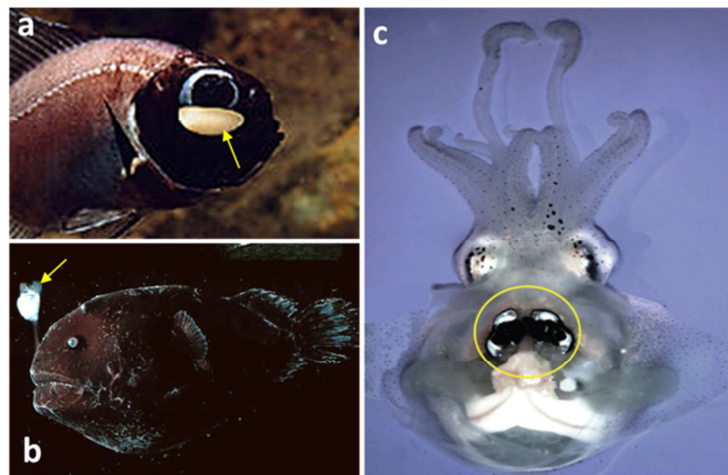


Fig. 9 Examples of symbiotic luminous organisms. **a** The flashlight fish *Photoblepharon* spp. **b** The anglerfish *Chaenophryne longiceps*. **c** The bobtail squid *Euprymna scolopes*. Yellow arrows and circle indicate light organ location. Picture: Seapics (www.seapics.com) (**a**); Haddock *et al.*, 2010 (**b**); Nyholm *et al.*, 2009 (**c**).

The most studied symbiosis occurs between the Hawaiian bobtail squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*, since both species are easily maintained in laboratory conditions or cultured. Once the young squid hatched, initial colonization by bacteria from surrounding water happened within hours (Rudy & Asato, 1993; McFall-Ngai & Ruby,

1998; Nyholm & McFall-Ngai, 2004). Moreover, the luminous organ is colonized with a remarkable specificity. Indeed, even if relatively rare in the environment (usually <0.1% of the bacterioplankton), only *V. fischeri* can enter the squid luminous organ, what has been proved to be in part related to the squid immune system (Nyholm & McFall-Ngai, 2004; Nyholm *et al.*, 2009). *Euprymna scolopes* luminescence is believed to serve for counter-illumination when the squid feed at night in shallower waters (Jones & Nishiguchi, 2004). In the meantime, the squid passes its daytime burrowed in sand and was shown to release until 95% of its symbionts, possibly to help neonates to be colonized. At night, remaining bacteria have multiplied and the cycle can start again (Nyholm & McFall-Ngai, 2004).

Vibrio fishery is also found in association with some monocentrid fishes such as *Monocentris japonica* (Ruby & Nealson, 1976; Haddock *et al.*, 2010) and *V. logei* has been found in association with other sepiolid squids (Dunlap & Kita-Tsukamoto, 2006). *Photobacterium leiognathi* is the exclusive symbiont of leiognathid fishes such as *Nucchequula nuchalis* (Wada *et al.*, 1999) or *Equulites rivulatus* in which bioluminescence is believed to serve in reproduction and sexual recognition (Ikejima *et al.*, 2008). *Photobacterium leiognathi* has also been found in symbiosis with some apogonid fishes (Fishelson *et al.*, 2005) and certain loligiroid squids (Dunlap & Kita-Tsukamoto, 2006). Yet, some luminous symbiotic bacteria remain to be identified, such as the ones of anglerfishes or anomalopid fishes, in which bacteria are believed to belong to a new genus (Hendry & Dunlap, 2011).

Physiological control of bioluminescence

Bioluminescence control mechanisms vary following light-emitting systems, and can be classified into four groups: (i) mechanical shuttering and physiological control of bacterial photophores; (ii) mechanical shuttering and optical filtering in intrinsic photophores (see **Fig. 4**); (iii) secretion of intrinsic molecules; and (iv) direct neuronal control (Case & Strause, 1978). Bacteria regulate their luminescence upon a population density-dependent mechanism (i.e. *quorum sensing*): many bacteria do not emit light in diluted culture, but as population is growing,

luminescence can be recorded and increases with cell density. Indeed, bacteria continuously release in media a specific molecule (called *autoinducer*) that specifically binds to autoreceptors and receptors from other cells, inducing in these latest expression of the *lux* operon (i.e. genes responsible for light emission in bacteria). Moreover, autoinducer binding on its receptor also induces its own gene expression, meaning that this molecule accumulates in the environment in a cell density-dependent manner (Dunlap & Kita-Tsukamoto, 2006; Meighen, 1993).

In symbiotic animals photophores (type i), bacteria rapidly reach a suitable population and glow continuously. For this reason, hosts had to develop particular control mechanisms, either physiological or mechanical, in order to maintain an adequate light production. *Photoblepharon* possesses a removable shutter which can cover its suborbital light organs while *Anomalops* is able to completely rotate its organs into dark pockets. Both mechanisms only permit an on/off light emission control (Herring, 1982). On the opposite, *Monocentris japonica* is believed to finely regulate bacteria light by controlling oxygen supply in its photophores (Tebo *et al.*, 1979), a mechanism also suggested in anglerfish esca light regulation (Munk, 1999). Excretory photophores (type iii) do not permit fine regulation of luminescence since animals bearing such organs can only control at what time molecules are realized in the environment.

Direct neural regulation (type iv) is the most widespread in organisms with intrinsic luminescence and has been particularly studied in fishes with, once again, *Porichthys* as a model species. Nicol found the fish to glow upon spinal cord electrical stimulation and adrenaline injection (Nicol, 1957). Later, photophore ultrastructure was studied and nerves were found to penetrate the organ and to form synaptic vesicles adjacent to photocytes. Ultrastructural observation of these nerves following spinal cord stimulation and noradrenaline or epinephrine injections indicates *Porichthys* luminescence to be controlled by sympathetic nervous system, via catecholaminergic nerves probably using adrenalin or noradrenalin as neurotransmitter. In the same way, most teleosts photophores are upon nervous control as well as various invertebrate photophores (coelenterates; scaleworms; echinoderms) (Strum, 1969a, b; Case & Strause, 1978; Ancil, 1979a).

GENERALITIES ON SHARKS

What is a chondrichthyan?

Chondrichthyans are cartilaginous fishes represented by sharks, rays and chimaeras. They are believed to be a monophyletic group (Heinicke *et al.*, 2009) and all chondrichthyans share two synapomorphic characteristics: the tessellate endoskeletal mineralization and the male pelvic claspers (Grogan *et al.*, 2012). Chondrichthyans possess a flexible cartilage skeleton. They have no true bone, contrary to their closest relatives, the bony fishes (Osteichthyes) (Grogan *et al.*, 2012). Cartilage is formed by chondrocytes producing hydroxyapatite crystals whose features (orientation, size, etc.) generate various density of mineralization, classified into three groups: the *areolar*, the *prismatic* and the *globular* calcification. Areolar calcification is found in vertebral centra, while other ones are found in particular calcified tissues called *tesserae* (Kemp & Westrin, 1979; Dean & Summers, 2006). The “tessellate” mineralization mode is regarded today as the key characteristic defining Chondrichthyans (Grogan *et al.*, 2012). Moreover, cartilaginous fishes also developed an internal fertilization. Males possess two claspers, extending from the pelvic girdle, and use one (or less frequently both) of those copulatory organs to fertilize females (Conrath & Musick, 2012; Grogan *et al.*, 2012).

Evolution of sharks

The oldest fossil record of a putative chondrichthyan comes from the Late Ordovician (450 MYA) and consists of small dermic denticles (Sansom *et al.*, 1996). The first chondrichthyans fossil teeth have been dated from the Lower Devonian (411 MYA). They were only 1 mm long and were assigned to the species *Leonodus carlsi* (Botella, 2006) (**Fig. 10**). Because sharks have a cartilaginous endoskeleton that poorly fossilizes, denticles and teeth are major fossil sources from this clade. *Doliodus problematicus*, a fossil from almost 409 MYA, is the first to exhibit anatomical parts in connection with the diagnostic chondrichthyans feature, i.e. tessellate mineralization mode (Miller *et al.*,

2003; Grogan *et al.*, 2012). The specimen was estimated to measure from 50 to 70 cm long and to possess tiny teeth of 0.5 mm high (Cuni, 2013). Chondrichthyans teeth were bicuspidate until the Middle Devonian. The first shape variation (the saw-shaped tooth) was recorded 385 MYA and allowed sharks to feed on bigger prey and settle in a higher trophic level. New shapes progressively emerged and their characteristics (tricuspidate teeth, durophagous plate-shaped teeth – **Fig. 10**) attest for the more and more specialized sharks diet (Burrow *et al.*, 2008; Darras *et al.*, 2008; Cuni, 2013).

The Frasnian-Famennian crisis (i.e. the second mass extinction event of our planet, 372 MYA) led to Placodermi (the Devonian oceans top-predators) extinction and permitted chondrichthyans to perform their first significant radiation. *Cladoseleache* fossils were the first to be sufficiently numerous and well conserved to enable a precise anatomical reconstitution: this 370 MYA shark predator measured until 2 m long and possessed a spindle-shaped body with two dorsal fins (preceded by prickles), large triangular pectoral fins and a powerful caudal fin (**Fig. 10**). Its sub-terminal mouth was fitted with tricuspidate teeth organized in conveyor belt (a feature still present in modern sharks) (Dean, 1894; Cuni, 2013).

During the Carboniferous (359-299 MYA), chondrichthyans reached a very high diversity (60% of fish species). This period delivered numerous very singular chondrichthyans fossils and it seemed to be a time where evolution has produced its most daring forms in this taxon (Cuni, 2013). As examples, we can quote *Falcatus falcatus* that possesses a kind of hook instead of the dorsal fin (Lund, 1985), *Stethacanthus productus* and *Akmonistion zangerli* that replaced their dorsal fin by a brush (Maisey, 2009), and *Helicoprion* whose teeth never fall but form a spiral in its lower jaw (Lebedev, 2009) (**Fig. 10**). Some species were very small (20 cm long) and developed efficient protective appendages such as large spines hard to digest, while others (some Ctenacanthiformes) were top-predators and could measure from 2 to 6 meters long (Cuni, 2013). Sharks progressively reached the highest food chain levels, a position that many of them still occupy today.

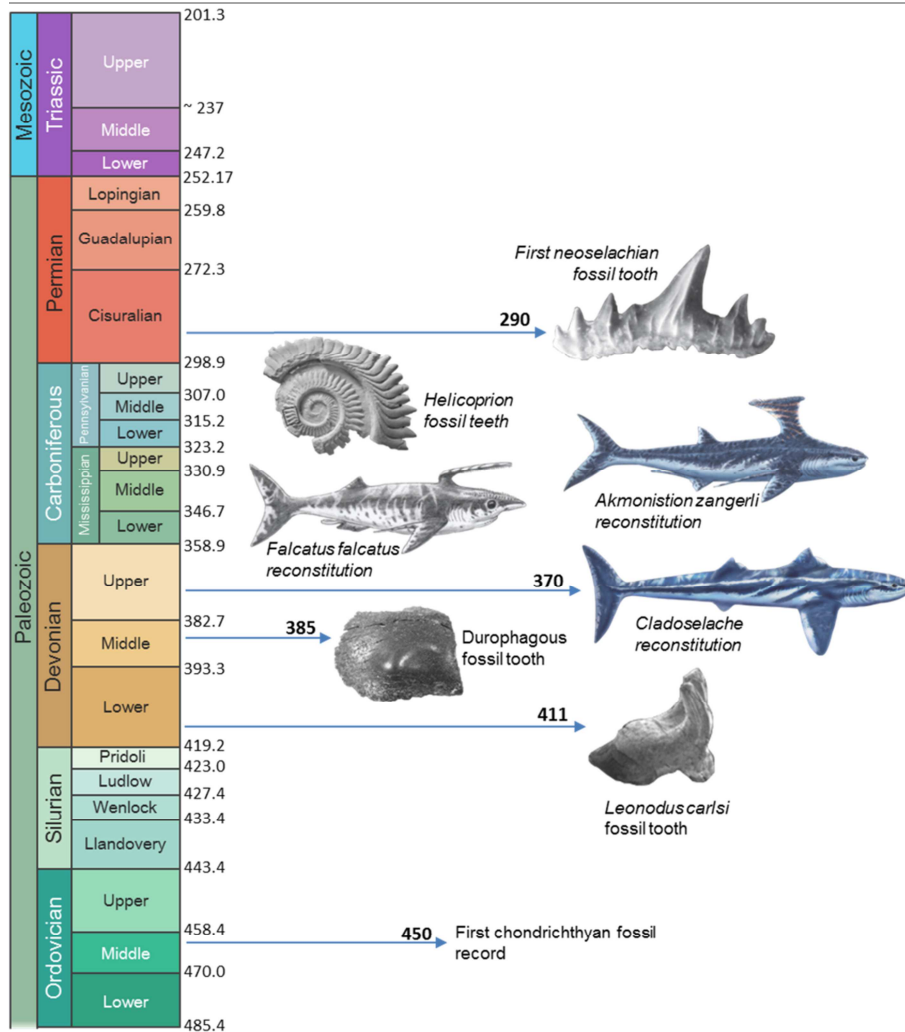


Fig. 10 Shark evolution diagram. **Left page:** illustrations of selected extinct species and particular evolution steps. **Right page:** current orders emergence and corresponding drawings of groups' morphology. Timeline modified from the International Commission on Stratigraphy (www.stratigraphy.org). Drawings and pictures modified from Botella, 2006; Compagno *et al.*, 2005; Cuni, 2013; Darras *et al.*, 2008; Ivanov, 2005; Lebedev, 2009.

The Early Permian witnessed the first modern shark emergence: *Synechodus antiquus* (Synchodontiformes) is a 290 MYA shark only known from its fossil teeth (**Fig. 10**) (Ivanov, 2005) whose feature permitted scientists to class the species among neoselachians: their

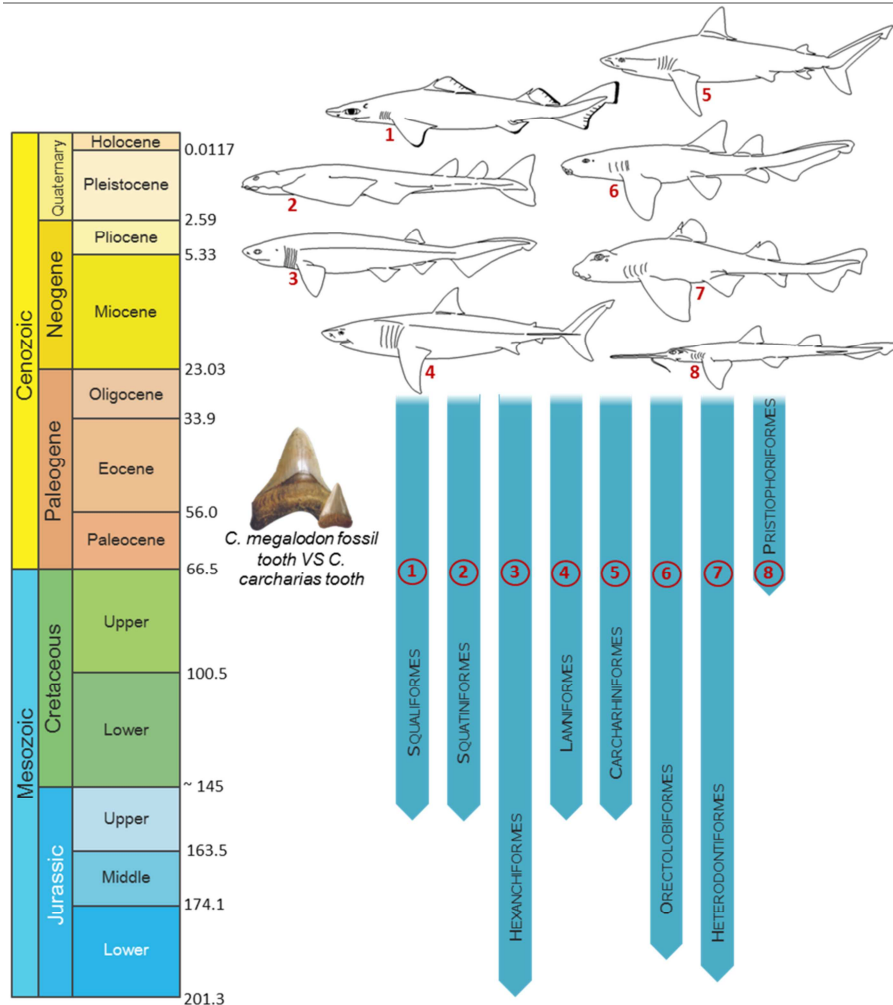


Fig. 10 Continued.

enamel was composed of three layers (Cuny & Risnes, 2005), a characteristic found in all living sharks (but not rays) (Cuni, 2013). Between the Permian and the Triassic, the third and most devastating mass extinction event (252 MYA) resulted in the extinction of 95% of the marine life. Later, the fourth extinction event also had an impact on chondrichthyans diversity so much that, at the Jurassic beginning (201 MYA), neoselachians started out with almost nothing and began the second large radiation event of the shark life history: the modern sharks diversification and the current groups appearance (Cuni, 2013).

Hexanchiformes (Sixgilled sharks) emerged during the Lower Jurassic and represent the oldest current order of which *Chlamydoselachus anguineus* (the Frilled shark) is the best known member (Compagno *et al.*, 2005). They were closely followed by Heterodontiformes (Bullhead sharks), Orectolobiformes (Carpetsharks) and, at the Jurassic end, by Lamniformes (Mackerel sharks), Carcharhiniformes (Ground sharks), Squaliformes (Dogfish sharks) and Squatiniformes (Angelsharks). By the end of Cretaceous (66.5 MYA), the last current shark order emerged (Pristiophoriformes, Sawsharks) (**Fig. 10**) and almost all current shark families were represented (Underwood, 2006; Cuni, 2013). Lamniformes, whose a today's member is the Great white shark (*Carcharodon carcharias*), were unquestionably the Upper Cretaceous (100.5-66.5 MYA) top-predators, as evidenced by *Cretalamna*, *Cretoxyrhina* or *Squalicorax* fossil records (Cuni, 2013). But they rapidly had to cope with new competitors ascension during Paleogene (66.5-23.03 MYA): the Carcharhiniformes, whose a today's member is the Tiger shark (*Galeocerdo cuvier*). Interestingly, some Lamniformes evolved to become oversized: *Otodus obliquus* could reach 8 meters long, and *Carcharocles megalodon*, which is known from its huge teeth (**Fig. 10**), has been estimated to reach 15 meters long (Gottfried *et al.*, 1996). Both orders still have tremendous predators today, but the size first prize goes to an orectolobid filter-feeder: the Whale shark (*Rhincodon typus*) (Compagno *et al.*, 2005). During Cenozoic era, modern sharks will reach the diversity we know today, with the last current family emergence and the Squaliformes radiation in the deep-seas (Cuni, 2013).

Shark current diversity is represented by eight orders encompassing 34 families, 107 genera and around 500 species, with new ones being described every year. Carcharhiniformes are the most diversified order (50 genera), followed by Squaliformes (25 genera). Sharks colonized almost all aquatic environments, from fresh waters (e.g. the Bull shark *Carcharhinus leucas* and the poorly known genus *Glyphis* – Compagno *et al.*, 2005) to seas and oceans, from the surface to the deep (52,7% of sharks are deep-water species and almost half of them belongs to the Squaliformes – Kyne & Simpfendorfer, 2010) and from high latitude seas (158 [32%] of the described species have been recorded in this environment – Ebert & Winton, 2010) to the tropics (199

[41%] of the described species – White & Sommerville, 2010). Some are confined to the continental shelf, being benthic (typically, the Wobbegongs –family Orectolobidae– Compagno *et al.*, 2005) or living in the water column. Others are oceanic and exclusively pelagic (among Lamniformes, 11 [73%] of the described species live offshore – Stevens, 2010). Their diet varies from marine mammals, sea birds, or fish (e.g. the White [*Carcharodon carcharias*] and the Tiger [*Galeocerdo cuvier*] sharks) to mollusks (e.g. Scyliorhinidae, Triakidae and Heterodontidae families) or krill (e.g. the Baskin [*Cetorhinus maximus*], Whale [*Rhincodon typus*] and Megamouth [*Megachasma pelagios*] sharks) (Wetherbee *et al.*, 2012).

Biology of sharks

External features

Shark general morphology is mainly related to their habitat and lifestyle: pelagic sharks have a hydrodynamic spindle-shaped body (large pectoral fins, almost symmetrical caudal fin, reduced pelvic, second dorsal and anal fins) in oceanic fast-swimmers (e.g. *Cacharodon*, *Isurus* or *Lamna*) to a more flattened body with developed fins in continental swimmers (e.g. *Carcharias*, *Galeocerdo* or *Alopias*). Truly benthic species often have a dorsoventrally flattened body with large head, pelvic fins located more anteriorly, dorsal fins located more posteriorly and a reduced or absent caudal fin ventral lobe (e.g. *Heterodontus*, *Chiloscyllium*, *Hemiscyllium* or *Squatina*) (**Fig. 11a**) (Thomson & Simanek, 1977; Maia *et al.*, 2012). Sharks total length (TL) varies from the small (<1 m) lanternsharks (Dwarf lanternshark [*Etmopterus perryi*] maximum size is 21 cm TL) to the giant filter-feeders Whale shark (*Rhincodon typus* - possibly reaching 20 m TL) and Basking shark (*Cetorhinus maximus* - over 10 m TL), but most species are around 1 m TL (Compagno *et al.*, 2005). All sharks have five gill slits except order Hexanchiformes whose members bear six (*Hexanchus*, *Chlamydoselachus* and *Notorynchus*) or seven (*Heptranchias*).

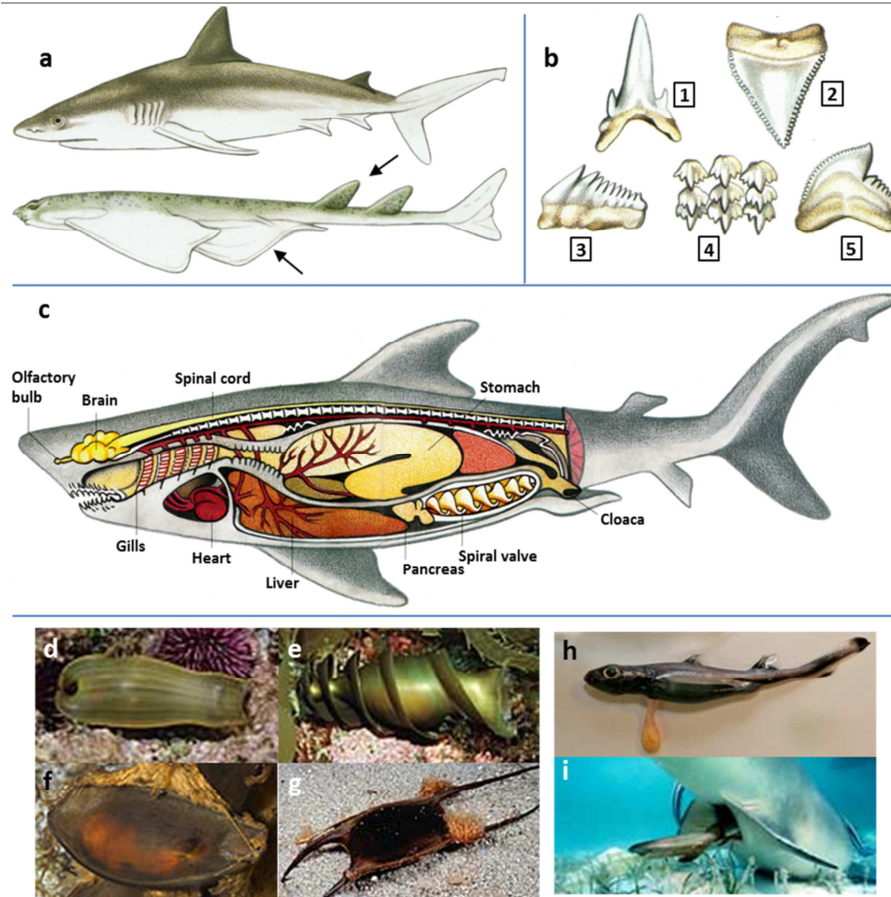


Fig. 11 **a** Shark external morphology. **Upper** The Blue shark (*Prionace glauca*), a pelagic species. **Lower** The Angelshark (*Squatina squatina*), a dorsoventrally flattened benthic species showing enlarged pectoral and pelvic fins and posteriorly located dorsal fins (arrows). **b** Shark tooth morphologies. (1) Sandtiger shark (*Carcharias taurus*); (2) Great white shark (*Carcharodon carcharias*); (3) Bluntnose sixgill shark (*Hexanchus griseus*); (4) Nurse shark (*Ginglymostoma cirratum*); (5) Tiger shark (*Galeocerdo cuvier*). **c** Shark biological systems. **d-i** Shark eggs and embryos. **d** Swellshark (*Cephaloscyllium ventriosum*) egg; **e** Horn shark (*Heterodontus francisci*) egg; **f** Arabian carpetshark (*Chiloscyllium arabicum*) egg; **g** Little skate (*Leucoraja erinacea*) egg; **h** Velvet belly lanternshark (*Etmopterus spinax*) embryos with the yolk sac. **i** Lemon shark (*Negaprion brevirostris*) giving birth. Modified from Mojette, 1998. Pictures: seapics (www.seapics.com) (**d,e,f,g,i**); M. Renwart (**h**).

Sharks' skin is covered by tiny protective denticles named *placoid scales*, structurally similar to teeth. They possess numerous teeth rows

that continually move forward as a conveyor belt and are thus constantly replaced: protruding teeth at the jaw edge fall while new ones grow in the back of the mouth. Teeth greatly differ among species and reflect sharks feeding habits (**Fig. 11b**): blade-like teeth are slender and suited for seizing and tearing (e.g. in the Sandtiger shark *Carcharias taurus*), cutting teeth are more triangular, sometime with denticulated edges (e.g. in the Great white shark *Carcharodon carcharias*), multicuspidate theet are well designed to grasp (e.g. in the Bluntnose sixgill shark *Hexanchus griseus*) and plate-like teeth are used to crush (e.g. in the Nurse shark *Ginglymostoma cirratum*). The particular asymmetrical teeth of the Tiger shark (*Galeocерdo cuvier*) might both slice using the curved side, bite on hard tissues using the notched side or even grab fish in a hook-like manner. Moreover, some species, such as lanternsharks, display a dignathic heterodonty, i.e. they bear different teeth types on their upper and lower jaw (Motta & Huber, 2012).

Anatomy and biological systems

Skeleton and nervous system: Shark cartilaginous endoskeleton comprises skull, jaw, gill arches, vertebral column, and cartilaginous structures supporting fins. For locomotion, sharks use axial undulatory propulsion that consists in lateral undulations of their axial skeleton. The skull contains and protects the brain as well as other associated structures such as optic lobes, eyes and olfactory bulbs. Together with the spinal cord (enclosed in vertebral column), they form the central nervous system, while other nerves form the peripheral one (**Fig. 11c**) (Compagno *et al.*, 2005; Maia *et al.*, 2012).

Respiration: Gill arches support the gills where oxygen exchanges take place thanks to a continuous water flow entering the mouth and going out through the gills (**Fig. 11c**). In order to maintain this water flow, some sharks (mainly active pelagic ones) have to swim constantly, while others (mainly benthic ones) are able to pump water by mouth movements (Compagno *et al.*, 2005).

Digestion: Shark digestive system starts with the U-shaped stomach that is involved in food storage and final acceptance, since many sharks have the ability to evert their stomach out of the mouth in order to reject unwanted food or unpalatable items. Accepted food then

moves to the intestine where prey digestion can take a long time, especially in cold-water species. Intestine is called “*spiral valve*” because of its numerous spiraled folds that increase absorption surface. This shark specific feature permits effective nutrients absorption despite a very short intestine length compared to mammals. Shark’s liver usually fills most of its internal cavity (it can represent a quarter of the body weight in some species such as the Basking shark [*C. maximus*]) and serves in energy storage and buoyancy (unlike most bony fishes, sharks lack swimbladder) thank to its high content in a low-density hydrocarbon called *squalene* (**Fig. 11c**) (Compagno *et al.*, 2005).

Reproduction: All elasmobranchs use internal fertilization, requiring the male to insert one (or rarely both) claspers in the female reproductive tract. In some species, female can store male sperm and delay eggs fertilization. Multiple paternity has been documented in various species such as the Nurse shark (*G. cirratum*) and the Lemon shark (*Negaprion brevirostris*), while parthenogenesis is thought to occur in the Bonnethead shark (*Sphyrna tiburo*) and in the Whitespotted bamboo shark (*Chiloscyllium plagiosum*), at least in captivity. Elasmobranchs reproduction modes are diverse and can be classified following fetal nutrition type. In lecithotrophic fetal nutrition, which includes oviparity and yolk sac viviparity, embryo entirely depends on yolk reserves for its nutrition. Oviparity, in which female releases encapsulated eggs in the environment, is used by approximately 40% of elasmobranchs species. Eggs greatly vary in size and shape (**Fig. 11d-g**) and usually have tendrils or filaments attaching them on the substrate. Incubation period is of 2 to 15 months at the end of which a fully formed embryo hatches. In yolk sac viviparity, embryos (**Fig. 11h**) grow inside females uterus but still entirely depend on their yolk sac to develop. Gestation period is of 2 to 12 months in those species, representing approximately 25% of elasmobranchs. In matrophic fetal nutrition, embryos receive nutrition from the female in addition to their yolk sac reserves. It can take the form of mucous (produced by uterus), unfertilized eggs (in Carchariniiformes and Lamniformes) or nutrients can be transferred to embryos via a placenta. In this last reproduction mode (**Fig. 11i**), called “true viviparity” and concerning approximately 10% of elasmobranchs species (including Blue shark [*Prionace glauca*], Lemon shark [*N. brevirostris*], Hammerheads sharks [*Sphyrnidae*]), gestation

time is usually equal or less than one year. On the contrary to lecithotrophic nutrition, matrophic one tends to produce larger and less vulnerable juveniles (Compagno *et al.*, 2005; Conrath & Musick, 2012).

Osmoregulation: In order to have internal fluids closely isotonic to sea water, sharks maintain a high chemical concentration inside their body: by retaining urea and secreting salts thanks to their rectal gland, they respectively prevent osmotic loss of water, and sodium and chloride entry via the gills (Gelsleichter & Evans, 2012).

Sensory physiology

Sharks have a reputation of being the ocean masters and are believed to be able to smell a drop of blood from kilometers around. Although their perfect predator image has been largely exaggerated (not all sharks settle on top of the food chain and their olfactory acuity is not necessarily better than teleosts one), they nevertheless have developed very sensitive sensory organs permitting them to successfully track a prey, detect a predator or locate themselves in the environment. Depending on their physiological mechanism, those organs are classified into mechanoreceptors, electroreceptors, chemoreceptors or photoreceptors.

Lateral line system: Functional units of this mechanoreceptive sense are the neuromasts that are found in variable quantity on Elasmobranchs skin and, according to their morphology and body location, are divided up the lateral line canals, the pit organs (both forming the lateral line system) and the vesicles of Savi (Gardiner *et al.*, 2012).

Lateral line is present in all fish and, in sharks, consists of a neuromasts epithelium occurring along pored (if in contact with water) or non-pored canals. Those canals form a web primarily spread over the animal head and extending on lateral sides until the tail for two of them (**Fig. 12a**). Neuromasts consist in sensory hair cells whose basal extremity is innervated while apical one is covered with a gelatinous capula protruding in the canal (**Fig. 12b**). As vibrations transmitted by water (pored canals) or skin surface (non-pored ones) reach the capula, stimulated sensory hair cells send a nerve impulse to the brain (Tester &

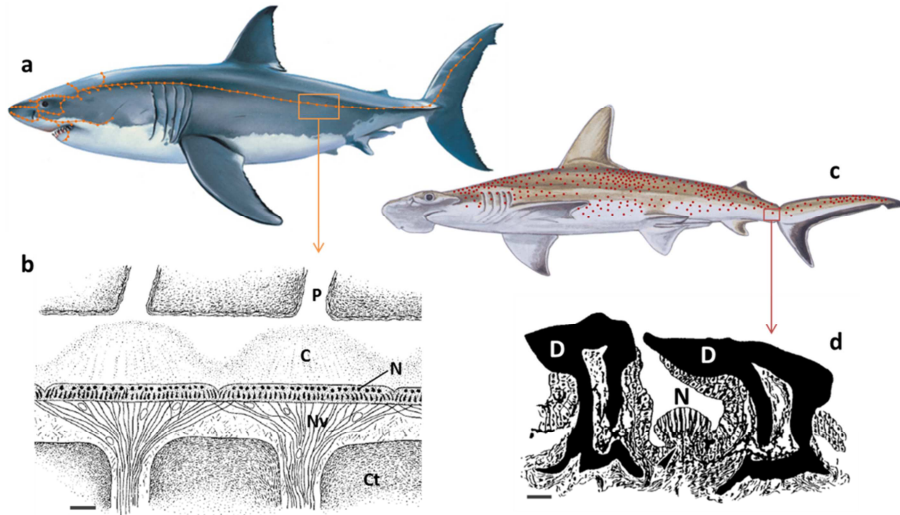


Fig. 12 Shark lateral line system. **a** Lateral line canals diagram of a Great white shark. **b** Schematic longitudinal section in a pored canal. Scale bar = 150 μm . **c** Free neuromasts diagram of a Hammerhead shark. **d** Schematic transverse section in a free neuromast. Scale bar = 50 μm . *P* pore; *C* cupula; *N* neuromast; *Nv* nerve; *Ct* connective tissue; *D* denticles. Modified from Tester & Kendall, 1969 and Maruska, 2001. Shark pictures: Encyclopédie Larousse online and Compagno *et al.*, 2005.

Kendall, 1969; Maruska, 2001; Gardiner *et al.*, 2012). In addition, numerous free (i.e. not along a canal) neuromasts, called *pit organ*, run over the whole body (**Fig. 12c**) and are enclosed between skin denticles with their capula being in contact with water (**Fig. 12d**). Their number (from 50 per side in benthic sharks to 600 per side in pelagic ones) and body pattern greatly vary among species and define shark sensory capacity. Conversely, *vesicles of Savi* are free neuromasts that are not in contact with water (Maruska, 2001; Gardiner *et al.*, 2012).

Each neuromast type encodes particular information. Lateral line canals (mainly the numerous head pored canals) are believed to play an important role in prey localization (and predator avoidance) by detecting their water flow. Indeed, some sharks have been proved to specifically detect their prey water disturbances (Montgomery *et al.*, 1995). Free neuromasts and posterior lateral line canals mainly provide for the shark general information on its position (direction, velocity, rheotaxy) and again on prey, predators or conspecifics behavior (e.g. for shoaling) (Maruska, 2001). Non-pored head lateral line canals could encode skin

movements (Maruska & Tricas, 1998). Role of vesicles of Savi remains unclear but could also act as specialized tactile receptors (Gardiner *et al.*, 2012).

Hearing: Hearing is the other shark mechanoreceptive sense. Inner ears (**Fig. 13a, b**) are classically composed of three semi-circular canals (involved in linear and angular acceleration perception) and three chambers: the *utricle*, the *sacculus* and the *lagena* (involved in acoustical and balance perception). Chambers are upholstered with a sensitive epithelium (the *macula*), covered by a mucilaginous matrix containing calcium carbonate granules (the *otoconia*). When a sound is emitted, resulting water vibrations encounter otoconia that act as otoliths and trigger an action potential to the brain (Myrberg, 2001; Gardiner, 2012). Shark auditory sensibility has been studied for few species, including the Bull shark (*Carcharhinus leucas*) and the Lemon shark (*Negaprion brevirostris*) that respectively show a maximal sensibility at 400 to 600 Hz and at 40 Hz (Kritzler & Wood, 1961; Nelson, 1967). Some sharks have also been proved to be attracted by specific sounds (e.g. irregular low frequency sounds emitted by speared fishes) and to go back up the source (Myrberg, 2001).

Electroreception: Elasmobranchs electroreceptive units are the *ampullae of Lorenzini*, mainly located on sharks head (**Fig. 13c**), and able to detect low-frequency electric stimuli (less than 5nV/cm). Each ampulla consists of a group of sub-dermal alveoli upholstered with a sensory epithelium and whose lumen open in a canal of about 1 mm wide, filled with a low resistance glycoproteins hydrogel with the same properties as sea water (ion content and conductivity) and reaching skin surface via a pore (**Fig. 13d**). The epithelium forms an electrical barrier having the apical extremity in contact with external environment via the canal, and the basal extremity with the shark inner fluid, what allows sensory cells to detect potential differences between shark internal and water external potentials (Tricas, 2001; Gardiner *et al.*, 2012). Such an electrosensitive system is believed to help in orientation and navigation by using Earth's magnetic field (Klimley, 1993), and for detecting prey (Kalmijn, 1971), predators (Sisneros *et al.*, 1998), or conspecifics as all living organisms emit a low electric field.

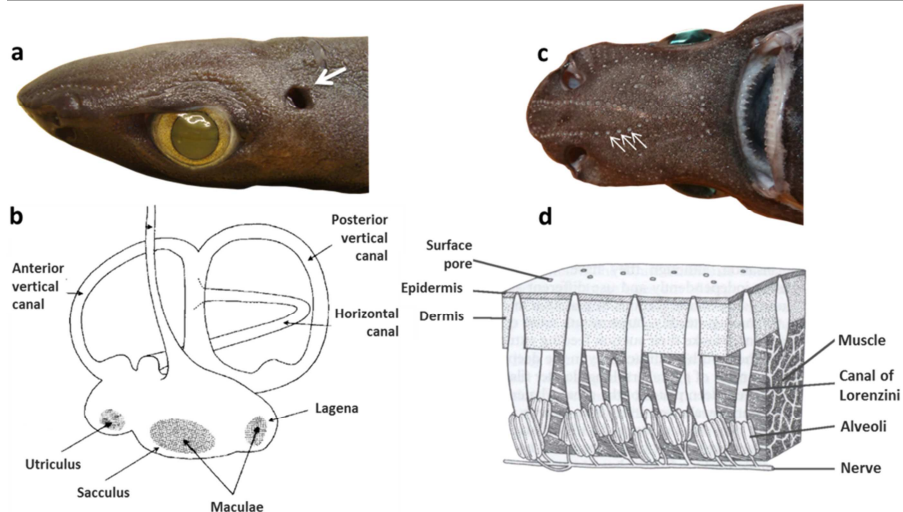


Fig. 13 Earing (**a** & **b**) and electroreception (**c** & **d**) in sharks. **a** Velvet belly lantern shark (*Etmopterus spinax*) head (lateral side) showing a spiracle (= inner ear opening, arrow). **b** Elasmobranchs inner ear diagram. Modified from Myrberg, 2001. **c** Velvet belly lantern shark (*E. spinax*) head (ventral side) showing ampullae of Lorenzini (arrows). **d** Elasmobranchs ampullae of Lorenzini diagram. Modified from Compagno *et al.*, 2005. Pictures: M. Renwart (**a,c**).

Chemoreception: Olfactory system is one of the keenest senses in elasmobranchs. Sharks possess two large olfactory organs (named *rosettes*) located on head ventral side in front of the mouth (**Fig. 14a**) and opening to external environment. Each consists of a succession of primary lamellae (wing-shaped plates attached to a central ridge) covered by a fold of secondary lamellae increasing the sensory area (**Fig. 14b**). The rosette is divided into two regions by the nasal flap: an incurrent area and excurrent area. This structural organization allows maintaining a continuous water flow along the olfactory epithelium covering the secondary lamellae. Epithelium receptor cells are bipolar neurons where odorant molecules can bind and whose axons form the olfactory nerve. Olfaction mainly helps sharks in food detection but it could also be involved in mating by allowing sex pheromones recognition (Theisen *et al.*, 1986; Gardiner *et al.*, 2012).

Chemoreception also includes gustation (taste buds are small papillae covering the entire oral cavity and involved in food final acceptance) and solitary chemosensory cells located in the epidermis

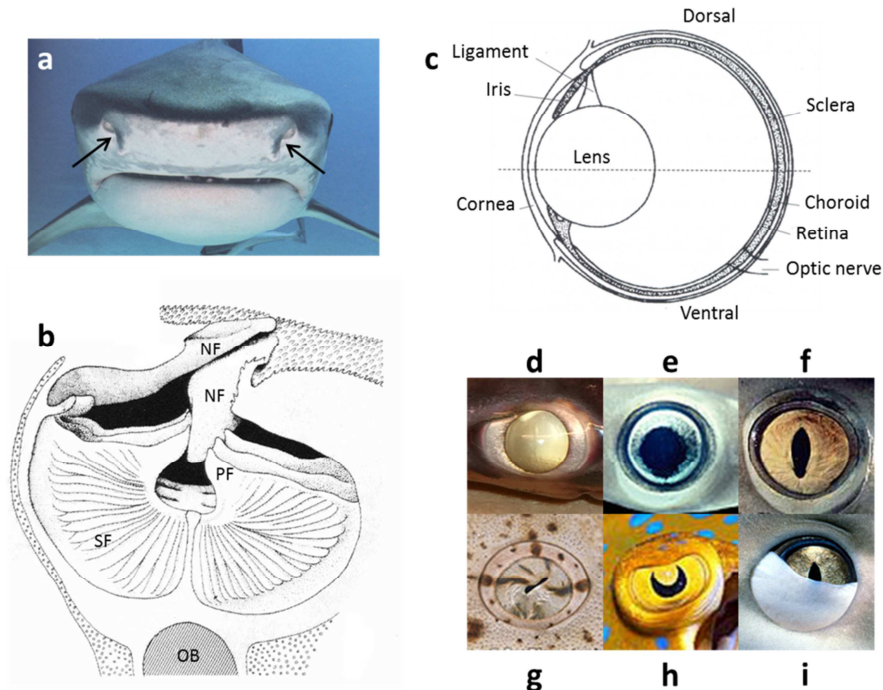


Fig. 14 Olfaction (**a & b**) and vision (**c-i**). **a** Tiger shark (*Galeocerdo cuvier*) snout showing both nostrils. Picture: Calcagno, 2013. **b** Olfactory rosette diagram. Modified from Theisen *et al.*, 1986. **OB** olfactory bulb; **NF** nasal flap; **PF** primary fold; **SF** secondary fold. **c** Schematic vertical section in a shark eye. Modified from Lebrun, 2001. **d-i** Various eye features: the Velvet belly lantern shark (*Etmopterus spinax*) eye (**d**) showing light reflection due to the *Tapedum lucidum*; the Scalloped hammerhead shark (*Sphyrna lewini*) oval pupil (**e**); the Blacktip shark (*Carcharhinus limbatus*) vertical pupil (**f**); the Bluespotted stingray (*Neotrygon kuhlii*) oblique pupil (**g**); the Bluespotted ribbontail ray (*Taeniura lymma*) crescent pupil (**h**); the Caribbean reef shark (*Carcharhinus perezii*) eye (**i**), showing the nictitating membrane. Pictures: seapics (**d-i**).

and believed to have a chemoreceptive function, although they are still very poorly understood in elasmobranchs (Gardiner *et al.*, 2012).

Photoreception: Vision is the main elasmobranchs photoreceptive organ, while pineal gland is also considered as such. Elasmobranchs eyes morphology is close to other vertebrates one: from the outer to the inner layer, they are composed of a cartilaginous sclera (extended in a transparent cornea in front of the pupil), a choroid (containing the *tapedum lucidum*) and a retina (covered with receptor cells) (**Fig. 14c**).

The *tapedum lucidum* (**Fig. 14d**) is made of cells full of guanine crystals with high reflective properties allowing reflecting until 90% of light toward the retina. Iris delimits the pupil that can be variously shaped, depending on species and light conditions, in order to enhance visual resolution, contrast or focusing ability (**Fig. 14e-h**): circular or oval in bright light and vertical, horizontal or oblique slit in dim light. Some can also be crescent-shaped or U-shaped, mainly in rays. Carcharhinid species have a third eyelid (the *nictitating membrane*) (**Fig. 14i**) located under the lower eyelid and having a protective role when shark attacks or is attacked. Sharks possess both rods and cones in specific quantity meaning they can see in color (unless for some isolated cases such as the Bluntnose sixgill shark [*Hexanchus griseus*] that only has rods). Eyes size and position in the body depend on shark habitat: eye represents 1% of the total length in clear and shallow waters species while it can reach 10% of the total length in deep-sea species. Pelagic or benthopelagic sharks have laterally located eyes while benthic ones have dorsally or dorso-laterally located eyes, as an adaptation to their dorso-ventral flattening. The roles of vision in sharks are highly diverse, including prey detection, predator avoidance, conspecifics recognition, rheotaxis, orientation and navigation, etc (Lebrun, 2001; Gardiner *et al.*, 2012).

Senses integration: Sharks constantly integrate the numerous signals they perceive thanks to their sensory organs, and adopt particular behaviors in response. Two behavior types are distinguished: the primary and the secondary orientation. Primary orientation refers to posture and locomotion and is defined as “reactions that guide an animal into its normal stance”. Vision and vestibular organ mainly act at this level. Secondary orientation refers to locomotion in normal habitat in response to middle-scale or long-distance stimuli. Middle-scale stimuli include vital behaviors such as finding food, escaping predators, rheotaxy and mate localization, which involved almost all sensory organs (Montgomery & Walker, 2001). Olfaction and hearing are the senses that carry further: sharks are able to detect an olfactory stimulus (e.g. mate pheromones) from kilometers and to orientate in consequence, following the nostril receiving the strongest stimulation. Sound perception can be divided into near field (about meters) and far field (about kilometers) (Myrberg, 2001; Gardiner *et al.*, 2012). Vision greatly varies with the light (bright or dim, day or night) and water (turbidity, particles) conditions: in

clear water, visual range has been estimated to be of approximately ten meters. Mechanoreception and electroreception work in a more limited field: the lateral line system approximately have one to two body lengths perception, while ampullae of Lorenzini perceive stimuli in a range of centimeters (except when use in long-distance navigation –Montgomery & Walker, 2001). Gustation and touch obviously require a contact with shark's body (Gardiner *et al.*, 2012).

Bioluminescence in sharks

As discussed in the first section, bioluminescence is a widespread phenomenon in deep environments where it provides a strong adaptive advantage. Although most deep-sea bony fish are luminous (70%), only 6% of deep-sea cartilaginous fish are (Claes & Mallefet, 2009c). Most of them are sharks (only one ray [*Benthobatis moresbyi*] and no chimaera have been described to be luminous) divided into two families, the Etmopteridae and the Dalatiidae, in which bioluminescence is believed to have appeared independently (Hubbs *et al.*, 1967, Claes & Mallefet, 2009c). Both families contain small sharks (less than one meter except for the Black dogfish [*Centroscyllium fabricii*] and the Kitefin shark [*Dalatias licha*]) usually living deeper than 200 m although some have been recorded in the epipelagic zone (<200 m). Etmopterids, which encompasses five genera, all having luminous members, are mainly bottom-dwelling species and radiated 90 to 65 MYA (depending on the approach, respectively morphological or molecular phylogeny – Adnet & Cappetta, 2001; Straube *et al.*, 2010). Dalatiids, of which at least six out of seven genera possess luminous members, are rather pelagic and evolved 65 MYA (Compagno *et al.*, 2005; Claes & Mallefet, 2009c). A total of more than fifty luminous sharks species have been described nowadays (Claes & Mallefet, 2009c), although this affirmation is mostly based on photophore presence but not on spontaneous bioluminescence observations. Indeed, only a few species have been observed to emit spontaneous natural luminescence (*Euprotomicrus bispinatus* [Dickens, 1956], *Isistius brasiliensis* [Widder, 1998], *Squaliolus aliae* [Claes *et al.*, 2012], *Etmopterus splendidus* [Claes *et al.*, 2011b] and *E. spinax* [Claes & Mallefet, 2008]) or

physiologically induced luminescence (*Etmopterus pusillus* [Ohshima, 1911]).

Photophore morphology

Etmopterid and dalatiid families display very different photophore patterns. In dalatiids, photophores are spread over the animal ventral and lateral faces with a density gradient: photophores number increases from the dorsal side (where almost no photophore is observed) to the ventral side (where density is the highest: 60 units/mm² in the Pygmy shark *Euprotomiscrus bispinatus* [Hubbs *et al.*, 1967]) (**Fig. 15a,b**). In etmopterids, pattern is usually more complex and presents various luminous areas located on ventral and lateral faces and displaying great variations among species, helping in their identification (**Fig. 15d,e**) (Iwai, 1960; Hubbs *et al.*, 1967; Reif, 1985, Claes & Mallefet, 2009c).

Etmopterids possess thousands of tiny photophores (110 µm on average [Claes *et al.*, 2014]), composed of a few photocytes (from 6 to 13 in number in *Centroscyllium* [Herring & Morin, 1978] and about 14 in the Smooth lanternshark [*Etmopterus pusillus*], Ohshima, 1911), surrounded by a sheath of pigmented cells (the *pigmented sheath*), and topped by one or two lens cells. Some pigmented cells extend between the photocytes and the lens to form a movable ring called the *iris-like structure* (**Fig. 15c**) (Ohshima, 1911; Iwai, 1960; Hubbs *et al.*, 1967). Dalatiid photophores are smaller (75 µm on average [Claes *et al.*, 2014]) and simpler than etmopterid ones in that they only have one photogenic cell, still encircled by a pigmented sheath and topped by a lens (**Fig. 15f**) (Hubbs *et al.*, 1967; Claes & Mallefet, 2009c).

Photophore control

Various attempts were made to physiologically stimulate etmopterid photophores with classical neurotransmitters (acetylcholine, adrenaline, noradrenaline, etc) with no success (Herring & Morin, 1978, Claes & Mallefet, 2009b). In 2009, Claes & Mallefet discovered sharks photophores to be under hormonal control, using the Velvet belly lanternshark (*Etmopterus spinax*) as a model species (Claes & Mallefet, 2009b). Subsequent studies demonstrated other etmopterid species (*Etmopterus splendidus* [Claes *et al.*, 2011b]) as well as a dalatiid

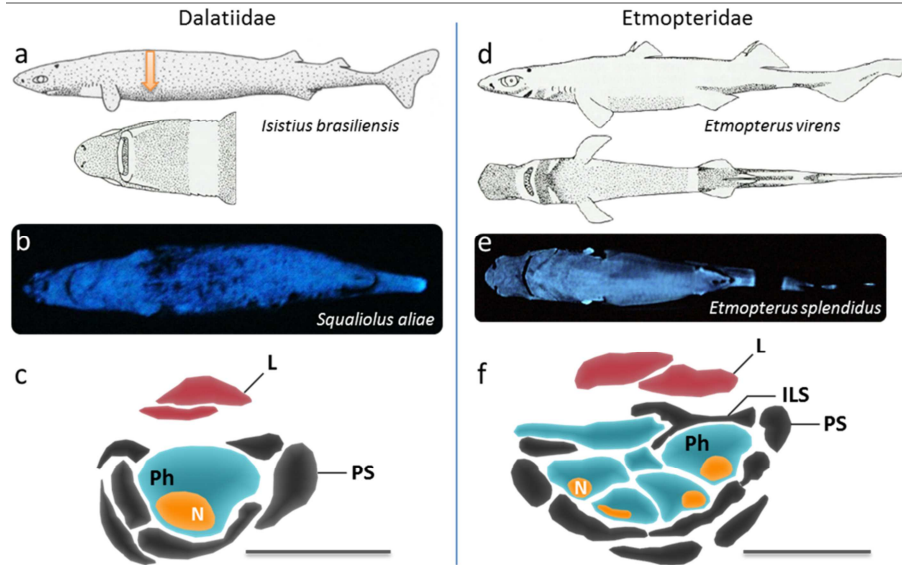


Fig. 15 Bioluminescence in Dalatiidae (**left**) and Etmopteridae (**right**). **a** photophore pattern diagram in the Cookiecutter shark (*Isistius brasiliensis*), showing photophores gradient (arrow). Modified from Reif, 1985. **b** Spontaneous luminescence from the Smalleye pygmy shark (*Squaliolus aliae*) ventral side. **c** Cross-section diagram in a dalatiid photophore. **d** photophore pattern diagram in the Green lanternshark (*Etmopterus virens*), showing specific luminous areas. Modified from Reif, 1985. **e** Spontaneous luminescence from Splendid lanternshark (*Etmopterus splendidus*) ventral side, showing specific pattern. **f** Cross-section diagram in an etmopterid photophore. Scale bars = 50 μ m (Based on dalatiid and etmopterid photophores average diameters - Claes *et al.*, 2014). ILS iris-like structure; L lens; N nucleus; Ph photocytes; PS pigmented sheath. Pictures: J. Mallefet (**b,e**).

species (*Squaliolus aliae* [Claes *et al.*, 2012]) to also possess a hormonal control of their light emission.

