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Swimming capabilities of deep-sea sharks through velocity, anatomical, physiological, and morphological analyses

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RÉSUMÉ

En biologie marine, un des principaux objectifs est de caractériser l'écologie des organismes : où ils vivent, se déplacent, chassent, se reproduisent, etc. Pour répondre à ces questions, l'étude des capacités natatoires reste une caractéristique fournissant plusieurs informations écologiques, encore plus pour les requins qui sont principalement des prédateurs actifs. Les études concernant les capacités natatoires de requins remontent à plusieurs années et ciblaient exclusivement les espèces de surface. Elles ont permis de mettre en évidence des différences entre les espèces pouvant expliquer leurs distributions, leurs lieux de vie mais aussi plusieurs adaptations internes en relation avec ces capacités locomotrices.

Alors que les capacités natatoires des requins de surface commencent à être très bien connues, les informations sur les espèces de grande profondeur restent peu documentées. En effet, à l'heure actuelle, seule les vitesses du requin du Groenland (*Somniosus microcephalus*) et du requin gris (et *Hexanchus griseus*) ont été mesurées au moyen de la télémétrie satellite. D'autres études ont utilisé des mesures indirectes (proxys) telles que le métabolisme musculaire pour estimer les capacités natatoires. Ces études ont montré que le métabolisme anaérobie des requins de grande profondeur est plus faible que celui de leurs congénères de surface. En ce qui concerne le métabolisme aérobie, aucune différence significative n'a été observée entre ces deux catégories de requins. Les auteurs de ces études ont donc conclu que les requins de grandes profondeurs devaient nager moins vite que leurs congénères de surface. Cette conclusion repose principalement sur deux hypothèses : (i) la première s'expliquant par l'impact de l'environnement froid sur le métabolisme de ces poissons ectothermes et (ii) la deuxième appelée hypothèse de l'interaction

visuelle, qui suggère que la pression de sélection pour une vitesse élevée diminue lorsqu'un environnement est plus sombre. Cependant, mentionnons que le faible nombre d'espèces étudiées ne permet pas aux auteurs de généraliser à toutes les espèces de grande profondeur.

Dans ce travail, nous avons étudié les capacités natatoires de plusieurs espèces de requins de profondeur provenant de l'ordre des Squaliformes. Les valeurs de vitesse de croisière mesurées ont été acquises au moyen de l'analyse de vidéos en stéréo. Ces données ont révélé que tous les requins de profondeur ne nageaient pas lentement. En effet, bien que le *Dalatias licha* présente la valeur de vitesse la plus faible jamais répertoriée et que les autres congénères non lumineux présentent de faibles vitesses intermédiaires, les requins lumineux de la famille des Etmopteridae nagent à une vitesse comparable à certains requins de surface. Ces valeurs de vitesse de croisière plus élevées chez les Etmopteridae lumineux pourraient être un avantage. En effet, les requins lumineux utilisent leur lumière ventrale pour disparaître des proies et prédateurs se trouvant en dessous d'eux, ce camouflage est appelé contre illumination. Cependant, la régulation de leur lumière se fait sous un contrôle hormonal lent et donc pas efficace avec l'idée de contre illuminateur qui doit moduler rapidement sa lumière. Dans ce contexte, une nage rapide pourrait être utile pour migrer dans la colonne d'eau dans le but de faire correspondre l'intensité de leur lumière ventrale avec celle de la lumière résiduelle environnante, cette hypothèse est appelée le « suiveur d'isolumes ».

De plus, des études sur le système musculaire ont aussi été faites à travers la quantification des muscles rouges et blancs ainsi qu'en analysant l'activité de certaines enzymes au sein de ces muscles. Les résultats ont mis en avant que les Etmopteridae

possédaient plus de fibres rouges et un métabolisme aérobique plus élevé que leurs congénères non lumineux et *D. licha*. A l'opposé, les Etmopteridae possèdent moins de muscles blancs et un métabolisme anaérobique plus faible que ces mêmes congénères. Cela suggère que les Etmopteridae ont un système musculaire plus efficace pour la vitesse de croisière alors que les autres espèces de grandes profondeurs ont misé sur un système musculaire ciblant les phases d'accélération. Cette dernière hypothèse pourrait expliquer pourquoi on retrouve des Etmopteridae dans les contenus stomacaux des plus grandes espèces de requins de profondeurs.

L'étude de la flottabilité des requins de grandes profondeurs a aussi confirmé que ceux-ci possédaient un foie fournissant plus de portance que les espèces de surface. Cette étude a aussi permis d'amener des preuves concernant la relation existante entre la portance que le foie fournit et la proportion de muscles rouges hypothétisée par Bone en 1979, mais qui n'a jamais été prouvée. En effet, les requins avec un foie fournissant plus de portance possèdent moins de muscles rouges au sein de leur corps. Cette étude est donc la première preuve de l'hypothèse de Bone.

Finalement, un grand nombre de variables morphologiques a aussi été étudiée. Cela a permis de montrer que les requins de l'ordre des Squaliformes ne partagent pas une forme de corps similaire alors que dans la littérature ils se retrouvent tous regroupés dans un même morphotype. La forme de l'épine pourrait aussi avoir un impact sur la vitesse de nage mais des études mécanistiques doivent être encore réalisées pour confirmer cela. Enfin, une nouvelle forme de nageoire caudale a été décrite dans ce travail pour *D. licha*. En effet, cette nouvelle nageoire caudale ne possède pas de fourche et semblerait être encore moins efficace

dans la propulsion que les nageoires caudales dites hétérocerques. Toutefois, ici encore, de futures analyses mécanistiques sont essentielles avant de tirer une conclusion.

Cette thèse se révèle comme la première étude multidisciplinaire concernant les capacités natatoires des requins de grandes profondeurs. Il en découle plusieurs informations, indices concernant l'écologie de ces espèces méconnues. Cette thèse a aussi amené plusieurs nouvelles questions, et des futurs projets et recherches sont en cours afin de mieux comprendre l'écologie de ces animaux fascinants.

ABSTRACT

In marine biology, one of the main objectives is to characterize the ecology of organisms: how they live, move, hunt, reproduce, etc. For that purpose, the study of swimming capabilities remains a feature providing several ecological information, especially for sharks which are mainly active predators. Studies regarding shark swimming capabilities have been initiated a long time ago for shallow-water species. They allowed highlighting differences between species explaining shark distribution, home range but also several internal adaptations related to their locomotor capabilities.

While swimming capabilities start to be well known for shallow-water species, information regarding deep-sea sharks is still poorly documented. Indeed, only the speeds of the Greenland shark (*Somniosus microcephalus*) and the bluntnose sixgills shark (*Hexanchus griseus*) were measured using satellite telemetry and revealed the slowest velocity recorded for a shark. Other studies have focused on indirect measurements (proxies) of the locomotor muscle metabolism to estimate the swimming capabilities. They have shown that anaerobic metabolism of deep-sea species is lower than their shallow-water counterparts. In contrast, the aerobic metabolism does not show significant difference. Therefore, authors have concluded that deep-sea sharks should have lower swimming capabilities than their shallow-water counterparts. This conclusion is mainly based on two hypotheses: (i) the effect of the cold environment decreasing muscle activity and (ii) the visual interaction hypothesis which suggests that the swimming capabilities of an organism decrease when it lives in a darker environment. However, the low number of deep-sea shark species considered in these studies does not allow to draw this general conclusion for all deep-sea sharks.

In this work, we studied the swimming capabilities of several deep-sea sharks from the Squaliformes order. Cruise swimming speed values were obtained using stereo-video analyses. Data analyses have revealed that not all deep-sea sharks swim slowly. Indeed, even if *Dalatias licha* displayed the lowest speed value ever recorded, and other non-luminous deep-sea sharks intermediate slow cruise swimming speeds, luminous sharks from the Etmopteridae family swim at similar velocities than some shallow-water sharks. This higher cruise swimming speed of Etmopteridae species could be an advantage. Indeed, luminous sharks use their ventral light luminescence to vanish from preys and predators underneath them. It is called counterillumination camouflage. However, their luminescence regulation is under hormonal control which is slow and do not fit with the counterillumination hypothesis which need to modulate quickly its light intensity. In this context, a high velocity might be useful to migrate in the water column in order to match their ventral light with the downwelling light of their environment, this hypothesis is called "isolume follower".

In addition to that, studies of the muscular system were done through red and white muscle fibers quantification and specific enzyme assays. Results highlighted more red muscle in Etmopteridae species with higher aerobic metabolism but lower white muscle and anaerobic metabolism than their non-luminous counterparts and *D. licha*. These results suggest that Etmopteridae display a muscular system design more for an efficient cruise swimming speed. In contrast, the higher white muscle proportion and anaerobic metabolism of other non-luminous deep-sea sharks suggest higher burst capabilities which might explain why Etmopteridae species are found in stomach contents of these other deep-sea sharks.

Study regarding the buoyancy of deep-sea sharks have allowed to confirm that the lift provided by the liver is higher for deep-sea shark species than shallow-water ones. It also provided evidence of relation between the hydrostatic lift and the proportion of red muscle fibers as suggested by Bone in 1979 but never proven. Indeed, sharks with a liver providing more buoyancy display less red muscle fibers across their body. This study is thus the first evidence of the Bone's hypothesis.

Finally studies of morphological features have revealed that Squaliformes sharks displayed different shapes while they were grouped in the same morphotype previously. The spine shape might also have an impact on the velocity but future mechanistic studies should be done on the subject. Also, a new shape of caudal fin was described for *D. licha*. This caudal fin does not display any fork and should be less efficient for the thrust than heterocercal fins but here again, mechanistic studies should be conducted before obtaining any strong conclusion.

This thesis sets up as the first multidisciplinary approach regarding the swimming capabilities of deep-sea sharks revealing differences which give information regarding the ecology of these poorly known species. It also brought new questions, future researches pathways and projects are currently in progress in order to still better understand the ecology of these fascinating animals.

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Introduction

INTRODUCTION

In this first part, a brief description of oceans zones is given using horizontal but mainly vertical criteria entailing the description of what deep-seas are. Secondly, the description of sharks in general then the studied species will be provided with a focus on deep-sea specimens and their adaptations to this particular environment. Finally, the importance of swimming capabilities for sharks will be presented, including information regarding physiological, anatomical, and morphological adaptations.

OCEANS AND DEEP-SEAS

In contrast with its name, our earth is primarily covered by water. In fact, 70% of our planet is water hence the name blue planet. With a mean depth of 3800 meters and the deepest place at 10 994 meters (Mariana trench), oceans and seas represent the largest habitat of our planet. In such a large environment, conditions and biodiversity vary greatly depending on the studied place (Herring, 2002; Ramirez-Llodra et al., 2010). Therefore, scientists have classified oceans and seas based on horizontal and vertical zones according to the physical, chemical, topographical features using specific words that are defined in the next section and will be of importance for the rest of the introduction to properly locate the studied species.

Horizontal zones

The horizontal zones are mostly classified depending on their topography (Fig. 1A). The *continental shelf* is the zone continuing the coastal terrestrial crust; this zone extends on average up to 60 kilometers from the coast. It is often characterized by low depths (35 to 250 meters) and represents only 5% of the total ocean surface. Currently, in terms of biodiversity, this zone represents the

richest part of the ocean and it is also the most studied due to its accessibility. At the end of the *continental shelf*, the oceanic floor goes down sharply. This is known as the *continental slope* and is characterized by a strong gradient (1:40). At around depth of 3000 meters, the slope gradient decreases (1:100); it is the *continental rise* which is the meeting region between the continental and oceanic tectonic plates. These last two zones represent together more than 20% of the total ocean surface. Trenches and canyons are often found in the *continental slope* but rarer in the *continental rise* which has an oceanic floor composed of sedimentary layers originating from the *continental slope* collapses and sliding events. After the *continental rise* starts the *Abyssal plain* characterized by a smaller slope (1:1000) depth from 4000 to 6000 meters. This zone represents more than the half of the total ocean surface yet very little is known about this zone. Some trenches and rifts are present in this abyssal territory which can be deeper than 10 000 meters (Gage and Tyler, 1992; Danovaro et al., 2014; Mengerink et al., 2014). In this work, we refer to neritic or coastal animals as marine organisms living in the water found on the *continental shelf* while oceanic animals correspond to organisms living in the other horizontal and vertical zones. Regarding benthic species will refer to animal which live close to the bottom, with bentho-pelagic species that spend part of their time near the bottom and demersal species that spend all of their time on the bottom

Vertical zones

Depending on the topography but also on the physical and chemical features oceans were also classified into different vertical zones. One parameter which has a constant logarithm change according to the depth is the pressure; every ten meters of water column the pressure increases of 1 atmosphere (Sverdrup et al., 1942; Gage and Tyler, 1992). The first and the shallowest layer is

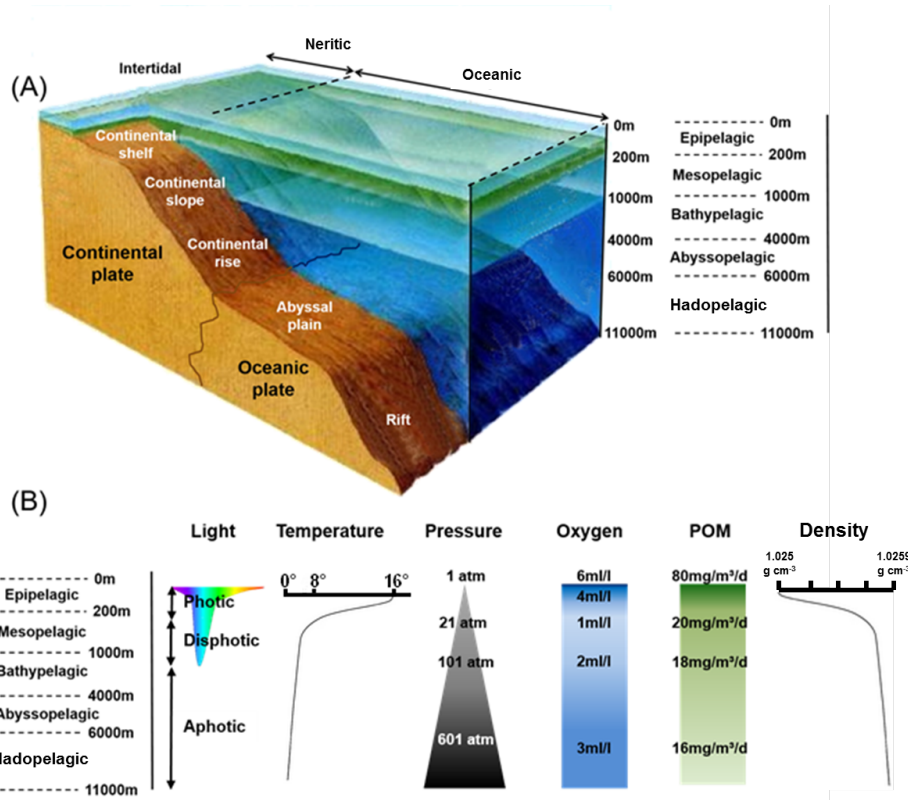


Fig. 1 (A) horizontal and vertical zones of ocean. (B) Physical (light, temperature, pressure, density) and chemical (oxygen, POM corresponding to Particular organic matter) changes according to the depth.

the *epipelagic zone*, which starts at the surface going down to 200 meter in depth (Töpke, 2018). This zone is characterized as photic zone; the light intensity is enough for algae to photosynthesize (Haedrich, 1996; Douglas and Partridge, 2011). The temperature and oxygen concentration are at their highest values (Ramirez-Llodra et al., 2010; Mallefet, 2017); the thermocline, corresponding to a strong decrease in the temperature according to the depth, often occurs at the end of this zone (Sverdrup et al., 1942; Haedrich, 1996). In contrast, the sea-water density is at the lowest value (1.025 g cm^{-3}) and the pycnocline corresponding to a strong

increase in the sea-water density according to depth, also often occurs at the end of this zone. All these features allow for a high biodiversity and therefore the particular organic matter (POM) displays its higher productivity in this layer (Gage and Tyler, 1992; Herring, 2002; Koslow, 2007). The next vertical layer is the *mesopelagic zone* (depth from 200 to 1000 meters) (Töpke, 2018). There is still a dim blue light in this zone but its intensity is too weak to allow for photosynthesis hence the informal name of twilight zone (Haedrich, 1996; Douglas and Partridge, 2011). After the thermocline and the pycnocline, temperatures decrease slightly from 8° to 2°C while the density increase from 1.026 to 1.28 g cm⁻³) (Sverdrup et al., 1942; Haedrich, 1996). Oxygen levels are at lowest concentrations (1-2 ml l⁻¹) in this zone with some anoxic regions occurring in some places (Levin, 2003; Ramirez-Llodra et al., 2010). The POM represents only a fourth of the one observed in the *epipelagic zone*; values are around 20 mg/dm³/l depending to the geographical place (Koslow, 2007). The third vertical layer is the *bathypelagic zone* from depth of 1000 to 4000 meters (Töpke, 2018). From this zone no residual light goes into the water; this zone and the next ones are aphotic (Haedrich, 1996; Douglas and Partridge, 2011). The temperature and POM are low but their changes according to the depth become non-significant (Sverdrup et al., 1942; 1996Gage and Tyler, 1992; Haedrich). In contrast, the oxygen concentration rises to reach the half of the surface level (± 3 ml l⁻¹). The two next zones: *abyssopelagic* (4000-6000 meters' depth) and *hadopelagic* (depth of 6000 - 11000 meters) are similar regarding their physical and chemical features, which in turn are comparable to the previous zone (*bathypelagic zone*); differences are more based on the topography (Töpke, 2018). The pattern of physical and chemical changes explained above correspond to general scenarios that we

can find in ocean. These general patterns can vary depending on the areas or seasons.

SHARKS

Generalities

Evolution and classification

Sharks are animals from the chondrichthyan class which comprises of sharks, rays, skates and chimeras (Carrier et al., 2012; Ebert et al., 2013). Chondrichthyans are a monophyletic group characterized by a cartilaginous skeleton and the presence of pelvic clasper as sexual organ for male individuals. They are the first gnathostomata vertebrates (Heinincke et al., 2009; Grogan et al., 2012).

It is hard to define the exact period when chondrycthyans first appeared due to the difficulty in finding well preserved fossils. Indeed, cartilage degrades faster than bone; therefore it is easier to find teeth and placoid scales of sharks (calcified skin structure) than their whole bodies (Miller et al., 2003, Grogan et al., 2012). The first occurrence of cartilaginous fishes seems to have appeared 450 million years ago during the inferior Devonian (Fig. 2). This first evidence of cartilaginous fishes was based on placoid scale and no teeth were found, it would suggest that the earliest forms could have been toothless (Sansom et al., 1996). Scientists are still debating if these were true sharks or shark-like animals.

The first main differentiation appeared around 420 million years ago with chimera's splitting from Elasmobranchii to form Holocephalii sub class (Brazeaux and Friedman, 2015; Coates et al., 2017). This sub class is represented by rabbit fishes (*Chimaera*) and elephant fishes (*Callorhynchus*), and are characterized by gills covered with an operculum like modern day

bony fishes. Elasmobranchii, on the other hand, are characterized by gills displaying individual slit (between 5 to 7 slits) (Fig. 2).

The first fossil teeth were dated around 410 million years ago belonging to an ancient fish called *Doliodus problematicus* (Cuni et al., 2013). The first entire fossil of a shark-like organism is aged around 380 million years, just in middle of the Devonian. The named of this organism is *Cladoseleache*, as active predators they had torpedo-shaped bodies, forked tails and dorsal fins (Dean, 1894; Cuni et al., 2013). It was right after the mass extinction at the end of the Devonian, which killed off at least 75% of all earth biodiversity, that shark diversification developed, this period is known as the golden age of sharks. This shark domination led to a whole variety of shapes and forms such as *Falcatus falcatus*, a cartilaginous fish growing up to 30 cm long. The males also displayed prominent fin spines that curved anteriorly over their head (Lund, 1985). Another example is, *Helicoprion*, a genus that appeared around 340 million years ago. Organisms from this genus are characterized by teeth arranged in a "tooth-whorl" strongly reminiscent of a circular saw (Fig. 2) (Lebedev, 2009).

The end of the Permian Period (252 million years ago) saw yet another mass extinction event, wiping out around 96% of all marine life but a handful of shark lineages persisted (Cuni et al., 2013). Around this period a numerous shark-extinct species lived in ocean such as sharks from the Hybodontiformes order characterized by their conical tooth shape (Carrillo-Briceño et al., 2016). This order was the closest relative to Neoselachii, which comprise of all current Elasmobranchii. Neoselachii are divided in three superorders: (i) Galeomorphii characterized by sharks with anal fin, (ii) Squaliformii which do not present anal fin, and (iii) Batoidea which are distinguished by their flattened bodies,

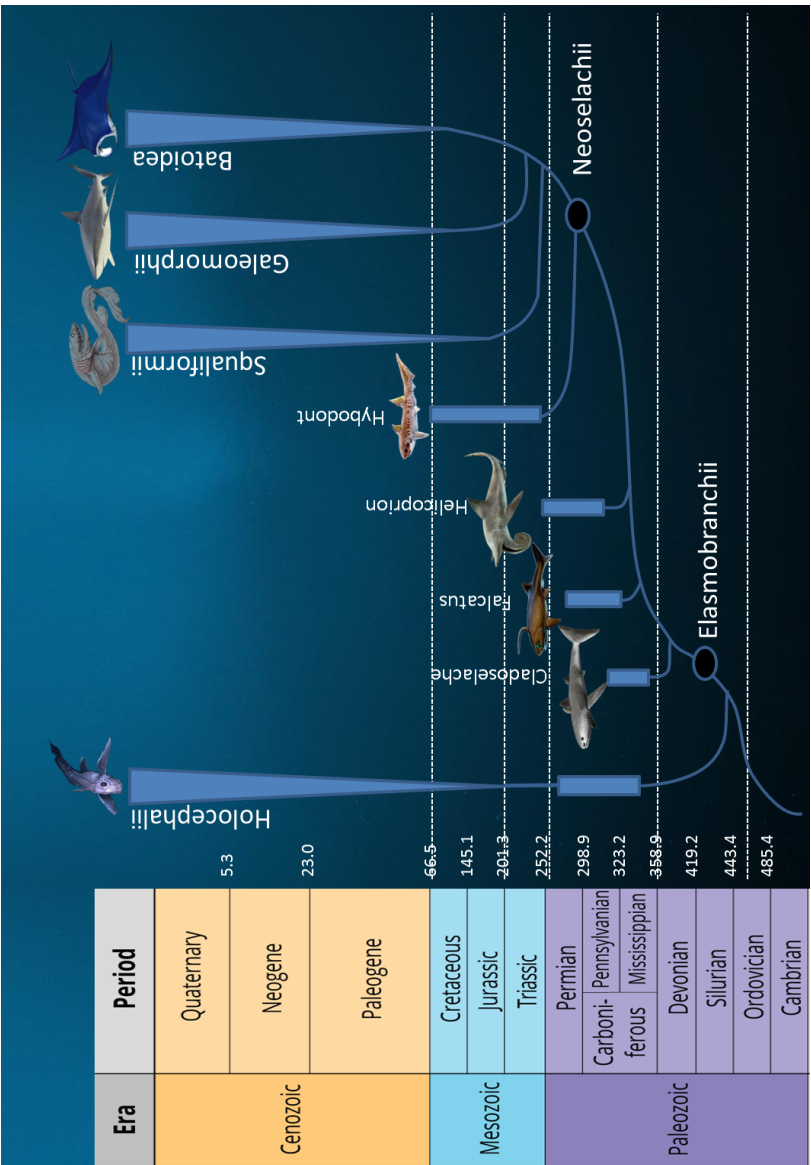


Fig. 2 Chondrichthyans evolution through a simplified tree of life, illustrations of selected extinct species and particular evolution step. White dotted lines correspond to massive extinction.

enlarged pectoral fins that are fused to the head, and gill slits that are placed on their ventral surfaces (Grogan et al., 2012; Ebert et al., 2013) (Fig. 2 and 3).

It is during the early Jurassic Period (195 million years ago) that the oldest-known order of modern sharks, the Hexanchiformes, evolved. They were followed during the rest of the Jurassic by most modern shark orders. Only the Pristiophoriformes sharks appeared a bit later, during the Cretaceous (Fig 3) (Grogan et al. 2012; Cuni et al., 2013).

Sharks are grouped in eight different orders mainly based on morphological differences. Galeomorphii are divided into four orders: (i) Carcharhiniformes which have five gill arches, a specific movable membrane (nictitating membrane) over their eyes to protect them when feeding, (ii) Orectolobiformes which possess five gill arches and a mouth in front of their eyes, (iii) Heterodontiformes which display spines on the base of both dorsal fins, and (iiii) Lamniformes which have five gill arches and no nictitating membrane. Squaliformii are also divided into four orders: (i) Squaliformes which present five gill arches a not flattened body a short snout and most of them harbor dorsal spines (ii) Squatiniformes displaying five gill arches and a flattened body, (iii) Hexanchiformes which present between six to seven gill arches, and (iiii) Pristiophoriformes which present five gill arches, a body not flattened with a long saw-shaped snout (Compagno et al, 2005; Grogan et al., 2012; Ebert et al., 2013).

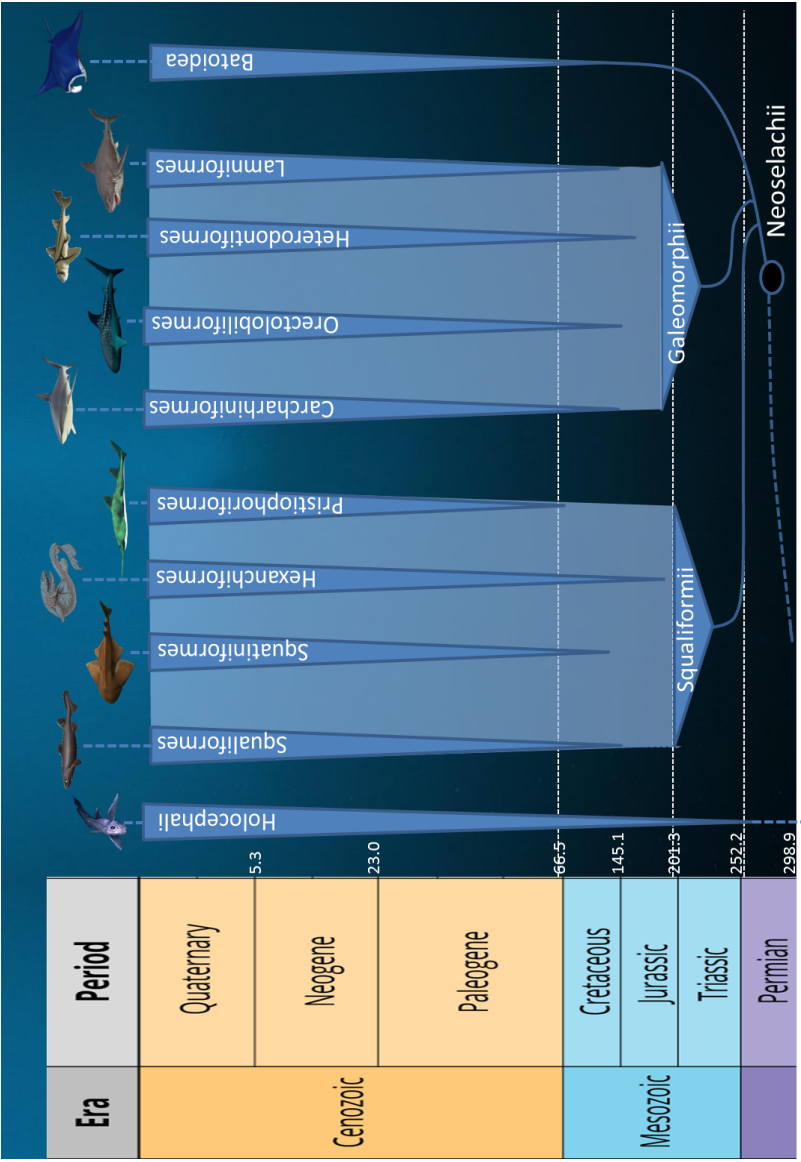


Fig. 3 Neoselachii evolution through a simplified tree of life with current orders emergence. White dotted lines correspond to massive extinction.

Body structure

The body structure of sharks presents several distinct features in comparison with bony fish. Firstly, their endoskeleton is composed of chondrocytes in an extracellular matrix surrounded by a fibrous perichondrium (Kemp and Westrin, 1979; Dean and Summer, 2006). This skeleton is composed anteriorly of a box-like skull (the chondrocranium); cartilaginous structure supporting gills (gill arches); and the jaw (Ebert et al., 2013). In the posterior part of the body we find a long vertebral column finishing with the upper or dorsal caudal lobe of the tail. Finally, cartilaginous structure supports the fins, for some species, the spines and for males, the claspers (Fig. 4) (Ebert et al., 2013). The advantage of a light softer skeleton is efficient swimming and the lack of any connection between most of these cartilaginous parts improves the flexibility. As results most species have high maneuverability (Lebrun, 2001).

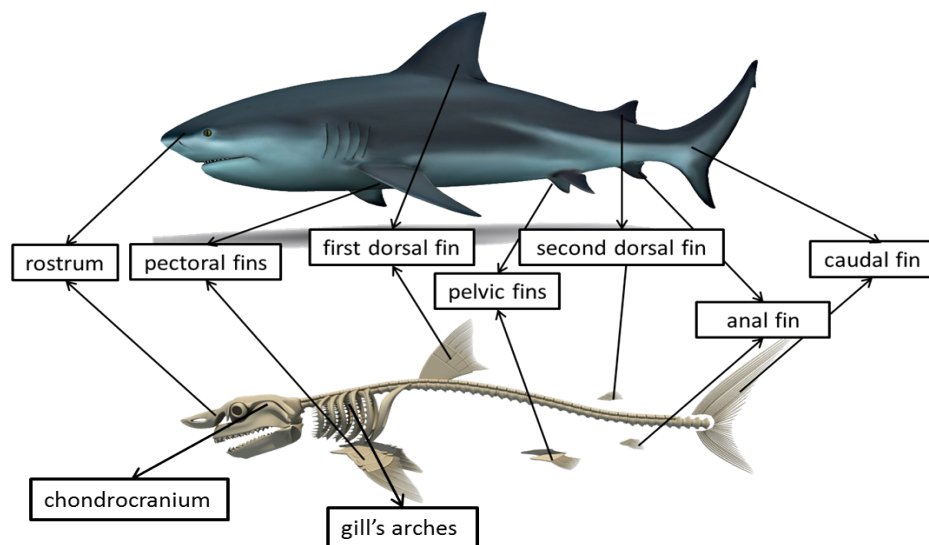


Fig. 4 Shark external anatomy and skeleton anatomy

Sharks display several fins which play a role in their swimming capabilities. These fins can be grouped in two categories: (i) the horizontal fins (pectoral and pelvic) primarily generate lift but act also against pitch, and (ii) vertical fins (first and second dorsal, anal, and caudal fin) counteract roll induced by water flow and yaw (Ebert et al., 2013) (Fig., 5). The caudal fin plays a major role in propulsion by beating. This tail can present a variety of shapes between species, which influences not only their velocity but also their maneuverability; this point will be discussed later in the caudal fin section (p36-38).

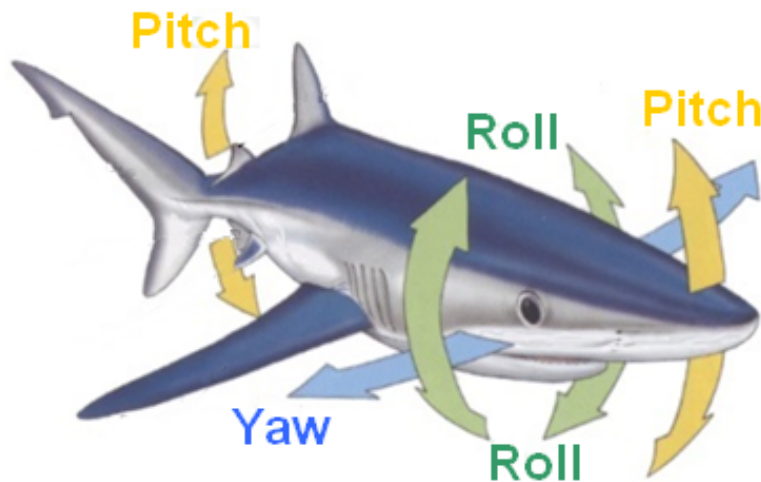


Fig. 5 Movements of shark and effect of the water on shark orientation (Ebert et al., 2013).

Although the majority of the shark skeleton is cartilaginous, some parts display calcified structure such as placoid scales, teeth and spines. Sharks are protected by a tough skin. Indeed, placoid scales or denticles are structures covering the entire body of sharks and even the buccal cavity (Ebert et al., 2013). These scales have a similar structure than teeth with a basal plate mooring the structure inside the dermis by fixing the collagen fiber

(Fig. 6A). The pulp is the first cavity which contains primary cells. This pulp is surrounded by the dentin which is a high vascular tissue which is in turn covered by the enameloid (Reif, 1985; Lang et al., 2012; Deynat, 2014). The enameloid is a high calcified tissue composed of calcium hydroxyapatite crystal network ($\text{Ca}_5[\text{PO}_4]_3\text{OH}$) which can fix Fluor changing it to calcium fluorohydroxyapatite crystal network ($\text{Ca}_5[\text{PO}_4]_3\text{F}$) (Frank, 1961; Lussi et al., 2015). The fact that it is similar to enamel, explains why these two are often compared and confused. The external shape of placoid scales is very variable depending on the species, the age, the sex and the position along the body (Reif, 1985). This variable shape is often associated with four different functions: (i) those that have spine or sharp structures are associated with mucus and are aimed at the defense against parasites; (ii) knob ones that provide abrasion resistance, for sharks living close to the bottom for example; (iii) those which have groove patterns are designed for drag-reduction and thus improve the swimming capabilities; and (iiii) the last shape associated with luminous shark is based on spaced denticles allowing photophores (luminous organs) to glow out (Fig. 6B). Placoid scales are replaced during the growth or when damaged (Reif, 1985; Raschit and Tabit, 1992; Claes and Mallefet, 2009a; Deynat, 2014).

The second calcified shark tissue is the tooth. Most sharks possess numerous rows of teeth that continually move forward like a conveyor belt. Primordial teeth are formed within a specific groove inside the dermis and grow along the conveyor belt to finally replace old teeth which fall out (Fig. 7A) (Motta and Huber, 2012; Klimley, 2013). Teeth display different shapes between species which reflect feeding habits: blade-like teeth are slender and suitable for seizing and tearing food such as fish and cephalopods (Fig. 7B), triangular with denticulate edges teeth are

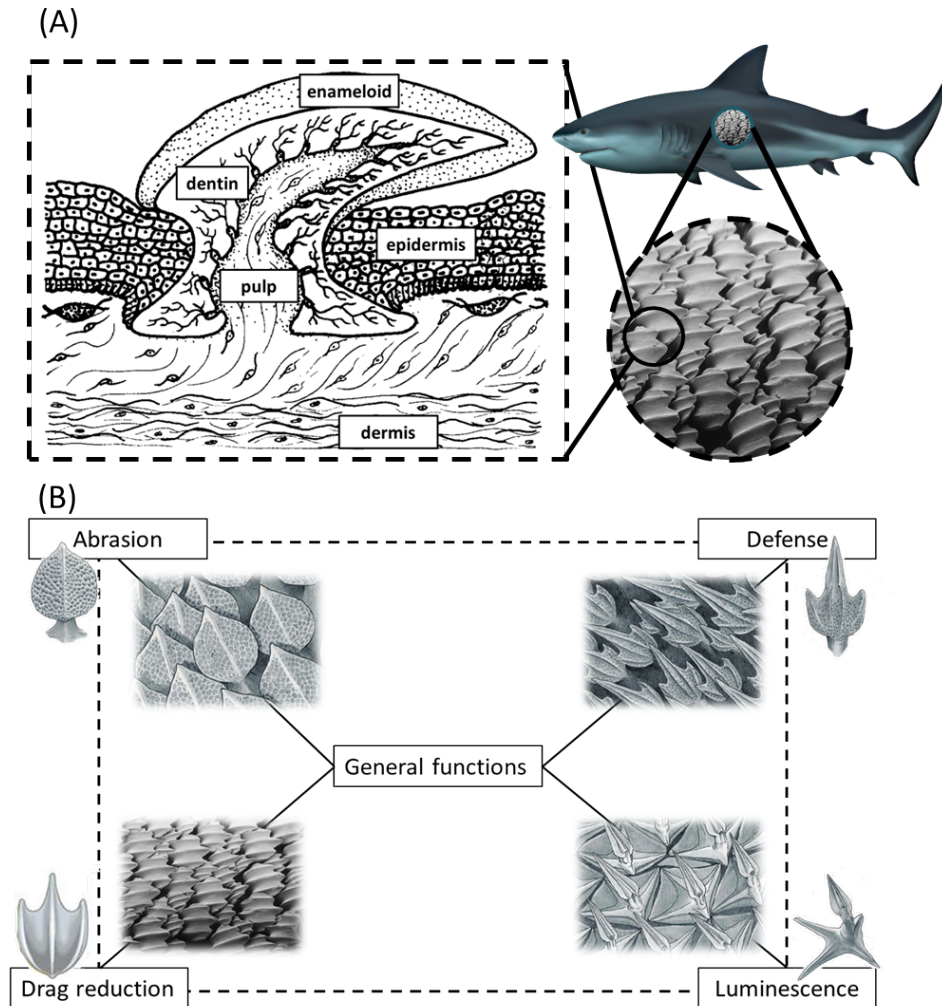


Fig. 6 Shark placoid scales: (A) internal anatomy of placoid scales; (B) function of placoid scales based on their morphology (modified from Reif, 1985).

specialized for cutting and are found in predators feeding stronger tissues of marine mammals (Fig. 7C), multicuspidate teeth are designed for grasping (Fig. 7D) and plate-like teeth are used to crush hard tissues such as gastropod or crustacean shells (Fig. 7E) (Motta and Huber, 2012; Klimley, 2013). Other shapes can be found in some species among which a few even display

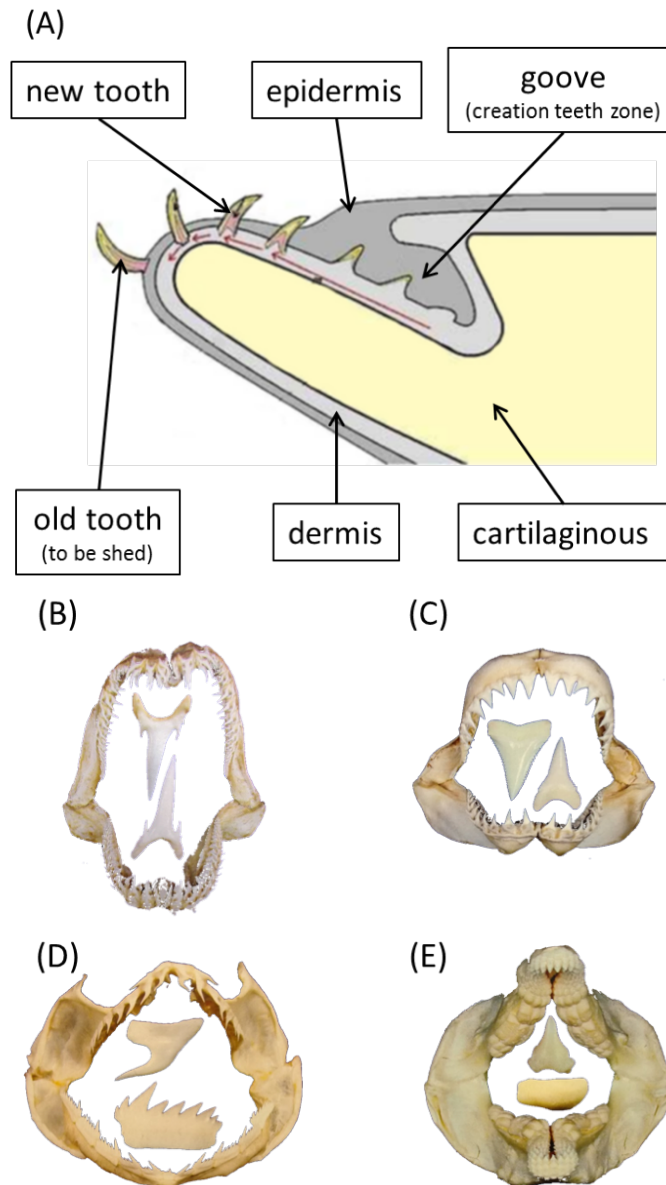


Fig. 7 Shark teeth and jaws: (A) simplified pattern of teeth appearing along the conveyor belt process; (B) Jaw and teeth of *Odontaspis ferox*; (C) Jaw and teeth of *Carcharodon carcharias*; (D) Jaw and teeth of *Heptanchias perlo*; (E) Jaw and teeth of *Heterodontus portuajacksoni* (Picture from Chappel and Seret, 2014).

heterodonty (i.e. shark displaying different teeth types on their upper and lower jaw) (Motta and Huber, 2012).

The last calcified structures which can be found in sharks are the finspines. Some shark species from Heterodontiformes and Squaliformes orders display spine connected to the dorsal fins (Maisey, 1979). In contrary with placoid scales and teeth, finspines are not replaced when they are damaged. It is believed that their main function is to protect the shark from predators (Maisey, 1979; Haddad et al. 2016; Duchatelet et al., 2019 – annex 4, p 241-251). Some species present venomous tissues at the base of their spines (Maisey, 1979; Haddad et al., 2016). It has also been suggested that the spine can play a role in the swimming capabilities of shark by increasing their maneuverability (Maisey, 1979; Lauder et al., 2004); this point will be discussed later in the finspine section (p 41). Transversal sections of the spine have been used as a tool to determine the age of shark. However, improvement of methodology still needs to be realized for more accurate results (Cailliet, 1990; Clarcke et al., 2002; Irvine et al., 2016; Clarcke and Irvine, 2006).

Senses

Most sharks are active predators tracking their preys in oceans. Active hunters require a diversity of tracking tools. For that purpose, sharks have developed several sensitive sensory organs that allow them to hunt, avoid possible predators, and locate themselves in their environment (Lebrun, 2001). These sensory organs are classified into mechanoreceptors, electroreceptors, chemoreceptors, or photoreceptors depending on physiological mechanism allowing for their activation. The range of senses vary greatly depending on the sensory organs, and include perceiving sound as far as 1000 meter to detecting electric fields less than 50 centimeters away using electroperception (Fig. 8A) (Lebrun 2001).

