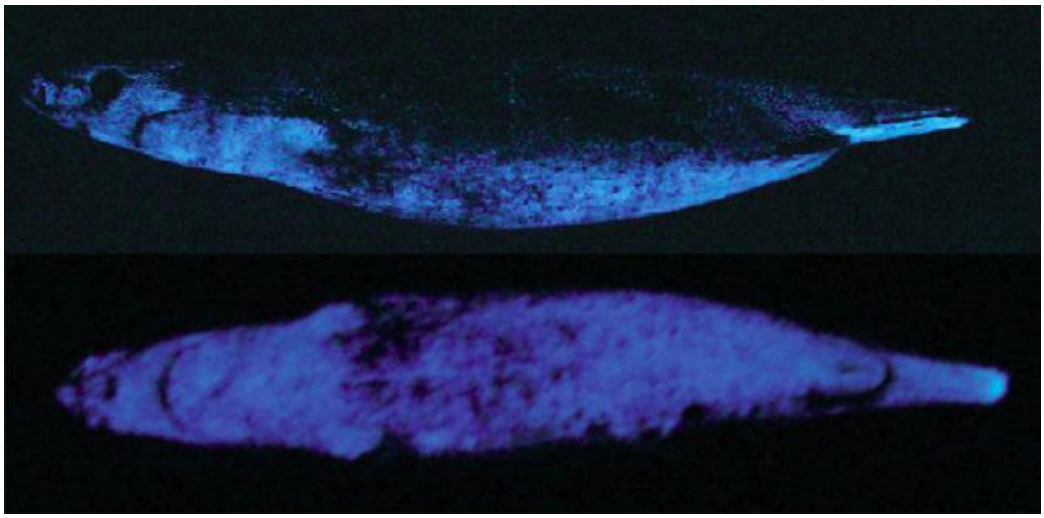
Université Catholique de Louvain

Faculté des Sciences

Laboratoire de Biologie Marine

Extraocular photoreception and photophore ontogeny during embryogenesis of a luminous shark from Dalatiidae family, *Squaliolus aliae*



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Abstract

Bioluminescence, the emission of visible light by a living organism through a chemical reaction, is a common phenomenon in marine organisms, especially in deep water where light is very weak or non-existent. Bioluminescence can have a variety of uses for the organisms that possess it. However, there are three main strategies using bioluminescence: i) food research; ii) the ability to protect oneself or avoid predator attacks; iii) intraspecific communication, especially when searching for a sexual partner. Among the Elasmobranchs, there are two families of sharks that have acquired the ability to emit light, the Etmopteridae and the Dalatiidae. These luminous sharks have thousands of luminous organs called photophores that allow them to produce light. One of the main functions of bioluminescence is to camouflage their silhouette by imitating the ambient light on their ventral surface in order to camouflage themselves from potential predators coming from the bottom. This technique is called counterillumination.

For this camouflage to be as effective as possible, it is necessary to be able to adjust the light emitted according to the ambient light. For this adjustment to be well managed, the shark must be able to perceive not only the ambient light, but also the luminescence emitted by itself in order to coordinate the two.

Recent studies have shown the presence of an extraocular opsin, encephalopsin (Opn 3), in the photophores of *Etmopterus spinax*, a luminous shark of the family Etmopteridae. This extraocular opsin allows the perception of light at the level of luminous organs, suggesting that this shark can perceive its own bioluminescence. Furthermore, it has been shown that in *E. spinax*, encephalopsin, located in a structure acting as an iris in the photophore (the ILS)), acts on the pigment cells of the ILS to increase light emission when the opsin is excited by light. This phenomenon suggests that there is indeed a feedback between the perception of light and the emission of light.

During this thesis, the presence of this same extraocular opsin in *Squaliolus aliae*, a species of luminous shark in the Dalatiidae family, was demonstrated through immunohistology. Encephalopsin is also present in the ILS of the photophores of this species, suggesting that it acts in the same way as in *E. spinax* on the regulation of light emission. The appearance of this opsin during the shark's embryonic development, while still in its mother's uterus, suggests that this opsin does allow the shark to perceive its own luminescence.

The ontogeny of the luminescent organ during embryonic development has also been studied for *S. aliae* and appears to be approximately identical to that already shown for *E. spinax*.

Résumé

La bioluminescence, l'émission de lumière visible par un organisme vivant au moyen d'une réaction chimique, est un phénomène courant dans les organismes marins, surtout en eau profonde où la lumière est très faible ou inexistante. La bioluminescence peut avoir diverses utilisations pour les organismes qui la possèdent. Cependant, il existe trois stratégies principales utilisant la bioluminescence : i) la recherche de nourriture ; ii) la capacité à se protéger ou à éviter les attaques de prédateurs ; iii) la communication intraspécifique, en particulier lors de la recherche d'un partenaire sexuel. Parmi les Elasmobranches, il existe deux familles de requins qui ont acquis la capacité d'émettre de la lumière, les Etmopteridae et les Dalatiidae. Ces requins lumineux possèdent des milliers d'organes lumineux appelés photophores qui leur permettent de produire de la lumière, dont l'une des principales fonctions est de camoufler leur silhouette en imitant la lumière ambiante sur leur surface ventrale afin de se camoufler des prédateurs potentiels venant du fond. Cette technique est appelée contre-illumination.

Pour que ce camouflage soit le plus efficace possible, il est nécessaire de pouvoir ajuster la lumière émise en fonction de la lumière ambiante. Pour que cette régulation soit bien gérée, le requin doit être capable de percevoir non seulement la lumière ambiante, mais également la luminescence émise par lui-même afin de coordonner les deux.

Des études récentes ont montré la présence d'une opsine extraoculaire, l'encéphalopsine (Opn 3), dans les photophores d'*Etmopterus spinax*, un requin lumineux de la famille des Etmopteridae. Cette opsine extraoculaire permet la perception de la lumière au niveau des organes lumineux, suggérant que ce requin est capable de percevoir sa propre bioluminescence. De plus, il a été montré que chez *E. spinax*, l'encéphalopsine, située au niveau d'une structure agissant comme une iris au niveau du photophore (l'ILS)), agit sur les cellules pigmentaires de l'ILS afin d'augmenter l'émission de lumière lorsque l'opsine est excitée par la lumière. Ce phénomène suggère qu'il y a effectivement une rétroaction entre la perception de la lumière et l'émission de lumière.

Au cours de ce mémoire, la présence de cette même opsine extraoculaire chez *Squaliolus aliae*, une espèce de requin lumineux de la famille des Dalatiidae, a été démontrée grâce à l'immunohistologie. L'encéphalopsine est également présente au niveau de l'ILS des photophores de cette espèce, ce qui suggère qu'elle agit de la même manière que chez *E. spinax* sur la régulation de l'émission lumineuse. L'apparition de cette opsine au cours du développement embryonnaire du requin, alors qu'il est encore dans l'utérus de sa mère, suggère que cette opsine permet bien au requin de percevoir sa propre luminescence.

L'ontogenèse de l'organe lumineux au cours du développement embryonnaire a également été étudiée pour *S. aliae* et semble être sensiblement identique à celle déjà montrée chez *E. spinax*.

Table of contents

Introduction

Bioluminescence

1. General

Bioluminescence is fairly common in oceans, particularly in the deep-water organisms. Haddock et al. define it as "the emission of visible light by an organism as a result of a natural chemical reaction" (Haddock et al., 2010). This phenomenon is quite widespread in marine organisms and more generally in deep-sea organisms for which it is the predominant form of communication (Haddock et al., 2010). Scientist estimate that more than 80% of bioluminescent organisms live in oceans, where the great depths induce dim light or even total darkness in stable environmental condition (Widder, 2010). Indeed, fewer luminescent organisms have been identified in freshwater where conditions are less stable than in the oceans. Furthermore, except for some specific sites such as lake Baikal in Russia, the depth of lakes or rivers is not great enough to have an environment in continuous darkness (Haddock et al., 2010). In the terrestrial ecosystem, even less luminescent organisms have been found. The permanent darkness of the environment has promoted the emergence of bioluminescence in the life history of most major marine phyla, from fishes to bacteria, and it seems that the ability to produce light has evolved between 40 and 50 times within existing organisms (Haddock et al., 2010; Widder, 2010).

In marine environment, the absorption of light by water depending on the depth prevents long wavelengths from reaching great depths, leaving the only sunlight visible in deep water in the blue or green spectrum. This has an impact on luminescent organisms, which therefore emit light mainly in the blue-green spectrum allowing them to be seen by organisms at such depths (Widder, 2010). However, it exists organisms emitting luminescence in all the visible wavelengths such as some dragonfishes that produce light in the red spectrum (Herring and Cope, 2005).

2. Biochemical mechanisms

Bioluminescence results from a biochemical reaction comprising a substrate, and an enzyme which promotes the reaction (Rees et al., 1998). Unlike fluorescence and phosphorescence, the chemical reaction involved in bioluminescence must be sufficiently energetic to produce a singlet molecule in an excited state. This excited molecule will emit a visible photon when it returns to its stable state. Such energy can be found in an oxidation reaction comprising a molecular oxygen which will break a peroxide bond (Widder, 2010). There are worldwide a remarkable number of enzymes and substrates which can be used for this biochemical reaction, but the most commonly used system is the "luciferin-luciferase" type. This system includes a species-specific luciferase, the enzyme, which will allow the

oxidation of a luciferin, the protein substrate, in presence of oxygen. The excited luciferin releases a photon to relax to its stable state.

Contrary to luciferase, which is species-specific, there are different types of luciferin which can be common to different species. For instance, coelenterazine, a widely found in luminous marine organisms luciferin (Shimomura et al., 1980; Rees et al., 1998; Shimomura and Teranishi, 2000), is present at least in nine phyla which are not necessarily phylogenetically related suggesting that coelenterazine, which has strong antioxidant properties, could be the evolutionary core of most bioluminescent systems (Rees et al., 1998). Indeed, originally, the luciferin substrate may have been used to protect organisms against reactive oxygen species such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-) with which it is highly reactive. Such reactive oxygen species are generated in presence of light and high oxygen concentration and could cause damages to the organism. However, when marine organisms have migrated to deeper waters where light irradiance and oxygen levels are lower, the function of the luciferin substrate has shifted from antioxidant to chemiluminescent properties (Rees et al., 1998; Widder, 2010). In deep water, where light is dim or even absent, light emission becomes more useful than antioxidant protection due to lower reactive oxygen species production.

In the oceans, at least four different types of luciferin have been found to be responsible for most of the light production (Haddock et al., 2010). However, many luciferins remain poorly known, if not totally unknown, such as the one involved in the luminescence of luminous sharks (Renwart and Mallefet, 2013). In some cases, luciferin and a catalytic protein, the equivalent of luciferase, are bound together forming a macromolecule called photoprotein. The type of macromolecule will facilitate the oxidation reaction by preloading molecular oxygen before carrying out its catalytic activity. In this system, the photoprotein requires a cofactor (often a calcium ion) to react and emit light.

Not every luminescent organism can produce their own substrate, such as coelenterazine, to produce luminescence. So, some organisms will have to provide the necessary substrate from their environment through food intake, which will make their light production dietary dependant (Frank et al., 1984; Mensinger and Case, 1991; Haddock et al., 2001; Widder,2010). This has been demonstrated for instance for Ophiuroids (Mallefet, personal communication), euphausiids (Haddock et al., 2010), cephalopods (Young et al., 1979 a), or fishes (Mallefet and Shimomura, 1995).

3. Different uses

Bioluminescence can have many functions (Figure 1) and it is quite common that one individual uses multiple applications of luminescence (Widder, 1998, 2010; Haddock et al., 2010; Claes et al., 2013). In oceans, below 1000 meters depth, the visible light disappears. So, to find food, partners, to escape or hide from predators, luminous organisms use light in different ways. The most widely used strategy seems to be the use of bioluminescence as a camouflage. This strategy, called counterillumination, is mainly used by pelagic organisms, which live in the water column with no place to hide. It allows them

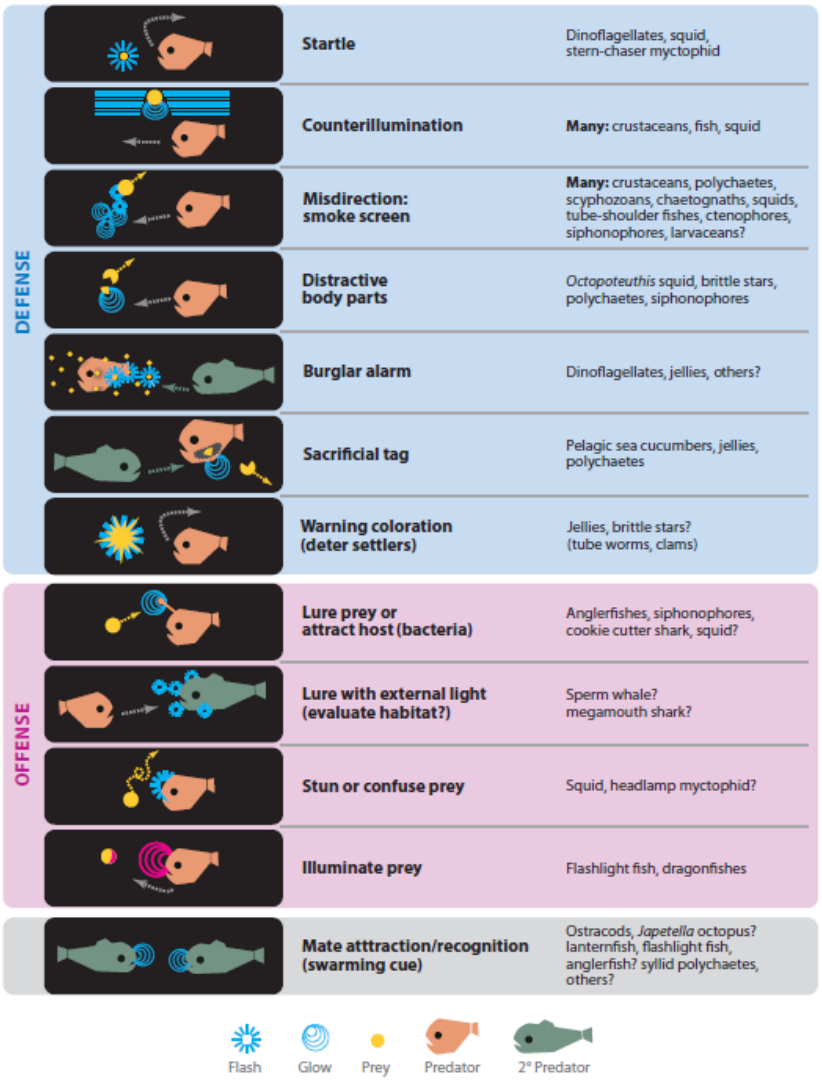

Figure 1: Schematic representations of the different bioluminescence uses in marine environment. Luminescence can be used for defence (blue), offense (red) and intraspecific communication (grey) (Haddock et al., 2010)

to dissimulate their silhouette and avoid being spotted by predators below. Therefore, they emit a ventral luminescence whose luminous intensity varies according to the environment light intensity, spectral and angular distribution which will hide their silhouette (Harper and Case, 1999; Jones and Nishiguchi, 2004; Claes and Mallefet, 2010b; Haddock et al., 2010; Widder, 2010). It has been shown,

for instance, that the velvet belly lantern shark (*Etmopterus spinax*) uses bioluminescence as a camouflage by counterillumination (Claes et al., 2010a; Claes and Mallefet, 2010b).