Bioluminescence functions

Photophore pattern of both luminous shark families, by having most luminous area ventrally located, strongly suggests a counter-illumination function of luminescence (Reif, 1985), a hypothesis experimentally proved for *E. spinax* (Claes *et al.*, 2010a). Moreover, bioluminescent sharks are believed to move up and down in the water column in order to remain cryptic, instead of changing their light characteristics in response to changing downwelling light (Claes *et al.*,

2014). Counter-illumination is likely the sole aim of bioluminescence in dalatiids, because of their very simple pattern. On the opposite, the very specific and complex etmopterid pattern probably serves other functions such as conspecific recognition for mating or hunting purposes (Reif, 1985, Claes & Mallefet, 2010a). A particular case is the Cookiecutter shark (*Isistius brasiliensis*) from dalatiid family, for which an unusual function of luminescence was proposed. This shark possesses a pigmented collar free from photophores that appears dark in its ventral luminous area. A first hypothesis proposes this dark collar to attract larger fishes that would associate it to a prey shadow. Knowing the particular feeding method of *I. brasiliensis* (the well-named “cookiecutter shark” extracts cookie-shaped plugs of flesh from larger animals), this technique would therefore attract the shark prey, a phenomenon called “predatory use of counter-illumination” by the author (Widder, 1998). Recently, another hypothesis proposed the dark collar to rather serves for intra-specific recognition (Claes *et al.*, 2014).

Etmopterus spinax

The Velvet belly lanternshark (*Etmopterus spinax*) is a small deep-sea shark (maximum total length (TL) = 60 cm [Compagno *et al.*, 2005]) from the Etmopteridae family, occurring along the continental shelf of the Eastern Atlantic Ocean (from Iceland to Gabon) and in the Mediterranean Sea (**Fig. 16a**). *Etmopterus spinax* can be found at depth from 70 to 2000 m but mainly lives between 200 to 500 m deep. Mature specimens are bottom-dwelling while juveniles are believed to live upper in the water column. *Etmopterus spinax* is an aplacental viviparous species: embryos (12.6 pups/litter on average, pers. obs.) grow inside female uterus and depend on their yolk reserve to develop (**Fig. 16b**). Juveniles are about 12 to 14 cm when hatching (**Fig. 16c**) and attain sexual maturity around 33 to 36 cm (Compagno *et al.*, 2005; Coelho & Erzini, 2008; Claes & Mallefet, 2009a).

Etmopterus spinax luminescence was first studied by Jordan (1899), while now it has become a model species in shark bioluminescence investigations. Its luminous pattern displays nine areas (**Fig. 16d**) that progressively appear during embryogenesis. Four of the

nine areas are already recorded in 5.5 cm TL embryos and all of them are present in the fully-formed ready to hatch ones (Claes & Mallefet, 2008). A continuous insertion of new photophores is observed as the juvenile is growing, but has been proved to not be sufficient to maintain a constant photophore density during life. Ventral photophore density, for instance, decreases from approximately 100 units/mm² in juveniles (~14 cm TL) to less than 10 units/mm² in a large female (~52 cm) in which photophore total number was estimated to reach ~440 000 units (Claes & Mallefet, 2009a).

During photophores development, pigmented sheath and iris-like structure appear first between the dermis and the epidermis; photocytes (from 4 to 6 – Jordan, 1899) and lens are formed in a second stage. Finally, completely developed photophores (**Fig. 16e**) have been shown to become functional (i.e. to emit light) when a fluorescent vesicle appears inside photocytes. In late pregnant females, ready to hatch embryos already show operational photophores and have been observed to be luminous (**Fig. 16f**) (Claes & Mallefet, 2008).

Hormonal control of luminescence in sharks has been first discovered in *E. spinax*. Melatonin and prolactin were found to activate luminescence, while α -MSH had an inhibitory effect. Both stimulating hormones showed different light-emission curves when injected on a skin patch: while prolactin induces a maximal emission peak after approximately 20 minutes and subsequently decreases within one hour, melatonin reaches emission peak after approximately 45 minutes and lasts until seven hours. Both hormones have been shown to act on the iris-like structure by opening the iris to let the light past (Claes & Mallefet, 2009b; 2010a; b). This slow hormonal kinetics contrasts with the rapid nervous light induction occurring in teleosts. Nevertheless, *E. spinax* photophores are innervated and an inhibitory GABAergic effect on light emission has been shown (Claes *et al.*, 2011a), as well as a modulatory effect of nitric oxide on the melatonin and prolactin light induction (Claes *et al.*, 2010b).

Bioluminescence main function in *E. spinax* is camouflage by counter-illumination since its light has been shown to closely match intensity, angular distribution and wavelength of the sun downwelling light (Claes *et al.*, 2010a). Other complementary functions were also

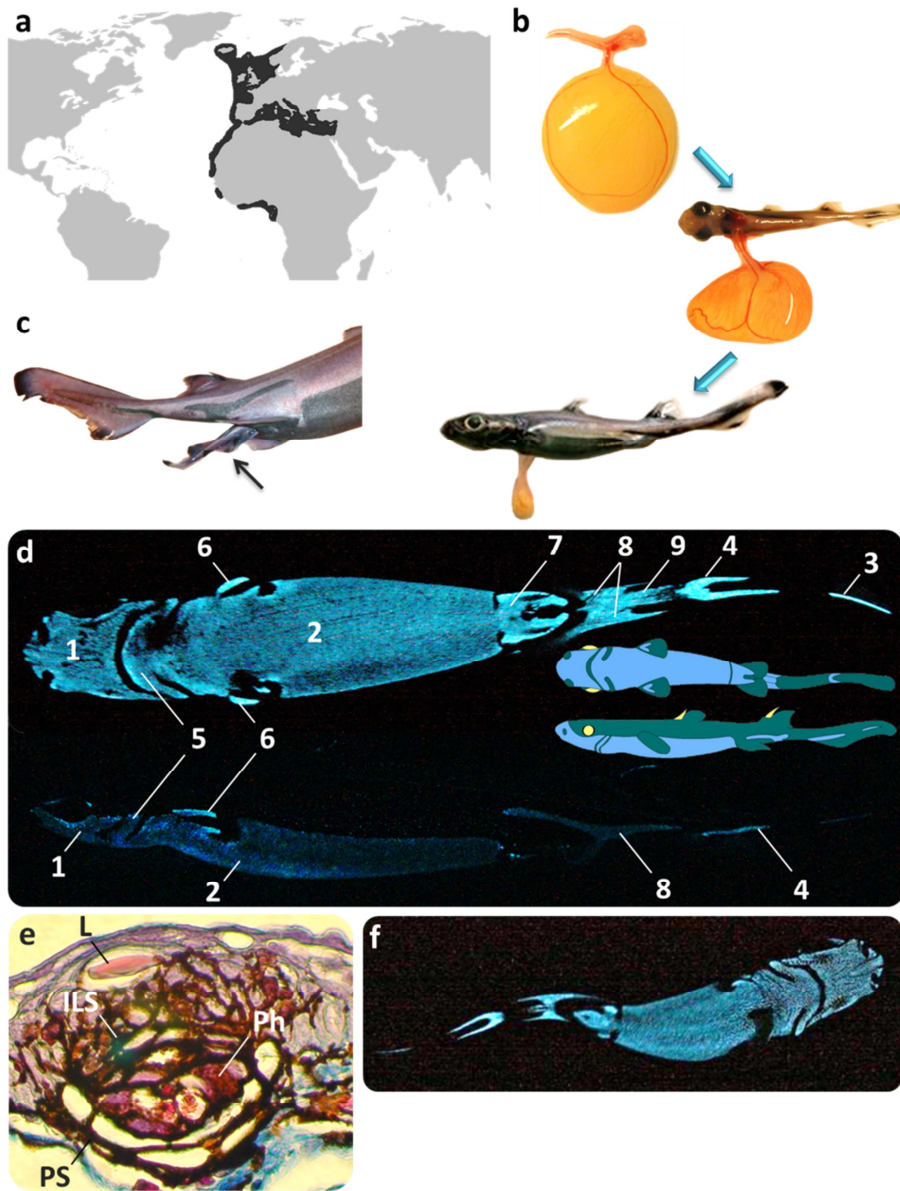


Fig. 16 *a* *Etmopterus spinax* geographic repartition. *b* *E. spinax* growing embryo showing gradual reduction of the yolk sac. *c* *E. spinax* female giving birth. Arrow shows the juvenile tail. *d* *In vivo* and schematic luminous areas of *E. spinax*, viewed from ventral (*upper*) and lateral (*lower*) side. 1 rostral; 2 ventral; 3 caudal; 4 infracaudal; 5 mandibular; 6 pectoral; 7 pelvic; 8 lateral; 9 infra-pelvic. *e* Cross-

section in a photophore of *E. spinax*. *ILS* iris-like structure; *L* lens; *Ph* photocyte; *PS* pigmented sheath. **f** Luminous embryo prior to birth. Pictures: R. De Jaegere (**c**); J. Mallefet (**b,d,f**); M. Renwart (**b,e**). Repartition world map from Compagno *et al.*, 2005.

proposed: because of their higher luminous intensity, lateral areas could help in schooling and social behavior. Moreover, males and females display differently-shaped pelvic luminous areas that could help in recognizing mates (Claes & Mallefet, 2009a). Finally, an aposematic function of the photophores associated with the two spines located anteriorly to dorsal fins was recently suggested since they were shown to signal the presence of these defensive appendages to potential predators (Claes *et al.*, 2013).

AIMS OF THE STUDY

Deep sea sharks are, in comparison with deep-sea teleosts, poorly studied, mainly due to their rarity and the limited accessibility of their environment. Bioluminescence in sharks has long been known, but very few information were gathered on this subject until recently. Because of its local abundance and the possibility of *in vivo* observations, *Etmopterus spinax* is a very convenient model species to study bioluminescence in sharks. Thanks to previous works, various aspects of its light emission system are now better understood, such as physiological control, ecological functions and photophores pattern and development. Yet, nothing is known on the photocyte intracellular light emission mechanism. The purpose of this study is therefore to provide key information on this subject by investigating photophores ultrastructural morphology on the one hand and the nature of chemiluminescent reaction leading to light production on the other hand.

First chapter addresses the ultrastructure of the luminous organ: fine organization of non-stimulated photophores is described in paper 1 while morphological changes during light emission in hormonally stimulated photophores are studied in paper 2. In paper 3 (second chapter), the possible involvement of a known luciferin, alimentary acquired, is approached by studying *E. spinax* feeding habits and performing cross-reactions with prey known to contain luminous compounds. The third chapter aims to provide a basis for future isolation and characterization of the molecules constituting the luminous system present in this shark.

Finally, in a general discussion, all experimental data are gathered and interpreted together, with regards to previous works on *E. spinax* light emitting systems, in order to draw a model of the photophore operating mode, which one seems to be one of the most complexes of the animal kingdom.

OUTLINE AND METHODOLOGY



*R.V. Hans Brattström deck and the trawling net used for sharks collection
(J. Mallefet)*

OUTLINE AND METHODOLOGY

Specimens collection

Specimens of *Etmopterus spinax* were caught in the Raunefjord (60°15.908N; 05°07.778O) and the Lysefjord (60°12.279N; 05°18.068O) in Norway (**Fig. 1a**), during a total of thirteen field missions from 2008 to 2013. Most sharks were caught by bottom longlines using fish pieces as baits (**Fig. 1b**), but some of them, from the 2008 field missions, were caught by deep trawling (**Fig. 1c**). They were transported in the Marine Biological Station of Espesgrend and maintain in 1m³ tanks with running sea water placed in a dark cold room (**Fig. 1d**), in which they can survive a few days. All sharks were sacrificed according to local rules for experimental fish care (i.e. a quick blow to the head) and were measured, weighted and sexed before being use for experimental studies.

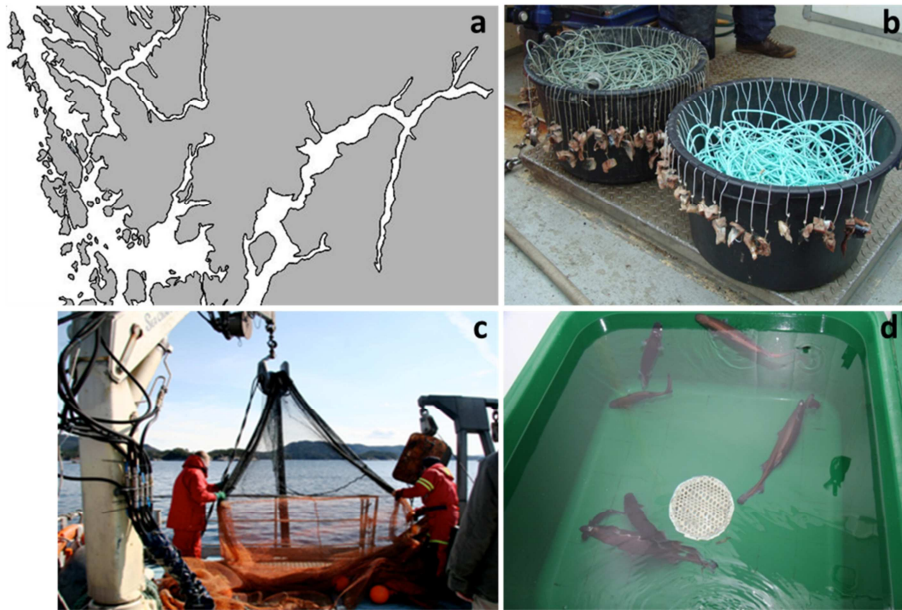


Fig. 1 **a** Location of scientific field missions undertook in this study. **b** Bottom longlines and **c** deep trawl used to catch *E. spinax* specimens. **d** Sea water tanks in which sharks were maintain before experiments. Pictures: J. Mallefet

Chapter 1

The first chapter aims to describe the ultrastructural components building up *E. spinax* photophore, their morphological changes during light event, as well as the possible involvement of bacterial symbionts in the light emission. Ultrastructural organization (Paper 1) and cytological changes (Paper 2) were investigated using transmission electron microscopy (TEM), while the study of bacterial symbionts presence (supplementary results) involved fluorescence *in situ* hybridization (FISH).

Ultrastructure

TEM is a microscopy technique that involves the transmission of an electron beam through an ultra-thin section of the sample. The differential electron absorption of biological structures allows visualizing cell components at a very high resolution. This technique was therefore convenient to observe photophore structural components and their evolution in the course of light emission. Before being observed, sample requires particular fixating, embedding and contrasting method.

Fixation: Fresh skin patches with photophores from shark ventral face were dissected and fixed in a solution of glutaraldehyde (3 % glutaraldehyde, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8) during three hours and were then rinsed in a cacodylate buffer (glutaraldehyde-fixed patches; 0.2 M sodium cacodylate, 0.31 M sodium chloride; pH 7.8). To get rid of the shark skin denticles, skin patches were immersed in a decalcifying solution (OsteoRAL R fast decalcifier, RAL diagnostics) for 10 days at room temperature with a continuous agitation. Samples were then post-fixed in osmium tetroxide (1 % osmium tetroxide, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8) during 45 minutes and then progressively dehydrated in a graded ethanol series (25 % / 10 min; 50 % / 10 min; 70 % / 20 min; 90 % / 30 min and 100 % / 1 h).

Embedding and sectioning: skin patches were embedded in a Spurr's resin (10g ERL 4206 resin, 6g DER 736 resin, 26g nonenyl succinic anhydride (NSA), 0.4g dimethylaminoethanol (DMAE)). After one night of continuous agitation at room temperature, the resin solution

was heated at 70 °C for 24 hours to trigger polymerization. Thin sections of 90 to 110 nm were obtained using an ultramicrotome and placed on copper grids.

Contrasting: Sections were first contrasted in uranyl acetate (0.18 M uranyl acetate solution:ethanol (2:1)) during 45 minutes and secondly in lead citrate (0.08 M lead nitrate, 0.12 M sodium citrate, 0.16 M sodium hydroxide) during 4 minutes. They were then ready to be observed in a transmission electron microscope.

In a first step, TEM permitted the observation of unstimulated photophores in order to describe their ultrastructure. In a second phase, evolution of photocyte components in the course of light emission was investigated by fixing skin patches at different times during the light event induced by two excitatory hormones, melatonin and prolactin.

Fluorescence in situ hybridization

FISH is a technique allowing localising a specific DNA sequence on a section. The section is hybridized with a probe that will specifically bind on the targeted DNA and that is, on the other hand, labelled with a fluorophore, permitting to observe the targeted DNA in fluorescence microscopy. This technique was used in this study in order to localise the presence/absence of bacterial DNA inside *E. spinax* photophores and therefore to explore the presence/absence of bacterial symbionts in the light-emitting organ. Shark skin sections were marked with the EUB338 probe (complementary to a 16S gene portion highly conserved in bacterial genome) labelled with the Cy3 fluorescent marker.

Fixation and sectioning: Fresh skin patches with photophores from shark ventral face were dissected and fixed in a solution of formaldehyde (4 % formaldehyde, 0.052 M sodium phosphate monobasic, 0.082 M sodium phosphate dibasic; pH 8.4) during three hours, and were then rinsed in a phosphate buffer (0.081 M sodium phosphate dibasic, 1.37 M sodium chloride, 0.015 M potassium phosphate monobasic, 0.027 M potassium chloride, 0.25 % sodium azide; pH 8.4). They were immersed in a decalcifying solution (OsteoRAL R fast decalcifier, RAL diagnostics) for 10 days at room temperature with a continuous agitation, and progressively dehydrated in

a graded ethanol series (70 % / 24 h; 90 % / 2 x 1 h / room temp.). They were then immersed in butanol baths (100 % / 1 h; 100 % / 12 h / 60 °C) and paraffin wax baths (100 % / 4 h; 100 % / 12 h / 60 °C). After cooling, paraffin blocks were cut in sections of 7 µm.

Hybridization: sections were deparaffinized by immersion in xylene baths (100 % / 2 x 10min / room temp.) and in a decreasing ethanol series (100 % / 10 min; 90 % / 30 sec; 70 % / 2 x 10 min / room temp.). Unembedded sections were firstly immersed in hybridization buffer during 1h at 46 °C in a humid chamber and secondly in the probe solution during 4 h (EUB338 probe labelled with the Cy3 fluorescent marker in a 5 ng / µl concentration). Tissues were finally rinsed with PBS during 15 min and mounted with fluorescence media, to be observed in fluorescence microscopy.

Chapter 2

Following results obtained in the chapter 1, *E. spinax* was thought to possess an intrinsic light-emitting system, based on chemicals (luciferin and luciferase or photoprotein) located inside photocytes. Because various luminescent organisms are thought to acquire their luciferin through the food chain, chapter 2 investigates the feeding habits of *E. spinax* in order to identify putative luminous or luciferin-carrying prey. Firstly, shark diet is analysed using various indexes (Paper 3 and supplementary results). Secondly, the presence of luciferins in the shark digestive tract and photophores was assayed and cross-reactions with the putative shark luciferase were performed, in order to test the hypothesis that *E. spinax* might use an alimentary acquired luciferin to produce its own bioluminescence.

Diet analysis

Shark stomach contents were collected and prey items were identified to the lowest taxonomic level possible based on non-digestible or less-digestible components. For instance, euphausiids eyes are characteristically bean shaped and digested slowly (**Fig. 2a**). Each pair of eyes was therefore counted as one unidentified euphausiid, unless a

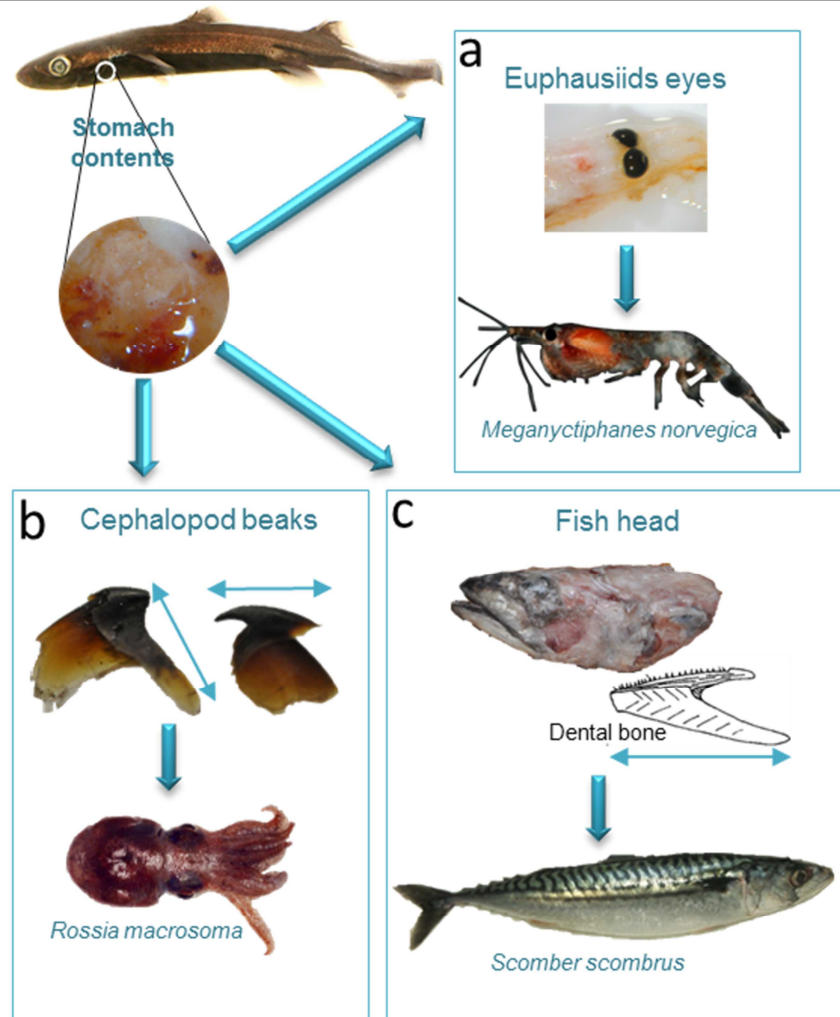


Fig. 2 Methodology for shark stomach contents analysis. **a** Euphausiids are identified based on their eyes shapes. **b** cephalopods are identified following beak measurement. **c** Large fishes are identified following dental bone measurement. Picture: M. Renwart & J. Mallefet.

more detailed morphological analysis allowed its identification until de species level. A morphological analysis of cephalopod beaks was used to identify the corresponding species (**Fig. 2b**). Shrimps and fishes were identified to the species level when digestion state was low and the

whole body was present in the stomach, or base on dental bone dimension for large fishes (**Fig. 2c**).

Four quantitative indexes were used to describe the importance of prey in the diet of *E. spinax*: (i) the numeric index (%N) as the abundance of a prey item in percent to the total abundance of prey, (ii) the gravimetric index (%W) as the remaining weight of a prey item in percent to the total weight of prey and (iii) the occurrence index (%O) as the number of stomachs containing a prey item in percent to all full stomachs. The relative importance index (iv) was calculated as follow: $IRI = (\%N + \%W) \times \%O$. (Cortés, 1997).

Cross-reactions

Results obtained in the diet analysis suggested *E. spinax* could alimentary acquire its luciferin. This was tested by performing various cross-reactions: luciferins presence in shark digestive tract and photophores was tested by mixing a specific luciferase solution with the corresponding luciferin extract. On the opposite, the use of a luciferin by the putative shark luciferase was tested by mixing a specific luciferin solution with the corresponding photophore luciferase extract.

Luciferin assay: a fresh sample from stomach/intestinal content or photophores from the ventral face was weighted and crushed with fivefold the weight of methanol, in order to extract the luciferin(s). Because methanol has the property to inactivate luciferase, this solvent is classically used for luciferin extraction, in order to avoid its irreversible oxidation by luciferase. The extract was then rapidly assayed versus three different luciferases: the purified *Renilla* luciferase (Nanolight, working dilution of 0.2 g/L in a TrisHCl buffer: TrisHCl 0.01 M, NaCl 0.5 M, BSA 0.1 %, pH 7.4) to test the presence of coelenterazine, the crude cypridinid luciferase (extracted with distilled water from dried *Vargula hilgendorfi* Muller, 1890) to test the presence of cypridinid luciferin, and the crude euphausiid luciferase (extracted with distilled water from fresh *M. norvegica*) to test the presence of euphausiid luciferin (**Fig. 3**). 20 µl of the extract was added to 180 µl of a TrisHCl buffer (TrisHCl 0.01 M, NaCl 0.5 M, pH 7.4) and mixed with 5 µl or 20 µl of purified or crude luciferase respectively, previously added to 195 or 180 µl of the same TrisHCl buffer. Light was recorded in a luminometer.

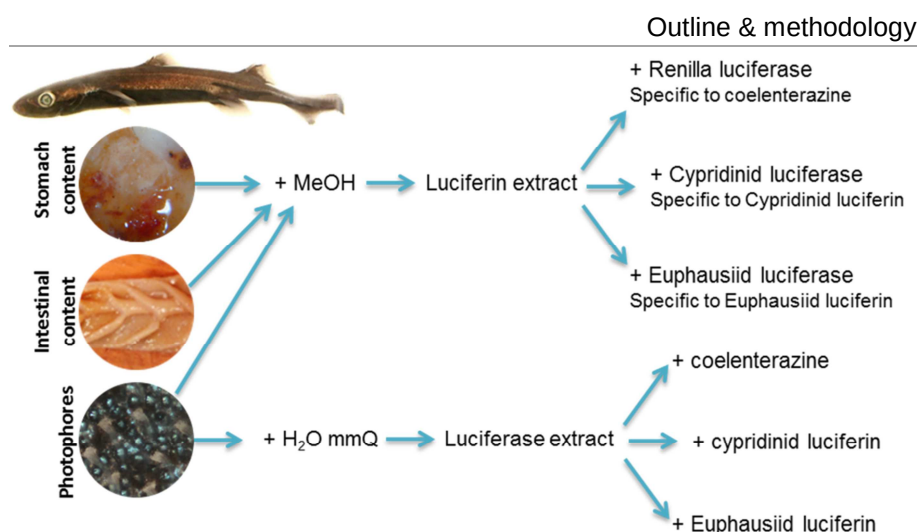


Fig. 3 Cross-reactions performed in the chapter 2, paper 3. Pictures: R. De Jaegere & J. Mallefet.

Luciferase assay: a fresh piece of shark skin with photophores from the ventral face was weighted and crushed with fivefold the weight of distilled water, in order to extract the catalyst of the shark luminous system. Distilled water is classically used for luciferase extraction to keep the enzyme active and allow the luciferin consumption. The distilled water extract was assayed versus three different luciferins: the purified coelenterazine (Nanolight, working dilution of 0.044 g/L in methanol), the purified cypridinid luciferin (purified by Y. Ohmiya's lab (AIST, Japan), working dilution of $4,05 \cdot 10^{-4}$ g/L in methanol) and the crude euphausiid luciferin (extracted with methanol from fresh *M. norvegica*) (**Fig. 3**). 20 μ l of the photophore extract was added to 180 μ l of a TrisHCl buffer (TrisHCl 0.01 M, NaCl 0.5 M, pH 7.4) and mixed with 5 μ l or 20 μ l of purified or crude luciferin respectively, previously added to 195 or 180 μ l of the same TrisHCl buffer. Light was recorded in a luminometer.

Chapter 3

Experiments carried out in the chapter 2 suggested *E. spinax* intrinsic luminous system to be not based on a known marine luciferin, at least on its active "free form" classically extracted with methanol. We therefore considered this luminous system as a novel mechanism

requiring to be purified and characterized. In a first part of the chapter 3, initial steps of luciferin isolation are carried out using reversed-phase HPLC coupled with a fluorimeter. In a second part, the hypothesis of luciferase to be a peroxidase is tested by using size-exclusion HPLC and peroxidase assays.

Luciferin purification

When isolating a new luciferin, a mean of assaying the molecule of interest is required. Because the shark luciferase could not be used for this purpose (the enzyme was impossible to isolate and stabilize), we chose to use a fluorimeter as detector as we assumed *E. spinax* luciferin or derivatives to be fluorescent.

Reversed-phase HPLC: Extraction of luciferin or derivatives was performed by crushing frozen skin patch in a volume of tenfold its weight in methanol, in order to inactivate luciferase if present. Extract was centrifuged (16,000 g; 4 °C; 3 min) and supernatant was collected and directly introduced in HPLC. An Atlantis T3 column (Waters) was used and the mobile phase was composed of water (H₂O mmQ) and acetonitrile (ACN HPLC) in a 95:5 proportion at elution beginning until a 10:90 proportion at elution end, following a gradient. Methanoic acid was added to water in order to keep a constant 0.1 % of these compounds in the total elution volume. Elution lasted 7 minutes and eluted molecules were detected with a fluorometer for four paired excitation/emission wavelengths (280/330; 280/450; 280/660; 370/450), chosen from pilot experiments results. Comparison with skin without photophore was made.

Luciferase characterisation

Based on *E. spinax* luminescence characteristic, we hypothesized that its luminous system might be based on an ancient reactive oxygen species (ROS) defence mechanism, and that luciferase might therefore have a peroxidase activity. In a first experiment, peroxidases from a skin extract with photophores were separated by size-exclusion HPLC and assayed in various collected peaks. In a second experiment, azide and cyanide were injected on fresh skin

patches with photophores in order to test their quenching effect on *in vivo* light production.

Size-exclusion HPLC: Extraction of luciferase was performed by cutting pieces of frozen skin with photophores in very thin slices and then crushed them in a volume of buffer (50 mM Na₂HPO₄; 0.1 M NaCl; pH 6.7) of fivefold their weight. Extract was centrifuged (16,000 g; room temp.; 15 min) and supernatant was collected and directly introduced in HPLC. The column used allowed separation of molecules by size if smaller than 200.000 Da. Mobile phase was the same buffer as used for extraction, and elution was isocratic and lasted 20 minutes. All peaks were collected just after elution and analyzed for their peroxidases content, thanks to chemiluminescent reaction produced by peroxidases when mixed with luminol and hydrogen peroxide. Ten µl of each peak fraction was mixed with 90 µl of a solution containing both luminol and H₂O₂ (TrisHCl 1 M; Ethanol 10 %; Luminol 7.5 mM; H₂O₂ 7.5 mM; pH 8.3) and the light was recorded in a multiplate luminometer. Light produces was considered to be proportional to peroxidases content and comparison with skin without photophore was made.

Azide and cyanide quenching effect was tested on the light produced by two excitatory hormones (melatonin and prolactin). Fresh skin patches with photophores from ventral face were immersed in the four corresponding solutions ([i] 15 mM NaN₃; 1 µM melatonin or [ii] prolactin in a buffered shark saline [Murphy & Sies, 1990; Bernal *et al.*, 2005]; [iii] 15 mM KCN; 1 µM melatonin or [iv] prolactin in a buffered shark saline) and the produced light was compared to controls where no inhibitor was added.

RESULTS



Etmopterus spinax gravid female (M. Renwart)

CHAPTER I

ULTRASTRUCTURAL MORPHOLOGY AND CYTOLOGICAL CHANGES OF *ETMOPTERUS* *SPINAX* PHOTOPHORES

CHAPTER I

ULTRASTRUCTURAL MORPHOLOGY AND CYTOLOGICAL CHANGES OF *ETMOPTERUS SPINAX* PHOTOPHORES

In the process of investigating a novel bioluminescent model organism, a key step is certainly to explore the morphology of the structures involved. This first chapter therefore aims to study the ultrastructural organisation of *E. spinax* photophores by using transmission electron microscopy (TEM) since this technique permits to explore this particular organ with accuracy.

The first paper aims to describe the structural components building up the photophore, as well as the intracellular organisation of the light-emitting cells, the photocytes. We found photophores to be composed of different interlocked pieces and one of them, a reflector-like structure, is described for the first time as it was until now believed to be absent in shark photophores. The photocytes present a regionalized cytoplasm that has been divided into three areas. The apical granular area, which is fluorescent and typically oriented toward the photophore centre, was hypothesized to be the site of the chemiluminescent reaction. The granular inclusions it contains were therefore assumed to be the shark microsources.

Our next step was to investigate the morphological changes occurring within photocytes as well as at photophore level during the light emission process. This is the purpose of the second paper. Pieces of shark skin with photophores were fixed at different time-points during light emission produced by two light-excitatory hormones, prolactin and melatonin. We found prolactin to generate the formation a new structure and both hormones induced morphological changes in granular

inclusions. The possible role of those structures in the light-emitting system of *E. spinax* is discussed. Moreover, hormones also played a role at the photophore level where they induced pigment retraction from the photophore centre.

The third part of this chapter examines in detail a possible involvement of bacterial symbionts in the light emission of *E. spinax*. Although this possibility is already discussed in the first paper, we present here supplementary results based on bacteria revelation using fluorescence *in situ* hybridization (FISH) technique. By comparing existing literature with our ultrastructural data and preliminary results on bacteria detection in shark skin, we came to the conclusion that *E. spinax* possesses an intrinsic mechanism of light emission since no bacteria were detected inside the photocytes.

PAPER 1

Ultrastructural organization of lantern shark (*Etmopterus spinax* Linnaeus, 1758) photophores

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Ultrastructural organization of lantern shark (*Etmopterus spinax* Linnaeus, 1758) photophores

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Abstract *Etmopterus spinax* Linnaeus, 1758 is a deep-sea lantern shark that emits blue light thanks to thousands of tiny cup-shaped organs made of a pigmented sheath enclosing light-emitting cells topped by an iris-like structure and a lens. In this study, we investigate the ultrastructure of these photophores in order to improve our understanding of the light emission process. The presence of a novel layer, a putative reflector upholstering the pigmented sheath, is highlighted. The intracellular organization of the photocytes is addressed. They appear as regionalized cells: their basal area is occupied by an ovoid nucleus, their medial area is highly vesiculated and their apical area, oriented toward the photophore center, displays small granular inclusions. We hypothesize this granular area to be the intracellular site of photogenesis in *E. spinax*, as it is also the most fluorescent part of the photocyte.

Keywords Bioluminescence · Shark · Electron microscopy · Photocyte · Microsource

Introduction

In the deep ocean, most taxa have bioluminescent members that display a wide range of mechanisms to emit light and take advantage of this particular interaction tool (Haddock et al. 2010). The organ involved in this chemical process is called photophore but this generic term includes many different structural organizations (Hastings and Morin 1991). A luminescent unit can be as simple as one unique light-emitting cell, the photocyte, or shows a much more complex arrangement with numerous photocytes grouped in the center of an organ—the photophore *stricto sensu*—and surrounded by various structures that enhance the light emission effectiveness, including protective pigmented layers, reflectors, focusing lenses and selective filters. In luminous symbiotic organisms (mainly some teleosts and cephalopods), photophores are specific structures containing luminous bacteria. In these bacterial photophores, the light reaching the outside can be controlled by a removable shutter or by physiological regulation (Herring 1982; Tong et al. 2009). Photophores also greatly differ in size, number and location: the teleosts *Photoblepharon palpebratus* Boddaert, 1781 and *Anomalops katoptron* Bleeker, 1856 display only two large suborbital symbiotic photophores of approximately 10 mm length (Haneda and Tsuji 1971); most euphausiid species bear ten photophores ranging from 0.1 to 0.6 mm in diameter (Herring and Locket 1978), while dalatiid sharks have thousands of 0.05-mm ventral photophores (Claes and Mallefet 2009).

Investigating the fine anatomy of photophores is a key step in the understanding of the light-emitting process and allows linking function with morphology (Strum 1969a, b). The ultrastructure of photophores has been described in various luminous species, and the knowledge of the photocyte's intracellular organization provided a better

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understanding of the mechanism of bioluminescence. Microsources are the intracellular structures responsible of the photogenesis. They are as diverse in their forms and operating modes as the photophores themselves and often difficult to identify with certainty. In dinoflagellates, microsources, called *scintillons*, are small organelles protruding in a low-pH vacuole. An action potential along the membrane of the vacuole triggers the light by creating a protons flux toward the scintillon that activates the luciferase and releases the luciferin, the two components of the chemical reaction (Fritz et al. 1990). Coelenterates microsources, termed *lumisomes*, are vacuoles that are supposed to contain all the molecules of the light-emitting reaction, as only the addition of calcium ions is needed to produce light (Anderson and Cormier 1973). In adult fireflies, microsources refer to vesicles containing dense inclusions and microtubules. These vesicles share the features of peroxisomes and were found to show a catalase activity possibly related to light emission (Hanna et al. 1976). The *photosomes* of the Polynoidae are tridimensional tubules of endoplasmic reticulum that form a paracrystalline network bearing the protein responsible for the light emission, the polynoidin. An action potential releases oxygen radicals that activate this protein (Bassot and Nicolas 1987). Osteichthyes and Chondrichthyes are the only vertebrates known to be luminous. The ultrastructure of photocytes in Chondrichthyes remains to be studied in detail. In midshipman fish *Porichthys spp.*, the cytoplasm of the photocytes contains numerous vacuoles that tend to coalesce during the light emission, which suggest their involvement in the bioluminescent process (Anctil 1979a, b).

In Chondrichthyes, all Dalatiidae (kitefin sharks) and Etmopteridae (lantern sharks), which currently represent 56 species, 46 etmopterids and 10 dalatiids, have been assumed to be luminous (Ebert et al. 2013); eleven out of the twelve genera listed in these shark families have been documented to bear photophores (Claes and Mallefet 2009). Ohshima (1911) studied the structure of photophores in two etmopterid species (*Etmopterus lucifer* Jordan and Snyder, 1902 and *E. pusillus* Lowe, 1839) and found that the cup-shaped organ is composed of photocytes surrounded by pigmented cells, which form a pigmented sheath and iris-like structure, and topped by lens cells. Studies on the ontogeny of photophores in *E. spinax* Linnaeus, 1758 (Claes and Mallefet 2008) support the aforementioned results.

The purpose of this paper is to investigate the ultrastructure of *E. spinax* photophores in order to describe their different structural components with an emphasis on the intracellular organization of the photocytes. This first ultrastructural description is essential to improve our

understanding of the light emission process occurring in these structures.

Materials and methods

Shark tissue collection

Specimens of *E. spinax* were caught by longlines in Norway (Raunefjord—60°15.908 N; 05°07.778 O) during two field missions in November 2012 and March 2013. Sharks were killed according to the local rules for experimental fish care. Fresh skin patches with photophores were dissected and fixed in a solution of either glutaraldehyde (3 % glutaraldehyde, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8) or formaldehyde (4 % formaldehyde, 0.052 M sodium phosphate monobasic, 0.082 M sodium phosphate dibasic; pH 8.4) during at least three hours. Skin patches were rinsed either in a cacodylate buffer (glutaraldehyde-fixed patches; 0.2 M sodium cacodylate, 0.31 M sodium chloride; pH 7.8) or in a phosphate buffer (formaldehyde-fixed patches; 0.081 M sodium phosphate dibasic, 1.37 M sodium chloride, 0.015 M potassium phosphate monobasic, 0.027 M potassium chloride, 0.25 % sodium azide; pH 8.4) and stored in the corresponding solutions. Glutaraldehyde-fixed patches were used for transmission electron microscopy (TEM) while formaldehyde-fixed patches were used for epifluorescence microscopy.

Transmission electron microscopy

Decalcification and post-fixation

Skin patches were immersed in a decalcifying solution (OsteoRAL R fast decalcifier, RAL diagnostics) for 10 days at room temperature with a continuous agitation (the solution was renewed every 2 days to avoid calcium saturation) in order to get rid of the elongated skin denticles that could jeopardize the sectioning process. Samples were then post-fixed in osmium tetroxide (1 % osmium tetroxide, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8) during 45 min and then progressively dehydrated in a graded ethanol series.

Embedding

Skin patches were embedded in a Spurr's resin [10 g ERL 4206 resin, 6 g DER 736 resin, 26 g nonenyl succinic anhydride, 0.4 g dimethylaminoethanol]. After one night of continuous agitation at room temperature, the resin solution was heated at 70 °C for 24 h to trigger polymerization.

Sections and contrast

Thin sections of 90–110 nm were obtained using an ultramicrotome (Leica Ultracut UCT—Leica Microsystems) and placed on copper grids. Sections were first contrasted in uranyl acetate [0.18 M uranyl acetate solution:ethanol (2:1)] during 45 min and secondly in lead citrate [0.08 M lead nitrate, 0.12 M sodium citrate, 0.16 M sodium hydroxide] during 4 min. After drying, sections were ready to be observed in a transmission electron microscope (Zeiss Leo 906E) where micrographs were taken. A 3D modeling of the structural components of the photophore was finally performed using the software Blender (Blender Foundation, Netherlands, software version 2.68).

Quantitative data

Areas of structures (i.e., lens, photocytes, pigments, photocyte vesicles and apical cells) were measured on the micrographs using Image J (National Institutes of Health, USA, software version 1.46r). These structures were assimilated to spherical elements with circular section, and their mean diameters with a standard error on the mean were post-calculated based on the measured areas. To measure the areas of the photocyte granules, the *watershed* option of Image J was first run to distinguish each individual granule from each other's. The *particle analysis* option was then applied to calculate the area of each granule, and one mean diameter was calculated per photocyte. The number of photocytes (N_{phot}) present in a photophore was estimated from selected photophore micrographs (those displaying at least two entire photocytes i.e., photocytes displaying their granular area in addition to their vesicular area and/or their nucleus, which indicate the section to pass through the photophore center), using the following formula:

$$N_{\text{phot}} = \left(\frac{A_{\text{phot}}}{A_{\text{tot}}} \right)^{\frac{3}{2}}$$

where A_{phot} is the area of an entire photocyte and A_{tot} is the whole area occupied by photocytes.

Fluorescence observations

Skin patches fixed with formaldehyde were decalcified and embedded as described before. Semi-thin sections of 600 nm were obtained using the ultramicrotome, put on a slide and directly observed with a fluorescence microscope (Leitz Diaplan). No post-fixation and contrast steps were applied.

Results

Structure of the photophore

The 3D modeling (Fig. 1) shows an overview of the structures constituting the photophore: a hemispherical cup-shaped layer of pigmented cells, the pigmented sheath, that encloses the other components surmounted by different cell types (I and II) that make the transition to the lens, placed in the apical region of the photophore (Fig. 1a). As the pigmented sheath is removed, a previously undescribed layer, the reticulated layer, is highlighted (Fig. 1b). Inside the reticulated layer, the light-emitting photocytes show a particular orientation toward the center of the photophore (Fig. 1c, d). In cross-section (Fig. 2), the pigmented sheath and the reticulated layer form an arch-like structure and photocytes appear as ovoid cells packed against each other, leaving few intracellular spaces. On each side of the photocytes, some pigmented cells form the iris-like structure. Number of photocytes per photophore (N_{phot}) was estimated to range from 5.22 to 18.66 (mean number = 13.16 ± 0.76 , $N = 18$).

Pigmented sheath and iris-like structure

The pigmented cells can be either elongated and piled on each other as a heap of leaves in the pigmented sheath (Fig. 3a, b) or branched and spread on each side of the photophore in the iris-like structure (Fig. 3a, c). Inside the pigmented cells, dark pigments are densely packed to form numerous oval melanosome-like organelles (diameter of $0.39 \pm 0.01 \mu\text{m}$, $N = 100$).

Reticulated layer

This layer enclosed the photocytes and upholds the inner face of the pigmented sheath. It is packed of thin cells with a flattened nucleus pushed against the cell wall and a lumen filled with a reticulated matrix (Fig. 4a). This three dimensional web is composed of fibrous material forming polygonal boxes that appear empty on the micrographs (Fig. 4b).

Photocytes

Photocytes (diameter of $15.46 \pm 0.51 \mu\text{m}$, $N = 34$) are regionalized cells grouped in the center of the photophore just above the reticulated layer (Fig. 5a). Their spherical nucleus, which presents deep cytoplasm protrusions (Fig. 5b), is usually located in their basal part and adjoins

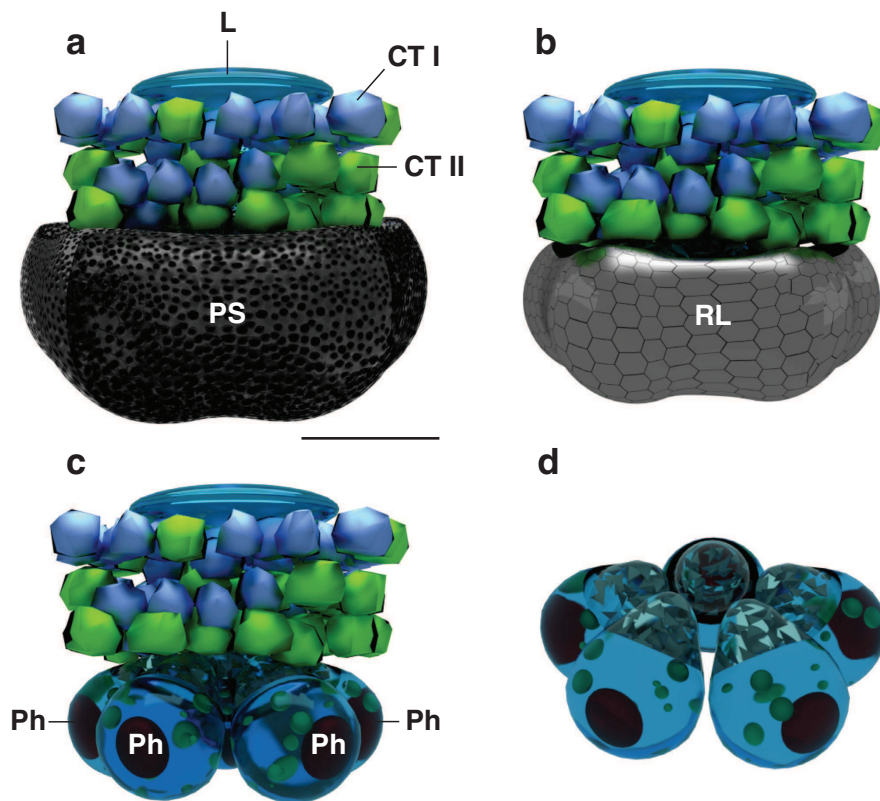


Fig. 1 3D modeling of the photophore. **a** Entire photophore with photocytes enclosed in the pigmented sheath (*PS*). **b** Reticulated layer (*RL*) revealed by pigmented sheath removal. **c** Photocytes (*Ph*)

revealed by reticulated layer removal. **d** Photocytes isolated from other photophore components, revealing their particular orientation. *CT I* cellular type I, *CT II* cellular type II, *L* lens

a highly vesiculated region that will be further referred as the “vesicular area.” In this intermediate zone, vesicles are numerous and can be separated into two types: ovoid (diameter of $2.12 \pm 0.13 \mu\text{m}$, $N = 36$) and polygonal vesicles (diameter of $0.49 \pm 0.02 \mu\text{m}$, $N = 100$), which are more numerous. The apical region of the photocyte is completely filled with granular inclusions (diameter of $0.38 \pm 0.01 \mu\text{m}$, $N = 44$) irregularly shaped and dense to electrons; it will further be referred as the “granular area.” By contrast, the lumen surrounding the granular inclusions appears white, revealing no electron

absorbance there (Fig. 5f). This area is not surrounded by a membrane although it forms a distinct part of the cytoplasm. The granules present in this region can also be found in smaller number in the vesicular area, packed between the vesicles. In this sense, both areas seem to be a continuum (Fig. 5d, e). Interestingly, the areas of the photocytes are not oriented randomly: The granular area of a photocyte is always oriented toward the center of the photophore (Figs. 2b, 3a, 6). This typical orientation is also visible in fluorescence microscopy as the granular area presents the highest fluorescence intensity (Fig. 7).

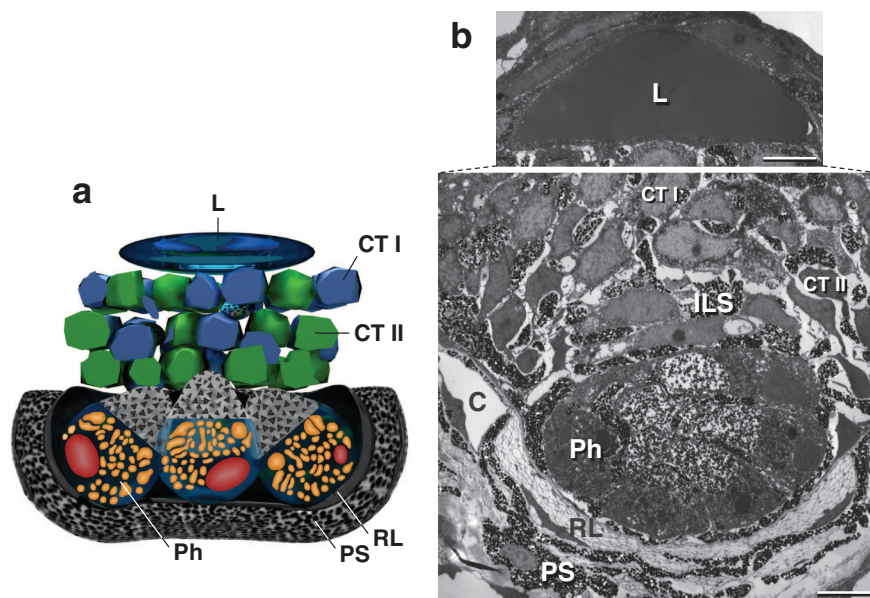


Fig. 2 Cross-sections of a photophore. **a** 3D modeling of a cross-section in a photophore. **b** Corresponding micrograph cross-section in a photophore. *C* capillary, *CT I* cellular type I, *CT II* cellular type II,

ILS iris-like structure, *L* lens, *Ph* photocyte, *PS* pigmented sheath, *RL* reticulated layer. Scale bar 10 μ m

Apical structures

Above the photocytes are located different cell types that make the transition between the photogenic and lens cells. In the first cellular type (diameter of $10.79 \pm 0.27 \mu\text{m}$, $N = 32$), the nucleus, located in the center of the cell, is surrounded by a fibrous material that constitutes a tridimensional web filling the whole cytoplasm (Fig. 8a, b). Those cells are preferentially located near the lens (Fig. 2b). Only the nucleus of the second cellular type—usually observed closer to the photocytes (Fig. 2b)—can be observed (Fig. 8c). For this reason, no quantitative data were taken. The lens (diameter of $27.7 \pm 1.2 \mu\text{m}$, $N = 20$) is composed of one or sometimes two cells situated at the apical end of the photophore. These cells are bigger than any other cells in the photophore and present a typical convex shape (Fig. 2b). The nucleus of the cell is pushed against the cell membrane, as the cytoplasm is entirely filled with a vacuole containing a homogeneous substance moderately dense to electron.