Underwater, sounds travel four times faster than in the air, moreover, lower frequencies dissipate slower than higher ones. Sharks have an inner ear which is involved in sound perception and balance. The shark's inner ear is composed of three semi-circular canals set at right angles to each other, three chambers (the utricle, the saccule, and the lagena) and two ampulla (Myberg, 2001; Gardiner, 2012) (Fig 8C). The sound detection takes place in the ampulla where the cupula structure gathers hair sensory cells which detect vibrations. It has been shown that sharks have a maximum sensitivity between 400 to 600 Hz and at 40Hz; the last frequency typically corresponds to irregular pulse of injured animals (Kritzler and Wood, 1961; Nelson, 1967; Myberg, 2001). The balance management takes place in the chambers covered with the macula (sensitive epithelium) holding calcium carbonate granules, called otoconia, a sort of otoliths. These otoconia move and stimulate hair-cells when the shark is in motion giving information regarding its orientation, acceleration and balance (Maisey, 2001).

Smelling is also important for long-distance sensing. Shark nostrils, located on the ventral side of the head, anteriorly of the mouth, present a close circulation, with the sole function of detecting olfactory molecules (Lebrun, 2001). They present two olfactory organs called olfactory sacs or “rosettes” enclosed in the nostril and are linked to the olfactory bulb. The water containing smelly molecules go inside through the incurrent aperture, which is separated from the excurrent aperture by a nasal flap. In the olfactory sacs, the water flows through a succession of folds, known as secondary lamellae, which cover the primary lamellae until the excurrent aperture (Fig 8D). The epithelium covering the secondary lamellae is composed of two kind of bipolar neuron (main olfactory system - MOS and the vomeronasal system - VNS) (Theisen et al., 1986; Gardiner et al., 2012). The main use of the olfactory sense is for predation but other uses such as mate finding (by sensing pheromones) or orientation during long-migrations have also been demonstrated (Lebrun, 2001; Gardiner and Atema, 2010; Gardiner et al., 2015).

Another type of mechanoreceptor is the presence of the lateral line all along the body on each lateral side. This lateral line acts as a sensory system that allows for the detection of movement in the surrounding water. The functional unit of this system is called the neuromast and is composed of the lateral line canal, and the pit organ. The water goes inside a small pore connected to the lateral line canal which is fluid-filled. This canal then continues to the pit organ where hair-cells are connected with the cranial nerve X (vagus nerve) (Maruska, 2001; Gardiner et al., 2012) (Fig 8F). Pressure changes in surrounding water, due for example to moving prey, will stimulate the hair-cells and, through a nerve impulse, will indicate to the shark where its prey is located (Montgomery et al., 1995).

The main visual organs of sharks are eye balls which display relatively similar morphology compared to other vertebrates. There are composed of the cartilaginous sclera which is transparent and forms the cornea in front of the eye; a choroid which contains the *tapedum lucidum*; the retina which is the light receptor linked with the optic nerve; and the iris which controls the light entrance through the lens (Lebrun, 2001; Gardiner et al., 2012) (Fig 8B). The motion of the eyes is under muscle control and some species protect their eyes using a nictitating membrane (Carcharhiniformes) or also by retracting their eyes (*Hexanchus griseus*) (Gardiner et al., 2012). The majority of sharks possess rods (sensitive to low light) and cones (color vision) in the retina (Reece et al., 2014). The *tapedum lucidum* is made of cells rich in guanine crystal allowing for second retina stimulation when the light intensity is dimer (Lebrun, 2001; Gardiner et al, 2012). Others photoreceptive extraocular structures allowing for light perception are worth mentioning such as the melanopsin in the pineal gland (circadian rhythm) or the encephalopsin spread all along the ventral epidermis of lanternsharks (Delroisse et al., 2018; Duchatelet et al., 2018). The uses of vision in shark are highly diversified from prey/predator detection to orientation (Lebrun, 2001).

The last sense, the electroreception, is specific to elasmobranchs. The functional unit is called the ampullae of Lorenzini located mainly on the head. It consists of a pore inside the epidermis, which leads to the canal of Lorenzini. This canal is fluid-filled with a glycoprotein hydrogel allowing for high ion conductivity to the ampullae of Lorenzini located inside the muscle layers (Fig 8E). In these alveoli, there is a sensory epithelium forwarding information to the brain through nerves (Tricas, 2001; Gardiner et al, 2012). This sense allows prey detection at low distance (around 50cm) even when living animals are hidden in the ocean floor (Kalmijn, 1971, Sisneros et al., 1998). It has also been

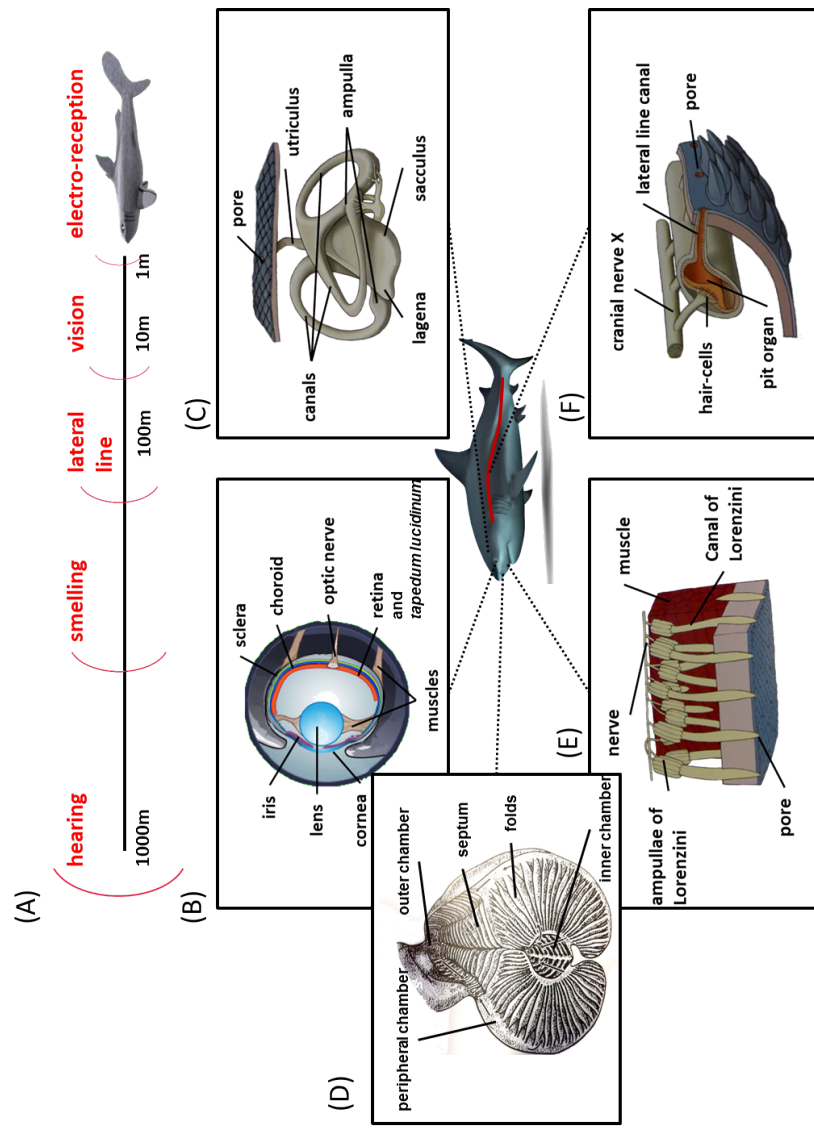


Fig. 8 Shark sensory systems: (A) range of detection in shark sensory systems; (B) eye structure (C) inner ear structure; (D) olfactory sac structure; (E) Lorenzini ampullae structure; (F) lateral line structure (Modified from Ebert et al., 2013 and Kimlev, 2013).

demonstrated that some sharks use this sense to migrate by following the earth's magnetic field (Klimley, 1993).

Internal systems

As a triploblastic, the whole internal system was developed from ectoderm, endoderm and mesoderm (Kuratani et al., 2001). The ectoderm forms the nervous system of sharks and is composed of a brain confined in the skull with openings to sensitive organ such as the eyes and olfactory bulbs. The spinal cord starts from the cerebellum to the end of the caudal fin; with the brain, this forms the central nervous system, with other nerves scattered along the body making up the peripheral nervous system (Lebrun, 2001; Compagno et al., 2005; Maia et al., 2012) (Fig 9).

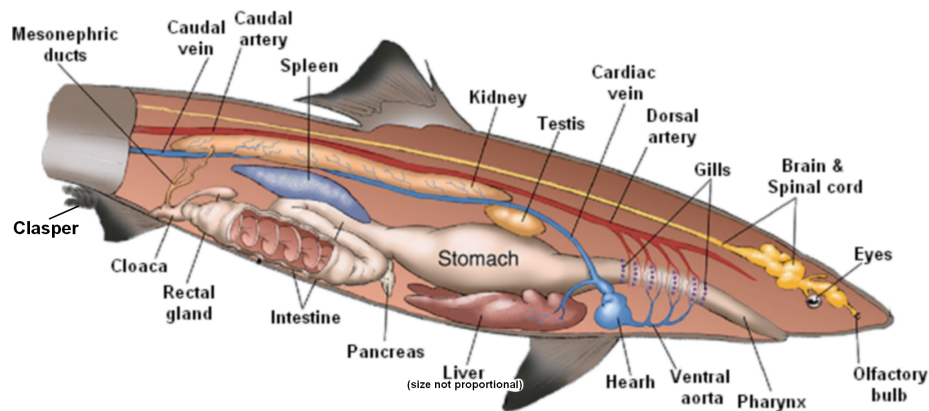


Fig. 9 Internal systems of sharks: nervous, respiratory, circulatory, digestive, excretory and reproductive organs (Modified from carlsonstockart.com).

Shark respiration is handled by five to seven gills depending on the species. Gas exchanges take place thanks to a continuous water flow entering the mouth and leaving through the gills via the gill slits. Depending of species, there are two ways that allows for the

continuous flow of water: (i) some shark have to swim constantly called “ram ventilation”; while (ii) others are able to pump the water. Some species use their spiracle to assist the process of taking water in, especially deep-sea shark and demersal species (Compagno et al., 2005; Ebert et al., 2013) (Fig 9).

The respiratory system is coupled to the circulatory system, allowing for the transport of gases and the nutrients across the body. It is a close circulatory system formed by a heart with only one auricle and one ventricle. The de-oxygenated blood is pumped from the cardiac vein to the gills through the ventral aorta. Then it goes out to the dorsal artery and circulates through the caudal artery (Lebrun, 2001; Compagno et al., 2005) (Fig 9).

The digestive system of sharks starts with the mouth followed by the pharynx. Then, the U-shaped stomach starts the digestion and carries the alimentary bolus to the short intestine (Lebrun, 2001; Compagno et al., 2005). The digestion is helped by the pancreas which secretes digestive enzymes (Papastamatiou and Lowe, 2005). One part of the shark’s intestine is called “spiral valve” due to the numerous spiraled folds improving nutrient absorption. The spleen is the place where red blood cells are created; it is also the main part of the immune system (Lebrun, 2001). Finally, the shark’s liver, which can occupy more than 20 % of the whole body and which is rich in low density lipids, serves in energy storage and buoyancy (Treberg and Speers-Roesch, 2016; Gleiss et al., 2017)

For adaptation to high salinity marine environments, cartilaginous fish have adopted a unique urea-based osmoregulation strategy. This osmoregulation is based on kidneys which retain urea inside internal fluid and the rectal gland secreting salt to avoid osmotic loss of water (Gelsleichter and Evan, 2012).

The reproduction of all shark species is based on internal fertilization. For that purpose, males insert one clasper in the female reproductive tract. Except for this generality, the reproductive system of sharks displays a large diversity. Indeed, three different reproduction mode can be observed.: (i) oviparity, for shark laying eggs; (ii) ovoviviparity, for shark which keep their pups inside the body but without direct connection with the mother (use of yolk sac); and (iii) viviparity, pups are linked with their mother which provides them nutrients, oxygen, and other vital elements (Lebrun, 2001; Compagno et al., 2005; Ebert et al., 2013). It has been recently revealed that parthenogenesis and gynogenesis occur in some shark species (Chapman et al., 2008; Robinson et al., 2011)

Deep-sea sharks

Proportion of deep-sea sharks:

Exept in Antartic, sharks are present in all of the world's oceans at all latitudes and longitudes, from neritic waters to the deep-sea waters (Simpfendorfer and Heupel, 2004). Being highly evolved predators, they occupy a relatively high position in the trophic chain. They feed on a wide range of species, even other sharks, and have a large array of foraging strategies (Heithaus, 2004). In 2018, 537 species of extant sharks were described (shark references, 2018). Since the twenty-first century, the proportion of deep-sea species has substantially changed from 61.2% in 2002, 52.7% in 2010, to 52.96% in 2015 (Martin and Treberg, 2002; Kyne and Simpfendorfer, 2010; Cotton and Grubbs, 2015). However, these figures should be considered cautiously since (i) the definition of deep-water sharks varies among authors; (ii) newly obtained information on the depth range and the biology of a species could change its classification; (iii) new species are still

being discovered, 54 extant sharks have been discovered since 2014, especially deep-sea species (Pollerspöck and Straube, 2019).

The IUCN Shark Specialist Group has defined deep-water chondrichthyans as sharks “whose distribution is (i) predominantly at, (ii) restricted to, or (iii) spend the majority of their lifecycle at, depths below 200 meters” (Kyne and Simpfendorfer, 2007, 2010). Still, the arbitrary separation of 200 meters’ depth is sometimes hard to apply specifically since some sharks undergo vertical movement, notably diel vertical migration, which defines daily vertical migration between deep waters during the day and shallower depths at night to feed (Haedrich, 1996; Heithaus, 2004). According to this definition, we measured the proportion of deep-sea sharks based on the most relevant literature (shark references, 2018). Among the 537 species, strict deep-sea sharks represents 47% (254 species), shallow-water ones counted for 41% (220 species) and 10% (53 species) are transitory species with an ambiguous range or vertical displacement which does not allow distinction between shallow or deep-water categories, finally vertical distributions in the water column for 2% (10 species) of shark species are still unknown (Fig. 10A). Typically, it corresponds to sharks found on the stalls of fishermen such as *Squalus formosus*.

The repartition of deep-sea sharks is different across the nine shark orders: Echinorhiniformes, Squaliformes and Hexanchiformes hold the highest proportion of deep-sea species (100%, 86% and 86% respectively). Then the proportion of deep-sea species is quite equivalent for Pristiophoriformes and Carcharhiniformes (50% and 40% respectively). The Lamniformes, the Squatiniformes, and the Orectolobiformes include also deep-sea species but in fewer

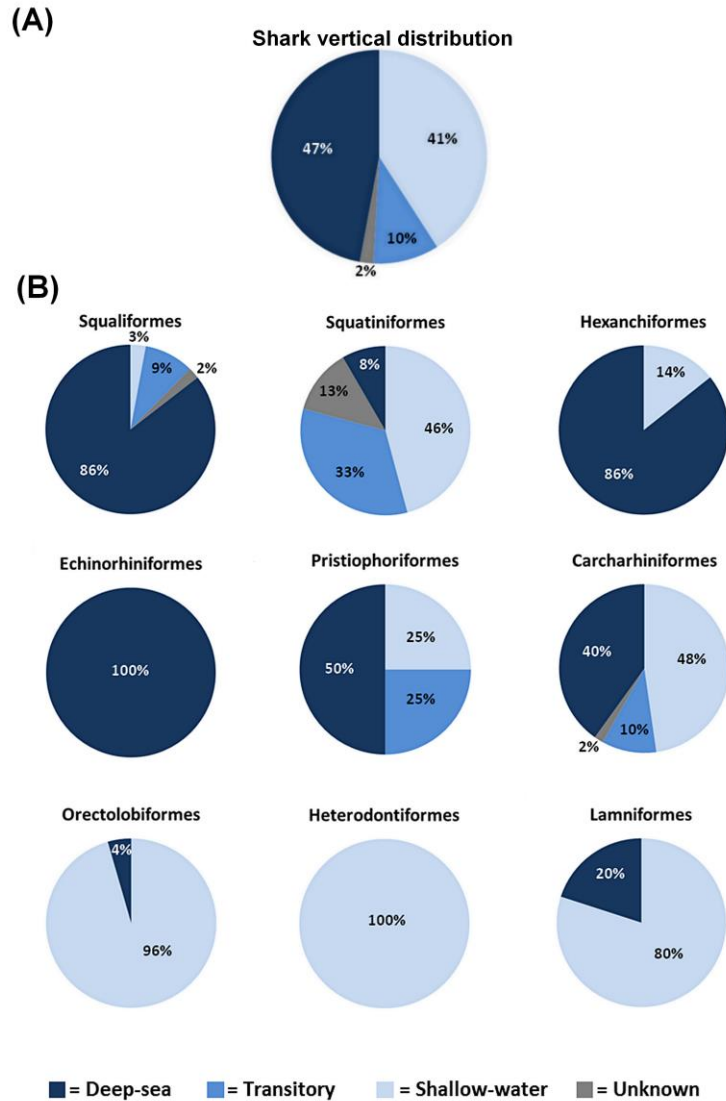


Fig. 10 Bathymetric classification of all sharks (A) and of the various orders (B) according to their lifecycle in ocean zone. Deep-sea corresponds to shark species living the majority of their life below 200 meters depth; shallow-water to shark species living the majority of their life above 200 meters depth; transitory for shark species migrating continually between the two previous zone; and unknown to species with no enough data regarding their vertical range; Data compiled from

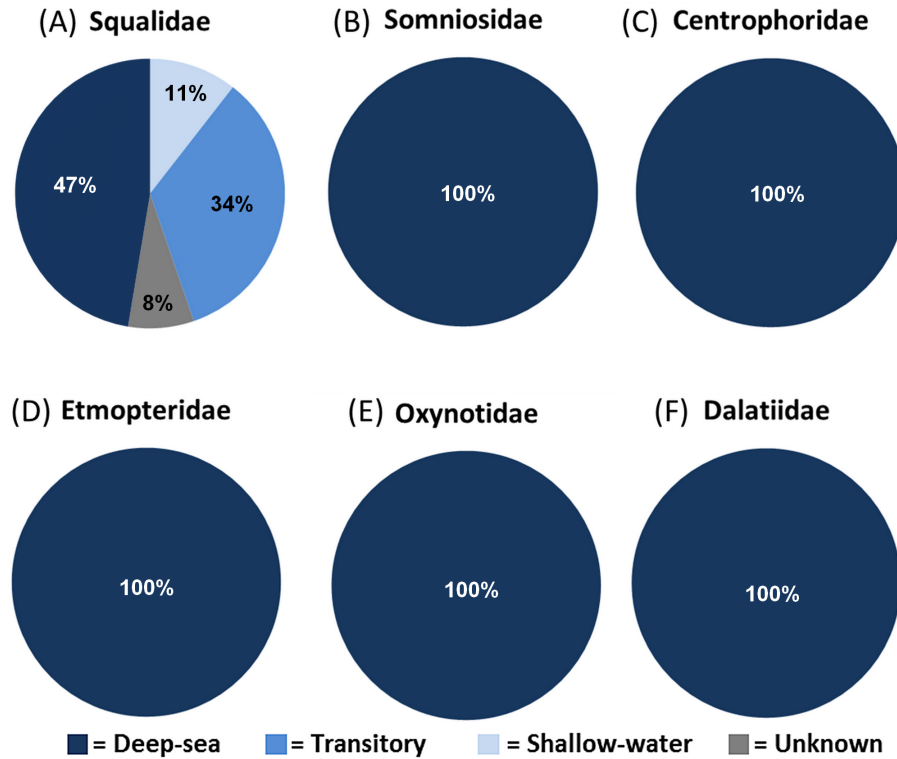


Fig. 11 Proportion of all sharks from Squaliformes order according to their lifecycle spent in vertical ocean zones with family cluster. Deep-sea corresponds to shark species living the majority of their life below 200 meters depth; Shallow-water to shark species living the majority of their life above 200 meters depth; Transitory for shark species migrating continually between the two previous zone; and Unknown to species with no enough data regarding their vertical range; Data compiled from (Pollerspöck and Straube, 2019).

proportion (20%, 8%, and 4% respectively). Finally, there is no deep-sea species in the Heterodontiformes order (Fig. 10B).

In the three orders holding the more deep-sea species, Squaliformes display the highest number of species, 138 in comparison with seven species of Hexanchiformes and two species of Echinorhiniformes. For this reason, this work only focused on species from this order. Squaliformes are separated in

six families (Squalidae, Somniosidae, Centrophoridae, Etmopteridae, Oxynotidae, and Dalatiidae). Except for Squalidae where the proportion of deep-sea shark is around 50%, every other families exhibit only deep-sea shark species (Fig. 11).

Deep-sea shark biology

While they represent about 50% of whole sharks species described so far, deep-sea shark species are still poorly studied (Martin and Treberg, 2002; Kyne and Simpfendorfer, 2010). The main reason is the fact that those species are elusive. However, the over-exploitation of shallow-water species led to a shift of fisheries from crashed shallow-water populations to preserved population in deep parts of ocean allowing to get more and more information regarding the extant fauna (Morato et al., 2006). Moreover, the improvement of technology has allowed for a better understanding of this environment with the benefit that these techniques are for the most non-destructive.

With deep-sea fisheries came the first information regarding deep-sea sharks' life-traits. Indeed, deep-sea sharks are often by-catch species from deep-sea trawling, thus data regarding their size, the sexual maturity, their vertical location have been collected (Priede et al., 2006; Coelho and Erzini, 2008; Graham and Dailey, 2011; Kousteni and Megalofonou, 2015; Rigby and Simpfendorfer, 2015). The distribution of deep-sharks in the water column is different between species but it seems that chondrichthyans do not live under depth of 4000 meter (Fig. 12).

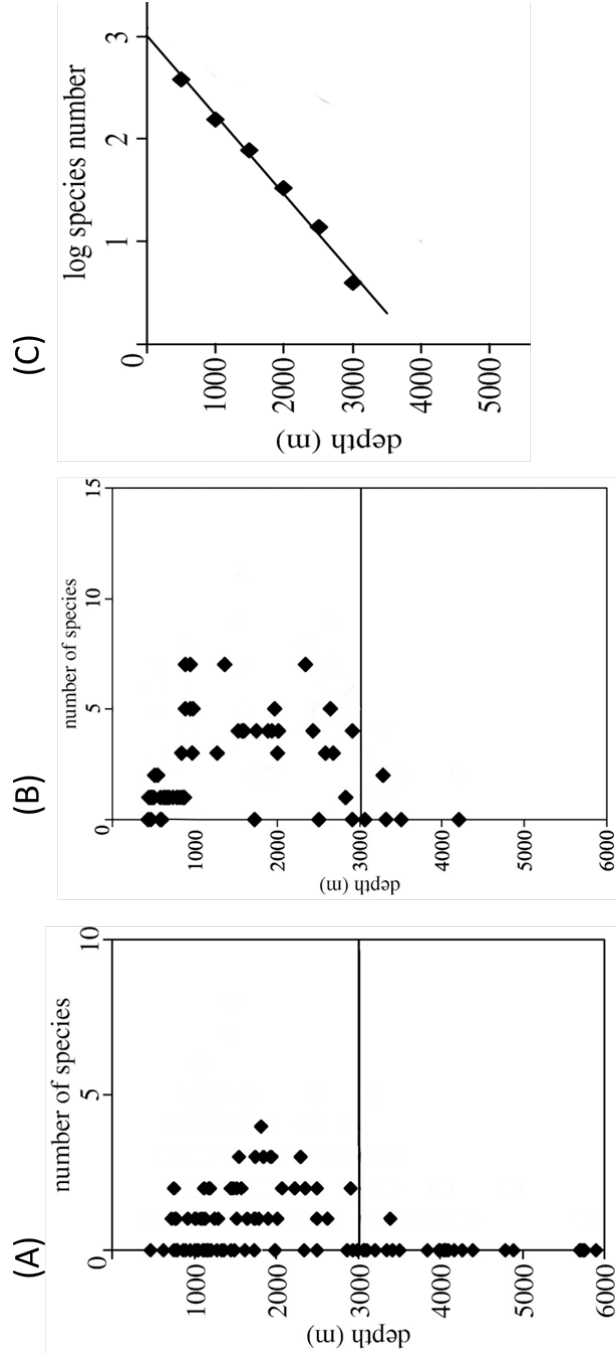


Fig. 12 Number of shark species according to the depth. (A) Atlantic, Pacific, Indian Oceans and Mediterranean Sea observed using baited camera deployment; (B) Mid-Atlantic Ridge, species captured on baited lines; (C) Rates of decrease in species numbers with depth, log of number of shark species depth maxima per 500 m stratum for the global data set ($\log N = -0.0008x + 2.9969$, $r^2 = 0.992$); dots correspond to each fisheries sampling, some of them fished sharks others no but all are represented (modified from Priede et al., 2006).

It has been suggested that sharks are not present in the abyssal region due to several physiological stresses: (i) firstly, due to scarce food, sharks would not be able to answer their high-energy demand, mainly regarding their oil-rich liver important for buoyancy; (ii) also linked with the food scarcity, their osmoregulation system based on nitrogen would be limited; (iii) Finally, the urea-based osmoregulatory strategy might conflict with the temperature and pressure acting on the protein and membrane. These three hypotheses are not exclusive and might act in concert (Priede et al., 2006; Treberg and Speers-Roesch, 2016).

Studies regarding the distribution also revealed segregation based on the sex or/and the maturity stages for several deep-sea species. For the majority of studied species, the segregation revealed immature male individuals in the shallower layer followed by mature male living in intermediate layer with immature females and mature females living in deeper zones such as for *Centroscymnus coelolepis* (Fig. 13) (Girard et al. 1999), but also for *Centrophorus squamosus* (Girard et al. 1999), *Centroscyllium fabricii* (Jakobsdottir, 2001), *Etmopterus princeps* (Jakobsdottir, 2001), *Etmopterus spinax* (Coelho and Erzini, 2010).

Regarding their ecological role and their trophic level inside their ecosystem, it is still poorly documented. We know that shallow-water sharks act mainly as top predator and regulate their ecosystem (Myers et al., 2007). However, deep-sea shark species are for the majority small shark (less than one meter) and there is evidence that they are hunted by other organisms (according to stomach contents and defense apparatus) (Maisey, 1979; Navarro et al., 2014). Several studies have analyzed stomach contents and revealed that small deep-sea sharks mainly feed on small teleostei and cephalopods while larger species also feed on small deep-sea

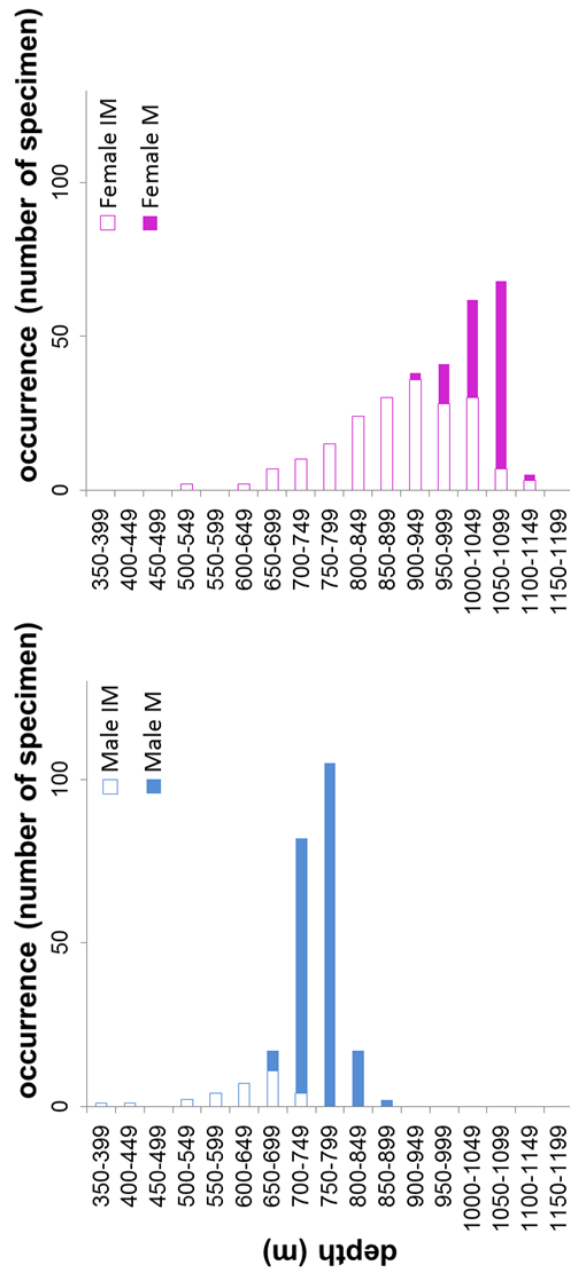


Fig. 13 *Centroscyllium coelelepis* occurrence (number of specimen) according to the depth (m) revealing sexual segregation. Empty blue bar charts correspond to immature male while pink ones represent immature female; Filled blue bar charts correspond to mature male while pink ones represent mature female (modified from Girard et al., 1999).

sharks (Ebert et al., 1992; Daley et al., 2002; Pethybridge et al., 2011; Navarro et al., 2014). In deep-sea, the carbon comes from the surface as carcasses or thin organic particles (Rowe and Staresinic, 1979). It has also been shown that several species go through circadian migration to feed in the shallower layers during the night (Papastamasiou and Lowe, 2012). Only recent studies, using stable isotope, have investigated the trophic level of deep-sea sharks and concluded that their primary organic sources is similar to pelagic shallow-water species (pelagic sources) in contrast with benthic species which display benthic primary carbon origin (Bird et al., 2018). We still need to better understand the ecological role of deep-sea species, especially since deep-sea fisheries impact the deep-sea shark population.

One specific trait of some deep-sea sharks is the ability to emit light, i.e. bioluminescence. Bioluminescence is the production of visible light by a living organism through biochemical reactions. In the animal kingdom, bioluminescence can be produced by two processes involving either a luciferin-luciferase reaction (see Hastings, 1983; Wilson and Hastings, 1998; Shimomura, 2006 for review) or an already stabilized complex, called photoprotein (i.e. Luciferin-Luciferase coupled) (see Shimomura, 1985; 1986; 2006 for review). All known luminous reactions need oxygen.

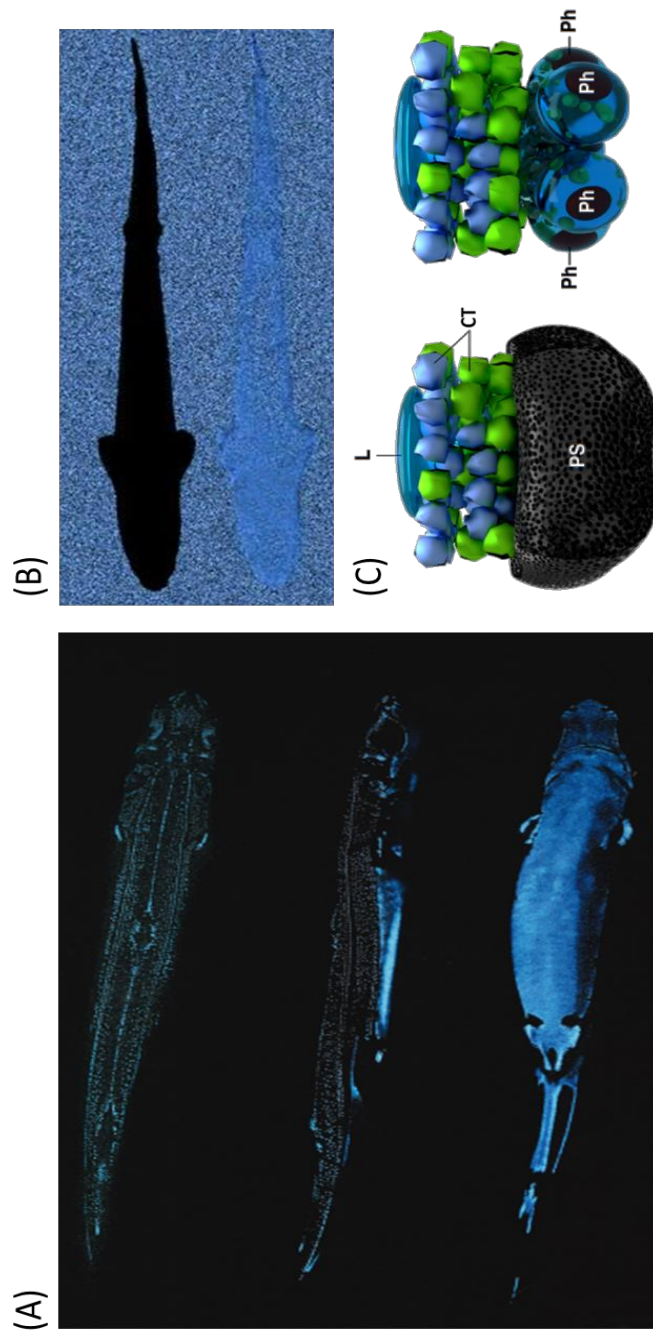
In the past fifteen years, studies about luminous capabilities of sharks increased to reach the current knowledge summarized in the Figure 14. Only two families display clear evidences of luminous capabilities (e.g. light emission records, luminous organ histology, light emission control) such as for Etmopteridae and Dalatiidae species (Claes and Mallefet, 2011; Claes et al., 2011a; Claes et al., 2012; Claes and Mallefet, 2015). Conversely, no such evidences are found for other species like *Zameus squamulosus*, Somniosidae (Straubes et al., 2015) and *Apristurus ampliceps*

(Ferrón et al., 2018) for which only indirect evidence suggest that they are potentially luminous. Shark produces light for different functions: (i) they produce a ventral blue-green (455-490nm) light to mimic the downwelling light of their environment and thus make their silhouette disappear this camouflage is called counterillumination (Claes and Mallefet, 2008; Claes et al., 2010; Claes et al., 2014a and 2014b); (ii) they produce sex-dependent light at the pelvic zone; (iii) some species also display specific flank marks suggesting a possible intra/inter specific communication use (Claes and Mallefet, 2010; CLaes et al., 2015); (iv) finally, recent study revealed a brighter light around the dorsal spine of Etmopteridae species which is used as defense apparatus, here the bioluminescence play a defense role as aposematic signal (Claes et al., 2013; Duchatelet et al., 2019 – Annex 4 p 241-251).

The shark luminescence takes place in a small luminous organ called photophore. This organ structure is composed from the deep inside towards the external epithelium of a thick pigmented sheath inside covered by a reflecting layer enclosing cells responsible for the light emission, called photocytes (one photocyte per photophore for Dalatiidae and from 6 to 14 for Etmopteridae depending species), surmounted by an iris-like structure (ILS) acting as the light organ shutter, and topped externally by one or two lens cells (Claes et al., 2012; Renwart et al., 2014). The study of luminescence control has revealed the first hormonal control of luminescence in vertebrate. In both luminous families, melatonin triggered a long lasting glow while adrenocorticotrophic hormone (ACTH) and alpha-melanocyte-stimulating hormone (α -MSH) inhibit it (Claes and Mallefet, 2011; Claes et al., 2011a; Claes et al., 2012; Claes and Mallefet, 2015; Ducahtelet et al., 2019 – Annex 5, p 252-278). Some classical neurotransmitters such as γ -aminobutyric acid (GABA) and nitric

oxide (NO) act as modulators of the light emission (Claes et al., 2011b). Finally, prolactin triggered a strong fast luminescence response in Etmopteridae while it inhibits the light emission of *Squaliolus aliae*, a representative of the Dalatiidae family (Claes and Mallefet, 2011; Claes et al., 2011a; Claes et al., 2012; Claes and Mallefet, 2015). Recent studies have shown the existence of an extraocular opsin co-localized with the ILS cells of the luminous organ. This opsin is suggested to act as light modulator through pigment aggregation control with ILS melanophores (Delroisse et al., 2018; Ducatelet et al., 2019). This slow hormonal control system seems to be in conflict with the counterillumination function. Indeed, several studies mentioned that good counterilluminator should be able to change their ventral light intensity quickly, (Clarke 1963; Young and Roper 1976; Case et al. 1977; Latz 1995), which is not the case for deep-sea sharks. Therefore, it has been suggested that luminous sharks move up and down in the water column to adjust their ventral light intensity. This hypothesis is called “isolume-follower” (Claes and Mallefet, 2014).

The physiology of deep-sea sharks was also investigated. Regarding their buoyancy, it has been shown that deep-sea sharks display a bigger liver that is richer in low-density lipids compared to their shallow-water counterpart (Treberg and Speers-Roesch, 2016). Therefore, livers of deep-sea species provide more buoyancy diminishing energy expenditure for slow swimmer (Gleiss et al., 2017). Other studies have focused on their muscle metabolism and it seems that deep-sea sharks have a lower anaerobic metabolism that powers white muscles which in turn plays a role in burst swimming. In contrast no significant differences were observed between deep-sea and shallow-water sharks regarding the aerobic metabolism which powers red muscle, which is important for cruise swimming mode (Treberg et



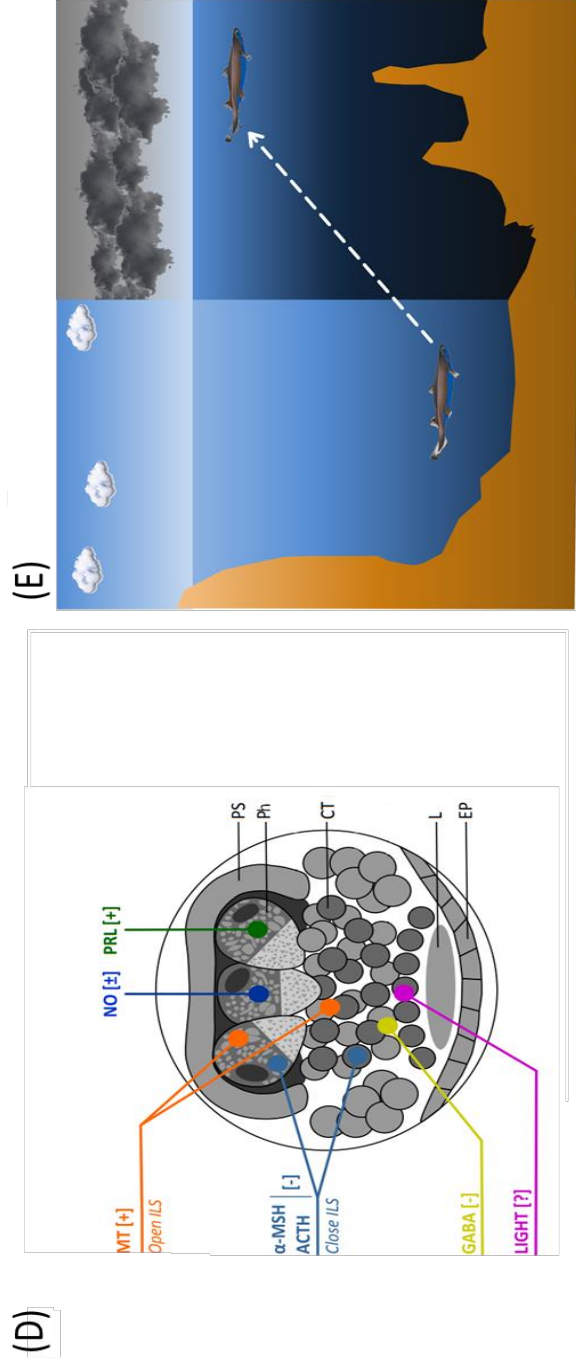


Fig. 14 Generalities regarding shark bioluminescence. (A) schematic view of the dorsal, lateral, and ventral luminous pattern of the slendertail lantern shark (*Etmopterus molleri*) , pictures from Jérôme Mallefet; (B) Schematic view of counter illumination camouflage; (C) simplified view of Etmopterid's photophores: L (lens), CT (cellular type, or Iris like structure - ILS), PS (pigmented sheat), Ph (photocyte), modified from Renwart et al., 2014; (D) schematic view of localization and control of known actors involved in bioluminescence of lanternshark: [-] activator, [±] modulator, [-] inhibitor, [?] unknown effect, L (lens), CT (cellular type, Iris like structure - ILS), PS (pigmented sheat), Ph (photocyte), MT (melatonin), PRL (prolactin), NO (nitric oxide), α-MSH (α-Melanocyte-stimulating hormone), ACTH (adrenocorticotrophic hormone), GABA (gamma-aminobutyric acid) ; (E) representation of the "isolume followers' hypothesis".

al., 2003; Condon et al., 2012). Physiological studies on deep-sea sharks will be discussed later in the introduction but despite some studies, there are still a lot of questions regarding deep-sea shark biology. Indeed, several ecological parameters are still unknown such as the reproduction, their movement inside the water column, and other examples. One missing interesting ecological information is the swimming capabilities of deep-sea sharks. Indeed, physiological studies using indirect measurements have suggested that deep-sea species should swim slower than their shallow-water counterparts but direct information on their swimming capabilities is still missing.

SWIMMING CAPABILITIES

Generalities

The majority of sharks are active predators. Therefore, their swimming capabilities in the ocean have crucial implications in survival behaviors as it is involved in many fundamental life-functions, such as hunting, escaping, migrating, and mating (Wilson et al. 2005; Watanabe et al. 2012). Swimming capabilities will also largely dictate the home range of a species (Sainte-Marie and Hargrave 1987). Study on the locomotor system of shallow elasmobranchs was initiated in 1930 by Sir James Gray (Shadwick and Goldbogen 2012) and numerous studies followed (Schadwick et al. 2016). Speed of locomotion is also an important factor for understanding the evolution and adaptation of sensory systems, as speed determines the rate at which sensory information is encountered and processed by the animal as it moves through its environment (Ryan et al. 2015). In sharks, the diversity of the locomotion between species is highly diversified, from *Somniosus microcephalus*, the Greenland shark displaying the lowest shark velocity observed to date to *Isurus oxyrinchus*, the mako shark which can reach up to 70km/h (Watanabe et al., 2012). The behavior and the life style are also linked with the swimming capabilities. Indeed, some species like the whale shark, *Rhincodon typus*, or the hammerhead shark, go through long distance migrations. Other species, on the other hand, like sharks from Scyliorhinidae family display a benthic residential life style (Wilson et al., 2005). Even if numerous studies conducted on this subject led to a deeper understanding of it, many aspects of elasmobranchs' locomotion are still not fully understood, especially for deep-sea sharks (Thomson and Simanek, 1977; Condon et al., 2012).