Another way to use light emission as a defensive strategy consist in the production of a luminescent smoke to blur or attract the predator in one direction while the potential prey disappears in the opposite direction (Chan et al., 2008; Haddock et al., 2010). Studies have shown that the deep-water pandalid shrimp (*Heterocarpus sibogae*) uses this stratagem by secreting the luminescent substrates from its mouth to attract potential predator on the luminous zone and escape it (Chan et al., 2008). Another kind of defence strategy using luminescence is the aposematic function. This strategy consists of deterring predators by bioluminescence. For instance, some brittle-stars such as *Ophiopsila riisei* emits luminescence to deter crustacean predators (Grober, 1988). *E. spinax* and *E. molleri* also use bioluminescence as an aposematic tool in addition to its use as a camouflage by counterillumination. With their luminous organs, they would highlight their dorsal spine, showing to predators that it can be dangerous to attack them and thus deter them (Claes et al., 2013; Duchatelet et al., 2019c).

Bioluminescence is not always used as defence mechanism but is also used as an offensive strategy by predators. Here again, various predatory strategies are used. The most known strategy is the one used by anglerfishes which exploit luminescent bacteria cultured in an organ to bait their prey closer to their mouth (Pietsch, 2009). Some cephalopods also use this strategy by illuminating one of their arms to lure the prey (Johnsen et al., 1999; Robison et al., 2003). Another predatory strategy is based on stunning or confusing effect on prey by using bright light (Haddock et al., 2010). The squid *Tamingia* uses this technique to hunt. It flashes its tentacles just before attacking to blur its prey but also to measure the well distance with prey in the dim light environment to successfully catch its target (Kubodera et al., 2007). A mix of hide strategy and prey attraction may also be used by some predators. It is suggested that the cookie-cutter shark, *Isistius brasiliensis*, combines both counterillumination and prey attraction according to the non-luminescent pigmented band beneath its jaw. The band is well seen by prey, looking as small prey (i.e. shadow made by the band contrasting with the environment), while the rest of the body is camouflaged by counterillumination (Widder, 1998; Warrant and Lockett, 2003). Finally, still another use of luminescence by predators is to illuminate the prey. Most deep-water bioluminescent organisms produce blue-green light, but some of them emit light in the red spectra. It is the case of species of three genera of midwater dragonfishes, *Malacosteus*, *Pachystomias* and *Aristostomias*. These fishes emit light at a wavelength of about 705 nm, in the red spectrum, which is not detected by other organisms at these depths. This enables the fish to illuminate their prey in order to find and attack them more easily (Herring and Cope, 2005).

The last identified bioluminescence use is the intraspecific communication. This application is still poorly known in marine environment, but well known in the terrestrial environment, notably for intraspecific and sexual communication in fireflies (Buck and Case, 2002; Woods et al., 2007; Takatsu et al., 2012). However, such intraspecific and sexual communications using luminescence have been studied in some marine species, such as Caribbean ostracods populations, where males attract females by luminescence (Morin, 1986; Morin and Cohen, 2010), or ponyfish populations where light emitted could be used for spatial organization within the population, courtship, predatory avoidance, etc (Woodland et al., 2002). Dragonfish species could also use light emitted in the red spectrum, as seen above, for intraspecific communication (Herring and Cope, 2005), while the planktonic annelid *Tomopteris helgolandica* emits yellow light serving for such intraspecific communication (Gouveneaux et al., 2018). In the lantern shark *E. spinax*, a sexual dimorphism has also been observed in the luminous pelvic area, where the sexual organs are located, suggesting a putative role of bioluminescence in mate recognition and sexual behaviour (Claes and Mallefet, 2010a).

4. Types of photophores

A photophore is a photogenic organ composed of one or more photocytes, the luminescent cell, associated with muscles, filters, optical machineries, etc, which are used to adjust, filter, reflect, or refract mechanically the light emission (Herring, 1985; Claes and Mallefet, 2010a; Widder, 2010). Among pluricellular organisms, two types of photophores exist: (i) extrinsic photophores (Figure 2A) and (ii) intrinsic photophores (Figure 2B, C). In extrinsic photophore, a luminescent bacterial population allows the organism to produce a symbiotic luminescence. These photophores are mainly present in deep-sea squids and bony fishes (Herring, 1985; Nyholm and McFall-Ngai, 2004; Claes and Mallefet, 2009a, 2010a; Mallefet et al., 2019). Intrinsic photophores, which are the most common in marine organisms, possess an endogenous luminescent system which allows the organisms to produce light by itself (Claes and Mallefet, 2010a; Haddock et al., 2010).

To use luminescence efficiently, this one must be well modulated, depending on the situation, the environment, the timing, and so forth. To this end, in addition to mechanical adjustments of the photophore, it exists (i) hormonal (figure 2C) and (ii) neural (figure 2B) regulations (physiological adjustments). For intrinsic photophores, luminescence regulation in Osteichthyes is mainly under neural control while in Chondrichthyes it is under hormonal control (Claes and Mallefet, 2009a, 2010a; Duchatelet et al., 2019 d); Mallefet et al., 2019). Different types of systems also exist for extrinsic photophores to modulate light emission (Haygood, 1993; Munk, 2001; Claes and Mallefet, 2010a).

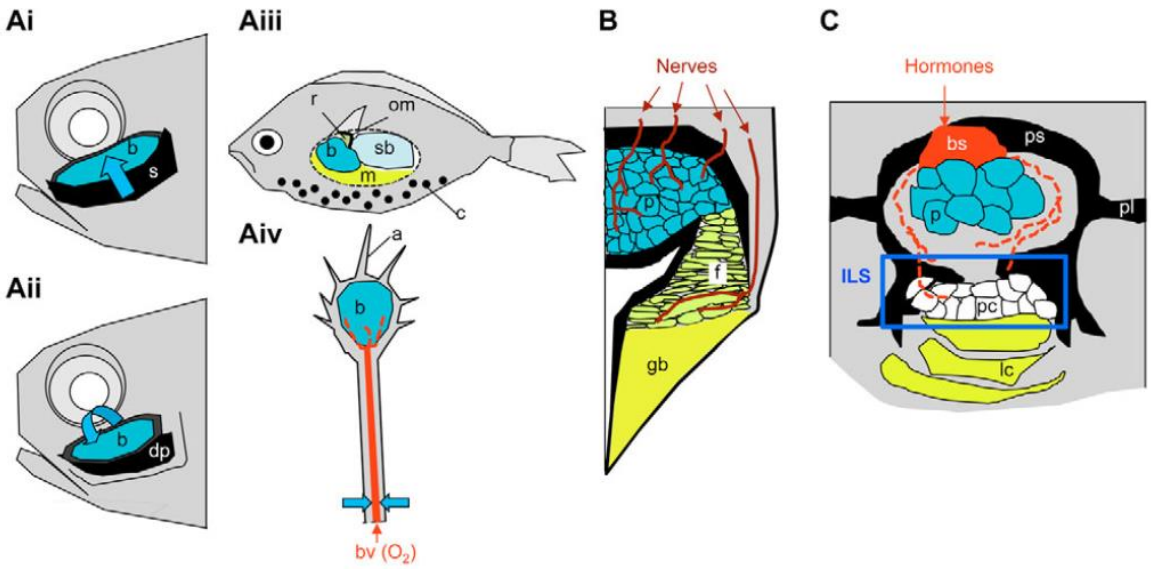

Figure 2: Luminescence control mechanisms in fishes. (A) Symbiotic luminescence (bony fishes). (Ai) The luminescence from the bacterial photogenic organ (b) of *Photoblepharon palpebratus* is controlled by the movement (blue arrow) of a dark shutter (s), which acts as an inverse eyelid. (Aii) The luminescence from the bacterial photogenic organ (b) of *Anomalops katoptron* is controlled by the movement (blue arrow) of the whole photogenic organ, which can rotate downward into a dark pocket (dp). (Aiii) Light organ system of a leiognathid. The bacterial photogenic organ (b) opens laterally into the oesophagus and its light emission is transmitted to the ventral surface by a translucent muscle (m). Expansion of chromatophores (c) on the ventral surface prevents the light from being emitted to the outside. (Aiv) A modulation (blue arrows) of the blood supply to the bacterial photogenic esca (b) of a ceratioid anglerfish could allow control of its luminescence by varying the amount of oxygen (O2) available for the chemiluminescent reaction. (B) Photophore section of the hatchetfish *Argyropelecus hemigymnus* (intrinsic luminescence; bony fishes). Like other luminous bony fishes endowed with intrinsic photophores, *A. hemigymnus* exhibits a neurally controlled luminescence. (C) Photophore section of the lantern shark *Etmopterus spinax* (intrinsic luminescence; cartilaginous fishes). The photophore luminescence from *E. spinax* is hormonally controlled, probably via movement of the pigmented cells (pc) comprising the iris-like structure (ILS, blue rectangle). Dashed red lines represent hypothetical movement of the blood inside the photogenic organ. The luminous tissue is shown in blue. a, appendage; bv, blood vessel; bs, blood sinus; f, filter; gb, gelatinous body; lc, lens cell; om, opaque membrane; p, photocyte; pl, pigmented layer; ps, pigmented sheath; r, guanine reflector; s, swimbladder (Claes and Mallefet, 2010a)

Bioluminescence in sharks

1. Families

Among all bioluminescent organisms, fishes are the only vertebrates capable of producing bioluminescence (Renwart and Mallefet, 2013). In fishes, Osteichthyes are the most numerous to be bioluminescent, with 70% of the deep-sea representants against only 6% of the deep-sea Chondrichthyes that can emit luminescence. Early studies suggest that bioluminescence would be appeared at least three times in the history of cartilaginous fishes: once in in basal Batoidea and twice in Squalean sharks (Figure 3). The first occurrence of bioluminescence in sharks would be in the Etmopteridae family as a response to the need for species recognition in a dim light environment, and the second one would be in Dalatiidae as a response to the need for effective camouflage in a pelagic

environment with no place to hide (Claes and Mallefet, 2009a). However, more recent researches based on the comparison of hormonal regulation of luminescence between Dalatiidae and Etmopteridae suggest that bioluminescence may have appeared only once in Squalean sharks (Claes et al., 2012).

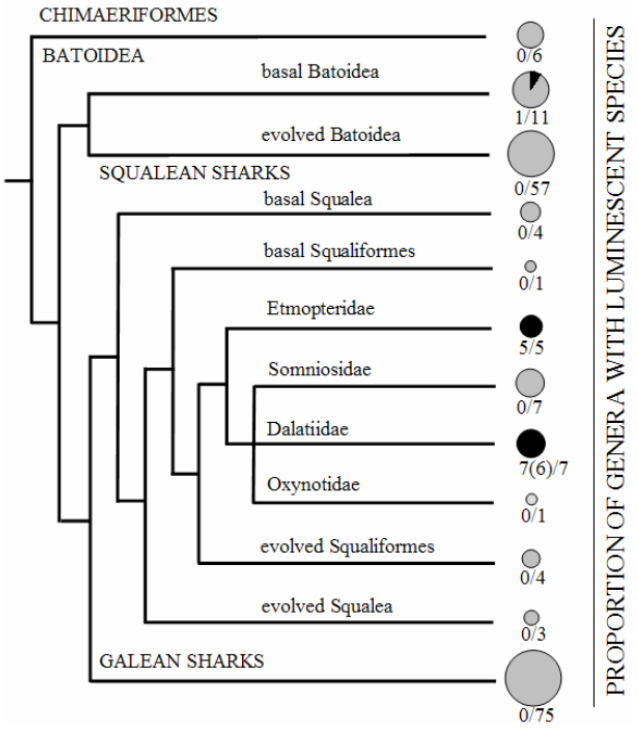

Figure 3: Cladogram showing the occurrence of bioluminescence in cartilaginous fishes. Circles to the right indicate the proportion of luminous genera containing luminous species in a taxonomic group (black shade, luminous; grey shade, non-luminous). Circles are scaled to the number of genera in a given taxonomic group (Claes and Mallefet, 2009 a)

As indicated above, two shark families are bioluminescent, Etmopteridae and Dalatiidae. In the Etmopteridae family, all five genera can emit luminescence while in the Dalatiidae family, counting seven genera, at least six are luminous (Figure 3). The seventh of the genus Dalatiidae is known by only with some luminescent characteristics, but additional information is needed to confirm its ability to produce light. The two families of luminescent sharks have different intrinsic photophore conformations (figure 4), which are smaller, simpler and more numerous than those of Osteichthyes. Dalatiidae have smaller photophores (100 µm) than Etmopteridae (150 µm), and they are also less complex than those of Etmopteridae. Dalatiidae photophores (figure 4a) are composed of a single photogenic cell, the photocyte containing the photogenic vesicles, with a pigmented sheath and on the apical surface, a lens composed of small cells (Claes and Mallefet, 2009a; Claes et al., 2012). Etmopteridae photophores (figure 4b), on the other hand, are more complex and consist of a group of six to thirteen photogenic cells surrounded by a pigmented circular black sheath (Claes and Mallefet, 2008; Renwart et al., 2014). The lens is composed by one or several great convex cells. Between this lens and the photocytes, a group of cells, including chromatophore-like cells, form an iris-like structure (ILS) (Claes and Mallefet, 2008, 2009a; Renwart et al., 2014, 2015). The photophore pattern of Etmopteridae is also more complex than in Dalatiidae with photogenic organs in ventral area, such as Dalatiidae, but also on the flanks, the tail, and for some of them, even on the upper eyelids (Claes and Mallefet, 2009a).