Discussion

Previous studies, using optical microscopy, revealed a simple organization of the etmopterid light-emitting organ: a cup-shaped hemisphere composed of a protective layer of pigmented cells, an iris-like structure, a few photocytes and a lens (Johann 1899; Ohshima 1911; Claes and Mallefet 2008). Using the TEM technique, we were able to investigate the ultrastructure of photophores of the lantern shark *Etmopterus spinax* and the presence of an undescribed layer as well as various intracellular components have been highlighted.

Fishes show a great diversity in the morphology and operating mode of their photogenic structures. The suborbital photophores of the teleosts *Photoblepharon* spp. and *Anomalops* spp. are large elliptical organs containing tubules full of luminous bacteria measuring from 2 to $3.3 \mu\text{m}$ long and identifiable on electron micrographs (Haneda and Tsuji 1971). So far, no bacterial symbionts have been reported in luminous sharks, and our results did

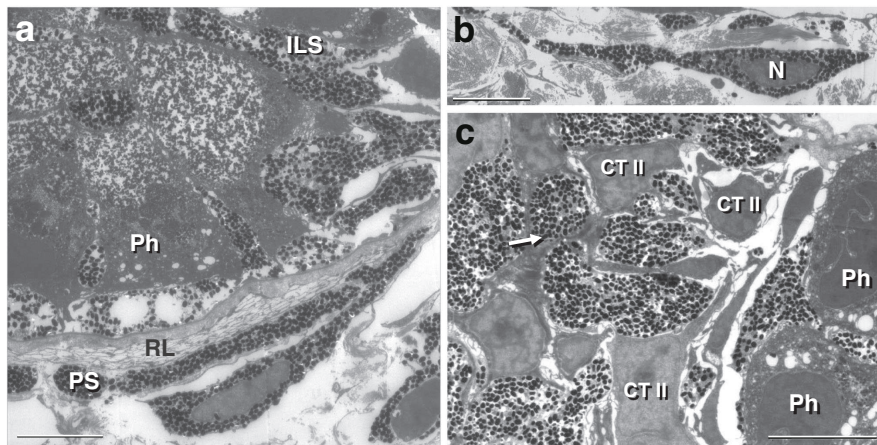


Fig. 3 Pigmented cell morphologies. **a** Photophore cross-section showing the pigmented sheath (PS) and the iris-like structure (ILS). **b** Typical shape of the pigmented cells when located in the pigmented sheath. **c** Typical shape of the pigmented cells when located in the

iris-like structure. Arrow shows a pigment organelle. CT II cellular type II, N nucleus, Ph photocyte, RL reticulated layer. Scale bar 10 µm

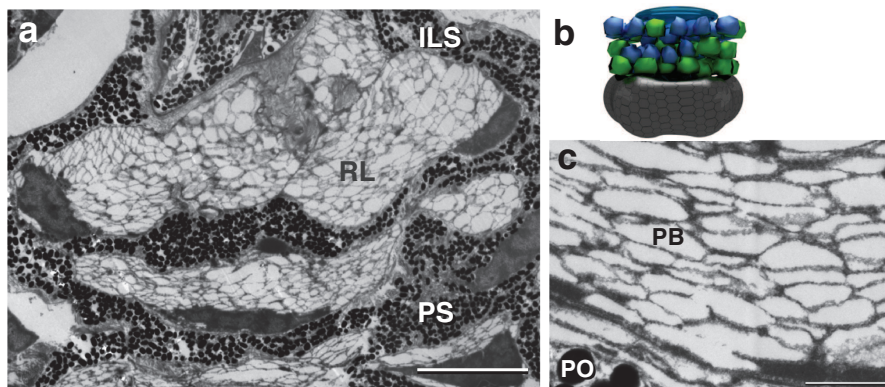
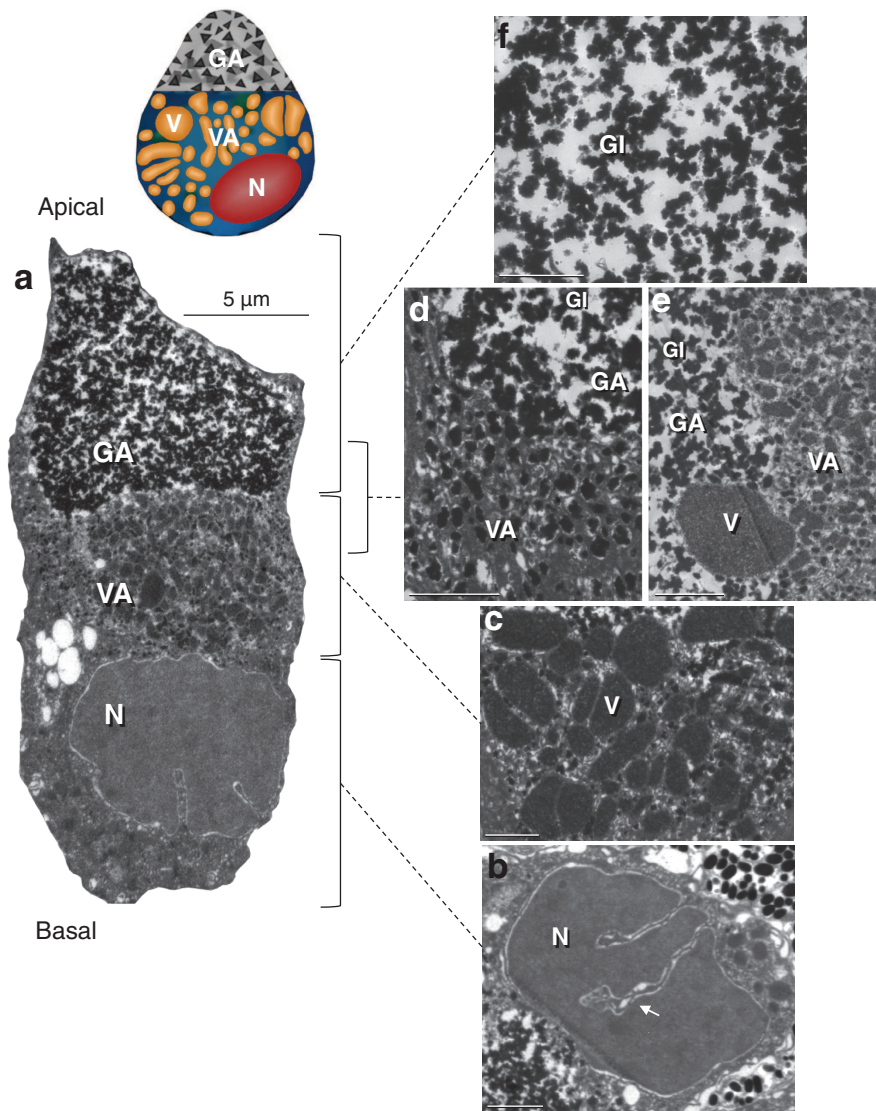


Fig. 4 Reticulated layer. **a** Photophore cross-section located before the photocytes and showing the reticulated layer (RL). Scale bar 10 µm. **b** The corresponding 3D modeling. **c** Magnification of the

reticulated matrix. ILS iris-like structure, PO pigment organelle, PB polygonal box, PS pigmented sheath. Scale bar 1 µm

not reveal the presence of bacteria inside the photocytes of *E. spinax*. Intrinsic luminescence, on the opposite, is the light produced by chemicals within an organism's intrinsic tissue and not by symbionts (Haddock et al. 2010). In most intrinsic photogenic structures, the luminous material

Fig. 5 Photocyte zonation. **a** Typical regionalization of the photocyte with the nucleus (N), the vesicular area (VA) and the granular area (GA). **b** Nucleus with its cytoplasmic protrusions (arrow). **c** Vesicular area showing numerous homogenous gray dense vesicles (V). **d** and **e** Transition between the vesicular and the granular areas showing a continuum between both. **f** Granular area with congregated granular inclusions (GI). Non-captioned scale bars, 2 µm



remains within the photocytes, but secretory luminescence can also be observed. In sharks, this particular light emission mechanism is reported for *Euprotomicroides zantedeschia* Hulley and Penrith, 1966, which has been hypothesized to eject luminescent secretion from the abdominal pouch (Munk and Jorgensen 1988). In teleosts, secretory luminescence has been studied in *Gonostoma elongatum* Günther, 1878. These particular photogenic structures are very different from what we observed in *E. spinax* photophores (Fig. 9), which did not show any excretory structure.

Shark photocytes are generally encircled by pigmented cells, in both etmopterid and dalatiid species (Claes and Mallefet 2009), and this structural characteristic is also present in Osteichthyes (Fig. 9). In *E. spinax*, we showed that pigmented cells present different morphologies according to their role in the light emission process. Flat and elongated pigmented cells form the sheath that

prevents light dispersion toward the dermis and underlying muscles, while branched pigmented cells form an iris-like structure that mechanically control the light emission. Ohshima (1911) studied the iris-like structure of etmopterid and found that pigmented cells display “pseudopodia-like projections” in three directions that decrease the light reaching the outside, which is supported by our results (Fig. 3a, c). The iris-like structure has been shown to open when the photophore is stimulated by adequate hormones, resulting in an increase in light intensity by retraction of the “pseudopodia-like projections” (Claes and Mallefet 2010).

To efficiently counterilluminate, pelagic organisms have to produce a ventral glow matching the angular distribution, intensity and spectrum of residual downwelling light (Denton et al. 1972). As a consequence, most counterilluminating teleosts and cephalopods have developed accessory structures such as specialized filters

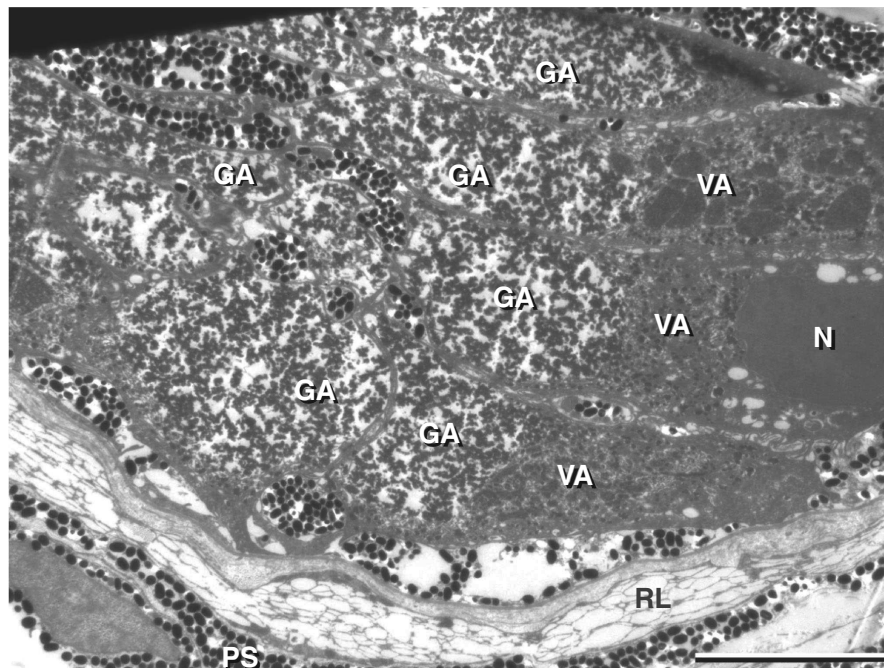
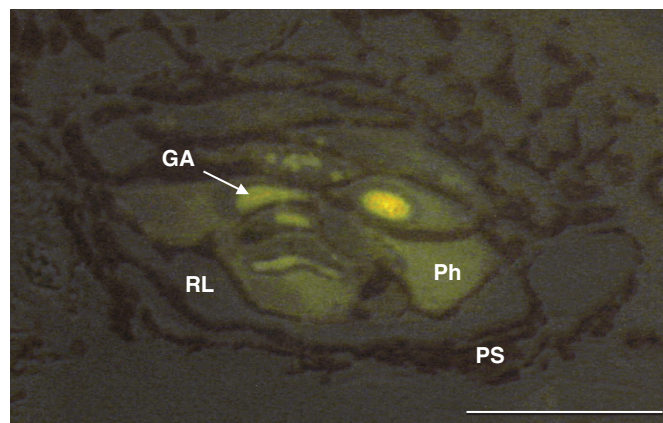


Fig. 6 Group of photocytes showing the characteristic orientation of the granular areas (GA). N nucleus, PS pigmented sheath, RL reticulated layer, VA vesicular area. Scale bar 10 μ m

Fig. 7 Superimposition of two pictures of a 600-nm photophore cross-section: a fluorescent picture showing the autofluorescence of the photocytes and a white light picture showing the delimitations of the photocytes. *Ph* photocyte, *PS* pigmented sheath, *RL* reticulated layer, *GA* granular area. Scale bar 50 μ m



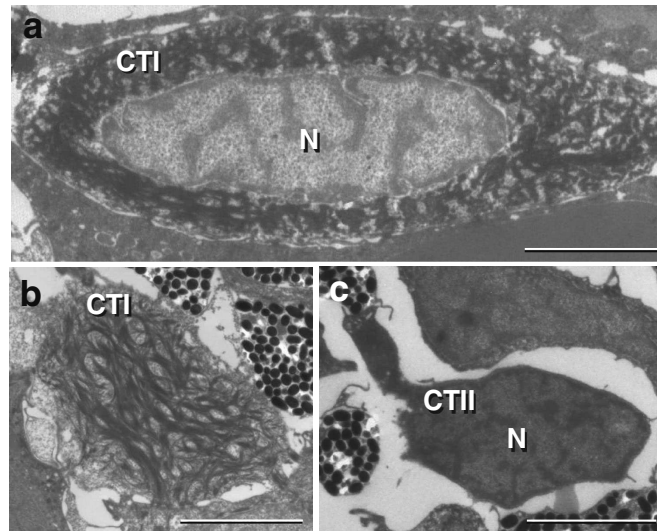
and reflectors to finely tune the physical characteristics of their luminescence (Nicol 1957; Denton et al. 1972, 1985; Harper and Case 1999; Jones and Nishiguchi 2004). These optical structures, on the other hand, are considered to be absent in shark photophores (Iwai 1960; Herring and Morin 1978), which is surprising since these organs can also be used in counterillumination as it was recently confirmed in *E. spinax* (Claes et al. 2010a). However, given the spatial organization and general morphology of the reticulated layer (Figs. 1, 2, 4), which are similar to previously described reflective structures (Denton 1970; Fishelson et al. 2005), we hypothesize this layer to fulfill the function of a reflector in *E. spinax* photophores. Indeed, the cells of the reticulated layer contain a reticulated matrix of polygonal boxes (Fig. 4b) that appear similar to those observed in others species such as the cardinal fish *Siphamia cephalotes* Castelnau, 1875 (Fishelson et al. 2005) or the midshipman fish *Porichthys notatus* Girard, 1854, although reflector cells of the latter appear more needle shaped (Strum 1969a, b). The presence of the reticulated layer in dalatiid species still remains to be confirmed. The polygonal boxes in *E. spinax* might have enclosed crystals with reflective properties. Reflector crystals usually refer to guanine (Denton 1970; Denton et al. 1985; Fishelson et al. 2005), which unfortunately have the property to solubilize when stained with uranyl acetate and lead citrate (Strum 1969a, b), a method used in this study. Attesting the reflective role of the reticulated matrix would therefore require unstained thin sections in order

to study the reflective properties and characteristics (thickness and orientation) of crystals.

The lens is a major component of fish photophores with internal light events. The typical convex shape of the lens covering *E. spinax* photophores (Fig. 2b) is perfectly designed to focus the light emitted outside. The role of the two cell types located between the photocytes and the lens is more difficult to assess. The cellular type I, which contains fibrous material and are often closely associated with the lens (Figs. 2b, 8a, b), probably ensure the stability of the lens even though they could also be involved in the control of the light emission. From the cellular type II, only the nucleus is still visible in the sections (Fig. 8c). For this reason, hypothesizing its function based on the morphology is impossible. Yet an involvement in the light emission control can also be suggested here. Claes et al. (2011) demonstrated the presence of γ -aminobutyric acid (GABA) in the cells surrounding photocytes and in the epidermal cells and argued for GABAergic modulation of the light emission in *E. spinax*. A closer examination of these cells using immunocytochemical techniques would be necessary to reveal the presence of GABA.

We found between 5 and 18 photocytes per photophore in *E. spinax*, which is slightly higher than previous estimation on this species (4–6, Johann 1899), but close to what was observed in two other etmopterid species, *E. lucifer* and *E. pusillus* (about 14, Ohshima 1911). Although the higher number found in this study regarding *E. spinax* might be linked to intraspecific difference,

Fig. 8 Cellular types I and II. **a** Typical nucleus (*N*) location in the center of the cellular type I (*CTI*). **b** Fibrous material filling the cytoplasm of the cellular type I. **c** Nucleus of the cellular type II (*CTII*). Scale bar 5 μ m



it might also be linked to our TEM-based three dimensional estimation method, which provides higher but more accurate values than simple two-dimensional countings. The main structural difference regarding etmopterid and dalatiid photophores lies in the presence of a single photocyte in this latest (Fig. 9), the ultra-structure of which remains to be studied. Interestingly, however, embryos of *E. spinax* also display photophores with single photocytes (Claes and Mallefet 2008; Claes et al. 2010b), a feature among others suggesting a unique origin of luminescence capability in sharks (Claes et al. 2012).

E. spinax photocyte ultrastructure revealed the presence of an apical area full of small granular inclusions i.e., the granular area, which is always oriented toward the center of the photophore, below the lens (Figs. 2b, 3a, 6). This feature has been observed in *P. notatus* (Strum 1969a, b). We suggest that such a physical conformation, which is not random, aims to optimize the amount of light captured by the lens. By superimposing a picture showing the autofluorescence of the photocytes with the same picture in white light, we observed that the granular area corresponds to the most intense fluorescent part of the photocyte (Fig. 7). In some cases (e.g., in the cephalopod

Vampyroteuthis infernalis Chun, 1903; Robison et al. 2003), bioluminescent compounds are autofluorescent and this fluorescence helps to locate the bioluminescence site (Bassot and Bilbaut 1977; Sweeney 1980). The granular area likely also corresponds to the previously described “fluorescent vesicle” (Claes and Mallefet 2008; Claes et al. 2010b). It must be pointed out that no membrane surrounding this area was observed, and therefore, it does not form a delimited vesicle *per se*. Because the apparition of this fluorescent area has been correlated with the functionality of the photophore in *E. spinax* embryos (Claes and Mallefet 2008), we assume the granular area to represent the site of photogenesis and hence the granular inclusions to be the microsources of *E. spinax* photophores. The role of the vesicles, located in the vesicular area, is still unknown. We cannot rule out their involvement in the bioluminescent process possibly by storing or releasing one of the components of the chemiluminescent reaction.

In conclusion, this study provides evidence of an intrinsic bioluminescence with no excretory activity in *E. spinax* photophores. In addition, it reveals the presence of a novel reflector-like layer and putative microsources in the apical granular area of the photocytes. Future work will

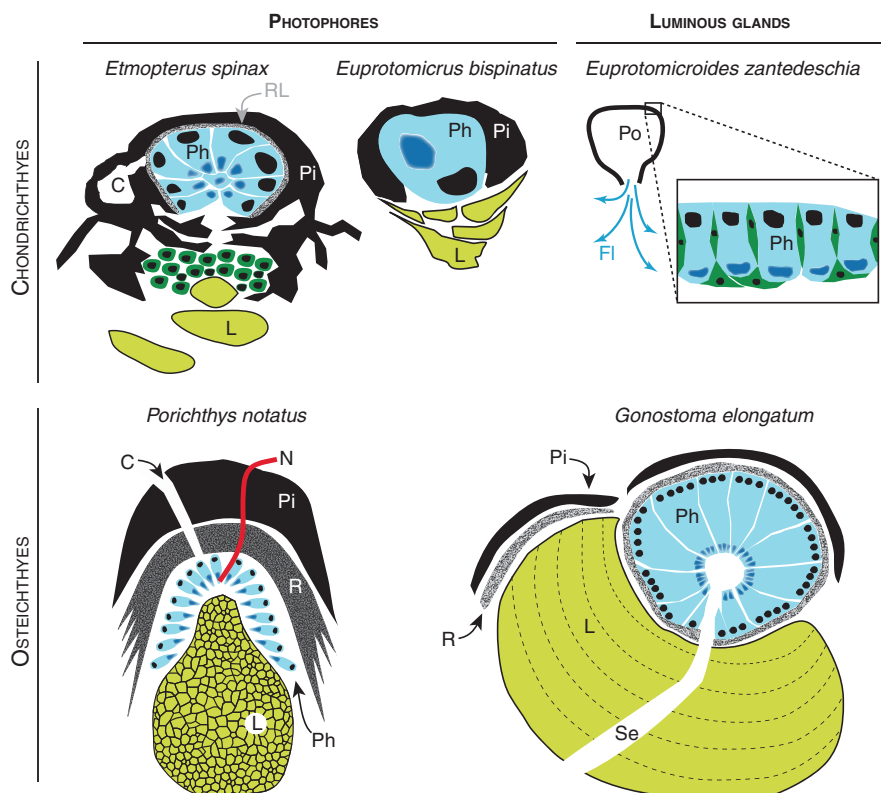


Fig. 9 Intrinsic ventral photophores of fishes. A similar color highlights analogous structures. Unlabeled cells in *E. spinax* and *E. zantedeschia* are cells closely associated with photogenic tissue whose function is uncertain. C capillary, Fi excreted luminous fluid,

L lens, N nerve, Ph photocyte, Pi pigmented cells, Po pouch with secretory photogenic epithelium, RL reticulated layer, R reflector, Se secretory duct

attempt to characterize the morphological changes occurring within the photocytes during the light emission process.

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References

- Ancil M (1979a) Ultrastructural correlates of luminescence in *Porichthys* photophores. I. Effects of spinal cord stimulation and exogenous noradrenaline. *Rev Can Biol* 38:67–80
- Ancil M (1979b) Ultrastructural correlates of luminescence in *Porichthys* photophores. II. Effects of metabolic inhibitors. *Rev Can Biol* 38:81–96
- Anderson JM, Cormier MJ (1973) Lumisomes, the cellular site of bioluminescence in coelenterates. *J Biol Chem* 248:2937–2943

- Bassot J-M, Bilbaut A (1977) Bioluminescence des élytres d'*Acholoe*. IV. Luminescence et fluorescence des photosomes. *Biol Cellulaire* 28:163–168
- Bassot J-M, Nicolas G (1987) An optional dyadic junctional complex revealed by fast-freeze fixation in the bioluminescent system of the scale worm. *J Cell Biol* 105:2245–2256
- Claes JM, Mallefet J (2008) Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark *Etmopterus spinax* (Squaloidea: Etmopteridae). *J Fish Biol* 73:1337–1350
- Claes JM, Mallefet J (2009) Bioluminescence of sharks: first synthesis. In: Meyer-Rochow VB (ed) *Bioluminescence in focus: a collection of illuminating essays*. Research Signpost, Kerala, pp 51–65
- Claes JM, Mallefet J (2010) The lantern shark's light switch: turning shallow water cryptis into midwater camouflage. *Biol Lett* 6:685–687
- Claes JM, Aksnes DL, Mallefet J (2010a) Phantom hunter of the fjords: camouflage by counterillumination in a shark (*Etmopterus spinax*). *J Exp Mar Biol Ecol* 388:28–32
- Claes JM, Krönström J, Holmgren S, Mallefet J (2010b) Nitric oxide in the control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J Exp Biol* 213:3005–3011
- Claes JM, Krönström J, Holmgren S, Mallefet J (2011) GABA inhibition of luminescence from lantern shark (*Etmopterus spinax*) photophores. *Comp Biochem Physiol* 153C:231–236
- Claes JM, Ho HC, Mallefet J (2012) Control of luminescence from pygmy shark (*Squaliolus aliae*) photophores. *J Exp Biol* 215:1691–1699
- Denton EJ (1970) Review lecture: on the organization of reflecting surfaces in some marine animals. *Philos Trans R Soc Lond* 258B:285–313
- Denton EJ, Gilpin-Brown JB, Wright PG (1972) The angular distribution of the light produced by some mesopelagic fish in relation to their camouflage. *Proc R Soc Lond* 182B:145–158
- Denton EJ, Herring PJ, Widder EA, Latz MF, Case JF (1985) The roles of filters in the photophores of oceanic animals and their relation to vision in the oceanic environment. *Proc R Soc Lond* 225B:63–97
- Ebert DA, Fowler SL, Compagno LJ (2013) *Sharks of the world: a fully illustrated guide*. Wild Nature Press, Plymouth
- Fishelson L, Gon O, Goren M (2005) The oral cavity and bioluminescent organs of the cardinal fish species *Siphamia permutata* and *S. cephalotes* (Perciformes, Apogonidae). *Mar Biol* 147:603–609
- Fritz L, Morse D, Hastings JW (1990) The circadian bioluminescence rhythm of *Gonyaulax* is related to daily variations in the number of light-emitting organelles. *J Cell Sci* 95:321–328
- Haddock SHD, Moline MA, Case JF (2010) Bioluminescence in the sea. *Annu Rev Mar Sci* 2:443–493
- Haneda Y, Tsuji FI (1971) Light production in the luminous fishes *Photoblepharon* and *Anomalops* from the Banda Islands. *Science* 173:143–145
- Hanna CH, Hopkins TA, Buc J (1976) Peroxisomes of the firefly lantern. *J Ultrastruct Res* 57:150–162
- Harper RD, Case JF (1999) Disruptive counterillumination and its anti-predatory value in the plainfish midshipman *Porichthys notatus*. *Mar Biol* 134:529–540
- Hastings JW, Morin JG (1991) Bioluminescence. In: Prosser CL (ed) *Neural and integrative animal physiology*. Wiley-Liss, New York, pp 131–170
- Herring PJ (1982) Aspects of the bioluminescence of fishes. *Oceanogr Mar Biol Ann Rev* 20:415–470
- Herring PJ, Locket NA (1978) The luminescence and photophores of euphausiid crustaceans. *J Zool* 186:431–462
- Herring PJ, Morin JG (1978) Bioluminescence in fishes. In: Herring PJ (ed) *Bioluminescence in action*. Academic Press, London, pp 273–329
- Iwai T (1960) Luminous organs of the deep-sea squaloid shark, *Centroscyllium ritteri* Jordan and Fowler. *Pac Sci* 14:51–54
- Johann L (1899) Über eigenthümliche epitheliale Gebilde (Leuchtorgane) bei *Spinax niger*. *Z Wiss Zool* 66:136–160
- Jones BW, Nishiguchi MK (2004) Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* berry (Mollusca: Cephalopoda). *Mar Biol* 144:1151–1155
- Munk O, Jorgensen JM (1988) Putatively luminous tissue in the abdominal pouch of a male dalatine shark, *Euprotomicroides zantedeschia* Hulley and Penrith, 1966. *Acta Zool* 69:247–251
- Nicol JA (1957) Observations on photophores and luminescence in the teleost *Porichthys*. *Q J Microsc Sci* 98:179–188
- Ohshima H (1911) Some observations on the luminous organs of fishes. *J Coll Sci Imp Univ Tokyo* 27:1–25
- Robison BH, Reisenbichler KR, Hunt JC, Haddock SHD (2003) Light production by the arm tips of the deep-sea cephalopod *Vampyroteuthis infernalis*. *Biol Bull* 205:102–109
- Strum JM (1969a) Fine structure of the dermal luminescent organs, photophores, in the fish, *Porichthys notatus*. *Anat Rec* 164:433–462
- Strum JM (1969b) Photophores of *Porichthys notatus*: ultrastructure of innervation. *Anat Rec* 164:463–478
- Sweeney BM (1980) Intracellular source of bioluminescence. *Int Rev Cytol* 68:173–195
- Tong D, Rozas NS, Oakley TH, Mitchell J, Colley NJ, McFall-Ngai MJ (2009) Evidence for light perception in a bioluminescent organ. *Proc Natl Acad Sci USA* 106:9836–9841

PAPER 2

Cytological changes during luminescence production in lanternshark (*Etmopterus spinax* Linnaeus, 1758) photophores

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Abstract Studying an organism's photogenic structures at the ultrastructural level is a key step in the understanding of its light-emission process. Recently, the photophore ultrastructure of the deep-sea lanternshark *Etmopterus spinax* Linnaeus, 1758 was described. The photocytes appeared to be divided into three areas including an apical granular area, which contains inclusions and was hypothesized to be the light-producing reaction site. In this study, we investigated the morphological changes occurring within the granular area during the bioluminescent emissions induced by two hormones: prolactin and melatonin. Prolactin provoked the formation of new structures in the granular area, the "grey particles", whose number was proportional to the amount of light produced by the reaction. An increase in the number of granular inclusions was also detected at the end of the prolactin-induced light emission. Conversely, melatonin induced a decrease in the number of granular inclusions and an increase in their diameter. An effect of hormones was also observed on the iris-like structure where they triggered pigment retraction and hence an increase in the iris aperture diameter. This is consistent with previous findings and is shown for the first time at the cellular level. The possible role of grey particles

in *E. spinax* light-emission mechanism is discussed, while granular inclusion is considered to be *E. spinax*'s intracellular luminescence site. Regarding typical shark long-lasting glows, a new term ("glowon") is proposed to characterize this novel membrane-free microsource.

Keywords Chondrichthyes · Glowon · Melatonin · Microsource · Photocyte · Prolactin

Introduction

Etmopterus spinax Linnaeus, 1758 is a small deep-sea shark from the Etmopteridae family that displays bioluminescence on its ventral and lateral sides thanks to thousands of tiny light-emitting organs (photophores) (Claes and Mallefet 2008; Ebert et al. 2013). Etmopteridae and Dalatiidae are the only shark families containing known luminous members (Claes and Mallefet 2009a). Until recently, bioluminescence of sharks was poorly studied mainly due to their rarity and/or the relative inaccessibility of their deep-sea environment. In the past 6 years, *E. spinax* was used as a model species to study shark bioluminescence, as this shark can be easily obtained and maintained in captivity. Different aspects of shark luminescence, such as its ecological function (Straube et al. 2010; Claes et al. 2010a, 2013, 2014) and physiological control (Claes and Mallefet 2009b, 2010a, b; Claes et al. 2010b, 2011), are now better understood. Very recently, we described the ultrastructure of *E. spinax* photophores (Renwart et al. 2014), since it is a key step in the understanding of this shark's bioluminescence process. The photophores of *E. spinax* appear as cup-shaped organs composed of a protective layer of pigments (the pigmented sheath) and a reflector-like structure that encloses on

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average 13 light-emitting cells (the photocytes), topped with different cell types including lens cells. Each photocyte is divided into three regions: the nucleus area, the vesicular area and the granular area. Because of its fluorescence capability, the presence of numerous granular inclusions as well as its particular orientation towards the photophore centre (Renwart et al. 2014), the granular area is believed to be the intracellular site of the luminous reaction (i.e. where light is produced). In this study, we aim to test this hypothesis by investigating the morphological changes occurring within the granular area during the light-emission process.

Materials and methods

Shark tissue collection

Specimens of *E. spinax* were collected by longlines in Norway (Raunefjord—60°15.908N; 05°07.778O) during two field missions, in November 2012 and March 2013. Sharks were killed according to the local rules for experimental fish care. Fresh ventral skin patches with photophores were dissected and placed in a buffered shark saline (Bernal et al. 2005) before the beginning of experiments.

Luminometry and electron microscopy

Skin patches were stimulated by two hormones (prolactin and melatonin) and fixed in glutaraldehyde at different stages of the light-emission process to be further observed in transmission electron microscopy. Four skin patches were dissected from each specimen and a total number of six sharks were used. One skin patch from each specimen was fixed before hormonal stimulation in a glutaraldehyde solution (3 % glutaraldehyde, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8) and was used as a control patch, the “time 0” sample. The remaining three patches were placed in chambers filled with 200 µl of hormone solution, either prolactin (10^{-6} M prolactin in a buffered shark saline; two patches) or melatonin (10^{-6} M melatonin in the same buffered shark saline; a single patch). The induced light was recorded during 1 h in a multiplate luminometer (Orion, Berthold Detection System, Pforzheim, Germany) connected to a computer. Consistent with previous findings (Claes and Mallefet 2009b), both hormones showed different light-emission pattern: prolactin induced a monophasic light curve that reached a peak after ~20 min and lasted a maximum of 60 min (Fig. 1); prolactin-stimulated skin patches were glutaraldehyde-fixed at the level of the peak (“PRL max”) and 1 h after the stimulation start (“PRL 60”). The melatonin-induced light curve reached a plateau after ~45 min and lasted over 60 min

(Fig. 1); melatonin-stimulated skin patch was glutaraldehyde-fixed after 1 h (“MT 60”).

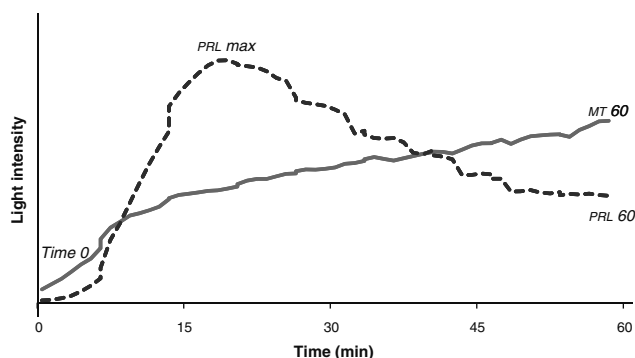
Fixed skin patches were processed using the method described in Renwart et al. (2014), in order to be observed in a transmission electron microscope (Zeiss Leo 906E). Briefly, skin patches were first decalcified (OsteoRAL R fast decalcifier, RAL diagnostics), and then post-fixed in osmium tetroxide (1 % osmium tetroxide, 0.1 M sodium cacodylate, 0.27 M sodium chloride; pH 7.8—45 min). Dehydrated patches were embedded in a Spurr’s resin (10 g ERL 4206 resin, 6 g DER 736 resin, 26 g nonenyl succinic anhydride, 0.4 g dimethylaminoethanol), and sections of 90–110 nm were obtained using an ultramicrotome (Leica Ultracut UCT—Leica Microsystems) and placed on copper grids. Sections were finally contrasted in uranyl acetate [0.18 M uranyl acetate solution:ethanol (2:1)—45 min] and lead citrate (0.08 M lead nitrate, 0.12 M sodium citrate, 0.16 M sodium hydroxide—4 min) before observation.

Data analysis

Micrographs of hormonally stimulated photophores (time 0, PRL max, PRL 60 and MT 60) were analysed using Image J v. 1.46 (National Institutes of Health, Bethesda, USA). Particular attention was given to the morphological changes occurring within the granular area of the photocytes, which may contain up to two distinct components, i.e. the “granular inclusions” and the “grey particles”. Granular inclusions were analysed using three morphological parameters: the mean diameter, the density (per square micrometre) and the coverage (i.e. the percentage of the granular area surface occupied by granular inclusions). The first two parameters were calculated using the *watershed* followed by the *particle analysis* options of Image J. The total surface area of inclusions, required for the third parameter calculation, derived from the number of pixels was represented on the micrograph and not from the *particle analysis*, in order to allow direct comparison with the grey particles coverage. Indeed, only the coverage parameter was calculated for the grey particles, using the number of pixels, since these particles are very small and aggregate in a way that the *watershed/particle analysis* option cannot discriminate them. Both structures (granular inclusions and grey particles) have been analysed separately when present on the same micrograph by adjusting the threshold in binarized micrographs: a threshold of 80 was assumed to separate the darker granular inclusions from the lighter grey particles.

Statistical analyses were performed to investigate the evolution of the three parameters during the light-emission process. For each parameter, the two time points (i.e. time 0 and MT 60) of the melatonin-induced light curve and the

Fig. 1 Prolactin (dashed line) and melatonin (grey line)-induced light curves with associated time points



three time points (i.e. *time 0*, *PRL max* and *PRL 60*) of prolactin-induced light curve were compared using Student's *t* tests. Normality and equality of variance were tested by Shapiro–Wilk tests and Levene's tests, respectively. When one or both of these parametric assumptions could not be met, the Student's *t* tests were replaced by a Student's *t* tests on log-transformed data, a Welch's *t* tests or a Wilcoxon tests, respectively. All analyses were performed using the software JMP v. 11 (SAS Institute Inc., Cary, USA) and considered to be significant at the 0.05 level.

Results

Prolactin stimulation

A total of 125 photocytes (*time 0*, $N = 44$; *PRL max*, $N = 41$; *PRL 60*, $N = 40$) from 32 photophores (*time 0*, $n = 13$; *PRL max*, $n = 9$; *PRL 60*, $n = 10$) were used to investigate the ultrastructural changes induced by prolactin in *E. spinax* photogenic structures. Figure 2a shows three pictures of the granular areas of representative photocytes corresponding to the three analysed time points: at *time 0*, only the granular inclusions previously observed by Renwart et al. (2014) were visible. When the light emission reached its maximum (at *PRL max*), another structure appeared and was still present after 1 h, at *PRL 60*. We named it “grey particles”, since their lower density to the electrons makes them appear lighter than granular inclusions.

Both granular inclusions and grey particles were quantified and compared between the three time points. The coverage of the grey particles showed a highly significant variation during the light-emission process (Fig. 2b). The coverage of the granular inclusions increased at the end of

the light-emission process (Fig. 2c) as a significant difference between *PRL 60* and *PRL max* was obtained. The mean diameter of the inclusions did not change (Fig. 2d), but their density was significantly higher at *PRL 60* (Fig. 2e).

Stimulated photocytes presented overall a homogeneous distribution of both grey particles and granular inclusions (Fig. 3a–c); on few occasions, however, granular inclusions adopted a heterogeneous distribution, forming dense aggregations (clusters) in the granular area (Fig. 3d, e). Interestingly, most of these clustered inclusions were observed at *PRL max*. Moreover, it must be pointed out that different stages sometimes cohabited in a same photophore; indeed, a few photocytes at *PRL max* and *PRL 60* stages did not show the grey particles (Fig. 3a). Finally, endoplasmic reticulum was also observed surrounding groups of granular inclusions in a few stimulated photocytes (Fig. 3e, f).

Melatonin stimulation

A total of 92 photocytes (*time 0*, $N = 44$; *MT 60*, $N = 48$) from 26 photophores (*time 0*, $n = 13$; *MT 60*, $n = 13$) were used to investigate the ultrastructural changes induced by melatonin in *E. spinax* photogenic structures. Contrary to what was observed in prolactin-stimulated patches, melatonin did not appear to induce the formation of grey particles, since no grey particle coverage difference was observed either visually (Fig. 4a) or statistically (Fig. 4b) between the two time points. Similarly, the analyses did not reveal any significant difference between the two time points for the granular inclusions coverage (Fig. 4c). Inclusions were nevertheless found to have a significantly larger diameter and a significantly lower density at *MT 60* (Fig. 4d, e).

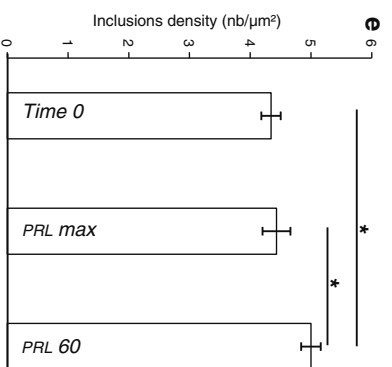
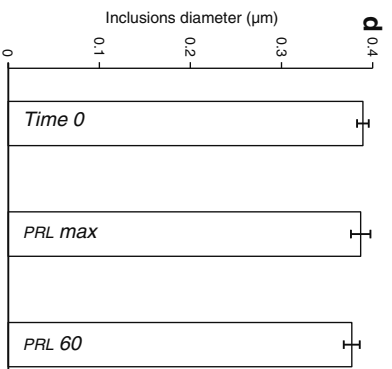
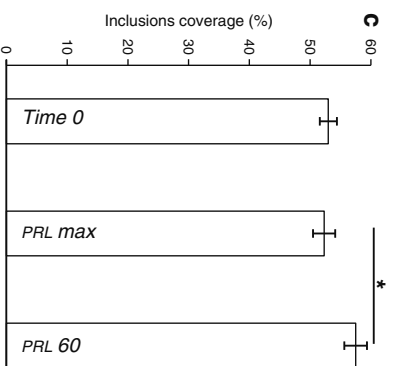
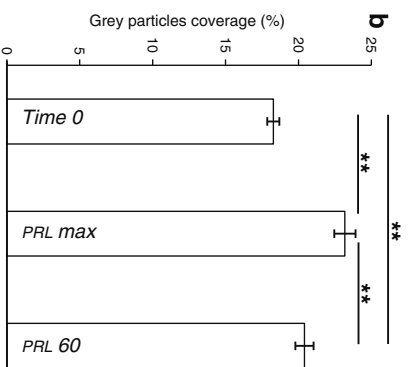
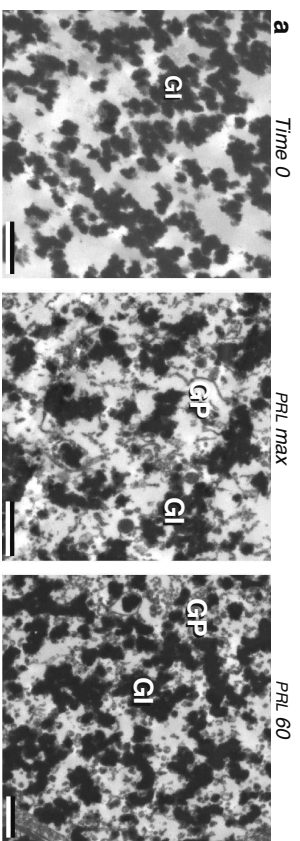


Fig. 2 Prolactin-induced morphological changes in *E. spinax* photocytes. **a** Granular area at the three time points. *GI* granular inclusions, *GP* grey particles. Scale bar 1 μ m. Photocyte morphological parameters at the three time points. **b** Grey particles coverage. **c** Granular inclusions coverage. **d** Granular inclusions diameter. **e** Granular inclusions density. Asterisks indicate statistically different means (* $P < 0.05$; ** $P < 0.01$)

Iris-like structure aperture

An action of hormones was also observed at another photophore level. At *time 0*, melanosomes were present inside the photophore centre, forming the iris-like structure and surrounding the photocytes and their overlying cells. In stimulated photophores, pigments were removed from the photophore centre to form clusters around it, as illustrated in the Fig. 5 in a melatonin-stimulated patch.

Discussion

Morphological changes occurring in *E. spinax* photophore have been studied at the ultrastructural level throughout prolactin- and melatonin-induced light-emission processes. Both hormones are known to produce different light pattern (Claes and Mallefet 2009b). We found they also induced different morphological variations, either inside the photocytes or at the melanosomes level within the photophore. This brings further evidence of their different but complementary roles in the control of *E. spinax* luminescence (Claes and Mallefet 2009b, 2010b).

The main action of prolactin occurs inside the photocytes and primarily consists in the transitory formation of grey particles, while these particles were never observed in melatonin-stimulated photocytes. At *time 0*, the grey particle value only corresponds to the narrow edges of the granular inclusions, which appear grey on the black and white pictures (Fig. 2a), but no grey particles were observed at that stage. Grey particle coverage displays a monophasic development that follows the prolactin-induced light curve, reaching a maximum value at the light peak (at *PRL max*, Fig. 2b). This correlation clearly suggests an involvement of the grey particles in the light-emission process, although the exact nature of these particles, which might represent more than one component with the same electron density, cannot be attested in this study. Because grey particles only appear in prolactin-stimulated photocytes, they probably do not represent a constituent (reagent or product) of the chemiluminescent reaction, although we cannot completely rule out the grey particles to be a non-recycled product accumulating in prolactin-stimulated photocytes only. Very few luminous animals have been found with two different intrinsic luminescences (e.g. the

Scyphozoan *Periphylla periphylla* Péron & Lesueur, 1810 and the teleost *Malacosteus niger* Ayres, 1848—Widder et al. 1984; Shimomura and Flood 1998), and in these cases, both luminescences were localized in different body parts and might involve the same light-emitting reaction. Occurrence of two light-emitting reactions in the same animal, and even more so in the same photogenic cell (one involving grey particles formation while the second do not), is very unlikely (Shimomura 2006). Therefore, we assume *E. spinax* photocytes to display one luminescence system and the grey particles to represent a component of the intracellular metabolic pathway, triggered by the binding of prolactin to its receptor, which consequently do not appear in melatonin-stimulated photocytes. Conversely, we assume granular inclusions—which are already present in the bioluminescence action site (i.e. the granular area) before prolactin stimulation—to be aggregations of stored/stabilized components (a substrate or a photoprotein, possibly combined with other molecular factors) waiting for a proper stimulus (possibly related to grey particles) to start the light-emitting reaction or increase the intensity of a basal emission.

Interestingly, both hormones showed a clear modification of the granular inclusions but acted on different parameters, arguing for their different roles in *E. spinax* light-emission process. Moreover, previous pharmacological experiments have shown an additive effect of both hormones on light intensity parameters (Claes and Mallefet 2009b), suggesting a close interaction at intracellular level in the shark photophore. In prolactin-stimulated photocytes, we found the granular inclusions to be more numerous after the reaction has been completed, which is also shown by their higher coverage at *PRL 60* (Fig. 2c, e). This higher density might reflect a restocking of reagents or an accumulation of products. In that context, non-activated photocytes at *PRL max* (i.e. showing no grey particles) might actually be recycling the products or rebuilding the reactive stock. This concept of a turnover of luminous material has already been proposed for another bioluminescent organism, the ophiuroid *Amphipholis squamata* Delle Chiaje, 1828 (Deheyn et al. 2000). In melatonin-stimulated photocytes, granular inclusions were found to have a significantly higher diameter 60 min after stimulation (at *MT 60*) concomitantly with a reduction in their density. This seems to indicate an aggregation of granular inclusions, since their coverage was shown to remain constant. Because melatonin induces a slow luminescence kinetics (Claes and Mallefet 2009b), effects of this hormone on photocyte ultrastructure may require a longer time to be clearly identified.