Water produces several physical coercions on shark bodies that must learn to move in this denser three-dimensional environment. The swimming mode of organisms have been split into two main types: (i) the first type is linked to burst activities – defined as when a fish reaches its maximum velocity during hunting or escaping behaviors, this swimming mode is powered by anaerobic metabolism and therefore can generally only last for a few seconds; (ii) the second is linked to cruise activities, and is used by fishes to travel long distances during the most common locomotion activities such as foraging, dispersal, locating a mate or circadian migration; it is powered by aerobic metabolism and can usually last for more than 200 hours (Watanabe et al. 2012; Ryan et al. 2015). Sharks, like the majority of fish, swim by undulating their body producing lateral waves while the caudal fin provides the power of the thrust (Ebert *et al.*, 2013). There are four different swimming categories for sharks: anguilliform, subcarangiform, carangiform and thunniform (Figure 15). The first one consists of full undulation of the entire body with more than one lateral wave along the body. This category is also referred as “eel-like” (Ebert *et al.*, 2013), used by species such as the frilled shark (*Chlamydoselachus anguineus*). It is believed to be the least efficient swimming category in terms of velocity (Lighthill, 1969; Ebert *et al.*, 2013). However, some studies assume that anguilliform swimming is as efficient, if not more efficient, than carangiform and thunniform swimings, at least in certain conditions and in terms of energy expenditure (Bone and Moore, 2008; Borazjani and Sotiropoulos, 2009). The subcarangiform and carangiform swimming also involves the oscillation of the body but concerns only the posterior third or the posterior half, respectively of the body (Ebert et al., 2013). There is less than one lateral wave along the body. These modes are the most common in sharks, used by species such as the blue shark, *Prionace glauca* (subcarangiform) and the

scalloped hammerhead shark, *Sphyrna lewini* (carangiform). Finally, the thunniform swimming is associated with rapid pelagic swimmers such as the mako shark, *Isurus oxyrinchus*. It involves only the caudal peduncle and caudal fin with less than one lateral wave present at a time (Ebert et al., 2013). Of course, *in situ* swimming sharks of one category display large modifications in swimming pattern as function of their activity (cruising, hunting...). Swimming by body undulation has some disadvantages but also some advantages. Some of the energy produced is wasted in lateral movements, causing the sharks to slightly move their head left and right. While not very advantageous, it does allow the shark to search for food and follow a track for example (Abedin et al., 2014). Also, greater undulations result in an increased drag (Shadwick and Goldbogen, 2012). Thus, sharks often use large body undulation to gain speed or investigate their habitat but come back to lower undulations for constant swimming (e.g. migration).

These undulations are measured as proxy to determine the sharks' velocity. This proxy is called the tailbeat which is defined as the time required for the tail to move from one extreme lateral position back to the same position. The tailbeat frequency is expressed in Hertz (Hz) (Watanabe et al. 2012). Therefore, a higher tail beat frequency leads to higher cruise swimming speed. For shallow-water sharks the tail beat is rarely less than 1Hz during the cruise swimming speed, while the majority of deep-sea sharks display values inferior to this (Graham et al. 1990). In addition to the tail beat, the tail amplitude is also a feature influencing the swimming speed: higher tail amplitudes provide more swimming force (Graham et al. 1990; Bainbridge 1958).

The locomotion system of sharks displays many diverse adaptations affecting its capacities. These adaptations can be separated into three categories: (i) anatomical adaptations which correspond to

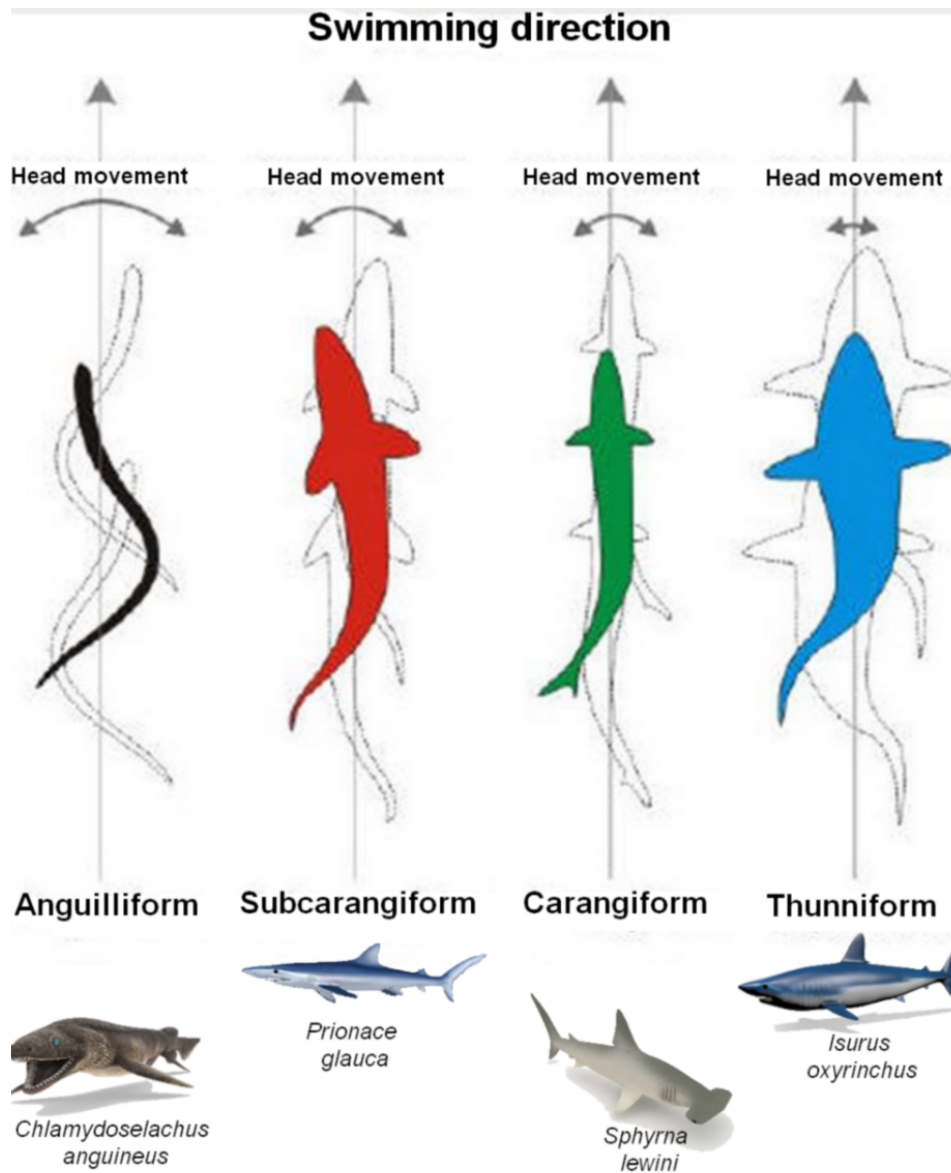


Fig. 15 Swimming mode diversity for extant sharks. (A) Anguilliform mode (e.g. *Chlamydoselachus anguineus*); (B) Subcarangiform mode (e.g. *prionace glauca*); (C) Carangiform mode (e.g. *Sphyrna lewini*); (D) Thunniform mode (e.g. *Isurus oxyrinchus*) (modified from kinefusion.com).

internal features, such as the red and white muscle volumes or also the muscle fiber structures; (ii) physiological adaptations which are the shark's way to produce and save energy through different metabolic processes; and (iii) morphological adaptations which correspond to every external change in morphology improving the locomotion of fish in its environment (Blake, 1983; Sfakiotakis et al., 1999).

These adaptations were well studied in shallow-water species allowing to identify different ways in which higher burst and cruise swimming speeds can be achieved. In contrast, the velocity of deep-sea sharks is unknown for more than 99 % of all deep-sea shark species. From the available data, it is suggested that they are slow and listless swimmers (Treberg et al., 2003; Watanabe et al., 2012; Condon et al., 2012; Treberg and Speers-Roesch, 2016).

Morphological adaptations

Sharks display different body shapes depending on their lifestyle. These differences have an impact on their swimming capabilities. The body and fin shapes have a strong impact on their penetration in the water but also generate drag perturbation acting to increase their stability. As previously mentioned in figure 5, all fins have an impact on the stability of shark: vertical fins counteract the yaw effect while horizontal fins manage the pitch and roll effect.

Caudal fin

The caudal fin plays the major role in swimming capabilities acting as the main propeller. The shape of this fin is one of the most important morphological aspects for shark locomotion. According to the literature, two shapes of caudal fin can be distinguished: the heterocercal caudal fin and the homo or isocercal caudal fin (Ebert et al., 2013). The distinctions between these two shapes are

measured using caudal fin ratio which corresponds to the value obtained by dividing the lengths of the lower lobe by the upper one. The closer the ratio is to one, the more the fin is homocercal (Sambilay, 1990; Low and Chong, 2010). The caudal fin shape can also be associated to the velocity (Ebert et al., 2013) (Fig 16).

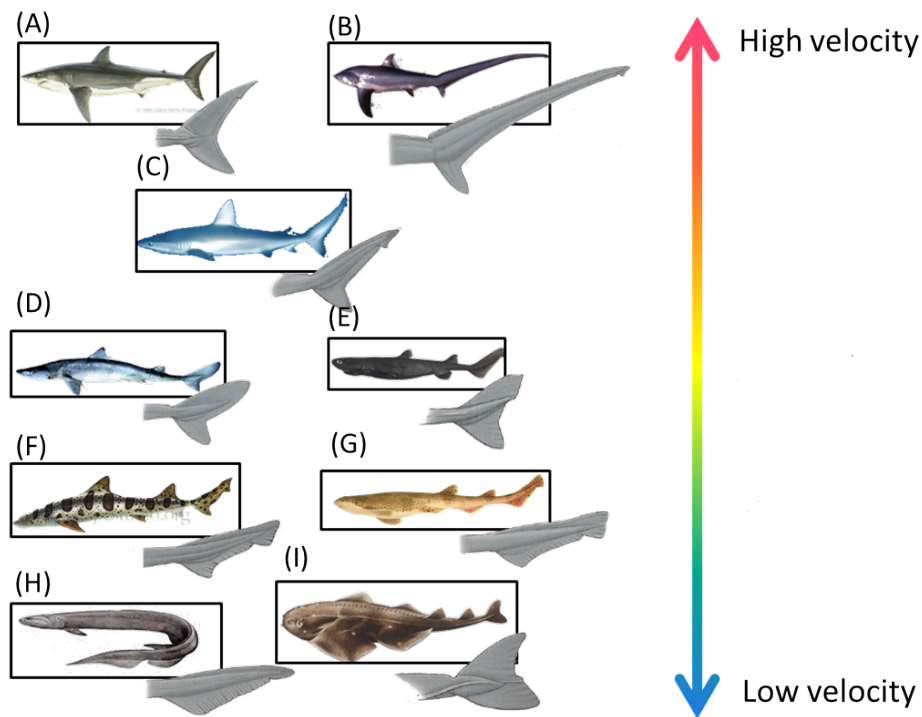


Fig. 16 Caudal fin diversity based on velocity. (A) Lamnidae; (B) Alopiidae; (C) Carcharhinidae; (D) Squalidae; (E) Dalatiidae; (F) Triakidae; (G) Scyliorhinidae; (H) Chlamydoselachidae; (I) Squatinidae (modified from Ebert et al, 2013).

Homocercal caudal fin (i.e. almost same length for the upper and lower lobe of the caudal fin) could allow a better swimming efficiency because more energy is involved in thrust. A ventral lobe as long as the dorsal one indicates a “thunniform” swim while a

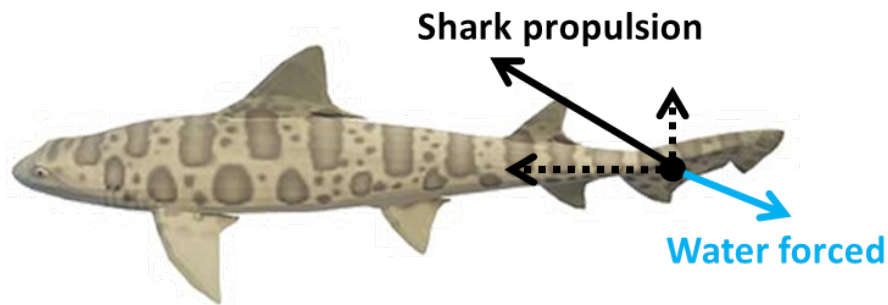
longer dorsal lobe is generally associated with “anguilliform” and “carangiform” swim (Thompson, 1976; Wilga & Lauder, 2002).

Heterocercal caudal fin (i.e. lower lobe shorter than the upper one) creates a dorso-ventrally water flow generating both an upward force and a forward force according to the classical model (Fig 17A). It could be explained by the wider wave of the upper lobe (more rigid) compared to the lower lobe (more flexible) which forces the caudal fin to be tilted most of the time with an angle nearly horizontal (Ferry & Lauder, 1996).

However, Thompson had previously developed in 1976 an antagonistic model in which the water is pushed backward and upward allowing the shark to be propelled forward and downward (Fig 17B). It is based on the fact that the upper lobe of the tail has a larger area than the lower lobe, and so, moves slightly slower. The consequence is that the caudal fin has a nearly horizontal move but with water pushed upward (Thompson, 1976; Ferry & Lauder, 1996). Further kinematics models are more nuanced (Ferry & Lauder, 1996). The lower lobe seems to move first but at the end of the movement it is the upper lobe which is farther to either side (right and left), corroborating the classical model. Wilga and Lauder in 2002 confirmed this theory by analyzing vorticity around the caudal fin and deducting a jet angle (water force) of -45° and -42° backward and downward for leopard shark (*Triakis semifasciata*) and bamboo shark (*Chiloscyllium plagiosum*).

Heterocercal caudal fin is then more useful for benthic sharks in order to rise in the water column and don't scrape the seabed with the ventral lobe of the fin (Ferry & Lauder, 1996). Although, homocercal caudal fin seems to be associated with swimming performance, some sharks like basking shark (*Cetorhinus maximus*) remain relatively slow (Sambilay, 1990). On other

(A) Classical model



(B) Thomson model

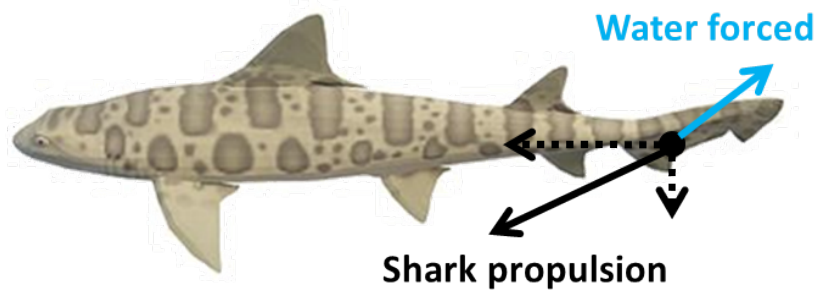


Fig. 17 Hypotheses explaining the forces generated by heterocercal tail during locomotion. (A) Classical model; (B) Thomson model (Modified from Ferry & Lauder, 1996).

exception is for thresher sharks (*Alopias spp*) which use the very long upper caudal lobe to strike preys but maintain a fast swim owing to their rigid lower caudal lobe (Aalbers et al., 2010). Their marked heterocercal caudal fin is not an encumbrance to swimming performance in this case.

Pectoral fins

To maintain a horizontal swim in water and not sinking, some sharks have to generate a lift by continual swimming in addition to their cartilaginous skeleton or their liver (bigger and rich in lipids). This lift is partially provided through pectoral fins and their “Aircraft Wing” shape. The cambered upper part of the pectoral fin forces water to rise and then to go down by accelerating. Consequently, water above the pectoral fin moves faster and decreases the pressure, by Venturi effect, generating a lift (Ebert et al., 2013).

During horizontal swimming, the pectoral fin has a concave downward shape which gives a positive angle of attack in the water generating a lift but almost negligible (Wilga & Lauder, 2001). During vertical swimming, they allow more dive stability at a higher (-5°) dihedral angle (corresponding to the angle between pectoral fin orientation and body axis) to counter the disturbance from lift caused by the heterocercal fin. During climbing, this angle is reduced (-35°) to allow more maneuverability by increasing the contact surface with the water located on the sides (Carrier et al., 2012; Wilga & Lauder, 2000).

Dorso-caudal fin distance

In addition to counteract the roll movement induced by the beat of the caudal fin (Klimley, 2013), dorsal fins have also a key role in propulsion. These fins are also able to generate thrust by accelerating water posteriorly (Wilga and Lauder, 2000) and creating a Venturi effect like explained for the pectoral fins. But the difference between sharks and teleost fishes may be related to the hydrodynamic interaction between the dorsal fins and the caudal fin (Webb & Keyes, 1982). Indeed, for thuniforms, carangiforms and sub-carangiform swimmers, the dorsal fins have a larger lateral displacement than the trunk indicating a controlled

movement to increase the volume of water moved backward for a more efficient thrust production (Maia & Wilga, 2013). The vortex of water created by the dorsal fin can then interact with the water flow incident to the caudal fin in order to let the tail beat easier (Lauder & Santo, 2016).

However, Squaliformes deep-sea sharks have also a second dorsal fin between the first one and the caudal fin, which impinge on the vortex created. If the distance between the two dorsal fins is small, there is little difference between the motion of the incident vortex and the motion of the leading edge of the downstream fin (Webb & Keyes, 1982). In this case, the interaction between median fins does not influence mean thrust. When the distance between the dorsal fins is large, a phase difference exists so that the incident vortex is not annihilated by the second dorsal fin (Lighthill, 1970).

As for the vortex created between the dorsal and caudal fins, a second vortex is created between the first and the second dorsal fins. Maia et al. in 2017 showed that the function of shark's dorsal fins is significantly different among the fins (first or second) and the species of sharks. The dorsal fins have not only a stabilizing or thrusting role but both can coexist. An example is for *Squalus acanthias* for which the first dorsal fin acts as a stabilizing because of its location near of the center of mass, and the second generates thrust by increasing longitudinal flow velocity (Maia et al., 2017). In contrast, both dorsal fins of the species *Chiloscyllium plagiosum* generate thrust by increasing flow velocity. Differences among sharks' lifestyles are important: *Chiloscyllium plagiosum* is a slower benthic species which reduces its need for a stabilizing dorsal fin in opposition with the faster *Squalus acanthias* for which roll and yaw are higher due to its fast swim.

Body shape

All sharks share similar body characteristics: the body is elongated with a fusiform (or sometimes flattened) shape for water penetration and broader on the median part due to an important muscular mass (Blake, 2004). Some differences exist, however, for the fin shapes, their locations or also the dorso-ventral flattening. Thompson and Simanek developed, in 1977, five main morphotypes differing by the shape of the body or fins and each adapted to particular lifestyles.

The first one is characterized by a low caudal ratio which is near of indicating a more homocercal caudal, dorsal fins are seriously reduced (Fig. 18A). This allows a faster swim but reduces maneuverability and is characteristic of fast pelagic sharks like Lamnidae or Carcharhinidae.

The second morphotype is similar to the first apart from the caudal fin with a higher ratio which is more heterocercal and presents sometimes a peduncle on the dorsal lobe (Fig. 18B). The body is less thick and the pelvic, anal and second dorsal fins are more developed what increases maneuverability. It's the typical of generalist pelagic sharks.

The third is significantly different of the two others with its dorso-ventral flattening more pronounced and its rounded rostrum (Fig. 18C). The tail is heterocercal with a ventral lobe sometimes absent. The pelvic fins are in a more anterior position and the first dorsal fin is more posterior. This flattened body is typical of benthic sharks that swim on the floor.

Then, the fourth morphotype is those of the Squaloids sharks in general including the majority of known deep-sea sharks up to now. The caudal fin is also heterocercal and anal fins are absent

(Fig. 18D). The two dorsal fins are well developed and are often topped with a dorsal spine while the pectoral and pelvic fins are much reduced. A fifth group exists for rays, chimeras or extremely flattened sharks (Fig. 18E)

(A) Morphotype 1



(B) Morphotype 2



(C) Morphotype 3



(D) Morphotype 4



(E) Morphotype 5

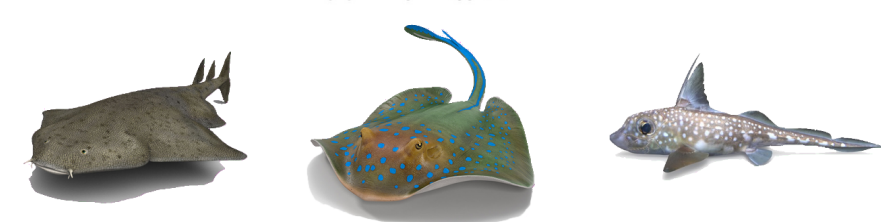


Fig. 18 Classification of sharks in four different morphotypes according to Thompson and Simanek, 1977. (A) Morphotype 1 (e.g. *Isurus oxyrinchus*); (B) Morphotype 2 (e.g. *Carcharhinus limbatus*); (C) Morphotype 3 (e.g. *Scyliorhinus sternalis*); (D) Morphotype 4 (e.g. *Dalatias licha*); (E) Morphotype 5 (e.g. *squatina* sp., *Taeniura lymma*, and *Hydrolagus colliei*).

Each morphotype is well adapted to swim differently. The first and second morphotype are more typical of thunniform and carangiform swimmers because of the rigidity of the body. The third is more anguilliform or sub-carangiform due to the length of

the tail and for some species, the absence of ventral caudal fin lobe. Then, the last is generally carangiform or sub-carangiform (Webb and Keyes, 1982). It is important to notice that some species do not fit in any of these categories because this classification is arbitrary and the first one carried out (Thompson & Simanek, 1977).

Webb in 1984 preferred a classification for all types of fishes and based on the fins involved in swimming, it describes not only sharks and therefore isn't directly connected to the Thompson and Simanek classification. The first and second group of this classification use the body and the caudal fin for locomotion, the first periodically (favouring cruising speed) and the second transiently (favouring fast-starts); in this group can be found all thunniforms, carangiforms, sub-carangiforms and anguiliforms swimmers. The third group use the median and paired fins to swim (favouring fine maneuver activities) while the last group just don't move most of the time or with occasional propulsion (Webb, 1984). This classification is also based on ecological approaches like the food characteristics. For example, preys of group 2 are evasive and locally abundant by opposition to them of group 1 which are non-evasive and widely dispersed. This classification, like that of Thompson, suggests that a specialization in one group reduces the capacity to perform another type of swimming.

It has been proved in previous studies (Graham et al., 1990; Sambilay, 1990) that body length was generally inversely correlated with swimming speed. The main explanation was that increasing velocity requires cardiac and muscles investment which are more difficult to maintain for bigger organisms (Graham et al., 1990). A second fact is that a bigger shark has a greater surface area in contact of water what increases the drag and slow the shark down. If smaller organisms are generally faster, lifestyle has

also to be taken into account, for example, pelagic sharks are generally faster than benthic sharks which favor maneuverability to swim easier in confined spaces. It is important to notice that speed increases at a slower rate than body size (Weihs, 1977) suggesting the impact of other potential factors. Indeed, size does not act alone on speed; its effect is species-specific because speed does not decrease with size in the same way for a 0.5 to 0.9 m bamboo shark than for a 5 to 8m basking shark. The best model to explain cruising speed takes length but also tail shape into account. It could be explained by the fact that tail shape is correlated to species, which is a major factor influencing speed, but also ecological factors related to it like habitat or trophic level (Ryan et al., 2015).

Other morphological features

As previously described in the introduction, sharks display numerous denticles on their skin. These denticles are assumed to provide some function to sharks. One of them is drag reduction through nearly horizontal scales. Dermal denticles, by their shape, generate little vortices reducing the flow and then the drag of water, it is called the Riblet effect. The keels also passively control flow by limiting the lateral transfer of force, training the vortices in the direction of the flow (Fletcher et al., 2014). The optimal ratio of the height of the keel divided by the inter-keel separation is 1:2 to reduce the drag (Raschi and Elsom, 1986).

Shark's dorsal spines are located ahead of the two dorsal fins for the majority of squaloid sharks. This spine has often been regarded as a defense attribute, especially for juveniles, but has little been considered as a locomotor function. However, Maissey in 1979 suggested its role as a "cutwater". The angle of spine insertion is probably related to the speed and habitat of the shark,

like at bottom-dwelling species for which finspines stand nearly at right-angles of the vertebral column (Maisey, 1979).

The anterior surface of the spine is sharper and then more hydrodynamic than a cylinder. The turbulent flow is confined immediately behind the spine while laminar flow from the other side is deflected behind the spine (Maisey, 1979). That decreases drag of water and helps shark to swim with rhythmic undulations. Indeed, the asymmetrical laminar flow around the spine is reversed after few moments and provides more maneuverability to shark. Another theory is that the presence of a spine in the anterior position of the dorsal fin limits the creation of a stronger wave vorticity (Maia et al., 2017)

Anatomical adaptations

In sharks three types of muscles can be found: (i) cardiac muscles which are found in the heart that pump blood and works continuously; (ii) visceral or smooth muscles, found in the guts, arteries and other organs, their contraction allows contents to pass flow through them; and (iii) skeletal muscles that move the skeleton. Only the last type will be discussed because shark's locomotion relies only on skeletal myotomal muscles (Seamone and Syme, 2015). Muscles which participate to the locomotion are localized from 25% of the fork length to the end of the caudal fin (Bernal et al., 2003). There are skeletal muscles on each basal, radials, rays or ceratotrichia of fins (Seamone and Syme, 2015). These muscle fibers allow moving fins more and less depending of species affecting therefore the orientation of the swimming. Regarding the thrust, as already mentioned, it is mainly provided by undulations of all body and especially the tail, the main propeller (Ebert et al., 2013). For that purpose, there are muscles all along the caudal vertebrae: muscles up to the lateral line are called

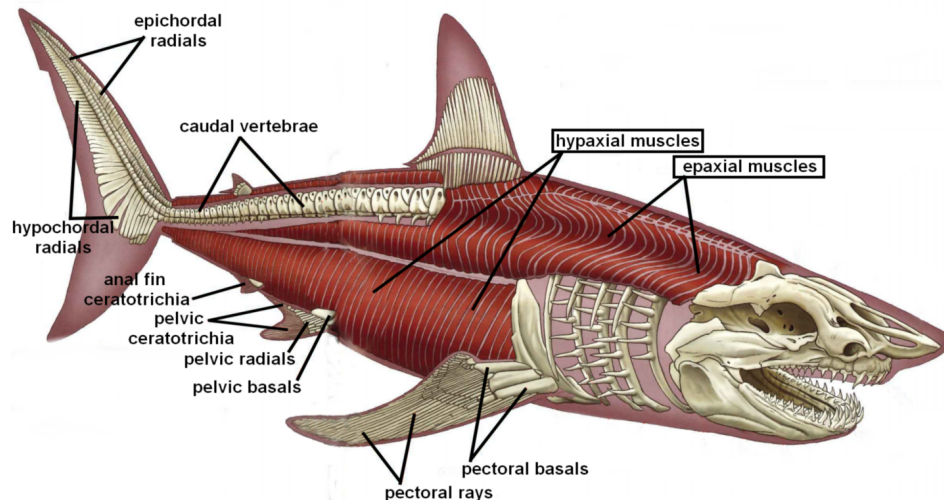


Fig. 19 Shark anatomy, distribution of muscle acting in the locomotion (hypaxial and epaxial muscles) and cartilaginous structure of fins (Modified from Ebert et al., 2013).

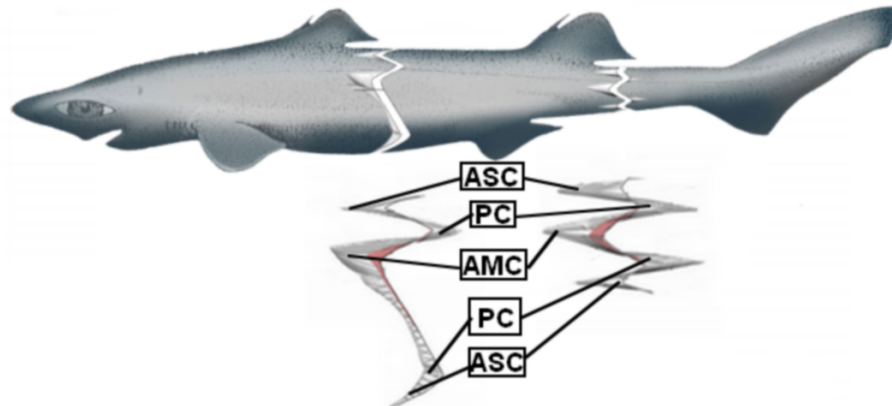
epaxial muscles while those under are called hypaxial muscles (Fig. 19) (Ebert et al., 2013). As fish, the volume of muscle in shark is around 50% of all the body. This high level is mainly due to the denser environment where they live (Seamone and Syme, 2015).

The muscle fibers are arranged into myomeres. Unlike myomeres of mammals that attach to bones, the myomeres of sharks are linked to a myoseptum made of collagen fibers (Shadwick and Goldbogen, 2012). The myosepta act as tendons that redirect the strength of the muscles to the skeleton (Gemballa *et al.*, 2003). Because the vertebral column is incompressible, the process results in lateral bending of the shark's body (Bone and Moore, 2008). The myomeres are folded in a complex three-dimensional way, forming three cones (one main and two secondary) pointing at the anterior part of the shark and two pointing at the posterior part for each myoseptum (Gemballa *et al.*, 2003) (Fig. 20). As for fish, the myomeres have a general W shape in sharks (Bone and Moore, 2008). However, the precise shape varies between species

and the position alongside the length of the body. The length of each cone has an impact on swimming capabilities. Indeed, shark swimming in thunniform mode, suggested to be the most efficient mode in term of velocity, display a more elongated anterior cones and shorter posterior cones than shark swimming by carangiform, subcarangiform and anguilliform mode (Fig. 21) (Gemballa *et al.*, 2003; Shadwick and Goldbogen, 2012). These elongated cones allow forwarding all the contraction in the posterior region and therefore focusing the undulations closer to the caudal fin (Gemballa *et al.*, 2006).

Shark muscle can be separate in two categories recognizable by the color: red and white muscle. In fact, the color difference is caused by the vascularization: white fibers are lowly vascularized compare to red fibers which reach their color by the high level of hemoglobin inside the tissue. Functionally, the two types are different with the white powered mostly by fast anaerobic metabolism and the red by slow aerobic metabolism. They have various histological and physiological differences, some of them presented in Table 1. The white muscles serve in rapid burst swimming. Because of their anaerobic metabolism, they can only be activated for a short time before needing a certain period of recovery. The red muscles serve in slow constant swimming and can be used during long periods of time (Shadwick and Goldbogen, 2012). Each type of muscle does not take action only in one type of movement, for example white muscle fibers follow the lead of red ones during cruise swimming speed. There also are muscle fibers that are in-between the two types presented here and called pink fibers (Bone and Moore, 2008).

Etmopterus spinax - carangiform swimming mode



Isurus oxyrinchus - thunniform swimming mode

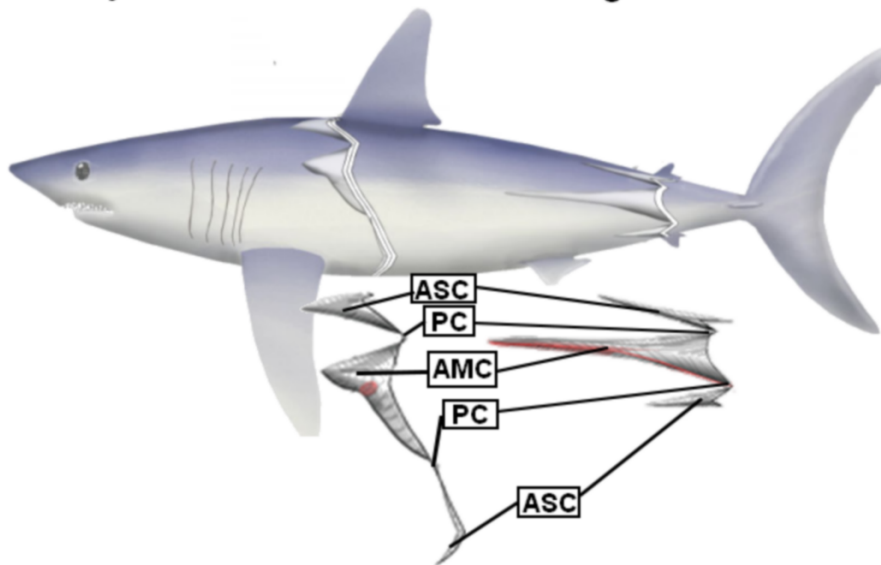
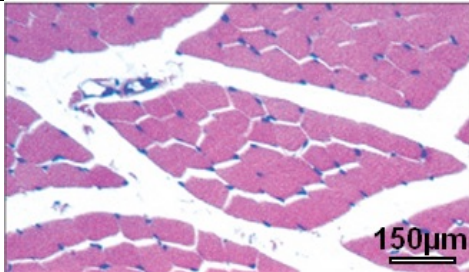
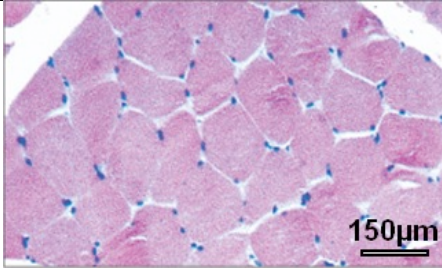


Fig. 20 Organization of lateral muscles in carangiform swimming mode species (*Etmopterus spinax*) and thunniform one (*Isurus oxyrinchus*). ASC corresponds to Anterior Secondary Cone; PC to Posterior Cone; and AMC to Anterior Main Cone (modified from Gemballa et al., 2006).

Table 1 Comparison of red and white muscle fiber in sharks (Modified from Shadwick et al., 2012)

Slow red muscle	Fast white muscle
	
Small diameter (20-50% of white fibers)	Large diameter (100-150µm)
Well vascularized, abundant myoglobin, abundant large mitochondria, red colour	Poorly vascularized, little or no myoglobin, low mitochondrial volume density, usually white
Oxidative enzyme system	High activity of myosin ATPase
Stored lipid and glycogen, sarcotubular system less in volume than in fast fibres	Glycogen stored, usually little lipid, relatively large sarcotubular system
Multiple (distributive) innervations	Focal, dual innervations
Low resting potential	High resting potential
No propagated action potentials, except under experimental conditions	Propagated action potentials
Long-lasting contractions evoked by depolarizing agents	Brief contractions evoked by depolarizing agents
Mainly acts in cruise swimming mode	Mainly acts in burst swimming mode

Across the body, red fibers are run alongside the body axis while white fibers are oriented with an axis of 40-45% of the body horizontal orientation. The proportion of red muscles was measured for shallow-water species and estimated around 4% of the total body mass which is lower than white muscle representing about 30% of total body mass (Bernal et al., 2003; Seamone and Syme, 2015). For deep-sea species, the proportion of each muscle type is still unknown. As for most fishes, the majority of sharks display red fibers at the lateral periphery of the body just under the skin (Fig 21A). But some species possess a different red muscle position. Indeed, high active swimmer such as the white shark (*Carcharodon carcharias*), the shortfin mako (*Isurus oxyrinchus*),

the salmon shark (*Lamna ditropis*), and one species of Alopidae, thresher shark (*Alopias vulpinus*) (Fig 21B) display red muscle interiorly close to the spine (Bernal et al., 2003; Bernal et al., 2005, Sepulveda et al., 2005).

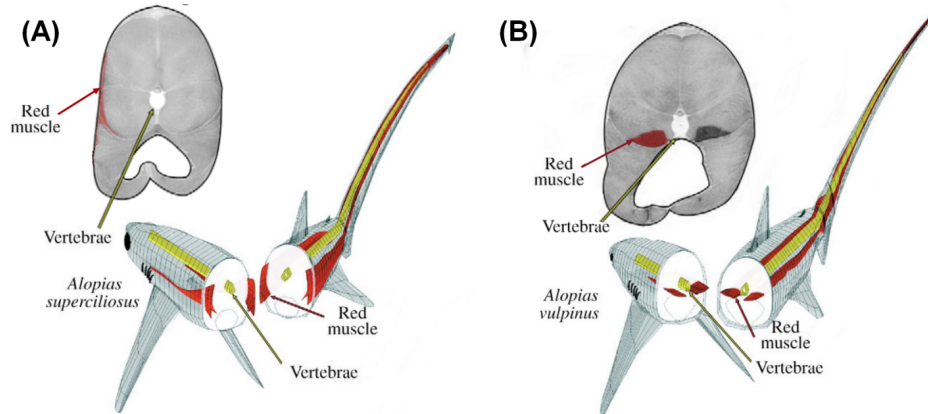


Fig. 21 Whole-body reconstructions of the two thresher shark species (Alopiidae), showing the position of the red aerobic locomotor muscle (red) and vertebral column (yellow). (A) *Alopias superciliosus*; (B) *Alopias vulpinus* (modified from Sepulveda et al., 2005)

This red muscle location change provides an efficient adaptation for shark highly active (Bernal et al., 2003; 2005). Indeed, most of sharks are ectotherm (i.e. this is an organism in which internal physiological sources of heat are of relatively small, such organisms rely on environmental heat sources). Sharks displaying regional endothermy can warm their blood at temperature superior than the environment one (Bernal et al, 2003; Bernal et al, 2005; Bernal and Sepulveda, 2005). This adaptation found also in high active swimmer fishes, such as tuna and swordfish, is correlated with the high swimming capabilities (Bernal et al., 2001; Bernal et al., 2003; Dickson and Graham, 2004). Thanks to this adaptation, shark can use the “waste” heat create by the red muscle contraction to warm their blood and the swimming muscle region

through vascular countercurrent heat exchangers (retia mirabilia). This increases the rate of enzymatic reactions, gas exchange and transport in their bodies which in turn allows them to swim at far faster speeds than many comparable sharks that are regular ectotherms (Bernal et al., 2005). While the locomotor muscle regional endothermy has an impact on swimming capabilities other tissues can also display regional endothermy such as the eyes, the brain or also the viscera aiming again to increase the efficiency of these organs. Regarding evolution issue, this adaptation appears separately in bony fish and in sharks. Currently, only sharks from Lamniformes order display this adaptation (Table 2) while the red muscle distribution of other species is still poorly documented (Dickson and Graham, 2004).

Table 2 Known or suspected endothermic shark species (modified from Dickson and Graham, 2004).

Order/family/species	Regional endothermy		
	Muscle	Eye/Brain	Viscera
<i>Lamna ditropis</i>	✓	✓	✓
<i>Lamna nasus</i>	✓	✓	✓
<i>Carcharodon carcharias</i>	✓	✓	✓
<i>Isurus paucus</i>	?	✓	✓
<i>Isurus oxyrinchus</i>	✓	✓	✓
<i>Alopias pelagicus</i>	✗	?	?
<i>Alopias superciliosus</i>	✗	?	?
<i>Alopias vulpinus</i>	✓	?	?

Physiological adaptations

Metabolism

The metabolism is defined as the process through an organism assimilates and consumes energy (Reece et al. 2014), it gave information on several biological aspects such as growth and feeding rates but also activity patterns such as swimming capabilities (Sullivan and Somero, 1980). Regarding the locomotor capabilities, the metabolism as energy producer is a key component to study: energy production is based on two main biochemical pathways, aerobic and anaerobic, allowing organisms to produce energy that supplies their needs (Reece et al. 2014). Both of these metabolic pathways create ATP from NADH oxidation reaction. Aerobic system produces more ATP (38) than anaerobic one (2) but the first system needs a high concentration of oxygen and it is a hundred times slower than the second system (Reece et al. 2014). Therefore, the aerobic metabolism powers mainly the red muscles, which are highly irrigated to provide in most case a long steady effort such as a cruise swimming mode. In opposite, the anaerobic metabolism powers mainly the white muscles which have few blood capillaries and produce a short-term but intense effort such as a burst swimming mode (Watanabe et al. 2012; Ryan et al. 2015).

Traditionally, the metabolism of sharks or fish is measured using: (i) the Standard Metabolic Rate (SMR) (Bril, 1987) which is defined as the minimal maintenance metabolic rate of fed resting ectotherm, below which physiological function is impaired (Brett and Groves, 1979; Priede, 1985); (ii) the Maximum metabolic rate (MMR), also named the Active Metabolic Rate (AMR) (Korsmeyer and Dewar, 2001) which is the maximal aerobic metabolic rate at a given temperature, provides the upper boundary for aerobic energy

metabolism (Norin and Clark, 2016); (iii) and finally, the subtraction between the standard and the maximal metabolic rate provides the absolute aerobic scope (AAS), which measures the total amount of aerobic energy available to the animal. All these measurements are taken using a respirometer which measures the oxygen consumption per kilogram of body per hour. Results on shallow-water sharks revealed that actively-swimming sharks which ventilate their gills by holding their mouth open during their displacements (called ram ventilation) have a higher SMR and MMR than benthic sharks which can pump the water through their gills (Carlson et al. 2004). Also for ram ventilator sharks, we observed that the SMR and MMR increase according to their swimming speed (see Fig. 22). For example, the SMR of mako shark (*Isurus oxyrinchus*) reach $240 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Bernal et al. 2001), the scalloped hammerhead shark (*Sphyna lewini*) is $189 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Sims, 1996), and the picked dogfish (*Squalus acanthias*) is $76.4 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Lowe, 2002). Same patterns are observed with the MMR of mako shark reaching $541 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Bernal et al. 2001), the scalloped hammerhead shark $500 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Sims, 1996), and the picked dogfish $250 \text{ mg O}_2 \text{ kg}^{-1}\text{h}^{-1}$ (Lowe, 2002). Additionally to the speed, temperature has also an effect on the SMR and MMR, both metabolic rate decreases according to temperature decrease. However, sharks displaying regional endothermy can counteract this decrease by holding higher internal temperature (Bernal and Sepulveda, 2005).

Even if the measurement of these metabolic indexes are the best way to characterized the metabolism of an organism, it displays two disadvantage: (i) first organism need to be alive in a confined environment (tank linked with a respirometer); (ii) secondly, it measures mainly the oxygen consumption which is linked with the aerobic metabolism and gives few information regarding the anaerobic one.

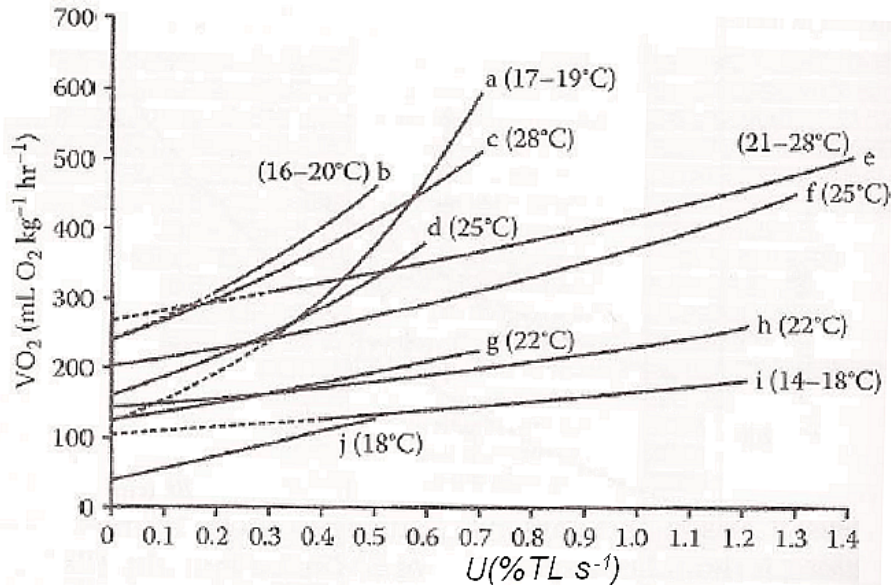


Fig. 23 Oxygen consumption – SMR (VO_2 – $\text{mL O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$) according to the temperature (T – $^{\circ}\text{C}$) and the relative velocity (U – $\% \text{TL s}^{-1}$) or ten species of shallow-water shark: a and b= *Isurus oxyrinchus* ($n=10$), c= *Carcharhinus acronotus* ($n=8$), d= *Negaprion brevirostris* ($n=7$), e= *Sphyrna lewini* ($n=17$), f= combined data of *N. brevirostris* ($n=1$) et ($n=7$), g) *N. brevirostris* ($n=13$), h) donnée combiné de *N. brevirostris* ($n=1$) et ($n=13$), i) *Triakis semifasciata* ($n=5$) et j) *Carcharhinus plumbeus* ($n=16$). (Modified from Carrier et al., 2012).