Due to their ventral photophore patterns, their small size and the pelagic environment in which they live, it is suggested that Dalatiidae mainly use bioluminescence as camouflage by counterillumination

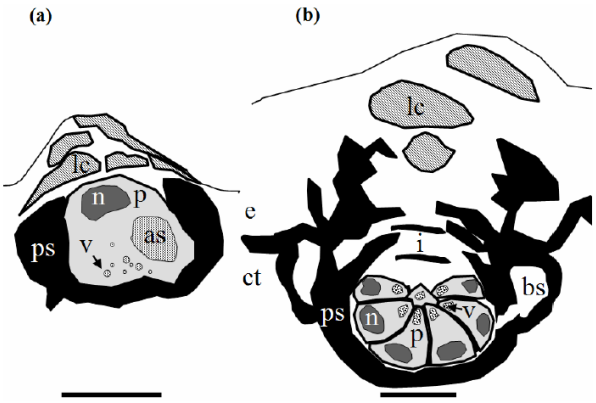

Figure 4: Photophores of luminescent sharks: (a) Dalatiidae (b) Etmopteridae. As, acidophilic secretion; bs, blood circulating sinus; ct, connective tissue; e, epidermis; i, iris; lc, lens cell; n, nucleus; p, photocyte; ps, pigmented sheath; v, vesicle presumed to be photogenic. Bars scale 50 µm (Claes and Mallefet, 2009a)

to avoid predators. An exception is the cookie-cutter shark, *Isistius brasiliensis*, which uses counterillumination as a predatory tool. The not luminescent pigmented band beneath its jaw play a role of bait, well visible by its prey, while the rest of its body is hidden by counterillumination (Widder, 1998; Warrant and Lockett, 2003). Etmopteridae, on the other hand, seem to use bioluminescence for a range of applications. It has been shown that *E. spinax* uses bioluminescence as a camouflage by counterillumination (Claes and Mallefet, 2008; Claes et al., 2010a), but also as an aposematic feature using its luminescence to highlight its dorsal spine (Claes et al., 2013; Duchatelet et al., 2019c). The photogenic patterns of Etmopteridae also suggest a usage of luminescence in schooling, species distinction, intraspecific and sexual communication (Claes and Mallefet, 2009a, 2010a; Renwart and Mallefet, 2013; Duchatelet et al., 2019c).

2. *E. spinax* model species

The velvet belly lantern shark, *Etmopterus spinax*, is a common luminescent ovo-viviparous shark, of the Etmopteridae family, living in the deep benthopelagic area of the Eastern Atlantic, from Iceland and Norway to Gabon, including the Macaronesian Islands and the Mediterranean waters. The species is often caught as by-catch from deep-water fishing, making it a good model species for scientific studies (Neiva et al., 2006). Moreover, *E. spinax* can be kept in captivity for several days in a water tank, which facilitates its study (Claes et al., 2010a; Claes and Mallefet, 2010b; Claes et al., 2013).

The velvet belly lantern shark possesses thousands of photogenic organs which emit light in the blue spectrum (maximum wavelength at 486 nm (Claes et al., 2010 a)). Recent research supports the idea that *E. spinax* uses intrinsic bioluminescence, as no bacteria or structures capable of hosting symbiotic luminous bacteria have been detected in photophores of this species (Duchatelet et al., 2019b). The bioluminescent pattern of *E. spinax* is divided into nine different zones appearing sequentially during the embryogenesis (Claes and Mallefet, 2008; Claes and Mallefet, 2009 a). Photophore morphogenesis is also depicted in three different stages (Figure 5). As first, a layer of pigmented cells appears between the dermal connective tissue and the epidermis (figure 5a, d1). Then, a not yet fluorescent photocyte, called protophotocyte, is formed, with a pigmented sheath, an iris-like structure and lens cells (figure 5b, d2, d3). Finally, the photophore is fully formed when green fluorescent vesicles appear inside the

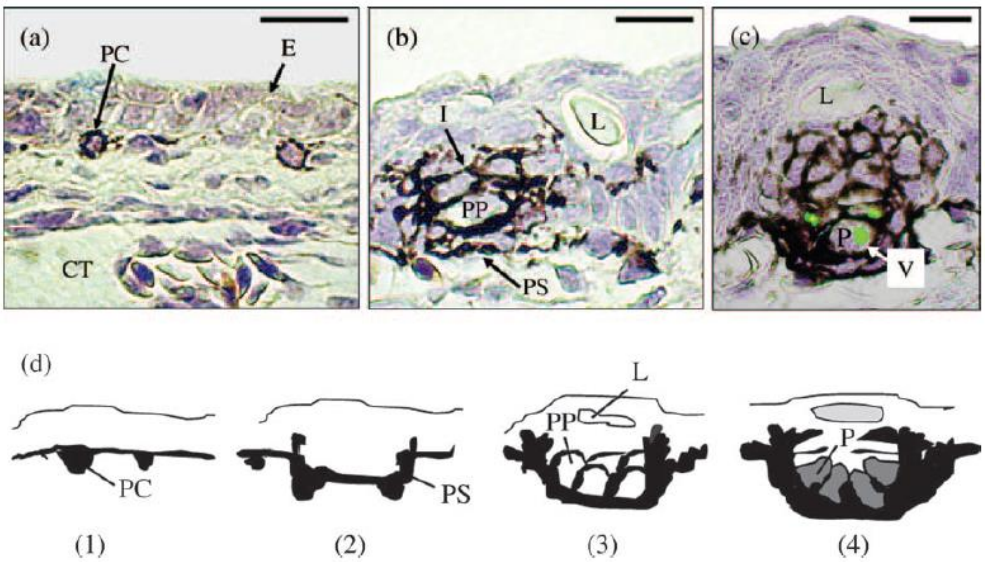

Figure 5: Development of *Etmopterus spinax* photophores. (a)–(c) Histological sections of photophores at different developmental stages, Masson's trichrome. (a) Stage A photophore (mandibular zone) from a 71 mm total length (LT) embryo. (b) Stage B photophore (lateral zone) from a 91 mm LT embryo. (c) Combined image (light and fluorescence microscopy) of a fully developed photophore (stage C, lateral zone) of a 96 mm LT embryo. (d) Schematic representation of photophore development: (1) appearance of pigmented cells (stage A); (2) and (3) formation of pigmented sheath, cluster of protophotocytes and appearance of lens cells (stage B); (4) maturation of the photophore, fluorescence is present inside the photocytes (stage C). CT, connective tissue; E, epidermis; I, iris; L, lens cell; P, photocyte; PC, pigmented cell; PP, protophotocyte; PS, pigmented sheath; V, fluorescent vesicle. Bars scale 50 µm (Claes and Mallefet, 2008)

photocyte, which enables it to produce luminescence (figure 5 c, d4). This development is fairly quick during embryogenesis, allowing the ready-to-hatch embryos to already emit bioluminescence (Claes and Mallefet, 2008, 2009a; Duchatelet et al., 2019 a). It is suggested that luminous compounds in embryo are transferred from the mother. Indeed, it has been shown that fluorescence is already present in the yolk sac before the development of photophores (Claes and Mallefet, 2008). However, in adults, scientists still do not know where the velvet belly lantern shark find its luminous substrate, or even the very nature of this substrate. Many luminescent marine organisms acquire their luminous substrate through their diet, but even if preys of *E. spinax*, which are essentially composed of cephalopods, euphausiids, teleosts and decapods, are mainly bioluminescent (Neiva et al., 2006), none of luciferins present in them have been detected in *E. spinax* photogenic organs, or are able to cross react with its supposed catalyst (Renwart and Mallefet, 2013). This suggests that an unknown luminous system, with a not yet found substrate or photoprotein, or a specific active form of known luciferin is present in the velvet belly lantern shark (Renwart and Mallefet, 2013).

To be as effective as possible in its various functions, bioluminescence must be precisely regulated and controlled according to the situation, the environment, etc. Many luminescent fishes regulate their light emission directly through the nervous system. However, the velvet belly lantern shark does not

respond to any classical neurotransmitters or KCl, suggesting that its photophores are not under direct nervous control. Further research has been conducted and a hormonal control of *E. spinax* photophores has been suggested (Claes and Mallefet, 2009b, 2010a, 2010b; Duchatelet et al., 2019d). The main hormones involved in the triggering of light emission are prolactin (PRL) and melatonin (MT). Both hormones appear to be dose-dependent stimulator of the light emission, but not at the same timing and sensitivity. A MT application induces a slow increase of luminous intensity over a long period of time (several hours) while PRL, on the other hand, induces a relatively faster light emission for a shorter period of time (one hour maximum). The different reaction between both hormones suggests that these hormones use a different pathway for the luminescence stimulation (Claes and Mallefet, 2009b; Duchatelet, 2019). Moreover, the action of PRL on light triggering is still mysterious, no PRL receptor mRNAs being detected in the ventral skin transcriptome of E. spinax (Duchatelet, 2019). Furthermore, MT and PRL are not the only molecules playing a role in the light emission (figure

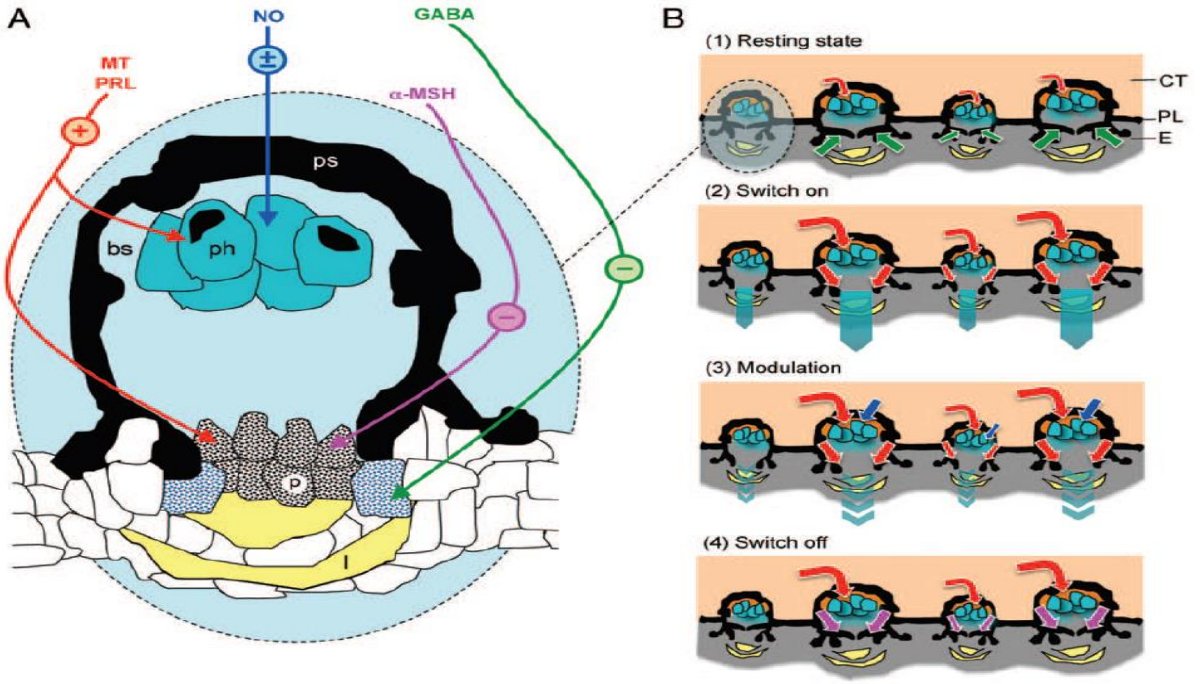

Figure 6: Model of photophore luminescence control in the shark *Etmopterus spinax*. (A) Luminescence control pathways present in a photophore (transversal section). Coloured arrows indicate the targets of the different substances involved in the control of *E. spinax*'s photogenesis. Symbols in colour circles indicate the effect of these substances on luminescence: +, activatory; -, inhibitory; ±, modulatory. (B) Different luminescence states in a group of photophores (transversal section): (1) resting state—photocytes are weekly stimulated to glow by low levels of circulating hormones (MT and PRL; red arrows) while GABA (green arrows) prevent light to be emitted outside the photophores by provoking pigment expansion in the pigmented cells topping the photocytes; (2) luminescence switch on—high levels of circulating hormones (MT and PRL; red arrows) stimulate the photocytes to glow and provoke pigment retraction in pigmented cells topping the photocytes, counterbalancing the effect of GABA; (3) luminescence modulation—NO (blue arrows) modulate the effects of stimulatory hormones, probably by acting directly on the photocytes; (4) luminescence switch off—α-MSH (purple arrows) inhibits the hormonally induced light, probably by acting on the pigmented cells topping the photocytes. α-MSH, α-melanocyte stimulating hormone; bs, blood sinus; CT, connective tissue; E, epidermis; GABA, γ-amino butyric acid; l, lens cell; MT, melatonin; NO, nitric oxide; p, pigmented cell; ph, photocyte; PL, pigmented layer; PRL, prolactin; ps, pigmented sheath (Claes and Mallefet, 2011)

6): The α-melanocyte-stimulating hormone (α-MSH), the adrenocorticotropic hormone (ACTH) and the γ-aminobutyric acid (GABA) act as light emission inhibitors on photophores, while NO applications tends to modulate the luminescence (Claes et al., 2010b; Claes and Mallefet, 2011; Duchatelet et al., 2019 d). Hormones are involved in the control of light production, but they can also induce a pigment retraction within melanophores, irising the pigmented layer and disclosing the luminescence of the underlying photocytes (Claes and Mallefet, 2010b; Duchatelet, 2019). This phenomenon shows that hormones can be involved in both physiological and mechanical regulation of luminescence in the lantern shark.

One of the main uses of bioluminescence in *E. spinax* is the camouflage by counterillumination (Claes and Mallefet, 2008; Claes et al., 2010a). Generally, organisms using this camouflage technique can modulate the light intensity produced according to the ambient light. However, hormonal control is slower than nervous control, which means that, counter to organisms with nervous control, the slow luminescence kinetic of the lantern shark could make it difficult to accurately adjust its luminescence over a wide range of intensity in a short period of time. To cope with this, it is assumed the shark maintains a diel vertical migration pattern following a constant isolume (Claes and Mallefet, 2009b; Claes et al., 2010a). Like other deep-sea shark species, *E. spinax* possess a "pineal window" which has been suggested to help the shark during its vertical migrations by collecting ambient light information (Claes and Mallefet, 2009 b). The pineal gland is associated with the release of MT into the bloodstream in vertebrates (Underwood, 1990; Arendt, 1997). Further, MT triggers light emission in a dose-dependent manner suggesting for this hormone that it is a direct connection between light emission and environmental luminosity perception (Claes and Mallefet, 2009b).

Bioluminescence and extraocular opsin

1. Light perception

The light perception is possible due to photopigments which are photosensitive molecules composed of an isomer of vitamin A chromophore (retinal) bound to an opsin protein. Opsins are G-protein-coupled receptor, a single transmembrane polypeptide chain forming seven transmembrane α-helices (figure 7c). The interaction with G-protein is made by intracellular domain of opsin while retinal chromophore is bound to the opsin at a cavity, formed by the seven α-helices, on the extracellular side (Peirson et al., 2009). It exists many opsins identified within vertebrates. They are subdivided into seven subfamilies which have been elaborated according to functional classification of opsins based partly on the type of G-protein-coupled with the opsin (figure 8).