Variations in shape, number and size of cytoplasmic components related to bioluminescent activity have been demonstrated in various luminous organisms (Table 1). In

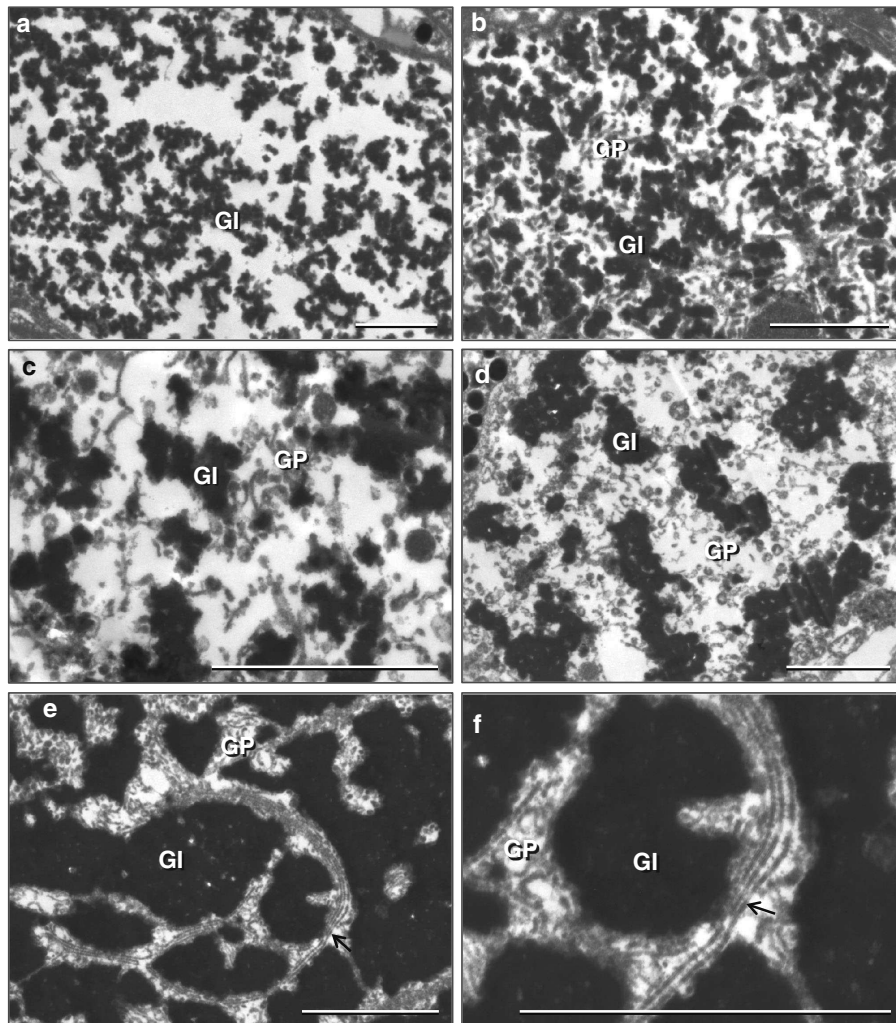


Fig. 3 Granular area from prolactin-stimulated photocytes. **a** A stimulated photocyte that does not show many grey particle. **b** Homogeneous distribution of granular inclusions. **c** Detailed view of the grey particles and the granular inclusions. Clustered

distribution of granular inclusions at low (**d**) and high (**e**) density. **f** Higher magnification view of the endoplasmic reticulum (arrows) present in (**e**). *GI* granular inclusions, *GP* grey particles. Scale bar 2 μ m

the ophiuroid *A. squamata*, the photocyte cytoplasm is full of vesicles that can be classified into six types (A–F). Only the vesicular type A is present before the luminous

reaction, while type A, B and C are present during luminescence and mostly types D–F were seen after the reaction has been completed. Vesicular type A was assumed to

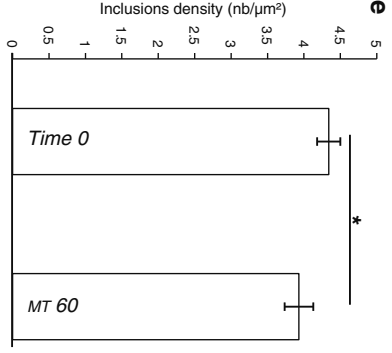
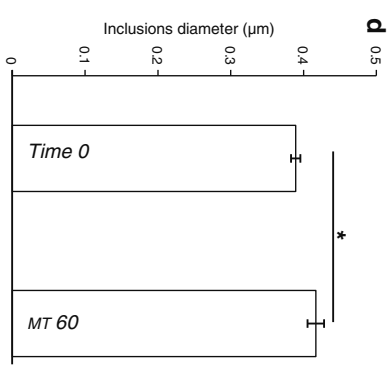
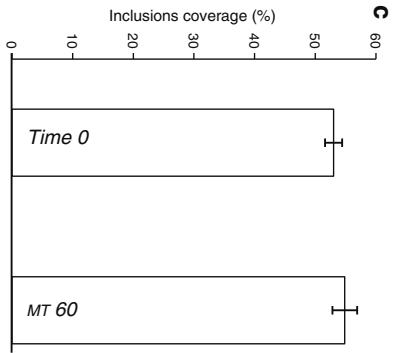
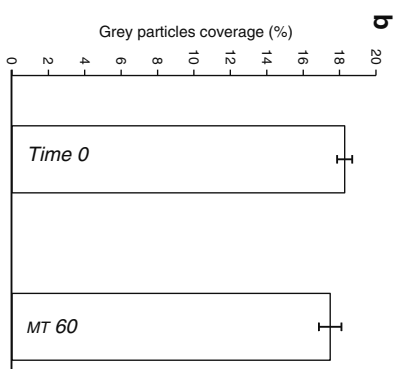
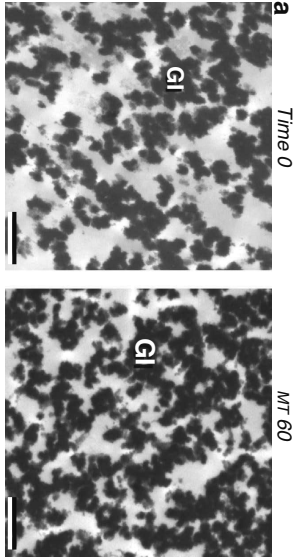


Fig. 4 Melatonin-induced photocyte morphological changes. **a** Granular area at the two time points. *GI* granular inclusions. *Scale bar* 1 μ m. Photocyte morphological parameters at the two time points. **b** Grey particles coverage. **c** Granular inclusions coverage. **d** Granular inclusions diameter. **e** Granular inclusions density. Asterisks indicate statistically different means (* $P < 0.05$)

contain one of the luminous component of the reaction, and the sequence of appearance of the other types allowed the authors to hypothesize the type C to be site of the luminous

reaction (i.e. the microsource), further transformed into the type D at the end of the process (Deheyn et al. 2000). The midshipman fish *Porichthys notatus* Girard, 1854 also has a highly vesiculated cytoplasm subject to variations under injection of noradrenaline, a luminescence-triggering neurotransmitter in this species (Ancil 1979). Contrary to what occurs in many luminous organisms, *E. spinax* light-producing reaction does not take place in a membranous compartment of the photocyte but inside a specific region of the cytoplasm: the apical granular area. In this sense,

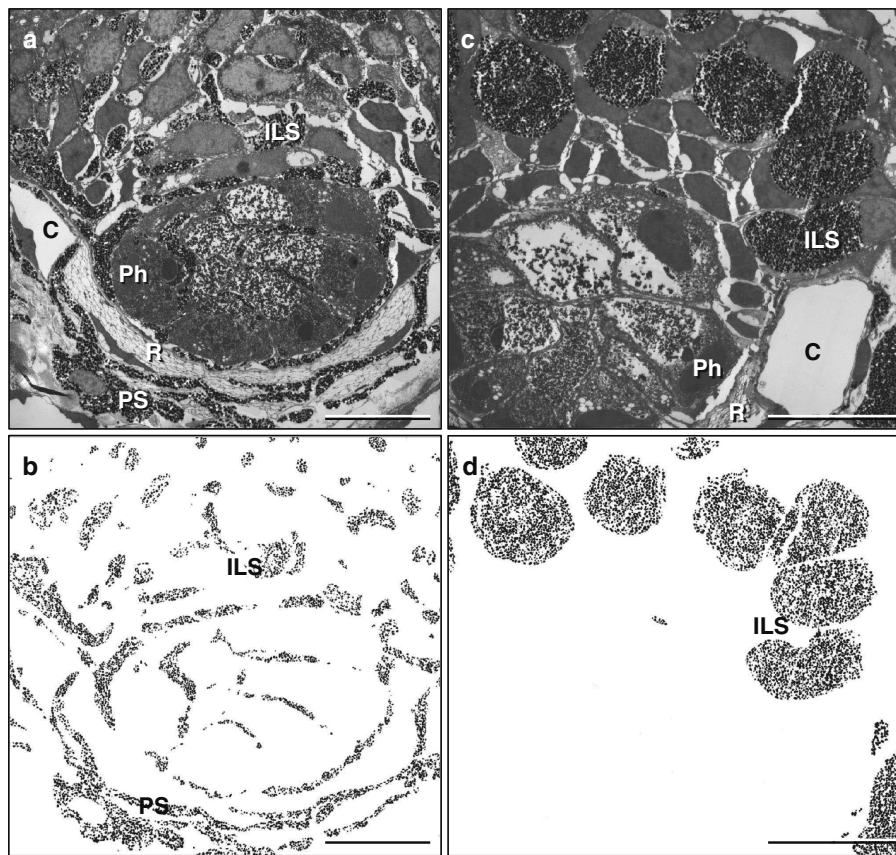


Fig. 5 Melatonin-induced pigment translocation. Whole structure (a) and pigment distribution (b) of a non-stimulated photophore. Whole structure (c) and pigment distribution (d) of a melatonin-stimulated photophore. Note the striking difference in the morphology

of pigments, which adopt a clustered distribution in the iris-like structure after melatonin stimulation. *C* capillary, *ILS* iris-like structure, *Ph* photocyte, *PS* pigmented sheath, *R* reflector-like structure. *Scale bar* 20 μ m

Table 1 Representative luminous species with corresponding microsource name, type and identification method

Taxa/selected species	Name	Type	Method
Dinoflagellata			
<i>Lingulodinium polyedrum</i> J. D. Dodge, 1989	Scintillons	Cytoplasmic granular structures	Microsources isolation, electron microscopy ¹
Cnidaria			
<i>Renilla reniformis</i> Pallas, 1766	Lumisomes	Membranous organelles	Microsources isolation, electron microscopy ²
Annelida, Polychaeta			
<i>Acholoe squamosa</i> Delle Chiaje, 1827	Photosomes	Membranous organelles	Histology, fluorescence and bioluminescence correlation ³
Arthropoda, Insecta			
<i>Photuris</i> sp.	Photocyte vesicles	Membranous organelles	Histology, fluorescence and bioluminescence correlation ^{4,5}
Mollusca, Cephalopoda			
<i>Watasenia scintillans</i> Berry, 1911	Rod-like crystals	Cytoplasmic crystals	Microsources isolation, electron microscopy ⁶
Echinodermata			
<i>Amphipholis squamata</i> Delle Chiaje, 1828	Cytoplasmic vesicles	Membranous organelles	Ultrastructural changes during luminous event ⁷
Chordata, Osteichthyes			
<i>Porichthys notatus</i> Girard, 1854	Photocyte vesicles	Membranous organelles	Histology, fluorescence and bioluminescence correlation in larval stage ⁸ Ultrastructural changes during luminous event ⁹
Chordata, Chondrichthyes			
<i>Etmopterus spinax</i> Linnaeus, 1758	Glowons	Cytoplasmic granular structures	Histology, fluorescence and bioluminescence correlation ^{10,11}

¹ DeSa et al. (1963);² Anderson and Cormier (1973);³ Bassot and Bilbaut (1977);⁴ Hanson et al. (1969);⁵ Smalley et al. (1980);⁶ Hamanaka et al. (2011);⁷ Deheyn et al. (2000); ⁸ Anctil(1977); ⁹ Anctil (1979);¹⁰ Claes and Mallefet (2008);¹¹ Renwart et al. (2014)

granular inclusions would therefore be the shark's microsources, as already proposed (Renwart et al. 2014). Given the particular hormone-dependent slow kinetics of shark luminescence, we propose here the term "glowons" for these particles, by analogy with the dinoflagellate's "scintillons", which are among the few other microsources lacking a surrounding membrane (Table 1).

In prolactin-stimulated photocytes, granular inclusions display either a homogeneous sparse distribution (Fig. 3a–c) or, in a few cases (mainly at *PRL max*), a heterogeneous distribution in which they form aggregations (clusters) of variable size (Fig. 3d, e). Since each investigated photocyte presented a unique combination of these two distribution types, we were not able to demonstrate whether these distinct morphologies represented chronological stages of the light-emission process. However, since clustered distributions of granular inclusions are mainly found at *PRL max*, they might represent a key step of the light production mechanism. The involvement of endoplasmic reticulum (ER) in bioluminescent reactions has been proved in several organisms, e.g. the brittle star *A. squamata*, whose ER

extends during light emission (Deheyn et al. 2000) and the scale worms (Polynoidae) in which ER forms a complex network building the microsources (the photosomes) and bears the protein of the bioluminescent reaction (Bassot and Nicolas 1987; Wilson and Hastings 1998). The ER observed in some excited photocytes of *E. spinax* might play a role in luminescence metabolism.

In addition to their action inside the granular area, hormones rearrange the pigmentation of the iris-like structure of photophores in order to mechanically control the light reaching outside. Although this capability has already been demonstrated (Claes and Mallefet 2010b), our micrographs allowed showing this process at the cellular level. After stimulation, melanosomes from the iris-like structure retract their cytoplasmic projections from the photophore centre, which allow more light to pass through the lens and to be recorded from the luminometer (note that light is recorded from the whole skin patch—i.e. from numerous photophores—while ultrastructural changes are studied at the photophore level).

In conclusion, this study aimed to improve our understanding of the light-emission process of the deep-sea shark *E. spinax* thanks to a dynamic morphological approach. We highlighted ultrastructural changes in the photocytes and in the pigmented cells of the iris-like structure during hormonally induced light events. In particular, our results support previous hypothesis following which granular inclusions were the microsources of the lanternshark photophores. A new term (“glowon”) has been proposed to characterize this novel intracellular membrane-free particle that produces a long-lasting glow under hormonal stimulation. Further research should investigate the chemical nature of *E. spinax*'s luminous system to elucidate its intracellular distribution and dynamics, in relation to glowon morphological changes.

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References

- Anctil M (1977) Development of bioluminescence and photophores in the midshipman fish, *Porichthys notatus*. *J Morphol* 151:363–396
- Anctil M (1979) Ultrastructural correlates of luminescence in *Porichthys* photophores. I. Effects of spinal cord stimulation and exogenous noradrenaline. *Rev Can Biol* 38:67–80
- Anderson JM, Cormier MJ (1973) Lumisomes, the cellular site of bioluminescence in coelenterates. *J Biol Chem* 248:2937–2943
- Bassot J-M, Bilbaut A (1977) Bioluminescence des élytres d'*Achiloe*. IV. Luminescence et fluorescence des photosomes. *Biol Cellulaire* 28:163–168
- Bassot J-M, Nicolas G (1987) An optional dyadic junctional complex revealed by fast-freeze fixation in the bioluminescent system of the scale worm. *J Cell Biol* 105:2245–2256
- Bernal D, Donley JM, Shadwick RE, Syme DA (2005) Mammal-like muscles power swimming in a cold-water shark. *Nature* 437:1349–1352
- Claes JM, Mallefet J (2008) Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark *Etmopterus spinax* (Squaloidea: Etmopteridae). *J Fish Biol* 73:1337–1350
- Claes JM, Mallefet J (2009a) Bioluminescence of sharks: first synthesis. In: Meyer-Rochow VB (ed) *Bioluminescence in focus—a collection of illuminating essays*. Research Signpost, Kerala, pp 51–65
- Claes JM, Mallefet J (2009b) Hormonal control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J Exp Biol* 212:3684–3692
- Claes JM, Mallefet J (2010a) Functional physiology of lantern shark (*Etmopterus spinax*) luminescent pattern: differential hormonal regulation of luminous zones. *J Exp Biol* 213:1852–1858
- Claes JM, Mallefet J (2010b) The lantern shark's light switch: turning shallow water crypsis into midwater camouflage. *Biol Lett* 6:685–687
- Claes JM, Aksnes DL, Mallefet J (2010a) Phantom hunter of the fjords: camouflage by counterillumination in a shark (*Etmopterus spinax*). *J Exp Mar Biol Ecol* 388:28–32
- Claes JM, Krönström J, Holmgren S, Mallefet J (2010b) Nitric oxide in the control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J Exp Biol* 213:3005–3011
- Claes JM, Krönström J, Holmgren S, Mallefet J (2011) GABA inhibition of luminescence from lantern shark (*Etmopterus spinax*) photophores. *Comp Biochem Physiol* 153C:231–236
- Claes JM, Dean MN, Nilsson D-E, Hart NS, Mallefet J (2013) A deepwater fish with 'lightsabers'—dorsal spine-associated luminescence in a counterilluminating lanternshark. *Sci Rep* 3:1308
- Claes JM, Nilsson D-E, Straube N, Collin SP, Mallefet J (2014) Iso-luminance counterillumination drove bioluminescent shark radiation. *Sci Rep* 4:4328
- Deheyn D, Mallefet J, Jangoux M (2000) Cytological changes during bioluminescence production in dissociated photocytes from the ophiuroid *Amphipholis squamata* (Echinodermata). *Cell Tissue Res* 299:115–128
- DeSa R, Hastings JW, Vatter AE (1963) “Crystalline” particles: an organized subcellular bioluminescent system. *Science* 141:1269–1270
- Ebert DA, Fowler SL, Compagno LJ (2013) *Sharks of the world: a fully illustrated guide*. Wild Nature Press, Plymouth
- Hamanaka T, Michinomae M, Seidou M, Miura K, Inoue K, Kito Y (2011) Luciferase activity of the intracellular microcrystal of the firefly squid, *Watasenia scintillans*. *FEBS Lett* 585:2735–2738
- Hanson FE, Miller J, Reynolds GT (1969) Subunit coordination in the firefly light organ. *Biol Bull* 137:447–464
- Renwart M, Delroisse J, Claes JM, Mallefet J (2014) Ultrastructural organization of lantern shark (*Etmopterus spinax* Linnaeus, 1758) photophores. *Zoomorphology*. doi:10.1007/s00435-014-0230-y
- Shimomura O (2006) *Bioluminescence: chemical principles and methods*. World Scientific, Singapore
- Shimomura O, Flood PR (1998) Luciferase of the scyphozoan medusa *Periphylla periphylla*. *Biol Bull* 194:244–252
- Smalley KN, Tarwater DE, Davidson TL (1980) Localization of fluorescent compounds in the firefly light organ. *J Histochem Cytochem* 28:323–329
- Straube N, Iglésias SP, Sello DY, Kriwet J, Schliwien UK (2010) Molecular phylogeny and node time estimation of bioluminescent Lantern Sharks (Elasmobranchii: Etmopteridae). *Mol Phylogenet Evol* 56:905–917
- Widder EA, Latz MI, Herring PJ, Case JF (1984) Far red bioluminescence from two deep-sea fishes. *Science* 225:512–514
- Wilson T, Hastings JW (1998) Bioluminescence. *Annu Rev Cell Dev Biol* 14:197–230

Supplementary results:

Are bacterial symbionts responsible for *Etmopterus spinax* light emission?

SUPPLEMENTARY RESULTS:**ARE BACTERIAL SYMBIONTS RESPONSIBLE FOR
ETMOPTERUS SPINAX LIGHT EMISSION?***Context*

Because various teleost fish have been shown to possess symbiotic bacterial photophores (Rudy & Nealson, 1976; Wada *et al.*, 1999; Fishelson *et al.*, 2005, Henry & Dunlap, 2011), the question arose if this particular bioluminescence mechanism might also have evolved in chondrichthyans. Most fishes and squids displaying symbiotic luminescence possess few large photophores containing the bacteria population (Hasting, 1995), while luminous sharks bear thousands of tiny (< 200 μm) photophores (Claes & Mallefet, 2008) and it seems unlikely that each of them might contain a population of luminous bacteria. Moreover, host initial colonization usually occurs by lateral transfer during the first host's life hours/days (Dunlap & Kita-Tsukamoto, 2006). *Etmopterus spinax* embryos are luminous before birth (Claes & Mallefet, 2008) what would involve luminous symbionts transfer from the female during embryogenesis. Wada *et al.* (1999) documented symbionts lateral transfer from parents to juveniles in the teleost *Nucifera nuchalis*, but a symbionts transfer from female to growing embryos in an ovoviparous species, such as *E. spinax*, has never been described so far. By combining data from transmission electron microscopy (Renwart *et al.*, 2014a, b) and from fluorescence *in situ* hybridization (described here), we aim to investigate the presence of bacterial symbionts in *E. spinax* photophores in order to definitely take a stance on this question.

This study has been carried out with the collaboration of Cyril Hammoud (MSc student), thanks to facilities provided by Prof. Jacques Mahillon and Olivier Henriot (Laboratory of Food and Environmental Microbiology, ELI/ELIM).

Materials and methods

Shark tissue collection

Specimens of *E. spinax* were caught by longlines in Norway (Raunefjord – 60°15.908N; 05°07.778O) during a field mission in March 2013. Sharks were sacrificed according to local rules for experimental fish care (i.e. a quick blow to the head) and fresh skin patches with photophores were dissected and fixed in formaldehyde [4 % formaldehyde, 0.052 M sodium phosphate monobasic, 0.082 M sodium phosphate dibasic; pH 8.4] during at least three hours. Skin patches were rinsed in a phosphate buffer [0.081 M sodium phosphate dibasic, 1.37 M sodium chloride, 0.015 M potassium phosphate monobasic, 0.027 M potassium chloride, 0.25 % sodium azide; pH 8.4] and stored in this solution to be further used for fluorescence *in situ* hybridization (FISH). A sample of *E. spinax* intestine was used as a positive control of bacteria presence in order to validate the method. This sample, previously frozen at -80 °C, was defrost and straight fixed in the same formaldehyde solution. It followed the same steps than the fixed skin patches.

Formaldehyde-fixed samples were firstly embedded in paraffin wax and sectioned, then processed to specifically mark bacterial DNA on sections, by using FISH method.

Embedding and sectioning

In order to decalcify *E. spinax* numerous skin denticles, skin patches were immersed in a decalcification solution (OsteoRAL R fast decalcifier, RAL diagnostics) during 10 days at room temperature with a continuous agitation and a solution renewal every two days. Samples were progressively dehydrated in a graded ethanol series (70 % / 24 h; 90 % / 2 x 1 h / room temp.) and then immersed in butanol baths (100 % / 1 h; 100 % / 12 h / 60 °C) and paraffin wax baths (100 % / 4 h; 100 % / 12 h / 60 °C). After cooling, paraffin blocks were cut in sections of approximately 7 µm (Reichert Juny 2030 microtome).

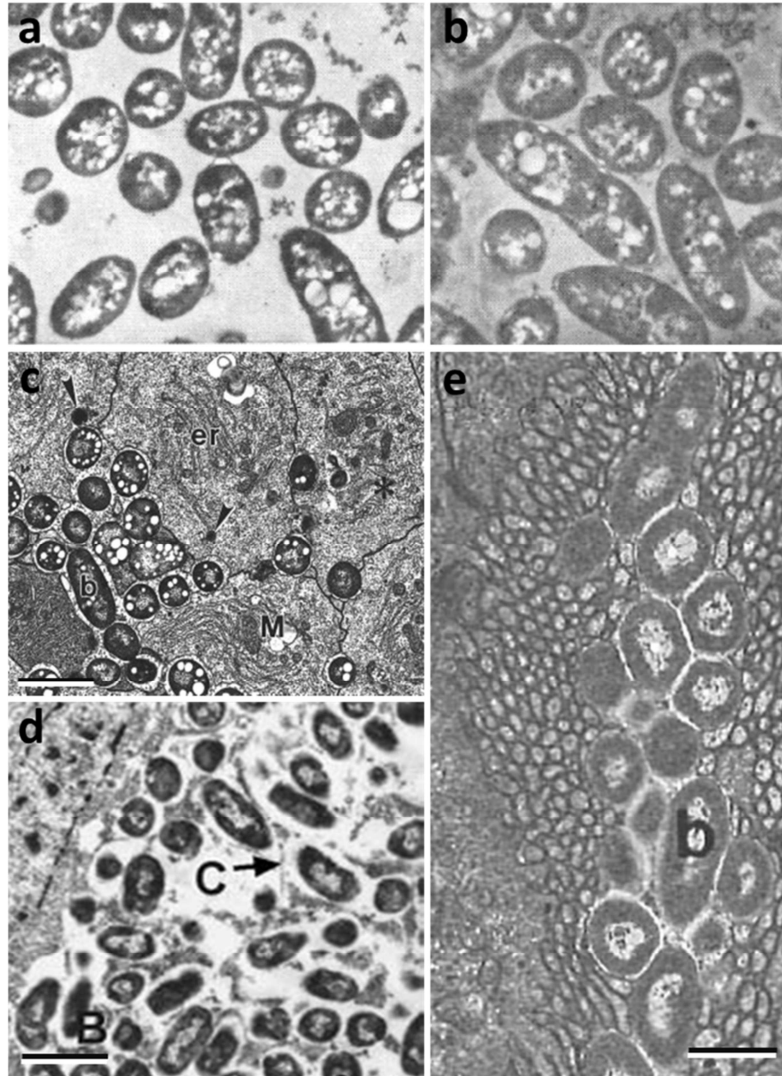


Fig. 1 Symbiotic luminous bacteria from different fish and squid species. **a** Unknown luminous bacteria from the fish *Photoblepharon* spp. Picture: Haneda & Tsuji, 1971. **b** Unknown luminous bacteria from the fish *Anomalops* spp. Picture: Haneda & Tsuji, 1971. **c** Unknown luminous bacteria from the fish *Melanocetus murrayi*. *Er* endoplasmic reticulum; *M* mitochondria; *b* bacteria. Scale bar, 2 μ m. Picture: Munk, 1999. **d** Luminous bacteria –*Photobacterium leiognathi*– from the fish *Siphamia permutata*. *B* bacteria; *C* cytoplasm extensions. Scale bar, 2 μ m. Picture: Fishelson et al., 2005. **e** Luminous bacteria –*Vibrio fischeri*– from the squid *Euprymna scolopes*. Scale bar, 1 μ m. Picture: McFall-Ngai & Ruby, 1998.

FISH processing

Before hybridization, sections were deparaffinized by immersion in xylene baths (100 % / 2 x 10min / room temp.) and in a decreasing ethanol series (100 % / 10 min; 90 % / 30 sec; 70 % / 2 x 10 min / room temp.). Shark skin tissues were marked with the EUB338 probe labelled with the Cy3 fluorescent marker. The EUB338 probe (here we used a commercial mix of EUB338 I, II and III probes-Sigma-Aldrich) is complementary to a 16S gene portion highly conserved in bacterial genome and which is therefore of usual application to specifically mark bacterial DNA. Cy3 fluorescent marker, coupled with the probe, permitted to observe bacterial DNA on epifluorescence microscope (Leica DMI6000B). Hybridization was performed as described in Greiter-Wilke *et al.* (2006): unembedded sections were first immersed in hybridization buffer during 1 h at 46 °C in a humid chamber. The labelled probe (16S rRNA Cy3-5'-GCT-GCC-TCC-CGT-3') was diluted in ultra-pure water to reach a concentration of 5 ng/μl and the solution was applied during 4 h on sections in the same conditions. Tissues were finally rinsed with PBS during 15 min and mounted with fluorescence media (Vectashield, Vector Labs).

DNA cloning and bacteria culture

In addition to FISH, we conducted some preliminary experiments to identify species present in *E. spinax* skin. Briefly, DNA from frozen skin patches with photophores was extracted and bacterial DNA was specifically amplified and cloned in *Escherichia coli* to be finally sequenced and identified. Other skin patches were gently grounded in a culture liquid medium and supernatant was cultured on three medium. The isolated colonies obtained were then sequenced.

DNA cloning: DNA from frozen shark skin patches with photophores was extracted using Dneasy Blood & Tissue (Qiagen) extraction kit. From the extracted DNA, the 16S gene was amplified in PCR in order to specifically target bacterial DNA. The PCR product was then purified using GenElute Clean-Up (Sigma-Aldrich) kit and DNA fragments were inserted in pCR4-TOPO plasmid following steps described in TOPO TA Cloning (Invitrogen) kit. Plasmids were inserted in *TOP10* chemically competent *Escherichia coli* by electroporation and

bacteria were cultured in a LB medium in order to obtain isolated colonies and therefore to separate DNA fragments. Plasmid DNA from 40 randomly chosen colonies was finally extracted using GenElute Plasmid Miniprep (Qiagen) kit and sequenced (Macrogen Inc.).

Bacteria culture: frozen skin patches with photophores were gently grounded in a LB liquid culture medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, 15 g/L agar). 100 µl from the solution were then cultured on three growing medium on Petri dishes: (i) LB medium enriched in sodium chloride to provide sea water osmotic conditions (35 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, 15 g/L agar); (ii) Alkaline peptone water (Conda) designed for *Vibrio* genus culture; (iii) Marine broth (Conda) designed for marine bacteria culture. DNA from isolated colony was extracted by lysis and sequenced (Macrogen Inc.).

Results and discussion

Photophores ultrastructural morphology of organisms harboring luminous bacteria has been investigated for some species. For comparison purposes, **figure 1** shows TEM micrographs of luminous bacteria isolated from four fish species (*Photoblepharon* sp., *Anomalops* sp., *Melanocetus murrayi* and *Siphamia permutata*) and one squid (*Euprymna scolopes*). Bacteria (respectively an unknown species (**Fig. 1a-c**), *Photobacterium leiognathi* (**Fig. 1d**) and *Vibrio fischeri* (**Fig. 1e**)) appear as dense ovoid cells usually containing grey-white vacuoles. The cytoplasmic content of *E. spinax* photocytes (**Fig. 2**) does not show such particular features especially not inside the granular area which is believed to be the site of action of luminescence (Renwart *et al.*, 2014b). Instead, this area is filled with granular inclusions that don't have any shared characteristic (size and shape) with bacteria. This morphological approach supports the hypothesis of an intrinsic light-emitting system in *E. spinax*, i.e. based on an intracellular chemiluminescent reaction.

In comparison with bony fishes, shark commensal bacterial flora has been poorly studied and mostly concerns the commensal populations of the digestive tract. In 1984, Buck published a report on bacterial populations present on skin surface of twelve elasmobranchs

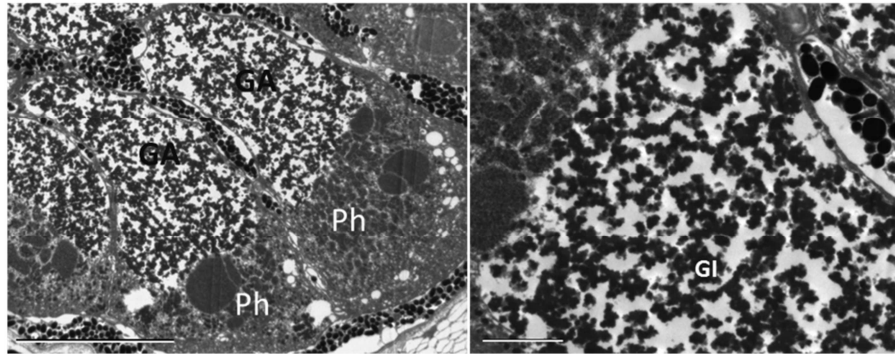


Fig. 2 *Etmopterus spinax* photocytes. **a** Global view of two photocytes (Ph) showing the apical granular area (GA). Scale bar, 10 μ m. **b** Magnification of the granular area. GI granular inclusion. Scale bar, 2 μ m.

from the Gulf of Mexico. Mainly gram-negative bacteria were identified and genus *Vibrio* and *Pseudomonas* accounted for 59% of the skin bacterial flora. *Photobacterium* genus was also detected, but none of the studied elasmobranchs were bioluminescent. Since free-living luminous bacteria are found in all oceans, it would not be surprising to find them in association with shark skin, even without symbiosis. Indeed, animal skin surface is recognized to represent an important ecological niche for bacterial populations (McFall-Ngai & Rudy, 1998).

From preliminary experiments conducted in addition to FISH (i.e. DNA cloning and bacteria culture), neither *Vibrio* nor *Photobacterium*, which are the two known marine genus making symbiosis with bioluminescent organisms, were identified whatever the technique used. Even so, different marine strains were found, such as *Marinococcus* sp., *Salinisphaera* sp., *Haliea* sp. and *Halomonas aquamarina*, along with some terrestrial strains, which probably came from experimental contamination (*Micrococcus lylae*, *Staphylococcus epidermis*). Although these experiments need to be repeated with care to avoid human contamination (note that the frozen skin patches used here were initially not devoted for bacterial analyses), the results present a supplementary clue of absence of known luminous bacteria in *E. spinax* skin.

Finally, in order to precisely locate putative bacteria in shark photophores, we used FISH technique to specifically mark bacterial DNA on skin sections. **Figure 3** summarizes results obtained: the intestine

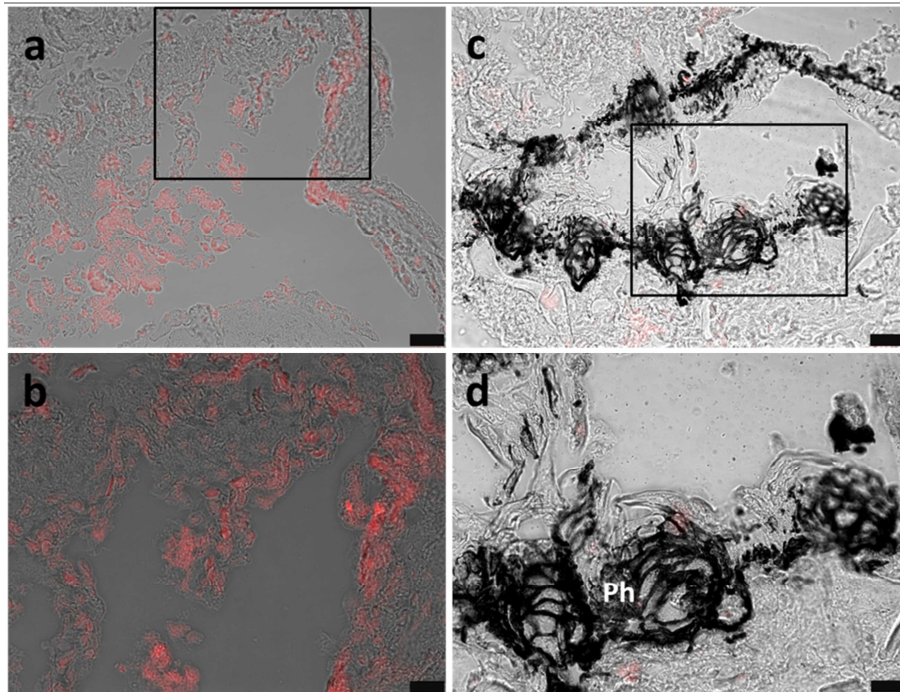


Fig. 3 *Etmopterus spinax* intestine and photophores market (red color) samples. **a** Intestine sample. Scale bar, 50 μm . **b** Magnification of the picture **a** (black rectangle). Scale bar, 25 μm . **c** Skin sample. Scale bar, 50 μm . **d** Magnification of the picture **c** (black rectangle). Ph photphore. Scale bar, 25 μm . Pictures: Cyril Hammoud.

sample (**Fig. 3a & b**) shows a good marking (red color) that confirms the validity of the FISH method we used. By comparison, skin sample (**Fig. 3c & d**) shows almost no marking, especially not inside de photophores. Although some supplementary adjustments of the technique would be required to enhance marking precision (red dot marking are sometimes too diffuse), acquired results are nonetheless sufficient to visualize absence of symbionts in *E. spinax* photophores, by comparison with intestine sample full of bacteria.

Conclusion

FISH and sequencing results, in accordance with morphological approach based on TEM micrographs, allow us to confirm the absence

of bacteria inside *E. spinax* photophores and to show this shark species to possess an intrinsic luminous system. Following this conclusion, next step in our investigation of *E. spinax* luminous system will be to explore characteristics of chemiluminescent reaction involved in the light emission.

CHAPTER II

DIETARY ACQUISITION HYPOTHESIS OF *ETMOPTERUS SPINAX* LIGHT-PRODUCING MOLECULE

CHAPTER II

DIETARY ACQUISITION HYPOTHESIS OF *ETMOPTERUS SPINAX* LIGHT-PRODUCING MOLECULE

In the first chapter, we argued *E. spinax* possesses an intrinsic light-emitting system, based on chemicals located inside the photocyte granular area. Intrinsic systems are the most common amongst luminous organisms and present a great variety of mechanisms. Taking into account the diversity of light emitting reactions and associated structures amongst luminous organisms, Haddock *et al.* (2010) suggested bioluminescence to have independently appeared more than 40 times in the course of evolution. However, all known chemiluminescent reactions are based on the same chemical principle: the oxidation of a substrate (the luciferin) by an enzyme (the luciferase) in the presence of oxygen and sometimes cofactor(s). Interestingly, despite the great diversity of intrinsic mechanisms and the presence of a specific luciferase in each luminescent organism, a few luciferins have been discovered so far since the same molecule is used as substrate in various non-sister taxa. To explain this phenomenon, scientists hypothesized (and demonstrated for some species) a transfer of these luciferins through the food chain; this would allow a luminous species to acquire its bioluminescent capability by feeding on luminous (or luciferin-carrying) prey.

When addressing a new luminous intrinsic system, investigating the feeding habits of the studied species in order to identify putative luminous or luciferin-carrying prey is of great interest. This is the goal of the paper 3. From all prey identified until the species level, three were found to be luminous by using a known luciferin and one was believed to carry luciferin. The hypothesis that *E. spinax* might use one of these luciferins to produce its own bioluminescence is tested: the presence of

luciferins in the shark digestive tract and photophores was assayed and cross-reactions with the putative shark luciferase were performed. As a result, none of luciferins tested were detected in shark photophore extracts and none seemed to react with the shark luciferase extract. We therefore came to the conclusion that *E. spinax* luminous system might be based on a novel luciferin or involved an unknown storage or active form of a known luciferin. When performing cross-reactions, no putative cofactors were added to trigger the reaction, as we assumed all components to be present in the shark luciferase extract. Yet, in order to support our results, supplementary cross-reactions on frozen tissues using coelenterazine and various possible cofactors were done (**Appendix A**). In addition, various coelenterazine analogues (i.e. laboratory synthesized molecules having the same imidazopyrazinone core but presenting different branching groups) were assayed for their cross-reaction with *E. spinax* luciferase (**Appendix B**).

The second part of the chapter is a comprehensive study of *E. spinax* diet using various indexes (paper 3 only provides one simple index along with prey identification). Here we used the index of relative importance (IRI), which is the most accurate and allows direct comparisons of dietary importance of each prey taxa. Euphausiids were found to be the most important food source of *E. spinax* while teleosts, decapods and cephalopods also represent a significant nutritional intake, a finding in accordance with previous studies.

PAPER 3

First study of the chemistry of the luminous system
in a deep-sea shark, *Etmopterus spinax* Linnaeus,
1758 (Chondrichthyes: Etmopteridae)

Marie Renwart • Jérôme Mallefet
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First study of the chemistry of the luminous system in a deep-sea shark, *Etmopterus spinax* Linnaeus, 1758 (Chondrichthyes: Etmopteridae)

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ABSTRACT

The chemistry of the luminous system in a shark is addressed for the first time. Assuming that many luminous species acquire their luminous substrate (luciferin) through the food chain, we investigated the feeding habits of a Norwegian population of the lantern shark *Etmopterus spinax* Linnaeus, 1758 in order to identify potential alimentary sources of luciferin in this species. Among the major groups of preys found in the diet of *E. spinax*, we identified three potential sources of luciferin: the euphausiid *Meganyctiphanes norvegica* Sars, 1857 (tetrapyrrole luciferin), the shrimp *Pasiphaea multidentata* Esmark, 1866 (coelenterazine) and the teleost *Maulolicus muelleri* Gmelin, 1789 (coelenterazine). Their luciferins were tested for their presence in the digestive tract and photogenic organs of *E. spinax* as well as for their cross-reaction with a putative shark catalyst. Although not detected in the diet, the cypridinid luciferin, the third most widespread marine luciferin, was also assayed. As a result, only the coelenterazine was unambiguously detected in the digestive tract of *E. spinax* and none of the tested luciferins reacted with the shark catalyst extract. Our results support the hypothesis of an unknown luminous system in this shark species, involving either a known luciferin in a specific active or storage form, or an unknown light-emitting molecule.

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1. Introduction

In the deep sea, where darkness is permanent, the capability to emit visible light is of major ecological role. This phenomenon called bioluminescence is widespread among marine taxa, from bacteria to fishes (Hastings, 1995). Bioluminescence arises from a chemical reaction carried out in specialized cells called photocytes (intracellular bioluminescence) or in the environment when luminous compounds are excreted (extracellular bioluminescence). The very first try to understand the chemistry of this reaction was made by Boyle in 1667, who demonstrated the oxygen requirement (Hastings, 1983). Nowadays, two types of chemical pathways have been shown to allow light emission in living organisms. In the first one, called luciferin–luciferase system, a substrate (luciferin) is oxidized by an enzyme (luciferase), in the presence of oxygen and sometimes cofactors (Henry and Michelson, 1978). In the second one, the luciferin, the enzymatic complex and the oxygen are bound together in a “pre-charged” compound named photoprotein (Shimomura, 1985). The symbiotic system is another way to emit light as the host (some squids and fishes) doesn’t produce light per

se but cultivates luminous bacteria in a particular organ (Hastings, 1995).

To date, more than 30 luminous systems have been discovered (Shimomura, 2006), involving various combinations of luciferins, enzymes and cofactors, and it is likely that many more remain to be discovered. Bioluminescence seems to be one of the most spectacular adaptive convergences as it is supposed to have appeared more than 40 times in evolution (Haddock et al., 2010).

Although luciferases are genome products, meaning that each of them is proper to the species it comes from, luciferins seem to be more ubiquitous: the same luciferin can be used as the substrate of the reaction in non-sister taxa, such as crustaceans and fishes (Shimomura et al., 1980; Thomson et al., 1997). From the four major marine luciferins currently characterized (Fig. 1), three (i.e. the coelenterazine, the cypridinid luciferin and the dinoflagellates luciferin) seem to be widespread in the oceans as they occur in at least two phyla, for the dinoflagellates (Nakamura et al., 1989) and cypridinid luciferins (Cormier et al., 1967) and up to nine phyla regarding the coelenterazine (Haddock et al., 2010; Widder, 2010). This phylogenetic diversity could be explained by a transmission of the luciferin through the food chain. Indeed, dietary acquisition of the luciferin has been experimentally proved for some species such as the teleost fish *Porichthys notatus* Girard, 1854 (Barnes et al., 1973), the mysid shrimp *Gnathopausia ingens* Dohrn, 1870 (Frank et al., 1984) and the jellyfish *Aequorea victoria* Murbach & Shearer, 1902 (Haddock et al., 2001). The alimentary acquisition of the substrate required for the chemiluminescent reaction was suggested in numerous

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¹ Contribution: collected the sample, performed the experiments, analyzed the data and wrote the article.

² Contribution: adviser, collected the sample, revising.

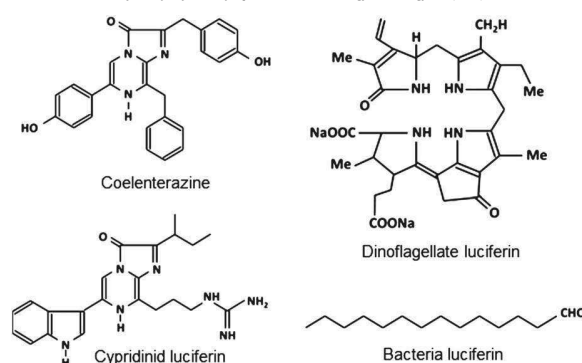


Fig. 1. Structures of the four marine luciferins known. Modified from Shimomura (2006).

marine groups including cephalopods (Young et al., 1979), shrimps (Shimomura, 1987), fishes (Mallefet and Shimomura, 1995), or euphausiids (Haddock et al., 2010), based on positive cross-reactions with extracts from their digestive tract and photophores.

The biochemistry of light emitting systems has been largely studied in invertebrates. Among vertebrates, only fishes are known to be luminous and exclusively Osteichthyes have been investigated for their light emission mechanism. Even if less known, some Chondrichthyes are also luminous. In sharks, two families show this ability: the lantern sharks (the Etmopteridae) and the dwarf mesopelagic sharks (the Dalatiidae). They are deep-sea sharks, rather rare and difficult to observe (Claes and Mallefet, 2009a). Although some features of their bioluminescence (photophores morphology, control and functions) have been recently studied (Claes and Mallefet, 2008, 2009b, 2009c, 2010a, 2010b; Claes et al., 2010a, 2010b, 2011), nothing is known about the biochemistry of their chemiluminescent reaction.

In this paper, we investigate for the first time the luminous system present in the velvet belly lantern shark *Etmopterus spinax* Linnaeus, 1758, a small common deep-sea shark occurring along the continental shelf of the Eastern Atlantic Ocean and in the Mediterranean Sea (Compagno et al., 2005).

According to the theory of the luciferin food transfer, we analyzed the feeding habits of this shark species in order to detect specific luciferin available for its luminous emission. Precisely, our goals are to (i) identify the preys involved in the diet of a Norwegian population of *E. spinax*, (ii) determine those containing luciferin in their tissues and (iii) test the presence of these luminous substrates in the digestive tract and the photogenic tissue of the shark as well as their cross-reaction with a potential chemiluminescent catalyst in this species.

2. Material and methods

2.1. Animals and stomach collection

Specimens of *E. spinax* were sampled during five scientific field missions (from December 2009 to December 2010) in the Raunefjord, a Norwegian fjord near Bergen (60°15.908 N; 05°07.7780 E) by bottom longlines. Animals were sacrificed, measured (total length (TL) and standard length (SL)), weighed and sexed. Their stomach contents were collected and directly frozen at -80°C for further prey's identification. From other specimens, stomach content, intestine content and

pieces of skin with photophores were taken as fresh samples and directly used for luminometry experiments.

2.2. Diet analysis

Prey items from full stomachs were identified to the lowest taxonomic level possible mostly based on non-digestible or less-digestible components (beaks of cephalopods, eyes or carapaces of crustaceans, skeleton of fishes). Number of each prey item (N) was calculated and expressed in percent to the total abundance of preys (%N).

2.3. Luminometry

Luciferins extracted from the shark digestive tract and photophores were tested for their capability to react with different specific luciferases by recording the light emitted from the mix of both components. Inversely, a putative shark catalyst extracted from the photophores was tested against known luciferins. The different cross-reactions tested with the shark samples are referred in Table 1. All the experiments were done on six specimens.

Extraction and assays from the stomach content

A fresh sample from the stomach content was weighted and crushed with fivefold the weight of methanol, in order to extract the luciferin(s). The coelenterazine and the cypridinid luciferin are known to be soluble in methanol (Shimomura, 2006), and the euphausiid luciferin was

Table 1
Cross-reactions between sharks extracted tissues and known luciferins or luciferases.

Luciferins	Luciferases
Crude extract from shark stomach	vs Purified <i>Renilla</i> luciferase
Crude extract from shark stomach	vs Crude euphausiid luciferase
Crude extract from shark stomach	vs Crude cypridinid luciferase
Crude extract from shark intestine	vs Purified <i>Renilla</i> luciferase
Crude extract from shark intestine	vs Crude euphausiid luciferase
Crude extract from shark intestine	vs Crude cypridinid luciferase
Crude extract from shark photophores	vs Purified <i>Renilla</i> luciferase
Crude extract from shark photophores	vs Crude euphausiid luciferase
Crude extract from shark photophores	vs Crude cypridinid luciferase
Purified coelenterazine	vs Crude extract from shark photophores
Crude euphausiid luciferin	vs Crude extract from shark photophores
Purified cypridinid luciferin	vs Crude extract from shark photophores

N = 6 specimens for each combinations.

tested for its solubility in this solvent through a positive cross-reaction between a methanol extract from *Meganyctiphanes norvegica* Sars, 1857 and a luciferase extract from the same species.

The extract was then rapidly assayed versus three different luciferases: the purified *Renilla* luciferase (NanoLight, working dilution of 0.2 g/L in a Tris–HCl buffer: Tris–HCl 0.01 M, NaCl 0.5 M, BSA 0.1%, pH 7.4) to test the presence of coelenterazine, the crude cypridinid luciferase (extracted with distilled water from dried *Vargula hilgendorfi* Muller, 1890) to test the presence of cypridinid luciferin, and the crude euphausiid luciferase (extracted with distilled water from fresh *M. norvegica*) to test the presence of euphausiid luciferin. These luciferases were first tested for their capability to react with their specific luciferin (positive cross-reactions, data not shown).

The assays between the stomach content extract and the three luciferases were done as follows: 20 µL of the stomach content extract were added to 180 µL of a Tris–HCl buffer (Tris–HCl 0.01 M, NaCl 0.5 M, pH 7.4). In another tube, 5 µL of purified *Renilla* luciferase or 20 µL of crude cypridinid or euphausiid luciferase was respectively added to 195 or 180 µL of the same Tris–HCl buffer. The weak self-emission of both reagents was independently controlled using a FB12 luminometer (Berthold Detection System). The background of the luciferase tube was recorded (Berthold Detection System, FB12/Sirius software 1.5) for 10 s, then the luciferin extract was quickly injected and the recording was continued for 2 min.

Results were considered to be significant when the injection of the luciferin generates a higher amount of light than the light produced by the luciferase alone during the first 10 s of its background recording, and when a subsequent decrease of the light is observed, which is due to the consumption of the substrate by the catalyst. Since mechanical injection of luciferin extract by a microsyringe generates a very short peak of light, this peak is not considered as a positive detection.

Different stabilized or active forms of the coelenterazine are known. In addition to the active free form, three of them have been assayed in this study: the protein-bound form, which is the precursor of the luciferin in the sea pansy *Renilla*, the enol-sulfate form, which is a storage form of coelenterazine in the sea pansy *Renilla*, and the dehydrocoelenterazine, which is a storage form in some squid liver such as *Watasenia* and *Symplectoteuthis* (Shimomura, 2006). The first form is included in the basic coelenterazine assay described just before, as the free-form of the luciferin is extracted by methanol. The enol-sulfate form can be converted to the free-form by heating the methanol extract at 95 °C with 0.5 M HCl, as described in Shimomura's protocols (Shimomura, 2006). In the same way, the free-form of coelenterazine can be obtained from the dehydrocoelenterazine by mixing the methanol extract with NaBH₄ (Shimomura, 2006). Assays on these two last forms have been done with frozen samples from the stomach and intestinal contents, and from photophores of the shark.

The extraction and assays from the intestinal content and photophores

Were done using the same protocol as for the stomach content. Photophore extracts were obtained using ventral skin patches known to contain numerous photophores per square centimeter (Claes and Mallefet, 2009b).

Extraction and assays with the putative shark catalyst

A fresh piece of shark skin with photophores from the ventral face was weighted and crushed with fivefold the weight of distilled water, in order to extract the catalyst of the shark luminous system. The shark catalyst is soluble in distilled water and in buffered shark saline (prepared from Bernal et al., 2005), as the crushed piece of skin in these solvents still emits light and shows a progressively decreasing curve, meaning that all components necessary to the reaction are present in such an extract. As the extraction in distilled water is the classical way to extract luciferases (Shimomura, 2006), we decided to use this solvent in this study. As we cannot attest for the stability of the shark

catalyst, the cross-reactions were performed within 1 h. The extract was kept in ice and in the dark between experiments.

The distilled water extract was assayed versus three different luciferins: the purified coelenterazine (NanoLight, working dilution of 0.044 g/L in methanol), the purified cypridinid luciferin (purified by Y. Ohmiya's lab (AIST, Japan), working dilution of $4.05 \cdot 10^{-4}$ g/L in methanol) and the crude euphausiid luciferin (extracted with methanol from fresh *M. norvegica*). These luciferins were first tested for their capability to react with their specific luciferase (positive cross-reactions, data not shown).

The assays between the photophore extract and the three luciferins were done as follows: 20 µL of the photophore extract was added to 180 µL of a Tris–HCl buffer (Tris–HCl 0.01 M, NaCl 0.5 M, pH 7.4). In another tube, 5 µL of purified coelenterazine or cypridinid luciferin, or 20 µL of crude euphausiid luciferin was respectively added to 195 or 180 µL of the same Tris–HCl buffer. The weak self-emission of both reagents was independently controlled in a FB12 luminometer. The background of the catalyst tube was recorded for 10 s, then the luciferin mixture was quickly injected and the recording was continued for 2 min.

3. Results

3.1. Animals and stomach collection

A total of 122 specimens of *E. spinax* were fished: 85 females and 37 males, ranging from 31 to 52 cm TL (Fig. 2). Out of them, 54 (44.3%) had full stomachs and were used for prey identification.

3.2. Diet analysis

Twelve prey taxa were identified for a total of 94 preys; among them, seven were determined at the species level. Euphausiids (%N = 41.49) were the main food source of *E. spinax* followed by cephalopods (%N = 10.64), teleost fishes (%N = 8.51) and decapods (%N = 7.45). It has to be noted that a non-negligible part of the intake was not identified (%N = 21.28) due to the high digested state of numerous prey items (Table 2).

Two heads from the species *Scomber scombrus* Linnaeus, 1758 were found twice in the stomachs. Based on dental bone dimensions, the total size of the fish was calculated as described in Scharf et al. (1998) and gives a result of 29.6 cm TL. In the same way, original mantle length (ML) of the five *Rossia macrosoma* Delle Chiaie, 1828 found was calculated based on the lower beak dimensions (Clarke, 1986) and gives a result from 3.76 cm to 8.22 cm ML. The whole body of the shrimp *Pandalus montagui* Leach, 1814 recorded was in the stomach and the measured size was 12.8 cm from the rostrum tip to the telson.

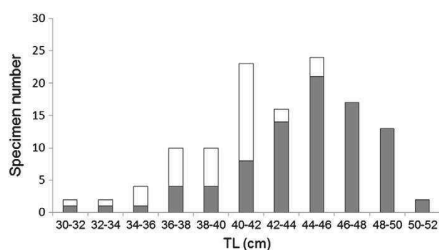


Fig. 2. Distribution of *Etmopterus spinax* specimen according to their total length (TL), for males (□) and females (■).

Table 2
Number (N) and numeric index (%N) for each prey item of the diet of *Etmopterus spinax*, and bioluminescence characteristics for identified species.

Prey items	N	%N	Substrate
Euphausiacea	39	41.49	
<i>Meganyctiphanes norvegica</i> ⁽⁺⁾	12	12.77	Tetrapyrrole ^a
Euphausiacea unid.	27	28.72	
Decapoda Natantia	7	7.45	
<i>Pasiphaea sivadoi</i> ⁽⁻⁾	2	2.13	Coelenterazine ^b
<i>Pasiphaea multidentata</i> ⁽⁻⁾	1	1.06	NT
<i>Pandalus montagui</i> ⁽⁻⁾	1	1.06	NT
Decapoda unid.	3	3.19	
Crustacea unid.	10	10.64	
Teleostei	7	8.51	
<i>Scomber scombrus</i> ⁽⁻⁾	1	2.13	NT
<i>Maurolicus muelleri</i> ⁽⁺⁾	2	2.13	Coelenterazine ^c
Teleostei unid.	4	4.26	
Cephalopoda	10	10.64	NT
<i>Rossia macrosoma</i> ⁽⁻⁾	5	5.32	
Cephalopoda unid.	5	5.32	
Unidentified prey	20	21.28	

(+) known to be luminous; (-) not known to be luminous; (NT) not tested for the substrate content.

^a Nakamura et al. (1989).

^b Thomson et al. (1995).

^c Pers. obs.

3.3. Luminometry

Regarding the seven prey taxa identified to the species level in the diet of *E. spinax*, three were of particular interest as potential sources of luciferin: the euphausiid *M. norvegica*, the shrimp *Pasiphaea multidentata* Esmer, 1866 and the teleost fish *Maurolicus muelleri* Gmelin, 1789. Substrates found in these species, i.e. the euphausiid tetrapyrrole and the coelenterazine (Table 2), were assayed for their presence in the shark's digestive tract and photophores (using crude euphausiid luciferase or purified *Renilla* luciferase), and for their utilization in the shark's photophores (using crude euphausiid luciferin or purified coelenterazine against crude shark luciferase).

Although euphausiids were the main food source for *E. spinax*, tetrapyrrole luciferin was not detected, neither in the digestive tract of the shark, nor in the photophore extract. Coelenterazine was present in the stomach and intestinal contents of most specimens but wasn't detected in the photophores of the shark (Fig. 3). The presence of two stabilized forms of coelenterazine, the dehydrocoelenterazine and the enol-sulfate form, was assayed on frozen photophore extracts and gave the same non-positive results (data not shown). Additional experiments were made with crude cypridinid luciferase and extracts from various tissues of the shark and failed to reveal the presence of cypridinid luciferin in the shark digestive tract, neither in the photophore extract (Fig. 3).

The use of specific luciferins against extracts from shark photophores never revealed the presence of a putative catalyst able to react with these substrates (Fig. 3).