Another method is also used to evaluate the metabolism of organism: the enzymatic assays. This method is based on the measurement of a proxy which is the enzyme activity (Drazen and Seibel, 2007). Although many enzymes can be measured, traditionally for the swimming capabilities, only enzymes producing energy in locomotor muscles are assayed (Childress and Somero, 1979; Sullivan and Somero, 1980; Drazen et al. 2011; Drazen et al. 2015; Launder and Di Santo, 2015). The energy is mainly provided by two metabolic ways: (i), the glycolysis, is the metabolic pathway which converts glucose in pyruvate. Then, the pyruvate can be transformed into lactate through the lactic fermentation

(anaerobic pathway) or (ii) be incorporated into the Krebs cycle, also known as the citric acid cycle (aerobic pathway) which take place into mitochondria. The Krebs cycle releases stored energy from carbohydrates, fats and protein using the oxidation of acetyl-CoA (Fig. 23) (Reece et al, 2014).

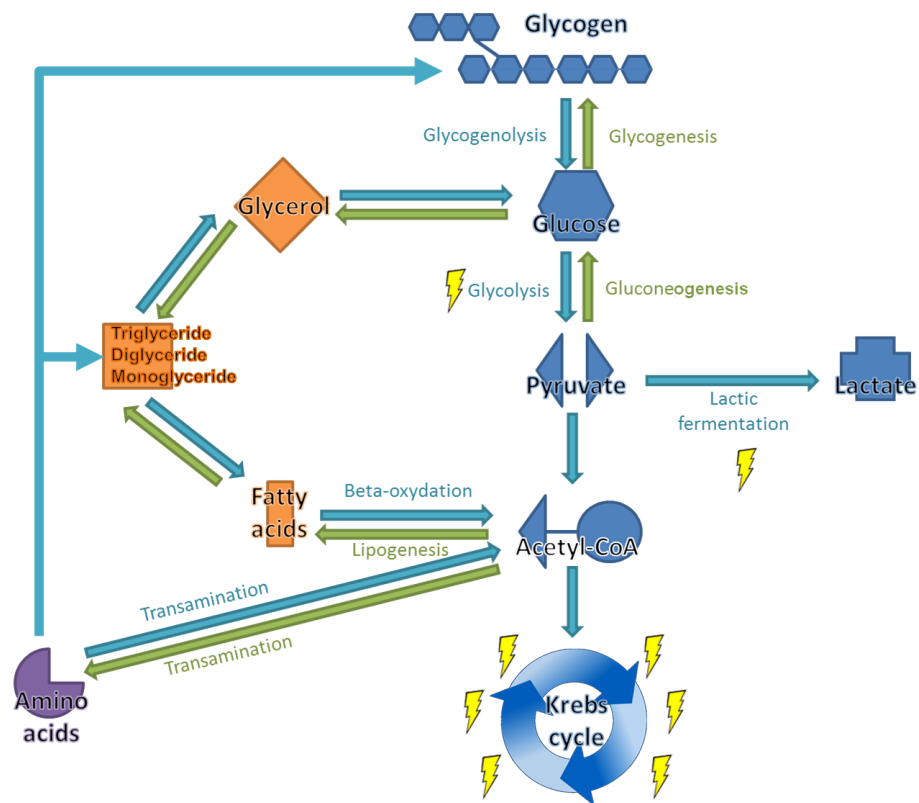


Fig. 23 Simplified pathway for energy production in organism from glycogen, fats and amino acid.

Both pathways create ATP from NADH oxidation reaction. Aerobic system produces more ATP (38) than anaerobic one (2) but the first system needs a high concentration of oxygen and it is a hundred times slower than the second system (Reece et al. 2014).

In most studies about the fish metabolism, anaerobic pathway is estimated using two enzymes as proxy: the pyruvate kinase (PK) and the lactate dehydrogenase (LDH) (Treberg et al., 2003; Drazen et al. 2011; Condon et al., 2012; Drazen et al. 2015; Launder and Di Santo, 2015). The PK is the enzyme which catalyzes the tenth and the last step of the glycolysis. It transforms the phosphoenolpyruvate (PEP) into pyruvate by transferring the phosphate group from PEP to adenosine diphosphate (ADP) creating one molecule of adenosine triphosphate (ATP) (Fig. 24). This reaction is catalyzed by magnesium (Mg^{2+}) acting as cofactor (Reece et al. 2014). After this last glycolysis reaction, the pyruvate can be incorporated either into the Krebs cycle, i.e. aerobic metabolism, occurring mainly in red muscle or into the lactic fermentation within the white muscle. This reaction determined by the LDH transform the pyruvate into lactate regenerating a NAD^+ from a NADH. Other cofactors can be used such as NADPH or b cytochrome but it is less common (Fig. 25). This new NAD^+ will be used for other reactions such as the sixth step of the glycolysis or also the transformation of the pyruvate into acetyl-CoA which is essential for its incorporation in the Krebs cycle (Reece et al. 2014).

Regarding studies about the aerobic metabolism, the citrate synthase (CS) and the malate dehydrogenase (MDH) are the most studied enzymes (Treberg et al., 2003; Drazen et al. 2011; Condon et al., 2012; Drazen et al. 2015; Launder and Di Santo, 2015). These enzymes correspond to the first and the last step of the Krebs cycle respectively. The CS catalyzes the transformation of acetyl-CoA with oxaloacetate to citrate. This reaction need water (H_2O) as cofactor and reject CoA-SH which is a reactive building block (Fig 25) (Reece et al., 2014). The citrate will be transform to malate along eight other step of the Krebs cycle generating numerous $NADH + H^+$ from NAD^+ . The malate will be finally

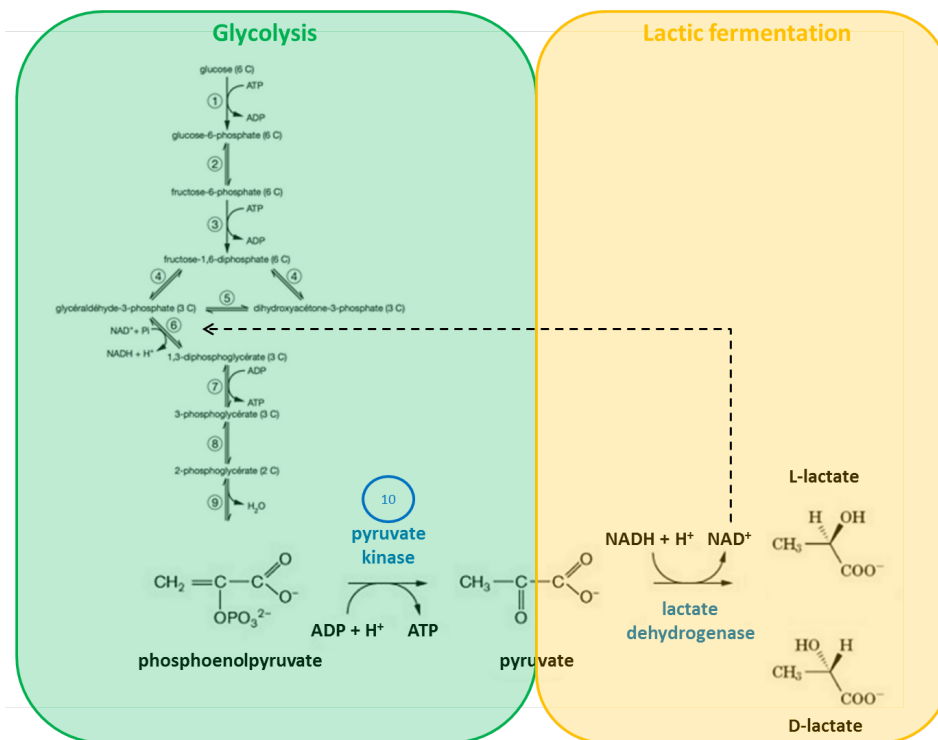


Fig. 24 Glycolysis and lactic fermentation steps with a focus on Pyruvate Kinase (PK) and Lactate Dehydrogenase (LDH) activities (inspired from Reece et al., 2014).

catalyzed by the MDH to regenerate the oxaloacetate essential for the first step of the cycle. This reaction will also convert a NAD⁺ to NADH + H⁺ (Fig. 25) (Reece et al., 2014). The ATPs produced by Krebs cycle is generated from the difference of pH delivered by H⁺ ions between the mitochondrial cytoplasm and the intracellular cytoplasm (Reece et al., 2014).

While numerous studies were conducted regarding the metabolism differences between deep-sea and shallow-water teleostei (Childress and Somero, 1979; Sullivan and Somero, 1980; Drazen et al. 2011; Drazen et al. 2015), the number of similar studies on chondrichthyans stay poorly documented (Treberg et al., 2003;

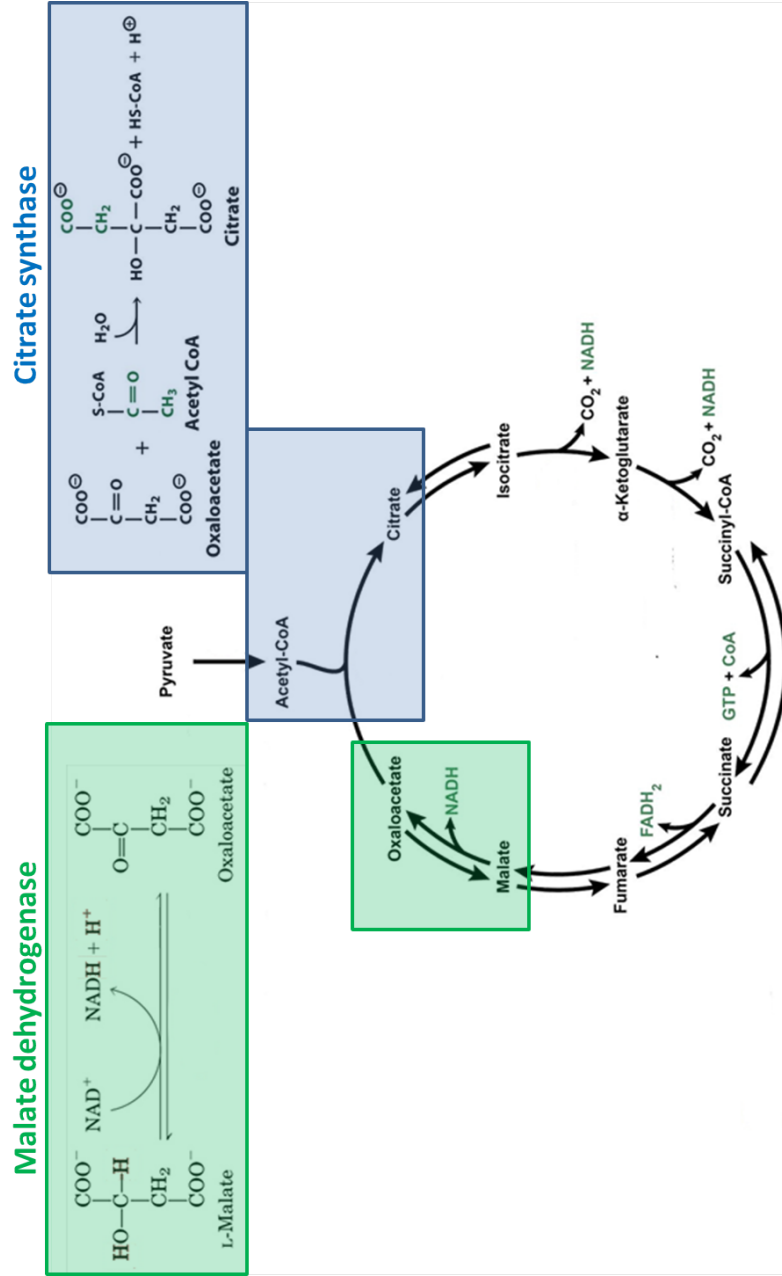


Fig. 25 Krebs cycle steps with a focus on Citrate Synthase (CS) and Malate Dehydrogenase (MDH) activities (inspired from Reece et al., 2014).

Condon et al., 2012). For teleostei it has been shown that deep-sea fish display a lower aerobic and anaerobic metabolism than shallow-water species. Authors conclude that the deep-sea fishes should therefore swim slower what has been confirmed by study on their velocity (Treberg and Speers-Roesch, 2016). Regarding shark, the metabolism of shallow-water species was mainly measured using enzyme activities proxy except for some species where measurement of SMR, AMR and AAS could be possible by respirometry. For high active species such as the mako shark, studies revealed a higher metabolism whether with enzyme proxy or with respirometry. There are only two studies on the metabolism of deep-sea shark species and they are based on enzyme assays only due to the impossibility to keep deep-sea shark alive without stress (which can interfere with the respirometry measurements) (Treberg et al., 2003; Condon et al., 2012). These studies have revealed a decrease of LDH and PK activities according to the depth in white and red muscle (Fig 26A and B). Regarding aerobic enzymes (CS and MDH), no decrease was observed according to the depth (Fig 26C and D). Nevertheless, authors have also concluded that swimming capabilities of deep-sea sharks should be lower than their shallow-water counterparts. This conclusion is mainly based on two hypothesis: (i) the cold water effect inhibiting the muscular contraction by decreasing the enzyme activities (Treberg et al. 2003); and (ii) the visual interaction hypothesis (VIH) which suggests that the evolutionary pressure for a good velocity decreases when the environment becomes darker because the reactive distances between predator and prey are reduced. Metabolism subsequently declines due to the cost of maintenance and operation associated with high-performance locomotion (Condon et al. 2012). It must be pointed out that, the first study focused only on one deep-sea specimen and authors clarified that no generality should be done on deep-sea sharks;

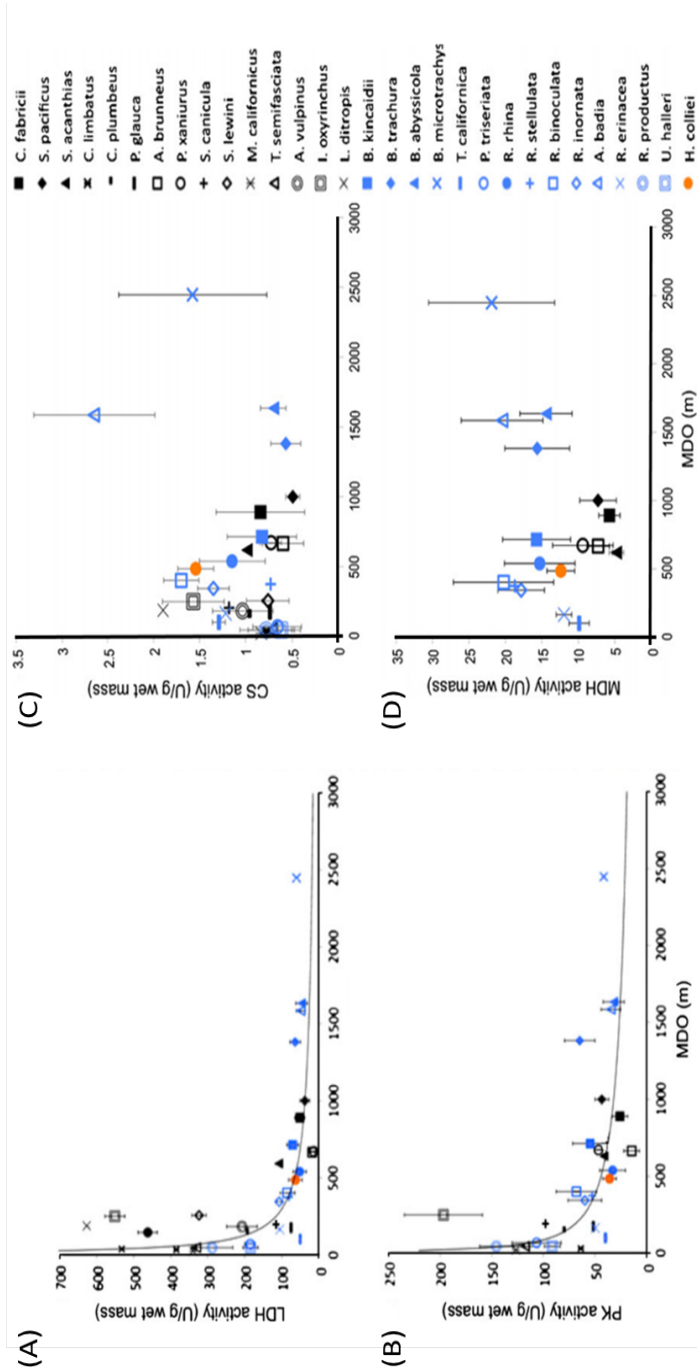


Fig. 26 (A) Mean white muscle LDH activity (U.g - 1 wet mass) per species at 10°C plotted versus median depth of occurrence (MDO) (m): $\log(y) = -0.830 \log^*(x) + 4.06$ ($r^2 = 0.29$, $p < 0.001$); (B) Mean WM PK activity (U.g - 1 wet mass) per species at 10°C plotted versus MDO (m): $\log(y) = -0.460 \log^*(x) + 2.86$ ($r^2 = 0.2$, $p < 0.001$); (C) Mean white muscle (WM) CS activity (U.g-1 wet mass) per species at 10°C plotted versus median depth of occurrence (MDO) (m); (D) Mean WM MDH activity (U.g-1 wet mass) per species at 10°C plotted versus MDO (m). No significant trends were seen; symbols: black sharks; blue skates and rays; orange chimaera (Condon et al., 2012).

while the second study focused more on rays than deep-sea sharks and authors also stated that visual interaction hypothesis takes not into account the bioluminescence which may have an effect on metabolism (increase rate for faster locomotion). .

Respiratory system

As previously said, the aerobic metabolism which powered continuous swimming such as the cruise swimming need oxygen. Assuming, all known bioluminescent reactions observed are based on oxygen (Haddock et al., 2010) and even if the nature of the luminous reaction for shark is still unknown, we can suppose that it is also based on an oxidation. Therefore, the respiratory system of shark might be adapted for better swimming capabilities or also light production.

The respiratory system of fish is typically gills which are the critical interface between the internal and external environment. They are the principal site of respiratory gas exchange, notably oxygen, and one of the main sites for osmoregulation, pH balance and nitrogenous waste excretion (Olson, 2011; Wegner, 2011, 2016). Gills are highly vascularized structures which form numerous folding to increase the exchange surface area. Since it is a fragile system, they are protected under an operculum in teleost fishes or several gill flaps, usually 5, in elasmobranchs (Lebrun, 2001). Although, the structure of the respiratory system has remained relatively similar across the fish evolution and diversification, some unique features characterize the elasmobranchs' gills (Wegner, 2016). The gills are bilaterally situated on either side of the orobranchial cavity and are composed of a series of arch-like structures that provide the physical support for the respiratory tissues (Wilson and Laurent, 2002). The gill arches are bow-shaped structures extending from the ventral surface to the dorsal

surface of the orobranchial cavity, the curved portion oriented posterior-laterally (Wilson and Laurent, 2002) (Fig. 27A). The hyoid arch is commonly accepted as the first arch (Fig. 27B). Each arch is supported by four skeletal elements: whereas the epibranchial and ceratobranchial cartilages form the arch-bearing gills itself, the pharyngobranchial and hypobranchial cartilages anchor the arch to the surfaces of the orobranchial cavity (Fig. 27C) (Wegner, 2016). In elasmobranchs, an interbranchial septum made of connective and muscular tissues extends from the gill arches posterior-laterally and is supported by skeletal branchial rays emanating from the gill arches (Fig. 27B and 27C). The interbranchial septum is attached and supports the filaments along almost their entire length, letting free the tips of the filament. The septa extend past the filament to the lateral edge of the body to form the gill flaps. Between the septa lays the parabronchial cavity which then open individually to the outside, forming the gill slits, characteristic of all elasmobranch (Fig. 27D) (Wilson and Laurent, 2002; Wegner, 2016). The filaments are dorsoventrally flattened and form secondary folding, called lamellae, constituting the basic respiratory unit (Wilson and Laurent, 2002; Olson, 2011). Each filament harbors a row of lamellae on either side and these arise perpendicularly to the axis of the filament and to the incoming water flow (Fig. 27E) (Wilson and Laurent, 2002; Wegner, 2016). Their flat, plate-like morphology furnish the support for effective gas exchange between the blood and water, allowed by a large surface area and a short diffusion distance. The lamellae are not continuous over the entire filaments, leaving a space called the septal channel, between the trailing lamellar edge and the lateral wall of the interbranchial septum (Fig. 27E) (Wilson and Laurent, 2002; Wegner, 2016). Probably to protect the lamellae from mechanical damage, a fleshy extension of the gill arch, called the branchial canopy extends across the filaments and binds to the

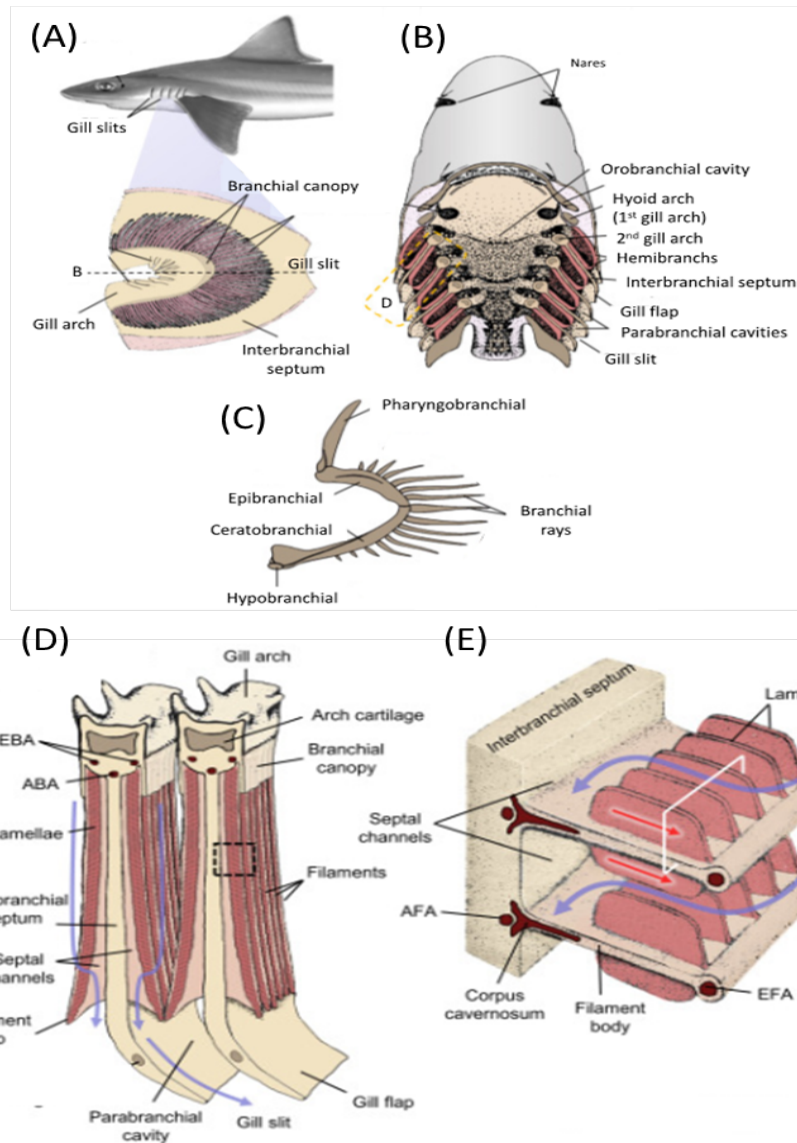


Fig. 28 Schematic overview of elasmobranch branchial morphology. (A) Lateral view of the anterior hemibranch of a single gill arch; (B) Ventral view of a frontal cross-section through the branchial region; (C) Lateral view of the main skeletal elements of the gill arch; (D) Magnified cross-section of gill's filament, ABA (afferent blood artery), EFA (efferent blood artery); (E) Cross-section of. Modified from Wegner (2016).

filament leading edge, covering the nearest lamellae to the arch (Fig. 27D). The lamellae are typically rectangular or semicircular, however, some variations have been observed between lamellae across one filament (Olson and Kent, 1980; Wegner, 2016). The thickness of lamellae epithelium is also an important factor regarding the oxygen absorption.

The rate of oxygen uptake by the gills depends of the metabolic demands and the availability in oxygen in the environment (Wegner, 2016). This rate has been described by the Fick equation (equation 1):

$$(1) V_{O_2} = (K \times A \times \Delta P_{O_2})/t$$

Where: V_{O_2} is the rate of oxygen uptake [$\text{mgO}_2 \cdot \text{min}^{-1}$]
 K is the diffusion (or Krogh) coefficient through a specific material [$\text{mgO}_2 \cdot \mu\text{m} \cdot \text{cm}^{-2} \cdot \text{mmHg}^{-1} \cdot \text{min}^{-1}$],
 A is the respiratory surface of the gills [cm^2],
 ΔP_{O_2} is the mean difference in the partial pressure oxygen between the blood and water [mmHg]
 t is the diffusion distance between the water and the blood [μm] (Wegner, 2011, 2016).

Therefore the capability of oxygen absorption depends of two factors: (i) the surface of the gills and (ii) the thickness of lamellae. The gills' surface can be measured following the next equation (equation 2):

$$(2) A = L_{fil} \times 2n_{lam} \times A_{lam}$$

Where= L_{fil} is the total length of all the filaments

n_{lam} is the lamellar frequency, that is the number of lamellae per unit length on one side of a filament (hence the multiplication by two to take in count the lamellae on the either side of the filament)

A_{lam} is the mean bilateral surface area of a lamella (Hughes, 1966; Muir and Hughes, 1969).

As sharks occupy a vast array of niche, both gill surface area and water-blood barrier thickness can vary greatly across the different taxa. Fast swimming oceanic sharks have elevated energetic demands in relation with their fast continuous swimming. These sharks have therefore large gill surface areas thanks to a high total filament length with large lamellae and thin water-blood barrier. It is also known that organisms that are exposed to low oxygen levels have large gill surface area to increase their oxygen uptake (Childress and Seibel, 1998). The largest gill surface area of a shark to be measured to date belongs to the bigeye thresher sharks (*Alopias superciliosus*, family Alopiidae) which is an active shark and carry out diel migrations within the oxygen minimum zone. It achieves such high surface area thanks to a higher total

filament length due to longer and more filaments than the other thresher sharks (*Alopias vulpinus* and *Alopias pelagicus*). These long filaments correspond to larger branchial chamber that expands laterally and affect the cranial streamlining reducing its hydrodynamism. However, this might not be a problem for the bigeye thresher sharks since its preys living in the oxygen minimum zone (OMZ) are probably slow-moving whereas for thresher shallow-water relatives, which feeds on fast-moving preys, the constraint of a highly streamlined cranium prevent them from having especially long filaments (Wootton et al., 2015). It is supposed that other sharks that dwell in depleted oxygen environment have a similar adaptation: increase of gills surface area but studies evaluating this parameters for OMZ inhabitants and deep-water sharks are lacking (Wegner, 2016; Bigman et al., 2018).

Buoyancy

Finally, the last feature influencing the swimming capabilities is the buoyancy. It explains the distribution of species across the different marine and fresh water environments and also their vertical distribution in the water column. Buoyancy (positive, negative or neutral) can constitute a good compromise to reduce the energy expenditure during locomotion. For example, the sperm whales use their spermaceti to deep dive at lower energy costs (Clarke et al., 1978), most fishes use a swim bladder to adjust their buoyancy and ease their 3D-locomotion provided by their fins (Phleger, 1998). Sharks does not possess swim bladder, therefore, their buoyancy mainly relies to two factors: (i) the hydrodynamic lift provided by the morphology of pectoral and pelvic fins; and (ii) the hydrostatic lift due to internal compounds less dense than water. The hydrostatic lift can be affected by osmolyte composition (TMAO and urea) (Treberg and Speers-Roesch, 2016). Studies

have shown than urea provides more hydrostatic lift than TMAO. However, the contribution of osmolyte represents only 10% while lipids represent 90% of all hydrostatic lift (Treberg and Speers-Roesch, 2016). Lipids are mainly localized in the liver which can represent 30% of all body mass for some shark species but they also can be found in muscle, hearth, kidney, spleen, pancreas and stomach (Table 3). The lipid implication in other tissues that liver is still poorly known, and shark liver is often called the hydrostatic organ in most of study regarding the buoyancy (Corner, 1969; Bone and Robert, 1969; Wetherbee and Nichols, 2000; Remme et al., 2005; Pethybridge et al. 2010; Gleiss et al., 2017).

Table 3 Lipid content according to tissues (mean % to the total body mass $\pm$ SE): hearth, kidney, pancreas, spleen, stomach and liver for three species of deep-sea shark (Remme et al., 2005).

	<i>Centroscymnus coelopsis</i>	<i>Centrophorus squamosus</i>	<i>Centroscyllium fabricci</i>
Hearth	5.41 $\pm$ 3.2	1.9 $\pm$ 0.25	2.07 $\pm$ 0.55
Kidney	4.78 $\pm$ 0.35	2.85 $\pm$ 0.9	2.89 $\pm$ 1.06
Pancreas	2.89 $\pm$ 0.29	3.01 $\pm$ 0.16	2.35 $\pm$ 0.05
Spleen	1.78 $\pm$ 0.05	2.01 $\pm$ 0.13	2.67 $\pm$ 0.45
Stomach	1.23 $\pm$ 0.49	1.22 $\pm$ 0.08	3.23 $\pm$ 0.37
Liver	37.37 $\pm$ 3.11	49.89 $\pm$ 11.79	35.38 $\pm$ 1.96

While shallow-water shark species are documented to be negatively buoyant with large pectoral fins to increase their hydrodynamic lift, deep-sea shark are suggested to have a hydrostatic lift close to the neutrality or even to be positively buoyant (Corner, 1969; Baldrige, 1970; Batista and Nunes, 1992; Pethybridge et al., 2010; Nakamura et al., 2015). Indeed, the hepatosomatic index (HSI); which corresponds to the ratio between the liver mass and the total body mass (expressed in percent); is higher for deep-sea species than shallow-water ones (Fig. 29A) (Treberg and Speers-Roesch, 2016) (See Table 4).

Similarly to the HSI, the liver lipid content also called oil content (% of wet mass); which corresponds to the total lipid fraction from the liver (expressed in percent); is higher for deep-sea sharks than their shallow-water counterparts (See Table 4).

Table 4 Contribution to hydrostatic lift from liver lipids in squaloid sharks (Treberg and Speers-Roesch, 2016).

	Shallow-water squaloid	Deep-sea squaloid
HSI (%)	10	25
Liver lipid (% wet mass)	60	80
Liver lipid (g · kg of shark ⁻¹)	60	200
Liver lipid density (g ml ⁻¹)	0.91 ^a	0.87 ^c
Lift per kg of shark (g) ^b	7.58	35.6

^a Assumes 50% TAG, 50% DAGE with a net density based on densities given in Table 1 (main text).

^b Lift = [liver lipid/liver lipid density] * [seawater density – lipid density], where seawater density is 1.025 g ml⁻¹.

^c (Corner et al. 1969)

However, the relation described shown a decrease of both after around 2000 meters depth which suggest that the high energy demand for a full-fill liver after 2000 meters is limited by the food availability and it could be one explanation why deep-sea sharks are so rare under these depths (Treberg and Speers-Roesch, 2016).

There are four main lipid forms in shark liver: squalene, alkydiaglycerol (diacylglycerol ether, DAGE), triacylglycerol (triglyceride, TAG), and wax ester (Lewis, 1970; Shadwick et al. 2016). They act mainly for two functions: (i) as energy and source of ketone body to provide oxidizable carbon for tissue. This function is not still proven for the squalene; and (ii) as hydrostatic compound due to their lower density in comparison with the shark environment. These two functions can be contentious for example during large migration or fast period as it was demonstrated with the great white shark which displays a more negative hydrostatic lift after a long migration (Shadwick et al. 2016; Raye et al. 2013).

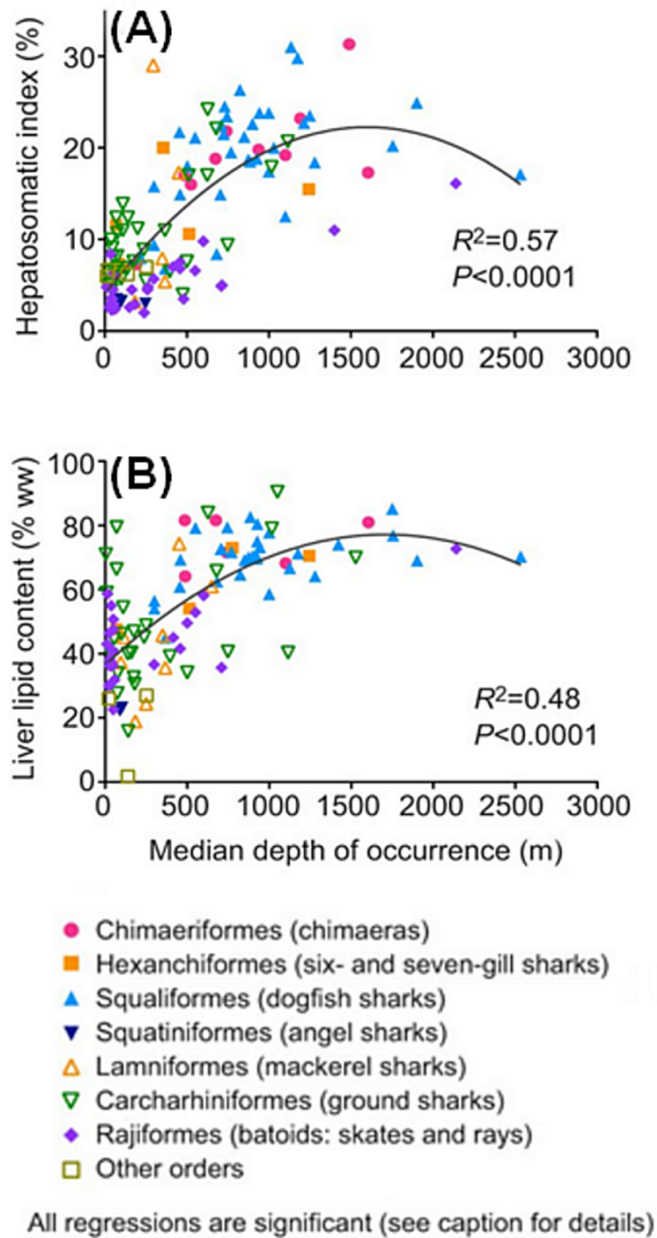


Fig. 29 (A) Hepatosomatic index (HSI, % of body mass) and (B) liver lipid content (% of liver wet mass) as a function of median depth of occurrence among chondrichthyans (Treberg and Speers-Roesch, 2016).

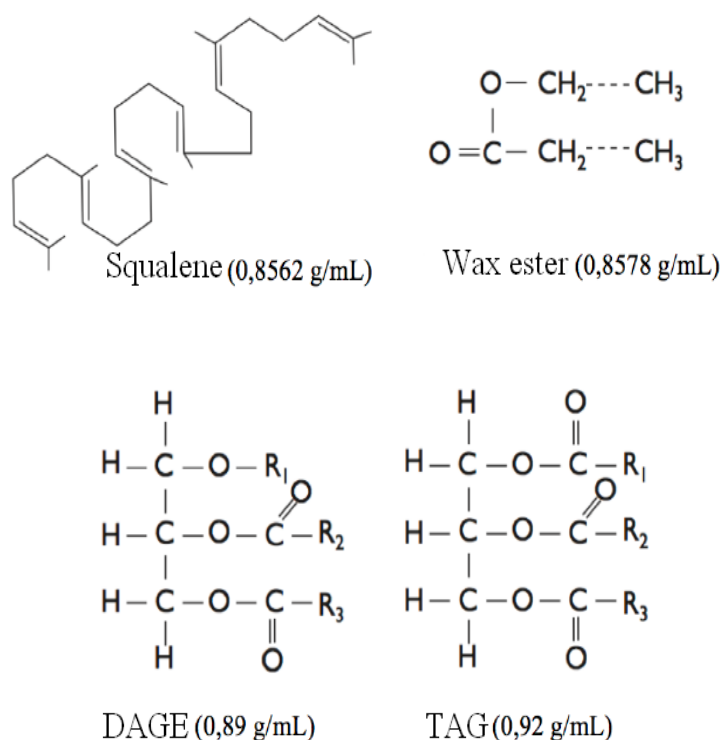


Fig. 30 Chemical structure and density of the main lipids in sharks' liver; DAGE correspond to diacylglycerol ether and TAG to triglyceride (Shadwick et al., 2016).

Squalene is a hydrocarbon with a density of 0.8562 g/ml. In shark body, it is often transformed in squalane by hydrogenation. Both lipids display similar physical properties: weak polarity, same molecular weight and high boiling point (Depez et al., 1990; Cecchi, 1998; Chabrol, 2012; Hall et al., 2016). The only function known currently for this lipid is its major implication in the hydrostatic lift (Batista et al., 1992; Bakes and Nichols, 1995; Shadwick et al., 2016). Results revealed also a higher squalene

concentration in deep-sea sharks' livers than the shallow-water sharks' ones (Treberg and Speers-Roesch, 2016) (Fig 32A).

Wax ester (0.8578g/ml) is the second less dense lipid found in shark organs. Despite its low density, it has been suggested that this lipid does not act in the hydrostatic lift of shark due to his low abundance in tissues (less than 2% of all lipid) (Van Vleet et al., 1984).

DAGE display a density of 0.89g/ml and it has been proposed to be the second most important lipid acting on the hydrostatic lift of sharks (Sargent et al., 1972). Indeed, it has been shown that this lipid is inversely correlated with the squalene concentration in liver (Fig 31) (Wetherbee et al., 2000; Hall et al., 2016). Moreover, this lipid displays the similar pattern to squalene concentration according to the depth. Indeed, deep-sea shark liver is filled with more DAGE than liver of shallow-water counterparts (Fig 32B) (Treberg and Speers-Roesch, 2016). In addition to its hydrostatic lift role, DAGE can be use as source of energy by the organism (Malin and Barone, 1970).

Finally, TAG with a density of 0.92g/ml is the lipid which provides the less of hydrostatic lift in shark liver. It has been proved that the main function of this form of lipid is to be a source of energy. In contrast with the squalene and DAGE, the TAG is more present in liver of shallow-water sharks than deep-sea species. Finally, Malin et al. in 1970 have also shown, with two groups of *Squalus acanthias*, one control and another one where sharks were weighted down, a change of lipid composition with a shift from TAG to DAGE in several hours for the second group, indicating a possible functional shift from energy to buoyancy.

As we saw, the temperature affects the metabolism of ectotherm organisms for example acting on the enzyme activities. Moreover,

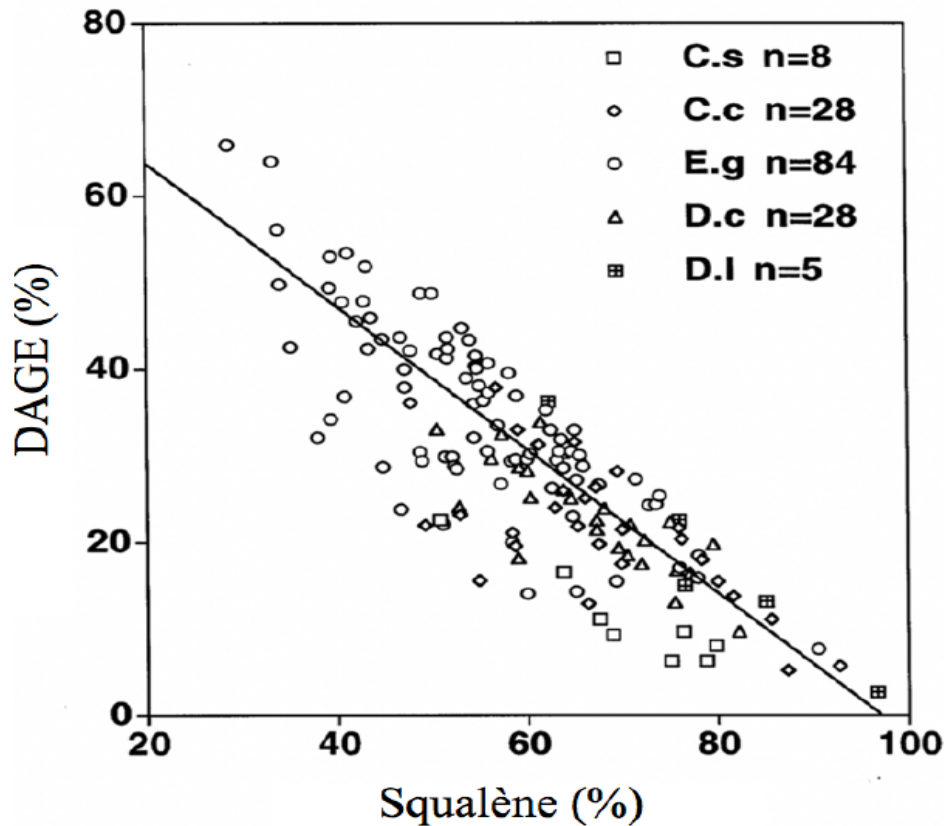


Fig. 31 Percentage of diacylglycerol ether (DAGE) according to the percentage of Squalene in the liver of four deep-sea shark species from Squaliformes order.; C.s, *Centrophorus squamosus*; C.c, *Centroscymnus crepidater*; E.g, *Etmopterus granulosus*; D.c, *Deania calcea*; D.l, *Dalatias licha* (Wetherbee et al., 2000).

the temperature has also an impact on tissues density especially with tissues containing lipids. For example, a lot of shark researcher have seen that deep-sea sharks, brought to the surface or put in water hotter than their living temperature, float and seem have difficulties to go down. Indeed the density of lipid decrease according to temperature increase. This was the reason why scientists suggested that deep-sea shark are neutrally buoyant in their cold environment.