The vertebrate visual and non-visual opsins, as well as the invertebrate G_q-coupled opsins and G_o-coupled opsins are bound to a 11-*cis*-retinal chromophore while photoisomerases and peropsins are

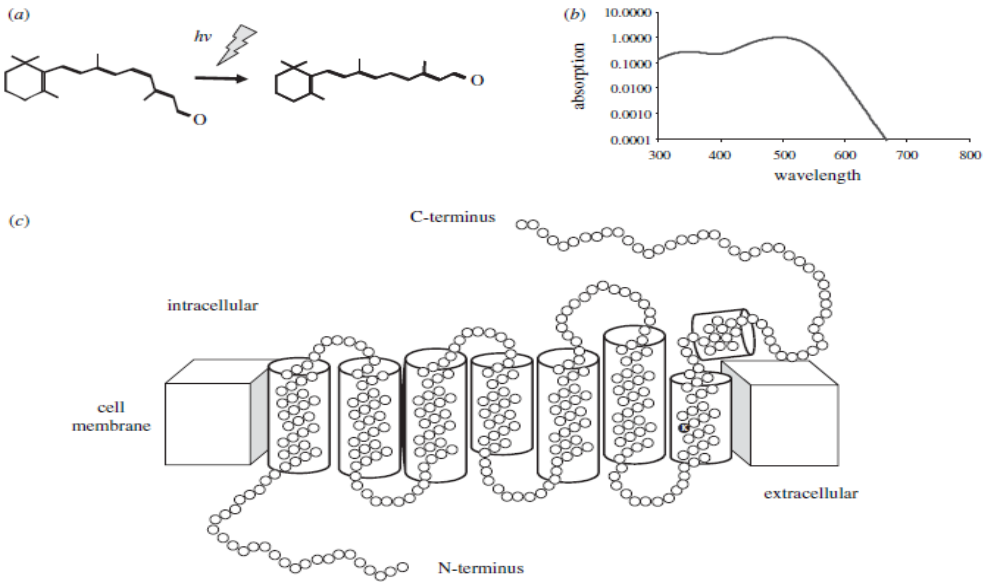

Figure 7: Structure and function of vertebrate photopigments. (a) The primary step in phototransduction is the absorption of a photon of light (hν) by the 11-cis isomer of retinal resulting in isomerization to the all-trans form. (b) All vitamin A/opsin-based photopigments have a characteristic absorption spectrum. (c) Opsins consist of a single oligopeptide chain forming seven a-helical transmembrane regions connected by cytoplasmic and extracellular loops. The intracellular domains mediate G-protein interactions. The retinal binding site (K) is indicated in the 7th transmembrane domain (Peirson et al., 2009)

bound to an all-*trans*-retinal. For other opsin subfamilies, the chromophore bound is still uncertain (Terakita, 2005).

Two photoreceptor cells situated within the vertebrate retina in lateral eyes are involved in the vision of most of vertebrates. These two photoreceptor cells, rods and cones, contain both different visual opsins. Cones contain cone opsins, which are subdivided into different groups, and underly high-resolution colour vision. On the other hand, rods contain a rhodopsin which is the base of the twilight vision, with a high sensitivity but a low-resolution (Terakita, 2005; Peirson et al., 2009; Delroisse et al., 2018). The first step of phototransduction, the cascade converting photic energy into neural responses (figure 9), is the absorption by the chromophore of a photon provoking the photoisomerization of the 11-*cis*-retinal to form an all-*trans* state (figure 7a). This conformation change allows the opsin to interact with G-protein activating it. The activation of G-protein will stimulate the cGMP phosphodiesterases which will decrease the intracellular cGMP concentration leading to closure of cGMP-gated cation channels. Finally, with the cation channel closure, hyperpolarization of photoreceptor cells appears, modulating the release of neurotransmitters which will transmit the information to ganglion cells and finally to the brain (Terakita, 2005; Peirson et al., 2009; Shichida and Matsuyama, 2009).

Photoreceptors can also be involved into a non-image-forming light detection, and in such case, photoreceptors outside the eyes are considered as extraocular or extraretinal. Like visual photopigments, non-visual photopigments are formed of an opsin protein linked to a retinal

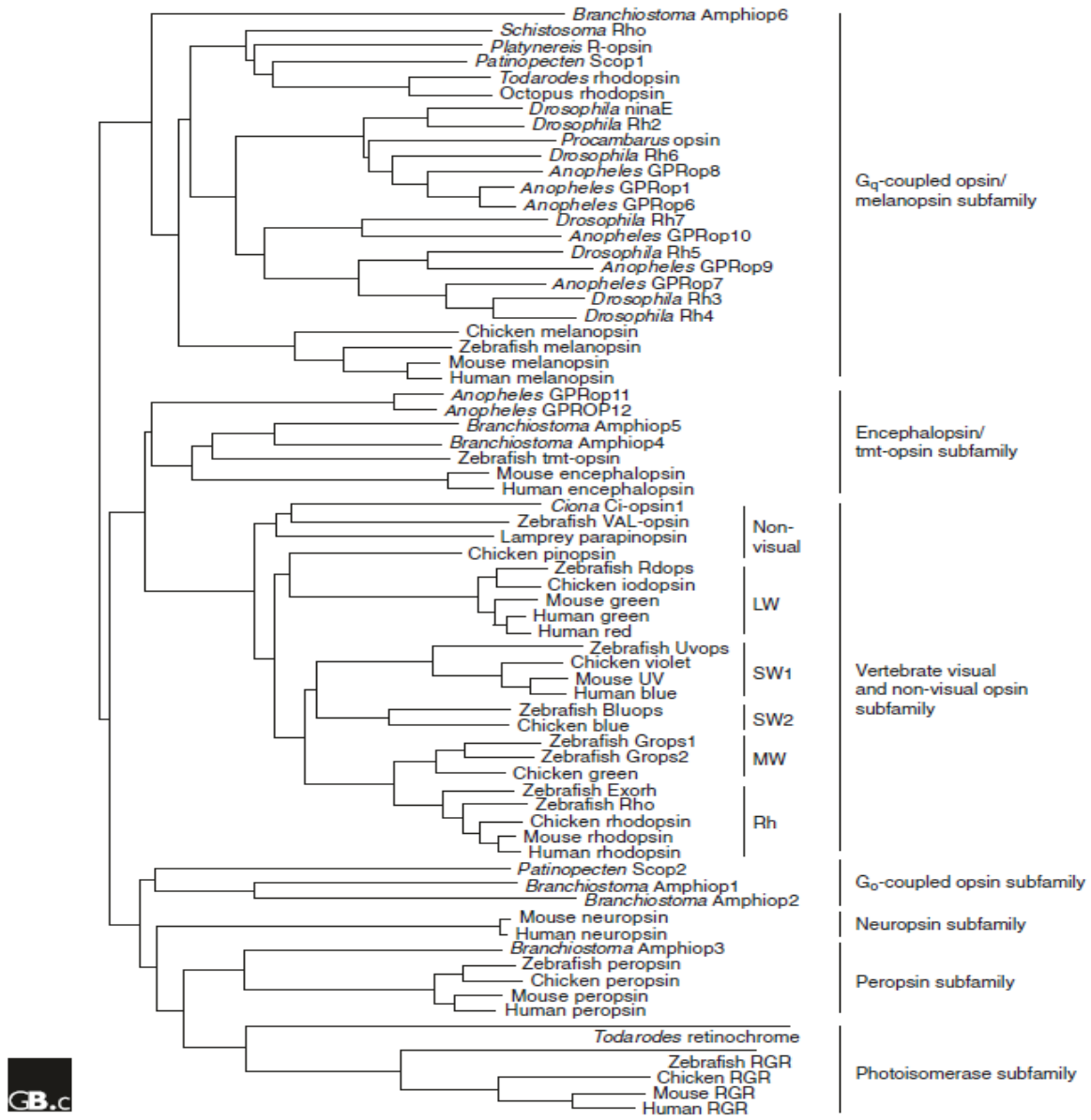

Figure 8: A molecular phylogenetic tree of the opsin family. Abbreviations: LW, long-wavelength-sensitive opsin; SW, short-wavelength-sensitive opsin; MW, middle-wavelength-sensitive opsin; Rh, rhodopsin; RGR, retinal G-protein-coupled receptor. Other abbreviations are protein names; where only a colour is given for a protein name, it refers to a cone opsin which detects that colour (Terakita, 2005)

chromophore which can differ depending on species and opsin subfamily (Nissilä et al., 2012; Delroisse et al., 2018). The most known extraretinal photoreceptive site in vertebrate is the pineal gland which synthetizes melatonin, a hormone involved, for instance, into the circadian rhythm regulation (Arendt, 1997). Different extraocular opsins have been detected in the pineal organ, among them, pinopsin, parapinopsin, extraretinal rod-like opsin/exo-rhodopsin, etc. In shark for instance, extraocular photoreception is commonly known to be associated to the pineal gland, like in lantern sharks which collect light information of ambient luminosity via the pineal gland, in addition to eyes, to help them into their vertical migration (Vigh-Teichmann et al., 1983; Claes and Mallefet, 2009b). Sometimes, extraocular opsins can be directly inside photophores/photocytes and provide information about light

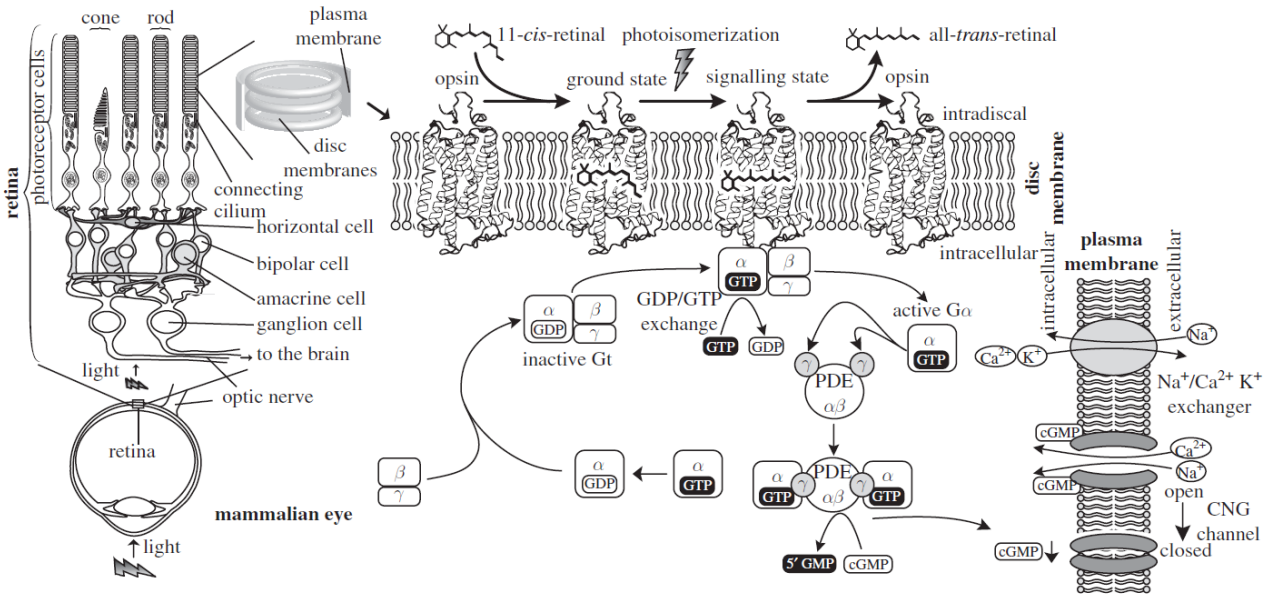

Figure 9: A diagram showing the mechanism of phototransduction in mammalian eyes. Light is captured by two specialized morphologically distinct photoreceptor cells derived from neurons: rods and cones which have the same molecular mechanism. Opsins in these cells absorb photons and form a signalling state, which can bind to and activate the G protein by catalysing the exchange of GDP to GTP. The GTP-bound Ga dissociates from Gbg exposing its active site. activated Ga binds to its effector, PDE (cyclic nucleotide phosphodiesterase), and activates it. PDE breaks the phosphodiester bond of cGMP producing 50GMP, and the decrease in the concentration of cGMP causes CNG (cyclic nucleotide gated) channels to close, which creates a hyperpolarization response in the photoreceptor cells. Light-activated rhodopsin is thermally unstable and the chromophore eventually detaches from the opsin. The hyperpolarization of the membrane potential of the photoreceptor cell modulates the release of neurotransmitters to downstream cells. The light signal is transmitted through different cells, finally reaching ganglion cells which form the optic nerve and project to the brain (Shichida and Matsuyama, 2009)

emission. This bioluminescence feedback has been suggested in some luminescent squid species which use this application to control the symbiotic luminescent bacteria population within their light organ and, thus, their luminescence (Young et al., 1979b; Tong et al., 2009).

2. Extraocular opsin

As indicated earlier, in addition to visual opsins present in retina, it exists extraretinal opsins involved into non-visual light perception. This non-visual phototransduction can occur in many extraretinal sites like skin, brain, testes, etc. The extraocular photoreceptor complexity can variate from a complex organ in a precise area to a diffusely distributed photoreceptive cells (Tong et al., 2009). These opsins are involved in diverse systems such as the circadian rhythm regulation (Nissilä et al., 2012), bioluminescence feedback (Tong et al., 2009; Schnitzler et al., 2012; Delroisse et al., 2014; Duchatelet et al., 2019a), or neural development (Beaudry et al., 2017). Extraretinal photoreceptors are quite common among non-mammalian vertebrates but can be found also in mammal such as encephalopsin which is strongly and specifically expressed in mouse and human brain (Blackshaw and Snyder, 1999; Terakita, 2005; Nissilä et al., 2012).

The phototransduction involving extraocular opsins is fairly similar to visual phototransduction, but contrary to opsins expressed in rods and cones cells which are monostable, most other opsins are bistable. A monostable opsin will bind to 11-*cis*-retinal form but will dissociate from all-*trans* form while bistable opsins can bind 11-*cis* form and all-*trans* form. This suggests that bistable opsins are less efficient G-protein activators than monostable opsins which are more specialized, according to their role in the vertebrate vision. However, bistable pigments in non-visual photoreceptors allow transition between dark state and light state via light exposure contrary to monostable pigment, visual opsins, which necessitate external input to return to the dark state (Beaudry et al., 2017; Pérez et al., 2019).

Variations into opsin structures allow a wide range of absorption spectrum, every opsin being characterised by a range of absorption spectrum (figure 10). This variation reflects the evolutionary pression from the environment to perceive only specific wavelength. For instance, in deep water, only blue and green spectrum penetrate the water column and organisms living in this environment only have to use photoreceptors sensitive to these wavelengths while shallow water organisms also necessitate photoreceptors sensitive to wavelength in the red and yellow spectrum.