4. Discussion

4.1. Animals and stomach collection

In this report, all sharks have been caught by lowered longlines, what explains the exclusive presence of large individuals in the sample. According to our observations, fishing gears have a clear impact on the catch, as trawl mainly provides small individuals (Claes and Mallefet, 2009b) while longline usually captures larger animals (from 31 to 52 cm TL, with 43.3 cm TL on average in this study). This observation probably reflects a size-related depth distribution of the specimens, as it has previously been suggested for this species (Coelho and Erzini, 2010) and for the related species *Etmopterus pusillus* Lowe, 1839 (Xavier et al., 2012).

4.2. Diet analysis

Euphausiids, cephalopods, teleosts, and decapods represent the main diet of *E. spinax*. Similar results are found by Bergstad et al. (2003) in the

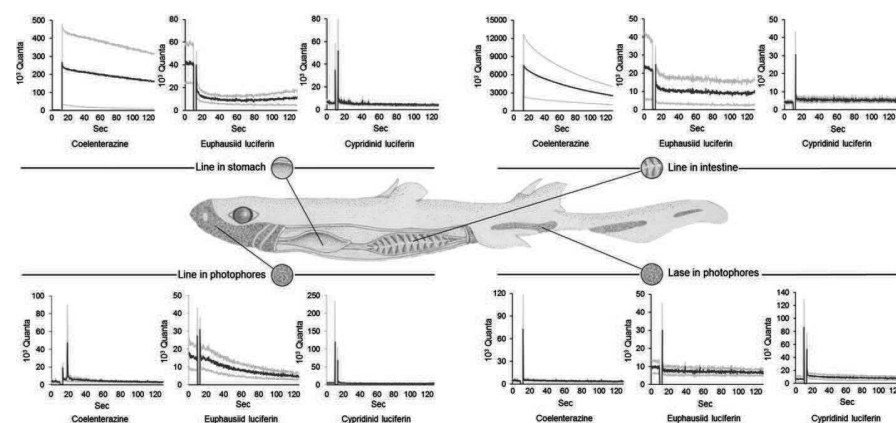


Fig. 3. Mean light emission (black lines) from the cross-reactions between shark crude extracts and known luciferins or luciferases. Stomach content, intestinal contents and photophores have been tested for their luciferin content: against purified *Renilla* luciferase to detect coelenterazine; against crude euphausiid luciferase to detect euphausiid luciferin and against crude cypridinid luciferase to detect cypridinid luciferin. Photophore luciferase extracts have been tested for their capability to react with the same luciferins: against purified coelenterazine, crude euphausiid luciferin and purified cypridinid luciferin. On each graph, the first 10 s are the recording of the luciferase alone. The luciferin injection is represented by the short vertical peaks as the luminometer is opened. Line = luciferin; lase = luciferase; SEM are represented by gray lines; N = 6 for each assays.

Skagerrak (Norway) and by Neiva et al. (2006) in the Algarve waters (Portugal).

The presence of large mackerel heads in shark stomachs is surprising. Nevertheless, these heads were fresh and clearly ripped up, which is not in favor of a scavenging act. This supports the idea that *E. spinax* hunts in groups to predate on large preys, as already suggested (MacPherson, 1980; Neiva et al., 2006).

4.3. Luminometry

As detailed in the Introduction, the objective of this study was to determine the luciferin sources available for *E. spinax*. Through its feeding habits, we identified two luciferin sources that can potentially be involved in the luminous system of this species: the tetrapyrrole luciferin (present in the euphausiid *M. norvegica*), and the coelenterazine (present in the teleost fish *M. muelleri* and, to a lesser extent, in the shrimp *P. multidentata*). Finally, the presence of cypridinid luciferin was also investigated.

The tetrapyrrole luciferin is found in the species *M. norvegica*, which counts for the main part of the krill in Norway. The same molecule (except for two oxygen atoms) was found to be the substrate of the bioluminescent reaction in dinoflagellates (Nakamura et al., 1989). It has been proved that the dinoflagellates luciferin cross-reacts with the krill catalyst and inversely, suggesting a food chain transfer of the tetrapyrrole luciferin (Dunlap et al., 1980; Haddock et al., 2010). However, the transit of this luciferin to a higher trophic level has never been documented. *E. spinax* being the next link in the food chain, we naturally tested the implication of the euphausiid luciferin in its luminous system. Despite the great incidence of euphausiids in the shark diet, none of the cross-reaction tested with the shark system produces light. Since tetrapyrrole is known to be a very unstable molecule inactivated by low pH (Dunlap et al., 1980), the molecule might therefore have been degraded by the acidic digestion process of the shark.

The coelenterazine is the most widespread luciferin among marine taxa (Shimomura, 1987; Shimomura et al., 1980) and is used for bioluminescence but probably also for other purposes, e.g. oxygen defense pathway (Rees et al., 1998). The fish *M. muelleri* emits blue light thanks to a coelenterazine-based system (Pers. observation). Coelenterazine has been detected in the stomach of the shrimp *P. multidentata*, which is not known to be luminous, revealing that it preys on organisms containing this molecule (Thomson et al., 1995). As a link in the food chain, this shrimp can be considered as another potential source of coelenterazine for the shark. Indeed, some non-bioluminescent organisms ingest coelenterazine and some are even able to store it in their tissues (e.g. in the hepatopancreas), making it available for their predators (Shimomura, 1987).

Cross-reactions demonstrate the presence of significant concentrations of coelenterazine in most of the digestive contents tested, which suggests the possibility of a transfer of this molecule to the shark tissues. However, negative cross-reactions with the photophore extracts of the shark reveal that the free-form of coelenterazine is not part of the luminous system in this species. Three stabilized forms of coelenterazine have been assayed for their presence in photophore extracts (the protein-bound form, the enol-sulfate form and the dehydrocoelenterazine), without success. We cannot exclude the presence of an unknown storage form or a different chemically active form of the coelenterazine in the shark system, or the presence of a photoprotein including the coelenterazine.

From literature, the cypridinid luciferin is present in two unrelated taxa: ostracods and fishes. It has been postulated that cypridinid luciferin can be transferred from preys to predators (Thompson et al., 1988). Since some luminous ostracods, such as *Vargula norvegica* Baird, 1860, are known to live in sympatry with *E. spinax* (IOC, 2012), the cypridinid luciferin could be present in the trophic web of the shark. However, our various attempts to detect cypridinid luciferin in tissues of the shark, as well as to perform cross-reactions between shark photophore extracts and cypridinid luciferin failed.

Work is in progress to investigate the involvement of a photoprotein in *E. spinax*, although this particular system has never been highlighted in fishes. In the same way, the bacterial symbiont hypothesis will be experimentally studied, but this possibility is even more unlikely. Indeed, embryos from *E. spinax* are known to be luminous before birth in the uterus of the female, where bacterial intrusion is unlikely (Claes and Mallefet, 2008). Moreover, this shark species presents thousands of tiny (<200 µm) photophores, each of them containing few photocytes (Claes and Mallefet, 2008); this kind of structural organization is far from the classical morphology of symbiotic luminous organs (Hastings, 1995).

5. Conclusion

In this study, various experiments were performed in order to detect the presence of known luciferins in different body compartments of the shark species *Etmopterus spinax*, with regard to the possibility of a food web transfer of these molecules. Our attempts to highlight the presence of a classical luciferin/luciferase system based on one of the known luciferins failed as none of the three luciferins tested (i.e. the dinoflagellate/euphausiid luciferin, the coelenterazine and the cypridinid luciferin) was detected in the photogenic organs of *E. spinax* or is able to cross-react with its putative catalyst. We suggest an unknown light-emitting system in this shark species, involving either a specific active (or storage) form of a known luciferin, or a new luciferin or photoprotein. Experiments will be conducted in order to characterize the luminous system of *E. spinax*.

Acknowledgments

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References

- Barnes, A.T., Case, J.F., Tsuji, F.I., 1973. Induction of bioluminescence in a luciferin deficient form of the marine teleost, *Parichthys*, in response to exogenous luciferin. *Comp. Biochem. Physiol.* 46A, 709–723.
- Bergstad, O.A., Wik, A.D., Hildre, O., 2003. Predator–prey relationships and food sources of the Skagerrak deep-water fish assemblage. *J. Northw. Atl. Fish. Sci.* 31.
- Bernal, D., Donley, J.M., Shadwick, R.E., Syme, D.A., 2005. Mammal-like muscles power swimming in a cold-water shark. *Nature* 437, 1349–1352.
- Claes, J.M., Mallefet, J., 2008. Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark *Etmopterus spinax* (Squaloidea: Etmopteridae). *J. Fish Biol.* 73, 1337–1350.
- Claes, J.M., Mallefet, J., 2009a. Bioluminescence of sharks: first synthesis. In: Meyer-Rochow, V.B. (Ed.), *Bioluminescence in Focus – A Collection of Illuminating Essays*. Research Signpost, Kerala, India, pp. 51–65.
- Claes, J.M., Mallefet, J., 2009b. Ontogeny of photophore pattern in the velvet belly lantern shark, *Etmopterus spinax*. *Zoology* 112, 433–441.
- Claes, J.M., Mallefet, J., 2009c. Hormonal control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J. Exp. Biol.* 212, 3684–3692.
- Claes, J.M., Mallefet, J., 2010a. The lantern shark's light switch: turning shallow water crypsis into midwater camouflage. *Biol. Lett.* 6, 685–687.
- Claes, J.M., Mallefet, J., 2010b. Functional physiology of lantern shark (*Etmopterus spinax*) luminescent pattern: differential hormonal regulation of luminous zones. *J. Exp. Biol.* 213, 1852–1858.
- Claes, J.M., Aksnes, D.L., Mallefet, J., 2010a. Phantom hunter of the fjords: camouflage by counterillumination in a shark (*Etmopterus spinax*). *J. Exp. Mar. Biol. Ecol.* 388, 28–32.
- Claes, J.M., Krönström, J., Holmgren, S., Mallefet, J., 2010b. Nitric oxide in the control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J. Exp. Biol.* 213, 3005–3011.
- Claes, J.M., Krönström, J., Holmgren, S., Mallefet, J., 2011. GABA inhibition of luminescence from lantern shark (*Etmopterus spinax*) photophores. *Comp. Biochem. Physiol.* 153C, 221–236.
- Clarke, M.R., 1986. *A Handbook for the Identification of Cephalopod Beaks*, first ed. Oxford University Press, Oxford.
- Coelho, R., Erzini, K., 2010. Depth distribution of the velvet belly, *Etmopterus spinax*, in relation to growth and reproductive cycle: the case study of a deep-water lantern shark with a wide-ranging critical habitat. *Mar. Biol. Res.* 6, 381–389.

- Compagno, L., Dando, M., Fowler, S., 2005. A Field Guide to the Sharks of the World, first ed. Harper Collins, London.
- Cormier, M.J., Crane, J.M., Nakano, Y., 1967. Evidence for the identity of the luminescent systems of *Porichthys porosissimus* (fish) and *Cypridina hilgendorffii* (crustacean). *Biochem. Biophys. Res. Commun.* 29, 747–752.
- Dunlap, J.C., Hastings, J.W., Shimomura, O., 1980. Crossreactivity between the light-emitting systems of distantly related organisms: novel type of light-emitting compound. *Proc. Natl. Acad. Sci. U. S. A.* 77, 1394–1397.
- Frank, T.M., Widder, E.A., Latz, M.I., Case, J.F., 1984. Dietary maintenance of bioluminescence in a deep-sea mysid. *J. Exp. Biol.* 109, 385–389.
- Haddock, S.H.D., Rivers, T.J., Robison, B.H., 2001. Can coelenterates make coelenterazine? Dietary requirement for luciferin in cnidarian bioluminescence. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11148–11151.
- Haddock, S.H.D., Moline, M.A., Case, J.F., 2010. Bioluminescence in the sea. *Ann. Rev. Mar. Sci.* 2, 443–493.
- Hastings, J.W., 1983. Biological diversity, chemical mechanisms, and the evolutionary origins of bioluminescent systems. *J. Mol. Evol.* 19, 309–321.
- Hastings, J.W., 1995. Bioluminescence. In: Sperelakis, N. (Ed.), *Cell Physiology Source Book*. Academic Press Inc., San Diego, pp. 665–681.
- Henry, J.P., Michelson, A.M., 1978. Bioluminescence: physiological control and regulation at the molecular level. *Photochem. Photobiol.* 28, 293–310.
- Intergovernmental Oceanographic Commission (IOC) of UNESCO, 2012. The Ocean Biogeographic Information System (OBIS) (Internet). Cited 2012 January 24. Available from <http://www.jobis.org>.
- MacPherson, E., 1980. Régime alimentaire de *Galeus melastomus* Rafinesque, 1810 *Etmopterus spinax* (L., 1758) et *Scymnorchinus licha* (Bonnaterre, 1788) en Méditerranée occidentale. *Vie. Milieu* 30, 139–148.
- Mallefet, J., Shimomura, O., 1995. Presence of coelenterazine in mesopelagic fishes from the Strait of Messina. *Mar. Biol.* 124, 381–385.
- Nakamura, H., Kishi, Y., Shimomura, O., Morse, D., Hastings, J.W., 1989. Structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products. *J. Am. Chem. Soc.* 111, 7607–7611.
- Neiva, J., Coelho, R., Erzini, K., 2006. Feeding habits of the velvet belly lantern shark *Etmopterus spinax* (Chondrichthyes: Etmopteridae) off the Algarve, southern Portugal. *J. Mar. Biol. Assoc. U. K.* 86, 835–841.
- Rees, J.F., De Wergifosse, B., Nolsen, O., Dubuisson, M., Janssens, B., Thompson, E.M., 1998. The origins of marine bioluminescence: turning oxygen defence mechanisms into deep-sea communication tools. *J. Exp. Biol.* 201, 1211–1221.
- Scharf, F.S., Yetter, R.M., Summers, A.P., Juanes, F., 1998. Enhancing diet analyses of piscivorous fishes in the Northwest Atlantic through identification and reconstruction of original prey sizes from ingested remains. *Fish. Bull.* 96, 575–588.
- Shimomura, O., 1985. Bioluminescence in the sea: photoprotein systems. *Symp. Soc. Exp. Biol.* 39, 351–372.
- Shimomura, O., 1987. Presence of coelenterazine in non-bioluminescent marine organisms. *Comp. Biochem. Physiol.* 86B, 361–363.
- Shimomura, O., 2006. *Bioluminescence: Chemical Principles and Methods*, first ed. World Scientific, Singapore.
- Shimomura, O., Inoue, S., Johnson, F.H., Haneda, Y., 1980. Widespread occurrence of coelenterazine in marine bioluminescence. *Comp. Biochem. Physiol.* 65B, 435–437.
- Thompson, E.M., Toya, Y., Nafpaktitis, B.G., Goto, T., Tsuji, F.I., 1988. Induction of bioluminescence capability in the marine fish, *Porichthys notatus*, by *Vargula* (crustacean) [¹⁴C]luciferin and unlabelled analogues. *J. Exp. Biol.* 137, 39–51.
- Thomson, C.M., Herring, P.J., Campbell, A.K., 1995. Coelenterazine distribution and luciferase characteristics in oceanic decapod crustaceans. *Mar. Biol.* 124, 197–207.
- Thomson, C.M., Herring, P.J., Campbell, A.K., 1997. The widespread occurrence and tissue distribution of the imidazolopyrazine luciferins. *Luminescence* 12, 87–91.
- Widder, E.A., 2010. Bioluminescence in the ocean: origins of biological, chemical, and ecological diversity. *Science* 328, 704–708.
- Xavier, J.C., Vieira, C., Assis, C., Cherel, Y., Hill, S., Costa, E., Borges, T.C., Coelho, R., 2012. Feeding ecology of the deep-sea lantern shark *Etmopterus pusillus* (Elasmobranchii: Etmopteridae) in the northeast Atlantic. *Sci. Mar.* 76, 301–310.
- Young, R.E., Roper, C.F.E., Mangold, K., Leisman, G., Hochberg, F.G., 1979. Luminescence from non-bioluminescent tissues in oceanic cephalopods. *Mar. Biol.* 53, 69–77.

Supplementary results:

Feeding habits of a Norwegian population of
the lanternshark *Etmopterus spinax*

SUPPLEMENTARY RESULTS:**FEEDING HABITS OF A NORWEGIAN POPULATION OF THE
LANTERNSHARK *ETMOPTERUS SPINAX****Context*

Studying feeding ecology of marine species in order to understand their role in the trophic web has become a common practice (Cortés, 1997). The easiest and standard way to do so is to analyse species stomach contents and to describe it using various indexes. Numeric, gravimetric and occurrence indexes respectively provide number and weight of prey in the stomachs, and number of stomachs containing a prey item. Although these indexes give very useful indications on predator feeding behavior and prey nutritional value, they do not provide information on relative importance of prey and so do not allow prey taxa to be compared. Indeed, if a predator is found with 10 euphausiids in its stomach for only 1 large sized fish, its main food source will be the euphausiids following numeric index. But taking the weight of prey into account will lead to assume fish to be the main food source of the predator. To overcome these biases, Pinkas *et al.* (1971) developed a new index, the index of relative importance (IRI), calculated from the three previous ones and thus expressing prey taxa as comparative global food intake.

Feeding ecology of *Etmopterus spinax* various populations has been addressed by several authors (Bello, 1998; Santos & Borges, 2001; Bergstad *et al.*, 2003; Klimpel *et al.*, 2003; Neiva *et al.*, 2006; Fanelli *et al.*, 2009; Preciado *et al.*, 2009). By studying the diet of a Norwegian population, our first goal was to identify prey that might be involved in *E. spinax* luciferin transfer (Renwart & Mallefet, 2013) but also to estimate their relative importance in the shark feeding ecology in order to make our data comparable with previous studies. For that reason, we decided to carry out a comprehensive analysis of *E. spinax* diet and to use the IRI. This study has been carried out with the collaboration of Romain De Jaegere (MSc student).

Materials and methods

Animals and stomachs collection

Specimens of *E. spinax* were sampled during eleven scientific field missions (from 2008 to 2012) in two Norwegian fjords near Bergen (60°15.908N; 05°07.778O-Raunefjord; 60°12.279N; 05°18.068O-Lysefjord) by lowered longlines and deep trawlings. Animals were sacrificed, measured (total length (TL) and standard length (SL)), weighed and sexed. Their stomach content were collected and straight frozen at -80°C for further prey identification.

Diet analysis

Prey items from full stomachs were identified to the lowest taxonomic level possible mostly based on non-digestible or less-digestible components (beaks, eyes or carapaces). In order to assess sample size sufficiency (i.e. stomachs number), a cumulative prey curve was performed by plotting the increasing number of randomly pooled stomachs with the corresponding increase of prey taxa (Cortés, 1997). Stomachs were randomly re-sampled one hundred times using Matlab (7.10.0.499 (R2010a)) and the resulting means on number of taxa were used to construct the curve.

Four quantitative index were used to describe the importance of prey in the diet of *E. spinax*: (i) the numeric index (%N) as the abundance of a prey item in percent to the total abundance of prey, (ii) the gravimetric index (%W) as the remaining weight of a prey item in percent to the total weight of prey and (iii) the occurrence index (%O) as the number of stomachs containing a prey item in percent to all full stomachs. The relative importance index (iv) was calculated as follow: $IRI = (\%N + \%W) \times \%O$. For comparisons, IRI index were then converted in percent (%IRI) (Cortés, 1997). During the 2007 and 2008 sampling, prey items were identified but not weighed and therefore not used to calculate relative importance index (IRI). The whole sample was, however, used to construct the cumulative prey curve.

Table 1 Sharks distribution (number of specimens) between sex and size (based on the alimentary shift proposed by Klimpel *et al.*, 2003), for the whole sample and the specimens with full stomachs. For each class, percentage of specimens with full stomachs is shown in brackets.

	Females		Males		Total
	< 19 cm TL	> 19 cm TL	< 19 cm TL	> 19 cm TL	
Total sample	7	165	8	75	255
Full stomachs	5 (71.43)	46 (27.88)	5 (62.50)	24 (32.00)	80 (31.37)

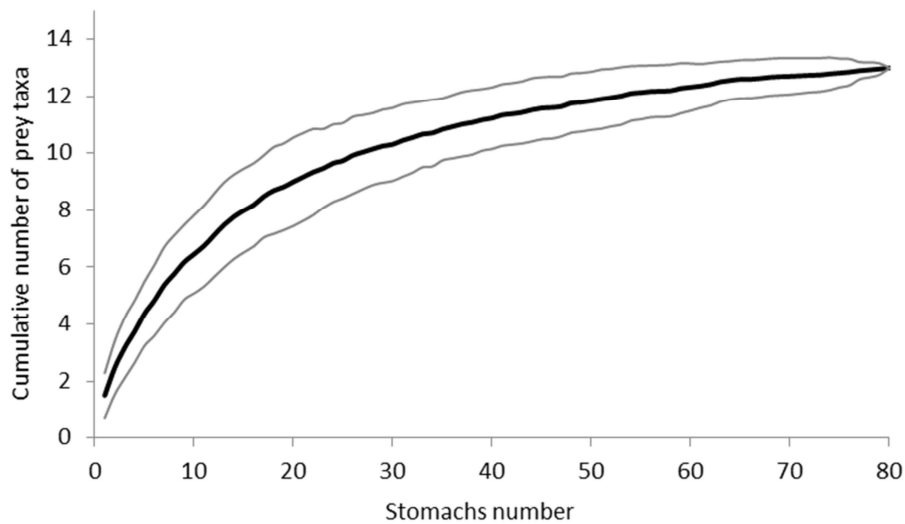


Fig. 1 Cumulative prey curve. Mean (black line) and standard deviations (grey lines) calculated for 100 repetitions of the randomly pooled stomachs.

Results

A total of 255 specimens of *E. spinax* were caught: 172 females and 83 males, ranging from 12.5 to 52.5 cm TL. Out of them, 80 (31.37%) had full stomachs and 49 (from which prey were weighed) were used in the diet analysis (**Table 1**).

Table 2 Number (N), numeric index (%N), gravimetric index (%W), occurrence index (%O) and index of relative importance (%IRI) for each prey item of *E. spinax* diet.

Prey items	N	%N	%W	%O	%IRI
Euphausiacea	39	41,94	7,04	55,10	45,55
<i>Meganyctiphanes norvegica</i>	12	12,90	3,29	18,37	7,63
Euphausiacea unid.	27	29,03	3,75	44,90	37,75
Decapoda Natantia	7	7,53	17,17	14,29	5,95
<i>Pasiphaea sivado</i>	2	2,15	1,71	4,08	0,40
<i>Pasiphaea multidentata</i>	1	1,08	1,28	2,04	0,12
<i>Pandalus montagui</i>	1	1,08	11,78	2,04	0,67
Decapoda unid.	3	3,23	2,40	6,12	0,88
Crustacea unid.	10	10,75	5,92	20,41	5,74
Cephalopoda	10	10,75	4,53	18,37	4,74
<i>Rossia macrosoma</i>	5	5,38	1,18	10,20	1,72
Cephalopoda unid.	5	5,38	3,35	10,20	2,28
Teleostei	7	7,53	52,76	14,29	14,54
<i>Scomber scombus</i>	1	1,08	50,00	2,04	2,67
<i>Maurollicus muelleri</i>	2	2,15	2,32	4,08	0,47
Teleostei unid.	4	4,30	0,45	8,16	0,99
Unidentified prey	20	21,51	12,57	40,82	23,48

Twelve prey taxa were identified for a total of 93 prey; among them, seven were determined at the species level (**Table 2**). As shown by the cumulative prey curve (**Fig. 1**), we sampled a sufficient stomachs number to reach the asymptote and represent accurately the diet of *E. spinax* in this region.

Table 2 and **figure 2** show that euphausiids were the main food source of *E. spinax* with the highest %IRI (45,55%). They were the most abundant (%N = 41,94) and the best represented in the stomachs (%O = 55,10). It must be pointed out that small individuals (< 30 cm TL) fed exclusively on this prey (data not shown). Although they were relatively rare (%N = 7,53), teleost fishes came in the second place (%IRI = 14,54) in the feeding habits of *E. spinax*, mainly due to their high gravimetric index (%W = 52,76). They were followed by decapods (%IRI = 5,95) and cephalopods (%IRI = 4,74). It has to be noted that a non-negligible part

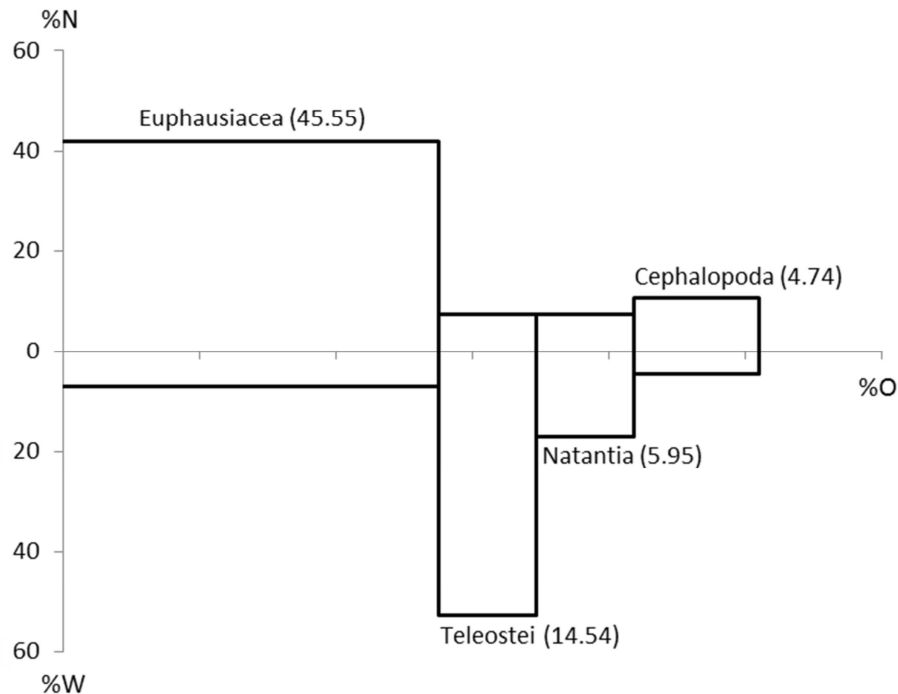


Fig. 2 Index of relative importance (IRI) diagram for the four main *E. spinax* prey categories. IRI correspond to areas of boxes. %IRI are shown in brackets. Each graduation of the %O axis represents 20%.

of the intake was not identified (%IRI = 23.48) due to the high digested state of numerous prey items.

Discussion

From our results, euphausiids, teleosts, decapods and cephalopods represent *E. spinax* major diet. The cumulative prey curve indicates stomach number is high enough to allow an accurate analysis. Nevertheless, total intake is probably slightly underestimated as some prey, especially the small-sized and the ones with soft bodies, might have been digested very rapidly and therefore not represented here. Indeed, some studies reported the presence of small-sized crustaceans such as isopods, mysids and larvae in *E. spinax* stomachs (Bergstad *et al.*, 2003; Fanelli *et al.*, 2009; Preciado *et al.*, 2009). Nevertheless, most

previous studies described the same major prey taxa as we do (euphausiids, teleosts, decapods and cephalopods) (Bello, 1998; Bergstad *et al.*, 2003; Neiva *et al.*, 2006; Preciado *et al.*, 2009) with the

Table 3 Prey categories % IRI for *E. spinax* and *E. pusillus* various populations.

Shark species and population location	%IRI			
	Euphausiids	Teleosts	Decapods	Cephalopods
<i>Etmoterus spinax</i>				
Cantabrian sea ¹	36,3	45,5	0,21	17,7
Algarve waters ²	37,4	36,6	18	7,1
Norwegian fjords ³	45,6	14,5	6	4,7
<i>Etmoterus pusillus</i>				
Algarve waters ⁴	-	27,9	0,26	10,3

¹Preciado *et al.*, 2009; ²Neiva *et al.*, 2006; ³This study; ⁴Xavier *et al.*, 2012.

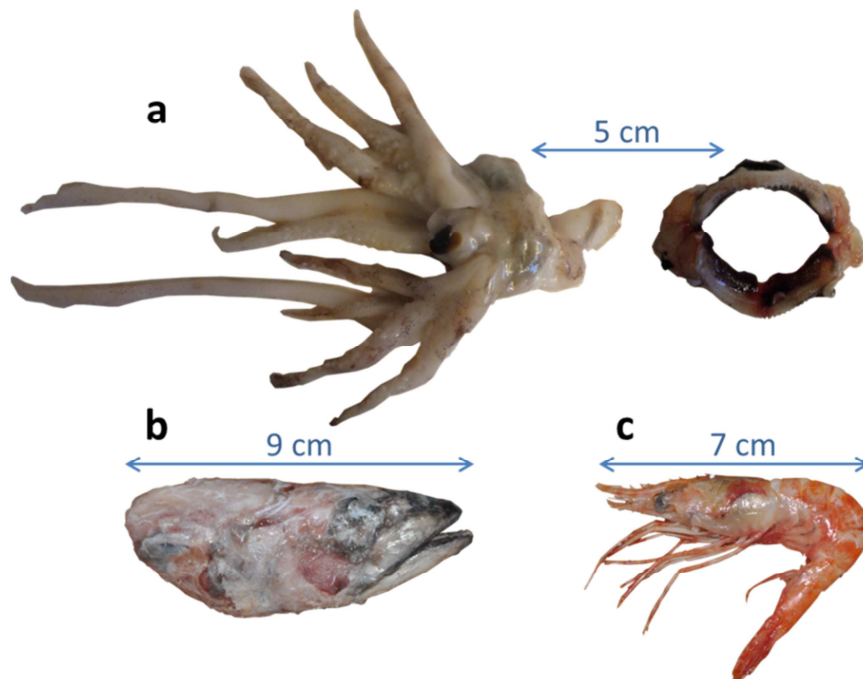


Fig. 3 Large prey found in stomachs of *E. spinax* from Norwegian fjords. **a** A cephalopod (prob. *Rossia macrosoma*) together with the shark's jaw who ate it for scale purpose. **b** A mackerel head (*Scomber scombrus*) (total length estimated to 29.6 cm) ate by a 43.5 cm shark. **c** A shrimp (*Pandalus montagui*, total length = 12.8 cm from the rostrum tip to the telson) ate by a 40.5 cm shark.

two first taxa accounting for the major part of the diet: similar %IRI were found by Neiva *et al.* (2006) in Algarve waters (**Table 3**) with euphausiids being the main food source, closely followed by teleosts and decapods, which ones represented a higher intake than what we found in this report. On the contrary, in Cantabrian sea (Preciado *et al.*, 2009), teleosts are the main intake, followed by euphausiids. The feeding habits of *Etmopterus pusillus*, a sister species of *E. spinax*, have been studied in Algarve waters. The study reports no euphausiids in the diet of this shark, while %IRI for decapods is very low. Nevertheless, it has to be noted that the %IRI of unidentified crustaceans is very high (50.7) and decapods importance from the **table 3** is therefore probably largely underestimated for this species.

The low trophic diversity of *E. spinax* (and *E. pusillus*), compared to other Algarve sympatric sharks such as the Spiny Dogfish (*Squalus acanthias*), suggests a very selective diet for both species (Xavier *et al.*, 2012). Moreover, the reason why no euphausiids were identified in *E. pusillus* diet might be related to a specific preference for other preys, although we cannot exclude euphausiids to be present as unidentified crustaceans. A differential diet related to ontogeny is also reported in most study, for both *E. spinax* and *E. pusillus*. Klimpel *et al.* (2003) mentioned two prey items from juveniles (< 30 cm) of *E. spinax* from a Norwegian population, i.e. *Meganyctiphanes norvegica* and *Maurolicus muelleri*. They observed an alimentary shift towards the fish *M. muelleri* for sharks larger than 19 cm TL, while sharks smaller than 19 cm TL mainly ate euphausiids. In the same way, Xavier *et al.* (2012) reported a decreasing importance of crustaceans and a progressively increasing importance of fish in the diet of *E. pusillus* according to the size of shark. Accordingly, euphausiids seem to be the only food source for *E. spinax* juveniles in this study (data no shown), while larger (> 30 cm) specimens feed on greater prey diversity. Those alimentary shifts toward a more diverse diet are most likely due to an increase in predatory capability correlated with size (increase in swimming capability and in stomach storage capacity) (Xavier *et al.*, 2012). Indeed, as discussed in Renwart & Mallefet (2013), mature specimens are able to feed on very large prey, whose some are illustrated in **figure 3**, arguing for their good hunting ability, possibly in groups (MacPherson, 1980; Neiva *et al.*, 2006).

The limited number of prey items recorded per stomach and the high number of empty stomachs (although this second parameter might be slightly overestimated due to the fishing gear – longlines with baits – that mainly attracts hungry sharks), suggest *E. spinax* to feed intermittently. Whether it is related to the low prey availability in the deep environment or to the long digestion time in this shark requires more investigations. In addition, some *E. spinax*/*E. pusillus* prey (euphausiids and *Pasiphaea* sp.) are vertically day/night migrating species and it has been suggested that sharks might perform the same migrations (some observations have recorded *E. spinax* at 70 m depth at night), in order to maintain the prey accessibility (Compagno *et al.*, 2005; Neiva *et al.*, 2006; Xavier *et al.*, 2012). Finally, no variation of prey availability have been recorded according to seasons since *E. spinax* main food sources (especially euphausiids) were equally present in analyzed stomachs throughout a year (data not shown). Nevertheless, some punctual prey items, such as *S. scrombus* or *P. montagui* have only been recorded during one field mission, what might be related to a relative rarity of this prey.

Conclusion

E. spinax diet analysis provided very useful information on its feeding habits, preferences and ability to hunt, with regard to ontogeny. Deep-sea shark feeding ecology is poorly investigated and this study is therefore a valuable contribution to this field. Indeed, by using the index of relative importance (IRI), we were able to compare our results with previous studies on this species and we found similar prey taxa between diverse populations.

CHAPTER III

TOWARD PURIFICATION AND CHARACTERIZATION OF *ETMOPTERUS SPINAX* LUMINOUS SYSTEM

CHAPTER III

*TOWARD THE CHARACTERIZATION AND THE PURIFICATION OF
ETMOPTERUS SPINAX LUMINOUS SYSTEM*

Previous chapters led to the conclusion that *E. spinax* luminescence was based on an intrinsic luminous system in which none known marine luciferins seemed to be involved, at least on their active “free forms”. Indeed, the possibility that an unknown active or storage form of a known luciferin would be used by the shark is not excluded. Nevertheless, *E. spinax* luminous system will be further considered as a new reaction, requiring to be purified and characterized. Because the isolation of a new system is not routine work, and might require years of work, the purpose of this chapter is to provide basis experiments and reflection for the future complete characterization of *E. spinax* luminous system.

The chapter has been divided into two parts: In the first one, initial steps of luciferin isolation are carried out. Reversed-phase HPLCs were performed in order to separate molecules present in a shark skin extract. Because most luciferins and/or their oxidized and storage forms are fluorescent, and knowing that fluorescence was observed in photocyte granular areas from luminous sharks (Claes & Mallefet, 2008; Renwart *et al.*, 2014a), molecules were detected with a fluorometer after elution. Skin with and without photophores were compared and the two first eluted peaks were found to be much higher in photophore extract. They probably represent the majority of molecules constituting photophores, but both peaks are not or much less retained by the column. Going further in the luciferin characterization requires exploring these two peaks by using various other chromatography methods. Moreover, it appears that luciferin and its derivatives are not the only

fluorescent compounds inside photophores. An additional detection mean of the luciferin is required in order to target the right compound with certainty.

The second part of the chapter investigates the identity of the luciferase. Our working hypothesis proposes *E. spinax* luminous system to be related to an ancient ROS detoxifying mechanism, and its luciferase to be a peroxidase, as it has been shown for various other luminescent animals (Dure & Cormier, 1963; Bellisario *et al.*, 1972). Molecules from a shark skin extract were separated using size-exclusion HPLC, and all peaks were collected after elution and assayed for their peroxidase content. It was found that one peak contains the majority of peroxidases, and no obvious difference was highlighted between skin with and without photophores. Once again, this peak requires to be analysed in details in order to reveal all peroxidases it contains. Subsequently, the quenching effect of two peroxidase inhibitors (azide and cyanide) was tested on *E. spinax* hormone induced luminescence. A clear light inhibition was observed, but at this stage, there is no evidence that both inhibitors acted on the chemiluminescent reaction itself. Additional experiments, discussed at the end of the chapter, are required to attest *E. spinax* luciferase is a peroxidase. Moreover, the involvement of hydrogen peroxide, which is the main peroxidase substrate, needs to be clarified.

PART 1

Etmopterus spinax luciferin purification: first
isolation step

***ETMOPTERUS SPINAX* LUCIFERIN PURIFICATION:
FIRST ISOLATION STEP**

Context

Experiments conducted in the chapter 2 led to the conclusion that *Etmopterus spinax* luminous system was not based on a known luciferin, at least on the “free forms” predominantly found in luminous marine organisms (see **fig. 2c** of the introduction for a drawing of these forms). Nevertheless, we cannot rule out the possibility for *E. spinax* to use coelenterazine, cypridinid or dinoflagellate luciferin in an unknown specific active or storage form. Indeed, some luminous organisms are known to use particular coelenterazine forms, such as the Sea Pansy *Renilla reniformis* who stabilizes coelenterazine by adding one sulfate group ($-\text{SO}_3\text{H}$) to the molecule. In the squid *Watasenia scintillans*, two sulfate groups are added to coelenterazine and the resulting molecule is not used for storage purpose but represents the active form of coelenterazine that reacts with the luciferase. Coelenterazine storage form in *W. scintillans* was found to be *dehydrocoelenterazine*, i.e. the coelenterazine with a hydrogen missing (Shimomura, 2006). Considering a particular storage form of coelenterazine (or any other luciferins) in *E. spinax*, its conversion into the active form might require a specific process; hence the classical methanol extraction might not be sufficient. In the same way, a slightly different active form of coelenterazine (or any other luciferins), such as in *W. scintillans*, might be enough to prevent cross-reactions between shark photophore aqueous extract and coelenterazine (Renwart & Mallefet, 2013). Note that assays on dehydrocoelenterazine and the coelenterazine sulfate were done with *E. spinax* photophore extracts and are presented in **appendix C**.

Whatever *E. spinax* luminous mechanism is based on an unknown form of a known luciferin or on a completely new substrate, it will be further considered as a novel luminous mechanism requiring to be purified in order to be characterized. Because of the large variety of luminous systems, there is not established protocols and methodologies for studying a new bioluminescent mechanism (Shimomura, 2006). In this chapter, we considered *E. spinax* luminous system to be a

luciferin/luciferase system, with the luciferin being a small molecule (molecular weight < 1.000 Da). The involvement of a photoprotein is not ruled out (and has been assayed on fresh tissues using various cofactors [**Appendix D**]), but because this system has never been documented in fish, we chose to not investigate this possibility first. Assuming a luciferin/luciferase system, we expected the “hot/cold cross-reaction” discovered by Dubois in 1885 (see introduction) to work. However, when mixing a heated aqueous photophore extract (which is supposed to destroyed luciferase and preserve luciferin) with a cold aqueous photophore extract (in which luciferase is preserved and progressively consumes luciferin), no light was produced. In most today’s experiments, the heated extract is replaced by a methanol extract, as methanol dissolves more efficiently small molecules. In the same way, we failed to obtain light by mixing both extracts. Such results might reflect the need of a cofactor in the reaction. Classical cofactors include Ca^{++} , Mg^{++} , ATP, H_2O_2 , cyanide ions, ferrous ions, FMNH₂ and NADH. Most of them were tested with no positive results (**Appendix E**).

So far, all attempts to make an *E. spinax* photophore aqueous extract glowing again after the light has been naturally quenched (consequently to luciferin consumption) failed. When isolating a new bioluminescent molecule, usually by using various chromatography methods, the first step required is a mean of assaying the molecule of interest. For instance, in a luciferin purification process using column chromatography, an easy way to know when luciferin is eluted is to collect elution fractions, add the corresponding luciferase (even as a crude extract) in each of them and observe which one glows. As we were unable to perform this assay, another detection mean was needed to initiate *E. spinax* luciferin purification: we decided to take advantage of its fluorescent property. Most luciferins and/or their oxidized product and stabilized forms are fluorescent. Because shark photophore apical area is fluorescent (and was shown to be the site of chemiluminescent reaction), we assumed *E. spinax* luciferin or derivatives to be fluorescent. We therefore decided to study fluorescence of molecules present in a methanol extract (assumed to extract luciferin and/or related compounds) from shark skin containing photophores. In order to exclude molecules present in shark skin but not in photophores, comparisons were made with skin samples without photophore, from shark dorsal

area. Only molecules present in a higher amount in skin with photophores were retained as putative luciferin or derivative candidates.

As “*the study of an unknown bioluminescent system is not routine work*” (Simomura, 2006), the purpose of this chapter is not to achieve the complete purification of *E. spinax* luminous system, what could require years of work. In this first part, we aimed to initiate the first step of luciferin isolation (or, more likely, a putative stabilized or oxidized form), by using chromatography coupled with fluorescence detection.

This study has been carried out with the collaboration of the Institute of Condensed Matter and Nanosciences (IMCN/MOST), thanks to facilities provided by Prof. Jacqueline Marchant-Brynaert, Prof. Benjamin Elias, Laurent Collard and Stéphanie Wautier.

Materials and method

Shark tissues collection and photophore extracts

Specimens of *E. spinax* were sampled during two scientific field missions (Augusts 2011 and February 2012) in a Norwegian fjord (60°15.908N; 05°07.778O) by lowered longlines. Fresh skin patches with (from animal ventral side) and without (from animal dorsal side) photophores were dissected from killed sharks and frozen at -80 °C until extraction. Extraction of luciferin or derivatives was performed by crushing skin patches in a volume of methanol (previously degased with argon during 30 min to reduce luciferin oxidation) of tenfold their weight in a cooled glass mortar, in order to inactivate luciferase if present. Note that methanol also has the property, in most case, to extract luciferin when bound to a protein for storage purpose (Shimomura, 2006). Extract was centrifuged (16,000 g; 4 °C; 3 min) and supernatant was collected and directly used for further experiments.

Thin-layer chromatography (TLC)

Different stationary and mobile phases were tested in TLC (**Table 1**) and only the protocol that gave the best migration and separation is detailed here. All other TLCs were performed using the same technique.

Eluents (acetonitrile [ACN HPLC] and water [H₂O mmQ], the latter containing 0.1 % of methanoic acid) were degased with argon during half an hour in order to avoid luciferin oxidation as far as possible. Mobile phase was obtained by mixing both eluents in a 2:8 proportion of acetonitrile and water respectively. The selected stationary phase was a reversed-phase composed of silica resin bearing alkyl chains covalently bonded. A small spot of extract was applied on the silica foil using a glass capillary tube and the foil was put in a glass beaker containing a small amount of mobile phase. By capillary action, mobile phase moves up and carries the sample above on the plate. TLC was stopped before solvent front reaches the end of the plate (approximately 0.5 cm ahead) and the natural spots fluorescence was revealed using a UV-lamp (350 to 400 nm) after the plate was dried.

	Type	Stationary phase	Eluent proportion (%)				Spot revelation
			ACN	H ₂ O	EtOH	MeOH	
Test A	1D	Alumine	-	-	100	-	UV-lamp
Test B	1D	Silica reversed	-	-	-	100	UV-lamp
Test C	1D	Silica reversed	50	50	-	-	UV-lamp
Test D	1D	Silica reversed	80	20	-	-	UV-lamp
Test E	1D	Silica reversed	20	80	-	-	UV-lamp
Test F	1D	Silica reversed	2	98	-	-	UV-lamp
Test G	1D	Silica reversed	20	80	-	-	Iodine
Test H	2D	Silica reversed	20	80	-	-	-
-	-	-	50	50	-	-	Iodine
Test I	2D	Silica reversed	20	80	-	-	-
-	-	-	80	20	-	-	UV-lamp

Table 1 Various combinations performed in thin-layer chromatography in order to improve migration and separation of shark skin extract. The best combination is highlighted in grey. *1D* one dimension, *2D* two dimensions, *ACN* acetonitrile, *H₂O* mmQ water, *EtOH* ethanol, *MeOH* methanol.

For some experiments, fluorescent spots were scrubbed from the foil after migration and the resulting silica powder was dissolved in 200 µl of methanol. Dissolved powder was finally filtered in order to be analyzed in high-resolution mass spectrometry (HRMS).

Fluorescence spectroscopy

Shark skin crude extract was analyzed in a fluorescence spectrophotometer (Varian Cary Eclipse, Agilent) in order to obtain its fluorescence emission and excitation spectra. Sample emission spectra were obtained for 26 excitation wavelengths (from 250 to 500 nm with a 10 nm step). The complete fluorescence spectrum was recorded each time, from the selected excitation wavelength + 50 nm until 800 nm in order to cover the entire visible range. Conversely, sample excitation spectra were obtained for 11 emission wavelengths selected around previously recorded emission peaks. The complete excitation spectrum was recorded each time, from 250 nm to the selected emission wavelength – 50 nm. For comparison purpose, emission spectra were recorded from skin with photophores as well as without photophore, while excitation spectra were recorded from skin with photophores only, in order to validate the emission results. Analyses were repeated on two specimens and results (for one specimen only) are presented as 3D diagrams (Matlab 8.2.0.701 (R2013b)).

Reversed-phase HPLC

Reversed-phase high-performance liquid chromatographies (HPLCs) were performed on an Alliance 2695 HPLC System (Waters) using two different columns designed for reversed-phase chromatography: an Atlantis T3 column and an XBridge C₁₈ column (Waters). Mobile phase was composed of water (H₂O mmQ) and acetonitrile (ACN HPLC) in a 95:5 proportion at elution beginning until a 10:90 proportion at elution end, following a gradient. Methanoic acid or ammonium acetate were added to water, respectively when using the Atlantis T3 or the XBridge C₁₈ column, in order to keep a constant 0.1 % of these compounds in the total elution volume. Elution lasted 7 minutes and eluted molecules were detected with a fluorometer, in a way that spectra obtained provided retention times and fluorescence intensities of molecules. Before HPLC, methanol extract was firstly filtered in order to eliminate components that might obstruct the column. Experiments were repeated on two shark specimens in order to validate the method, but only one series of spectra are presented since both specimens showed very similar results. Moreover, additional HPLCs were run in order to collect various peaks just after elution and to analyses them in HRMS.

High resolution mass spectrometry (HRMS)

HRMS were performed from two sample types (i.e. fluorescent spots scrubbed from the TLC foil after migration, and fluorescent peaks collected from HPLC after elution) in order to have a first idea of the molecules present in our samples. Only skin with photophores was tested. Sample was introduced directly on the device (Orbitrap Mass Spectrometer - Thermo Scientific), and analysis was run using the Full-scan MS option. Spectrum was recorded on a large molecular weight range (at least from 200 to 1500 Da).

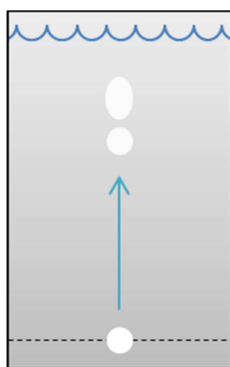
*Results and discussion**Thin-layer chromatography*

Fig. 1 Drawing of a thin-layer chromatography on *E. spinax* skin with photophores. White and grey spots represent fluorescent spots before and after migration respectively.

Before HPLCs were performed, a few pilot experiments were conducted to have a general idea of molecules fluorescence characteristics. Firstly, TLCs (**Table 1**) were done in order to define conditions required for the sample to migrate. First TLCs were performed on normal-phase alumina foil. After solvent (100% ethanol) reached the foil upper edge, sample appeared as a white fluorescent spot easily visible under the UV-lamp, but no migration was observed. We concluded the fluorescent spot to be composed of very polar molecules bound to the stationary phase and not eluted, even when eluent was polar such as ethanol (water was not tried). We therefore moved to a hydrophobic stationary phase (silica reversed) and various eluents were tested. The best migration/separation was obtained when using acetonitrile and water (containing 0.1% of methanoic acid) in a 2:8 proportion, since two fluorescent spots were observed close to the solvent front (**Fig. 1**). Attempt to reveal non-fluorescent spots was done by using iodine vapours but no extra-spot was observed. Finally, 2D TLCs were performed in

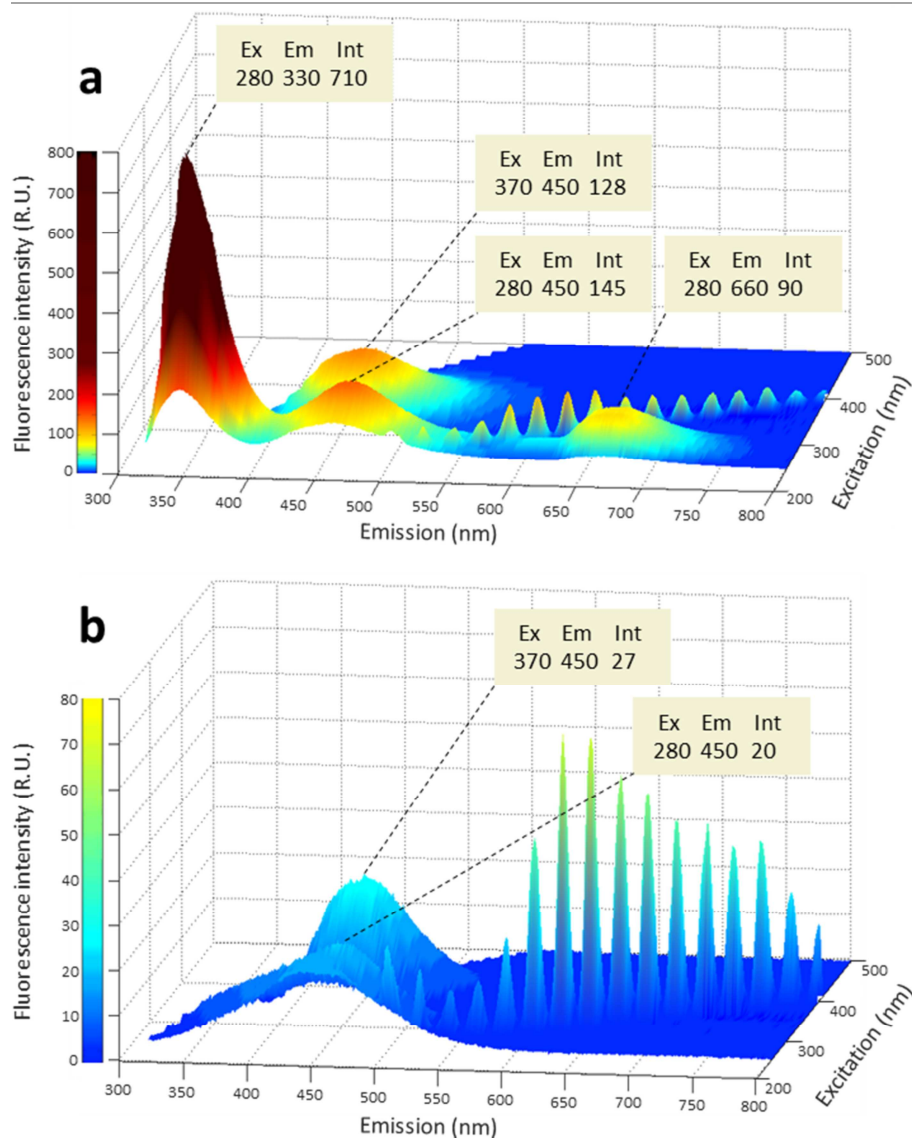


Fig. 2 3D diagrams of fluorescence spectra from skin with (a) and without (b) photophores. Peak coordinates are given in boxes: *Ex* excitation wavelength; *Em* emission wavelength; *Int* fluorescent intensity in relative units (*R.U.*). Note that fluorescence intensity is lower in skin without photophore. Non labelled peaks are device artefacts and should not be taken into account.

order to increase spots separation, but we did not obtained better results. TLCs were only performed on skin with photophores samples.