Even if the mechanism how shark can move and adjust is buoyancy in the water become well and well known, there still need for improvement. Indeed, Treberg and Spoers in 2016 suggested an equation characterizing the hydrostatic lift provided by sharks' liver (equation 3):

$$(3) LHF = \left(\frac{\text{liver lipid (g.kg}^{-1} \text{ of shark)}}{LLD \text{ (g.ml}^{-1})} \right) \times (\text{sea water density (g.ml}^{-1}) - LLD \text{ (g.ml}^{-1}))$$

Where:

Liver lipid is a combination of HSI and oil content data

$$(4) HSI (\%) = \left(\frac{\text{Liver mass (g)}}{\text{Total body mass (g)}} \right) \times 100$$

$$(5) OC (\%) = \left(\frac{\text{Total lipid volume (ml)}}{\text{liver mass (g)}} \right) \times 100$$

Sea water density correspond to 1.025 g.ml⁻¹

The liver lipid density (LLD, corresponding to the weighting of each lipid class proportion and their respective density)

$$(6) LLD = (0.92 \times \%TAG) + (0.89 \times \%DAGE) + (0.86 \times \%HC) + (0.86 \times \%WE)$$

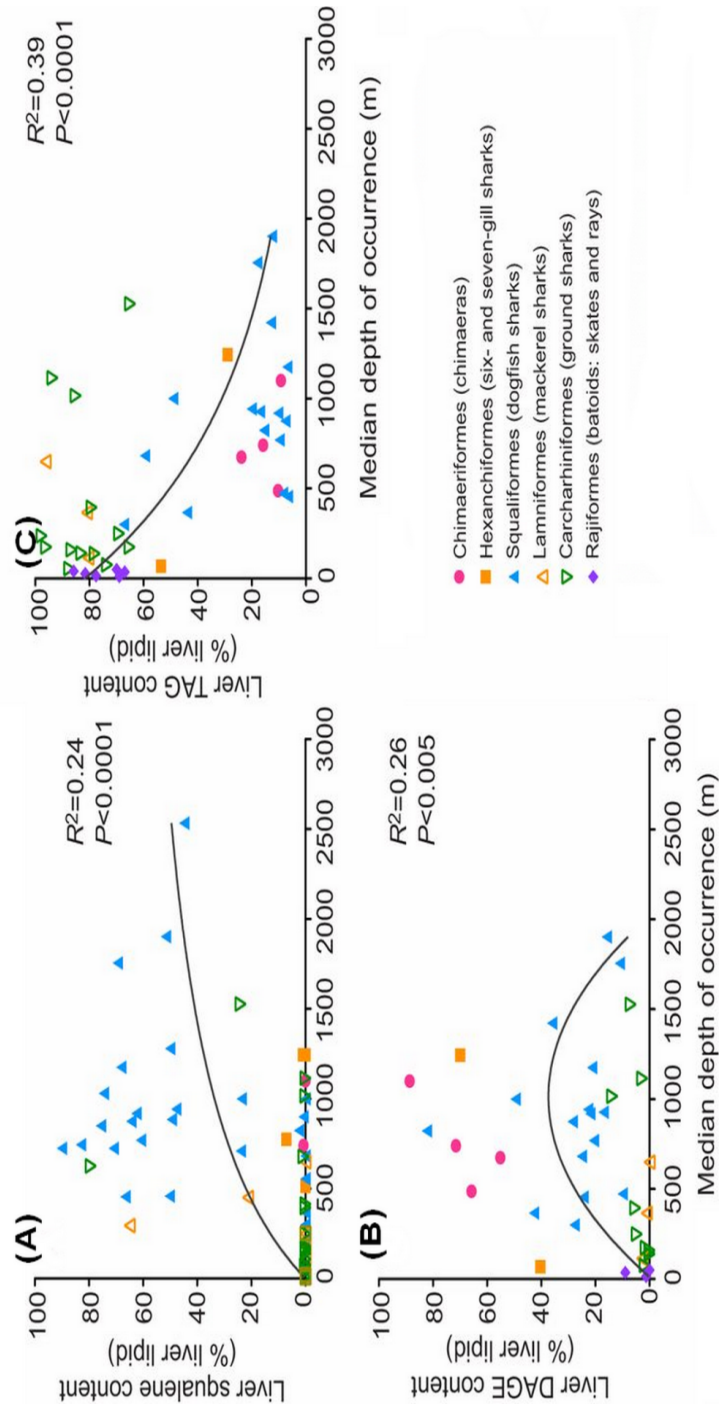


Fig. 32 (A) Liver squalene, (B) diacylglycerol ether and (C) triacylglycerol contents as a function of median depth of occurrence among chondrichthyans (Treberg and Speers Roesch, 2016).

More and more complex modeling appeared but there still no one taking into account all parameters which have an impact on sharks' buoyancy: temperature, osmolytes composition, other tissue densities, etc. However, the main role of liver in the hydrostatic buoyancy and the change of its features through species make it a good proxy for buoyancy estimation.

Nevertheless a lot of different hypothesis were formulated to justify the possible neutral buoyancy of deep-sea sharks. For example, in 1966, Bone hypothesized that sharks that are close to neutral buoyancy should have fewer red aerobic muscle fibers. Indeed, it is difficult for marine organisms to swim slowly when they are negatively buoyant. For this reason and also to have high swimming capabilities, shallow-water sharks developed higher proportion of red aerobic muscle, whereas deep-sea sharks do not need a high proportion for their low swimming capabilities. While this hypothesis has been cited numerous times in the literature, it has never been directly tested through direct evidences (Shadwick and Goldbogen, 2012; Shadwick et al., 2016).

AIMS OF STUDY

While the swimming capabilities of shallow-water sharks have become well known, it is not the case for their deep-sea counterparts. Numerous studies have shown the importance of the locomotor system in understanding the ecology of organisms. It has implications in several essential behaviors such as hunting, mating or even escaping for smaller species. Swimming capabilities can be determined by measuring different proxies: enzyme assays, buoyancy, muscle analyses and morphological features such as body shape, caudal fin shape among others. However, while these physiological and morphological adaptations can be linked with the velocity of shallow-water sharks, for deep-sea sharks it only gives rise to some hypotheses without any real value of velocity measured hence, deep-sea sharks are all considered to be slow and listless swimmers.

In this general context, my thesis aims to characterize the swimming speed of deep-sea sharks in order to not only determine if they swim slower than their shallow-water counterparts but also to reveal if they display speed difference between them. A pluridisciplinary approach will be developed: swimming speed analysis of *in situ* video combined with the measurements of different proxies (i) muscle enzyme assays, (ii) muscle proportion, (iii) hydrostatic lift estimation, and (iv) morphological features. Results from the various studied parameters will be presented in four scientific papers what represent the core of the three chapters of this thesis.

Chapter I:

In literature, deep-sea sharks are described as slow and listless swimmers. This statement is mainly based on indirect measurement of their velocity and two hypotheses: the cold

environment in which these sharks live reduces the effectiveness of muscle contractions; and the visual interaction hypothesis (VIH) which suggests that when the light in an environment decreases, the selection pressure for a high swimming speed also decreases.

Considering the lack of any measured values of deep-sea sharks swimming speed, I decided to use available *in situ* video recordings to measure the velocity of deep-sea sharks. 3D reconstruction of shark motion at depth was done through a newly developed script and allowed accurate estimate of cruise swimming speed on 8 deep-sea shark species. Obtained results not only permitted a comparison between shallow-water and deep-sea shark swimming speeds but also authorized the comparison between different deep-sea sharks speeds in order to verify the hypothesis that differences might exist according to shark's lifestyles, i.e. different lifestyles inducing different swimming capabilities (in our case, luminous versus non-luminous deep-sea sharks).

Chapter II:

In literature, the swimming speed of deep-water sharks was only indirectly estimated for 3 species by proxies such as muscle metabolism, buoyancy, and red/white muscle proportion. Indeed, two studies showed that anaerobic muscle metabolism activity decrease according to the depth, while anaerobic metabolism did not vary. It has also been suggested that deep-water sharks have buoyancy close to neutrality and Bone in 1976 hypothesized that sharks close to neutrality have less red muscles, reasons why they should swim slower.

Although former studies point in the direction that deep-water sharks swim slowly, my first chapter shows speed differences between deep-water sharks with some species (Etmopteridae)

swimming as fast as coastal benthic species. The purpose of the second paper is to correlate the measured velocities with anatomical (muscle proportion) and physiological adaptations (metabolism proxies) in deep-sea shark species. With the hypothesis that aerobic metabolism and red muscle proportion are related to the cruise swimming speed observed. Assuming the “isolume follower” hypothesis which suggests that luminous sharks will continuously move up and down in the water column to correlate their ventral light intensity with the downwelling light intensity of their environment to properly countershade, Etmopteridae species would display a higher aerobic metabolism and red muscle proportion. Furthermore, the third paper relies on Bone’s hypothesis. In this paper, the hydrostatic lift provided by deep-sea sharks’ livers is evaluated to reveal a possible correlation with the red muscle proportion. Our hypothesis, like Bone, is that the more sharks’ liver provide lift, the less red muscle they will harbour.

To answer the question about shark’s metabolism, the red and white muscles of 10 species of deep-water sharks were collected, their proportions were measured and the aerobic metabolism was characterized by two key enzymes: citrate synthase and malate dehydrogenase while anaerobic metabolism was characterized by one key enzyme: lactate dehydrogenase. The lift provided by the liver was measured using an index that takes into account the hepatosomatic index, the oil content and the lipid composition. The lipid composition was evaluated to a new methodology (FT Ramman and MIR spectroscopy).

Chapter III:

In shallow-water sharks, the relationship between body morphology and swimming capability is established and accepted

shape of the body and caudal fin has an important implication in water penetration and thrust, respectively. It has also been suggested that the rostrum shape and the dorsal spine could have an impact but the potential effect is still poorly known. In contrast, the morphology of deep-sea sharks in relation to ecological features is poorly documented. Indeed, all deep-sea sharks from the Squaliformes order are classified into a single morphotype, according to Thomson and Simanek in 1977. However, since the first paper in this thesis shows differences in swimming speed, it is interesting to take a closer look at morphological differences that could be associated to these different swimming speeds.

The last study of this thesis highlights the possible impact of morphological adaptations on the swimming speed deep-sea sharks. Two hypotheses will be tested: (i) Squaliformes sharks display different body shapes and therefore should not be grouped in the same morphotype; (ii) morphological features related to velocity (caudal fin, rostrum, spine and body shape) would change between slow deep-sea species and faster ones.

This paper represents the first multivariate morphological study done on Squaliformes sharks. Twenty-six morphological variables, three morphological ratios and geometric morphometric will be measured on nine species in order to identify the potential morphological differences which could separate Squaliformes deep-sea species and be related to the velocity previously recorded.

Methodology

METHODOLOGY

Sampling

While several sharks were observed using stereo-Baited Remote Underwater Videos (stereo BRUVs), only some of them were sampled through four field campaigns:

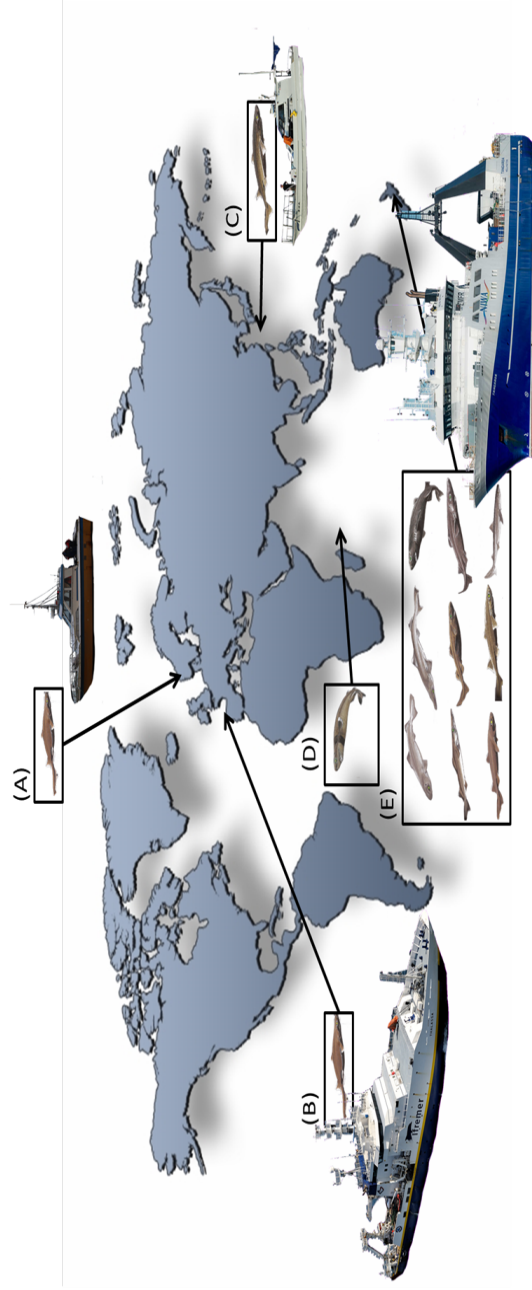
- (i) *Etmopterus spinax* and *Galeus melastomus* were sampled firstly from the 11th January 2016 to the 18th January 2016 at Espeyrend marine field station (Bergen university), in Norway (60.260623°N, 5.162056°E). Sharks were fished aboard the *R.V. Aurelia* using baited bottom long-line at 210 meter's depth (Fig. 33A); *E. spinax* has been the model species of UCLouvain marine biology lab since more than 10 years. Sharks were kept in a dark tank on the boat and brought back in the lab. They were transferred to bigger tanks standing in a dark cold room (6°C). Tissues were sampled from sharks and stored at -80°C for many experiments. Some other specimens of *E. spinax* were also collected during the EVHOE campaign aboard the *R.V. Thalassa* in the November 2018 using deep-trawl at 350 meter's depth in the Celtic Sea (48.575909°N, -6.978180°O) (Fig. 33B). Tissues were directly sampled after the trawling and stored at -80°C.
- (ii) *Etmopterus molleri* were sampled in December 2016 and November – December 2017 at Okinawa Churaumi Aquarium Research Center. Sharks were fished aboard the *R.V. Kaykomaru* by longliners at depth of 500 meters in the East China Sea (26.32468°N, 127.41207°E) (Fig. 33C). All specimens were transferred to oxygen saturated plastic bags

filled with seawater and transferred in a refrigerated box to the Okinawa Churaumi Aquarium where they were kept alive in a cold dark tank filled with seawater (13 °C) until manipulations. Sharks were dissected and all tissues were frozen at -80°C for future analyses.

- (iii) *I. brasiliensis* came from by-catch provided by fisherman from La Reunion Island. Individuals were stored directly at -80°C and dissected in the lab before manipulations (Fig. 33D).
- (iv) *Etmopterus baxteri*, *Etmopterus lucifer*, *Deania calcea*, *Centrophorus squamosus*, *Centroscymnus owstonii*, *Scymnodon plunketi*, *Dalatias licha* (deep-sea species) and *Squalus acanthias* (shallow-water species) were caught by trawling during the Hooki survey along the Chatham rise, New Zealand, in January 2018 aboard the *R.V. Tangaroa* (42.5907°S, 176.3978°E). Sharks were directly dissected onboard, and tissues were frozen at -80°C (Fig. 33E).

Stereo video analyses

For video analyses, deployments of stereo-BRUVS were carried out in New Zealand between 2009 and 2012 during summer months at daylight (Zintzen et al., 2012 and 2017). Videos were taken at seven different locations and seven different depths (50-100-300-500-700-900-1200 meter's depth). Only videos collected from 300 to 1200 meter's depth were considered in this work. After shark identification, the stereo videos were analyzed using Matlab to measure the speed and the size of deep-sea sharks. Analyzing steps started with the calibration of cameras using a cube in swimming pools. The cubes spots from the right and the left images were associated



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2016	A											C
2017											C	
2018	E										B	

Fig. 33 Map and schedule of sampling fieldwork. (A) Espegrend marine field station, Norway (60.2606 5.162056°E) aboard the *R.V Aurelia*; (B) EVHOE campaign, IFREMER, France (48.575909°N, -6.9781°E) aboard the *R.V Thalassa*; (C) Okinawa Churaumi Aquarium Research Center, Japan (26.32468°N, 127.412°E) aboard the *R.V Kaykomaru*; (D) L'île de la Réunion, GPS localization unknown; (E) Hooki survey, NIWA, Zealand (42.5907°S, 176.3978°E) aboard the *R. V Tangaroa*.

to 3D coordinates allowing to extract the internal parameters of the cameras. Then, we could measure several points on sharks to reconstruct them and their displacements in three dimensions and therefore take all measurement we needed (Fig. 34A) (for more details, see Paper I – p 81).

Muscle analyses

For muscles analyses, sharks were directly cut in slices after fishing. Each section corresponds to five percent of their fork length (FL) starting at 25%. We measured the red and white muscle surface areas on the anterior and posterior sides of each section using ImageJ software. Both side areas were averaged and multiplied by the thickness of sections to measure the volume. With this volume and theoretical density of red and white muscle, it is possible to measure the mass of each fiber and therefore determine the proportion of each one according to the total body mass (Fig. 34B) (for more details, see Paper II – p 121) The longitudinal red muscle distribution was also determined by transforming the area (cm²) measured at 50% of the FL as a reference value of 1. Other positions along the body are thus calculated accordingly to this reference point (Fig. 34B) (for more details, see Paper III – p 141).

Muscle enzyme assays

Red and white muscles used for enzyme assays were stored at -80°C. Frozen muscle samples were weighed (+/- 0,25g) and homogenized on ice. The homogenates were centrifuged at 5000 g for 5 minutes at 4°C and the supernatant was used for the enzyme assay. The three enzymes analyzed (LDH, CS, MDH) were based on the measurement of kinetic reaction with a spectrophotometer during five minutes. The spectrophotometer records the absorbance of some molecules

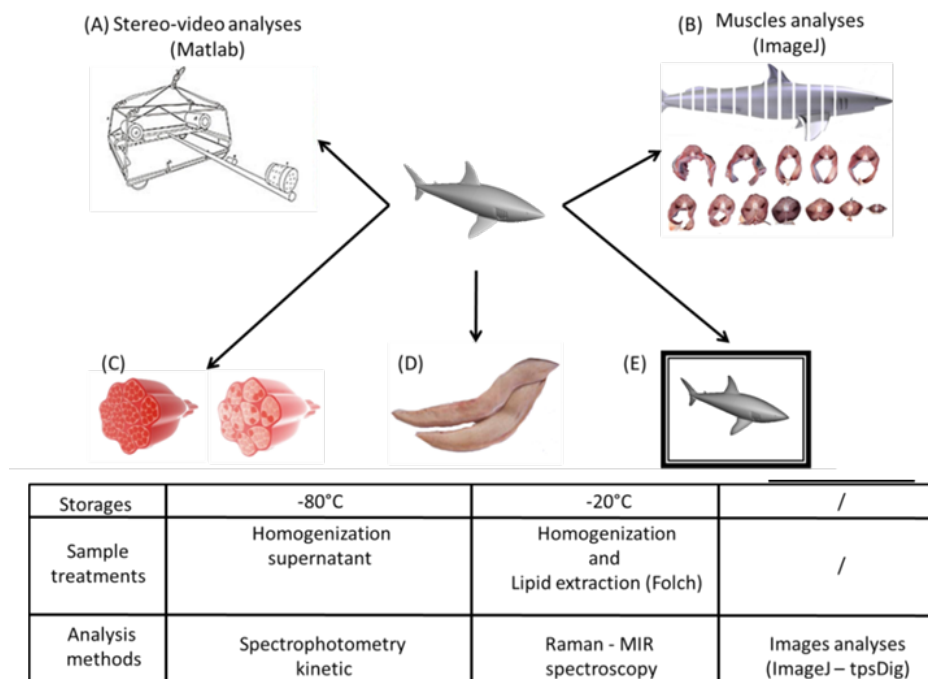


Fig. 34 All manipulation summary. (A) Stereo video analyses based on stereo BRUVs for swimming speed measurement; (B) Red and white muscle analyses using imageJ software to measure de muscle proportion and the red muscle distribution; (C) Muscle enzyme assays using spectrophotometry kinetic to estimate the aerobic and anaerobic shark metabolism; (D) Buoyancy analyses using RT-Ramman and MIR spectroscopy to evaluate the lift provided by the liver; (E) Morphological analyses using ImageJ and tpsDig software evaluating possible morphological adaptations.

which are specific co-factor of each enzyme reaction: NADH for the LDH and MDH (absorbance at 340nm) and DTNB for CS (absorbance at 412nm). The measurements of enzymes activities were done at 10°C to compare results with the literature (Fig. 34C) (for more details, see Paper II– p 121).

Liver buoyancy analyses

Liver of each shark were stored at -20°C onboard and directly stored at -80°C on land for long term reservation before assays. They were homogenized and Folch method has allowed the lipid extraction. Lipids were characterized using FT-Ramman and MIR spectroscopy. This method is based on the identification using specific absorbance of molecular links. When the structure of molecules is known, it is possible to identify and quantify those ones. The HSI, the oil content and the lipid composition of the liver have allowed to calculate the Liver hydrostatic force (LHF) which is a proxy of the lift provided by the liver (Fig. 34D) (for more details, see Paper III – p 141).

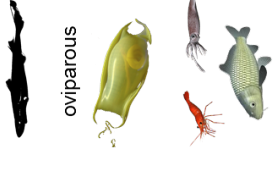
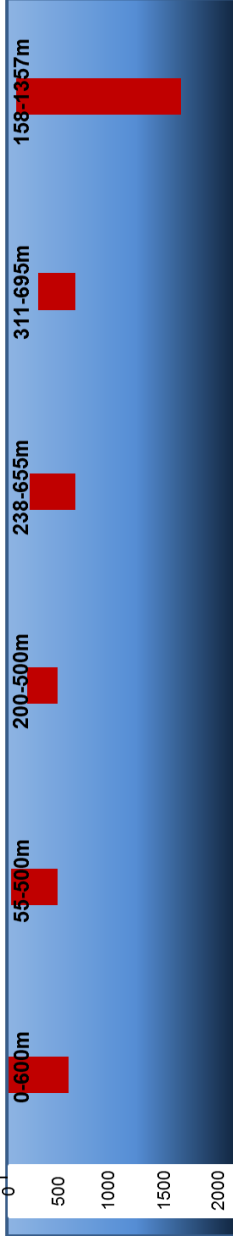
Morphological feature analyses

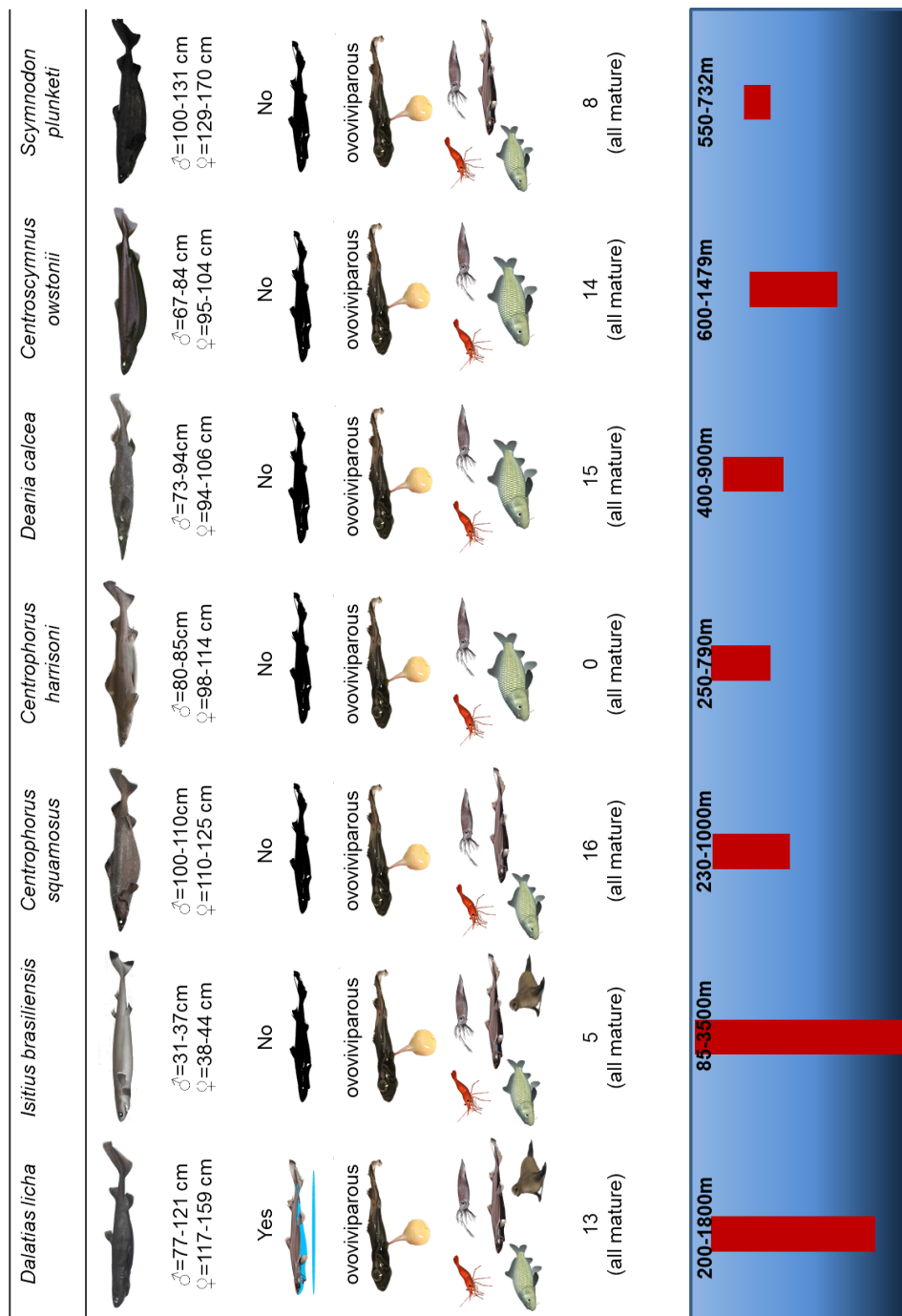
Pictures of fished sharks were directly taken on board dorsally, ventrally and laterally with a reference scale. Classical morphological features were measured using ImageJ software. Additionally morphological feature of the caudal fin and dorsal fin spines were analyzed as geomorphological unit using tpsGig and Rstudio software (Fig. 34E) (for more details, see Paper IV – p 177).

Studied species

The general data of eleven deep-sea and two shallow-water sharks studied in this work are described in the Table 1.

Table 1 deep-sea and shallow-water studied sharks, ecological

<u>Name</u>	<i>Squalus acanthias</i>	<i>Galeus melastomus</i>	<i>Etmopterus spinax</i>	<i>Etmopterus molleri</i>	<i>Etmopterus granulosus</i>	<i>Etmopterus lucifer</i>																																			
<u>Size (mature stage)</u>	 ♂=52-100cm ♀=66-120 cm	 ♂=34-42cm ♀=39-45 cm	 ♂=24-34cm ♀=30-41 cm	 ♂ & ♀ = 33-45cm	 ♂=55-60 cm ♀=75-85 cm	 ♂=29-42 cm ♀=34-47 cm																																			
<u>Luminescence</u>	No	No	Yes	Yes	Yes	Yes																																			
<u>Reproduction</u>	ovoviviparous	oviparous	ovoviviparous	ovoviviparous	ovoviviparous	ovoviviparous																																			
<u>Diet</u>																																									
<u>Number of specimen fished</u>	14 (all mature)	7 (all mature)	56 (all mature)	47 (all mature)	15 (all mature)	17 (all mature)																																			
<u>Depth</u>	 <table border="1"> <caption>Depth Distribution Data</caption> <thead> <tr> <th>Depth Range (m)</th> <th><i>S. acanthias</i></th> <th><i>G. melastomus</i></th> <th><i>E. spinax</i></th> <th><i>E. molleri</i></th> <th><i>E. granulosus</i></th> <th><i>E. lucifer</i></th> </tr> </thead> <tbody> <tr> <td>0-600</td> <td>14</td> <td>7</td> <td>56</td> <td>47</td> <td>15</td> <td>17</td> </tr> <tr> <td>600-1000</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>1000-1500</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>1500-2000</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> </tr> </tbody> </table>						Depth Range (m)	<i>S. acanthias</i>	<i>G. melastomus</i>	<i>E. spinax</i>	<i>E. molleri</i>	<i>E. granulosus</i>	<i>E. lucifer</i>	0-600	14	7	56	47	15	17	600-1000	0	0	0	0	0	0	1000-1500	0	0	0	0	0	0	1500-2000	0	0	0	0	0	0
Depth Range (m)	<i>S. acanthias</i>	<i>G. melastomus</i>	<i>E. spinax</i>	<i>E. molleri</i>	<i>E. granulosus</i>	<i>E. lucifer</i>																																			
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1000-1500	0	0	0	0	0	0																																			
1500-2000	0	0	0	0	0	0																																			



Results

Chapter I:
Study of deep-sea shark swimming
capabilities through stereo-video
analyses

Due to their elusiveness, direct estimates of deep-sea sharks swimming speed are lacking. Taking advantage of the availability of video footage done by Dr V. Zintzen (Te papa , New Zealand) we intend to measure, for the first time, the cruise swimming and burst speed of eight deep-sea shark species from Squaliformes family (*D. licha*, *C. owstonii*, *P. plukenti*, *C. squamosus*, *D. calcea*, *C. harrisoni*, *E. granulosus*, and *E. molleri*) using stereo BRUVs footages analyses with Matlab to reconstruct sharks and their displacements in three dimensions. Also, the measurement of tailbeat will be done in order to put it in relation with the speeds measured.

These data will allow revealing if their velocity is lower than their shallow-water counterparts. Also, it will show if all deep-sea sharks swim slowly or if some display higher speed values according to their life-styles.

Paper 1:

Ecological features and swimming
capabilities of deep-sea sharks from
New Zealand.

Nicolas Pinte, Pascaline Parisot, Ulrich Martin, Vincent Zintzen,
Christophe De Vleeschouwer, Clive D. Roberts & Jérôme Mallefet

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Ecological features and swimming capabilities of deep-sea sharks from New Zealand.

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ABSTRACT

Currently the ecology of deep-water sharks is poorly documented, especially in situ information for these elusive species are lacking. In this study, stereo-Baited Remote Underwater Videos (stereo-BRUVs) were deployed to collect ecological data from New Zealand deep-sea sharks. The results showed differences in abundance between species, with *Etmopterus granulosus* (Etmopteridae) found in greatest numbers. Moreover, the known depth range increased for *Scymnodon macracanthus* (Centrophiridae). Deep-sea shark species were generally found to swim at slower cruise speeds (0.36 ± 0.04 m.s⁻¹) than their shallow-water counterparts (0.63 ± 0.05 m.s⁻¹). However, the swimming speed of deep-sea sharks was clearly not uniform, with some species displaying higher cruise swimming speeds than others. The fastest sharks (*Centrophorus harrissoni*, *Etmopterus*

granulosus and *Etmopterus molleri*) had swimming abilities comparable to benthic shallow water sharks (0.48 ± 0.02 m.s⁻¹). The higher cruise swimming speed in the family Etmopteridae could be an advantage for these luminous sharks if they follow isolumines to match their ventral light intensity with the down-welling light of their environment. This study revealed that alternative non-destructive methods can be effective for ecological studies of deep-sea marine fauna

KEYWORDS: Deep-sea sharks, swimming speed, stereo-BRUVs, Squaliformes, bioluminescent, isolume-followers

INTRODUCTION

We still know very little about the ecology of deep-sea sharks. They live at depths characterized by high pressure, darkness, low temperature and low oxygen concentration, which are factors making life observations of specimens difficult (Chiswell et al. 2015; Gallo et al. 2014). Few studies identified some key elements of the basic ecology for some of these species (Finucci et al. 2018; Coelho and Erzini 2010; Andrews et al. 2009; Clarke et al. 2002; Girard and Du Buit 1999; Nelson et al. 1997).

One important aspect that has not yet been very well studied is the swimming capabilities of deep-sea sharks. The swimming capability of an organism in the ocean has essential implications in survival behaviors such as hunting, escaping, migrating, and mating (Watanabe et al. 2012; Wilson et al. 2005). Swimming capability will also largely dictate the home range of a species (Sainte-Marie and Hargrave 1987). Schadwick et al. (2016) split the swimming speeds of fishes into two main types: The first is linked to burst activities – when fishes reach their maximum velocity quickly during hunting or escaping behaviors. The second is linked to cruise activities, and is used by fishes to travel long

distances during the most common locomotion activities such as foraging, dispersal, locating a mate or circadian migration (Ryan et al. 2015; Watanabe et al. 2012).

Swimming capabilities studies of shallow-waters species showed that elasmobranchs possess a large diversity of morphological, anatomical and physiological adaptations linked to their locomotion system (Lauder and Santo 2015; Shadwick and Goldbogen 2012; Gembala et al. 2006; Bernal et al. 2003; Thomson and Simanek 1977). In contrast, few studies have looked at swimming behaviors of deep-water sharks. These sharks are often described as slow and listless swimmers (Condon et al. 2012; Treberg et al. 2003; Bagley et al. 1994), as for the Greenland shark recorded as the slowest (Watanabe et al. 2012) but some of them still perform long vertical migration such as *Centrophorus squamosus* (Rodriguez-Cabello et al. 2016; Rodriguez and Sanchez 2014). Two main hypotheses have been put forward to explain this. The first is that cold water directly affects the muscular contraction metabolism of these ectothermic fishes (Treberg et al. 2003). The second, called the visual interaction hypothesis (VIH), suggests that declining light levels with depth decreases the selective pressure for efficient locomotor capacity by species with visual capabilities, because the reactive distances between predator and prey are reduced (Condon et al. 2012). The majority of data about the swimming performance of deep-sea sharks have so far been inferred indirectly from measurements of their metabolisms (Condon et al. 2012; Treberg et al. 2003). These two studies have shown that anaerobic metabolism is lower for deep-sea species than their shallow-water counterparts while no difference is observed for the aerobic metabolism

In our study, we evaluated the occurrence and relative abundance of sharks on the slopes off New Zealand at depths from 300 to 1200m at latitudes of 29.15 to 50.91°S using stereo-BRUVs. These deployments enabled to measure ecological features such as the size, the swimming speed and the tail-beat frequency of eight deep-sea shark species. The results obtained allow us not only to compare absolute values of cruise swimming speeds of deep-sea sharks with cruise swimming speed values of shallow-water species found in the literature; but also to study the difference in cruise swimming speeds and tail beat frequency between the deep-sea species.

MATERIALS AND METHODS

Video recording

Video deployments were carried out in New Zealand waters between 2009 and 2012 during daylight of the austral summer months (see Zintzen et al. 2012, 2017 for details on video deployments). The videos were recorded at seven different locations spanning a latitudinal range between 29.15°S and 50.91°S, at five different depths (300, 500, and 700, 900 and 1200 m) at each of these locations. A minimum of six video replicates were achieved at each depth by location cell, yielding a total of 247 deployments. The camera systems were stereo-baited remote underwater video systems (stereo-BRUVs) that included two Sony

Handycam HD cameras of the models HDR-CX7 or HDR-CX500. They were placed 70 cm apart on the base bar with an 8° rotation toward the central bait. Each system was illuminated using Royal Blue Cree XLamps XP-E LEDs. Parameters were selected to provide sufficient vision range (clearly observed until five meters from cameras) and minimizing fish repulsion, backscatter, and light reflection from silvery fishes (Zintzen et al. 2012, 2017). Ten additional red LED lamps, to enable the synchronization of the right and left cameras, were placed in the vision field of the two cameras and turned on one after one regularly during the whole recording period.

Shark identification and General ecological features

Sharks were identified in the high definition digital images coupled with large fish traps deployed simultaneously to the video systems, to capture voucher specimens. Species level identifications were based on Roberts et al. (2015) and Ebert et al. (2013).

Following shark identification, we measured species richness according to the depth, which is defined as the number of different species of sharks for a particular depth stratum (300, 500, 700, 900, and 1200 m) (Zintzen et al. 2012). We also evaluated the relative abundance of each squaliform family. For that purpose, we distinguished and counted individuals for each family over the course of the deployment. Then, we reported this number according to the total number of sharks observed. Length (total,

fork, and precaudal length) was also measured and aggregation was assessed to determine if some species occurred in large numbers, or were singletons. For this purpose, all individuals of each species were counted over the course of each deployment.

Swimming speed and tail beat frequency

The swimming speed and length values were measured using MatLab software (MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States). A short summary of the method is given below.

Pairs of video cameras were calibrated using a calibration cuboid (1000x1000x500mm with high contrast white spots) in a swimming pool. The first step was the modeling of the cuboid and its spots in MatLab. For that, each spot was converted as a number and related with 3D coordinates. Then, a MatLab code was developed to calibrate cameras by associating each spot from the right and the left images with their matching spots using the pinhole camera model. This method provided parameters of each of them for next measurements. (Hartley and Zisserman 2004; Heikkilä 2000; Zhang 1999; Heikkilä and Silken 1997) (For more details see supplementary data 1 and 2).

For size and speed measurements, we needed to measure the “x, y” shark coordinates in pixels for the left and right images. For the size, four specific points representing total, fork and precaudal

lengths were selected (rostrum, precaudal pit, posterior notch and terminal margin). For velocity, three points visible on the videos from the left and the right cameras were selected along the body. The use of three different points allows accounting for speed differences in function of the selected point. For example, if one point is the rostrum, a left or right movement of the head will change the distance travelled every few seconds. One image per second was extracted to measure the swimming speed of a shark during its displacement. The points selected on the body of the shark remained identical through the entire sequence. The 2D coordinates of the body of the shark obtained with MatLab were inserted into another MatLab code. This new code converted the 2D coordinates of each point into 3D coordinates, thanks to the calibration matrix of the stereo-camera systems. With the coordinates in three-dimensions, it was possible to measure the size and speed of a shark by taking the vector length between the points. Measurement repeatability was estimated by measuring several times the LED synchronization lamp and the error of measurement was calculated to 0.91cm (see supplementary data 2).

The speed recorded was classified in two categories: (i) burst swimming speed when acceleration of shark was identified due to predation or predation avoidance behaviors; when such behaviors are not observed we considered burst speed to be when speed

excess 6 to 18 body length s⁻¹ (Ryan et al. 2015). And (ii) the cruise swimming speed when the shark swam without any interaction with other organisms or the BRUVs system. Therefore, sharks swimming with a direct bait interest (touching, smelling, and biting) were not taken into account. However, it should be noted that bait is the reason the shark is here. If we do not perceive a burst and consider this as a cruising speed, it must also be considered as an investigation speed.

To determine whether swimming species of deep-sea sharks differ from their shallow-water counterparts, the swimming speed values obtained in our study were compared with similar results from compiled literature (Ryan et al. 2015; Watanabe et al. 2012) (Supplementary data 3). We used these velocity values for comparison because they were collected using the same criteria as ours: (i) measurements were obtained during day time, and (ii) only on mature individuals. A total of 20 shallow-water sharks and a deep-water one, grouped in six different orders were compiled. Sharks were classified in general groups: surface (i.e., living most of their time above 200m depth) and deep (i.e., living most of their time deeper than 200m). A second deeper classification was defined according to the habitat of surface sharks (pelagic or neritic) and the velocity groups obtained by the first test for deep-sea sharks.

Finally, for each velocity value obtained, we measured the corresponding tail beat frequency. Tail beat frequency is defined as the time required for the tail to move from one extreme lateral position back to the same position and it is expressed in Hertz (Hz). In this study, the tail beat frequency was calculated from the time difference between the two images corresponding to this complete motion (Watanabe et al. 2012). Thanks to the speed and tail beat frequency, it is also possible to measure the stride length (corresponding to the ratio of the speed to the tail beat frequency, expressed as a fraction of body length) for each species at a given speed (0.3 m s^{-1}). This speed was chosen because it is the speed common to almost all species except *Dalatias licha*, which never reached this speed.

Statistical analyses

All statistical analyses (univariate parametric and non-parametric tests) were performed with JMP pro 14 (JMP®, Version <14>. SAS Institute Inc., Cary, NC, 1989-2007). The normality and the homoscedasticity were confirmed for each parametric test. When the Gaussian distribution was not proved, non-parametric equivalent tests were used.

RESULTS

Video analyses

The 267 hours of video recording allowed more than 1400 shark observations (number of shark individuals observed) to be made. Twenty-eight species of sharks from three orders (Carcharhiniformes, Hexanchiformes, and Squaliformes) were identified (Table 1). Eight deep-sea shark species (*Dalatias licha*, *Centroscymnus owstonii*, *Proscymnodon plukenti*, *Centrophorus squamosus*, *Centrophorus harrissoni*, *Deania calcea*, *Etmopterus granulosus*, and *Etmopterus molleri*) were common enough in video recordings to enable accurate swimming speed measurements. Of these, a total of 253 speed measurements and 135 size measurements were obtained, with a minimum of seven measurements of swimming speed for each species. The lower number of size measurements is explained by frames where individuals were not entirely visible as for some bigger sharks, such as the bluntnose sixgill shark (*Hexanchus griseus*), which were common enough, but too large for the camera field of view.

Table 2 Summary of data collected on the 22 species of sharks observed from stereo baited underwater video surveys in New Zealand EEZ; total number of observations (# obs), number of replicate for size measurement (n1), mean total length $\pm$ SE (TL cm), number of replicate for speed measurement (n2), mean time in motion during a measurement (MTM s), cruise swimming speed (CS m.s⁻¹) and burst swimming speed (BS m⁻¹). * Luminous shark based on our observations and literature. Taxa classification is based on Roberts et al. (2015) and Ebert et al. (2013).