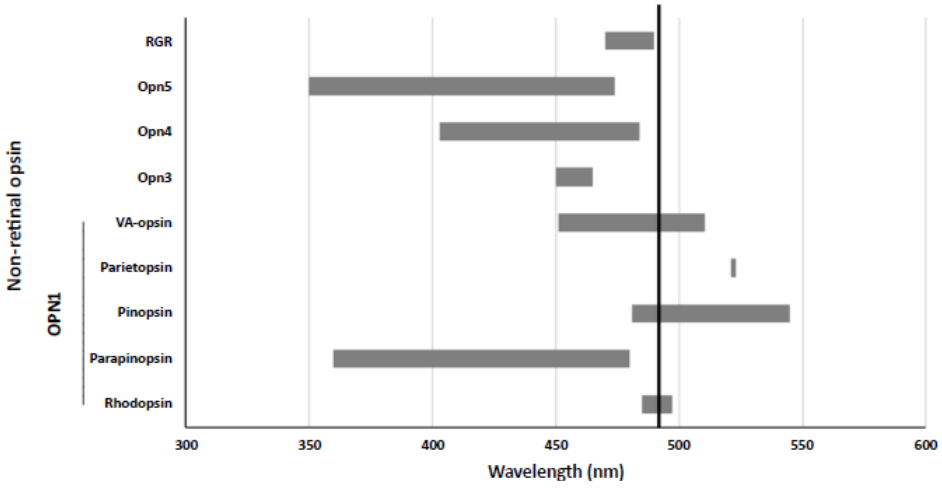

Figure 10: Spectrum maxima for the main "super" families of opsins. Bars represent the full range of values reported in the current literature under a variety of test conditions (Pérez et al., 2019)

Extraocular opsins are divided into different subfamily as shown on the figure 8. Pinopsins, involved into the photoreception in the pineal organ of birds and lizards, parapinopsins, also present in the pineal complex of several fish species including lamprey, and "vertebrate ancient" opsins (VA-opsins), present in salmon retina are all three vertebrate non-visual opsins belong to the same subfamily of visual opsins. Other opsin subfamily are the encephalopsin/tmt-opsin subfamily, present in mouse, humans, teleosts and some invertebrates ; melanopsin, present in many vertebrates including human and fishes, which is quite similar to invertebrate G_q-protein-coupled visual opsin; G_o-protein-coupled opsins, present in molluscs and in chordate amphioxus; G_s-protein-coupled opsin from cnidarian; neuropsins, identified in human and mouse; peropsins, expressed in diverse vertebrates; and finally

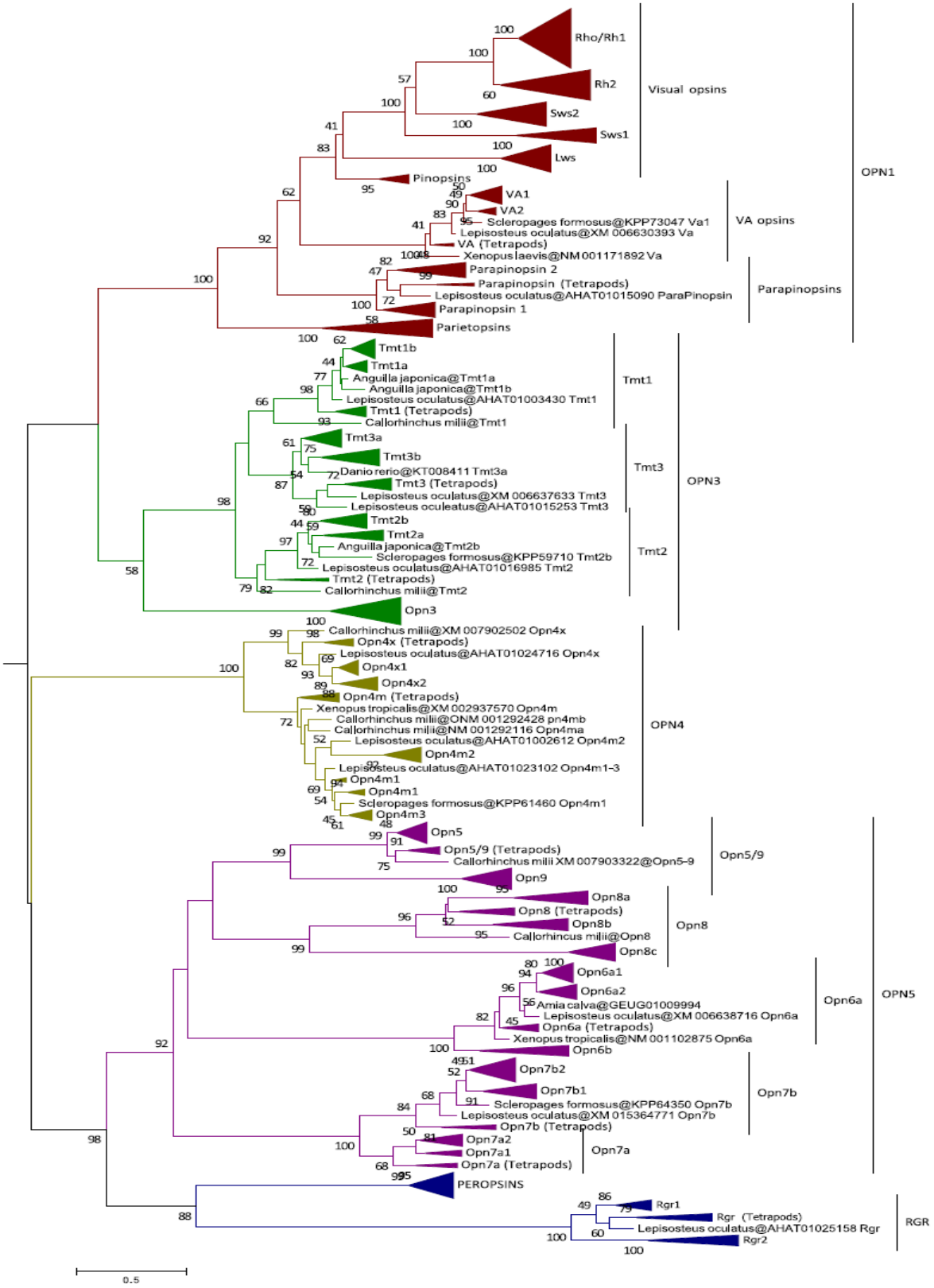

Figure 11: Evolutionary relationships of vertebrate visual and non-visual opsin families and subfamilies. The ML tree was inferred by using the Maximum Likelihood method based on the GTR model with four gamma-distributed substitution rates. The percentage of trees in which the associated taxa clustered together is shown next to the branches based on 100 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Beaudry et al., 2017)

the retinal-photoisomerase subfamily involving retinal G-protein-coupled receptors (RGR) and retinochromes, present in vertebrates and molluscs (Terakita, 2005). However, recent studies have shown that this opsin classification was not exactly correct for some lineage and have proposed a new classification for extraretinal photoreceptors (Figure 11). These studies were based on opsin sequences analyses from more than 500 species representing most of vertebrate lineages suggesting a common vertebrate ancestor possessing at least eighteen non-visual opsins, divided into five groups (OPN1, OPN3, OPN4, OPN5 and RGR), and five visual opsins (Beaudry et al., 2017 ; Pérez et al., 2019).

3. Encephalopsin and bioluminescence of *E. spinax*

Encephalopsin, also called panopsin or opsin 3 (Opn3), is an extraocular opsin belonging to the OPN3 subfamily. It is a non-visual opsin which play a role in the central nervous system tissue photosensibility by acting as a G-protein-coupled receptor and mediating phototransduction. Encephalopsin has been

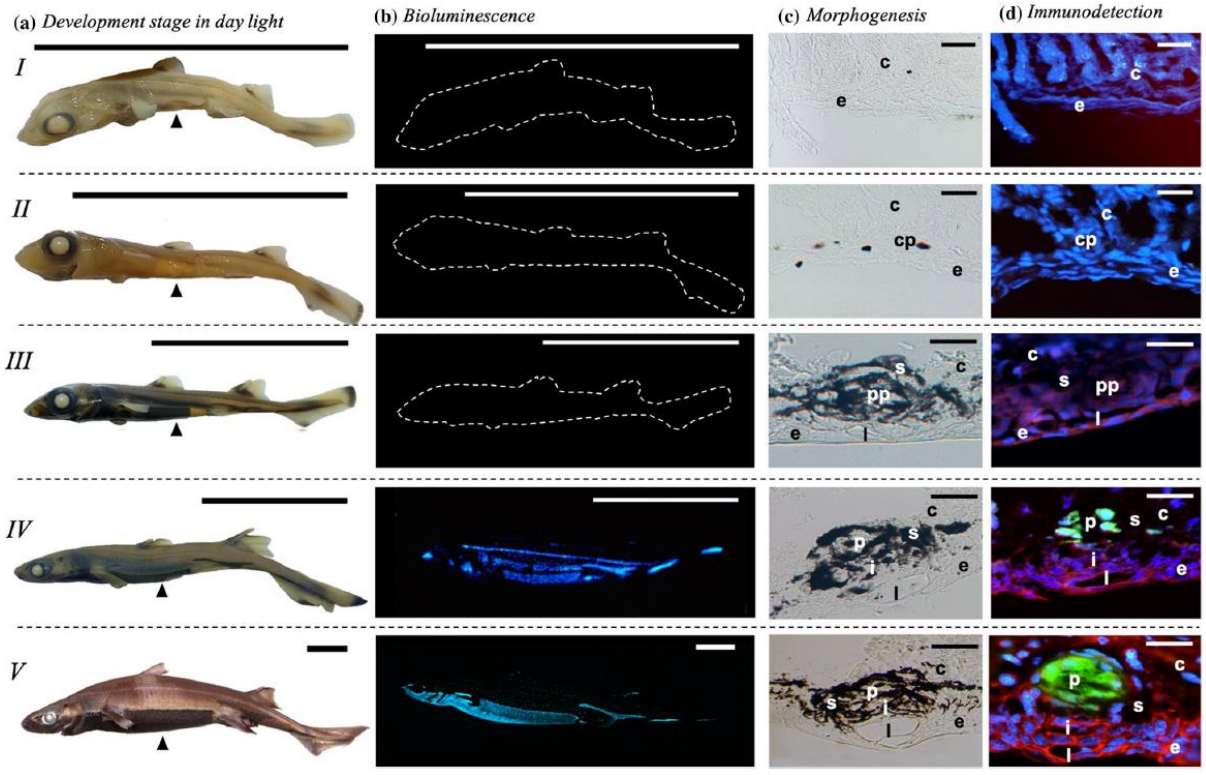

Figure 12: Joint appearance of encephalopsin and photophores during *Etmopterus spinax* photophore embryogenesis within the ventral skin epidermis. (a) Developmental series of *E. spinax* in natural light (stages I–IV come from four distinct litters collectively constituting an embryonic series, while stage V is an adult female specimen). Arrowheads depict the ventral location sampled for histological and immunodetection experiments. (b) Images taken in the dark highlighting spontaneous luminescence, when present. Contour dot line depicted the non-luminous embryo from stage I to III. (c) Histological section of the ventral epidermis under bright light highlighting the presence and the ontogeny of photophore structures, when present. (d) Histological section of the ventral epidermis under UV stimulation highlighting the presence of photophore structures, when present. The autofluorescence of the photocyte vesicles (green fluorescence, only present in mature photocytes) and encephalopsin immunodetection (red labelling) is shown. DAPI blue staining associated with to the cell nucleus. *c* connective tissue, *cp* pigmented cells, *e* epidermis, *i* irislike structure cells, *l* lens cells, *p* photocytes, *pp* protophotocytes, *s* pigmented cell layer. Scale bars represent 5 cm in (a) and (b); 50 µm in (c) and (d) (Duchatelet et al., 2019)

detected abundantly in the mouse brain but is also expressed in diverse vertebrate extra-retinal and retinal tissues (Nissilä et al., 2012; Delroisse et al., 2018).

Transcriptomic analyses have shown that encephalopsin mRNAs are present in the ventral skin transcriptome of *E. spinax*. In addition to that, immunodetection shows that encephalopsin has a widespread expression within epidermal cell membranes around photophores and no expression is detected inside photocytes (figure 12d V). These results strongly suggest that this opsin could have a functional relation with photophores, and an extraocular photoreception might be involved into light emission control by shutter opening, regulated by pigments dispersion in ILS, and/or photocyte activity regulation (Renwart et al., 2014, 2015; Delroisse et al., 2018; Duchatelet, 2019). Moreover, encephalopsin has a blue light sensitivity (absorbance spectrum between 410 to 490 nm with a peak at 445 nm) overlapping with the light emission spectrum of *E. spinax*, emitting blue-green luminescence at a maximum value of 486 nm (Claes et al., 2010a; Duchatelet, 2019). More recent study has shown that development of luminous organs during embryogenesis is in correlation with encephalopsin expression within epidermal cells (figure 12). This is another argument supporting the hypothesis that encephalopsin is well associated with the emitted light perception and that a physiological interaction between light emission and light perception exists, acting as a bioluminescence feedback control mechanism (Duchatelet et al., 2019a).

Species studied: *Squaliolus aliae*

The smalleye pygmy shark, *Squaliolus aliae*, is a dwarf pelagic shark from the Dalatiidae family, encompassing shark species of small size except for *Dalatias licha* which can reach up to two meters long. It exists 10 species describe in Dalatiidae family, but some of them are poorly known. *S. aliae*, with a total length of approximatively 15 cm at sexual maturity and a maximum of 22 cm, is the smallest species of its family and one of the smallest shark species in the world (Compagno et al., 2004). It migrates from deep-water up to 2000 meters during the day to epipelagic zone during the night. These migrations are probably made to follow its preys, mainly composed of small crustaceans, cephalopods and fishes (Seigel, 1978). During its migration, *S. aliae* uses bioluminescence, with a maximum emission value of 457 nm (Claes et al., 2014), as a counterillumination camouflage to avoid predators in the water column and do not be seen by its preys. This hypothesis is made according to its photophores pattern, homogeneously distributed on the ventral side. According to its relative abundance in some places, its ability to be maintained in captivity for short periods of time, and because ventral skin patches can be used to study its luminescence, *S. aliae* seem to be a good model species for dalatiid bioluminescence studies.

Like other bioluminescent sharks, pygmy shark luminescence is under hormonal control. It has been shown that MT has a stimulatory effect on photophores (figure 13) increasing slowly the light emission for a long period of time (more than one hour). In addition to increase light emission, MT also induces retraction of pigments present inside the melanophores overlying photocytes, letting pass more luminescence. On the other hand, PRL and α-MSH are two hormones with an inhibitory effect on spontaneous light emission and MT-induced luminescence. Inhibitory effect of ACTH on light emission, as suggested for *E. molleri* (Duchatelet et al., 2019d), could also play a role in the bioluminescence control for *S. aliae*. As *E. spinax* hormonal control, studied by Claes et al. (Claes et al., 2010 b), GABA has also an inhibitory effect on luminescence in *S. aliae*, and bicuculline (BICU) (a GABA antagonist) has a stimulatory effect on photophores. The light production inhibition induced by GABA seems to be partially inhibited in presence of high MT concentration levels. Contrary to *E. spinax*, NO does not have any effect on light emission induced by MT in *S. aliae*.