Fluorescence spectroscopy

Fluorescence emission and excitation spectra were recorded from skin extracts with and without photophores. Emission spectrum from skin with photophore revealed four fluorescent peaks (330, 450x2 and 660 nm) corresponding to two excitation wavelengths (280 and 370 nm): excitation at 280 nm gave rise to all three fluorescent peaks, while excitation at 370 nm gave rise to another 450 nm peak (**Fig. 2a**). Conversely, excitation spectra were recorded around the three emission wavelengths: 330 and 660 nm emission peaks led to 280 nm excitation peak, while 450 nm emission peak led to both 280 and 370 nm excitation peaks (data not shown). Emission spectrum from skin without photophore showed only the two 450 nm emission peaks (with a lower intensity), one at 280 nm of excitation, the other at 370 nm (**Fig. 2b**), and excitation spectra at 450 nm of emission led to both 280 and 370 nm excitation peaks (data not shown). Emission and excitation spectra were performed on two shark specimens and the same results were obtained.

Reversed-phase HPLC

Reversed-phase HPLCs were performed in order to separate fluorescent compounds present in samples of skin with and without photophores. Pilot experiments previously described (TLCs and fluorescence spectroscopy) permitted us to determine conditions to use in HPLC: (i) because samples did not migrate in normal phase TLC, but required a reversed-phase, we followed the same conditions in HPLC by using an Atlantis T3 column (Waters) designed for a balanced retention of polar and non-polar molecules from mixtures containing diverse analytes such as crude extracts. In agreement with TLC results, water (H₂O mmQ) and acetonitrile (ACN HPLC) were used as eluents, as described in the method. (ii) Following peaks obtained in fluorescence spectroscopy on skin with photophores (**Fig. 2a**), eluted compounds were detected at four paired excitation/emission wavelength (i.e. 280/330; 280/450; 280/660; 370/450, for both skin with and without photophores), since only one excitation/emission couple can be set at a time in the fluorometer.

A total of 16 fluorescent peaks (**Table 2**) have been recorded from the eight emission spectra (**Fig 3 – 6**). Peaks in the same color are

Table 2 Presence (✓)/absence of the 16 recorded peaks in the eight spectra presented at figures 3 to 6. The first column allows peaks identification based on color (i.e. the same as in fig. 3-6) and their mean retention time $\pm$ standard deviation.

Tissue $\Rightarrow$	Skin with photophores				Skin without photophores			
Excitation $\Rightarrow$	280			370	280			370
Emission $\Rightarrow$	330	450	660	450	330	450	660	450
0,62 $\pm$ 0,002	✓	✓	✓	✓	✓	✓	✓	✓
0,94 $\pm$ 0,073	✓	✓	✓	✓	✓	✓	✓	✓
2,27 $\pm$ 0,014	✓	✓	✓		✓	✓	✓	
2,87 $\pm$ 0,011	✓		✓		✓		✓	
3,26 $\pm$ 0,015					✓		✓	
3,4 $\pm$ 0,015	✓	✓	✓		✓	✓	✓	
3,6 $\pm$ 0,008	✓		✓		✓		✓	
4,05 $\pm$ 0,006	✓		✓		✓		✓	
4,38 $\pm$ 0,003	✓		✓					
4,41 $\pm$ 0,006					✓		✓	
4,54 $\pm$ 0,013	✓		✓					
4,74 $\pm$ 0,008	✓		✓		✓		✓	
5,13 $\pm$ 0,005	✓		✓		✓		✓	
5,53 $\pm$ 0,015	✓		✓		✓		✓	
5,82 $\pm$ 0,005	✓		✓		✓		✓	
5,99 $\pm$ 0,034	✓	✓	✓	✓	✓	✓	✓	✓

considered to be the same molecule or group of molecules from one to another spectrum. Fluorescence intensity (Y axis) was considered to be proportional to the amount of molecule(s) in the peak and is used for comparison between skin with and without photophores since both samples undergone the same extraction method. Regarding the four “skin with photophores” spectra (**Fig. 3a-6a**), two (i.e. the 280/330 and the 280/660 spectra) look very similar since all 14 peaks were recorded on both of them. Five of those peaks were also recorded on the 280/450 spectrum, 3 on the 370/450 spectrum, and 3 were present on all four spectra (**Table 2**). The same observation can be made from the four “skin without photophore” spectra (**Fig. 3b-6b**). From these results, we

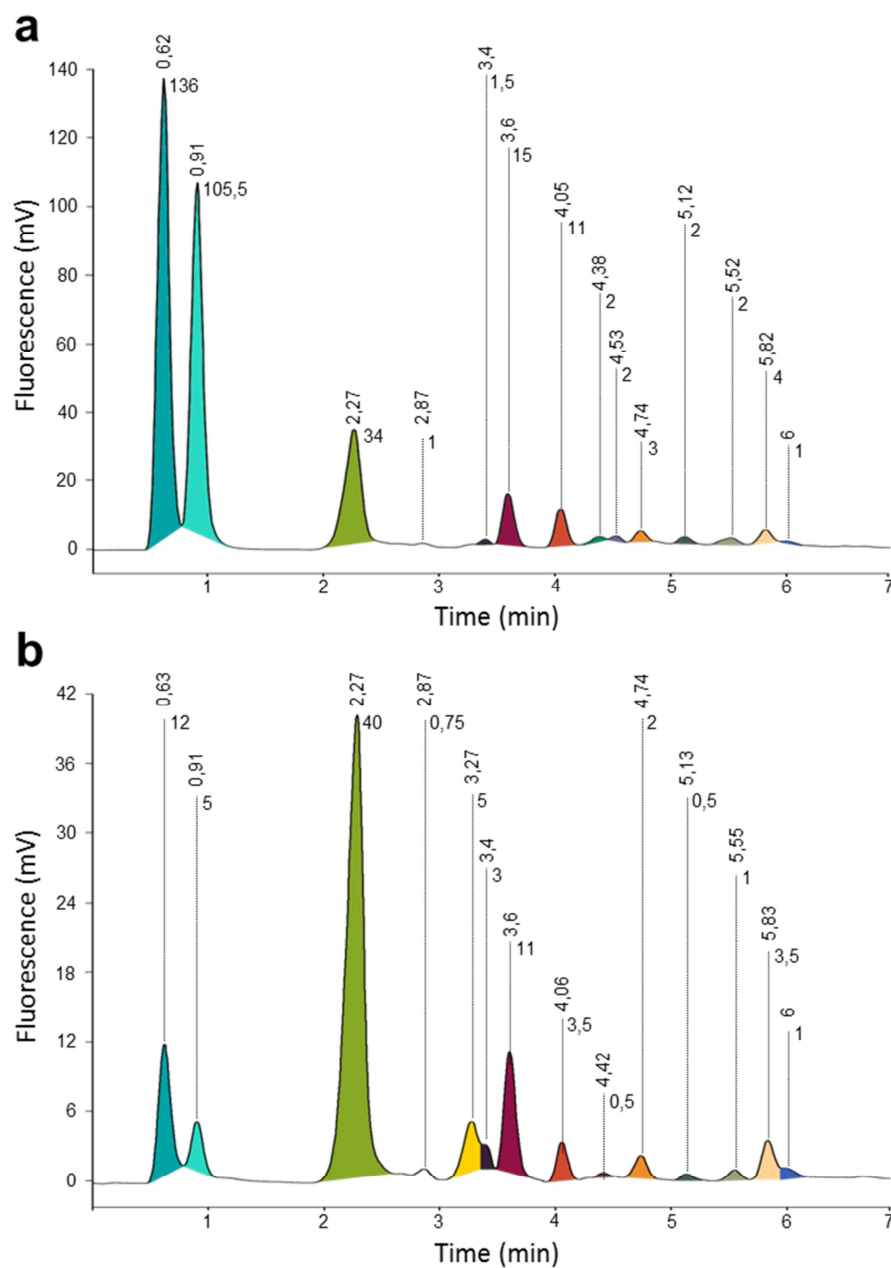


Fig. 3 Reversed-phase HPLC spectra of fluorescent compounds present in *E. spinax* skin with (a) and without (b) photophores, at 280 nm of excitation and 330 nm of emission.

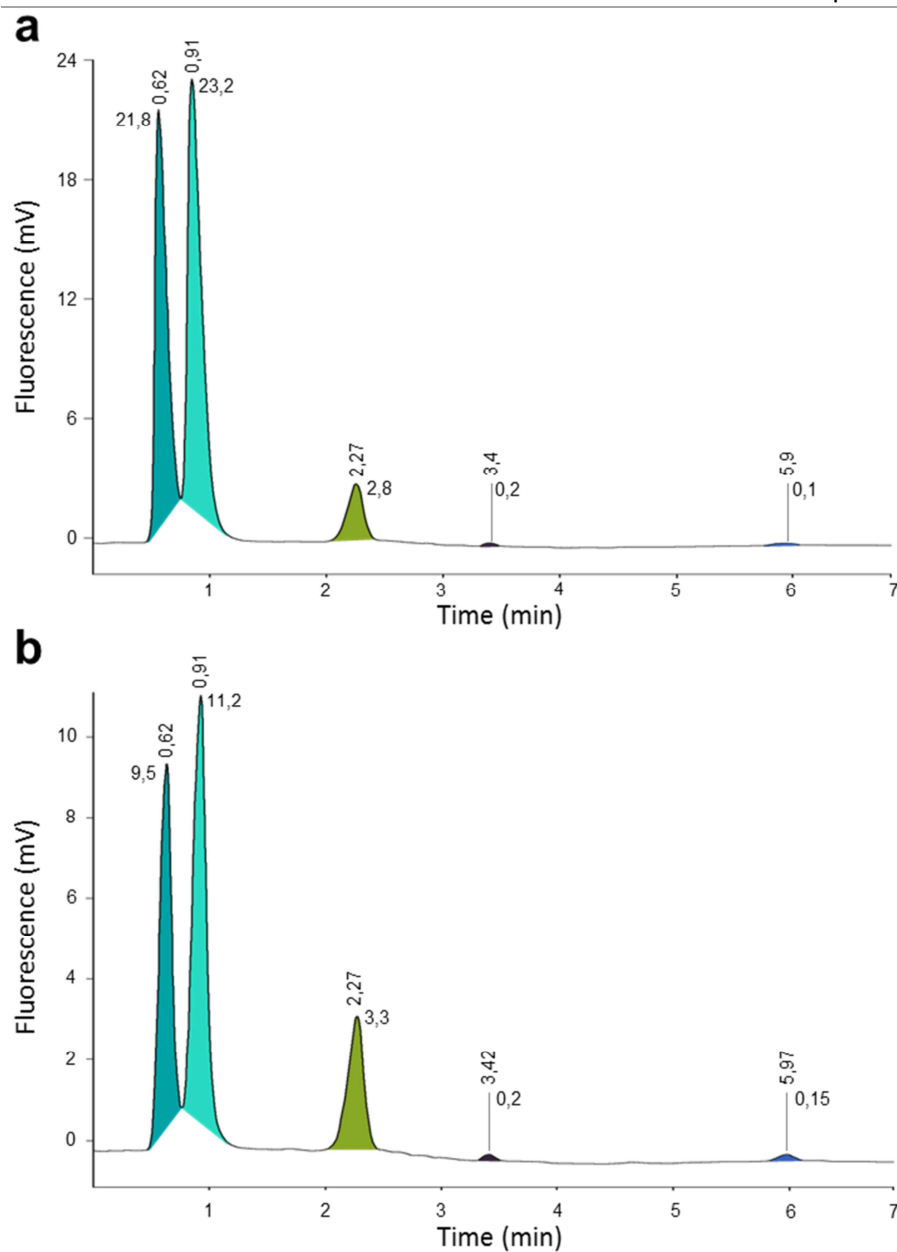


Fig. 4 Reversed-phase HPLC spectra of fluorescent compounds present in *E. spinax* skin with (a) and without (b) photophores, at 280 nm of excitation and 450 nm of emission.

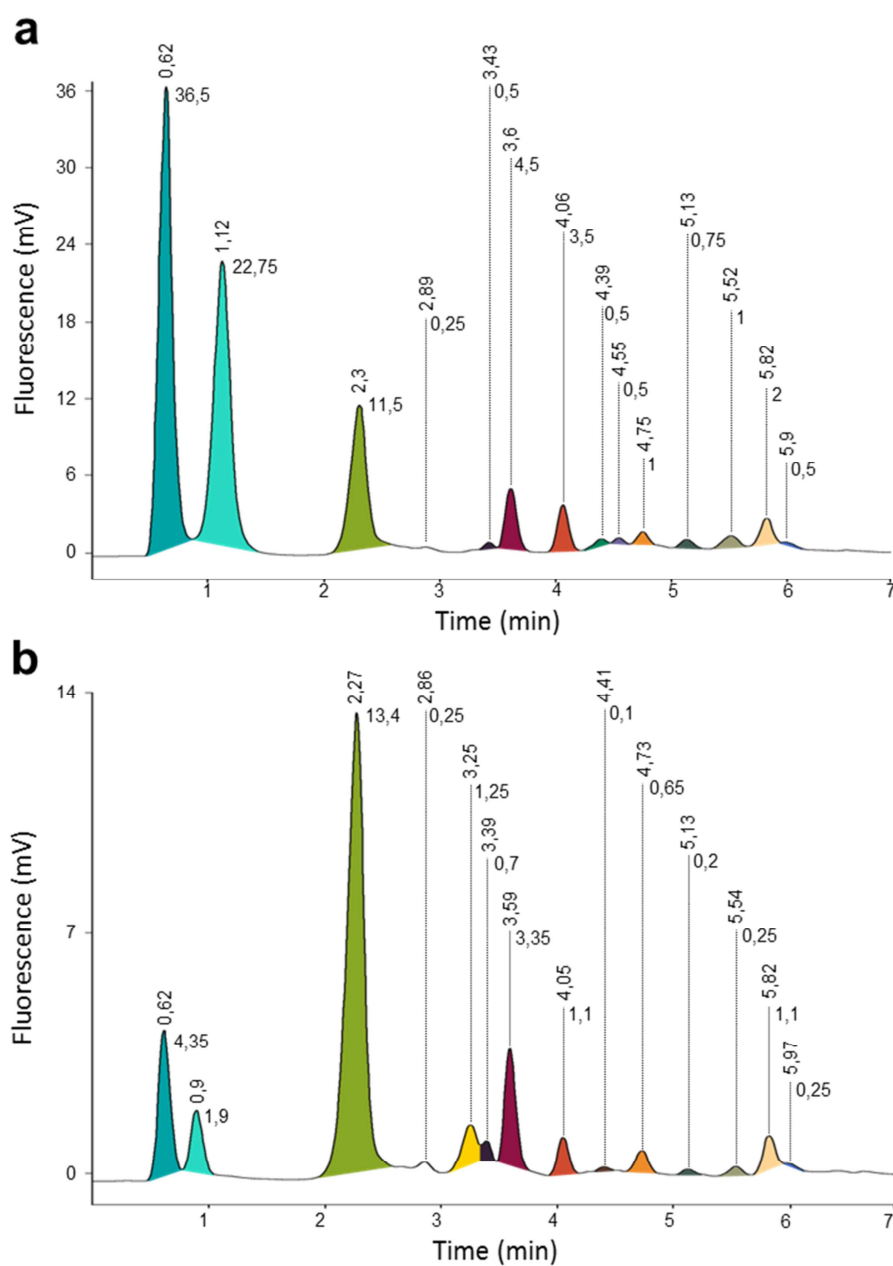


Fig. 5 Reversed-phase HPLC spectra of fluorescent compounds present in *E. spinax* skin with (a) and without (b) photophores, at 280 nm of excitation and 660 nm of emission.

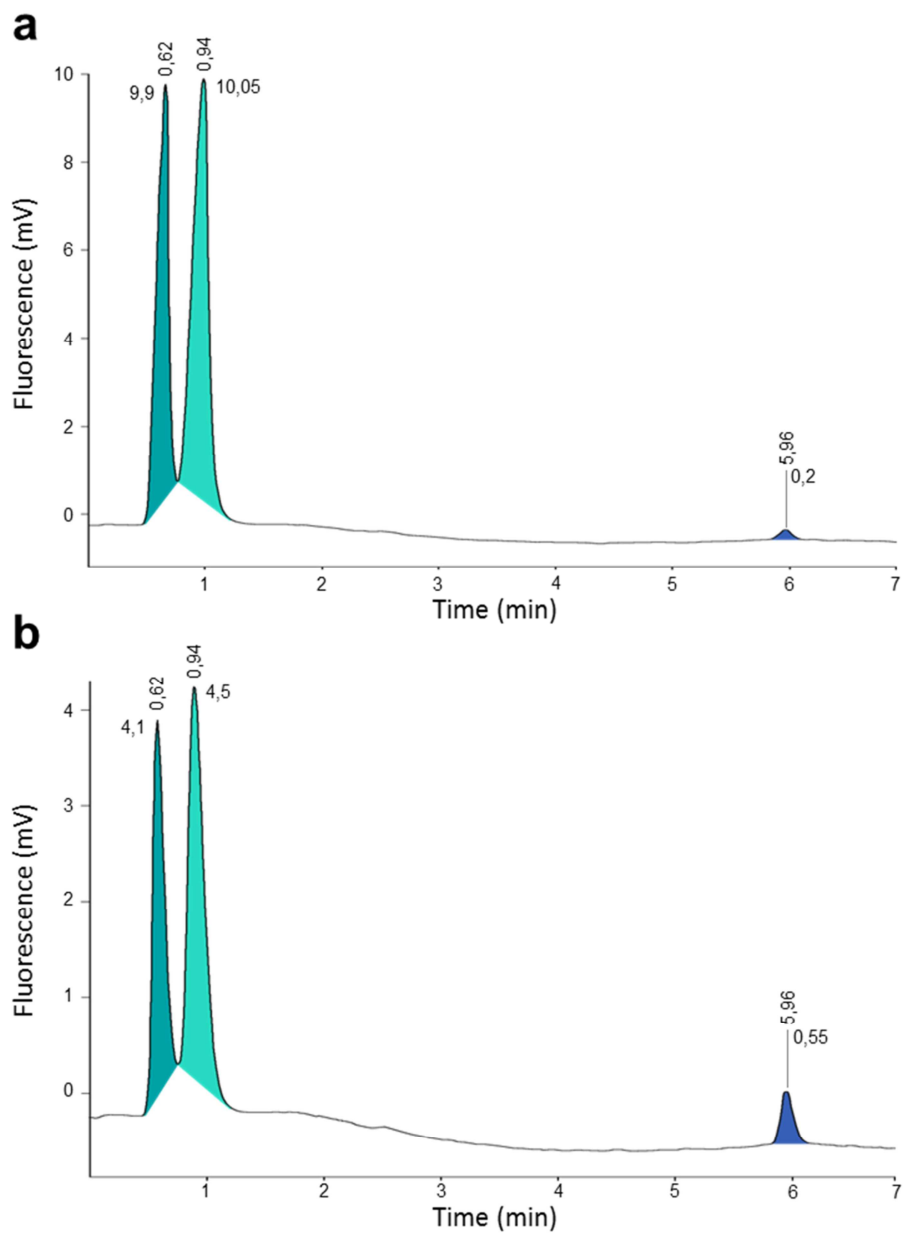


Fig. 6 Reversed-phase HPLC spectra of fluorescent compounds present in *E. spinax* skin with (a) and without (b) photophores, at 370 nm of excitation and 450 nm of emission.

can conclude that many peaks probably contain more than one molecule and that the HPLC method is not sufficient to completely separate all fluorescent molecules. Indeed, it seems unlikely for one molecule to have three or four fluorescent peaks, and even peaks that only appeared in two spectra might cover two or more molecules. Even more so, the two first peaks, which showed a very short retention time (respectively 0.62 and 0.94 on average), probably contain all molecules that are not retained by the column and straight eluted.

Regarding differences between skin with and without photophores, two peaks (4.38 and 4.54 time of retention on average) are present in the first but not in the second, both appearing in the 280/330 and 280/660 spectra. Instead, a 4.41 peak appears in skin without photophore. Moreover, the two first retention peaks are higher in photophore samples, being at least double the intensity, until ten times or even twenty-one times higher regarding the second peak in the 280/330 spectra. This means that some fluorescent molecules are only present in photophores and not in the surrounding skin. Obviously, luciferin and derivatives are not the only photophore compounds to exhibit fluorescence.

Fluorophores are chemical compounds able to re-emit light upon light excitation. Among amino acids, three are known for their ultraviolet fluorescent capability, i.e. tryptophan, phenylalanine and tyrosine (Teale & Weber, 1957). Tryptophan is an important precursor of biochemical molecules, such as melatonin which is also fluorescent (Muñoz *et al.*, 2009) and, for recall, is one of the excitatory hormones of *E. spinax* luminescence and should consequently be present in photophores. Tryptophan is also precursor of the cypridinid luciferin which was proved to be *de novo* synthesized from this amino acid in the ostracod *Vargula hilgendorffii* (Oba *et al.*, 2002). In the same way, coelenterazine was shown to be *de novo* synthesized by the copepod *Metridia pacifica*, from two tyrosines and one phenylalanine (Oba *et al.*, 2009). Finally, dinoflagellate luciferin belongs to tetrapyrroles, i.e. another large family of fluorescent molecules (Nakamura *et al.*, 1989). All three luciferins and/or their derivatives present fluorescent capabilities and there is a good probability to observe the same characteristic in *E. spinax* luciferin. Melanin, whose precursors are phenylalanine and tyrosine, is also

fluorescent (Na *et al.*, 2001). This skin pigment is known in sharks to be involved in physiological color change (Gelsleichter, 2012) and in bioluminescence control *via* the iris-like structure in lantern shark photophores (Claes & Mallefet, 2010b; Renwart *et al.*, 2014b). Although melanosomes present in extracts are precipitated during centrifugation, and the supernatant is filtered before HPLC, we cannot rule out that some diluted melanin might be still present. Nucleobases represent another family of fluorescent compounds (Udenfriend & Zaltzman, 1962) and photophore reflectors are one of the structures build by these compounds. Reflectors are present in a lot of marine animal photophores (but also in various other structures such as eyes and scales), including *E. spinax* photophores as demonstrated in Renwart *et al.* (2014a). Purines have been shown to compose reflective material in most marine animals, and specifically guanine and, in a lesser extent, hypoxanthine most often compose fish reflective structures (Denton, 1970; Fishelson *et al.*, 2005). Following this observation, there is a good probability that *E. spinax* reflector contains purine crystals, although it still have to be demonstrated. Meanwhile, we have to consider that fluorescent reflective material might be diluted in our samples. The last structure of *E. spinax* photophores that possibly contributes to HPLC fluorescent peaks is the lens. Lenses are usually designed to focus light produced by photocytes, but might also be involved in filtering this light in order to change its wavelength, especially in counter-illuminating animal such as *E. spinax* (Denton *et al.*, 1985). Indeed, in these animals, the light produced by photophores requires to perfectly match the downwelling one, and filters have a predominantly role in the final wavelength tuning. Filters are composed of pigments that are in many cases porphyrins (such as in the teleost *Valenciennellus* or the cephalopod *Histioteuthis*) or other fluorescent pigments (such as in the teleost *Chauliodus*). Porphyrins are known as fluorophores emitting in the red range (Denton *et al.*, 1985), and considering that *E. spinax* lens might also have a filtering purpose, these molecules could account for a part of peaks we observed in 660 nm range of emission.

Although we cannot attest that all these fluorescent molecules were efficiently extracted by our method, peaks observed in skin with photophores that were absent or of lower intensity in skin without photophore represent “photophore fluorescent molecules” and are

therefore luciferin good candidates. The two first peaks of spectra (respectively at 0.62 and 0.94 time of retention) accounted for the major part of these “photophore fluorescent molecules”, as well as, in a lesser extent, the two peaks which were never observed in skin without photophores (respectively at 4.38 and 4.54 time of retention). The main difficulty encountered at that stage was to investigate the two first peaks. Indeed, both of them are not or very poorly retained by the column and probably contain a lot of molecules. Two reasons might explain that these compounds are not retained by the Altantis T3 column: firstly, big molecules, such as fluorescent proteins, cannot enter the pores of the stationary phase with which they are therefore unable to make covalent bound. They are consequently eluted first. If *E. spinax* luminous system is based on a photoprotein containing a fluorophore, it might be present in one of these peaks. Secondly, very polar molecules are not retained by the column as this latest is hydrophobic. Moreover, Altantis T3 column requires to work in acidic conditions (pH ~ 3) and some molecules (for instance, if containing an amine group such as cypridinid luciferin) might consequently be protonated and straight eluted. To get rid of this possible protonation, we tried to work in basic media by using an XBridge C₁₈ column (Waters) allowing working at pH 10 (data not shown). Yet, we still had two large peaks (or sometimes three or only one) straight eluted and showing a higher intensity in skin with photophores, meaning that a majority of “photophore fluorescent molecules” were still not retained by the column. This could be explained by the high pH that, on the opposite to low pH, deprotonates some molecules. The first peaks might be composed of different molecules than when using Atlantis T3 column, or the same ones if they possess both protonable and deprotonable groups.

High resolution mass spectrometry

Two sample types (i.e. the fluorescent spots scrubbed from the TLC foil after migration, and the fluorescent peaks collected from HPLC after elution) were used in HRMS. Regarding samples coming from TLC, we were unable to obtain reliable results mainly because of a very high contamination of our methanol diluted sample with molecules coming from the silica of the foil. Even when subtracting a blanc of silica from the sample spectra, no reliable peak was observed, what is disconcerting

since the sample should have contained almost all fluorescent molecules. Experiments were therefore stopped at this stage.

HRMSs were run for three HPLC peaks collected after elution, i.e. peaks at 0.62, 0.94 and 2.27 time of retention. As a general conclusion, we judged our results (data not shown) to not be reliable enough since (i) spectra obtained were very close to the device background and it seemed impossible to be certain that HRMS peaks came from our sample and not from previously analyzed samples; and (ii) we failed to obtain a good repetition of these data when using another shark specimen or event with the same one. For these reasons, no comparison with skin without photophore was made. Moreover, not a lot of peaks were observed on HRMS spectra, what could be surprising knowing that analyzed samples are supposed to contain a lot of molecules. Indeed, both peaks at 0.62 and 0.94 time of retention probably contain all molecules non-retained by the HPLC column, and all of them (not only the fluorescent ones) are analyzed in HRMS. Nevertheless, in mass spectrometry, peak height and number do not reflect molecules abundance but their capacity to be ionized. If a very abundant molecule doesn't have a good ionization potential, it will not be seen on HRMS spectrum. Since we don't have any idea of shark luciferin ionization potential, it is impossible to be certain of its presence on our spectra. Consequently, we considered HRMS should not be used at that stage of purification, but rather at the end of the process with an almost purified sample containing the molecule of interest.

Conclusion

Experiments performed in order to characterize fluorescent compounds of a shark skin extract gave us very useful information in that there is a lot of fluorescent molecules present in *E. spinax* photophore, among which luciferin and/or its derivatives might be present. The main difficulty encountered during this preliminary purification process was to investigate molecules present in the HPLC peaks straight eluted. Indeed, they cover most of "photophore fluorescent molecules" and are therefore of great interest. In normal phase, these molecules did not migrate and stayed bound to the stationary phase, while in reversed-phase, they are

straight eluted whatever the pH used. To try solving this problem, other chromatography technics should be attempted, such as hydrophilic interaction liquid chromatography (HILIC), ions exchange chromatography or size-exclusion chromatography. This will be further discussed at the end of this work in a “perspective” paragraph.

PART 2

Is *Etmopterus spinax* luminous system based
on an ancient ROS detoxifying mechanism?
The peroxidase hypothesis

**IS *ETMOPTERUS SPINAX* LUMINOUS SYSTEM BASED ON
AN ANCIENT ROS DETOXIFYING MECHANISM?
THE PEROXIDASE HYPOTHESIS**

Context

Two uncommon features distinguish *Etmopterus spinax* luminescence from most other luminescent organisms: its relative low intensity and its long lasting production. While many luminous animals emit bright luminescence visible to the eyes even when not in complete darkness (such as the brittle star *Ophiopsila californica*, pers. obs.), seeing *E. spinax* luminescence requires observing freshly captured specimens in complete darkness. In these conditions, shark luminescence becomes gradually visible as eyes become dark adapted (within a few minutes, pers. obs.). *E. spinax* is also one of the rare animals to emit a luminescence that lasts at least several hours (pers. obs.). Only bacteria (and therefore fishes in symbiosis with bacteria) are known to emit a continuous light. Some fishes (such as *Porichthys notatus* – Greene, 1899) have been recorded producing light during several minutes to one hour, but many luminescent animals produce rapid flashes or no more than several seconds lasting light. These two particular *E. spinax* luminescence features led to a new hypothesis on its mechanism, based on an “adventitious biological chemiluminescence”.

Three to 2.5 billion years ago, the first oxygen-producing organisms appeared and started to increase environmental oxygen *via* photosynthesis. As a result, other unicellular organisms had to cope with the damaging effect of this molecule and its chemically reactive derivatives, known as reactive oxygen species (ROS). ROS are formed when oxygen reacts with cellular components and, in significant concentration, can cause damages to the cell. They mainly include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\text{OH}^\bullet$), singlet oxygen (O_2^*) and peroxynitrite (ONOO^-). To face ROS, organisms developed two defense strategies: either preventing intracellular oxygen to increase thanks to oxygen consuming pathways; or, on the opposite, allowing both external and internal O_2 pressure to equilibrate and detoxifying resulting ROS. The first mechanism is

believed to have been firstly used by unicellular organisms, and might have generated the first bioluminescent reactions. However, the continuous increase of environmental oxygen probably made them unable to prevent oxygen accumulation in their cells, and the second mechanism was therefore adopted (Timmins *et al.*, 2001). ROS detoxifying mechanisms include superoxide dismutase, catalase and peroxidases, acting with various antioxidants such as glutathione and ascorbic acid. In ocean, oxidative stress varies with depth and consequently so does the need of a highly effective ROS detoxifying system. Animals inhabiting the upper part of the water column deal with a higher ROS concentration than the ones living in deeper layers. Indeed, in deep-ocean, the lower light irradiance and the reduced oxygen level substantially decrease the oxidative stress undergone by organisms. Superoxide anion (O_2^-) and its predominant decay product, hydrogen peroxide (H_2O_2), also show a decreasing concentration with increasing depth, mainly because the first one is photochemically formed at ocean surface. Finally, the reduced metabolism often adopted by deep-sea organisms probably also restricts their ROS production (Rees *et al.*, 1998). Consequently, when organisms colonized the still empty deep-sea niche, such as Etmopteridae 90 to 65 MYA, they faced a reduced oxidative stress and had the opportunity to harness their ROS detoxifying mechanisms to other purposes, such as bioluminescence. Following this theory, bioluminescence evolution is an exaptation, i. e. the current use of a character in a particular function which was not the function for which the character was originally shaped (Gould & Vrba, 1982). This hypothesis is based on the observation that coelenterazine, the most widespread luciferin, has great antioxidant properties since it has been shown to successfully replace other antioxidants, such as α -tocopherol, in ROS detoxifying reactions with the same if no more efficiency (Dubuisson *et al.*, 2000,2005). Because this molecule occurs in non-sister taxa, in various luminescent systems but also in non-luminous organisms (with some of them being able to store it in their tissues), coelenterazine was hypothesized to have previously played a role in ROS defense pathways of marine organisms (Shimomura *et al.*, 1980; Shimomura, 1987; Rees *et al.*, 1998). All cells express very low chemiluminescence as a by-product of various oxidative reactions, and luciferins have such endogenous chemiluminescent properties. In low oxidative stress conditions, this ultraweak light production might have

turned from a by-product to the main purpose of the reaction. The numerous ecological advantages of producing light created a selective pressure on such mechanisms, in which a more effective yield, intensity, kinetics or spectrum were selected, leading to the various luminous systems known today (Slawinska & Slawinski, 1983; Rees *et al.*, 1998). This has been defined as secondary adaptation, occurring after primary exaptation (Gould & Vrba, 1982). Although this hypothesis is still in debate and not demonstrated yet, this is the most cited theory to explain the numerous appearances of bioluminescence in the course of evolution, predominantly in deep-oceans.

Various reactions of the ROS detoxifying system generate *ultraweak luminescence*, sometimes also called *adventitious biological chemiluminescence* (Cadenas & Sies, 1984; Murphy & Sies, 1990; Vladimirov & Proskurnina, 2009). Some luminous animals provided supplementary clues of the relationship between current bioluminescent mechanisms and detoxifying mechanisms: some annelids require a ROS instead of oxygen to emit light (e.g. hydrogen peroxide [H₂O₂] in *Diplocardia* or superoxide [O₂⁻] in *Harmothoë*), the hemichordate *Balanoglossus* also needs hydrogen peroxide and the luminescence of the bivalve *Pholas* is enhanced either by hydrogen peroxide or superoxide. Moreover, luciferases from *Diplocardia*, *Pholas* and *Balanoglossus* showed peroxidase activity (Cormier & Dure, 1963; Dure & Cormier, 1963; Henry & Michelson, 1970; Bellisario *et al.*, 1972; Henry *et al.*, 1975; Nicolas *et al.*, 1982; Rees *et al.*, 1998; Shimomura, 2006). Because *E. spinax* bioluminescence main role is to continuously counter-illuminate at depths where very few light remains, the emission necessarily has to be low (*In vivo* maximal emission of a 1 cm² piece of skin with photophores vary from 10² to 10⁴ mega quanta per second, what correspond to their environment downwelling light – Claes *et al.*, 2010a). If the shark's luminous system is based on an ancient ROS detoxifying mechanism, the original chemiluminescent reaction might not have evolved to a highly effective luciferin/luciferase system exhibiting a high light intensity, as observed in many other luminous organisms able to emit bright flashes of light. In this section, preliminary experiments are conducted in order to test the hypothesis of *E. spinax* luciferase to be a peroxidase, and its luminous system to be related to an ancient ROS detoxifying pathway. In a first experiment, peroxidases from skin extracts

were separated by size-exclusion HPLC and assayed in various collected peaks. In a second experiment, azide and cyanide were injected on fresh skin patches with photophores in order to test their quenching effect on *in vivo* light production, since both components are known to inhibit peroxidases.

This study has been carried out with the collaboration of the Department of Biochemistry and Microbiology from the Rutgers University (USA), thanks to facilities provided by Prof. William Ward, and with the collaboration of Cyril Hammoud (MSc student).

Materials and method

Shark tissues collection and photophore extracts

Specimens of *E. spinax* were sampled during three scientific field missions (February and November 2012 and March 2013) in a Norwegian fjord (60°15.908N; 05°07.778O) by lowered longlines. Fresh skin patches with (from animal's ventral side) and without (from animal's dorsal side) photophores were dissected from killed sharks and either frozen at -80 °C until extraction in the laboratory of Prof. William Ward at Rutgers University, or straight used in luminometry experiments (i.e. no extraction was performed) in the Espegrend Marine Biological Station (Bergen, Norway). Extracts were done as follow: pieces of frozen skin were cut in very thin slices and crushed in a volume of buffer (50 mM Na₂HPO₄; 0.1 M NaCl; pH 6.7) of fivefold their weight. Extract was centrifuged at 16,000 g during 15 minutes and supernatant was collected and checked for its peroxidase content, using ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]): 10 µl of supernatant were mixed with 200 µl of a solution containing ABTS conjugated with hydrogen peroxide. If peroxidases were present in the supernatant, they oxidized H₂O₂, what produced singlet oxygen molecules that, in their turn, oxidized ABTS which consequently turned blue. This experiment therefore constitutes a rapid and visual peroxidases assay. Remaining supernatant was used in size-exclusion HPLC in Prof. Ward laboratory.

Size-exclusion HPLC

Size-exclusion HPLCs were performed on a Millipore Waters 600 Multisolvant delivery system (Waters) and molecules were detected with a Spectro Monitor 3200 (LDC Analytical). The column used was a BioSep-SEC-S 2000 (Phenomenex) allowing separation of molecules by size if smaller than 200.000 Da. Mobile phase was the same buffer as used for extraction (Na_2HPO_4 50 mM; NaCl 0.1 M; pH 6.7), and elution was isocratic and lasted 20 minutes. All peaks were collected just after elution and analyzed for their peroxidase content.

Peroxidase assay

Peroxidase detection in fractions collected from the size-exclusion HPLC was not performed with ABTS because fractions were too diluted to allow detecting the ABTS blue signal by naked eyes. Instead, peroxidases were detected *via* the chemiluminescent reaction they produce when mixed with luminol and hydrogen peroxide: when peroxidases oxidized H_2O_2 , resulting singlet oxygen molecules oxidized luminol, which emits light as a result. Ten μl of each fraction were mixed with 90 μl of a solution containing both luminol and H_2O_2 (TrisHCl 1 M; Ethanol 10 %; Luminol 7.5 mM; H_2O_2 7.5 mM; pH 8.3) and the light was recorded in a multiplate luminometer (Wallac Victor 2, Perkin Elmer). Comparisons were made between skin with and without photophores and experiments were repeated on the three shark specimens.

Luminometry

The effect of two peroxidase inhibitors (sodium azide [NaN_3] and potassium cyanide [KCN]) was tested on light induced by two excitatory hormones (melatonin and prolactin). Four fresh skin patches with photophores (i.e. no extraction was performed) were immersed in the four corresponding solutions ([i] 15 mM NaN_3 ; 1 μM melatonin or [ii] prolactin in a buffered shark saline [Murphy & Sies, 1990; Bernal *et al.*, 2005]; [iii] 15 mM KCN; 1 μM melatonin or [iv] prolactin in a buffered shark saline) and two skin patches were immersed in two control solutions (1 μM melatonin or prolactin in a buffered shark saline) in which no inhibitor was added. Light was recorded during one hour in a

multiplate luminometer (Orion, Berthold Detection System, Pforzheim, Germany). The experiment was repeated on three shark specimens.

Results and discussion



Fig. 1 Peroxidase visual assay in extracts from skin with (a) and without (b) photophores, using ABTS-H₂O₂ protocol.

Shark skin peroxidases were extracted and separated from other molecules using size-exclusion HPLC, and finally assayed in order to compare their abundance in skin with and without photophores. After extraction, supernatants showed a blue color when mixed with ABTS-H₂O₂ (**Fig. 1**), meaning that peroxidases are present in both skin with and without photophores, what seems normal for such ubiquitous molecules. The size-exclusion HPLC showed a good separation of molecules and the same nine peaks were found in skin with (**Fig. 2a**) and without (**Fig. 2b**) photophores. The first peak of both spectra represent molecules bigger than 200.000 Da that are not retained by the column. Spectra show that peaks 4 and 5 are slightly higher in skin without photophores, but this result was not repeatable on the two other specimens studied (data not shown). Such small differences can be due to the extraction process since it was not automated but performed by the experimenter (the more the tissue is crushed, the more molecules can be extracted). More replicates would thus be needed in order to highlight differences in molecules relative abundance.

The nine fractions corresponding to the nine peaks were collected and assayed for their peroxidases content. The fourth peak contained the majority of peroxidases, in both skin with and without photophores (**Fig. 3**), although peaks 1, 2, 3 and 5 might also contain a small peroxidases amount. Same results were found for the two other specimens tested (data not shown). The relative light intensity, which is considered to be proportional to peroxidases abundance, is higher in skin without photophore in peak 4, at least for the specimen assayed in the **figure 3**. Indeed, **table 1**, which presents the relative light intensity

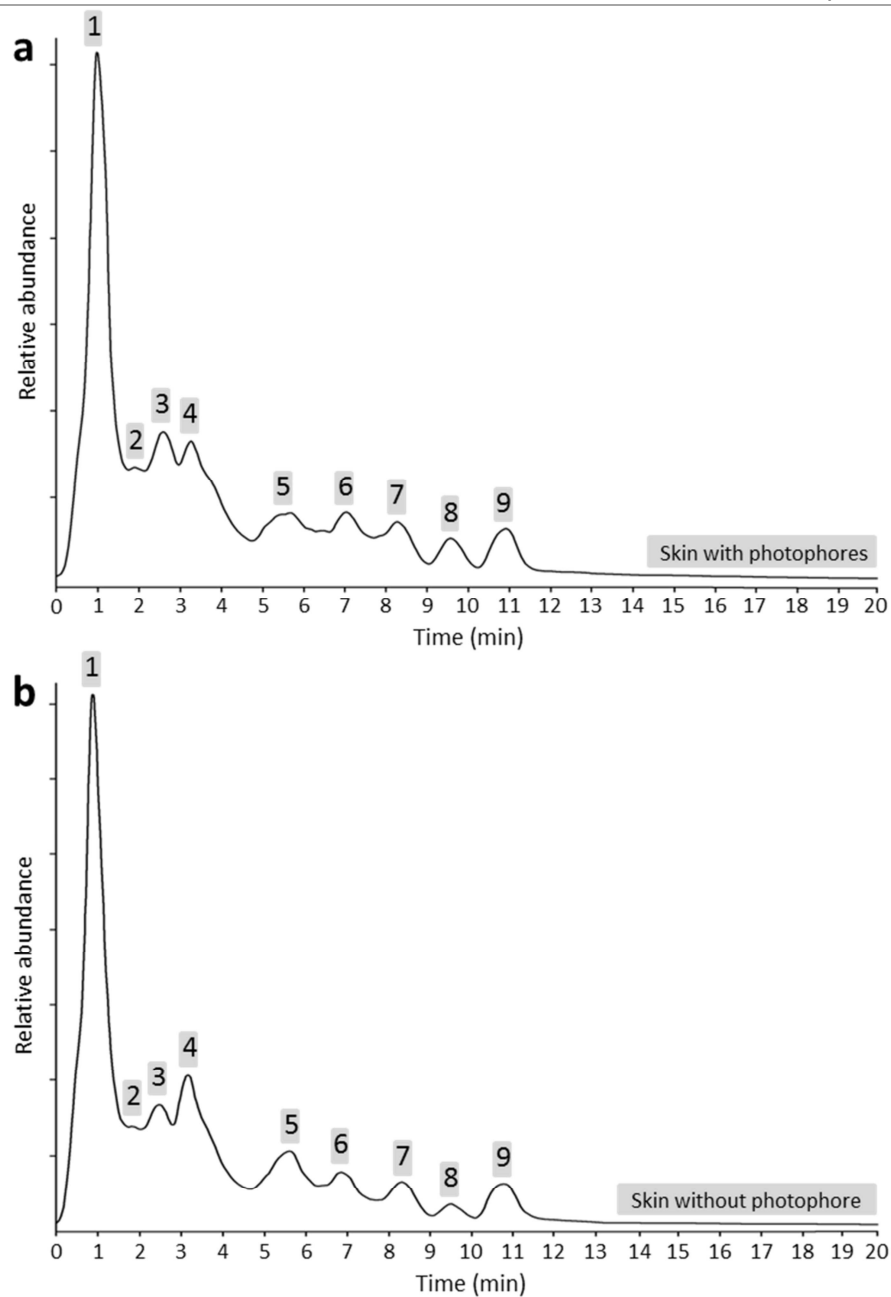


Fig. 2 Size-exclusion HPLC spectra of compounds present in *E. spinax* skin with (a) and without (b) photophores. All peaks are numbered, from 1 to 9.

obtained for the fourth peak in two more specimens, shows that this is not the rule, since the third specimen has a higher peroxidases content (almost double) in skin with photophores. However, great variations occur between specimens and results don't have a good repeatability (**table 1**), despite all exactions were standardized to skin patch weight. Once again, a bias can come from the extraction process and a mean to standardize it should be found to improve results.

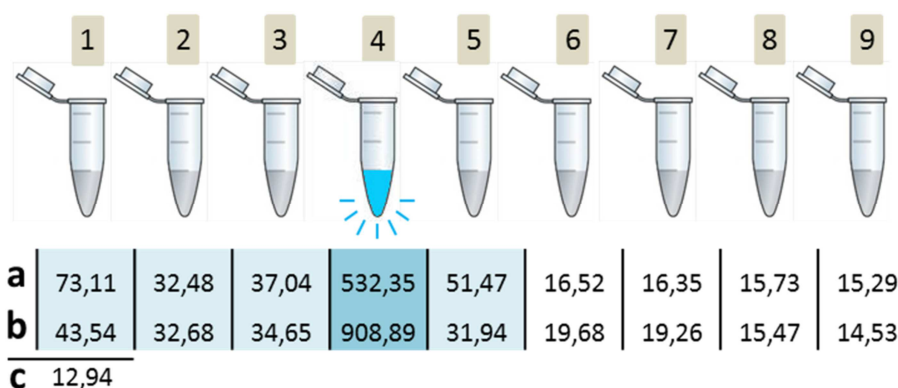


Fig. 3 Peroxidase assays in the nine fractions collected from size-exclusion HPLC. Results are presented in relative light intensity in skin with (**a**) and without (**b**) photophores. The (**c**) value represents a control containing H_2O_2 and luminol only.

Table 1 Peroxidase abundance (based on relative light intensity) in the fourth fraction for the three specimens assayed

	Skin with photophores	Skin without photophore
Specimen 1	532,35	908,89
Specimen 2	334,58	702,76
Specimen 3	1489,83	757,98

Determining peroxidases occurrence between skin with and without photophores would require to go further in the separation process and to perform HPLC on the fourth fraction, in order to see all peroxidases it contains. Experiments should be performed on several specimens and extractions have to be improved, in order to avoid great variations between specimens. Finally, although the fourth fraction contains the majority of peroxidases, the other ones should be

investigated too, especially the first fraction which might contain peroxidases bigger than 200.000 Da. Indeed, *Diplocardia* and *Pholas* luciferases, which are peroxidases composed of several sub-units, respectively are 200.000 and 310.000 Da (Bellisario *et al.*, 1972; Henry *et al.*, 1975).

Another clue of peroxidase involvement in a luminous system is the light quenching by cyanide and azide, since both components are known to bind with peroxidases heme group (Dure & Cormier, 1963; Bellisario *et al.*, 1972). We therefore investigated the effect of cyanide and azide on *E. spinax in vivo* luminescence. In its attempt to prove the balanoglossid luciferase to be a peroxidase, Dure and Cormier (1963) used the same inhibitors but worked with a luciferin/luciferase mixture extracted from animals. Because luminescence of *E. spinax* photophore extract decay to zero within a few minutes (regardless of the liquid used for extraction: water, shark saline or any buffer) and could not be restored, it was impossible to work in the same conditions here. The only known mean to activate *E. spinax* luminescence during minutes to several hours is to inject excitatory hormones on physiologically active patches of skin with photophores, what has been done in this experiment. It might be seen from the **figure 4** that both inhibitors induced a strong inhibition of the light produced by the two hormones (especially for prolactin) compared to the control curves. These results constitute a clue that light production in *E. spinax* might involve a peroxidase, but they should nevertheless be interpreted with extreme caution. Indeed, since we worked on physiologically active skin patches, and not on luminescent extracts, it is impossible to attest that both inhibitors acted on the light-producing reaction. Because azide and cyanide have many effects on various biological processes (i.e. they disrupt respiratory chain by inhibiting cytochrome C oxidase), they might have quenched luminescence *via* another pathway. For instance, inhibition might have occurred on the intracellular pathway triggered by melatonin and prolactin, but not on the chemiluminescent reaction itself. Inhibition might even have occurred at another photophore level than photocytes. One could assume that azide and cyanide acted on the iris like structure by closing it. However, this possibility seems unlikely since no variation of the iris-like structure aperture was visually observed in azide/melatonin stimulated skin patches, as attested by the **figure 5**.

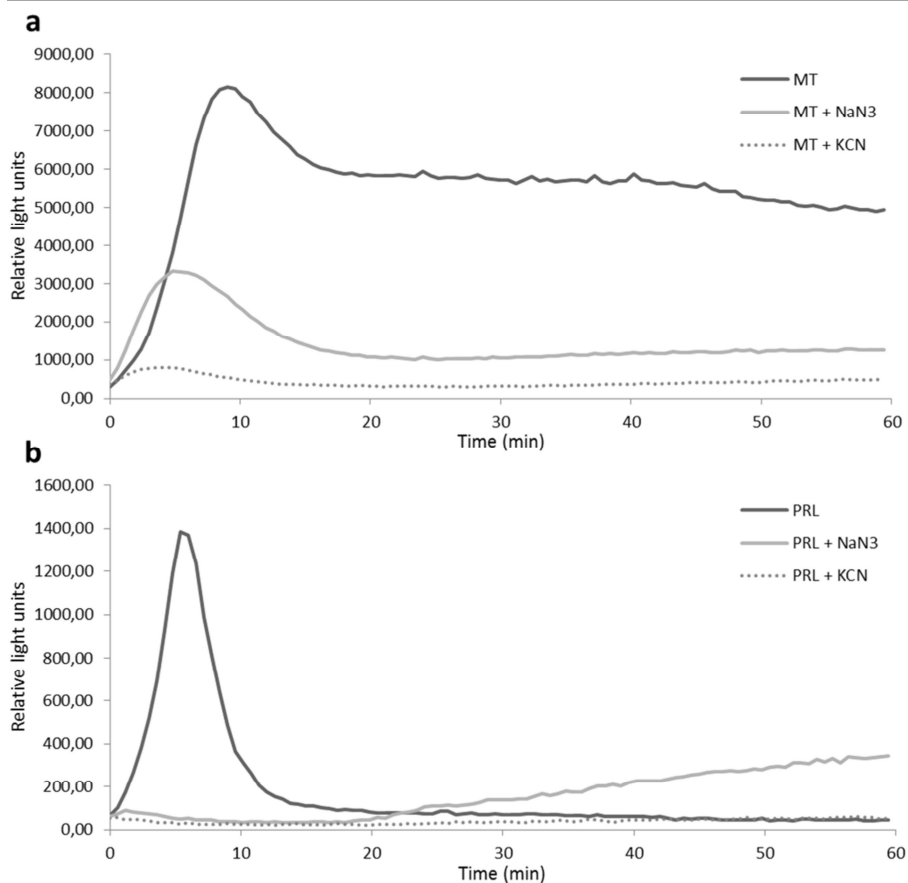


Fig. 4 Inhibition action of azide (NaN_3) and cyanide (KCN) on light produced by two excitatory hormones, melatonin (MT) (a) and prolactin (PRL) (b).

If *E. spinax* luciferase is a peroxidase, several assays can be tried in order to corroborate the inhibitory effect of azide and cyanide on the chemiluminescent reaction. Both compounds have metal-binding properties, the reason why they inhibit various peroxidases. It represents one of the pathway inhibitors can use to quench *E. spinax* luminescence and, to support this hypothesis, various other metal-binding agents can be tested for their quenching effect: 1,10-phenanthroline, sodium diethyldithiocarbamate or sodium oxalate (Bellisario *et al.*, 1972). Moreover, during peroxidation reactions, singlet oxygen molecules are produced which are known to be quenched by azide. By using other singlet oxygen trapping compounds, such as hydroquinone, tocopherol

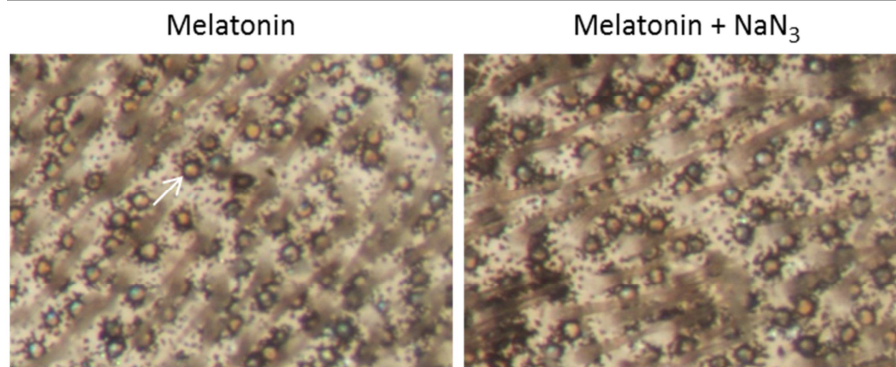


Fig 5 Iris-like structure aperture in a melatonin stimulated skin patch (**left**) compared to a melatonin + azide (NaN_3) stimulated skin patch (**right**). All black rings (*arrow*) are opened photophores. Pictures: J. Mallefet.