Order	Family	Species	# obs	Depth range (m)	n1	TL (cm)	n2	MTM (s)	CS (m s ⁻¹)	Burst (m s ⁻¹)
<i>Squaliformes</i>										
<i>Dalatidae</i>										
		<i>Dalatias licha</i> *	27	300-900	10	110.82 $\pm$ 22.59	22	6.81 $\pm$ 4.57	0.13 $\pm$ 0.04	
<i>Somniosidae</i>										
		<i>Centroscyllium owstonii</i>	84	700-1200	6	97.96 $\pm$ 19.22	19	2.55 $\pm$ 1.17	0.29 $\pm$ 0.10	1.03
		<i>Centroscyllium coelelepis</i>	2	900	-	-	-	-	-	-
		<i>Centroselachus crepidater</i>	2	700-900	-	-	-	-	-	-
		<i>Prosomnodon plukenti</i>	264	300-1200	26	121.76 $\pm$ 29.64	38	3.74 $\pm$ 1.61	0.32 $\pm$ 0.11	1.32
		<i>Scymnodon macracanthus</i>	13	300-900	-	-	-	-	-	-
<i>Centrophoridae</i>										
		<i>Centrophorus squamosus</i>	55	500-900	24	134.80 $\pm$ 13.49	37	3.29 $\pm$ 1.49	0.32 $\pm$ 0.07	0.76
		<i>Centrophorus harrissoni</i>	115	500-900	14	78.95 $\pm$ 7.75	35	2.02 $\pm$ 0.78	0.53 $\pm$ 0.17	
		<i>Deania calcea</i>	69	500-900	8	79.69 $\pm$ 13.86	20	3.19 $\pm$ 1.14	0.33 $\pm$ 0.12	
<i>Etmopteridae</i>										
		<i>Etmopterus granulosus</i> *	759	500-1500	45	52.60 $\pm$ 13.45	61	2.69 $\pm$ 1.13	0.45 $\pm$ 0.14	1.59
		<i>Etmopterus molleq</i> *	21	300-700	2	38.15 $\pm$ 1.87	7	1.13 $\pm$ 0.57	0.51 $\pm$ 0.14	
		<i>Etmopterus lucifer</i> *	2	1200	-	-	-	-	-	-

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Biodiversity analyses

Species richness of sharks changed with depth (Fig. 1A). The number of species from all locations was ten at 300m and increased to reach a maximum of twelve species at 700m. It then decreased to ten species at 900m, and six species at 1200m. At the two shallower depths (300-500m), three orders

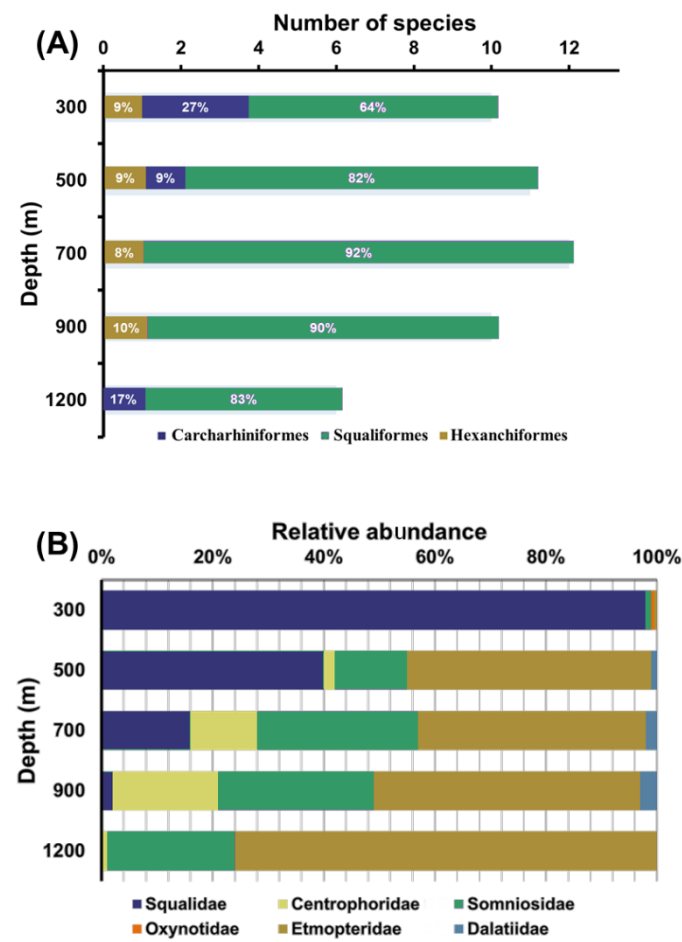


Fig. 1 (A) Shark species richness according to depth, observed from video deployments. Percentage indicates the proportion of the total number of shark observations within the four orders of sharks identified. (B) Relative abundance of families of Squaliformes according to depth. The percentage represents the proportion of all the specimen observations belonging to one of six families.

were represented. Deeper (700-900m) Carcharhiniformes disappear, but at the greatest depth studied (1200m) one Carcharhiniformes species (*Apristurus* sp.) was found. Squaliformes was the most represented order in the deep water (i.e. under 200 meters depth), and accounted for 91% of all shark observations. Family analysis of Squaliformes showed that at 300m, 98% of sharks were Squalidae, with the rest belonging to Somniosidae, Etmopteridae and Oxynotidae (Fig. 1B). Between 500 and 900m, Etmopteridae individuals were the most represented group, with a stable relative abundance across the range of 44%. The proportion of Squalidae individuals decreased with depth (40% at 500m, 16% at 700m, 2% at 900m), while the proportion of Centrophoridae and Somniosidae individuals increased (2% and 13% at 500m, 12% and 29% at 700m, 19% and 28% at 900m, respectively). In the deepest layer (1200m), Etmopteridae represented 76% of all sharks observed, while the percentage of Centrophoridae and Somniosidae decreased to 1% and 23%, respectively.

Length analyses

The relationship between total length of each shark species and depth revealed no significant difference (ANOVA, $P > 0.05$); except for *C. harrissoni* (see Supplementary Data 4). However, three different distribution patterns were observed: (i) sizes greater at shallower and deeper depths, with intermediate values in mid depth range, found for *C. owstonii* and *P. plunketi*; (ii) longer specimens measured in deep waters, found for *C. harrissoni* and *D. calcea* and (iii) sizes decreasing progressively with depth for *E. granulosus* and *C. squamosus*. Regarding *Dalatias licha* and *Etmopterus molleri* no relation could be done due to the lack of occurrence at different deep layers.

Aggregation pattern

Results showed that *E. granulosus* occurred in much larger numbers (12.35 ± 1.04 individuals) than other species (average for all other species: 3 ± 0.69 individuals) (Kruskal-Wallis test, $P < 0.0001$) (see bar chart representing these values in Supplementary Data 4).

Swimming speed

There were statistically significant differences between cruise swimming speeds of the eight deep-sea shark species (Welch test, $P < 0.0001$) (Fig. 2). Paired comparison isolated three significant groups (Tukey test, $P < 0.01$). The first group comprised just *D. licha*, displaying the slowest cruise swimming speed (group deep A: 0.13 ± 0.01 m.s⁻¹). The second group was represented by *C. owstonii*, *P. plunketi*, *C. squamosus* and *D. calcea* having intermediate cruise swimming speeds (group deep B: 0.31 ± 0.01 m.s⁻¹). The last group, with *E. granulosus*, *E. molleri* and *C. harrissoni*, showed the highest cruise swimming speeds (group deep C: 0.48 ± 0.02 m s⁻¹) (Fig. 2).

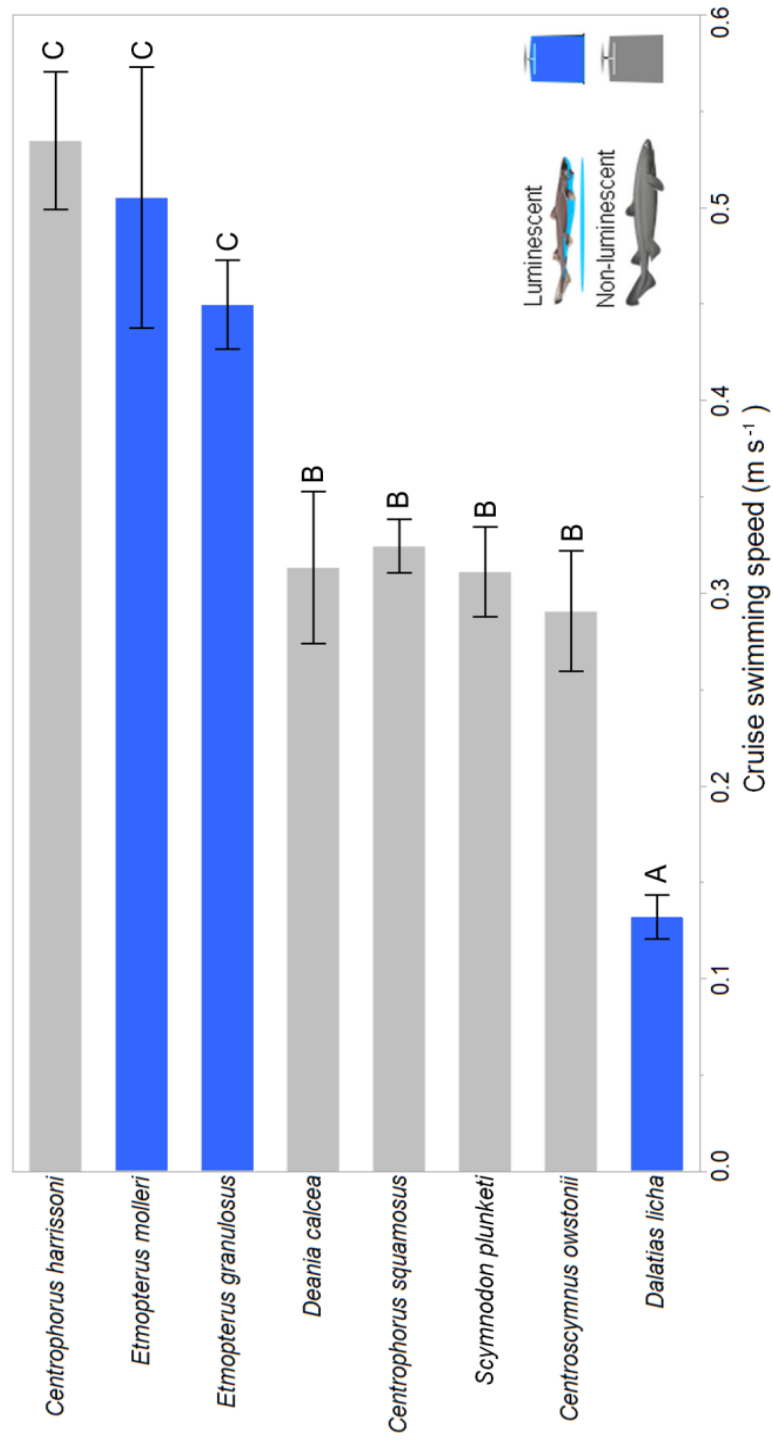


Fig. 2 Mean cruise swimming speed (m.s⁻¹ ± SE) in deep-sea shark species measured by stereo-BRUVs; different capital letters indicate significant differences of cruise swimming speeds (Tukey test <0.05). Blue bars correspond to luminous deep-sea sharks, while grey bars correspond to non-luminous ones.

Comparison between deep-sea shark species and shallow-water ones from the literature showed a significant difference in cruise swimming speed (Student t test, $P < 0.0012$), with shallow-water sharks displaying higher cruise swimming speeds ($n=20$, 0.63 ± 0.05) than deep-sea sharks ($n=9$, 0.36 ± 0.04) (Fig. 3).

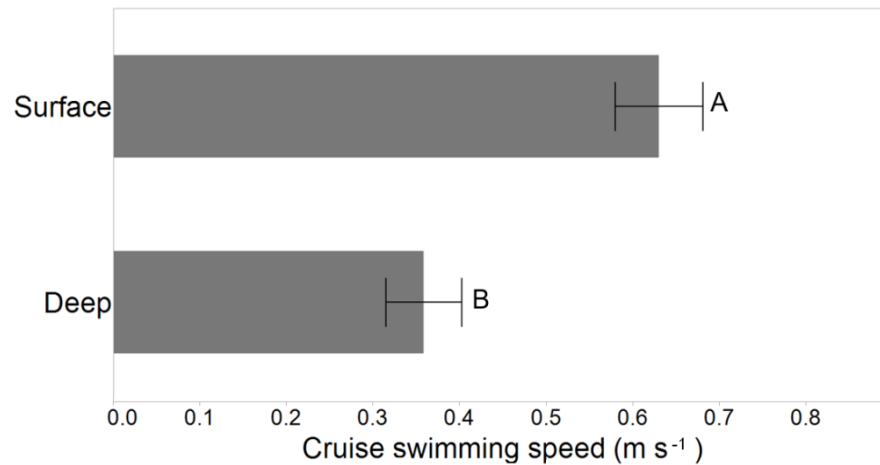


Fig. 3 Mean cruise swimming speed ($\text{m.s}^{-1} \pm \text{SE}$) difference between deep-sea sharks (Deep) and shallow-sea sharks (Surface), Deep: see table 1 and 2, Surface: see table 2; A, B close to the bar correspond to significant difference while same letter are not shared (Student t test > 0.05). $N = 20$ number of surface species and $N = 9$ number of deep sea sharks species.

Looking into the details of this difference between shallow and deep-water species revealed that not all deep-water sharks have slower cruise swimming speeds than their shallow counterparts (Fig. 4). There were significant differences between cruise swimming speeds of pelagic shallow-water sharks, neritic shallow-water sharks, and the three groups of deep-sea sharks identified on Fig. 2 (ANOVA, $P < 0.0024$). However, post-hoc Tukey test separated sharks cruising swimming speeds into three groups: the first, with the highest value ($n=7$, 0.75 ± 0.08), were the pelagic sharks; the second, benthic shallow water sharks and deep-sea sharks from velocity

group Deep C ($n=13$, 3; 0.56 ± 0.06 ; 0.51 ± 0.03 , respectively); and the third group, with the lowest cruise swimming speed value ($n= 5$, 1; 0.31 ± 0.008 ; 0.13, respectively), deep-sea sharks from velocity groups Deep A and Deep B (Fig. 4).

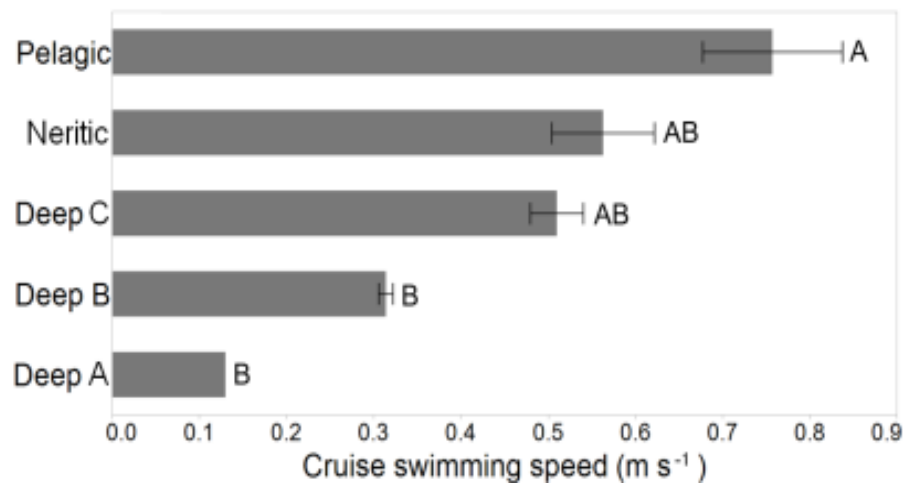


Fig. 4 Mean cruise swimming speeds ($m.s^{-1} \pm SE$) of pelagic shallow-water sharks, benthic shallow-water sharks and deep-sea sharks. Deep-sea sharks are classified into three groups ($C > B > A$) based on their average swimming speed (Fig. 2). Different capital letters indicate significant differences (Tukey test < 0.05). $N = 13$ for Benthic species, $N = 7$ for Pelagic species, $N = 3$ for Deep C, $N = 5$ for Deep B and $N = 1$ for Deep A.

A linear model described the relationship between the cruise swimming speed of sharks as a function of tail beat frequency, species and tail beat frequency/species interaction. There was a significant effect of each factor (tail beat frequency, species and tail beat frequency/species on the velocity) (LM, $r^2 = 0.71$, $P < 0.0001$) (Fig. 5). This model showed that, for a given speed (0.3 and 0.6 $m.s^{-1}$ chosen because it fits with the minimum and the maximum velocity of the majority of deep-sea sharks, dotted lines on Fig. 5), *E. granulosus* and *E. molleri* had a higher

tailbeat frequency than other deep-sea species; the mean frequency at 0.3 m s⁻¹ is 0.5 Hz for most deep sea species while it reached about 1 Hz for Etmopteridae. Again at 0.6 m s⁻¹ the mean was close to 0.65 Hz for most of the deep sea species while it was 1.45 Hz for Etmopteridae. Comparison of regression line slopes revealed that, in comparison to other deep-sea sharks, Etmopteridae species needed a larger increase of their tailbeat (shown by a slope <0.5) to increase their speeds. For a given speed of 0.3 m s⁻¹, the stride length was lower for the two Etmopteridae species (0.29 and 0.35 body length), higher for *Scymnodon plunketi* and *Centrophorus squamosus* (0.72 and 0.63 body length respectively) and other species displayed intermediate values (Table 2).

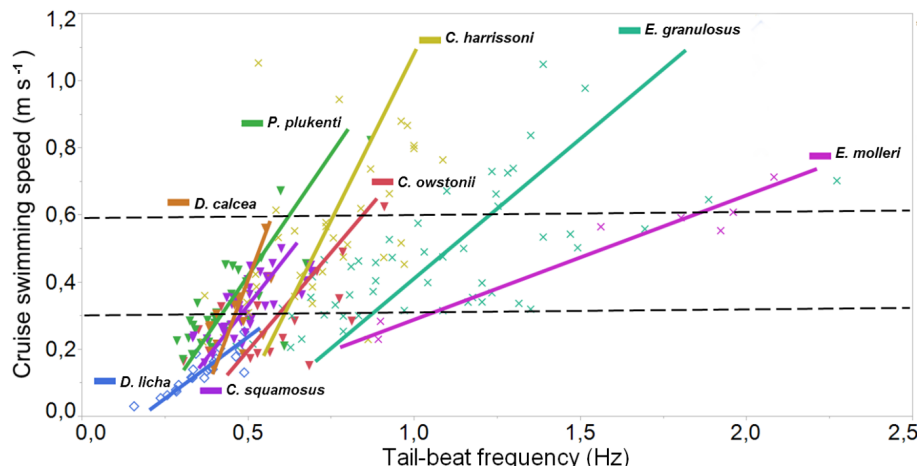


Fig. 5 Relationship between tail beat frequency (Hz) and cruise swimming speed (m s⁻¹) for eight Squaliformes deep-sea shark species. The line represents the linear regression; pink: *E. molleri*, turquoise: *E. granulosus*, red: *C. owstonii*, orange: *D. calcea*, yellow: *C. harrissoni*, purple: *C. squamosus*, green: *P. plukenti*, blue: *D. licha*; the $\diamond$, $\blacktriangledown$ and $\times$ correspond respectively to sharks from the group Deep A, Deep B and Deep C; the two dotted lines correspond from to 0.3, 0.6 m.s⁻¹ respectively.

Linear regression on each species does not show any evidence of a relationship between cruise swimming speed and the total length of the shark.

Table 2 Tail beat frequencies (Hz) and stride lengths (body length) for a given cruise swimming speed (0.3 m s⁻¹) for eight deep-sea shark species from Squaliformes order.

Species	Cruise swimming speed (m s ⁻¹)	Tail beat frequency (Hz)	Stride length (body length)
<i>Scymnodon plunketi</i>	0.3	0.42	0.72
<i>Deania calcea</i>	0.3	0.56	0.53
<i>Centrophorus squamosus</i>	0.3	0.48	0.63
<i>Centroscymnus owstonii</i>	0.3	0.62	0.48
<i>Dalatias licha</i>	0.3	0.59	0.51
<i>Centrophorus harrissoni</i>	0.3	0.61	0.49
<i>Etmopterus granulosus</i>	0.3	0.87	0.35
<i>Etmopterus molleri</i>	0.3	1.04	0.29

Finally, regarding burst activities, only four species showed behavior in agreement with the burst definition (Table 1). Behaviors producing burst activities were often hunting activities for *C. owstonii*, *P. plunkenti* and *D. calcea*, while the small lanternshark, *E. granulosus*, had more burst activities triggered by escaping behaviors.

DISCUSSION

Biodiversity, length, and aggregation

Our results showed a clear prevalence of species of Squaliformes in New Zealand deep-sea waters at 300–1200m, where they represented more than 91% of all shark occurrences observed. It is in this deep layer that the shark species richness increased to reach its maximum at 700m. Thanks to video analyses, the vertical range of one species is

increased by the present study: *Scymnodon macracanthus* was observed at 300m, while its minimum depth was previously reported as 545m (Stewart & Last 2015). Based on the most recent classification of Squaliformes in New Zealand (Roberts et al. 2015), our data reveal a shift of families starting at 300m depth, where the number of sharks from Squalidae decreased, while sharks from Somniosidae and Centrophoridae increased. In contrast, during this shift, the proportion of sharks from Etmopteridae did not change. Squalidae, Centrophoridae and Somniosidae sharks are relatively similar in terms of size, morphology, diet, and trophic level; while Etmopteridae sharks are smaller and are sometimes hunted by these first three families (Ebert et al. 2013; Pethybridge et al. 2012). Therefore, the shift observed could avoid competition between close counterparts sharing potentially the same trophic role. It is important to mention that the number of all counted Centrophoridae and Somniosidae were lower than the number of Squalidae, which is logical because food availability decreases with depth.

Regarding size measurements collected, except for *C. harrissoni*, no significant effect of depth and velocity could be found for species taken individually. This observed lack of relationship could be explained theoretically by experimenter selection, the video quality, and also light intensity. Indeed, to collect data, the chosen points of reference for length measurement should be easy to localize for all individuals. However, it was easier to label these points of reference on big sharks, rather than small ones. Therefore, there is a shift to bigger sharks observed in our study. Nevertheless, we observed three different size distribution modes, depending on the species. Moreover, the significant relationship between the three different morphometric lengths shows that, in big specimens, the measurements taken are very accurate.

Finally, *E. granulosus* occurrence revealed that this species is always present in higher number of individuals, around twelve, than other species where only two - three are observed. . Nevertheless, it was already described from fishing surveys that this species as an aggregative behavior (Finucci et al. 2018). Therefore, our results are consistent with previous findings in literature but with non-destructive methodology

Swimming speed

In our study, the swimming speeds of several deep-sea sharks were measured for the first time in situ, bringing some new insights about their ecology. In situ measurements remain the best way to characterize organisms in their environment but bias might be possible due to environment fluctuations. Indeed, here the velocity of sharks were measured in deep-sea water where the current was not taken into account and our results should be consider this way with all environmental factors influencing sharks. However, bias should be considered minimal in our conditions since: (i) presence of weaker currents at greater depth are well known (Heath 1972); (ii) video footages showed that every time the sediment was disturbed (i.e. when the set-up landed on the bottom or when numerous hagfishes attacked the bait or even during strong interactions between larger organisms), it took more than ten minutes for the silt and sand to sediment; what suggest low or no current. Previously, deep-sea sharks were considered as slow and lazy swimmers compared with their shallow-water counterparts. These conclusions come from indirect measurement of their metabolism. Authors argue this conclusion mainly by the cold water effect of the environment, but also because they do not need a high velocity for prey capture in dark environments (visual interaction hypothesis) (Condon et al. 2012; Treberg et al. 2003). Studies on sharks' buoyancy also show that deep-sea shark with liver providing more buoyancy is ideal adaptation for slow swimmer (Pinte et al. 2019; Gleiss et al. 2017). Our results

confirmed the conclusions of previous studies, with deep-sea shark species swimming at slower cruise speeds than their shallow-water counterparts. However, the results of our study allow us to refine a hypothesis on these broad patterns because the swimming speed of deep-sea sharks inferred is clearly not uniform. Some groups of species displayed higher cruise swimming speeds than others, with the high speed group (*C. harrissoni*, *E. granulosus* and *E. molleri*; group Deep C) even showing swimming abilities comparable to benthic shallow-water sharks while pelagic shallow-water sharks remain the fastest species (See data from Ryan et al., 2015 and Watanabee et al., 2012).

These differences in cruise swimming speed between deep-sea species could be related to their living mode. In group Deep C (Fig. 2), two of the three sharks were luminous sharks from family Etmopteridae. Like other lanternsharks, *E. granulosus* and *E. molleri* have thousands of ventral photogenic organs called photophores that produce blue-green light (Claes and Mallefet 2009b). This bioluminescence has been shown to be primarily involved in camouflage through counter-illumination, producing light on the ventral side of the animal at the same intensity, wavelength, and angular distribution as residual down-welling light of its environment. This ventral bioluminescence hides their silhouette from below (Claes and Mallefet 2014; Claes et al. 2010). Luminous organisms must be able to change ventral light intensity quickly to be efficient counter-illuminators (Latz 1995; Case et al. 1977; Young and Roper 1976; Clarke 1963). However, the luminescence of lanternsharks is under hormonal control, which is relatively slow in comparison with nervous control, as seen in many other counter-illuminating organisms (Duchatelet et al. 2019; Claes and Mallefet 2015; Claes et al. 2011). To compensate for this slow adapting control system, it is possible that luminous deep-sea sharks have to move up and down the water column to align their ventral light intensity with their environment; this is

the postulate of the “isolume followers’ hypothesis” (Claes and Mallefet 2014). Being a fast swimmer would then represent a significant adaptive advantage if these sharks perform this behavior. Vertical movement of lanternsharks is also supported by the observation of circadian migration for other species of Etmopteridae (Xavier et al. 2012; Neiva et al. 2006) and stomach content analyses which revealed fishes and squids from shallower layers (Daley et al. 2002). However, this circadian migration and the presence of food from near the surface, is also observed for non-luminous deep-sea sharks, such as *Centrophorus harrissoni* (group Deep C) and *Centrophorus squamosus* (group Deep B), suggesting that other reasons exist for differences in swimming abilities of deep-sea sharks.

In contrast, *Dalatias licha* displayed the slowest cruise swimming speed ever measured for a shark, i.e. lower than the Greenland shark, *Somniosus microcephalus* (Watanabe et al. 2012). *D. licha* is assumed to be luminescent since ventral photophores were described (Straube et al. 2015), supporting the hypothesis that ventral luminescence might be used to light up the ocean floor while searching preys. Therefore, its luminescence would be useful to hunt, rather than for predation avoidance, since it is a relatively large species (attaining 185 cm TL) and unlikely to have many predators. In this context, this species is not assumed to be an “isolume-follower” as it displays a benthic living mode while Etmopteridae are benthopelagic species. Moreover, *D. licha* is not known to perform circadian cycle since fishing reports and stomach contents revealed that this shark remains at the same depth, feeding mainly on benthic organisms (Jones Emma, personal comm.; Dailey et al. 2002). Surprisingly, fast lanternsharks are often found in *D. licha* stomach contents (Navarro et al. 2014), what suggests that it has better burst capabilities allowing a slow and discrete approach towards its prey, before hitting fast when

close as it has been observed on one footage (see Zintzen et al. 2011 for footage and description of this attack).

Our results also showed that the tail beat frequency of deep-sea shark species was positively correlated with their cruise swimming speed, which agrees with literature (Graham et al. 1990; Bainbridge 1958). Except for the two Etmopteridae, the deep-sea shark tail beat frequency was not higher than 1 Hz, while it is often more than 1Hz for shallow-water species (Graham et al. 1990). Our results revealed that Etmopteridae species displayed higher tail beat for a given speed than other deep-sea sharks, and that they also increase their tailbeat faster when they need to increase their cruise swimming speed. Regarding the stride length, Etmopteridae species showed the lowest values which agree with the literature on fish swimming stating that higher tail beat frequency means lower stride length. However, stride length also depends on swimming type and body shape, therefore it is species dependent. Indeed, *D. licha* which had the lowest tail beat frequency did not have the highest stride length (Svendsen et al. 2016; Videler and Wardle 1991).

One species, *C. harrissoni*, displayed a counterintuitive pattern: it presents a similar regression line to *D. licha*, while its cruise swimming speed placed it in the fastest group. One possible explanation relies on the morphology of its caudal fin. Indeed, this species had a higher proportion of the caudal region (20% of the total length – see supplementary data 2) than for the other deep-sea sharks studied. These results suggest that Etmopteridae sharks reach high-velocity due to higher tail beat frequency, which are probably related to their metabolism and muscular properties, while *C. harrissoni* goes faster due to the efficiency of its caudal fin. More work will, however, be necessary to better understand swimming abilities of deep-sea sharks using a combination of both morphological and physiological features (work in progress).

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AUTHOR CONTRIBUTION

N.P. participated at the conceptualization, the methodology making, the formal analyses, and provided the funding for this

research. U.M. was a master student helping during all the formal analyses. V.Z. and C.R. from Te Papa conceptualized and realized all steps of the fieldwork BRUVS deployed in New Zealand and provided all videos with deep-sea sharks. P.P. and C.V. from the ICTEAM-ELEN, helped for the script creation allowing the 3D measurement of size and speed. The initial draft of this manuscript was written by N.P. and was reviewed by U.M., V.Z., C.R. and J.M.

ETHICAL ISSUES

For this kind of analyses ethical issues were not applicable.

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Supplementary data

Supplementary data 1 - Methodology

Videos were collected in New-Zealand between 2009 and 2012 during summer days using a baited remote underwater stereo-video system (BRUVs). A total of 267 hours of video were recorded at 7 different locations. Sequences containing deep-sea sharks were conserved for speed and length analysis. First, the cameras used at each sampling sites were calibrated using a calibration cube. Then, points of interest, allowing length and velocity measurements, were manually annotated on the left and right images of each sequence, recording their coordinates in pixels. These coordinates were used to represent the sharks in three-dimensions, and to measure their size and speed. The generated data were used to assess deep-sea shark ecology (see main paper).

CALIBRATION MATRIX

The cameras were calibrated using a calibration cube (cube with specific white spots) in a swimming pool (Fig. 1). Calibration consists in estimating, for each camera, the parameters of the model defining how a 3D point projects on the image associated to the camera. Those parameters are estimated from a set of 3D↔2D correspondences. In our case, those correspondences are defined based on the cube white spots. The Matlab script written to associate each 3D cube spot to its 2D image projection is available in supplementary data 3.



Fig. 1 Calibration cube used in swimming pool to create the calibration matrix on MatLab. Cube from SeaGIS EventMeasure.

The pinhole camera model describes the mathematical relationship between the coordinates of a point in the three-dimensional space and its projection onto the image plane of an ideal pinhole camera, where the camera aperture is described as a point and no lenses are used to focus light (Hartley and Zisserman 2004). Hence, the pinhole camera model is based on the principle of collinearity, where each point in the 3D space is projected by a straight line through the camera optical center into the image plane.

Formally, a projective pinhole camera maps a point in space $\mathbf{X}$ to an image point $\mathbf{x}^P$ according to the projection $\mathbf{x}^P = \mathbf{P} \cdot \mathbf{X}$, with $\mathbf{P}$ being a 3×4 matrix acting as the projective operator, and $\mathbf{X}$ and $\mathbf{x}^P$ denoting the homogeneous coordinates associated to the 3D point and to its corresponding projected image point, respectively. Homogeneous coordinates define the conventional Cartesian coordinates relatively to an additional coordinate. They have been introduced because they allow implementing a vector translation through matrix multiplication. Since homogeneous coordinates are defined up to a multiplicative factor, the projection matrix $\mathbf{P}$ is also defined up to

a multiplicative factor, and has thus 11 degrees of freedom. Physically, those 11 parameters correspond to 6 extrinsic parameters, defining the optical center location and the orientation of the camera in the 3D space, and to 5 intrinsic ones, defining the origin and skewing factor of the pixel grid in the image plane, as well as the pixel size, measured along lines and columns, relatively to the focal length (= distance between optical center and image plane). The intrinsic parameters are thus internal and fixed for a given camera. They define the mapping between camera coordinates and pixel coordinates in the image plane.

Given $n \geq 6$ world to image point correspondences $\{x_i \leftrightarrow X_i\}_{i < n}$ with Gaussian measurement errors, the Maximum Likelihood Estimate (MLE) of the camera projection matrix is determined by computing the projection matrix P that minimizes the geometric error

$$(1) \quad \varepsilon = \sum_{i=1}^n d(x_i, P \cdot X_i)^2$$

with $d(x_i, x_j)$ measuring the Euclidian distance between two image points x_i and x_j .

Minimizing ε corresponds to a non-linear least-squares optimization problem, which has to be solved using gradient-descent with good initialization.

In practice, this is done using the so-called Gold Standard algorithm. After normalization of the inputs, this algorithm first computes an approximate solution using a linear method, known as the Direct Linear Transformation (DLT) algorithm. In this formulation, each correspondence $x_i \leftrightarrow X_i$ is written in the form of two linear equations, obtained by zeroing, in homogeneous coordinates, the cross product between x_i and the projection $P \cdot X_i$ of its corresponding 3D point X_i . This results in an (overcomplete) linear system of $2 \cdot n$ equations with 11 unknowns (= the entries of P , up to a multiplicative factor),

which writes $A \cdot \mathbf{p} = 0$, with $\mathbf{p}$ being a column vector stacking the rows of P . The approximate DLT solution is then defined to be the unit singular vector corresponding to the smallest singular value of the $2.n \times 12$ matrix A . In a second step, an iterative gradient-descent algorithm, typically the Levenberg–Marquardt algorithm is used to minimize the geometric error, using the approximate solution derived from DLT to initiate the convergence process.

The pinhole camera model does not account for lens distortion. Hence, in practice, it serves as a basis model that is extended with some corrections to derive the distorted image coordinates. The most commonly encountered distortions are radially symmetric. They cause the actual image point to be displaced radially in the image plane. In addition, another common type of distortion, named decentering distortion, introduces tangential displacement components. The decentering distortion is a non-symmetric distortion due to the misalignment of the lens elements when the camera is assembled. It may occur when the centers of curvature of lens surfaces in the lens system are not strictly collinear. Formally, most prominent distortions are modelled as follows. Let denote (x_1^P, x_2^P) the image coordinates of a point under ideal (undistorted) pinhole projection. Then, the distorted coordinates $(\tilde{x}_1, \tilde{x}_2)$ are modelled as:

$$(2) \quad \tilde{x}_1 = x_1^P \cdot (1 + \rho \cdot r^2) + 2 \cdot \tau \cdot x_1^P \cdot x_2^P + \tau' \cdot (r^2 + 2x_1^P \cdot x_1^P)$$

$$(3) \quad \text{and } \tilde{x}_2 = x_2^P \cdot (1 + \rho \cdot r^2) + \tau \cdot (r^2 + 2x_2^P \cdot x_2^P) + 2 \cdot \tau' \cdot x_1^P \cdot x_2^P$$

with $r = \sqrt{x_1^2 + x_2^2}$ denoting the radial distance from the image center, and ρ, τ and τ' defining the coefficients associated to the radial and tangential distortions. More complex models are possible to capture the distortion (www.vision.caltech.edu/bouguetj/calib_doc/), but the above one has appeared to be sufficiently accurate, leading to very small geometrical error.

Given the above distortion model, the Levenberg–Marquardt algorithm is used to jointly optimize the projection matrix P and the distortion parameters ρ, τ and τ' , so as to minimize the geometric error

$$(4) \quad \varepsilon = \sum_{i=1}^n d(x_i, \tilde{x}_i)^2$$

with x_i denoting the image point measurement, and $\tilde{x}_i$ being the distorted coordinates associated to the undistorted projection $x_i^P = P \cdot X_i$ of the corresponding 3D point X_i .

The publically available ‘Camera Calibration Toolbox for Matlab’ (www.vision.caltech.edu/bouguetj/calib_doc/) has been used as reference software to calibrate each camera from our stereo setup (script available in supplementary data 3.1 & 3.2).

POINT ANNOTATION

To estimate the size of a shark individual, we need to estimate the 3D coordinates of specific points of interest at a given time instant. In contrast, the shark speed estimation requires the definition of 3D coordinates for pairs of corresponding points at distinct time instants. We needed to measure the x and y shark coordinates in pixels for the left and right images. For the size, four specific points representing total, fork and precaudal length were selected (rostrum - precaudal pit - posterior notch - terminal margin). For the velocity, three points visible on the videos from the left and the right cameras were selected along the body (Fig. 2A). These points had to be placed on recognizable body parts which remain fixed and conserved through the entire sequence (one image per second). In most cases rostrum and dorsal fin bases fitted the best for these requirements. The use of three different points allows to characterize the whole body displacement while less points would only characterize one body part displacement. In addition, three points account for points speed differences.

3D RECONSTRUCTION

We now explain how to compute the position of a point in 3D-space given its projection position in the two views, and the projection matrices P and P' and associated lens correction parameters for both cameras (Fig. 2B). In projective geometry, the set of 3D points that map to a given a pixel x in an image corresponds to a ray of light passing through the camera center. It is assumed that the calibration parameters are known with great accuracy, but that errors in the measured image coordinates cannot be avoided due to the manual identification of corresponding points in presence of finite image resolution. As a consequence of these errors, a triangulation by back-projecting rays from the measured image points will fail, because the rays will not intersect in most cases. For simplicity, in our case, we adopt the midpoint method to estimate the 3D point. This method finds the midpoint of the common perpendicular to the two rays that are associated in space to the image measurements. Formally, two projection lines L_1 and L_2 are associated to the image points x_1 and x_2 , respectively. Letting $d(L, X)$ denote the Euclidian distance between the line L and the 3D point X , the midpoint X^* associated to the two lines L_1 and L_2 is defined to be

$$(5) \quad X^* = \underset{X}{\operatorname{argmin}} \left(d^2(L_1, X) + d^2(L_2, X) \right)$$

This estimator is not projective-invariant, in the sense that the projection set-up affects the noise distribution for which the estimator is a Maximum Likelihood Estimator (MLE). However, it is simple to implement and it is close to the MLE associated to a Gaussian error distribution in the common and desirable use case where the rays associated to each image measurement are reasonably aligned with the principal axis of the camera.

More importantly, to exploit this stereo system properly, it is worth considering the errors (mainly in depth when rays are

aligned with principal axes) induced by the limited accuracy associated to image measurements. Formally, the depth error ε_z in stereo can be shown to be

$$(6) \quad \varepsilon_z = \frac{z^2}{b \cdot f} \cdot \varepsilon_d$$

with z , b , and f respectively denoting the depth of the 3D point, the stereo setup baseline, and the camera focal length measured in pixels (Gallup et al. 2008). In this equation, ε_d corresponds to the matching error in pixels, also named disparity error. Hence, the error in depth estimation caused by a small shift in the point image measurement increases as the square of distance to the camera. In our case, the baseline is about 0.8 meter, while the focal length is about 3100 pixels. This means that the depth error caused by a one pixel disparity error is about 25 cm when the shark is at 25 meters from the camera. Since the visibility also decreases with the depth, only the sharks that are at less than 10m from the camera will thus be considered in our study, to keep the error smaller than a few centimeters (script for triangulation is available in the supplementary data 4).

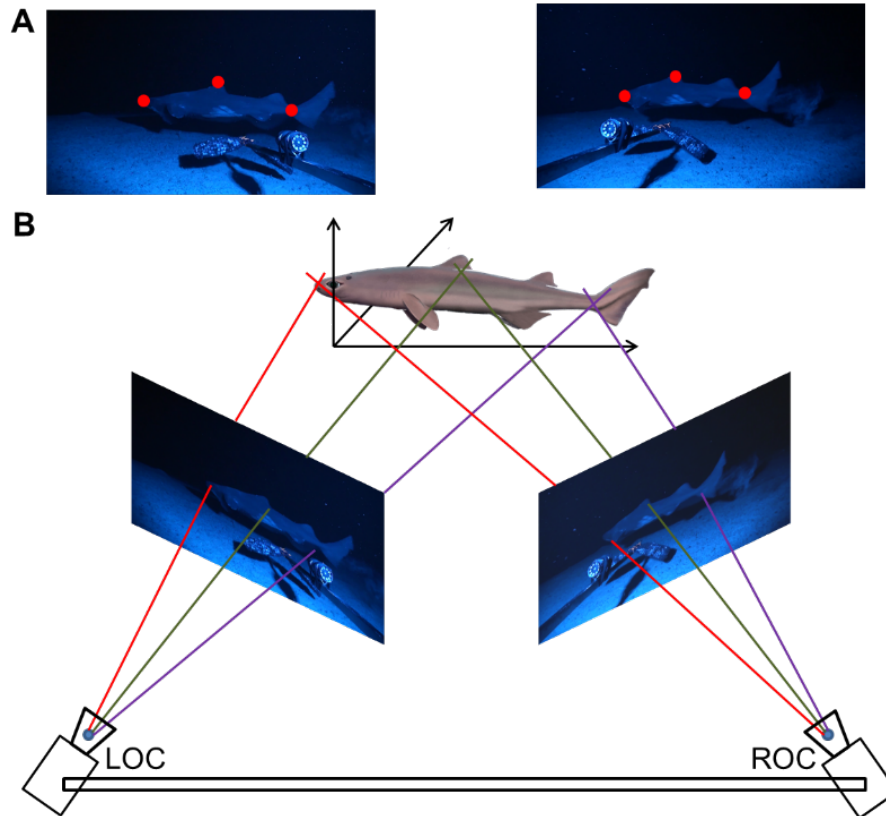


Fig. 2 Representation of the basic principle of stereo-video. (A) Choice of three points: rostrum, posterior base of the anterior dorsal fin and posterior base of the posterior dorsal fin. (B) Crossing of the projecting beams for the three points, LOC: Left Optical Center ROC: Right Optical Center

Supplementary data 2 - Methodology analyses: source of error

The repetitiveness of our results was tested using the synchronization diode length (i.e. the led lamp used in the middle of the video frame to adjust and synchronize the time of both video from left and right camera). Indeed, its diameter was known a priori and was measured three times in each video deployment showing no significant difference between the measures (Anova, $p>0.05$). Sometimes, the lengths of a same shark individual were also measured several times and reveal no difference inside the same sequence even at different position. The annotation errors (in pixels) were estimated by considering the distribution of manual annotations around their mean value. The maximal annotation error was of 5 pixels. In addition, it appeared that the maximal calibration error, as measured on the calibration white spots, was of 5 pixels on the x-axis and 6 pixels on the y-axis. It resulted in a maximal error of 10 pixels on the x-axis and 11 pixels on the y-axis. In the end, such errors in pixels led to a maximal error of ± 0.91 cm for the size values and ± 0.91 cm/s for the speed values. These errors were calculated assuming that the organism was located in the center of both left and right images, at a relatively short distance to the camera and parallel to the stereo baseline. Constant linear displacement on a period of one second was assumed for speed error measurements.

In all these measurements, four percent were considered as outliers with a value sitting outside the 1.5 quartile (quartile 1 and quartile 3). The examination of the videos associated to the cases producing those outliers revealed two scenarios: (i) sharks were swimming in an orientation perpendicular to the camera axis (Fig 3). The shark orientation was measured as the

shark displacement vector and the camera viewing direction. When the angle between the shark motion vector and the stereo baseline was superior to 30° , more than 45 % of measurements were outliers. Beyond 75° , all measurements were large outliers. This can be explained by the fact that, when the shark is parallel to the baseline, the error in size/speed estimation is dominated by the angular/azimuthal error. In contrast, when it is perpendicular to the baseline, the error in size/speed is dominated by the depth error. At 10 meters from the cameras, and for a matching error of one pixel, the azimuthal error is given by $\frac{z}{f}$, which is about 0.3 cm in our case (focal length is about 3100 pixels), while the depth error is given by equation (6) and is equal to about 4 cm, which is more than one order of magnitude than the azimuthal error.

(ii) Sharks were swimming on the edges of at least one camera where the calibration was done with a cube in the center of both camera images (Fig 4). . Two reasons explain why the error becomes larger when getting closer to the image border: (1) The projection model is more accurate in the center of the image, where the white spots used to estimate calibration parameters are located; (2) At a given depth, since the depth error remains independent of the viewing direction (as defined by equation 6), the Euclidian displacement of the 3D point induced by a matching error is inversely proportional to the cosine of the angle θ between the viewing direction of the stereo setup and the position of the observed point. The 3D localization error affecting a point lying on the border of the image is thus larger than the one affecting a point in the center of the image.

For further acquisitions, the problem resulting of the organism location at the edge of the image could be avoided by using a calibration tool covering both cameras entire field of view. It would integrate the stronger distortion effect occurring on the border of the images.