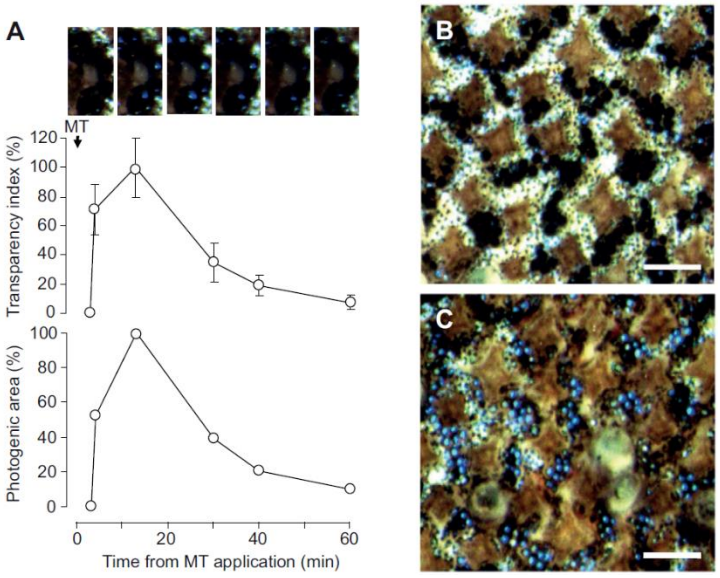

Figure 13: Iris-like movement of pigmented cells underlying the photophores of *Squaliolus aliae*. (A) Evolution of the "transparency index" and photogenic area of photophores after melatonin (MT) application (arrow). Serial pictures (top) illustrate the group of photophores (*N*=6) from which the data points were calculated. (B) Photophore-containing skin patch before MT stimulation (i.e. not producing light). (C) Photophore-containing skin patch producing light after MT application. Scale bars, 250 µm (Claes et al., 2012)

Like in Etmopteridae, bioluminescence of pygmy sharks is controlled by as hormonal (MT, PRL, α-MSH, ACTH) as nervous (GABA) substances. However, this control differs in some points from the one of Etmopteridae, previously seen with *E. spinax* (Claes and Mallefet, 2009b). Indeed, PRL has an opposite effect between *E. spinax* with which it triggers light emission while it plays a role of inhibitor in *S. aliae*. This difference is probably due to the different application of bioluminescence in these two species, *S. aliae* using light emission only for counterillumination and Etmopteridae using it in more functions, necessitating a quicker on/off light switching (Claes et al., 2012).

Question remain on the potential implication of an extraocular opsin and the luminescence photoreception in the mechanical control of light emission in *S. aliae*, such the one demonstrated recently by Duchatelet (2019) for *E. spinax*.

Goals and working assumptions

The main goals of this study are:

- The highlighting of the supposed presence of encephalopsin, an extraocular opsin, in the luminous ventral skin of *S. aliae.*
- The localization of this predicted extraocular opsin at the level of shark skin and in relation to photophores.
- The establishment of relations between photophores formation and photoreceptors apparition during the embryogenesis.

Responses to those questions may bring new information about the mechanism of light perception in relation with photoemission of Dalatiidae and may help to compare mechanisms between both luminous shark families.

Materials and methods

Specimen collection

Nineteen gravid female smalleye pygmy sharks, *S. aliae* (14.3—22.5 cm TL, 19—74 g W), were captured by several midwater trawls (50—150 m depth) in inshore waters off Donggang harbour in southwest Taiwan (22°26' N, 120°23' E). All specimens were captured during two field seasons, first in July 2011 (nine gravid females) and second in December 2018 (ten females, including seven gravid females). Living specimens were brought to the National Museum of Marine Biology and Aquarium (Pingtung, southern Taiwan) and were kept in tank filled with 18—19°C placed in the dark. All shark caught alive were euthanized by a blow on the head followed by a full incision into the spinal cord at the back of the head. After which, sharks were sized, weighed and female uteri were excised to free embryos. A total of sixty embryo (1.7—11.7 cm TL) were extracted from sixteen different litters. They were euthanized following the same method used for the adults, fixed in PFA 4%-PBS, and then stored in phosphate-buffered saline (PBS).

Morphology and photophore development

All embryos were measured and photographed with a digital camera (Panasonic DMC-FZ300; Panasonic, Kadoma, Japan) to establish a classification of six different development stages considering that the size of embryos is correlated to their relative age (larger specimens supposed to be older).

To determine the establishment and density of luminous organs, placoid scales and skin pigmentation, embryos or ventral skin patches (1 cm^2), depending on the size of the specimen, were placed in a small transparent recipient containing PBS. Photographs of the ventral skin (between the pectoral and pelvic fins) of embryos and free-swimming specimens were taken using a light microscope (Leitz Diaplan; Wild Leitz, Germany) coupled with a digital camera (ToupTek UCMOS 03100 KPA; Hangzhou ToupTek Photonics, Zhejiang, China). The pictures were analysed with ImageJ (ImageJ; National Institutes of Health, Maryland, USA) to determine the photophore and placoid scales density and the mean size of the scales (S_{Si}). To calculate the density, three pictures (at a 10X magnification) per specimen, covering a skin area of 6,81 10^5 μm^2 each, were randomly taken. Photophores and scales present in the image (completely or partially) were counted and an average density was made per individual. Then the density was transposed into number of photophores per cm^2 (P_{De}) and number of placoid scales per cm^2 (S_{De}). Mean size of placoid scales (S_{Si}) were calculated using 4X magnification pictures. A line was drawn at the centre of the image and every scale touching the line was measured by averaging the measurements of the two large widths of the scale. These measures were repeated three time per specimen. For both, density and scales size, measurements were taken on five specimens per stage of development except for stage two and three which contain respectively four and two different specimens.

Embryonic classification

The embryonic classification was established according to several criteria. As shown in the Table 1, different relative powers were assigned to each of these criteria. Six embryonic stages and one free-swimming stage were established based on total length (TL), skin pigmentation, luminous organs development and placoid scales development.

Table 1: Classification criteria, possible values and relative weights to establish the different stages of development.

Classification criterion	***Available choices***					***Relative power of criterion***
Total lenght (cm)	< 4	[4 ; 6[	[6 ; 8[	[8 ; 9[	9 <	2
Development stage of scales	Absent	Not fully formed round scales	Some fully formed placoid scale	Mostly fully formed		3
Development stage of photophores	Absent	Not fully formed	Mostly fully formed			3
Pigment cells	Absent	present				3
Photophore density	Relative density					1

Relative weight of criterion: 1 < 2 < 3

Encephalopsin detection

Skin patches (1 cm^2) from ventral area between pectoral and pelvic fins were dissected from at least two embryos per development stage and from 3 adult females with a pair of surgical scissors. An eye was also taken from three different ready to hatch embryos with a pair of surgical scissors as a control. Skin patches and eyes were cryoprotected by a series of three PBS baths with an increasing concentration of sucrose (10% for 1h, 20% for 1h, 30% overnight). Then, tissues were embedded with an optimal cutting temperature compound (OCT compound, Tissue-Tek, Netherlands) in specifically designed cuvettes and immediately frozen at -80°C. After few hours, 10 µm sections were made with a cryostat microtome (CM3050 S; Leica Microsystems GmbH, Wetslar, Germany), laid on coated Superfrost slides (Thermo Scientific) and left overnight to dry.

To detect and localise encephalopsin within the luminous organs, an immunofluorescence technique was employed on *S. aliae* embryos and adults ventral skin sections and on ready to hatch embryo eye sections following method describe by Delroisse et al. (Delroisse et al., 2018). First, slides were rinsed in Tris buffer saline 1% Tween [TTBS: Trizma base (Sigma) 20 mM, NaCl 150 mM, pH 7.5 + 1% Tween 20 (Sigma)] baths for 15 minutes before being left 40 minutes in a trisodium citrate [trisodium citrate, pH 6 + 1% Tween 20 (Sigma)] bath to defolde protein. Slides were rinsed again in TTBS baths for 15

minutes and then blocked with TTBS containing 10% bovine albumin serum (BSA, Amresco). After that, slides were incubated overnight in refrigerator with the anti-encephalopsin primary antibody (anti-encephalopsin Pab in *Homo sapiens*, Genetex, GTX 70609) at a dilution of 1/400 in TTBS 5% BSA. The following day, slides were rinsed during 30 minutes in TTBS baths before being incubated 1h at room temperature in the dark with the secondary antibody Alexa Fluor ® 594 Goat Anti-Rabbit IgG (Goat Anti-Rabbit, Alexa Fluor ® 594, Life Technologies Limited) at a dilution of 1/200 in TTBS 5% BSA and bathed another 15 minutes in TTBS. Lastly, the slides were put 15 minutes in DAPI (DAPI nucleic acid stain, Invitrogen) nucleus staining, bathed 10 minutes in TTBS and mounted with Mowiol (Mowiol® 4-88, Sigma).

Nikon DS-UI digital camera (Nikon, Tokyo, Japan) coupled with NIS-Elements FW software (Nikon, Tokyo, Japan) mounted on a Polyvar SC epifluorescence microscope (Leica Reichert Jung; Leica Microsystems GmbH, Wetzlar, Germany) was used to observe sections using different kind of filter. The pictures taken were joined and analysed with the ImageJ software.

The immunodetection was performed on at least two embryos of each embryonic development stage and on three adult female sharks and applied on at least fifteen sections for each specimen. Control slides were made by omission of the primary antibody and by immunodetection experiment on eyes of ready to hatch embryos.

Statistical analysis

All statistical analyses were performed using RStudio v. 1.2.5019 software (RStudio Inc, Boston, USA) and considered significant for P-value < 0.05. Normality and equality of variances were tested by a Shapiro-Wilk test and a Barlett's test respectively. As the data do not follow the normal distribution, a non-parametric Kruskal-wallis ANOVA test was performed to determine whether there are significant differences between the different groups. When the Kruskal-wallis test indicated that a significant difference existed between the groups, all pairwise comparisons were tested using a post hoc Dwass-Steele-Critchlow-Fligner comparison test (dscf all pairs test). For linear regression, the normality and homogeneity of the residues were tested by a Shapiro-Wilk test and a Breush-Pagan's test respectively. For each stage of development, N = 5, corresponding to the number of sharks used and an average of three measurements were made on each specimen to calculate P_{De} and S_{De} as well as an average of the sizes made on twenty scales randomly selected per shark specimen.

Results

Morphology

A total of sixty embryos (1.7-11.7 cm TL) were extracted from sixteen different litters. These embryos were classified into six different stages of embryonic development (stage I to stage VI), with stage VII

being adult individuals (Figure 14). The classification was made according to different criteria with a specified relative weight for each criterion, as shown in Table 1. The first criterion considered was the size of the embryos. Specimens less than 4 cm tall were classified in the first stage. From 4 cm to 6 cm, individuals are classified in stage II. Stage IV includes specimens from 6 cm to 8 cm. Embryos with intermediate size in stage II and stage IV, but with a different morphology in terms of placoid scale and photophore development, were placed in stage III. Then, sharks between 8 cm and 9 cm and those over 9 cm determine stages V and VI respectively.

The appearance of scales (Figure 14b) begins at stage II, but at this stage they are not yet fully formed and are in the form of fine circular scales. From stage IV onwards, some scales begin to look final in the form of pavement-like scales. It is only at stage VI that most scales are in their final pavement form. The appearance of skin pigmentation occurs from stage II and increases importantly during embryonic development. The first drafts of luminous organs appear in stage III and it is only in stage IV that the first fully formed photophores appear. During embryonic development and therefore in subsequent stages, the number of fully formed photophores is increasing and the density of the latter also increases.

Table 2: Means of TL, P_{De}, S_{De} and S_{Si} for every *S. aliae* development stages and comparison with three Etmopteridae species (when data available) + Réf!.

Development stages	Mean TL ± s.d. (mm)	Mean P_{De} ± s.d. (photophores cm^{-2})	Mean S_{De} ± s.d. (scales cm^{-2})	Mean S_{Si} ± s.d. (mm)
Stage I	33,4 ± 6,9	0	0	/
Stage II	52,8 ± 3,6	0	1353 ± 178	0,1617 ± 0, 0454
Stage III	59,4 ± 2,9	1363 ± 418	1569 ± 270	0,1579 ± 0,0482
Stage IV	72,4 ± 3,8	2745 ± 464	1265 ± 282	0,2013 ± 0,0582
Stage V	81 ± 4,0	4020 ± 682	1225 ± 227	0,2139 ± 0,0645
Stage VI	98,4 ± 2,3	5873 ± 855	971 ± 174	0,2111 ± 0,0517
Stage VII (adult)	192,8 ± 24,9	7863 ± 636	1265 ± 199	0,2079 ± 0,0488
E. spinax	450	2883 ± 232	NA	NA
E. molleri	400	3862 ± 193	NA	NA
E. splendidus	220	4620 ± 360	NA	NA

TL, total length; P_{De}, photophores density; S_{De}, scales density; S_{Si}, scales relative size.
Data are mean ± s.d. (standard deviation)

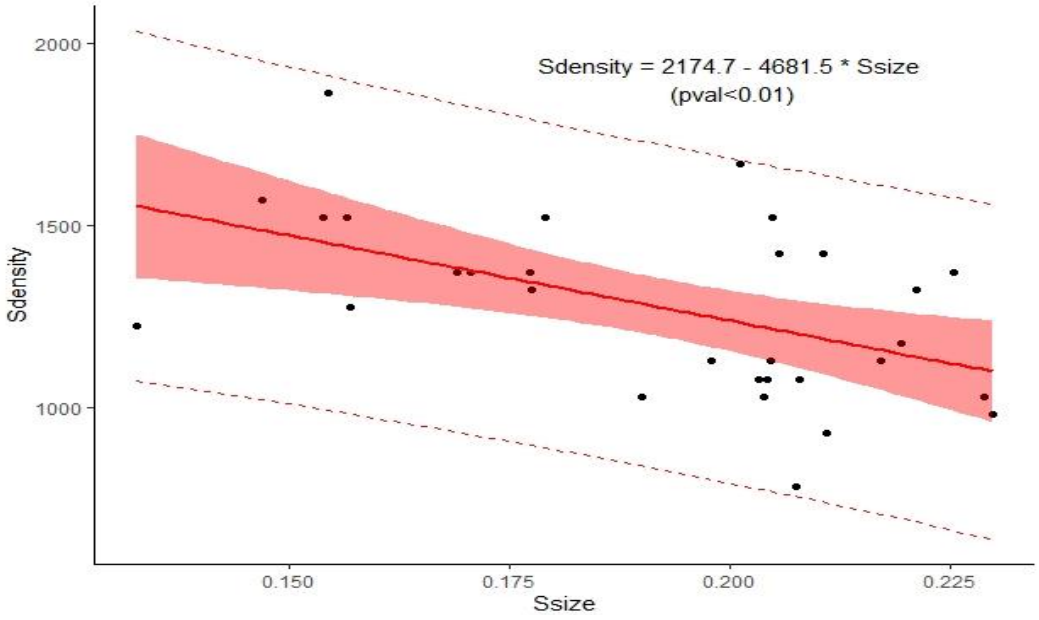

Figure 15: Linear relation between scales density and scales relative size.