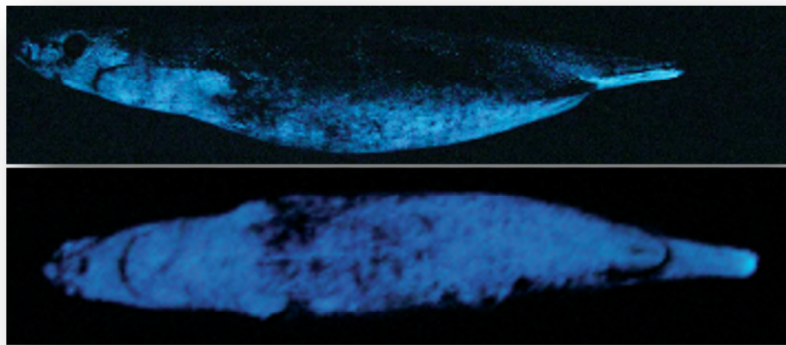
and β -carotene, light inhibition should also be observed (Murphy & Sies, 1990). This experiment was performed using various antioxidants (L-ascorbic acid [0.5 mM]; α -tocopherol [0.5 mM]; β -carotene [5 μM]; Glutathione [0.5 mM]) by simply immersing skin patches in corresponding solutions as we did with azide and cyanide, but not quenching effect was observed (data not shown). Nevertheless, it supposes antioxidant molecules to have reached the light-producing site (i.e. the glowons of the photocytes granular area), what cannot be proved here. These negative results thus have to be interpreted with caution since they might not reflect the reality. Other widespread final acceptors in peroxidase reactions are catechol, indoleacetic acid and pyrogallol. They might inhibit *E. spinax* luminescence, if related to a peroxidation reaction, by competing with luciferin (note that in such a reaction, the specificity of the luciferase for its luciferin is much less strong than what is observed in classical luciferin/luciferase system [Dure & Cormier, 1963]). Once again, this is true considering substrates can reach the light-producing site, what we cannot be sure. Nevertheless, these molecules, as well as luminol we previously used for peroxidase detection, should be tested for their quenching effect. Conversely, the commercially available horseradish peroxidase is sometimes used to activate luminescence instead of luciferase. Such experiment has been assayed with success in *Balanoglossus* and *Diplocardia* luciferase characterization, by mixing the horseradish peroxidase with extracted

luciferin and H_2O_2 (Dure & Cormier, 1963; Bellisario *et al.*, 1972). It was attempted on frozen *E. spinax* photophore extracts but we failed to obtain light with this method (data not shown) and it would be interesting to try it again on fresh extracts. Finally, the addition of hydrogen peroxide, as the main peroxidase substrate, should have an activating effect. Such tests have been made on fresh photophore extracts and gave no light (data not shown). Nevertheless, Claes and Mallefet (2008) used H_2O_2 injections on fresh non-extracted skin patches and successfully triggered luminescence, meaning that our extraction method might inhibit/disrupt the chemiluminescent reaction. Still, the role of H_2O_2 in *E. spinax* luminescence has to be clarified.

Conclusion

The hypothesis of *E. spinax* luciferase to be a peroxidase requires more experiments to be supported. While the light inhibition by azide and cyanide is in favor of this assumption, the result that no more peroxidases are present in skin with photophores than without photophore is puzzling. Nevertheless, peroxidases are widespread molecules and almost all of them were eluted together. Supplementary chromatography steps might be necessary in order to separate them and be able to see any difference between skin with and without photophore.

DISCUSSION AND PERSPECTIVES



Squaliolus aliae (Dalatiidae) *in vivo* bioluminescence (J. Mallefet)

DISCUSSION AND PERSPECTIVES

This work aims to investigate the fine structure and biochemistry of *Etmopterus spinax* bioluminescent system. The first chapter was devoted to photophore ultrastructural description and dynamics and allowed to identify shark microsource, which we named *glowon*. The second chapter gathered evidences suggesting *E. spinax* luminous system was not based on a known luciferin, but might rather involve a new luminous reaction. Finally, the third chapter provided basis experiments to characterize such an unknown luminous mechanism. All results have been discussed in respective sections and the purpose of this discussion is to integrate our data in order to interpret them all together, in the light of previous findings on shark luminescence control and functions. Perspectives for future works are also suggested.

On integrated model of luminescence control in E. spinax: connecting ultrastructure, functions and control

An original aspect of shark bioluminescence lies in its hormonal control, while most other known luminous organisms possess a nervous control. Nervous control allows very rapid changes in light emission and, on the opposite, hormones involve slow onset of luminescence. In *E. spinax*, both melatonin (MT) and prolactin (PRL) have a light-stimulatory effect, while α -melanocyte-stimulating hormone (α -MSH) has an inhibitory effect. Because MT and PRL show different light pattern and because simultaneous injection of both hormones has an additive effect on light intensity, they are believed to use different pathways to induce luminescence (Claes & Mallefet, 2009b, Claes *et al.*, 2011a). We found they also generate different ultrastructural changes inside the photophore (Renwart *et al.*, 2014b), which brings further evidence in accordance with the hypothesis of a differential control mechanism.

MT main action consists in triggering the iris-like structure (ILS) opening, when binding to its receptor (MT₂). MT₂ is coupled with an inhibitive protein G which prevents cAMP production by adenylyl cyclase (Claes & Mallefet, 2009b). The decrease in cAMP induces the complete

retraction of melanosomes from photophore top and center *via* a still unknown intracellular signal transduction (Claes & Mallefet, 2010b; Renwart *et al.*, 2014b). This observation can be correlated with MT light curve generated in luminometry: when MT reaches melanophores, light intensity increases as ILS progressively opens. ILS reaches its maximum diameter after approximately 45 minutes, when a plateau is observed on the MT light curve. The light produced by photocytes can thereafter pass through ILS during several hours.

When PRL binds to its receptor (PRLr), the Janus kinase 2 to which PRLr is coupled triggers the intracellular signal transduction (Claes & Mallefet, 2009b). As for MT, signal transduction also induces ILS opening (Claes & Mallefet, 2010b) but not all melanosomes are removed from the photophore center (pers. obs. on TEM micrographs). Instead, photocytes seem to be PRL main action site, since the hormone was found to induce glowons morphological changes and to generate new particles appearance (the grey particles), proportionally to the emitted light (Renwart *et al.*, 2014b).

Assuming MT and PRL to take different pathways to trigger light emission and to mainly target different cell types (respectively melanophores and photocytes), they also can be hypothesized to act during different luminescence-related behaviors, as already proposed by Claes & Mallefet, 2009b. Counterillumination is the predominant luminescence function in *E. spinax*, and requires a continuous light production from photophores. Accordingly, we can postulate photocytes to emit light continuously (Claes *et al.*, 2011a) and counterillumination intensity to be mediated by ILS, *via* MT. This is consistent with the long lasting kinetics of MT light curve. Moreover, MT is known to be released by pineal gland (Underwood, 1989), which is involved in the collection of downwelling light information. This hormone therefore appears as a suitable candidate to regulate counterillumination (Claes & Mallefet, 2009b). How a continuous light production might be achieved by photophores is still unknown (and requires to understand the chemiluminescent reaction) but, as previously suggested by Claes & Mallefet (2010b), might also involve MT since the hormone was found to induce glowons morphological changes in addition to ILS aperture (Renwart *et al.*, 2014b).

Because of the very slow kinetics of counterillumination regulation in sharks, it is impossible for them to adapt their light intensity within a few minutes as do counterilluminating teleosts endowed with a nervous control. To overcome this situation, sharks rather go up and down in the water column to stay at iso-luminance depth, as recently demonstrated by Claes *et al.* (2014).

PRL displays a more rapid kinetics than MT since its light curve reaches a peak after 20 to 30 minutes and lasts approximately one hour. Consequently, it has been postulated that PRL might be released in situations requiring quicker on/off of light emission, such as in intraspecific communication events (cohesive swimming or hunting and sexual signaling –Claes & Mallefet, 2009a), when being seen by conspecifics becomes a priority on counterillumination. Indeed, Claes & Mallefet (2010a) found sexual differences in PRL-induced luminescence for three luminous areas surrounding cloaca, including the pelvic area which is also differently shaped in males and females. Another interesting clue lies in *Squaliolus aliae* luminescence control: in this dalatiid shark, PRL has an inhibitory effect on light emission (Claes *et al.*, 2012), on the contrary to *E. spinax* and *E. splendidus* (from the etmopterid family) in which a stimulating effect is observed (Claes & Mallefet, 2009b; Claes *et al.*, 2011b). Since dalatiids lack a complex luminous pattern but rather show a homogeneous photophores distribution on ventral and lateral sides, *S. aliae* luminescence is believed to be only devoted to counterillumination. Hence, this species might not had to develop a stimulating effect of PRL, assuming this role is related to intraspecific communication.

In addition to hormonal regulation, a neuronal modulatory effect was highlighted in *E. spinax* luminescence control. γ -Aminobutyric acid (GABA) was shown to inhibit light induced by MT and PRL, probably by closing the ILS since (i) GABA immunoreactivity was located at ILS level but not inside photocytes, and (ii) injection of bicuculline (a GABA_A receptor antagonist) induces ILS aperture by pigments retraction (Claes *et al.*, 2011a). Nitric oxide (NO) was found to have a modulatory effect on light induced by MT and PRL but, on the opposite to GABA, seemed to only target photocytes since NO synthase immunoreactivity was only found inside this cell type (Claes *et al.*, 2010b). Interestingly, such as

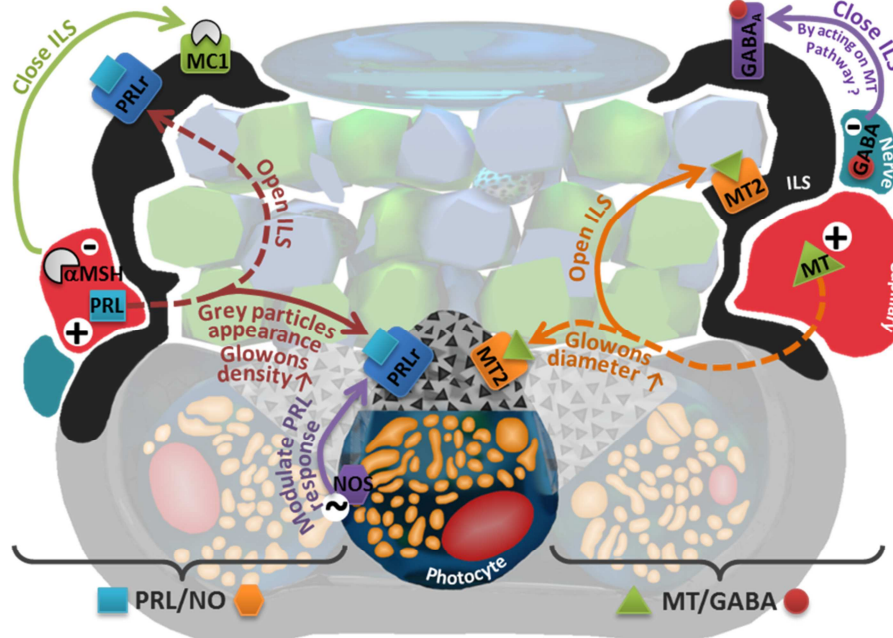


Fig. 1 Proposed model for luminescence control mechanism in relation with ultrastructure. **Left** Coupled action of prolactin (PRL) and nitric oxide (NO, via NO synthase - NOS): PRL main action (solid arrow) is located at photocycle level (where it induces grey particles appearance and glowons density increase) and is mediated by NO. Another role of PRL (dashed arrow) is to open iris-like structure (ILS). On the contrary, α -melanocyte-stimulating hormone (α -MSH) closes ILS. **Right** Coupled action of melatonin (MT) and γ -aminobutyric acid (GABA): MT main action (solid arrow) is to open ILS, but it also has an action at photocycle level (dashed arrow), where it induces glowons diameter increase. GABA main action is to close ILS, possibly by acting on MT pathway. The symbols (+) and (-) respectively indicate a triggering or an inhibitive action of molecules on light emission, while symbol (~) indicates a modulatory action. MC1 melanocortin receptor 1; MT2 melatonin receptor 2; PRLr prolactin receptor; GABA_A GABA receptor A.

PRL showed a different effect on light emission in *S. aliae*, NO doesn't have any effect on its luminescence. Consistent with these clues, we can hypothesize NO to mainly modulate PRL response (Claes *et al.*, 2012).

In conclusion, by integrating all data, we can hypothesize (i) counterillumination in *E. spinax* to be primarily controlled by the pair MT/GABA, of which the target is the ILS, together with an inhibitory action of α -MSH; (ii) intraspecific communication to be primarily

controlled by the pair PRL/NO, although MT/GABA might also act in the background during communication events in order to regulate ILS aperture (**Fig. 1**). Moreover, NO also has an impact on MT-induced light emission (Claes *et al.*, 2010b), either inside photocytes (where MT probably also plays a role) or at the ILS level via an intercellular metabolic pathway. To corroborate this hypothesis, it is needed to precisely locate the cellular types targeted by both MT and PRL, by locating their respective receptors: PRLr and MT₂ (Claes & Mallefet, 2009b). Also, it would be interesting to investigate the ultrastructural effect of a simultaneous injection of MT and PRL, and check whether a morphological additive effect is observed (i.e. complete pigment retraction and grey particles appearance), simultaneously to what is observed in luminometry (Claes & Mallefet, 2009b).

Perspective: precisely locate MT and PRL receptors inside photophore by using immunogold labelling.

Perspective: perform a simultaneous injection of MT and PRL in order to check whether an ultrastructural additive effect is observed.

On glowons chemical nature and the next steps in E. spinax luminous system purification

Glowons, the cytoplasmic granules found in photocyte granular area, have been proved to be the intracellular sites of bioluminescence (i.e. microsources) in *E. spinax* (Renwart *et al.*, 2014b). On the opposite to most other known microsources, glowons are not surrounded by a membrane and therefore do not appear as vesicles but rather look like concretions of material which are truly parts of the cytoplasm. The squid *Watasenia scintillans* is one of the rare other luminous organism in which microsources are not vesicles, but rod-like micro-crystals (Hamanaka *et al.*, 2011). In the same way as they did, studying the chemical nature of glowons will certainly create new trails to elucidate *E. spinax* luminescent system.

The major difficulty encountered during *E. spinax* luminous system characterization was the impossibility to trigger a cross-reaction between shark luciferin and luciferase or between a putative

photoprotein and its cofactor (see **appendices**). It has to be pointed out that the photophore ultrastructural study was performed after the initiation of the luminous system purification, implying that we did not have any idea of glowon morphology when we started to purify the putative shark luciferin. At that time, shark microsources were considered by default to be membranous organelles such as in most other organisms, and the luminous material was therefore considered to be extracted using classical solvents such as methanol or distilled water (Shimomura, 2006). Retrospectively, knowing shark microsources are actually concretions and not vesicles, it appears this methodology is possibly not suitable to extract and dissolve them. Therefore, the centrifugation step we performed (usually between 10,000 and 16,000 g) when assaying various cross-reactions as well as in all HPLC experiments (in order to avoid blocking the device), might have been sufficient to precipitate glowons, meaning luminous material was maybe not present in these cross-reaction/HPLC processes. Although this would invalidate all results from chapter 3, the methodology performed in this section remains nevertheless valid.

Before pursuing *E. spinax* luminous system purification, it is definitely needed to be certain that crude extract used in HPLC contains the (dissolved) glowons. Even more, the ideal preparation to work on would be a solution containing isolated glowons, what would provide the great advantage to get rid of all other photophore components.

Perspective: isolate glowons by differential centrifugation in order to work on a solution free from other photophore and skin components.

Isolating microsources to exclusively work on these structures was regularly done in the purification process of new luminous systems (DeSa *et al.*, 1963; Anderson & Cormier, 1973; Deheyn *et al.*, 2000; Hamanaka *et al.*, 2011). In most cases, however, microsources were bigger than glowons. In *W. scintillans*, which we consider to have the closest microsource morphology with *E. spinax*, rod-like crystals (from 2 to 6 μm long) were isolated by centrifugation through sucrose density gradient (a differential centrifugation technique). This method, or any alternative method allowing cellular structures to be separated (e.g. Percoll gradient), should be assayed on *E. spinax* photophore extract. Note that the caudal and pectoral areas of *E. spinax* luminous pattern

exclusively contain photophores in a very high density with no denticles in between. Although they represent the smallest luminous area, they might be suitable for glowons preparation. Between each differential centrifugation steps, the presence/absence of glowons should be checked using TEM, as this microscopy technique is the only one known to reveal these microsources. Moreover, one of the trickiest steps might be to separate glowons from melanosomes, as they display a close electron absorbance. All the process will probably necessitate an important adjustment phase but, if successful, would allow a great advance in *E. spinax* luminous system purification. Once glowons will have been isolated and concentrated, a mean to solubilize them will be required (this step is achieved on *W. scintillans* rod-like crystals by using sodium dodecyl sulfate -SDS- but irreversibly ceased the luciferase activity, Hamanaka *et al.*, 2011) and all the processes described in the first section of chapter 3 should be retried.

In the meanwhile, efforts to find an assay to trigger the light from a crude shark photophore extract by cross-reaction should be pursued, particularly by using alternative extraction solvents (such as SDS or mercaptoethanol), in order to dissolve glowons if methanol is not enough. All cross-reaction experiments should be performed on fresh skin, as the effect of freezing on luminous material is unknown.

Perspective: find an assay to trigger the light in *E. spinax* photophore crude extract by assaying various extraction solvents.

W. scintillans rod-like bodies were proved, thanks to X-ray diffraction, to be crystals of two different proteins (one being the luciferase) together with the luciferin, since ATP in addition to Mg^{++} triggered the light. Investigating the possible crystalloid nature of glowons might, in the same way, allow learning more on their chemical nature. This technique should be performed on a glowon preparation and will therefore only be possible if this first step is achieved. As in *W. scintillans*, we believe all components necessary to the chemiluminescent reaction to be present inside glowons (grey particles only appear in PRL-stimulated photocytes and probably do not represent a component of the reaction –Renwart *et al.*, 2014b), waiting for a proper stimulus to trigger light emission. As a consequence, there is a good

probability for the luciferin to be on a stabilized non-active form, maybe bound to a protein.

Perspective: investigate the crystalloid nature of glowons using X-ray diffraction, in order to learn more on their chemical nature.

On the chemiluminescent reaction features and the luminous material transfer through the food chain

The capability to emit a continuous luminescence is a rare phenomenon and bacteria (and fungi on earth) are believed to be the sole organisms able to do so (Hastings, 1978). Most luminous teleosts having an intrinsic light production emit a transitory luminescence which can last several minutes to one or two hour maximum but is not continuous *stricto sensu*. When a continuous emission was really valuable to fish, however, a symbiosis with bacteria was developed and was mainly controlled with removable shutters. Luminous sharks are, in our knowledge, the sole multicellular animal displaying such a long lasting luminescence (at least several hours, pers. obs.) by using an intrinsic system.

In the case of a long lasting light emission, all components required for chemiluminescent reaction must be available all the time and must therefore either be stored in a huge quantity or, more likely in our opinion, recycled and *de novo* produced by the organism.

Most luminescent animals are believed to acquire their luminous substrate by feeding on other luminescent or “luciferin-carrying” prey (Barnes *et al.*, 1973; Young *et al.*, 1979; Frank *et al.*, 1984; Shimomura, 1987; Mallefet & Shimomura, 1995; Haddock *et al.*, 2001), while only some of them, at the beginning of the food web, synthesize those components for all others (Oba *et al.*, 2002; 2009). On few occasions (such as for *Porichthys notatus* – Thompson *et al.*, 1988), it has been postulated that a first contact with the luciferin is necessary (either via alimentation or by parents transfer) to trigger its *de novo* production or recycling. The first step in the investigation of *E. spinax* luminous reaction was therefore to test whether a known luciferin was used in the

reaction and whether this luciferin was alimentary acquired (only the known and assayable forms of luciferin were tested). We showed this was not the case (Renwart & Mallefet, 2013) and, in a sense, this is not surprising. Indeed, it seems very hazardous, for an animal living in the deep sea where food intake might be low and sporadic, to only rely on its diet for emitting such a long lasting light. Although we cannot completely exclude this possibility (for instance if an irregular contact with luciferin *via* the diet is sufficient to trigger its recycling), our data rather suggest *E. spinax* is able to produce its own luminous material (which might be unknown molecules) and possibly to recycle it. For this reason, we decided to initiate the purification of this unknown luminous system, and because we were unable to trigger the light from a crude photophore extract by performing cross-reaction, an alternative detection mean, based on glowons fluorescence, was developed.

On the purification process and the chromatography method

First results in luciferin purification process showed that photophore crude extract contains many more fluorescent molecules than we expected. It was therefore decided to compare fluorescent molecules from skin with *versus* without photophores, since luminous material was assumed to be less present (and so its fluorescence to be weaker) in the second sample. Assuming glowons were dissolved in our HPLC samples (see discussion above), two major peaks were found to be good luciferin candidates. Unfortunately, those two peaks were not retained by the columns, whatever the pH used (acidic with an Atlantis T-3 column or basic with an XBridge C₁₈ column). This is the main reason why isolation process had to be stopped. In order to pursue the purification (particularly if the problem persists when using a glowons preparation in which right compounds are targeted with certainty), other column types and chromatography methods should be assayed. Both Atlantis T-3 and XBridge C₁₈ columns are designed for a balanced retention of polar and non-polar molecules and are well suited for analysis of crude extracts, the reason why we used them. However, both peaks of interest seemed to be very polar, and were therefore not eluted on normal phase and straight eluted on reversed phase. This is

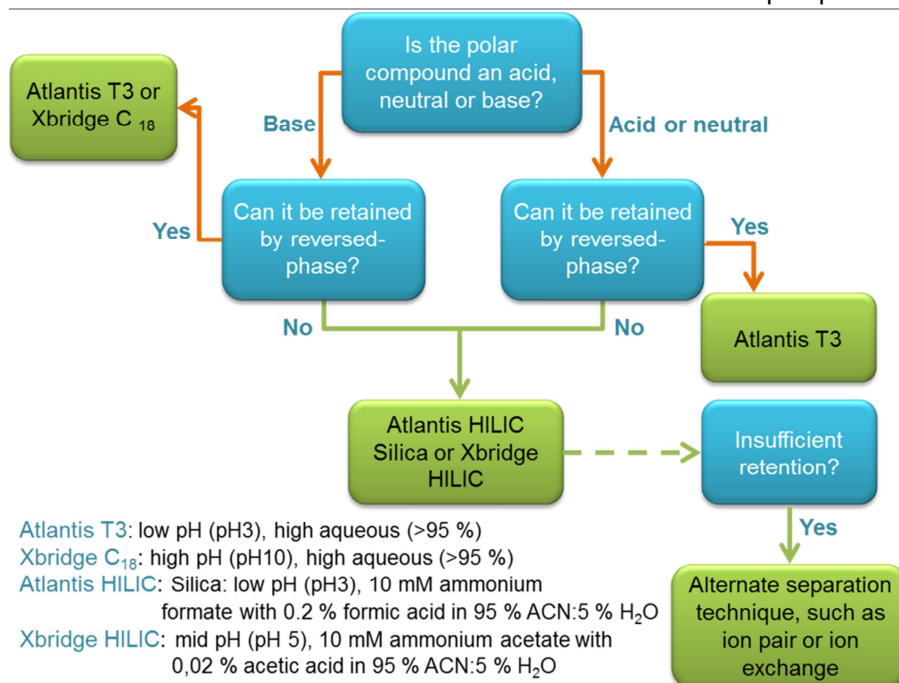


Fig. 2 Diagram of polar compound retention. Red arrows represent what have been tried in chapter 3, green arrows represent what should be tried to improve results. Modified from Waters website (http://www.waters.com/waters/fr_FR/AtlantisColumns/nav.htm?cid=513211).

illustrated in **figure 2**, as well as solutions proposed in this particular case.

A first solution would be to perform hydrophilic interaction liquid chromatography (HILIC) in which a polar stationary phase (such as in normal phase) is combined with a polar mobile phase (such as in reversed phase). Both Atlantis and Xbridge columns exist for this particular chromatography method and have been specifically designed to retain molecules that are not or poorly retained in reversed-phase. If compounds are still not retained, alternate separation techniques should be used. The nature of both peaks are still unclear in that we don't know if they represent big molecules or charged molecules. Performing a size-exclusion chromatography on a crude extract in order to eliminate macro-molecules and successively performing a reversed phase chromatography on collected elution peaks might inform us on the nature

of the two peaks: (i) charged molecules if peaks are still present or (ii) big molecules if they are absent. In the first case, separation should be pursued using ion exchange or ion pair chromatography. In the second case, components constituting peaks might be fluorescent proteins (luciferase, luciferin-binding protein, or any fluorescent protein constituting photophore). Once again, those results would be greatly improved by working on glowons preparation only, in order to be certain peaks contain luminous material.

Perspective: perform hydrophilic interaction liquid chromatography (ILIC), size-exclusion chromatography and ion exchange chromatography, coupled with a fluorescence detector, on *E. spinax* photophore crude extracts in order to improve peak retention and separation.

On luciferase characterization and peroxidase hypothesis

The hypothesis developed in the second section of chapter 3, proposed *E. spinax* luciferase to be a peroxidase and its luminous system to be related to an ancient ROS detoxifying mechanism. Although we were unable to detect a different peroxidase activity between skin with and without photophore, we found sodium azide (NaN_3) and potassium cyanide (KCN), which are known to inhibit peroxidases, to block hormonally-induced light production. The question arise whether those molecules acted on the chemiluminescent reaction itself by quenching luciferase, or at any other level of the light-production mechanism, since both NaN_3 and KCN are known for their versatile action inside cells. To answer this question, it would be useful to investigate the ultrastructural changes inside photophore caused by both molecules when added to MT and PRL. NaN_3 does not seem to prevent MT to fix on its receptor, since tested photophores showed an opened ILS under binocular. This can be confirmed at ultrastructural level by checking whether melanosomes are still present or not between photocytes. In the same way, if grey particles still appear in PRL + NaN_3 /KCN conditions, it would mean NaN_3 /KCN does not prevent PRL action. On the contrary, no change on glowon ultrastructure (diameter and/or density), would suggest NaN_3 /KCN act at this level.

Perspective: investigate the ultrastructural action of NaN_3 and KCN on *E. spinax* hormonally-induced light production.

Finally, a genetic approach for studying *E. spinax* luciferase would be to analyse its skin transcriptome. Indeed, some specific portions of luciferase DNA are highly conserved among marine luminous animals. Therefore, specifically search for a luciferase-like RNA might allow finding luciferase candidates and orient future research.

Perspective: studying *E. spinax* skin transcriptome in order to find a luciferase-like RNA.

Implication of this study in the knowledge of deep-sea sharks biology and ecology

Because deep-sea shark biology and ecology is a still poorly investigated research field, this work represents a valuable contribution to the subject. Bioluminescence is one of the most astonishing aspects of lanternsharks and its study permits to better understand the biology of deep-sea sharks. In particular, the ultrastructure of *E. spinax* photophores, interpreted in regards to control and functions of these organs, allowed to propose a theory of light emission mechanism related to the shark behavior in its natural environment. When counterilluminating, *E. spinax* uses its coverage ventral luminous areas to completely hide its shadow from predators below (Claes *et al.*, 2010a; Claes & Mallefet, 2010a). This behavior is assumed to be primarily controlled by MT and GABA that act on iris-like structure of photophores (Claes & Mallefet, 2009b; Claes *et al.*, 2011), what is confirmed by our ultrastructural study (Renwart *et al.*, 2014b). The photophore was found to be perfectly designed for this purpose, with the discovery of a reflector upholstering the pigmented sheath (Renwart *et al.*, 2014a). Luminescence in sharks, at least in the etmopterid family, also helps in intraspecific communication. For schooling, hunting, or finding a mate, *E. spinax* uses its brighter (lateral and ventral) luminous areas to attract the attention of conspecifics (Claes & Mallefet, 2010a). This behavior is assumed to be primarily controlled by PRL and NO (Claes & Mallefet,

2009b; Claes *et al.*, 2010b) which mainly act on photocytes, as described in this thesis (Renwart *et al.*, 2014b).

E. spinax feeding habits also gave very useful information on its ecology. The shark mainly feed on euphausiids, while fishes, decapods and cephalopods are also part of the diet for older specimens, in a more sporadic manner (Renwart & Mallefet, 2013). It has been postulated that *E. spinax* might perform day/night vertical migrations to follow its main prey, a hypothesis that remains to be proved. Large mackerels found in two stomachs suggested *E. spinax* is able either to hunt in group, or at least is a scavenger.

Finally, biochemical study revealed that *E. spinax* luminous capacity is not based on a known mechanism to date. The future complete characterization of this system opens up perspectives of new discoveries in the field of bioluminescence and shark biology.

Conclusion

E. spinax, and probably all other luminous sharks, displays very original features (i.e. a hormonally controlled long-lasting light emission) that are not or rarely observed in the field of bioluminescence. In the same way, we believe this shark can surprise us again with new luminescent molecules, or even a completely unknown luminescent reaction. For this reason, efforts to fully understand its luminous mechanism and the underlying chemiluminescent reaction should be pursue. Thereafter, a comparative study on the chemiluminescent system of other luminescent sharks will be of great interest. Particularly, the question arise whether dalatiid photophores, which are morphologically different from etmopterid ones in that they only enclose one unique photocyte, also contain glowons.

REFERENCES



The sampling boats *Aurelia* (front) and *Hans Brattström* (behind)
(M. Renwart)

REFERENCES

- Adnet, S., Cappetta, H.** (2001). A palaeontological and phylogenetical analysis of squaliform sharks (Chondrichthyes: Squaliformes) based on dental characters. *Lethaia*. **34**, 234-48.
- Anctil, M.** (1979a). Ultrastructural correlates of luminescence in *Porichthys* photophores. I. Effects of spinal cord stimulation and exogenous noradrenaline. *Rev. Can. Biol.* **38**, 67-80.
- Anctil, M.** (1979b). Ultrastructural correlates of luminescence in *Porichthys* photophores. II. Effects of metabolic inhibitors. *Rev. Can. Biol.* **38**, 81-96.
- Anctil, M., Case, J.F.** (1976). Pharmacomorphological study of denervation induced by 6-hydroxydopamine in *Porichthys* photophores. *Cell Tissue Res.* **166**, 365-88.
- Anctil, M., Shimomura, O.** (1984). Mechanism of photoinactivation and re-activation in the bioluminescence system of the ctenophore *Mnemiopsis*. *Biochem. J.* **221**, 269-72.
- Anderson, J.M., Cormier, M.J.** (1973). Lumisomes, the cellular site of bioluminescence in coelenterates. *J. Biol. Chem.*, **248**, 2937-43.
- Barnes, A.T., Case, J.F., Tsuji, F.I.** (1973). Induction of bioluminescence in a luciferin deficient form of the marine teleost, *Porichthys*, in response to exogenous luciferin. *Comp. Biochem. Physiol.* **46A**, 709-23.
- Bassot, J.-M., Bilbaut, A.** (1977). Bioluminescence des élytres d'*Acholoe*. IV. Luminescence et fluorescence des photosomes. *Biol. Cellulaire*. **28**, 163-8.
- Bassot, J.M., Nicolas, M.T.** (1978). Similar paracrystals of endoplasmic reticulum in the photoemitters and the photoreceptors of scale-worms. *Experientia*. **34**, 726-8.
- Bassot, J.-M., Nicolas, G.** (1987). An optional dyadic junctional complex revealed by fast-freeze fixation in the bioluminescent system of the scale worm. *J. Cell. Biol.* **105**, 2245-56.
- Bellisario, R., Spencer, T.E., Cormier, M.J.** (1972). Isolation and properties of luciferase, a non-heme peroxidase, from the bioluminescent earthworm, *Diplocardia longa*. *Biochemistry*. **11**, 2256-66.
- Bello, G.** (1998). The feeding ecology of the velvet belly, *Etmopterus spinax* (Chondrichthyes: Squalidae), of the Adriatic Sea on the basis of its stomach contents. *Atti della Società italiana di scienze naturali e del museo civico di storia naturale di Milano*. **139**, 187-93.

References

- Bergstad, O. A., Wik, A. D., Hildre, O.** (2003). Predator-prey relationships and food sources of the Skagerrak deep-water fish assemblage. *J. Northwest Atl. Fish. Sci.* **31**, 165.
- Bernal, D., Donley, J.M., Shadwick, R.E., Syme, D.A.** (2005). Mammal-like muscles power swimming in a cold-water shark. *Nature*. **437**, 1349-52.
- Botella, H.** (2006). The Oldest Fossil Evidence of a Dental Lamina in Sharks. *J. Vertebr. Paleontol.* **26**, 1002-3.
- Buck, J.D.** (1984). Quantitative and qualitative bacteriology of elasmobranch fish from the Gulf of Mexico. *Fish. Bull.* **82**, 375-9.
- Burrow, C.J., Hovestadt, D.C., Hovestadt-Euler, M., Turner, S., Young, G.C.** (2008). New information on the Devonian shark *Mcmurdodus*, based on material from western Queensland, Australia. *Acta. Geol. Pol.* **58**, 155-63.
- Cadenas, E., Sies, H.** (1984). Low-level chemiluminescence as an indicator of singlet molecular oxygen in biological systems. *Methods Enzymol.* **105**, 221-31.
- Calcagno, R.** (2013). Requins: au-delà du malentendu. Edition du Rocher.
- Case, J.F., Strause, L.G.** (1978). Neurally controlled luminescent systems. In: *Bioluminescence in action* (ed. P.J. Herring), pp 331-366. New York: Academic Press.
- Campbell, A.K.** (1989). Living light: biochemistry, function and biomedical applications. *Essays Biochem.* **24**, 41-81.
- Claes, J.M., Aksnes, D.L., Mallefet, J.** (2010a). Phantom hunter of the fjords: Camouflage by counterillumination in a shark (*Etmopterus spinax*). *J. Exp. Mar. Biol. Ecol.* **388**, 28-32.
- Claes, J.M., Dean, M.N., Nilsson, D.E., Hart, N.S., Mallefet, J.** (2013). A deepwater fish with 'lightsabers'—dorsal spine-associated luminescence in a counterilluminating lanternshark. *Sci. rep.* **3**, 1308.
- Claes, J.M., Ho, H.C., Mallefet, J.** (2012). Control of luminescence from pygmy shark (*Squaliolus aliae*) photophores. *J. Exp. Biol.* **215**, 1691-99.
- Claes, J.M., Krönström, J., Holmgren, S., Mallefet, J.** (2010b). Nitric oxide in the control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J. Exp. Biol.* **213**, 3005-11.
- Claes, J.M., Krönström, J., Holmgren, S., Mallefet, J.** (2011a). GABA inhibition of luminescence from lantern shark (*Etmopterus spinax*) photophores. *Com. Biochem. Physiol.* **153C**, 231-6.

References

- Claes, J.M., Mallefet, J.** (2008). Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark *Etmopterus spinax* (Squaloidea: Etmopteridae). *J. Fish. Biol.* **73**, 1337-50.
- Claes, J.M., Mallefet, J.** (2009a). Ontogeny of photophore pattern in the velvet belly lantern shark, *Etmopterus spinax*. *Zoology* **112**, 433-41.
- Claes, J.M., Mallefet, J.** (2009b). Hormonal control of luminescence from lantern shark (*Etmopterus spinax*) photophores. *J. Exp. Biol.* **212**, 3684-92.
- Claes, J.M., Mallefet, J.** (2009c). Bioluminescence of sharks: first synthesis. In: *Bioluminescence in focus - A collection of illuminating essays* (Ed. V.B. Meyer-Rochow), pp 51-65. Kerala (India): Research Signpost.
- Claes, J.M., Mallefet, J.** (2010a). Functional physiology of lantern shark (*Etmopterus spinax*) luminescent pattern: differential hormonal regulation of luminous zones. *J. Exp. Biol.* **213**, 1852-58.
- Claes, J.M., Mallefet, J.** (2010b). The lantern shark's light switch: turning shallow water crypsis into midwater camouflage. *Biol. Lett.* **6**, 685-7.
- Claes, J.M., Nilsson, D.-E., Straube, N., Collin, S.P., Mallefet, J.** (2014). Iso-luminance counterillumination drove bioluminescent shark radiation. *Sci. Rep.* **4**, 4328.
- Claes, J.M., Sato, K., Mallefet, J.** (2011b). Morphology and control of photogenic structures in a rare dwarf pelagic lantern shark (*Etmopterus splendidus*). *J. Exp. Mar. Biol. Ecol.* **406**, 1-5.
- Clarke, W.D.** (1963). Function of bioluminescence in mesopelagic organisms. *Nature* **198**, 1244-47.
- Coelho, R., Erzini, K.** (2008). Life history of a wide-ranging deepwater lantern shark in the North-East Atlantic, *Etmopterus spinax* (Chondrichthyes: Etmopteridae), with implications for conservation. *J. Fish Biol.* **73**, 1419-43.
- Compagno, L., Dando, M., Fowler, S.** (2005). A field guide to the sharks of the world. London: Harper Collins.
- Conrath, C.L., Musick, J.A.** (2012). Reproductive biology of elasmobranchs. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-29. Second edition, Boca Raton (Florida): CRC Press.
- Copeland, J., Moiseff, A.** (1994). The occurrence of synchrony in the North American firefly *Photinus carolinus* (Coleoptera: Lampyridae). *J. Insect Behav.* **8**, 381-94.

References

- Cormier, M.J., Crane, J.M., Nakano, Y.** (1967). Evidence for the identity of the luminescent systems of *Porichthys porosissimus* (fish) and *Cypridina hilgendorffii* (crustacean). *Biochem. Biophys. Res. Commun.* **29**, 747-52.
- Cormier, M.J., Dure, L.S.** (1963). Studies on the bioluminescence of *Balanoglossus biminensis* extracts: I. Requirement for hydrogen peroxide and characteristics of the system. *J. Biol. Chem.* **238**, 785-9.
- Cortés, E.** (1997). A critical review of methods of studying fish feeding based on analysis of stomach contents: application to elasmobranch fishes. *Can. J. Fish. Aquat. Sci.* **54**, 726-38.
- Cuni, G.** (2013). *Requins de la préhistoire à nos jours*. France: Editions Belin.
- Cuny, G., Risnes, S.** (2005). The enameloid microstructure of the teeth of Synechodontiform sharks (Chondrichthyes: Neoselachii). *PalArch J. Vertebr. Palaeontol.* **3**, 8-19.
- Darras, L., Derycke, C., Blieck, A., Vachard, D.** (2008). The oldest holocephalan (Chondrichthyes) from the Middle Devonian of the Boulonnais (Pas-de-Calais, France). *C. R. Palevol.* **7**, 297-304.
- Dean, B.** (1894). Contributions to the morphology of *Cladoselache* (Cladodus). *J. Morphol.* **9**, 87-114.
- Dean, M.N., Summers, A.P.** (2006). Mineralized cartilage in the skeleton of chondrichthyan fishes. *Zoology.* **109**, 164-8.
- Deheyn, D., Mallefet, J., Jangoux, M.** (1998). Bioluminescence in *Amphipholis squamata*: An anti-predator aposematic deterrent function? In: *Echinoderms: Proceedings of the Ninth International Echinoderm Conference* (eds. R. Mooi, M. Telford), pp 351. Rotterdam: Balkema.
- Deheyn, D., Mallefet, J., Jangoux, M.** (2000). Cytological changes during bioluminescence production in dissociated photocytes from the ophiuroid *Amphipholis squamata* (Echinodermata). *Cell. Tissue Res.* **299**, 115-28.
- Denton, E.J.** (1970). On the organization of reflecting surfaces in some marine animals. *Philos. Trans. R. Soc., B.* **258**, 285-313.
- Denton, E.J., Herring, P.J., Widder, E.A., Latz, M.F., Case, J.F.** (1985). The roles of filters in the photophores of oceanic animals and their relation to vision in the oceanic environment. *Proc. R. Soc. London, Ser. B.* **225**, 63-97.

References

- DeSa, R., Hastings, J.W., Vatter, A.E.** (1963). "Crystalline" Particles: An Organized Subcellular Bioluminescent System. *Science*. **141**, 1269-70.
- Dickens, D.A.G., Marshall, N.J.** (1956). Observations on *Euprotomiscus*. *Mar. Obs.* **26**, 73-4.
- Dubuisson, M.L.N., de Wergifosse, B., Trouet, B., Baguet, F., Marchand-Brynaert, J., Rees, J.F.** (2000). Antioxidative properties of natural coelenterazine and synthetic methyl coelenterazine in rat hepatocytes subjected to *tert*-butyl hydroperoxide-induced oxidative stress. *Biochem. Pharmacol.* **60**, 471-8.
- Dubuisson, M.L.N., Rees, J.F., Marchand-Brynaert, J.** (2005). Coelenterazine (marine bioluminescent substrate): A source of inspiration for the discovery of novel antioxidants. *Drug Dev. Ind. Pharm.* **31**, 827-49.
- Dunlap, J.C., Hastings, J.W., Shimomura, O.** (1980). Crossreactivity between the light-emitting systems of distantly related organisms: Novel type of light-emitting compound. *Proc. Natl. Acad. Sci. U. S. A.* **77**, 1394-7.
- Dunlap, P.V., Kita-Tsukamoto, K.** (2006). Luminous bacteria. In *The Prokaryotes* (eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt), pp. 863-92). New York: Springer.
- Dure, L.S., Cormier, M.J.** (1963). Studies on the bioluminescence of *Balanoglossus biminensis* extracts: II. Evidence for the peroxidase nature of balanoglossid luciferase. *J. Biol. Chem.* **238**, 790-3.
- Ebert, D.A., Winton, M.V.** (2010). Chondrichthyans of high latitude seas. In: *Sharks and their relatives II: biodiversity, adaptive physiology and conservation* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-35. Boca Raton (Florida): CRC Press.
- Fanelli, E., Rey, J., Torres, P., Gil de Sola, L.** (2009). Feeding habits of blackmouth catshark *Galeus melastomus* Rafinesque, 1810 and velvet belly lantern shark *Etmopterus spinax* (Linnaeus, 1758) in the western Mediterranean. *J. Appl. Ichthyol.* **25**, 83-93.
- Fishelson, L., Gon, O., Goren, M.** (2005). The oral cavity and bioluminescent organs of the cardinal fish species *Siphamia permutata* and *S. cephalotes* (Perciformes, Apogonidae). *Mar. Biol.* **147**, 603-9.
- Fleisher, K.J., Case, J.F.** (1995). Cephalopod predation facilitated by dinoflagellate luminescence. *Biol. Bull.* **189**, 263-71.
- Frank, T.M., Widder, E.A., Latz, M.I., Case, J.F.** (1984). Dietary maintenance of bioluminescence in a deep-sea mysid. *J. Exp. Biol.* **109**, 385-9.

References

- Fritz, L., Morse, D., Hastings, J.W.** (1990). The circadian bioluminescence rhythm of *Gonyaulax* is related to daily variations in the number of light-emitting organelles. *J. Cell. Sci.* **95**, 321-8.
- Fuqua, W.C., Winans, S.C., Greenberg, E.P.** (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**, 269-75.
- Gardiner, J.M., Hueter, R.E., Maruska, K.P., Sisneros, J.A., Casper, B.M., Mann, D.A., Demski, L.S.** (2012). Sensory physiology and behavior of Elasmobranchs. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-29. Second edition, Boca Raton (Florida): CRC Press.
- Gelsleichter, J., Evans, A.N.** (2012). Hormonal regulation of elasmobranchs physiology. In: *Biology of Sharks and their Relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 313-48. Second edition, Boca Raton (Florida): CRC Press.
- Gottfried, M.D., Compagno, L.J.V., Bowman, S.C.** (1996). Size and skeletal anatomy of the giant "megatooth" shark *Carcharodon megalodon*. In: *Great White Sharks: the Biology of Carcharodon carcharias* (Eds. A.P. Klimley, D.G. Ainley), pp 55-66. San Diego: Academic Press.
- Greene, C.W.** (1899). The phosphorescent organs in the toadfish *Porichthys notatus girard*. *J. Morphol.* **15**, 667-96.
- Greiter-Wilke, A., Scanziani, E., Soldati, S., McDonough, S.P., McDonough, P.L., Center, S.A., Rishniw, M., Simpson K.W.** (2006). Association of *Helicobacter* with Cholangiohepatitis in Cats. *J. Vet. Intern. Med.* **20**, 822-27.
- Grober, M.S.** (1988). Brittle-star bioluminescence functions as an aposematic signal to deter crustacean predators. *Anim. Behav.* **36**, 493-501.
- Grogan, E.D., Lund, R., Greenfest-Allen, E.** (2012). The origin and relationships of early chondrichthyans. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-29. Second edition, Boca Raton (Florida): CRC Press.
- Haddock, S.H.D., Moline, M.A., Case, J.F.** (2010). Bioluminescence in the sea. *Annu. Rev. Mar. Sci.* **2**, 443-93.
- Haddock, S.H.D., Rivers, T.J., Robison, B.H.** (2001). Can coelenterates make coelenterazine? Dietary requirement for luciferin in cnidarian bioluminescence. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 11148-51.

References

- Hamanaka, T., Michinomae, M., Seidou, M., Miura, K., Inoue, K., Kito, Y.** (2011). Luciferase activity of the intracellular microcrystal of the firefly squid, *Watasenia scintillans*. *FEBS Lett.* **585**, 2735-38.
- Haneda, Y., Johnson, F.H., Sie, E.H.C.** (1958). Luciferin and luciferase extracts of a fish, *Apogon marginatus*, and their luminescent cross-reactions with those of a crustacean, *Cypridina hilgendorffii*. *Biol. Bull.* **115**, 336.
- Haneda, Y., Tsuji, F.I.** (1971). Light production in the luminous fishes *Photoblepharon* and *Anomalops* from the Banda Islands. *Science* **173**, 143-5.
- Hansen, K., Herring, P.J.** (1977). Dual bioluminescent systems in the anglerfish genus *Linophryne* (Pisces: Ceratiidae). *J. Zool.* **182**, 103-24.
- Harper, R.D., Case, J.F.** (1999). Disruptive counterillumination and its anti-predatory value in the plainfish midshipman *Porichthys notatus*. *Mar. Biol.* **134**, 529-40.
- Harvey, E.N.** (1952). Bioluminescence. New York: Academic Press.
- Harvey, E.N.** (1957). A history of luminescence from the earliest times until 1900. Philadelphia: The American Philosophical Society.
- Hastings, J.W.** (1978). Bacterial and dinoflagellate luminescent system. In: *Bioluminescence in action* (ed. P.J. Herring), pp 129-70. London: Academic Press.
- Hastings, J.W.** (1983). Biological diversity, chemical mechanisms, and the evolutionary origins of bioluminescent systems. *J. Mol. Evol.* **19**, 309-21.
- Hastings, J.W.** (1995). Bioluminescence. In: *Cell physiology Source Book* (ed. N. Sperelakis), pp 665-81. San Diego: Academic Press Inc.
- Hastings, J.W., Morin, J.G.** (1991). Bioluminescence. In: *Neural and integrative animal physiology* (ed. C.L. Prosser), pp 131-70. New York: Wiley-Liss.
- Heinicke, M.P., Naylor, G.J., Hedges, S.B.** (2009). Cartilaginous fishes (Chondrichthyes). In: *The timetree of life* (Eds. S.B. Hedges, S. Kumar), pp. 320-27. New York: Oxford University Press.
- Hendry, T.A., Dunlap, P.V.** (2011). The uncultured luminous symbiont of *Anomalops katoptron* (Beryciformes: Anomalopidae) represents a new bacterial genus. *Mol. Phylogenet. Evol.* **61**, 834-43.
- Henry, J.P., Michelson, A.M.** (1970). Studies in bioluminescence IV. Properties of luciferin from *Pholas dactylus*. *Biochim. Biophys. Acta, Bioenerg.* **205**, 451-8.

References

- Henry, J.P., Michelson, A.M.** (1978). Bioluminescence: physiological control and regulation at the molecular level. *Photochem. Photobiol.* **28**, 293-310.
- Henry, J.P., Monny, C., Michelson, A.M.** (1975). Characterization and properties of *Pholas* luciferase as a metalloglycoprotein. *Biochemistry.* **14**, 3458-66.
- Herrera, A.A.** (1979). Electrophysiology of bioluminescent excitable epithelial cells in a polynoid polychaete worm. *J. Com. Biol.* **129**, 67-78.
- Herring, P.J.** (1982). Aspects of the bioluminescence of fishes. *Oceanogr. Mar. Biol. Ann. Rev.* **20**, 415-70.
- Herring, P.J.** (2007). Sex with the lights on? A review of bioluminescent sexual dimorphism in the sea. *J. Mar. Biol. Assoc. U. K.* **87**, 829-42.
- Herring, P.J., Dilly, P.N., Cope, C.** (2002). The photophores of the squid family Cranchiidae (Cephalopoda: Oegopsida). *J. Zool.* **258**, 73-90.
- Herring, P.J., Locket, N.A.** (1978). The luminescence and photophores of euphausiid crustaceans. *J. Zool.* **186**, 431-62.
- Herring, P.J., Morin, J.G.** (1978). Bioluminescence in fishes. In: *Bioluminescence in action* (ed. P.J. Herring), pp 273-329. New York: Academic Press.
- Hubbs, C.L., Iwai, T., Matsubara, K.** (1967). External and internal characters, horizontal and vertical distribution, luminescence, and food of the dwarf pelagic shark, *Euprotomicrus bispinatus*. *Bull. Scripps Inst. Oceanogr.* **10**, 1-64.
- Ikejima, K., Wada, M., Kita-Tsukamoto, K., Yamamoto, T., Azuma, N.** (2008). Synchronized development of gonad and bioluminescent light organ in a highly sexually dimorphic leiognathid fish, *Photoplagios rivulatus*. *Mar. Biol.* **153**, 1009-14.
- Inoue, S., Okada, K., Kakoi, H., Goto, T.** (1977). Fish bioluminescence I. Isolation of a luminescent substance from a Myctophina fish, *Neoscopelus microchir*, and identification of it as *Oplophorus* luciferin. *Chem. Lett.* **6**, 257-8.
- Ivanov, A.** (2005). Early Permian Chondrichthyans of the middle and south Urals. *Rev. Bras. Paleontol.* **8**, 127-38.
- Iwai, T.** (1960). Luminous organs of the deep-sea squaloid shark, *Centroscyllium ritteri* Jordan and Fowler. *Pac. Sci.* **14**, 51-4.
- Gould, S.J., Vrba, E.S.** (1982). Exaptation-a missing term in the science of form. *Paleobiology.* **8**, 4-15.