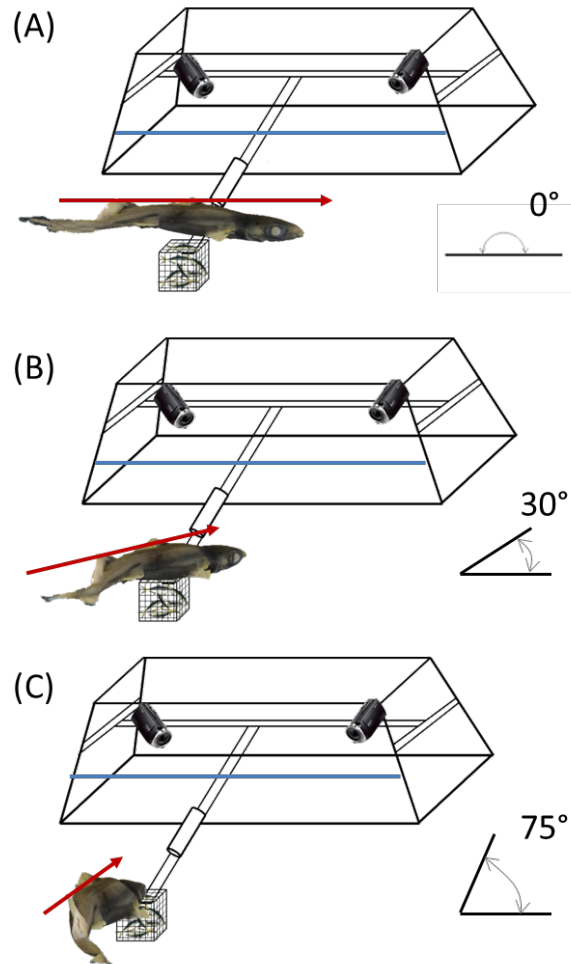
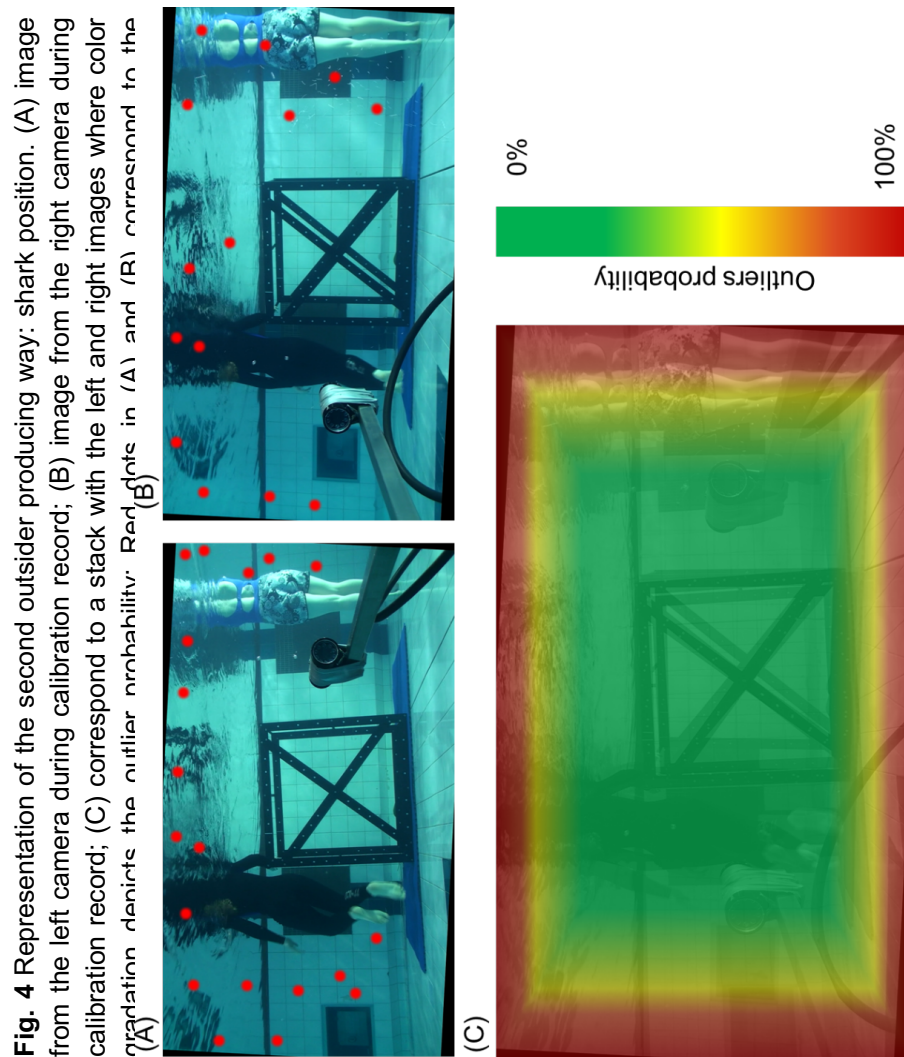


Fig. 3 Representation of the first scenario resulting in large errors: shark orientation. (A) corresponding to a shark swimming in a direction totally parallel to the camera recording axis; (B) corresponding to a shark swimming in a direction 30° in the perpendicular to the camera recording axis; (C) corresponding to a shark swimming in a direction 75° in the perpendicular to the camera recording axis; blue line correspond to the stereo set-up baseline; red line corresponds to the movement vector of the shark.



Supplementary data 3 – Matlab script – Cube modeling

**Supplementary data 4.1 – Matlab script – Cube point
matching**

**Supplementary data 4.2 – Matlab script – Camera
calibration**

Supplementary data 5 – Matlab script – Point annotation

Found on the USB flash drive provided because it is MatLab
file. It can also be opened using .txt software.

Supplementary data 3: cruise swimming speed from literature

Table 1 Summary of cruise swimming velocity for shark species obtained from the literature; phylogenetic order (Order/species), general living habitat (Habitat), number of replicate (n) and cruise swimming speed (CS ms⁻¹).

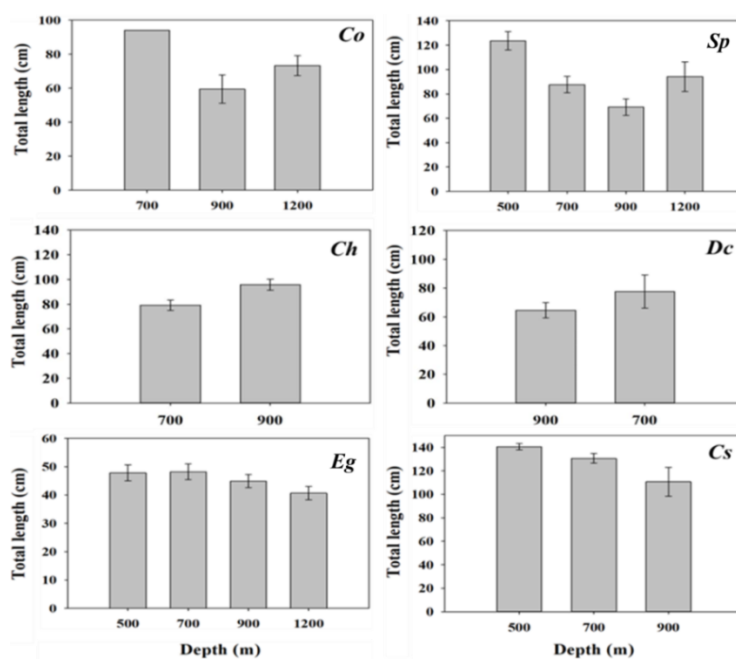
Order/species	Habitat	n	CS (m s ⁻¹)	Source
Carcharhiniformes				
<i>Carcharhinus albimarginatus</i>	Benthic	8	0.73	Ryan et al. 2015
<i>Carcharhinus amblyrhynchos</i>	Benthic	51	0.64	Ryan et al. 2015
<i>Carcharhinus brevipinna</i>	Benthic	5	0.65	Ryan et al. 2015
<i>Carcharhinus obscurus</i>	Benthic	11	0.5	Ryan et al. 2015
<i>Carcharhinus plumbeus</i>	Pelagic	5	0.73	Ryan et al. 2015
<i>Furgaleus macki</i>	Benthic	9	1.09	Ryan et al. 2015
<i>Galeocerdo cuvier</i>	Pelagic	4	0.63	Ryan et al. 2015
<i>Hemitriakis falcata</i>	Benthic	3	0.53	Ryan et al. 2015
<i>Mustelus antarcticus</i>	Benthic	27	0.52	Ryan et al. 2015
<i>Negaprion acutidens</i>	Benthic	6	0.53	Ryan et al. 2015
<i>Negaprion brevirostris</i>	Benthic	3	0.63	Watanabe et al. 2012
<i>Prionace glauca</i>	Pelagic	2	0.42	Watanabe et al. 2012
<i>Sphyrna lewini</i>	Pelagic	5	0.69	Ryan et al. 2015
<i>Triaenodon obesus</i>	Benthic	49	0.5	Ryan et al. 2015
Lamniformes				
<i>Cetorhinus maximus</i>	Pelagic	2	1.08	Watanabe et al. 2012
<i>Isurus oxyrinchus</i>	Pelagic	1	0.90	Watanabe et al. 2012
Heterodontiformes				
<i>Heterodontus portusjacksoni</i>	Benthic	52	0.34	Ryan et al. 2015
Orectolobiformes				
<i>Nebrius ferrugineus</i>	Benthic	4	0.32	Ryan et al. 2015
<i>Parascyllium variolatum</i>	Benthic	9	0.26	Ryan et al. 2015
<i>Rhincodon typus</i>	Pelagic	9	0.85	Watanabe et al. 2012
Squaliformes				
<i>Somniosus microcephalus</i>	Deep	2	0.34	Watanabe et al. 2012

Supplementary data 4: General ecological features

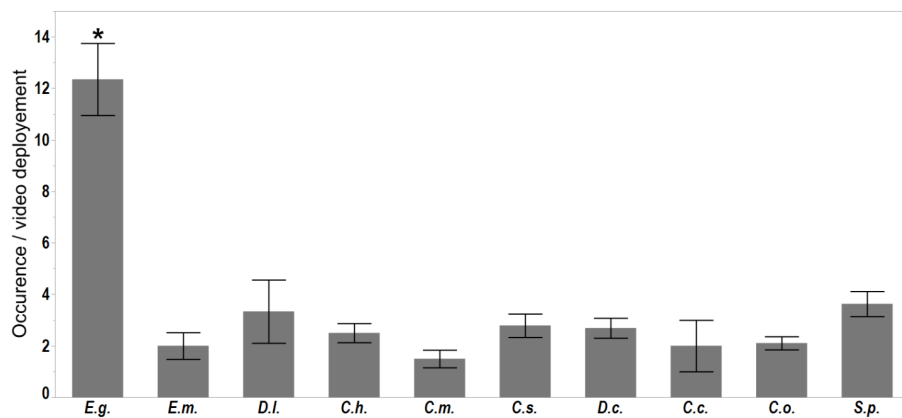
Results of linear regressions between the three lengths (total length, fork length and pre-caudal length) for six deep-sea shark species.

	<i>Centrophorus harrissoni</i>	<i>Centroscymnus owstonii</i>	<i>Centrophorus squamosus</i>	<i>Etmopterus granulosus</i>	<i>Dalatias licha</i>	<i>Scymnodon plunketi</i>
Total length in function of precaudal length						
p-value	5.76e-05	7.46e-04	2.35e-08	< 2.20e-16	2.55e-08	3.18e-13
R square	0.733	0.945	0.770	0.925	0.980	0.900
Intercept	9.51	4.98	5.07	-2.25	-10.86	3.14
Slope	0.706	0.810	0.779	0.888	0.917	0.797
Total length in function of fork length						
p-value	7.95e-09	3.14e-07	1.82e-14	< 2.20e-16	1.53e-08	< 2.20e-16
R square	0.938	0.999	0.939	0.973	0.983	0.960
Intercept	9.95*	-2.88	1.27	-0.09	-6.42	3.31
Slope	0.774	0.971	0.923	0.922	0.973	0.898
Precaudal length in function of fork length						
p-value	8.13e-06	8.39e-04	1.24e-10	< 2.2e-16	1.12e-09	< 2.20e-16
R square	0.806	0.941	0.860	0.971	0.991	0.967
Intercept	13.48	7.53	15.21	3.91*	5.58	4.95
Slope	0.888	0.833	1.003	0.999	1.055	1.075

Bar charts representing the total length of six different squaloid deep-sea shark according the depth where they were found during video deployments; Co correspond to *Centroscymnus owstonii*, Sp corresponds to *Scymnodon plukenti*, Ch corresponds to *Centrophorus harrissoni*, Dc corresponds to *Deania calcea*, Eg corresponds to *Etmopterus granulosus* and Cs corresponds to *Centrophorus squamosus*.



Bar chart representing the mean occurrence of different individuals observed during video deployment; E.g. corresponds to *Etmopterus granulosus*, E.m. corresponds to *Etmopterus molleri*, D.l. corresponds to *Dalatias licha*, C.h. corresponds to *Centrophorus harrissoni*, C.m. corresponds to *Centroscymnus macracanthus*, C.s corresponds to *Centrophorus squamosus*, D.c. corresponds to *Deania calcea*, C.c. corresponds to *Centroselachus crepidater*, C.o. corresponds to *Centroscymnus owstonii*, S.p. corresponds to *Scymnodon plukenti*; Error bars correspond to the standard error (SE); * corresponding to significant difference.



Results of linear regressions between the tail beat and the cruise swimming speed for eight deep-sea shark species.

	C. <i>harrissoni</i>	C. <i>owstonii</i>	C. <i>squamosus</i>	E. <i>granulosus</i>	D. <i>licha</i>	S. <i>plunketi</i>	D. <i>calcea</i>	E. <i>molleri</i>
Tail beat according cruise swimming speed								
p-value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
R square	0.733	0.39	0.55	0.43	0.75	0.62	0.30	0.93
Intercept	9.51	0.10	0.24	0.46	0.18	0.21	0.36	0.22
Slope	0.706	1.71	0.79	0.50	1.36	0.67	0.31	2.70

Chapter II:

Physiological and anatomical
adaptations relative to deep-sea shark
swimming capabilities: Metabolism,
muscle proportions and buoyancy

The first chapter of this thesis (**Paper 1**) has shown that even if deep-sea sharks swim slower than their shallow-water pelagic counterparts, some of them, particularly luminous shark from Etmopteridae family, display cruise swimming speed similar to benthic shallow-water species.

In contrast to previous studies which focused on the metabolism of only three species of deep-sea sharks, without any information regarding their velocity, the first paper of this chapter will study the metabolism of nine deep-sea sharks and one shallow-water species (*D. licha*, *C. squamosus*, *D. calcea*, *P. plukenti*, *C. owstonii*, *E. Lucifer*, *E. granulosus*, *E. molleri*, *E. spinax*, and *S. acanthias*) using the same traditional enzyme assays (**Paper 2**). Therefore, the aim will be to verify if proxies for enzyme activities, such as for shallow-water species, can be related to swimming capabilities in deep-sea sharks. We also measured, for the first time, the red and white muscle proportions aiming for the same relative analyses (shallow water/deep-sea). Enzyme activities were studied using spectrophotometer protocol and muscle proportions values were measured according to the literature.

In the second paper of this chapter, the buoyancy of deep-sea sharks was investigated using new approaches to characterize liver features based on Raman and MIR spectroscopy. Indeed, in literature, several studies have mentioned that deep-sea sharks should be close to the neutrality and it was suggested by Bones, in 1964, that more a shark was close to the neutrality, the less red muscles it should have. This hypothesis has been cited numerous times and increased the idea that deep-sea shark are slow swimmers. However, the muscle proportion of deep-sea sharks was never measured and therefore this hypothesis was never challenged. In that context, the second paper will study the buoyancy provided by the liver of three deep-sea species (*E. spinax*, *E. molleri*, and *I. brasiliensis*) and one shallow-water one (*G. melastomus*) (**Paper 3**). Our study

will also measure the red muscles proportion of several deep-sea sharks and combine it with previous measurement of liver features found in literature. Aims of this study are: (i) to confirm that deep-sea sharks are indeed closer to the neutrality and (ii) to provide evidence that confirm or deny Bone's hypothesis.

Paper 2:

Enzymatic activities in the aerobic and anaerobic metabolism of mesopelagic sharks and impact on their swimming capabilities

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Enzymatic activities in the aerobic and anaerobic metabolism of mesopelagic sharks and impact on their swimming capabilities

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ABSTRACT

There has been an increase in the study of the ecology of deep-sea organisms in the last decade, in part driven by the recent exploitation of these resources requiring an understanding of the impacts of our activities on this ecosystem. One way to understand an organism's ecology is the study of their metabolism. Unlike deep-sea bony fish, the metabolism of deep-sea cartilaginous fish is still poorly documented. Deep-sea sharks possess a lower glycolytic metabolism than their shallow-water counterparts, but no difference has been observed regarding their aerobic metabolism. Although, these metabolic studies have suggested deep-sea sharks should be slow and listless swimmers. However, other studies based on video observations have revealed differences in cruise swimming speed between different species. Therefore, present study paired muscle enzyme assays with measurement of red and white muscle proportions to reveal a possible correlation between metabolism and swimming capabilities. Results have shown that non-luminous species (*Centrophorus squamosus*, *Centroscymnus owstonii*, *Deania calcea*, *Procymnodon*

plunketi) displayed lower levels of aerobic metabolism than their shallow-water counterparts (*Squalus acanthias*) whilst luminous Etmopteridae species were found to have similar aerobic metabolism to shallow-water species. In contrast, Etmopteridae species displayed a low glycolytic metabolism whilst the glycolytic metabolism of non-luminous sharks was similar to *S. acanthias*. Thanks to this study, red muscle proportion and aerobic metabolism were positively related to the cruise swimming speed measured previously. This correlation confirms the results that Etmopteridae display higher cruise swimming speed than other deep-sea sharks, specifically *D. licha*, which swims at the slowest value never recorded. In contrast, the values obtained for white muscle proportion and anaerobic metabolism suggest higher burst capacity for non-luminous sharks and *D. licha* than for Etmopteridae

KEYWORDS Deep-sea, enzyme assays, metabolism, muscles, sharks, velocity

INTRODUCTION

Metabolism is defined as the process through which an organism assimilates and consumes energy (Reece et al. 2014). Studies of metabolic processes provide information on locomotion, growth and feeding rates, and also activity patterns (Sullivan and Somero, 1980). For deep-sea organisms, measurement of whole animal metabolic capacity is complicated by difficulties in accessing the environment. A commonly applied method is measuring the metabolism indirectly using proxies such as enzyme activity assays (Drazen and Seibel, 2007). The aerobic and anaerobic metabolic pathways allow organisms to produce energy that supplies different needs (Reece et al. 2014). Both pathways create adenosine triphosphate (ATP) from nicotinamide adenine dinucleotide (NADH) oxidation. The aerobic system produces more ATP (38) than the anaerobic one (2), but the former

requires a higher concentration of oxygen, and is one hundred times slower than the second system (Reece et al. 2014). Aerobic metabolism is used mainly to power red muscle, which is highly vascularized with blood vessels to provide, in most cases, steady and sustained effort (e.g. for cruise swimming). In contrast anaerobic metabolism powers mainly white muscle, which has few blood capillaries and produces short-term but intense effort (e.g. for burst swimming mode) (Watanabe et al. 2012; Ryan et al. 2015).

The metabolism of many deep-sea teleosts has been studied in the last 40 years (Childress and Somero, 1979; Sullivan and Somero, 1980; Drazen et al. 2011; Drazen et al. 2015). To date, most deep-sea teleosts that have been studied have a lower anaerobic metabolism in comparison with their shallow-water counterparts. The same trend has been observed for the aerobic metabolism in red muscle (Childress and Somero, 1979; Sullivan and Somero, 1980; Drazen et al. 2011, Drazen et al., 2013; Drazen et al. 2015). A smaller number of studies have looked at elasmobranchs, which display a quite different metabolism: they have a lower anaerobic metabolism than their shallow-water counterparts, but the aerobic metabolism is similar between deep-sea and shallow-water species (Treberg et al. 2003; Condon et al. 2012; Treberg et al. 2016). These studies suggest that deep-sea sharks possess less efficient swimming capabilities than shallow-water species, based on two key hypotheses: (i) the lower water temperature inhibits muscular contraction (Treberg et al. 2003) and (ii) the reduced advantage of faster swimming capability in a low light environment, the visual interaction hypothesis (VIH) (Condon et al. 2012). However, these studies are based on two shark species along with deep-sea skates, and the authors emphasized that generalities should not be across this highly diverse group. A recent study based on *in situ* stereo video observations has revealed differences in the cruise swimming speed of several Squaliformes deep-sea sharks (eight species)

(Pinte et al. 2019a). The video observations of sharks attracted to bait demonstrated that luminous sharks from the Etmopteridae family displayed a higher cruise swimming speed than non-luminous counterparts (*Centrophorus squamosus*, *Centroscymnus owstonii*, *Deania calcea*, *Procymnodon plunketi*) and it was hypothesized that higher swimming speed could be necessary if these sharks were “isolume followers”. In contrast, *Dalatias licha*, another luminous shark from the Dalatiidae family, displayed the slowest swimming speed ever measured for sharks (Pinte et al. 2019a).

In this study, we investigated the muscular system involved in the locomotion of deep-sea sharks. This approach allowed us to compare these data with swimming speed, already known for most species (Pinte et al., 2019a). Aerobic metabolism is measured using the activities of key enzymes from the Krebs cycle: citrate synthase (CS) and malate dehydrogenase (MDH). Anaerobic metabolism is measured from the activity of the key enzyme lactate dehydrogenase (LDH) that catalyzes the step (lactic fermentation) following the glycolysis. These enzymes were studied in red and white muscle fibers and the proportion of both fibers was also measured. The aim was to explore the relationship between aerobic metabolism and the cruise swimming speed mainly powered by it in both luminous and non-luminous deep-sea sharks.

MATERIALS AND METHODS

Sampling

A total of ten species of deep-sea shark were collected from three different locations. *Etmopterus spinax* were caught using long-line in Raudenfjord (60.260623°N, 5.162056°E; Espegrend, Norway) (Figure 1a), in January 2016 at specific depth of 225 meters. Sharks were euthanized by a blow to the head followed by a full incision of the spinal cord at the level of

the first vertebrae, following the local rules for experimental vertebrate care (Permit 12/14048).

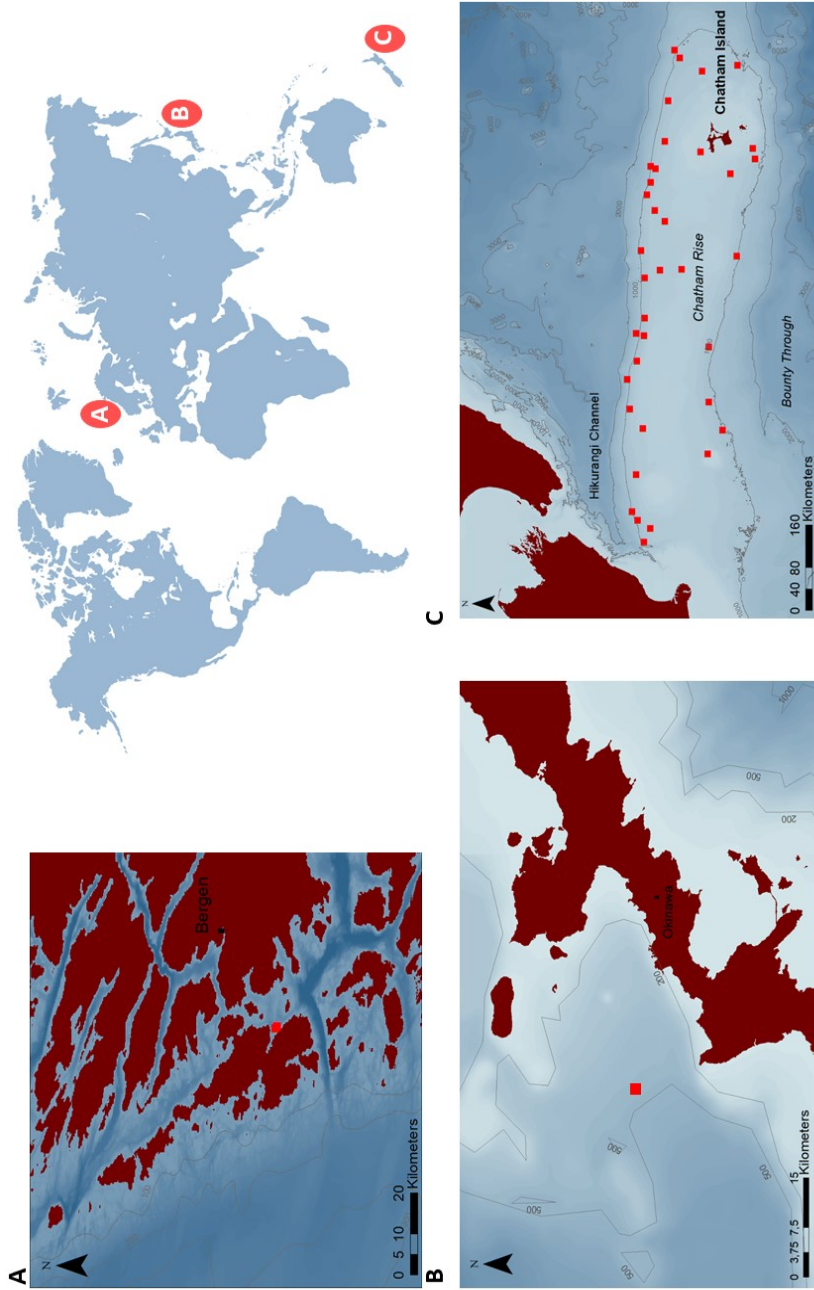
Etmopterus molleri were caught on longlines during a survey in December 2016 at 500 meters depth in the East China Sea (26.32468°N, 127.41207°E; Okinawa, Japan) (Figure 1b). All shark specimens were euthanized according to the rules of OCA for experimental fish care before experimentation took place.

A further eight species of shark, *Etmopterus granulosus*, *Etmopterus lucifer*, *Deania calcea*, *Centrophorus squamosus*, *Centroscymnus owstonii*, *Scymnodon plunketi*, *Dalatias licha* (deep-sea species) and *Squalus acanthias* (shallow-water species) were sampled by trawl during a fisheries research survey for hoki on the Chatham rise, south-western Pacific in January 2018 (42.5907°S, 176.3978°E; New Zealand) (Figure 1c).

For each species, up to 10 specimens were collected. From each specimen, white and red muscles were removed on ice and stored at -80°C. Enzyme assays were carried out up to 3 months after the tissues had been collected, with no significant differences observed between samples with different storage times. Additional whole shark specimens were used for the red and white muscle quantification.

A total of 103 sharks were sampled from three locations. Data related to size, number of specimens by species, red/white muscle enzyme activities and proportion are summarized in Table 1.

Fig. 1 Maps showing sampling location in Norway (A), Japan (B) and New Zealand (C).



White and red muscle volume quantification

The protocol used to determine the red and white muscle proportions are based on one used by Bernal et al. (2003). Except for *E. lucifer*, all 3-4 specimens were sampled from each species. Given all of the studied sharks display a carangiform mode of swimming, we started the transverse section cuts at a quarter of the fork length (FL), continuing to the caudal peduncle (85-95 % FL). Each transverse section (5% of the fork length) was photographed on its anterior and posterior sides. Photographs were analyzed with ImageJ2[®] software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2018), measuring the red muscles area on the anterior and posterior sides of the section using a reference scale placed on each picture. The red and white muscle volume for each cross-section is the product of the section thickness and its red/white muscle areas (mean between the anterior and the posterior area of the transverse section). The muscle mass was the product of muscle volume and density (literature value confirmed on our shark samples: 1.05 g.cm⁻³, Bernal et al., 2003). The quantification of the total red muscle mass (TRMM) and the total white muscle mass (TWMM) were determined by the sum of each section mass value. Finally, the TRMM and the TWMM were compared with the total body mass to quantify the red and white muscles proportion. This methodology was validated by removing and weighing these different muscle fibers for one specimen.

Enzyme Assays

The activities of the enzyme citrate synthase (CS - E.C. 4.1.3.7), malate dehydrogenase (MDH - E.C. 1.1.1.37), lactate dehydrogenase (LDH - E.C. 1.1.1.27) were measured using a protocol based on those used in previous studies (Treberg et

al., 2003; Condon et al., 2012). Results from *S. acanthias* were compared with published values to validate the methodology. Frozen muscle samples were weighed (+/- 0,25g) and homogenized using T-10 basic ultraTurax IKA on ice in nine-volume of cold 10mM Tris-HCl buffer (pH 7.4 at 10°C). The homogenates were centrifuged at 5000 g for 5 minutes at 4°C and the supernatants were used for the enzyme assays. Bicinchoninic acid assay (BCA) was carried out to check that the quantity of protein extracted was equal.

All assays were performed in a total of 2.0 ml mixture using Genesis™ 10S UV-Vis ThermoFisher Scientific spectrometer® at 10°C. This temperature was chosen based on water temperatures recorded at each station by CTD. All sharks' species are known to experience this temperature during some part of their life. Enzymatic assay is proportional to the decrease of absorbance in 340 nm (MDH, and LDH), and to the rise of absorbance in 412 nm (CS) over time. The results are given in international units of UE: μ mole of substrate converted to product per minute and gram of wet tissues. Before starting assays, it is important to be certain that the substrate is fully saturated. For that, we used the previous dilution of our samples with homogenization buffer. The final conditions for each enzymatic assay were as follows: Citrate synthase: 100mM Tris-HCl (pH 8.1), 0.1% Triton X100, 0.1 mM DTNB and 0.15mM of Acetyl-CoA. The reaction was initiated by 0.5 mM Oxaloacetate. Malate dehydrogenase: 100mM Tris-HCl (pH 8.1); 20mM MgCl₂ and 0.15 NADH. The reaction was initiated by 0.5mM oxaloacetate. Lactate dehydrogenase: 80mM Tris-HCl (pH 7.4); 8mM pyruvate. The reaction was initiated by 0.10 mM NADH. Negative control without cofactor and samples confirmed the need for these two compounds to start the reaction. Positive control with pure enzyme extract confirmed the enzyme role in the observed reaction.

In contrast to temperature, it has already been shown that pressure changes do not affect the activity of these enzymes.

Therefore, all measurements were carried out at a pressure of 1atm.

Statistical analyses

All statistical analyses were performed with JMP Pro 14 (JMP®, Version <14>. SAS Institute Inc., Cary, NC, 1989-2007). Normality and the homoscedasticity were confirmed for each parametric test (ANOVA, post hoc Tukey test). When the Gaussian distribution was not apparent, non-parametric equivalent tests were used (Wilcoxon, post hoc Steel-Dwass test). Data on enzyme activities and muscle proportions were related to the cruise swimming speed of several deep-sea species using Principal Component Analysis (PCA), based on correlation and by Pearson's test of correlation. The swimming speeds used in this study were based on published observations of sharks attracted to baited cameras placed *in situ* (Pinte et al. 2019a).

RESULTS

Red and white muscle proportion

The results concerning the red muscle proportion show four groups of different significance from sharks with the smallest proportion to the shark with the highest (Fig. 2A), the letters associated with each group make it possible to distinguish significant differences: When no letters are common, the groups are significantly different (ANOVA, $F(8,35)=7.03$, $p < 0.05$, Tukey test post hoc). The Group 1 (A) is represented by *Dalatias licha* and *Deania calcea*; then comes Group 2 (ABC) where we find *Centrophorus squamosus*, *Etmopterus lucifer*, *Etmopterus granulosus* and *Etmopterus spinax*; Group 3 (BC)

contains *Centroscymnus owstonii* and *Squalus acanthias*; finally Group 4 (C) which contains only *Etmopterus mollerii*.

Regarding the white muscle proportion, four groups can also be highlighted from the one with the highest to the one with the lowest (Fig. 2B), the statistical differences are presented in the same letter format as for the previous analysis (ANOVA, $F(8,35)=7.37$, $p<0.05$, Tukey test post hoc). The first group (A) is represented by *D. calcea*; the second group (AB) contains *D. licha*, *C. squamosus*, *C. owstonii*, and *S. acanthias*; the third group (BC) is represented by *E. spinax* and *E. granulosus*; finally, the last group (C) contains *E. lucifer* and *E. mollerii*.

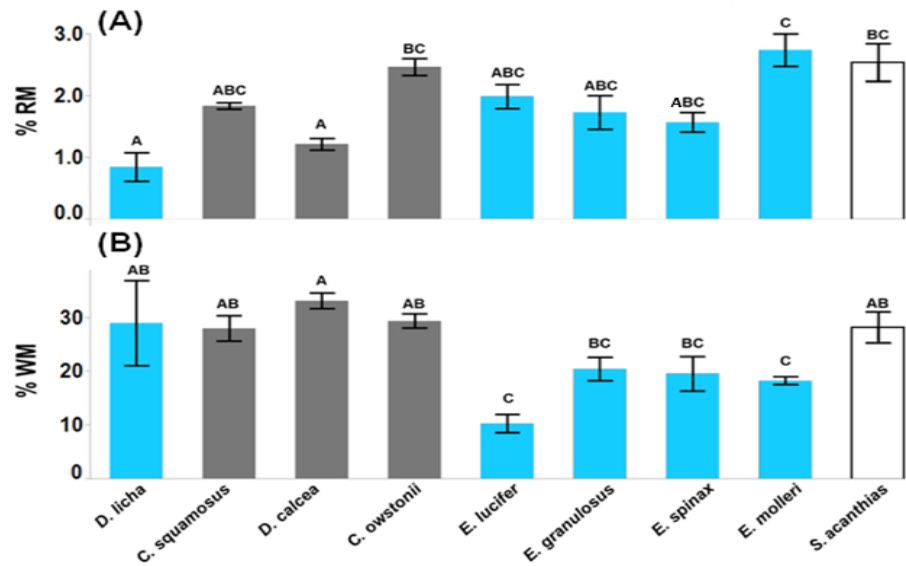


Fig. 2: (A) Red muscle proportion of eight deep-sea sharks and one shallow-water species. (B) White muscle proportion of eight deep-sea sharks and one shallow-water species; Red/white muscle proportion corresponding to the red/white muscle weight compared to the total body mass (%RM / %WM); Bars represent the mean value of each muscle proportion, with error bars corresponding to the standard error; Blue bars represent luminous shark species, grey bars correspond to non-luminous species, and white bar to the shallow-water species; Letters up to the bars describe the significant differences between species.

Table 1 Summary of data measured on the 10 species of sharks; number of replicate (n), mean total length $\pm$ SE (Total length, cm), mean Citrate synthase (CS), Malate dehydrogenase (MDH), Lactate dehydrogenase (LDH) activities $\pm$ SE in red and white muscles (UE corresponding to $\text{mmol min}^{-1}\text{g wet tissue}^{-1}$); mean red and white muscles proportion $\pm$ SE (% RM and %WM) corresponding to the red and white muscle weight compared to the total body weight respectively; *Luminous shark based on our observations and literature; } corresponding to shallow-water species. Taxa classification based on Ebert et al. (2013) and Roberts et al. (2015).

Species	n	Total length (cm)	Red muscles				White muscles			
			CS (UE)	MDH (UE)	LDH (UE)	CS (UE)	MDH (UE)	LDH (UE)	% RM	%WM
<i>Dalatias licha</i> *	9	84.1 $\pm$ 25.4	0.8 $\pm$ 0.2	39.3 $\pm$ 4.8	56.7 $\pm$ 4.5	0.4 $\pm$ 0.1	19.6 $\pm$ 0.7	76.4 $\pm$ 10.3	0.8 $\pm$ 0.3	28.9 $\pm$ 2.4
<i>Centrophorus squamosus</i>	11	109.6 $\pm$ 12.4	2.5 $\pm$ 0.4	71.7 $\pm$ 4.6	59.3 $\pm$ 5.3	0.8 $\pm$ 0.2	19.6 $\pm$ 1.9	77.6 $\pm$ 10.8	1.8 $\pm$ 0.1	27.9 $\pm$ 3.6
<i>Deania calcea</i>	11	85.3 $\pm$ 7.7	2.4 $\pm$ 0.3	82.3 $\pm$ 10.2	65.9 $\pm$ 5.2	0.4 $\pm$ 0.1	26.6 $\pm$ 1.8	31.7 $\pm$ 4.7	1.2 $\pm$ 0.1	33.1 $\pm$ 2.1
<i>Proscymnodon plunketi</i>	7	118.1 $\pm$ 7.7	2.14 $\pm$ 0.9	85.9 $\pm$ 9.7	66.2 $\pm$ 6.9	0.1 $\pm$ 0.02	25.7 $\pm$ 0.8	101.2 $\pm$ 12.9	/	/
<i>Centroscyrmus owstonii</i>	11	92.2 $\pm$ 14.5	1.5 $\pm$ 0.5	80.5 $\pm$ 11.6	74.6 $\pm$ 6.1	0.2 $\pm$ 0.1	28.8 $\pm$ 2.0	107.3 $\pm$ 8.8	2.5 $\pm$ 0.2	29.3 $\pm$ 1.9
<i>Etmopterus lucifer</i> *	10	32.4 $\pm$ 3.7	9.1 $\pm$ 1.1	101.9 $\pm$ 9.6	62.7 $\pm$ 4.0	0.7 $\pm$ 0.1	27.5 $\pm$ 2.4	57.8 $\pm$ 7.5	1.9 $\pm$ 0.2	10.2 $\pm$ 1.9
<i>Etmopterus granulosus</i> *	11	65.1 $\pm$ 5.9	9.7 $\pm$ 0.9	116.8 $\pm$ 6.9	83.3 $\pm$ 5.4	0.5 $\pm$ 0.1	23.1 $\pm$ 1.8	55.7 $\pm$ 5.6	1.7 $\pm$ 0.4	20.4 $\pm$ 1.8
<i>Etmopterus molleri</i> *	12	45.2 $\pm$ 1.59	12.6 $\pm$ 0.4	178.2 $\pm$ 4.4	52.5 $\pm$ 2.3	0.5 $\pm$ 0.1	40.6 $\pm$ 4.3	70.5 $\pm$ 5.9	2.9 $\pm$ 0.7	18.2 $\pm$ 1.5
<i>Etmopterus spinax</i> *	11	41.9 $\pm$ 4.6	9.3 $\pm$ 1.1	164.6 $\pm$ 13.5	54.5 $\pm$ 8.4	0.9 $\pm$ 0.1	78.8 $\pm$ 3.7	60.3 $\pm$ 8.0	1.6 $\pm$ 0.21	19.6 $\pm$ 4.3
<i>Squalus acanthias</i>	10	68.3 $\pm$ 7.2	9.5 $\pm$ 0.8	91.8 $\pm$ 12.9	36.1 $\pm$ 3.2	0.9 $\pm$ 0.2	34.8 $\pm$ 2.4	123.7 $\pm$ 16.0	2.5 $\pm$ 0.5	28.1 $\pm$ 4.2

Enzyme activities analyses

In red muscle, the level of LDH activity (Fig. 3A) was lowest for the shallow-water species, *S. acanthias*. In contrast, deep-sea species displayed similar higher levels in their red muscle, the highest value from *E. granulosus* (ANOVA, $F(9,102)=5.92$, $p<0.05$, Tukey test post hoc). With regard to MDH activity, a gradient is observed between deep-water sharks from the lowest to the highest activity (Fig. 3B): *D. licha* (A), *C. squamosus* (B), *D. calcea* (BC), *S. plunketi* (BC), *C. owstonii* (BC), *E. lucifer* (BCD), *E. granulosus* (CD), *E. spinax* (DE), *E. molleri* (E). The shallow-water species, *S. acanthias* has similar values (BC). Once again, Letters up to the bars describe the significant differences between species (ANOVA, $F(9,102)=19.16$, $p<0.05$, Tukey test post hoc). Finally, the activity of CS in red muscle shows two significant groups (letter symbol) (Fig. 3C): the group 1 (A) with the lowest activity value is represented by *D. licha*, *C. squamosus*, *D. calcea*, *S. plunketi*, *C. owstonii*; and group 2 (B) with *E. lucifer*, *E. granulosus*, *E. spinax*, *E. molleri* and *S. acanthias* has the highest activity values (Wilcoxon, $H(9)=80.56$, $p<0.05$, Post hoc Steel-Dwass).

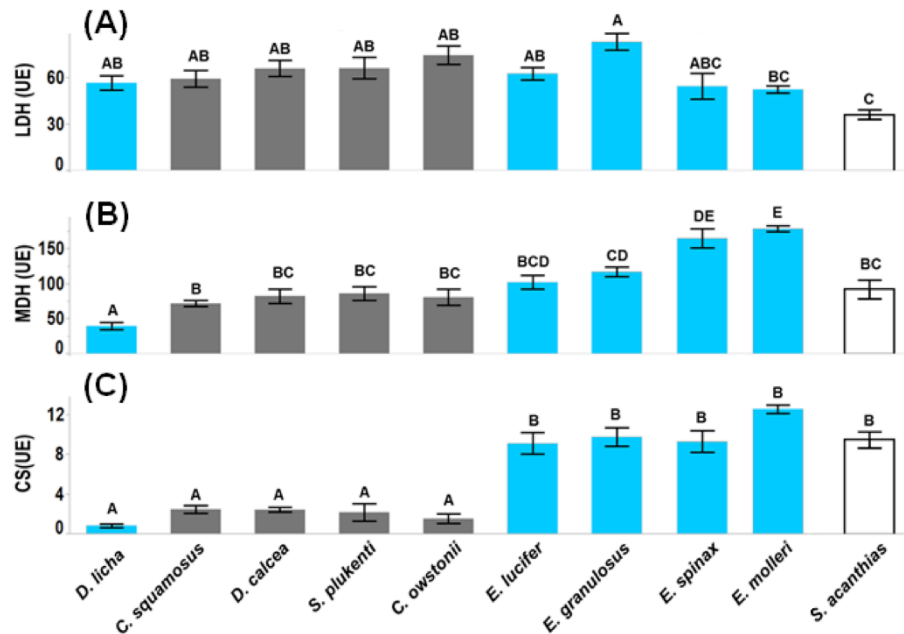


Fig. 3 Red muscle enzyme activities for (A) lactate dehydrogenase, (B) malate dehydrogenase and (C) citrate synthase in nine species of deep-sea sharks and one shallow-water species (*S. acanthias*); Bars represent the mean value of each enzyme activities and error bars correspond to the standard error; Blue bars represent luminous shark species, gray ones correspond to non-luminous species and white bar to the shallow-water shark; Letters up to the bars describe the significant differences between species.