The ANOVA Kruskal-Wallis showed that there was a significant difference between the different stages of development for the photophores density P_{De} (χ^2 = 101.69, P-value < 0.0001), for the scale density S_{De} (χ^2 = 63.29, P-value < 0.0001) and for the relative scale size S_{Si} (χ^2=323.97, P-value < 0.0001). Even eliminating the zero data from stage I for all three tests and from stage I and II for the P_{De} test, the results show that there are significant differences between the stages with a P-value < 0.0001. The averages of the three parameters (P_{De}, S_{De} and S_{Si}) for each stage and the comparison with three species of Etmopteridae are given in Table 2.

The Dwass-Steele-Critchlow-Fligner all-pairs test makes it possible to compare the different stages two by two in order to know which stages are significantly different from each other (Table 3; figure 15 (Appendix 1)). Concerning the density of photophores, except for stage I and II where data are equal to zero, there is significant differences from one stage to another with a tendency to increase during the development of the shark (Figure 15a). The scales density, from the second stage of development when it begins to appear, seems to remain more or less constant during development (Figure 16 a), even if some significant differences between the groups are present, as for stage III and V seem different (P-value = 0.029), or stage VI which seems to have a significantly lower density than the other stages (stage I not included because equal to zero) (Figure 15b). Finally, the relative scales size tends to increase during the individual's development and a significant difference between stages II, III and stages IV, V, VI, VII (P-value < 0.0001) appears (Figure 15 c).

Linear regression is done to see if there is a linear relationship between scale density and scale size. The model (Appendix 2) shows that the slope is significant (P-value = 0.00373) and that the Fisher test

does not reject the linear model (P-value = 0.003732). The graph in Figure 15 shows the linear relationship between S_{De} and S_{Si}. As the size of the scales increases, their density decreases with a negative slope of -4681.5.

Photophore histology

The luminous organ in *S. aliae* is made up of different parts. In adults (Figure X a), the photophore is delimited by a layer of pigmented cells, called the pigmented sheath. Within this pigmented sheath is the photocyte, which has the luminescent vesicles which emit light. The photophore is capped by an ILS, formed by the juxtaposition of chromatophore-like cells, which is surmounted by several lens cells.

During the development of the photophore, different steps can be distinguished (Figure X). Firstly, pigmented cells appear between the epidermis and the connective dermal tissue (Figure X c). Then pigmented cells develop and form the pigmented sheath. ILS and lens cells appear as well as a non-fluorescent photocyte, called the protophotocyte (Figure X d). The final step of the photophore development occurs when fluorescent vesicles appear in the photocyte, allowing it to produce light (Figure X a).

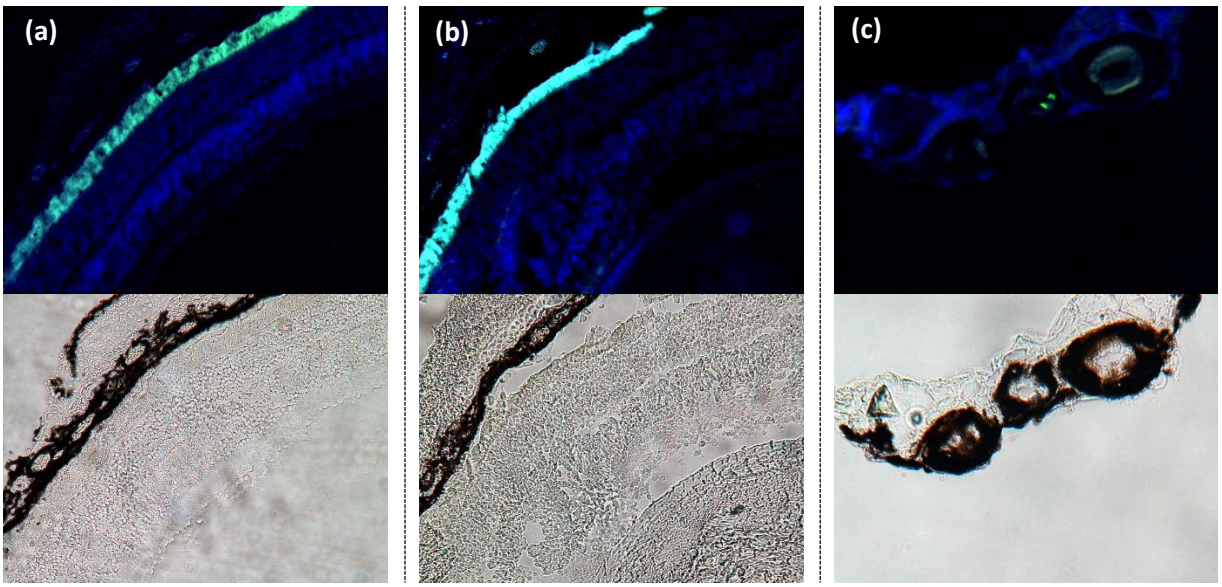

Figure 17: Control test for encephalopsin immunodetection for *S. aliae*. (a) encephalopsin immunodetection within retina. (b) encephalopsin immunodetection within retina with primary antibody omission. (c) encephalopsin immunodetection within ventral skin (photophore) with primary antibody omission.

As shown in the Figure 14 (c) the first pigmented cells appear at stage II. In stage III, the first protophotophores appear lacking luminous vesicles within the organ. From stage IV onwards, the first photophores with luminescent vesicles appear and produce luminescence. The luminous organs of the following stages (stage V to VII) are larger and the different structures are more visible.

Luminous shark encephalopsin immunodetection

The control test performed on the retina of *S. aliae* eye shows that the primary antibody used to detect encephalopsin does not bind to other components, or other opsins (Figure 17a), as no labelling was detected. In addition, a second control was performed by omitting to add the primary antibody on a section of retina and skin, to see if the secondary antibody with the red fluorescent marker attached

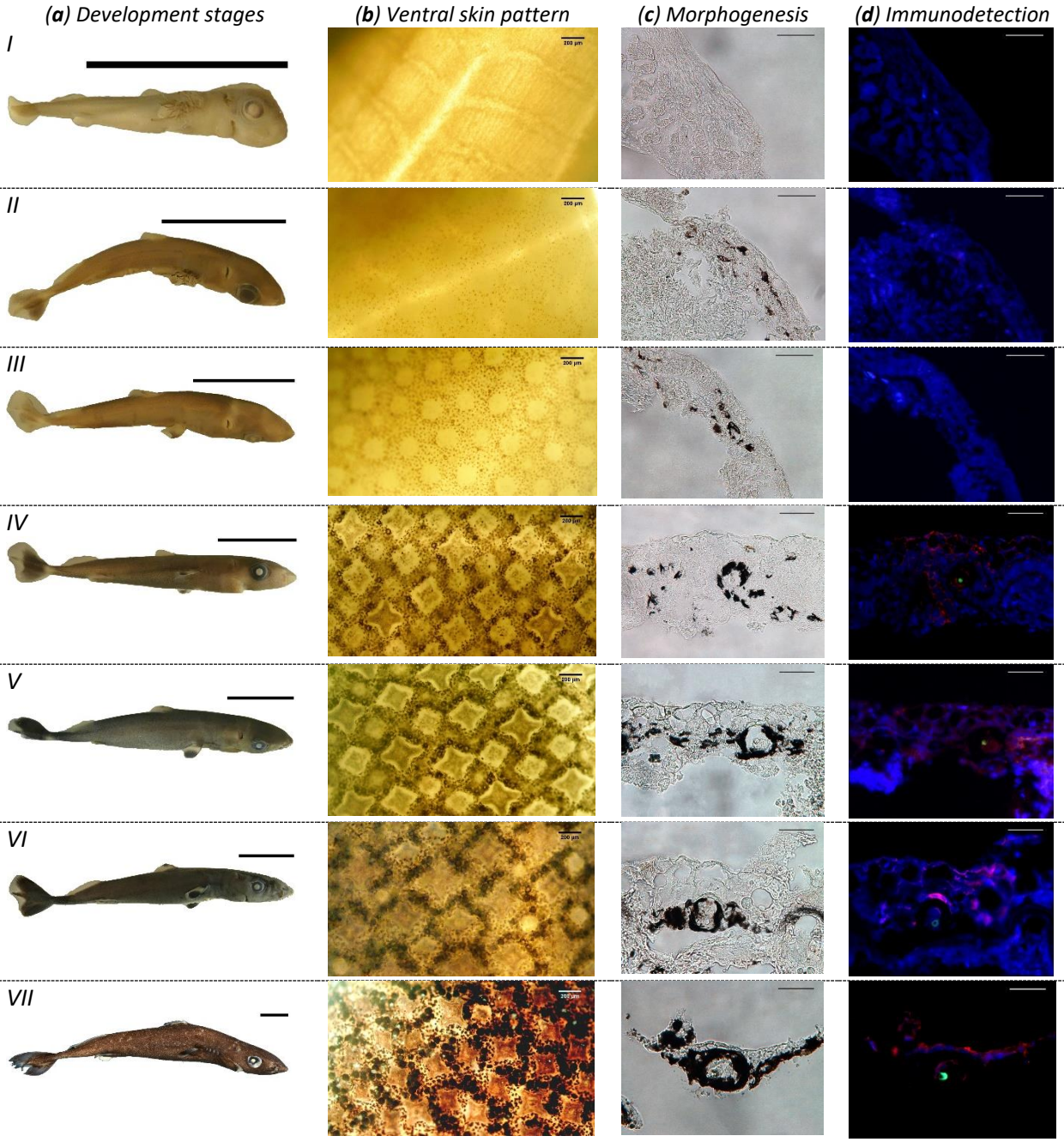

Figure 14. Skin pattern, appearance of photophores and encephalopsin during *Squaliolus aliae* embryogenesis. (a) Developmental series of *S. aliae* (Stage I-VI are embryonic series while stage VII corresponds to an adult specimen. (b) Ventral skin pattern showing scales and photophores development. (c) Histological section of photophores from ventral epidermis showing ontogeny of photophore structures. (d) Histological section of ventral epidermis under UV stimulation highlighting presence of photophore structure. Autofluorescence of luminescent vesicle (green fluorescence), Dapi blue staining associated with the cell nucleus and encephalopsin immunodetection (red labelling) are shown. Scale bars represent 2 cm in (a); 200 µm in (b); 100 µm in (c) and (d).

itself only to the primary antibody and not to certain compounds of the shark. The results show that no labelling appear, both on the retina (Figure 17b) and on the skin or around the photophores (Figure 17c). This confirms the correct labelling and targeting of the primary and secondary antibody pair on encephalopsin.

Figure 14 shows the evolution of light organs (Figure 14c) and encephalopsin expression (Figure 14d) during the different stages of embryonic development (stage I to stage VI) to the adult stage (stage VII) in *S. aliae* (Figure 17a). The expression of encephalopsin appears at stage IV and is mainly expressed around the photophore between the epidermal and dermal layers. Encephalopsin continues to be expressed in later stages of development. Expression always occurs around the luminous organs but seems to express itself more strongly around the ILS and lens cells as can be seen for stage VI marking (Figure 14d) while its expression is weaker when moving away from the photophore. Very little expression of encephalopsin is detected within the photophore but it could be due to the noise of the labelling.

Discussion

The data collected during this study show that during embryogenesis in *S. aliae*, the size and density of the scales vary only slightly (Figure 16a). The average relative size of the scales, once they have appeared in the form of a fine, circular blank, increases only slightly with the growth of the shark. However, there is a relationship between scale size and density. Indeed, the results show that when the relative size of the scales increases, the density tends to decrease, which seems to follow a logical pattern, the space available for their development being more limited with their growth. The scale density remains relatively constant during embryonic development. Little information is available on the development of scales in luminous sharks, which does not allow a comparison between the two families of luminous sharks (Etmopteridae and Dalatiidae). Nevertheless, according to Reif (1985), the difference in scale shape between Dalatiidae (pavement-like) and Etmopteridae (cross-, bristle- or hookshaped) would allow a higher light emission among Etmopteridae, the latter's scales allowing the light from photophore to pass more easily through the epidermis. This suggests that the total coverage of the body by scales is greater in Dalatiidae than in Etmopteridae.

The photophores density, in comparison with scales density, increases significantly during ontogenesis (Figure 16b). These results contrast with those obtained by Claes and Mallefet in *E. spinax*, where the density of photophores tends to decrease during development as the diameter of the light organs increases (Claes and Mallefet, 2008). This difference could be explained by the difference in the size of

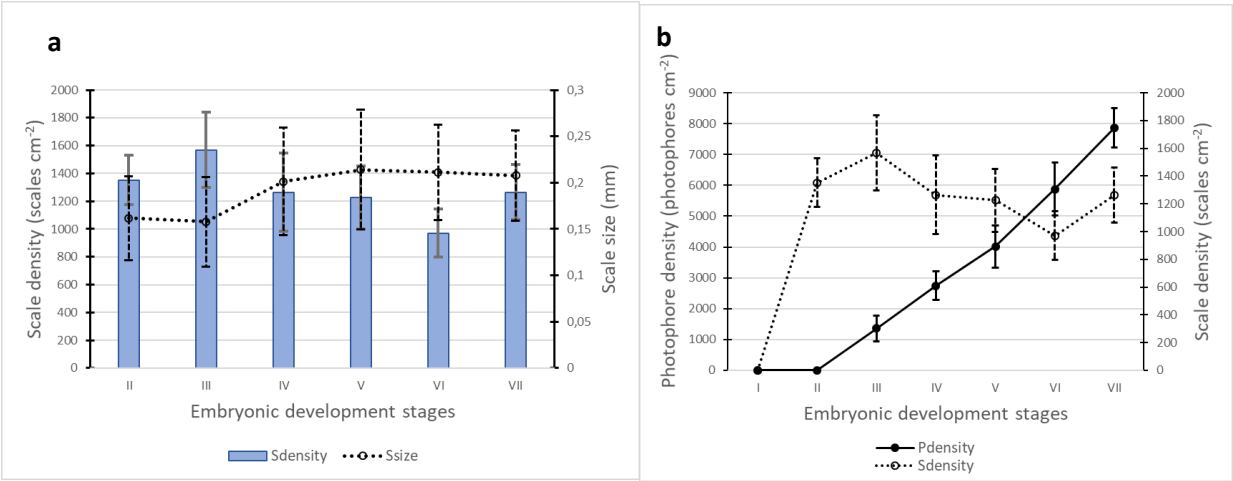

Figure 16: Graphs showing (a) the evolution of scales density and scales relative size during the embryogenic development. The dashed line represents the evolution of scales relative size and the columns represent the scales density. (b) the evolution of photophores density and scales density during the embryogenic development. The solid line represents the evolution of photophores density, and the dashed line represents the evolution of scales density. Data are means ± s.d. (standard deviation).

the light organs in Etmopteridae and Dalatiidae, with Dalatiidae photophores being on average 30% smaller than those of Etmopteridae (Claes and Mallefet, 2009a). In addition, when comparing P_{De} in *S. aliae* and different adults of Etmopteridae species (*E. spinax* (Claes and Mallefet, 2010a), *E. molleri* (Claes and Mallefet, 2015) and *E. splendidus* (Claes et al., 2011)), a significantly higher density is observed in *S. aliae* compared to Etmopteridae species (Table 2). On the other hand, unlike Etmopteridae such as *E. spinax*, smalleye pygmy shark does not have divers luminescent regions, with differences in photophores density depending on the regions of the body whose bioluminescence is used for other more complex uses than just counterillumination (Claes and Mallefet, 2015). In *S. aliae*, the density of photophores, although increasing centripetally from the edge to the median area of the ventral side, is relatively uniform, suggesting a use of light emitted only as camouflage by counterillumination (Seigel, 1978; Reif, 1985; Claes and Mallefet, 2008; Claes et al., 2014).