References

- Johnson, F.H., Sugiyama, N., Shimomura, O., Saiga, Y., Haneda, Y.** (1961). Crystalline luciferin from a luminescent fish, *Parapriacanthus beryciformes*. *Proc. Natl. Acad. Sci. U. S. A.* **47**, 486-9.
- Jones, A., Mallefet, J.** (2010). Aposematic use of bioluminescence in *Ophiopsila aranea* (Ophiuroidea, Echinodermata). *Luminescence* **25**, 155-6.
- Jones, A., Mallefet, J.** (2013). Why do brittle stars emit light? Behavioural and evolutionary approaches of bioluminescence. *Cah. Biol. Mar.* **54**, 729-34.
- Jones, B.W., Nishiguchi, M.K.** (2004) Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar. Biol.* **144**, 1151-5.
- Johann, L.** (1899). Über eigenthümliche epitheliale Gebilde (Leuchtorgane) bei *Spinax niger*. *Z. Wiss. Zool.* **66**, 136-60.
- Kalmijn, A.J.** (1971). The electric sense of sharks and rays. *J. Exp. Biol.* **55**, 371-83.
- Kemp, N.E., Westrin, S.K.** (1979). Ultrastructure of calcified cartilage in the endoskeletal tesserae of sharks. *J. Morph.* **160**, 75-102.
- Kishi, T., Goto, T., Hirata, Y., Shimomura, O., Johnson, F.H.** (1966). *Cypridina* bioluminescence I: Structure of *Cypridina* luciferin. *Tetrahedron Lett.* **7**, 3427-36.
- Klimley, A.P.** (1993). Highly directional swimming by scalloped hammerhead sharks, *Sphyrna lewini*, and subsurface irradiance, temperature, bathymetry, and geomagnetic field. *Mar. Biol.* **117**, 1-22.
- Klimpel, S., Palm, H. W., Seehagen, A.** (2003). Metazoan parasites and food composition of juvenile *Etmopterus spinax* (L., 1758) (Dalatiidae, Squaliformes) from the norwegian deep. *Parasitol. Res.* **89**, 245-51.
- Kritzler, H., Wood, L.** (1961). Provisional audiogram for the shark, *Carcharhinus leucas*. *Science.* **133**, 1480-2.
- Kyne, P.M., Simpfendorfer, C.A.** (2010). Deepwater Chondrichthyans. In: *Sharks and their relatives II: biodiversity, adaptive physiology and conservation* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-35. Boca Raton (Florida): CRC Press.
- Latz, M.I.** (1995). Physiological mechanisms in the control of bioluminescent countershading in a midwater shrimp. *Mar. Freshwater Behav. Physiol.* **26**, 207-18.

References

- Lebedev, O.A.** (2009). A new specimen of *Helicoprion* Karpinsky, 1899 from Kazakhstaniian Cisurals and a new reconstruction of its tooth whorl position and function. *Acta Zool.* **90**, 171-82.
- Lebrun, P.** (2001). Requins, raies et autres chondrichthyens fossiles. France: éditions CEDIM.
- Lund, R.** (1985). The morphology of *Falcatus falcatus* (St. John and Worthen), a mississippian Stethacanthid Chondrichthyan from the Bear Gulch Limestone of Montana. *J. Vertebr. Paleontol.* **5**, 1-19.
- MacPherson, E.** (1980). Régime alimentaire de *Galeus melastomus* Rafinesque 1810, *Etmopterus spinax* (L., 1758) et *Scymnorhinus licha* (Bonnaterre, 1788) en Méditerranée occidentale. *Vie Milieu.* **30**, 139-48.
- Maia, A.M.R., Wilga, C.A.D., Lauder, G.V.** (2012). Biomechanics of locomotion in sharks, rays, and chimaeras. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 125-51. Second edition, Boca Raton (Florida): CRC Press.
- Maisey, J.G.** (2009). The spine-Brush complex in Symmoriiform sharks (Chondrichthyes; Symmoriiformes), with comments on dorsal fin modularity. *J. Vertebr. Paleontol.* **29**, 14-24.
- Mallefet, J., Shimomura, O.** (1995). Presence of coelenterazine in mesopelagic fishes from the Strait of Messina. *Mar. Biol.* **124**, 381-5.
- Maruska, K.P.** (2001). Morphology of the mechanosensory lateral line system in elasmobranch fishes: ecological and behavioral considerations. *Environ. Biol. Fishes.* **60**, 47-75.
- Maruska, K.P., Tricas, T.C.** (1998). Morphology of the mechanosensory lateral line system in the Atlantic stingray, *Dasyatis sabina*: The Mechanotactile Hypothesis. *J. Morphol.* **238**, 1-22.
- McFall-Ngai, M.J., Morin, J.G.** (1991). Camouflage by disruptive illumination in leiognathids, a family of shallow-water, bioluminescent fishes. *J. Exp. Biol.* **156**, 119-37.
- McFall-Ngai, M.J., Ruby, E.G.** (1998). Sepioids and vibrios: when first they meet. *Bioscience.* **48**, 257-65.
- Meighen, E.A.** (1991). Molecular biology of bacterial bioluminescence. *Microbiol. Rev.* **55**, 123-42.
- Meighen, E.A.** (1993). Bacterial bioluminescence: organization, regulation and application of the *lux* genes. *The FASEB journal.* **7**, 1016-22.
- Mesinger, A.F., Case, J.F.** (1992). Dinoflagellate luminescence increases susceptibility of zooplankton to teleost predation. *Mar. Biol.* **112**, 207-10.

References

- Miller, R.F., Cloutier, R., Turner, S.** (2003). The oldest articulated chondrichthyan from the Early Devonian period. *Nature*. **425**, 501-4.
- Miller, S.D., Haddock, S.H.D., Elvidge, C.D., Lee, T.F.** (2005). Detection of a bioluminescent milky sea from space. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 14181-4.
- Mojetta, A.** (1998). Les requins. France: Gründ.
- Montgomery, J.C., Coombs, S., Halstead, M.** (1995). Biology of the mechanosensory lateral line in fishes. *Rev. Fish Biol. Fish.* **5**, 399-416.
- Montgomery, J.C., Walker, M.M.** (2001). Orientation and navigation in elasmobranchs: which way forward? *Environ. Biol. Fishes.* **60**, 109-16.
- Morin, J.G.** (1983). Coastal bioluminescence: patterns and functions. *Bull. Mar. Sci.* **33**, 787-817.
- Morin, J.G., Cohen, A.C.** (2010). It's all about sex: bioluminescent courtship displays, morphological variation and sexual selection in two new genera of Caribbean ostracodes. *J. Crustacean Biol.* **30**, 56-67.
- Morin, J.G., Harrington, A., Nealson, K., Krieger, N., Baldwin, T.O., Hastings, J.W.** (1975). Light for all reasons: versatility in the behavioral repertoire of the flashlight fish. *Science* **190**, 74-6.
- Motta, P.J., Huber, D.R.** (2012). Prey capture behavior and feeding mechanics of elasmobranchs. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 153-209. Second edition, Boca Raton (Florida): CRC Press.
- Munk, O.** (1999). The escal photophore of ceratioids (Pisces; Ceratioidei) - a review of structure and function. *Acta. Zool.* **80**, 265-84.
- Muñoz, J.L.P., Ceinos, R.M., Soengas, J.L., Míguez, J.M.** (2009). A simple and sensitive method for determination of melatonin in plasma, bile and intestinal tissues by high performance liquid chromatography with fluorescence detection. *J. Chromatogr. B.* **877**, 2173-77.
- Murphy, M.E., Sies, H.** (1990). Visible-range low-level chemiluminescence in biological systems. *Methods Enzymol.* **186**, 595-610.
- Myrberg, A.A.** (2001). The acoustical biology of elasmobranchs. *Environ. Biol. Fishes.* **60**, 31-45.

References

- Na, R., Stender, I.-M., Henriksen, M., Wulf, H.C.** (2001). Autofluorescence of human skin is age-related after correction for skin pigmentation and redness. *J. Invest. Dermatol.* **116**, 536-40.
- Nakamura, H., Musicki, B., Kishi, Y., Shimomura, O.** (1988). Structure of the light emitter in krill (*Euphausia pacifica*) bioluminescence. *J. Am. Chem. Soc.* **110**, 2683-5.
- Nakamura, H., Kishi, Y., Shimomura, O., Morse, D., Hastings, J. W.** (1989). Structure of dinoflagellate luciferin and its enzymic and nonenzymic air-oxidation products. *J. Am. Chem. Soc.* **111**, 7607-11.
- Neiva, J., Coelho, R., Erzini, K.** (2006). Feeding habits of the velvet belly lanternshark *Etmopterus spinax* (Chondrichthyes: Etmopteridae) off the Algarve, southern Portugal. *J. Mar. Biol. Assoc. U. K.* **86**, 835-41.
- Nelson, D.R.** (1967). Hearing thresholds, frequency discrimination, and acoustic orientation in the lemon shark, *Negaprion brevirostris* (Poey). *Bull. Mar. Sci.* **17**, 741-68.
- Nicol, J.A.** (1957). Observations on photophores and luminescence in the teleost *Porichthys*. *Q. J. Microsc. Sci.* **98**, 179-88.
- Nicolas, M.-T., Bassot, J.-M., Shimomura, O.** (1982). Polynoidin: A membrane photoprotein isolated from the bioluminescent system of scale-worms. *Photochem. Photobiol.* **35**, 201-7.
- Nyholm, S.V, McFall-Ngai, M.J.** (2004). The winnowing: establishing the squid–vibrio symbiosis. *Nat. Rev. Microbiol.* **2**, 632-42.
- Nyholm, S.V, Stewart, J.J., Ruby, E.G., McFall-Ngai, M.J.** (2009). *Environ. Microbiol.* **11**, 483-93.
- Oba, Y., Kato, S.I., Ojika, M., Inouye, S.** (2002). Biosynthesis of luciferin in the sea firefly, *Cypridina hilgendorffii*: L-tryptophan is a component in *Cypridina* luciferin. *Tetrahedron Lett.* **43**, 2389-92.
- Oba, Y., Kato, S.I., Ojika, M., Inouye, S.** (2009). Biosynthesis of coelenterazine in the deep-sea copepod, *Metridia pacifica*. *Biochem. Biophys. Res. Commun.* **390**, 684-8.
- Ohshima, H.** (1911). Some observations on the luminous organs of fishes. *J. Coll. Sci., Imp. Univ. Tokyo.* **27**, 1-25.
- Pinkas, L.** (1971). Food habits of albacore, bluefin tuna, and bonito in California waters. Resources agency department of fish and game, State of California. *Fish Bull.* **152**, (5-10). Online document (http://content.cdlib.org/view?docId=kt8290062w&brand=c...here&doc.view=entire_text).

References

- Poisson, J.** (2010). Raphael Dubois, from pharmacy to bioluminescence. *Rev. Hist. Pharm.* **58**, 51-56.
- Preciado, I., Cartes, J. E., Serrano, A., Velasco, F., Olaso, I., Sánchez, F., Frutos, I.** (2009). Resource utilization by deep-sea sharks at the Le Danois Bank, Cantabrian Sea, north-east Atlantic Ocean. *J. Fish Biol.* **75**, 1331-55.
- Rees, J. F., De Wergifosse, B., Noiset, O., Dubuisson, M., Janssens, B., Thompson, E. M.** (1998). The origins of marine bioluminescence: turning oxygen defence mechanisms into deep-sea communication tools. *J. Exp. Biol.* **201**, 1211-21.
- Rees, J.F., Thompson, E.M., Baguet, F., Tsuji, F.I.** (1990). Detection of coelenterazine and related luciferase activity in the tissues of the luminous fish, *Vinciguerria attenuata*. *Comp. Biochem. Physiol.* **96A**, 425-30.
- Rees, J.F., Thompson, E.M., Baguet, F., Tsuji, F.I.** (1992). Evidence for the utilization of coelenterazine as the luminescent substrate in *Argyrops leucostictus* photophores. *Mol. Mar. Biol. Biotechnol.* **1**, 219-25.
- Reif, W.-E.** (1985). Functions of scales and photophores in mesopelagic luminescent sharks. *Acta Zool.* **66**, 111-18.
- Renwart, M., Mallefet, J.** (2013). First study of the chemistry of the luminous system in a deep-sea shark, *Etmopterus spinax* Linnaeus, 1758 (Chondrichthyes: Etmopteridae). *J. Exp. Mar. Biol. Ecol.* **448**, 214-19.
- Renwart, M., Delroisse, J., Claes, J.M., Mallefet, J.** (2014a). Ultrastructural organization of lantern shark (*Etmopterus spinax* Linnaeus, 1758) photophores. *Zoomorphology*. **133**, 405-16.
- Renwart, M., Delroisse, J., Flammang, P., Claes, J.M., Mallefet, J.** Cytological changes during luminescence production in lanternshark (*Etmopterus spinax* Linnaeus, 1758) photophores (2014b). *Zoomorphology*. Published online: DOI 10.1007/s00435-014-0235-6.
- Robison, B.H., Reisenbichler, K. R., Hunt, J. C., Haddock, S. H.** (2003). Light production by the arm tips of the deep-sea cephalopod *Vampyroteuthis infernalis*. *Biol. Bull.* **205**, 102-9.
- Ruby, E.G., Asato, L.M.** (1993). Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch. Microbiol.* **159**, 160-7.
- Ruby, E.G., Nealson, K.H.** (1976). Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. *Biol. Bull.* **151**, 574-86.

References

- Sansom, I.J., Smith, M.M., Smith, M.P.** (1996). Scales of thelodont and shark-like fishes from the Ordovician of Colorado. *Nature*. **379**, 628-30.
- Santos, J., Borges, T.** (2001). Trophic relationships in deep-water fish communities off Algarve, Portugal. *Fish. Res.* **51**, 337-41.
- Shimomura, O.** (1985). Bioluminescence in the sea: photoprotein systems. In: *Physiological adaptations of marine animals*. (ed. M.S. Laverack), pp 351-372. Cambridge: University Press.
- Shimomura, O.** (1987). Presence of coelenterazine in non-bioluminescent marine organisms. *Comp. Biochem. Physiol.* **86B**, 361-3.
- Shimomura, O.** (1995). The roles of the two highly unstable components F and P involved in the bioluminescence of euphausiid shrimps. *J. Biolumin. Chemilumin.* **10**, 91-101.
- Shimomura, O.** (2005). The discovery of aequorin and green fluorescent protein. *J. Microsc.* **217**, 3-15.
- Shimomura O.** (2006). Bioluminescence: chemical principles and methods. Singapore: World Scientific.
- Shimomura, O., Flood, P.R.** (1998). Luciferase of the scyphozoan medusa *Periphylla periphylla*. *Biol. Bull.* **194**, 244-52.
- Shimomura, O., Inoue, S., Johnson, F.H., Haneda, Y.** (1980). Widespread occurrence of coelenterazine in marine bioluminescence. *Comp. Biochem. Physiol.* **65B**, 435-7.
- Shimomura, O., Johnson, F.H.** (1975). Regeneration of the photoprotein aequorin. *Nature*. **256**, 236-8.
- Shimomura, O., Johnson, F.H.** (1978). Peroxidized coelenterazine, the active group in the photoprotein aequorin. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2611-15.
- Shimomura, O., Johnson, F.H., Saiga, Y.** (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell. Comp. Physiol.* **59**, 223-39.
- Shimomura, O., Kishi, Y., Inouye, S.** (1993). The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues. *Biochem. J.* **296**, 549-51.
- Sisneros, J.A., Tricas, T.C., Luer, C.A.** (1998). Response properties and biological function of the skate electrosensory system during ontogeny. *J. Comp. Physiol.* **183A**, 87-99.
- Slawinska, D., Slawinski, J.** (1983). Biological chemiluminescence. *Photochem. Photobiol.* **37**, 709-15.

References

- Stevens, J.D.** (2010). Epipelagic oceanic Elasmobranchs. In: *Sharks and their relatives II: biodiversity, adaptive physiology and conservation* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-35. Boca Raton (Florida): CRC Press.
- Straube, N., Iglésias, S.P., Sellos, D.Y., Kriwet, J., Schliewen, U.K.** (2010). Molecular phylogeny and node time estimation of bioluminescent lantern sharks (Elasmobranchii: Etmopteridae). *Mol. Phylogenet. Evol.* **56**, 905-17.
- Strum, J.M.** (1969a). Fine structure of the dermal luminescent organs, photophores, in the fish, *Porichthys notatus*. *Anat. Rec.* **164**, 433-62.
- Strum, J.M.** (1969b). Photophores of *Porichthys notatus*: ultrastructure of innervation. *Anat. Rec.* **164**, 463-78.
- Sweeney, B.M.** (1980). Intracellular source of bioluminescence. *Int. Rev. Cytol.* **68**, 173-95.
- Teale, F.W.J., Weber, G.** (1957). Ultraviolet fluorescence of the aromatic amino acids. *Biochem. J.* **65**, 476-82.
- Tebo, B.M., Scott Linthicum, D., Nealson, K.H.** (1979). Luminous bacteria and light emitting fish: ultrastructure of the symbiosis. *BioSystems.* **11**, 269-80.
- Tester, A.L., Kendall, J.I.** (1969). Morphology of the lateralis canal system in the shark genus *Carcharhinus*. *Pac. Sci.* **23**, 1-16.
- Theisen, B., Zeiske, E., Breucker, H.** (1986). Functional morphology of the olfactory organs in the spiny dogfish (*Squalus acanthias* L.) and the small-spotted catshark (*Scyliorhinus canicula* (L.)). *Acta Zool.* **67**, 73-86.
- Thomson, K.S., Simanek, D.E.** (1977). Body Form and Locomotion in Sharks. *Integr. Comp. Biol.* **17**, 343-54.
- Thompson, E.M., Toya, Y., Nafpaktitis, B.G., Goto, T., Tsuji, F.I.** (1988). Induction of bioluminescence capability in the marine fish, *Porichthys notatus*, by *Vargula* (crustacean) [¹⁴C]luciferin and unlabelled analogues. *J. Exp. Biol.* **137**, 39-51.
- Timmings, G.S., Jackson, S.K., Swartz, H.M.** (2001). The evolution of bioluminescent oxygen consumption as an ancient oxygen detoxification mechanism. *J. Mol. Evol.* **52**, 321-32.
- Tricas, T.C.** (2001). The neuroecology of the elasmobranch electrosensory world: why peripheral morphology shapes behavior. *Environ. Biol. Fishes.* **60**, 77-92.
- Tsuji, F.I., Barnes, A.T., Case, J. F.** (1972). Bioluminescence in the marine teleost, *Porichthys notatus*, and its induction in a non-luminous form by *Cypridina* (ostracod) luciferin. *Nature.* **237**, 515-6.

References

- Tsuji, F.I., Haneda, Y.** (1971). Luminescent system in a myctophid fish, *Diaphus elucens* Brauer. *Nature* **233**, 623-4.
- Udenfriend, S., Zaltzman, P.** (1962). Fluorescence characteristics of purines, pyrimidines, and their derivatives: Measurement of guanine in nucleic acid hydrolyzates. *Anal. Biochem.* **3**, 49-59.
- Underwood, C.J.** (2006). Diversification of the Neoselachii (Chondrichthyes) during the Jurassic and Cretaceous. *Paleobiology*. **32**, 215-35.
- Underwood, H.** (1989). The pineal and melatonin: regulators of circadian function in lower vertebrates. *Experientia*. **45**, 914-22.
- Vladimirov, Yu.A., Proskurnina, E.V.** (2009). Free radicals and cell chemiluminescence. *Biochemistry*. **74**, 1545-66.
- Wada, M., Azuma, N., Mizuno, N., Kurokura, H.** (2009). Transfer of symbiotic luminous bacteria from parental *Leiognathus nuchalis* to their offspring. *Mar. Biol.* **135**, 683-7.
- Ward, W.W., Seliger, H.H.** (1974). Extraction and purification of calcium-activated photoproteins from the ctenophores *Mnemiopsis* species and *Beroe ovata*. *Biochem.* **13**, 1491-9.
- Wetherbee, B.M., Cortés, E., Bizzarro, J.J.** (2012). Food consumption and feeding habits. In: *Biology of sharks and their relatives* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-29. Second edition, Boca Raton (Florida): CRC Press.
- White, W.T., Sommerville, E.** (2010). Elasmobranchs of tropical marine ecosystems. In: *Sharks and their relatives II: biodiversity, adaptive physiology and conservation* (Eds. J.C. Carrier, J.A. Musick, M.R. Heithaus), pp 3-35. Boca Raton (Florida): CRC Press.
- Widder, E.A.** (1998). A predatory use of counterillumination by the squaloid shark, *Isistius brasiliensis*. *Environ. Biol. Fishes.* **53**, 267-73.
- Widder, E.A.** (2010). Bioluminescence in the ocean: origins of biological, chemical, and ecological diversity. *Science*. **328**, 704-8.
- Wilson, T., Hastings, J.W.** (1998). Bioluminescence. *Annu. Rev. Cell Dev. Biol.* **14**, 197-230.
- Xavier, J.C., Vieira, C., Assis, C., Cherel, Y., Hill, S., Costa, E., Borges, T.C., Coelho, R.** (2012). Feeding ecology of the deep-sea lanternshark *Etmopterus pusillus* (Elasmobranchii: Etmopteridae) in the northeast Atlantic. *Sci. Mar.* **76**, 301-10.
- Young, R.E., Roper, C.F.E., Mangold, K., Leisman, G., Hochberg, F.G.** (1979). Luminescence from non-bioluminescent tissues in oceanic cephalopods. *Mar. Biol.* **53**, 69-77.

APPENDICES



Espegrend Marine Biological Station (M. Renwart)

**APPENDIX A: ASSAY ON PUTATIVE SHARK LUCIFERASE WITH
COELENTERAZINE, USING VARIOUS COFACTORS AND
EXTRACTION METHODS**

Method

Extraction: putative shark luciferase was extracted by crushing a piece of frozen skin in a volume of distilled water of tenfold its weight in a cooled agate mortar. Various components were added to water in order to improve extraction: (i) *Triton X-100* (1%) is a detergent with the property to solubilize proteins when bound to a membrane; (ii) *Bovine serum albumin* (1 g/L) help in stabilize proteins during extraction; and (iii) *Complete* (1:2000) is an antiprotease which avoid protein degradation. For some experiments, skin was frozen within the extraction liquid before crushing, and/or extract was centrifuged (16,000 g; 4°C; 3 min) after crushing, and supernatant was used in luminometry (based on Shimomura, 2006).

Luminometry: 20 µl of extract and 5 µl of coelenterazine (0.5 µM) were each respectively added to 80 µl and 95 µl of buffer. Diluted luciferase and luciferin were mixed together and the reaction was recoded within a luminometer (FB12, Berthold Detection System) during 3 minutes. Various cofactors (Ca^{++} , Mg^{++} , ATP, H_2O_2 , CN) and pH were essayed and the buffer used varied in consequence. All experiments were repeated on at least two shark specimens and controls were done in order to check the activity of coelenterazine (based on Shimomura, 2006).

Buffer used

Buffer A: TrisHCl 0.02 M; NaCl 0.5 M

Buffer B: TrisHCl 0.02 M; NaCl 0.5 M; CaCl_2 0.02 M

Buffer C: TrisHCl 0.02 M; NaCl 0.5 M; MgCl_2 0.02 M

Buffer D: TrisHCl 0.02 M; NaCl 0.25 M; MgCl_2 0.02 M

Buffer E: TrisHCl 0.02 M; NaCl 0.5 M; MgCl_2 0.02 M; ATP 0.005 M

Buffer F: TrisHCl 0.02 M; NaCl 0.25 M; MgCl_2 0.02 M; ATP 0.005 M

Buffer G: TrisHCl 0.02 M; NaCl 0.25 M; H_2O_2 0.0044 M

Buffer H: TrisHCl 0.02 M; NaCl 0.25 M; H₂O₂ 0.0044 M; K₃(FeCN₆) 0.0044 M

Results

All experiments are summarized in the following table. None of them gave satisfying results in term of light intensity and data repeatability.

Luciferase extraction			Luminometry		
Freezing	Extraction liquid	Centri.	Buffer	pH	Cofactor
x	H ₂ O	x	A	7.7	-
x	H ₂ O	✓	A	7.7	-
✓	H ₂ O	✓	A	7.7	-
x	H ₂ O/BSA	x	A	7.7	-
x	H ₂ O/BSA	✓	A	7.7	-
✓	H ₂ O/BSA	✓	A	7.7	-
x	H ₂ O/BSA/TX	x	A	7.7	-
x	H ₂ O/BSA/TX	✓	A	7.7	-
✓	H ₂ O/BSA/TX	✓	A	7.7	-
x	H ₂ O/COMP	✓	A	7.7	-
✓	H ₂ O/COMP	✓	A	7.7	-
x	H ₂ O/COMP/BSA	✓	A	7.7	-
✓	H ₂ O/COMP/BSA	✓	A	7.7	-
x	H ₂ O/COMP/TX	✓	A	7.7	-
✓	H ₂ O/COMP/TX	✓	A	7.7	-
x	H ₂ O/COMP/BSA/TX	✓	A	7.7	-
✓	H ₂ O/COMP/BSA/TX	✓	A	7.7	-
x	H ₂ O/COMP/BSA/TX	✓	B	7.7	Ca ⁺⁺
x	H ₂ O/COMP/BSA/TX	✓	C	7.7	Mg ⁺⁺
x	H ₂ O/COMP/BSA/TX	✓	D	7.7	Mg ⁺⁺
x	H ₂ O/COMP/BSA	✓	E	7.7	Mg ⁺⁺ /ATP
x	H ₂ O/COMP/BSA/TX	✓	E	7.7	Mg ⁺⁺ /ATP
x	H ₂ O/COMP/BSA/TX	✓	F	7.7	Mg ⁺⁺ /ATP
x	H ₂ O	✓	G	7.7	H ₂ O ₂

Appendices

x	H ₂ O	x	G	7.7	H ₂ O ₂
x	H ₂ O/BSA	✓	G	7.7	H ₂ O ₂
x	H ₂ O/BSA	x	G	7.7	H ₂ O ₂
x	H ₂ O/COMP	✓	G	7.7	H ₂ O ₂
x	H ₂ O/COMP	x	G	7.7	H ₂ O ₂
x	H ₂ O/COMP/BSA	✓	G	7.7	H ₂ O ₂
x	H ₂ O/COMP/BSA	x	G	7.7	H ₂ O ₂
x	Shark saline/BSA	✓	G	7.7	H ₂ O ₂
x	H ₂ O/COMP/BSA/TX	✓	G	7.7	H ₂ O ₂
x	H ₂ O/COMP/BSA	✓	H	7.7	H ₂ O ₂ /CN
x	H ₂ O/COMP/BSA/TX	✓	H	7.7	H ₂ O ₂ /CN
x	H ₂ O/BSA	✓	G	6	H ₂ O ₂
x	H ₂ O/BSA	✓	G	6.4	H ₂ O ₂
x	H ₂ O/BSA	✓	G	6.8	H ₂ O ₂
x	H ₂ O/BSA	✓	G	7.2	H ₂ O ₂
x	H ₂ O/BSA	✓	G	7.4	H ₂ O ₂
x	H ₂ O/BSA	✓	G	7.6	H ₂ O ₂
x	H ₂ O/BSA	✓	G	7.8	H ₂ O ₂

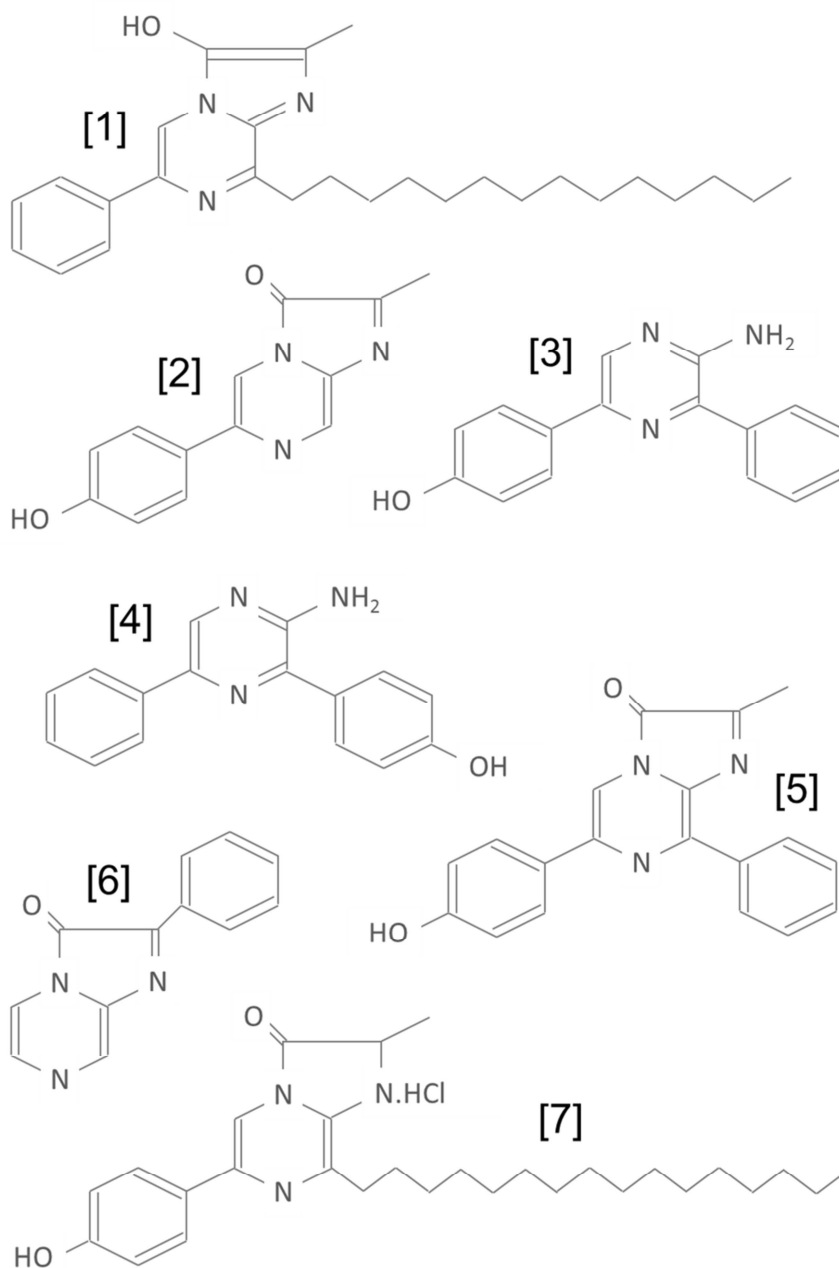
H₂O mmQ distilled water; BSA Bovine serum albumin; TX Triton X-100; COMP Complete; Centri Centrifugation.

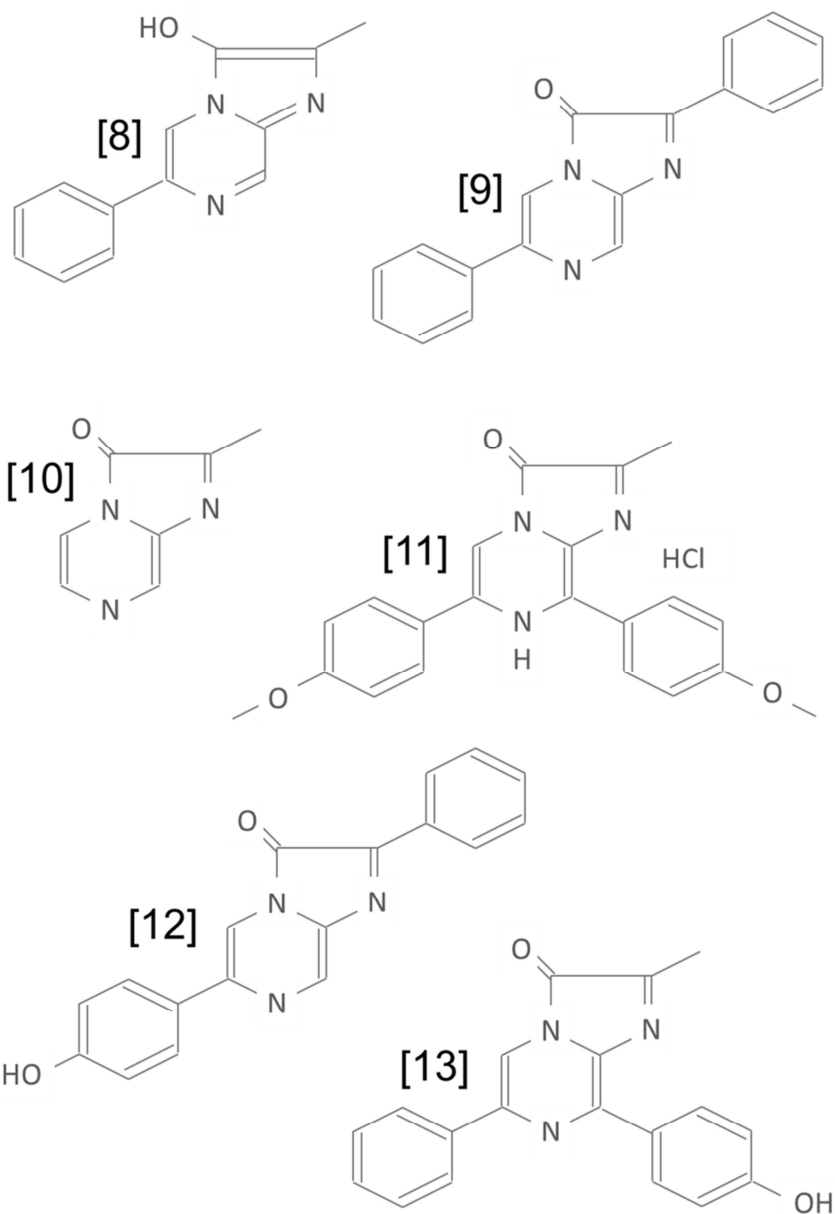
APPENDIX B: ASSAY ON PUTATIVE SHARK LUCIFERASE WITH COELENTERAZINE ANALOGUES

Method

Extraction: putative shark luciferase was extracted by crushing a piece of fresh skin in 200 µl of distilled water (mmQ H₂O) in a cooled glass mortar. Extracted liquid was then straight used in luminometry without centrifugation.

Luminometry: the 200 µl of extract were mixed with 100 µl of analogue solution and the reaction was recoded within a luminometer (FB12, Berthold Detection System) during 3 minutes. Because analogue purity was unknown, a stock solution was obtained by diluting a very small quantity of analogue powder (a tip extremity) in 500 µl of methanol.





Five μl of stock solution were diluted in 95 μl of a buffer (TrisHCl 0.02 M, NaCl 0.5 M, pH 7.5) to obtain working solution. Experiments were repeated with the 13 analogues (see molecules drawing), on at least two shark specimens for each of them.

Results

Coelenterazine analogues possess the same imidazopyrazinone core as coelenterazine and cypridinid luciferin (except for molecules n° 3 and 4), but present different branching. Because imidazopyrazinone core is responsible for bioluminescence in both luciferins, and because both of them are widespread in luminous organisms, it seemed possible for *E. spinax* to use a different luciferin but having an imidazopyrazinone core. Nevertheless, none of the 13 analogous assayed emitted light when in contact with shark luciferase extract.

APPENDIX C: ASSAY ON PUTATIVE SHARK LUCIFERASE WITH COELENTERAZINE STABILIZED/STORAGE FORMS

C1: ASSAY WITH DEHYDROCOELENTERAZINE

Method

Extraction: putative shark luciferase was extracted by crushing a piece of frozen skin in a volume of distilled water of tenfold its weight in a cooled agate mortar. Triton X-100 (1%) (a detergent with the property to solubilize proteins when bound to a membrane) was added to water for some experiments. Extract was centrifuged (16,000 g; 4°C; 3 min) and straight used in luminometry. Putative shark dehydrocoelenterazine was extracted by crushing a piece of frozen skin in a volume of methanol of tenfold its weight in a cooled agate mortar. Extract was centrifuged (16,000 g; 4°C; 3 min) and 50 µl of supernatant were mixed with 1 mg of sodium borohydride (NaBH₄) powder in order to transform dehydrocoelenterazine, if present, into coelenterazine (based on Shimomura, 2006).

Luminometry: 20 µl of both extracts were each added to 180 µl of buffer (TrisHCl 0.02 M; NaCl 0.5 M) and then mixed together within a luminometer (FB12, Berthold Detection System) where reaction was recorded during 3 minutes. Experiments were repeated with eleven buffer pH values and on at least two shark specimens for each of them.

Results

All experiments are summarized in the following table. None of them gave satisfying results in term of light intensity and data repeatability.

Luciferase extraction liquid			Buffer pH
H ₂ O	and	H ₂ O/TX	3
H ₂ O	and	H ₂ O/TX	4
H ₂ O	and	H ₂ O/TX	5
H ₂ O	and	H ₂ O/TX	6
H ₂ O	and	H ₂ O/TX	7
H ₂ O	and	H ₂ O/TX	7.5
H ₂ O	and	H ₂ O/TX	8
H ₂ O	and	H ₂ O/TX	9
H ₂ O	and	H ₂ O/TX	10
H ₂ O	and	H ₂ O/TX	11
H ₂ O	and	H ₂ O/TX	12

H₂O mmQ distilled water; TX Triton X-100

C2: ASSAY WITH COELENTERAZINE ENOL-SULFATE

Method

Extraction: putative shark luciferase was extracted by crushing a piece of frozen skin in a volume of distilled water of tenfold its weight in a cooled agate mortar. Triton X-100 (1%) was added to water for some experiments. Extract was centrifuged (16,000 g; 4°C; 3 min) and straight used in luminometry. Putative shark enol-sulfate coelenterazine was extracted by crushing a piece of frozen skin in a volume of methanol of tenfold its weight in a cooled agate mortar. Extract was centrifuged (16,000 g; 4°C; 3 min) and 10 µl of supernatant were heated with 100 µl of 0.5 M HCl at 95°C for 1 minutes. Sample was then cooled on ice and

neutralized by adding a very small quantity of sodium bicarbonate (NaHCO_3) powder (based on Shimomura, 2006).

Luminometry: 20 μl of both extracts were each added to 180 μl of buffer (TrisHCl 0.02 M; NaCl 0.5 M) and then mixed together within a luminometer (FB12, Berthold Detection System) where reaction was recorded during 3 minutes. Experiments were repeated using eleven buffer pH values and on at least two shark specimens for each of them.

Results

Experiment table is the same as for dehydrocoelenterazine. None of them gave satisfying results in term of light intensity and data repeatability.

APPENDIX D: ASSAY ON PUTATIVE SHARK PHOTOPROTEIN

Method

Extraction: putative shark photoprotein was extracted by crushing a piece of fresh skin in a volume of buffer of fivefold its weight in a cooled glass mortar, and not centrifuged.

Luminometry: 100 μl of extract were mixed either (i) with 1 ml of buffer containing one or two cofactor(s) or (ii), when using $\text{H}_2\text{O}_2/\text{Fe}^{++}$ as cofactors, with 100 μl of a buffer containing H_2O_2 in a first time and after 30 seconds, 200 μl of a buffer containing Fe^{++} were added. Reactions were recorded within a luminometer (FB12, Berthold Detection System) during 3 minutes. Experiments were repeated using three buffer pH values and on at least two shark specimens for each of them.

Buffer used

Buffer A: TrisHCl 0.02 M; NaCl 0.25 M; EDTA 0.015 M

Buffer B: TrisHCl 0.02 M; NaCl 0.25 M; CaCl_2 0.01 M

Buffer C: TrisHCl 0.02 M; NaCl 0.25 M; H_2O_2 0.006 M

Buffer D: TrisHCl 0.02 M; NaCl 0.25 M; FeSO_4 0.0001 M

Buffer E: TrisHCl 0.02 M; NaCl 0.25 M; MgCl_2 0.001 M; ATP 0.0001 M

Results

All experiments are summarized in the following table. None of them gave satisfying results in term of light intensity and data repeatability.

Photoprotein extraction liquid	Luminometry		
	Buffer	pH	Cofactor
Buffer A	B	6	Ca ⁺⁺
Buffer A	B	7,5	Ca ⁺⁺
Buffer A	B	9	Ca ⁺⁺
Buffer A	C/D	6	H ₂ O ₂ /Fe ⁺⁺
Buffer A	C/D	7,5	H ₂ O ₂ /Fe ⁺⁺
Buffer A	C/D	9	H ₂ O ₂ /Fe ⁺⁺
Buffer A	E	6	Mg ⁺⁺ /ATP
Buffer A	E	7,5	Mg ⁺⁺ /ATP
Buffer A	E	9	Mg ⁺⁺ /ATP

APPENDIX E: ASSAY ON PUTATIVE SHARK LUCIFERASE WITH PUTATIVE SHARK LUCIFERIN

E1: EXTRACTED FROM FRESH PHOTOPHORES

Method

Extraction: putative shark luciferase or luciferin were extracted by crushing a piece of fresh skin in a volume of distilled water, methanol or buffer of fivefold their weight in a cooled glass mortar, and not centrifuged. To improve luciferase extraction, Triton X-100 (0.1%) (a detergent with the property to solubilize proteins when bound to a membrane) or NaCl (1 M) were sometimes added to water. Luciferin was extracted either with methanol or with heated (95°C) distilled water.

Luminometry: two protocols were used in luminometry: (1) 20 µl of both extracts were each added to 180 µl of buffer and both diluted extracts were then mixed together within a luminometer (FB12, Berthold Detection System) where reaction was recorded during 3 minutes. (2) 50 µl of luciferin extract were added to 950 µl of buffer and then mixed with 100 of luciferase extract in the same luminometer. Experiments were repeated on at least two shark specimens.

Buffer used

Buffer A: TrisHCl 0.02 M; NaCl 0.25 M

Buffer B: TrisHCl 0.02 M; NaCl 0.25 M; EDTA 0.015 M

Buffer C: TrisHCl 0.02 M; NaCl 0.25 M; CaCl₂ 0.01 M

Buffer D: TrisHCl 0.02 M; NaCl 0.25 M; H₂O₂ 0.006 M

Buffer E: TrisHCl 0.02 M; NaCl 0.25 M; FeSO₄ 0.0001 M

Buffer F: TrisHCl 0.02 M; NaCl 0.25 M; MgCl₂ 0.001 M; ATP 0.0001 M

Results

All experiments are summarized in the following table. None of them gave satisfying results in term of light intensity and data repeatability.

Luciferase extraction liquid	Luciferin extraction liquid	Luminometry			
		Prot	Buffer	pH	Cofactor
H ₂ O	MeOH*	1	A	7.5	-
H ₂ O/TX	MeOH	1	A	7.5	-
H ₂ O/NaCl	MeOH	1	A	7.5	-
H ₂ O	Hot (95°C) H ₂ O	1	A	7.5	-
H ₂ O/TX	Hot (95°C) H ₂ O	1	A	7.5	-
H ₂ O/NaCl	Hot (95°C) H ₂ O	1	A	7.5	-
Buffer B	MeOH	2	C	7.5	Ca ⁺⁺
Buffer B	MeOH	2	D/E	7.5	H ₂ O ₂ /Fe ⁺⁺
Buffer B	MeOH	2	F	7.5	Mg ⁺⁺ /ATP

H₂O mmQ distilled water; TX Triton X-100; MeOH Methanol; Prot Protocol used.

* Extractions have also been done on stomach and intestinal contents in order to see if shark alimentary acquired its luciferin.

E2: EXTRACTED FROM FROZEN PHOTOPHORES

Method

Extraction: putative shark luciferase or luciferin were extracted by crushing a piece of frozen skin in a volume of distilled water or methanol of fivefold their weight in a cooled agate mortar. To improve luciferase/luciferin extraction, Triton X-100 (0.1 %) (a detergent with the property to solubilize proteins when bound to a membrane) or 2-mercaptoethanol (0.002 M) were sometimes added to water. 2-mercaptoethanol sometimes helps in luciferin stabilization or creates a reductive environment that helps in breaking disulfide bonds which might be involved in luciferin storage forms.

Luminometry: 20 µl of luciferase and 10 µl of luciferin were each respectively added to 200 µl of buffer. Both diluted extracts were then mixed within a luminometer (FB12, Berthold Detection System) where reaction was recorded during 3 minutes.

Buffer used

Buffer A: TrisHCl 0.02 M; NaCl 0.5 M

Buffer B: TrisHCl 0.02 M; NaCl 0.5 M; CaCl₂ 0.01 M

Buffer C: TrisHCl 0.02 M; NaCl 0.5 M; MgCl₂ 0.01 M

Buffer D: TrisHCl 0.02 M; NaCl 0.5 M; ATP 0.001 M

Buffer E: TrisHCl 0.02 M; NaCl 0.5 M; CaCl₂ 0.01 M; ATP 0.001 M

Buffer F: TrisHCl 0.02 M; NaCl 0.5 M; MgCl₂ 0.01 M; ATP 0.001 M

Buffer G: TrisHCl 0.02 M; NaCl 0.5 M; FeSO₄ 0.01 M

Results

All experiments are summarized in the following table. None of them gave satisfying results in term of light intensity and data repeatability.

Appendices

Luciferase extraction		Luciferin extraction		Luminometry		
Extr. liquid	Centri.	Extr. liquid	Centri.	Buffer	pH	Cofactor
H ₂ O	x	MeOH	x	A	7.5	-
H ₂ O	✓	MeOH	✓	A	7.5	-
H ₂ O/TX	✓	MeOH	✓	A	7.5	-
H ₂ O	✓	MeOH	✓	A	3	-
H ₂ O	✓	MeOH	✓	A	3.5	-
H ₂ O	✓	MeOH	✓	A	4	-
H ₂ O	✓	MeOH	✓	A	4.5	-
H ₂ O	✓	MeOH	✓	A	5	-
H ₂ O	✓	MeOH	✓	A	5.5	-
H ₂ O	✓	MeOH	✓	A	6	-
H ₂ O	✓	MeOH	✓	A	6.5	-
H ₂ O	✓	MeOH	✓	A	7	-
H ₂ O	✓	MeOH	✓	A	7.5	-
H ₂ O	✓	MeOH	✓	A	8	-
H ₂ O	✓	MeOH	✓	A	8.5	-
H ₂ O	✓	MeOH	✓	A	9	-
H ₂ O	✓	MeOH	✓	A	10	-
H ₂ O	✓	MeOH	✓	A	11	-
H ₂ O	✓	MeOH	✓	A	12	-
H ₂ O	x	MeOH	✓	A	3	-
H ₂ O	x	MeOH	✓	A	4	-
H ₂ O	x	MeOH	✓	A	5	-
H ₂ O	x	MeOH	✓	A	6	-
H ₂ O	x	MeOH	✓	A	7	-
H ₂ O	x	MeOH	✓	A	8	-
H ₂ O	x	MeOH	✓	A	9	-
H ₂ O	x	MeOH	✓	A	10	-
H ₂ O	x	MeOH	✓	A	11	-
H ₂ O	x	MeOH	✓	A	12	-
H ₂ O/TX	✓	MeOH	✓	A	3	-
H ₂ O/TX	✓	MeOH	✓	A	4	-
H ₂ O/TX	✓	MeOH	✓	A	5	-

Appendices

H ₂ O/TX	✓	MeOH	✓	A	6	-
H ₂ O/TX	✓	MeOH	✓	A	7	-
H ₂ O/TX	✓	MeOH	✓	A	7.5	-
H ₂ O/TX	✓	MeOH	✓	A	8	-
H ₂ O/TX	✓	MeOH	✓	A	9	-
H ₂ O/TX	✓	MeOH	✓	A	10	-
H ₂ O/TX	✓	MeOH	✓	A	11	-
H ₂ O/TX	✓	MeOH	✓	A	12	-
H ₂ O	✓	MeOH/TX	✓	A	3	-
H ₂ O	✓	MeOH/TX	✓	A	4	-
H ₂ O	✓	MeOH/TX	✓	A	5	-
H ₂ O	✓	MeOH/TX	✓	A	6	-
H ₂ O	✓	MeOH/TX	✓	A	7	-
H ₂ O	✓	MeOH/TX	✓	A	7.5	-
H ₂ O	✓	MeOH/TX	✓	A	8	-
H ₂ O	✓	MeOH/TX	✓	A	9	-
H ₂ O	✓	MeOH/TX	✓	A	10	-
H ₂ O	✓	MeOH/TX	✓	A	11	-
H ₂ O	✓	MeOH/TX	✓	A	12	-
H ₂ O	✓	MeOH/MERC	✓	A	3	-
H ₂ O	✓	MeOH/MERC	✓	A	4	-
H ₂ O	✓	MeOH/MERC	✓	A	5	-
H ₂ O	✓	MeOH/MERC	✓	A	6	-
H ₂ O	✓	MeOH/MERC	✓	A	7	-
H ₂ O	✓	MeOH/MERC	✓	A	8	-
H ₂ O	✓	MeOH/MERC	✓	A	9	-
H ₂ O	✓	MeOH/MERC	✓	A	10	-
H ₂ O	✓	MeOH/MERC	✓	A	11	-
H ₂ O	✓	MeOH/MERC	✓	A	12	-
H ₂ O	x	MeOH/MERC	✓	A	3	-
H ₂ O	x	MeOH/MERC	✓	A	4	-
H ₂ O	x	MeOH/MERC	✓	A	5	-
H ₂ O	x	MeOH/MERC	✓	A	6	-
H ₂ O	x	MeOH/MERC	✓	A	7	-
H ₂ O	x	MeOH/MERC	✓	A	8	-

Appendices

H ₂ O	x	MeOH/MERC	✓	A	9	-
H ₂ O	x	MeOH/MERC	✓	A	10	-
H ₂ O	x	MeOH/MERC	✓	A	11	-
H ₂ O	x	MeOH/MERC	✓	A	12	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	3	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	4	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	5	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	6	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	7	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	7.5	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	8	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	9	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	10	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	11	-
H ₂ O/TX	✓	MeOH/MERC	✓	A	12	-
H ₂ O	✓	MeOH	✓	B	3	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	4	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	5	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	6	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	6.5	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	7	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	7.5	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	8	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	B	9	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	3	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	4	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	5	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	6	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	6.5	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	7	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	7.5	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	8	Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	B	9	Ca ⁺⁺
H ₂ O	✓	MeOH	✓	C	3	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	4	Mg ⁺⁺

Appendices

H ₂ O	✓	MeOH	✓	C	5	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	6	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	6.5	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	7	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	7.5	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	8	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	C	9	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	3	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	4	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	5	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	6	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	6.5	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	7	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	7.5	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	8	Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	C	9	Mg ⁺⁺
H ₂ O	✓	MeOH	✓	D	6	ATP
H ₂ O	✓	MeOH	✓	D	6.5	ATP
H ₂ O	✓	MeOH	✓	D	7	ATP
H ₂ O	✓	MeOH	✓	D	7.5	ATP
H ₂ O	✓	MeOH	✓	D	8	ATP
H ₂ O/TX	✓	MeOH	✓	D	6	ATP
H ₂ O/TX	✓	MeOH	✓	D	6.5	ATP
H ₂ O/TX	✓	MeOH	✓	D	7	ATP
H ₂ O/TX	✓	MeOH	✓	D	7.5	ATP
H ₂ O/TX	✓	MeOH	✓	D	8	ATP
H ₂ O	✓	MeOH	✓	E	7	ATP/Ca ⁺⁺
H ₂ O	✓	MeOH	✓	E	7.5	ATP/Ca ⁺⁺
H ₂ O	✓	MeOH	✓	E	8	ATP/Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	E	7	ATP/Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	E	7.5	ATP/Ca ⁺⁺
H ₂ O/TX	✓	MeOH	✓	E	8	ATP/Ca ⁺⁺
H ₂ O	✓	MeOH	✓	F	7	ATP/Mg ⁺⁺
H ₂ O	✓	MeOH	✓	F	7.5	ATP/Mg ⁺⁺
H ₂ O	✓	MeOH	✓	F	8	ATP/Mg ⁺⁺

Appendices

H ₂ O/TX	✓	MeOH	✓	F	7	ATP/Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	F	7.5	ATP/Mg ⁺⁺
H ₂ O/TX	✓	MeOH	✓	F	8	ATP/Mg ⁺⁺
H ₂ O	✓	MeOH	✓	G	3	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	4	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	5	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	6	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	6.5	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	7	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	7.5	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	8	Fe ⁺⁺
H ₂ O	✓	MeOH	✓	G	9	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	3	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	4	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	5	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	6	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	6.5	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	7	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	7.5	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	8	Fe ⁺⁺
H ₂ O/TX	✓	MeOH	✓	G	9	Fe ⁺⁺

Extr Extraction; *H₂O* mmQ distilled water; *TX* Triton X-100; *MeOH* methanol; *MERC* 2-mercaptoethanol; *Centri* centrifugation.