In *S. aliae*, the organogenesis of photophores follows the same developmental stages as in *E. spinax* described in Claes and Mallefet (2008). First, pigmented cells appear between the epidermis and the dermis. Then, pigmented cells will gather to form the pigment layer of the protophotophore with an ILS and differentiated cells forming the lens cells. The last step in the formation of the photophore is the appearance of a luminescent vesicle within the protophotophore, which then becomes a functional photophore, capable of emitting light (Claes and Mallefet, 2008; Duchatelet et al, 2019a). Unlike etmopterid photophores, *S. aliae* photophores contain only one photocyte within the luminescent organ, which is smaller, as explained earlier (Claes and Mallefet, 2009a). In lantern sharks, Claes and Mallefet (2008) suggest that the acquisition of light compounds should be done by maternal transfer via the yolk sac as it is the case for other luminescent fish species (Rees et al., 1990, 1992; Mensinger

& Case, 1991). The same process of acquisition of luminescent compounds may be suggested in *S. aliae*, however, further studies should be done to confirm this hypothesis. Photophore organogenesis comparison between Dalatiidae and Etmopteridae suggests that photophore may have a common ancestral development. The ability to emit light would have appeared first among the Dalatiidae, allowing them to escape predators. Subsequently, Etmopteridae would have split and colonized deep waters, which would have been accompanied by a reorganization and fusion of light organs to allow, in addition to counterillumination, intraspecific communication and aposematic function in dark benthopelagic waters (Claes and Mallefet, 2008, 2009c, 2015; Straube et al, 2010; Claes et al., 2010c).

Figure 14 shows that the photophores are fully formed and already capable of emitting light while the embryo is still in the maternal uterus (Figure 14 c, d). This suggests that, as in *E. spinax* (Claes and Mallefet, 2008; Duchatelet et al., 2019a), juveniles are able to produce luminescence from birth and use it as camouflage by counterillumination.

The expression of encephalopsin around photophores (Figure 14d) is associated with the development of light organs during ontogenesis. Its expression, more intense close to lens cells and ILS, suggests that it could have an effect on this structure, as seems to be the case with *E. spinax* (Duchatelet et al., 2019a; Duchatelet, 2019). ILS is closely linked to the regulation of light emission, acting like a shutter to allow more or less light to pass through by melanophore pigments motion (Claes and Mallefet, 2010a; Claes et al, 2011 Duchatelet, 2019). The expression of encephalopsin and the emission of luminescence by the embryo into the female's uterus suggest a close relationship between extraocular perception of light and luminescence emission by the photophores of *S. aliae*. Furthermore, the overlap of the emission spectrum of *S. aliae* (peaking at 457 nm (Claes et al. 2014)) and the absorbance spectrum of the encephalopsin (between 410 and 490 nm with a maximum value of 445 nm (Sugihara et al., 2016; Duchatelet, 2019)) assumes that the shark is able to perceive its own emitted light.

According to Haltaufderhyde et al (2015), light could induce signalling pathways via encephalopsin present in the skin and the work of Sety et al (2018) shows that in the presence of blue light, encephalopsin triggers a pathway inducing hyperpigmentation of the skin. Duchatelet (2019) has shown that, in *E. spinax*, light perception by opsin 3 resulted in a reaction cascade involving G protein, inositol triphosphate (IP3) which will act on the concentration of CA^{2+} ions, CA^{2+}-dependent calmodulin and calcineurin phosphatase. This transduction pathway acts on the cytoplasmic dynein-based cellular motor which will allow to move the pigment granules from the periphery to the nuclei of the ILS melanophores, thus allowing more light to pass out of the photophore (Duchatelet, 2019). On the other hand, another transduction pathway using α-MSH and ACTH (Duchatelet et al., 2019 d) will disperse the ILS pigment granules in order to reduce the passage of light. This transduction pathway

involves a G protein which, once activated by a specific ligand (e.g. α-MSH, ACTH) will stimulate an adenylate cyclase allowing an increase in the level of intracellular cAMP. This cAMP will allow the activation of a protein kinase A (PKA) which in turn will induce the phosphorylation of a protein allowing plus-end cellular motor kinesin to act on pigment displacement (Duchatelet, 2019). MT has also been shown to act on light regulation by inhibiting adenylate cyclase activity and thus preventing pigments from moving to the periphery of ILS cells, thereby allowing greater light emission (Duchatelet et al., 2019 d). Thus, encephalopsin, in concert with hormones and neuromodulators, acting on pigment location modulation in the ILS and directly on photocyte light emission, (Duchatelet et al., 2019d) affects the pigment motion in the ILS of photophores in order to control more precisely the light emission by the luminous organ. However, the origin of the light perceived by the encephalopsin is still unknown. It could come from the luminous organ itself, but also from ambient light, or a combination of the two. As suggested in lanternsharks by Duchatelet (2019), and based on the observations made on *S. aliae* in this these, extraocular perception of emitted bioluminescence may act as a feedback control mechanism in *S. aliae* allowing precise control of bioluminescence, concomitantly with hormones and neuromodulators, and effective camouflage by counter-illumination. However, further research will be needed to clarify these points and confirm this hypothesis.

Perspectives and improvement

The results of this work have revealed a possible involvement of an extraocular opsin, OPN3, in the perception and regulation of the bioluminescence emitted by *S. aliae*. However, more precise and detailed studies should be carried out to confirm this hypothesis and clarify the potential role of this opsin on luminescence emission.

A transcriptomic analysis such as that carried out in *E. spinax* by Delroisse et al (2018) would make it possible to show whether the sequence, or at least part of the sequence, associated with encephalopsin is found in the transcriptome of the smalleye pygmy shark and thus support this hypothesis.

A pharmacological analysis could also be done on the photophores of *S. aliae* in order to try to better understand the different mechanisms and the involvement of the various components in the regulation and emission of light as was done for *E. spinax* (Duchatelet, 2019). This would provide a better understanding of the complex regulatory mechanisms of luminescence emission in *S. aliae* and more generally in Dalatiidae.

As the photoemission compound of the smalleye pygmy shark are still unknown to date, as are those of other species of light sharks, the pathway leading to light emission within the photocyte remains

partly unknown. To try to remedy this problem, a mass spectroscopic analysis could be envisaged, as has been carried out on the squid *Watasenia scientillans* (Gimenez et al., 2016), which revealed the luciferase involved in the light emission reaction of this species.

One improvement which could be applied to this study would be to increase the number of individuals per stage. Indeed, during this work, too few samples were used to carry out statistical studies on the different embryonic stages. An increase in the number of replicas per stage and the possibility of making measurements at a precise and constant point on each individual, the photophore density varying centripetally from the edge to the median area (Claes et al., 2014), would allow more precise and powerful results to be obtained concerning the evolution of the development of photophores and scales during ontogenesis.

A series of additional immunodetection for each stage of development could also be performed and observed with more accurate equipment to better distinguish the intensity and location of encephalopsin expression. Indeed, the size of the photophores being reduced, the labelling and precise observation of the tissues has proved in some cases to be inaccurate. To cope with this, an analysis of the photophore via semi-fine sections could be considered, or the use of transmission electron microscopy (TEM) for even more details.

Finally, additional measurements such as the average surface area of the scales, the diameter of the photophores, the light intensity emitted, etc., for each stage could be interesting to understand more precisely how the development of these organs takes place during the growth of the shark.

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Appendices

Appendix 1

Table 3. Pairwise comparisons using Dwass-Steele-Critchlow-Fligner all-pairs test.

	Scale density by development stage					
Stade	I	II	III	IV	V	VI
II	< 0.0001***	-	-	-	-	-
III	< 0.0001***	0.29929	-	-	-	-
IV	< 0.0001***	0.92832	0.12971	-	-	-
V	< 0.0001***	0.63592	0.02914.	0.99983	-	-
VI	< 0.0001***	0.00101*	0.00018**	0.04857.	0.04220.	-
VII	< 0.0001***	0.91795	0.07528	0.99996	0.99557	0.01107·
	Photophore density by development stage					
Stade	I	II	III	IV	V	VI
II	-	-	-	-	-	-
III	< 0.0001***	< 0.0001***	-	-	-	-
IV	< 0.0001***	< 0.0001***	< 0.0001***	-	-	-
V	< 0.0001***	< 0.0001***	< 0.0001***	0.00173*	-	-
VI	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	-
VII	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***	0.00018**
	Relative scale length by development stage					
Stade	I	II	III	IV	V	VI
II	< 0.0001***	-	-	-	-	-
III	< 0.0001***	1.00	-	-	-	-
IV	< 0.0001***	< 0.0001***	< 0.0001***	-	-	-
V	< 0.0001***	< 0.0001***	< 0.0001***	0.79	-	-
VI	< 0.0001***	< 0.0001***	< 0.0001***	0.96	1.00	-
VII	< 0.0001***	< 0.0001***	< 0.0001***	1.00	0.87	0.98

Significatif code : 0 '***' ; 0.001 '**' ; 0.01 '*' ; 0.05 '.' ; 0.1 '' ; 1

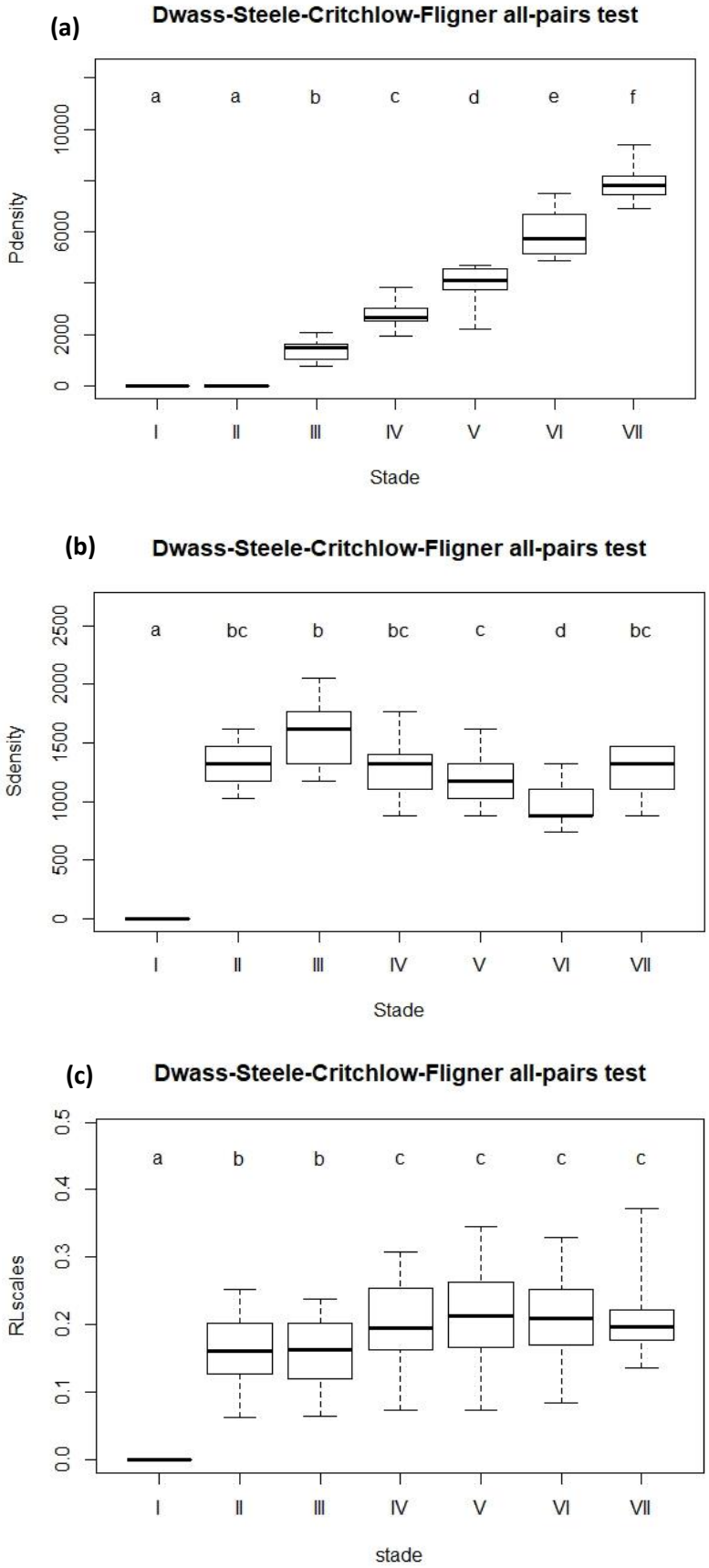

Figure 15. Dwass-Steele-Critchlow-Fligner all-pairs test for photophores density in (a); scales density in (b); scales relative size in (c).

Appendix 2

Table 4. Output RStudio for linear regression between S_{Si} and S_{De}.

```
Call:
lm(formula = SDe ~ SSi, data = table)

Residuals:
    Min      1Q  Median      3Q     Max
-419.49 -135.84  -15.34  157.60  433.72

Coefficients:
            Estimate   Std. Error   t value   Pr(>|t|)
(Intercept)   2174.7        287.2     7.572   3.01e-08 ***
SSi          -4681.5       1479.8    -3.164    0.00373 **
---
Signif. codes: 0‘***’ 0.001‘**’ 0.01‘*’ 0.05‘.’ 0.1‘’ 1

Residual standard error: 213.4 on 28 degrees of freedom
Multiple R-squared:  0.2633,     Adjusted R-squared:  0.237
F-statistic: 10.01 on 1 and 28 DF,  p-value: 0.003732
```