In white muscles, LDH activity is different and the statistical test differentiates four groups (letter symbol) from the lowest to the highest activity (Fig. 4A) (ANOVA, $F(9,102)=8.91$, $p<0.05$, Tukey post hoc test). The first group (A) is composed only of *D. calcea*; then the second group (AB) contains all Etmopteridae; the third group (BCD) is represented by *D. licha*, *C. squamosus* and *S. plunketi*; the fourth group (CD) with *C. owstonii*; and finally the last group (D) with the surface species, *S. acanthias*. The study of MDH activity shows four significantly different groups, from the lowest activity to the highest (Fig. 4B) (Wilcoxon, $H(9)=62.63$, $p<0.05$, Post hoc Steel-Dwass). In the first group (A), with the lowest activity, *D. licha*, *C. squamosus*

and *E. granulosus*; in the second group (AB), *D. calcea*, *S. plunketi*, *C. owstonii*, *E. lucifer* and *S. acanthias*; the third group (B) contains only *E. molleri*; while the last group (C) is represented only by *E. spinax*. Concerning the activity of CS, the statistical test distinguished three significant groups, from the lowest to the highest activity (Fig. 4C) (Wilcoxon, $H(9)=41.02$, $p<0.05$, Post hoc Steel-Dwass). The first (A) represented by *S. plunketi* and *C. owstonii*; then the second intermediate group (AB) which contains the species of *D. licha*, *C. squamosus*, *D. calcea*, *E. granulosus*, and *E. molleri*; Finally, the last group (B) with *E. lucifer*, *E. spinax* and *S. acanthias*.

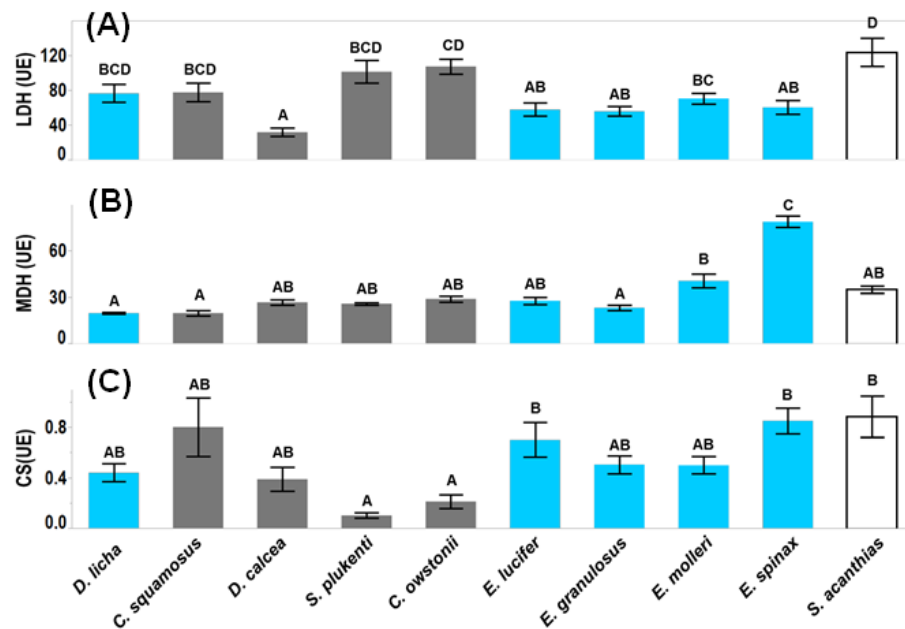


Fig. 4 White muscle enzyme activities for (A) lactate dehydrogenase, (B) malate dehydrogenase and (C) citrate synthase in nine species of deep-sea sharks and one shallow water species (*S. acanthias*); Bars represent the mean value of each enzyme activities and error bars correspond to the standard error; Blue bars represent luminous shark species, gray ones correspond to non-luminous species and white bar to the shallow-water shark; Letters up to the bars describes the significant differences between species.

Relation between cruise swimming speed, muscle proportion, and enzyme activities

For those species where information on cruise swimming speed was available to correlate with enzyme activity, Principal Component Analysis (PCA) revealed that the dispersion of species was mainly related to the axis 1 (49.9% of species dispersion) (Figure 5a), while axis 2 (19.9% of species dispersion) largely explained the dispersion of *C. owstonii* in relation to other deep-sea species. The PCA loading graph indicated how numerical variables scattered the species the correlation between each one (Figure 5b). The loading graph paired with Spearman's correlation test (Figure 5c) revealed that the cruise swimming speed was significant positively correlated with the CS and MDH activity in red muscle, a lower non-significant positive correlation is shown with MDH activity in white muscle, and the red muscle proportion. In contrast, no correlation was apparent between swimming speed and the LDH activity in red and white muscle and the CS activity in white muscle. The white muscle proportion was non-significantly correlated with swimming speed, but negatively this time. Finally, the CS and MDH activities in red muscle displayed a significant positive correlation, while other enzymes did not show any correlation.

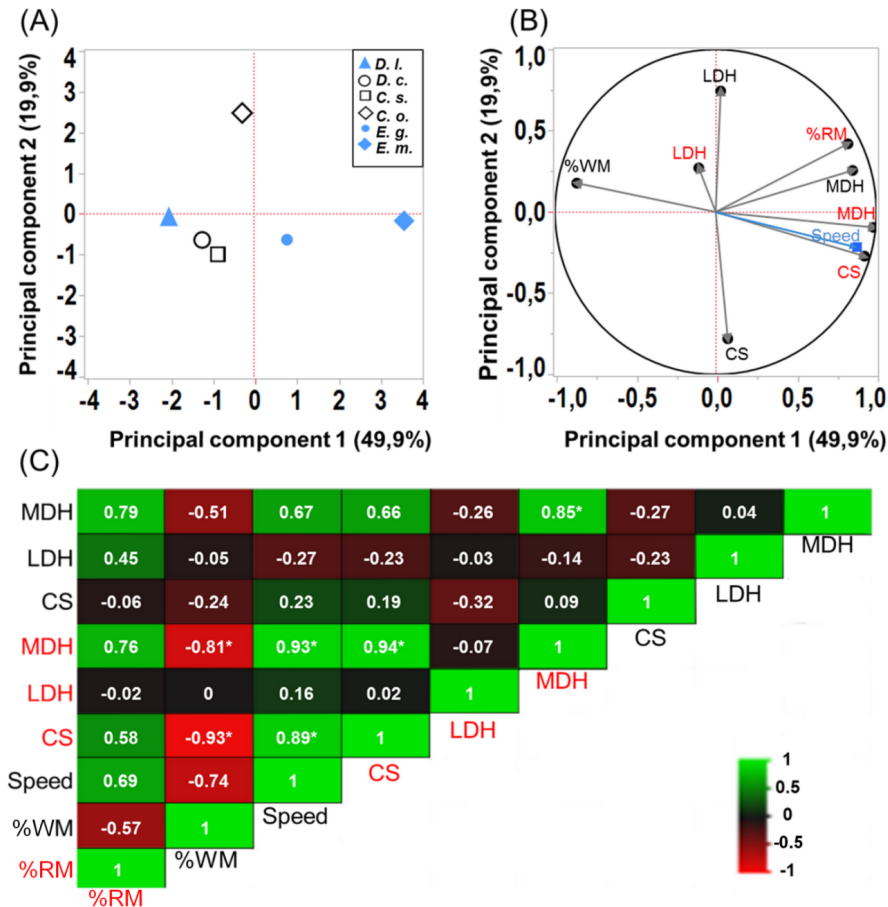


Fig. 5 (A) PCA score graph revealing species dispersion according to eight variables (speed is only a variable projection) where *D.l.*= *Dalatias licha*, *D.c.*= *Deania calcea*, *C.s.*= *Centrophorus squamosus*, *C.o.*= *Centroscymnus owstonii*, *E.g.*= *Etmopterus granulosus*, *E.m.*= *Etmopterus molleri*. (B) PCA loading graph illustrating the influence of eight variables on species dispersion; red color corresponds to red muscle enzymes activities and proportion; black color corresponds to white muscle enzyme activities and proportion; blue axis corresponding to the cruise swimming speed is a projection and does not take part in the species dispersion. (C) Matrix correlation based on Spearman's test; red color corresponds to red muscle enzymes activities and proportion; black color corresponds to white muscle enzymes activities and proportion; value in each square corresponds to the "R", and * reveals the significant differences.

DISCUSSION

Red and white muscle proportion

As for fish, the red muscle proportion of sharks was lower for deep-sea species compared to their shallow-water counterparts (Drazen et al., 2013; Bernal et al., 2003b; Laughlin and Kramer, 1991), apart from two deep-sea species (*Etmopterus molleri* and *Centroscymnus owstoni*). The lowest proportion was recorded for *Dalatias licha* while other sharks display superior values but still inferior to red muscle proportion of deep-sea fishes (Drazen et al., 2013; Laughlin and Kramer, 1991). For fish, it was suggested that lower red muscle proportion indicate sedentary behavior while higher red muscle proportion indicate mobile behavior (Drazen et al., 2013). Furthermore for sharks, it has been recently shown that the proportion of red muscle can be related to the lift provided by the shark liver (Pinte et al., 2019b). Sharks do not possess swim bladder, but a large liver enriched in low-density lipid that mainly provides buoyancy. While shallow-water species are still negatively buoyant, deep-sea sharks are thought to be neutrally or even positively buoyant thanks to their liver providing more lift (Bone and Roberts, 1969, Corner et al. 1969; Wetherbee and Nichols, 2000; Iosilevskii and Papastamatiou, 2016; Gleiss et al. 2017). Bone's hypothesis proposes that sharks that are close to neutral buoyancy should have fewer red aerobic muscle fibers because their tailbeat only need to provide the thrust, whereas sharks that have less hydrostatic lift need to provide thrust and lift with their tailbeat (Bone, 1966; Shadwick and Goldbogen, 2012; Pinte et al., 2019b). In their study, Pinte et al. (2019b) found a negative relationship between the red muscle proportion and the lift provided by the liver for different species and concluded that deep-sea shark with a more buoyant liver displayed less red muscle fiber. In contrast, except for the Etmopteridae, the white muscle proportion was not different between deep-sea species and the shallow-water shark

species. Etmopteridae displayed less white fiber than their deep-sea relatives. These results suggest that deep-sea sharks could have the same burst swim capabilities as shallow-water species except Etmopteridae, which could be less efficient. Finally, the white muscle proportion showed a negative correlation with the red muscle proportion, *i.e.* sharks with more red muscle hold less white fiber and vice versa.

Muscle enzyme activities

In previous studies, the red muscle aerobic activity has been described as relatively similar between species from both deep-sea and shallow-water environments. In this study, results suggested a different conclusion. The MDH activities are similar between non-luminous deep-sea species and *S. acanthias*. However, *D. licha* displayed the lowest significant value compared to other species, and the Etmopteridae had even higher value than *S. acanthias*. The CS activities revealed a stronger difference with lower values recorded for *D. licha* and other non-luminous deep-sea species compared to *S. acanthias* and all Etmopteridae species, which were quite similar. The LDH activities were quite similar between all deep-sea species and higher than the shallow-water species.

Regarding white muscle, the MDH activities were similar between all studied species except *E. molleri*, which displayed the highest value. The CS activities were different between all species but without any clear separation between deep-sea and shallow-water species, or between luminous and non-luminous species. Our results were in agreement with previous studies of both bony and cartilaginous fish, showing that the activities of these both aerobic enzymes were significantly lower than in a red muscle (Childress and Somero, 1979; Sullivan and Somero, 1980; Treberg et al., 2003; Drazen et al., 2011; Condon et al. 2013). This is due to the weaker vascularization of white tissue, and the lower importance of aerobic metabolism in this tissue.

In contrast, the LDH activities were low for *Deania calcea* and Etmopteridae species, whilst the other non-luminous species, *D. licha* and *S. acanthias* had higher values. LDH activities have been mentioned to decrease with the median depth occurrence (MDO) (Condon et al. 2013; Treberg et al., 2016). However, few deep-sea species were used in these studies. In the current study, results showed that some deep-sea sharks displayed values of glycolytic metabolism similar to *S. acanthias*, but still lower compared to values of other shallow-water sharks (Dickson et al., 1988; Dickson et al. 1993; Bernal et al., 2003).

Relation with the cruise swimming speed

This study reports the first measurements of enzyme activities in red and white muscle of deep-sea species combined with the muscle proportions and known cruise swimming speeds. Principal component analysis and correlation matrix revealed a strong positive relationship between the cruise swimming speed of deep-sea species and the CS - MDH activities in red muscle. These two enzymes are also strongly correlated in red muscle, suggesting an efficient Krebs cycle. Etmopteridae species displayed the highest values of these two aerobic enzymes in red muscle, and also swim at the highest cruise speeds. Species in this family use the intensity of their ventral light to appear invisible from prey and predator below; a strategy called “counter-illumination” camouflage (Claes et al. 2010; Claes and Mallefet 2014). Organisms displaying counter-illumination need to change light intensity quickly (Clarke 1963; Young and Roper 1976; Case et al. 1977; Latz 1995). However, luminous sharks regulate their ventral light intensity by slow hormonal control (Claes et al. 2011; Claes and Mallefet 2015; Duchatelet et al. 2019). Therefore, it has been suggested that luminous Etmopteridae may migrate up and down continuously in the water column to match their ventral light intensity with the downwelling light intensity of their environment, the “isolume-follower” hypothesis (Claes and Mallefet, 2014). The higher

cruise swimming velocities measured in this family support this hypothesis (Pinte et al. 2019a) and the results in this study reveal possible physiological adaptations in aerobic metabolism to enable this increased cruise swimming speed. In contrast, *D. licha* swims at the slowest speed recorded for any shark (Pinte et al., 2019a) and the aerobic metabolism reported in this study, especially in the red muscle, was the weakest measured. However, this shark and other non-luminous sharks are thought to be ambush predators on smaller sharks such as the Etmopteridae (Navarro et al., 2014), and should display more effective burst capabilities than Etmopteridae species. The results reported here show a higher proportion of white muscles for *D. licha* and other non-luminous species than Etmopteridae species, with higher activity of LDH in white muscle that supports this hypothesis. *D. licha* has been observed to be capable of a very fast swimming action once close to its prey (see Zintzen et al. 2011 for footage and description of this), but further burst swimming speed observations are required to reveal if anaerobic muscle metabolism in white fiber and the proportion of this fiber could be correlated with burst swimming capability.

CONCLUSION

Our study was one of the first to measure the metabolism of nine deep-sea shark species. Results revealed that: (i) while previous studies have shown non-significant differences of aerobic metabolism between deep-sea shark species and their shallow-water counterparts, this study showed similar aerobic metabolism between Etmopteridae and the shallow-water species studied, but a lower aerobic metabolism in non-luminous deep-sea species and *D. licha* (ii) In contrast, while previous studies have shown a weaker glycolytic metabolism in deep-sea species compared to shallow-water species, this study observed a weaker glycolytic metabolism for

Etmopteridae species whereas non luminous deep-sea species and *D. licha* displayed similar values to *S. acanthias*, the shallow-water example in this study. However, glycolytic values are still lower than other shallow-water pelagic shark species. The aerobic metabolism seems correlated with reported cruise swimming speeds, indicating a possible physiological adaptation for Etmopteridae species to enable the “isolume-follower” hypothesis. Finally, white muscle proportion and glycolytic metabolism of *D. licha* and non-luminous species suggest higher burst capabilities than Etmopteridae but values regarding their burst speed are still required.

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Paper 3:

Deep-sea sharks: Relation between the liver's buoyancy and red aerobic muscle volumes, a new approach

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Deep-sea sharks: relation between the liver's buoyancy and red aerobic muscle volumes, a new approach.

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ABSTRACT

Shark's buoyancy depends on two types of force: (i) the hydrostatic force which is mainly provided by their liver filled with low density lipids and (ii) the hydrodynamic force which is provided by the morphology of their body and fins. Shallow-water shark species are usually negatively buoyant, whereas deep-sea shark species have been suggested to display neutral buoyancy. It has been suggested that species that are close to the neutrality would have less red aerobic muscle fibers. Here, we investigated several liver features (the hepatosomatic index, the oil content and the lipid composition) playing a major role regarding the buoyancy of three deep-sea shark species (*Etmopterus molleri*, *Etmopterus spinax* and *Isistius brasiliensis*) and one shallow-water counterpart (*Galeus melastomus*). We used FT-Raman and FT-MIR spectroscopy to qualify/quantify the lipid composition of their liver. Our results showed that most deep-sea shark species studied have liver features providing more buoyancy than their shallow-water counterparts, apart

from *E. molleri* which shows liver's features that resemble more shallow-water shark species (e.g. *G. melastomus*). Finally, data regarding liver features of several deep-sea shark species from the literature were added and the red aerobic muscle distribution/proportion of nine species was measured, to reveal how these parameters might be related. Our results showed that sharks characterized by a liver providing more hydrostatic force possess proportionally less red aerobic muscles than sharks having a liver that contributes less to their buoyancy. Therefore, our results i.e. deep-sea shark displaying less red aerobic muscle with a liver providing more buoyancy, support low metabolic rates hence slow swimming speed.

KEYWORDS Squaliformes –lipids – liver - muscle - FT-Raman - FT-MIR

INTRODUCTION

The buoyancy of marine organisms remains an essential feature to their locomotion. Buoyancy (positive, negative or neutral) can constitute a good compromise to reduce the energy expenditure during locomotion. For example, the sperm whales use their spermaceti to deep dive at lower energy costs (Clarke et al., 1984), most fishes use a swim bladder to adjust their buoyancy and ease their 3D-locomotion provided by their fins (Phleger, 1998). As regards to sharks, their buoyancy depends on two types of force: the hydrodynamic force is provided by the shape of the body and the fins; and the hydrostatic force depends on the density of the body, that is mainly influenced by the liver of sharks, also previously called the hydrostatic organ (Treberg and Speers-Roesch, 2016). Although previously mentioned in the literature as hydrodynamic and hydrostatic lifts due to the lift they provide to the whole shark body, in this paper, we will refer to the terms hydrostatic and hydrodynamic force. Other tissues may also act on the buoyancy of the shark's body but their implications on the buoyancy remains

equivalent/vary less across species, compared to the role played by the liver (Malins et al., 1965; Sargent et al., 1972; Treberg and Speers-Roesch, 2016).

The relative proportion of the elasmobranch liver, measured through the hepatosomatic index (HSI; the liver weight divided by the body weight) is greater than for other marine organisms, it represents between 10 to 30% of the total body mass for sharks (Corner et al., 1969; Pethybridge et al., 2010; Nakamura et al., 2015). On top of that, the skeleton of sharks is cartilaginous and thus less dense than calcified skeletons (Bone et al., 2008). Therefore, the main factors affecting the hydrostatic force are (i) the mass of the liver, (ii) its oil content (OC) and (iii) the lipid classes in the oil and their respective densities (Shadwick et al., 2016). The shark liver oil is obtained through different lipid extractions. The percentage of OC ranges from 36 to 83% between species (Wetherbee and Nichols, 2000; Pethybridge et al., 2010). Four major lipid classes are mainly found in shark oil: the squalene (HC, hydrocarbon) which is the main hydrocarbon found in shark liver (Tsujimoto, 1916), the triacylglycerides (TAG), the diacylglycerides ether (DAGE) and the wax esters (WE) (Shadwick et al., 2016). All of them provide a specific hydrostatic force thanks to their respective densities which are lower than the surrounding seawater density (1.025g/ml), in order of their relative contribution on the hydrostatic force: squalene (0.8562 g/ml), WE (0.8578 g/ml), DAGE (0.89 g/ml) and TAG (0.92 g/ml) (Bone et al., 2008; Shadwick et al., 2016). They also play a second function as ketone body source which acts as an energy reserve for the shark, apart from squalene that does not display evidences of these functions (Phleger, 1998; Bone et al., 2008; Watson et al., 2015; Shadwick et al., 2016). Studies have shown that osmolytes may also contribute to the hydrostatic force (mainly trimethylamine N-oxide [TMAO] and urea, for sharks) but that this contribution (around 10% of the liver contribution) is less important for deep-sea species than for shallow-water ones

(Treberg and Speers-Roesch, 2016). Moreover, deep-sea shark species tend to have a greater proportion of TMAO than of urea and studies suggested that this accumulation by deep-sea animals may allow the protection of proteins against the destabilizing effects of high hydrostatic pressure (Yancey et al., 2018). Due to the minor role of osmolytes compared to liver features in the hydrostatic force, their contribution was not considered in this study.

Despite this, the buoyancy of shallow-water sharks is still believed as being negative. Indeed, several pelagic species display large pectoral fins that allow them to improve their hydrodynamic force by increasing their lift-drag ratio (Iosilevskii and Papastamatiou, 2016). Others, such as the hammerhead sharks, swim in a rolled manner so that the dorsal fin is approximately perpendicular to the gravity and provides a hydrodynamic force (Payne et al., 2016). Benthic sharks have a highly heterocercal caudal fin (dorsal lobe longer than ventral one), this shape also provides a hydrodynamic force in addition to the thrust (Thompson and Simanek., 1977; Shadwick and Goldbogen, 2012). In contrast, deep-sea shark species are known to be closer to neutral buoyancy (Bone and Roberts, 1969; Corner et al. 1969; Wetherbee and Nichols, 2000; Gleiss et al. 2017), as many of these sharks living in an oligotrophic environment display larger liver that contains higher oil content, richer in low-density lipids than their shallow-water counterparts. Neutral buoyancy would allow them to reduce their energy loss in such environment where food availability is low (Corner et al., 1969; Bakes and Nichols, 1995; Wetherbee and Nichols, 2000; Pethybridge et al., 2010). It has already been shown that the bluntnose sixgill shark (*Hexanchus griseus*) is characterized by positive buoyancy (Nakamura et al., 2015). However, it is important to note that no modelling of the sharks buoyancy has ever been made, implying that all papers to date built their conclusions on indirect measurements, without considering all the features playing a role in the buoyancy of the sharks

(hydrostatic force, hydrodynamic force, tail beat, swimming speed, temperature of the surrounding environment, etc.).

The beat of the caudal fin of the shark is an important feature to consider. Indeed, most sharks have a heterocercal caudal fin providing force during each tailbeat (Thompson et al., 1977; Shadwick and Goldbogen, 2012). This tail motion, taking part in the hydrodynamic force, is directly dependent on the composition of the shark's musculature. Fish muscles are present in higher proportion (40-60% of the total body mass) than for terrestrial vertebrates (20-40% of the total body mass) as they live and move in a denser environment (Bone, 1978). Sharks display two different types of muscle fibers: the white fibers, taking part during burst activities, are powered by the anaerobic metabolism and the red fibers, taking part during cruise swimming activities (main swimming mode used by the sharks) are powered by aerobic metabolism (Bone, 1966; Syme, 2005; Shadwick and Goldbogen, 2012; Shadwick et al., 2016). Bernal et al. in 2003 have shown that the red aerobic muscles distribution across the posterior part (25-95% of the fork length) is in higher proportion around 70% of the fork length (Bone et al., 1978; Bernal et al., 2003). They also evaluated the red aerobic muscles proportion being between 2 and 3% of the total body mass (Bernal et al., 2003). In 1966, Bone hypothesized that sharks that are close to neutral buoyancy should have fewer red aerobic muscle fibers. Indeed, it is difficult for marine organisms to swim slowly when they are negatively buoyant. For this reason and also to have high swimming capabilities, shallow-water sharks developed higher proportion of red aerobic muscle, whereas deep-sea sharks do not need a high proportion for their low swimming capabilities. While this hypothesis has been cited numerous times in the literature, it has never been directly tested through direct evidences (Shadwick and Goldbogen, 2012; Shadwick et al., 2016). Therefore, the study of deep-sea sharks, often

suggested to be close to neutral buoyancy, should allow us to test this hypothesis.

In this work, we investigated the hydrostatic force provided by the liver of three deep-sea shark species (*Isistius brasiliensis*, *Etmopterus molleri* and *Etmopterus spinax*) and one shallow-water counterpart (*Galeus melanostomus*). For this purpose, we measured the HSI of the liver, its oil content (OC) and the diversity and abundance of the lipid classes within the liver oil through Fourier transform Raman (FT-Raman) and Mid-infrared (MIR) spectroscopy. We also completed our results with data from the literature, applying the liver hydrostatic force equation from Treberg and Speers-Roesch (2016) that characterizes the force provided by the liver. Finally, we measured the proportion of red aerobic muscles and described their distribution along the body of the studied species, aiming to correlate the total red aerobic muscle mass with the hydrostatic force provided by the liver and get a first experimental evidence supporting the hypothesis made by Bone in 1966.

MATERIAL AND METHODS

Samples

The individuals *Etmopterus spinax* and *Galeus melastomus* were collected with deep longlines at ~220 meters depth in the Raunefjord (Norway; 60.260623°N; 5.162056°E), during scientific surveys in January 2016 and February 2017. Specimens were then directly brought back to the Marine Research Field Station (Espegrend, Bergen University, Norway) and transferred into tanks maintained at 6°C in a dark room. Sharks were euthanized following the local rules for experimental vertebrate care (Permit 12/14048). The liver was extracted and stored at -80°C. The individuals *Etmopterus molleri* were caught by a longline at 500 meters depth in the East China sea (Japan; 26.555543°N; 127.713359°E), in November 2016 and November 2017. Samplings and ethical

procedures are similar than sharks from Norway. Finally, the individuals *Isistius brasiliensis* (N=6) come from by-catch provided by fisherman from La Reunion island. Individuals were stored directly at -80 °C.

For each individual, the liver weight, total body weight, size of the body, sex and reproductive status were determined. The liver was collected for 44 samples: 13 *E. spinax*, 7 *G. melastomus*, 5 *I. brasiliensis* and 19 *E. molleri*. The data are summarized in table 1. All individuals selected in this study were mature to avoid bias due to maturity stages. Given the low proportion of females in this study and the fact that in the data from literature, no information on sex is provided, the difference between females and males has not been taken into account. However, preliminary tests on *E. spinax* indicate that there is a difference of squalene concentration between pregnant and non-pregnant females but no difference between males and females tested for squalene and the HSI (Supplementary data 1).

Table 1 Sampling data summarize

Species	Total length (cm)	Weight (g)	Liver weight (g)	Sex ratio (M:F)
<i>E. spinax</i>	44,35 ± 1,51	367,85 ± 43,65	75,39 ± 9,66	4:9
<i>G. melastomus</i>	/	494,83 ± 36,65	50,61 ± 6,03	6:2
<i>I. brasiliensis</i>	33,19 ± 2,15	166,85 ± 36,87	30,48 ± 5,89	4:1
<i>E. molleri</i>	41,75 ± 1,02	199,52 ± 13,36	11,58 ± 1,15	15:4

In addition, other deep-sea shark species were collected only to obtain the red aerobic muscles quantification and distribution. These six other species (*Centrophorus squamosus*, *Deania calcea*, *Centroscymnus owstonii*, *Etmopterus granulosus*, *Dalatias licha* and *Squalus acanthias*) were collected as by-catch fishes during the Hoki survey, NIWA scientific cruise from January to February 2018 aboard the RV Tangaroa.

Spectroscopic analysis: Sample preparation

Liver oil was extracted based on Folch's protocol (lipid extraction) (Folch et al., 1957). Preliminary, 1g of the anterior part and 1g of the posterior part of the liver were collected from complete livers (different lobes included) maintained at -80°C. Both parts of the liver were then mixed together and this process was repeated in triplicate on the liver of each individual. Folch's protocol was then applied to each sample individually. Homogenates were centrifuged in chloroform/methanol (2:1) solution and the supernatant containing the lipids was then extracted. Methanol contamination was removed in a saline phase and at the end of the process, samples were left overnight in an incubator at 40°C to allow the total evaporation of the chloroform. Finally, the total volume of the lipids extracted from the 2g-liver sample was measured to approximate the percentage of lipids present in the whole liver as percentage of volume by weight of tissue. 500µl of the lipid extract was then transferred in a closed glass cylinder and maintained at 4°C until analysis.

FT-MIR analysis

IR absorption spectra were acquired using a FT-MIR (Fourier Transform Mid Infrared) spectrometer VERTEX 70 (Bruker, Germany) connected to a diamond attenuated total reflectance accessory (diamond ATR) on which are presented the oils. Absorbance spectra are obtained using OPUS 7.2 software (Bruker Optics Inc., Billerica, MA, USA) in a range of 4000-

600cm⁻¹ and 64 co-added scans were acquired with a spectral resolution of 4 cm⁻¹. Three replicates were collected for each sample and spectral backgrounds were acquired between each triplicate with a cleaning step between each analysis. Air was considered as a background.

FT-Raman analysis

Fourier transform Raman (FT-Raman) spectra were acquired using a FTIR (Fourier Transform Infrared) spectrometer VERTEX 70 (Bruker, Germany) connected to a module Ram II. Raman scattering was generated using a 1064nm diode laser. A laser power of 600mW was used. FT-Raman spectra were measured in the range of 3600-200cm⁻¹ with a spectral resolution of 4cm⁻¹ (128 scans were co-added per spectrum), using OPUS 7.2 software (Bruker Optics Inc., Billerica, MA, USA). Liquid samples of liver lipid extracts (500µL) were presented in glass tubes and analyzed in duplicate.

Spectral pre-processing and multivariate analysis

Qualitative analysis

Unscrambler v10.2 software from CAMO (Computer Aided Modelling, Trondheim, Norway) was used to perform spectral pre-processing and multivariate analysis. To be able to identify each absorption band of the mean spectrum of each species, the spectra of several standards were first analyzed (Squalene, Triolein, Tripalmitin, Dipalmitin and Chloroform, all sourced from Sigma-Aldrich, Belgium). Following spectral analyses and absorption bands identifications, the peaks relevant for the different lipid classes of interest (squalene, TAG and DAGE) were selected for multivariate analysis. In this regard, FT-Raman spectra were used to generate a PCA by selecting the ranges of the spectra containing the peaks associated to squalene (1667cm⁻¹, from 1765 to 1628cm⁻¹; 1382 and 1282cm⁻¹, from 1404 to 1233cm⁻¹) (Baeten et al., 2001), allowing to

separate the samples based on this component. FT-MIR spectra were used to generate a PCA based on the ranges of the spectra associated with peaks corresponding to DAGE and TAG (3380cm^{-1} , 1740cm^{-1} and 1118cm^{-1}).

Quantitative analysis

Squalene concentration was measured using partial least square regression (PLS-R), as usually used in the literature (Tewari et al., 2004; Cozzolino et al., 2005; Meissl et al., 2007; Hall et al., 2016). For this purpose, six different squalene concentrations were made by serial dilution (100%, 50%, 25%, 12.5%, 6.25%, 3.125%) and used to allow the calibration of the PLS-R model. Several spectral pre-processing were tested (no pre-processing, standard normal variate and first derivative) and their performance were compared by measuring the root mean standard error cross validation (RMSECV) and the determination coefficient (R^2) of each model. Based on these data, PLS-R without any pre-treatment was selected among the three models.

TAG and DAGE quantification was uncertain because peaks used to describe these lipid classes display a large, unspecific range (wide band at 3300cm^{-1}), were localized in the fingerprint region (many information; 1118cm^{-1}) or were common to both classes (TAG and DAGE contain C=O groups; 1744cm^{-1}).

Red aerobic muscle distribution and quantification

The protocol used for red aerobic muscles quantification and distribution is identical to the one from Bernal et al. in 2003, to allow the comparison of our results with the literature. Ten different species (*E. spinax*, *E. molleri*, *E. granulosus*, *I. brasiliensis*, *D. licha*, *D. calcea*, *C. squamosus*, *C. owstonii*, *S. plukenti* and *S. acanthias*) were analyzed with 3 to 4 specimens by species. We started to cut the transverse sections from 25% of the fork length (FL) because the quantity of red aerobic

muscles was insignificant at the anterior part of the body. Red aerobic muscles are also found in the pectoral fins but do not generate thrust and thus were not considered in this study. Each transverse section measured 5% of the FL, until the caudal peduncle (85-95% FL) was photographed on their anterior and posterior sides.

Pictures were analyzed with ImageJ2[®] software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2018) to measure the red aerobic muscles area on the anterior and posterior sides of the section. The longitudinal red aerobic muscle distribution was obtained by transforming the area (cm²) measured at 50% of the FL as a reference value of 1. Other positions along the body are thus calculated accordingly to this reference point. The red aerobic muscle volume for each cross section is the product of the section thickness and its red aerobic muscles area (mean between the anterior and the posterior area of the transverse section). The red aerobic muscle mass was measured as the product of red aerobic muscle volume and red aerobic muscles density (literature value confirmed on our shark samples: 1.05g.cm⁻³). The quantification of the total red aerobic muscle mass (TRMM) was determined by the sum of each section mass value. Finally, this TRMM is compared with the total body mass to quantify the red aerobic muscles proportion. This methodology was validated by removing and weighing the red aerobic muscles of four *I. brasiliensis* previously analyzed.

Buoyancy index

Due to the lack of an existing 'model of buoyancy', we used a proxy to evaluate the contribution of the shark's liver to the hydrostatic force (Treberg and Speers-Roesch, 2016). This proxy relies on (1) the hepatosomatic index (HIS, % of liver mass according to the total body mass); (2) the oil content of the liver (OC, % of lipid oil by wet mass); and (3) the liver lipid

density (LLD, corresponding to the weighting of each lipid class proportion and their respective density).

$$(1) HSI (\%) = \left(\frac{\text{Liver mass (g)}}{\text{Total body mass (g)}} \right) \times 100$$

$$(2) OC (\%) = \left(\frac{\text{Total lipid volume (ml)}}{\text{liver mass (g)}} \right) \times 100$$

$$(3) LLD = (0.92 \times \%TAG) + (0.89 \times \%DAGE) + (0.86 \times \%HC) + (0.86 \times \%WE)$$

By combining the HSI and the OC we calculated the liver lipid (g.kg⁻¹ of shark) and finally inferred the hydrostatic force provided by the liver (Liver Hydrostatic Force -LHF) of the shark by the following equation (4), as previously mentioned by Treberg and Speers-Roesch, 2016:

$$(4) LHF = \left(\frac{\text{liver lipid (g.kg}^{-1} \text{ of shark)}}{LLD \text{ (g.ml}^{-1})} \right) \times (\text{sea water density (g.ml}^{-1}) - LLD \text{ (g.ml}^{-1}))$$

Where sea water density is 1.025 g.ml⁻¹.

Based on this proxy, a shark with a large liver that is rich in oil of low density lipids will display a high LHF value from its liver (deep-sea squaloid sharks were evaluated with a LHF value around 35g per kg of shark by Treberg and Speers-Roesch, 2016). In contrast, a shark with a small liver with low oil content and rich in high density lipids will show a low LHF value (shallow-water squaloid sharks were evaluated with a LHF value around 7g per kg of shark by Treberg and Speers-Roesch, 2016). The hydrostatic force contribution of shark livers was tested and validated using overall tissue density and liver density of sharks from the literature (Treberg and Speers-Roesch, 2016; Gleiss et al. 2017). Strong linear relations were obtained with r²= 0.84 between the LHF and the overall tissue

density (Linear regression, $F_{1, 10} = 2912.84$, $P < 0.001$) and $r^2 = 0.80$ between the LHF and the liver density (Linear regression, $F_{1, 10} = 2912.84$, $P < 0.001$) (additional information and data available in supplementary data 2 and 3). For four species (*E. spinax*, *E. molleri*, *I. brasiliensis* and *G. melastomus*) the quantitative analysis of DAGE and TAG was not possible. Only semi-quantitative values were obtained, with a prevalence of one or the other lipid class. Therefore, every analysis was based on with the minimum and maximum concentration of each lipid class and graph representations were done using a 50/50 proportion of each class. The LHF values calculated from our results and literature data (*E. granulosus*, *D. licha*, *C. owstonii*, *C. squamosus*, *D. calcea*, *S. acanthias*, *P. glauca*, *L. ditropis* and *Alopidae sp.*) were related to the median depth of occurrence corresponding to the mean between the maximal and the minimal recorded depth for each species (Ebert et al., 2013).

Statistical analyses

All univariate statistical analyses (univariate parametric test) were performed with JMP pro 14 (JMP®, Version <14>. SAS Institute Inc., Cary, NC, 1989-2007). The normality and the homoscedasticity were confirmed for each parametric test.

RESULTS

All data collected from our research and literatures are summarized in the table 2, detail data for the liver hydrostatic force calculation are in the supplementary data 3.

Lipid composition: spectroscopic analyses

The mean spectra obtained by FT-Raman and FT-MIR spectroscopy are presented on Fig. 1. Letters allow the differentiation between the spectral regions of interest based on their molecular vibrations. Each absorption band was identified

based on its molecular vibrations and the preliminary analyses of the spectra of the standards (Table 3).

The complete analysis of the spectra of the standards and the samples allowed the identification of the spectral region of interest (containing the three relevant lipid classes: squalene, TAG and DAGE) in FT-Raman and FT-MIR data. In this way, on one hand, FT-Raman spectroscopy allowed a better resolution of the peaks associated to squalene (1667cm^{-1} , corresponding to the additive intensity of the 6 double bonds $\text{C}=\text{C}$; 1382cm^{-1} , corresponding to several molecular vibrations such as stretching of $\text{C}-\text{C}$; 1282cm^{-1} , corresponding to bending vibrations of the methyl or methylene group) (Baeten et al., 2001). On the other hand, FT-MIR spectroscopy allowed the distinction of the peaks associated to the DAGE (wide band around 3300cm^{-1} and peak at 1118cm^{-1} corresponding to $\text{O}-\text{H}$ and $\text{C}-\text{O}$ stretching vibrations, respectively) and to the TAG (1744cm^{-1} , corresponding to $\text{C}=\text{O}$ stretching vibrations) (Vermeulen et al., 2015).

Table 2 Summary of data collected from this study and the literature on the 14 species of sharks; median depth occurrence (MDO) corresponds to the mean between the maximum and the minimum depth where shark was observed (m), Hepatosomatic index (HSI % $\pm$ SD), Oil content correspond to the percentage of lipid g wet mass⁻¹ (OC % $\pm$ SD), Percentage composition of main lipid classes in shark liver: TAG = Triacylglyceride, DAGE= Diacylglyceride, HC= Hydrocarbon, mainly squalene (Tsujiimoto, 1916) and WE= Wax Ester (% $\pm$ SD), g of hydrostatic force per kg of shark (LHF), Red aerobic muscle position (RML) where HLP = Highly Lateral and Posterior and PL= Posterior and Lateral, proportion of red aerobic muscles (RM % $\pm$ SD), Ref. corresponds to the literature sources: ¹ Wetherbee and Nichols, 2000; ² Pethybridge et al., 2010; ³ Bernal et al., 2003; ⁴ Jayasinghe et al., 2003a; ⁵ Jayasinghe et al., 2003b; ⁶ Sepulveda et al. 2005; ⁷ Treberg and Speers-Roesch 2016; ⁸ Mull et al. 2013.

Order/species	Habitat	MDO (m)	HSI (%)	OC (%)	TAG (%)	DAGE (%)	HC (%)	WE (%)	LHF (g kg ⁻¹ of shark)	RML	%RM	Ref.
<i>Squaliformes</i>												
<i>Isistius brasiliensis</i>	deep	1793	18.8 $\pm$ 0.6	71.5 $\pm$ 4.9	<29	>29	41.9 $\pm$ 1.6	<1	20.8	HPL	1.14	Present study
<i>Dalatias licha</i>	deep	918	20.9 $\pm$ 5.2	79 $\pm$ 9	2 $\pm$ 2	18 $\pm$ 13	79 $\pm$ 13	<1	30.1	PL	0.84 $\pm$ 0.30	Present study, 1,2
<i>Etmopterus spinax</i>	deep	1035	20.2 $\pm$ 0.9	69.7 $\pm$ 1.9	<31	>31	36.9 $\pm$ 1.8	<1	21.3	PL	1.57	Present study
<i>Etmopterus granulosus</i>	deep	860	18.7 $\pm$ 2.7	72 $\pm$ 7	9 $\pm$ 7	35 $\pm$ 11	55 $\pm$ 12	<1	22.8	PL	1.73 $\pm$ 0.36	Present study, 1,2
<i>Etmopterus molleri</i>	deep	447	5.9 $\pm$ 0.5	51.2 $\pm$ 2.4	<50	>50	<1	<1	3.8	PL	2.89 $\pm$ 0.67	Present study

<i>Centroscyrmnus owstonii</i>	deep	805	20.1±0.2	65±5	22±7	23±8	54±13	<1	21.1	PL	2.47±0.21	Present study, 1,2
<i>Deania calcea</i>	deep	770	19.3±1.3	81±6	9±5	24±6	67±8	<1	27.1	PL	1.21±0.14	Present study, 1,2
<i>Centrophorus squamosus</i>	deep	1315	21.4	83±3	18±5	11±6	70±6	<1	30.2	PL	1.84±0.08	Present study, 1,2
<i>Squalus acanthias</i>	shallow	300	8.7±2.5	41.3±4.6	74.7±4.3	15.7±1.2	<1	<1	3.9	PL	2.54±0.52	Present study, 1,2
Carcharhiniformes												
<i>Galeus melastomus</i>	shallow	528	10±0.6	69.9±1.9	>50	<50	<1	<1	8.9	/	/	Present study
<i>Prionace glauca</i>	shallow	175	6.7±1.1	49.9±0.6	72.3±1.1	2.7±0.4	11±0.4	<1	3.9	PL	2.65±0.56	3,4
Lamniformes												
<i>Lamna ditropis</i>	shallow	188	7.3±0.9	48.3±10	80.4±4.6	2.8±1.4	4.4±1.8	<1	3.9	AC	2.09	3,5
<i>Alopias superciliosus</i>	shallow	200	5.4±1.2	36±0.5	80.7±1.4	1.1±0.3	5±0.5	<1	2.2	AC	2.31±0.21	4,6
<i>Carcharodon carcharias</i>	shallow	650	12.7±3.1	63±15.5	93.3	<1	<1	<1	7.9	AC	2.06±0.12	3,7,8

Table 3 FT-Raman and FT-MIR absorption bands identification of the mean spectra of the four studied species, based on bands identification of the standards (O'Connor et al., 1955; Baeten, 1998; Socrates, 2001; Chun et al., 2013; Czamara et al., 2014; Laachari et al., 2015; Hall et al., 2016).

Region	Wavenumber (cm ⁻¹)								Vibration
	Em		Es		Ib		Gm		
	Raman	MIR	Raman	MIR	Raman	MIR	Raman	MIR	
A		~3300		~3300		~3300		~3300	Stretching O-H
B	3009	3007	3009	3003	3009	3003	3014	3011	Stretching C-H
B	2924	2921	2927	2921		2921	2924	2921	Symetrical Stretching CH ₃ / A: CH ₂
B			2913		2913				Symetrical Stretching CH ₃ / A: CH ₂
B	2893		2893		2893		2893		Asymetrical Stretching CH ₃
B	2875		2875		2875		2875		Asymetrical Stretching CH ₂
B	2852	2852	2852	2852	2852	2852	2852	2852	Symetrical Stretching CH ₂
C	1746	1744	1746	1744	1746	1744	1746	1744	Stretching C=O
D			1667	1668	1667	1668			Stretching <i>trans</i> C=C
D	1657	1655					1657	1655	Stretching <i>cis</i> C=C
E	1451	1460	1451	1456	1451	1452	1451	1430	Bending CH ₂ (Scissoring)
E	1439		1436		1439		1439		Bending CH ₂ (Scissoring)
									Bending CH ₃ symetrical/CH ₂ (Wagging)
E			1382		1382				C-H (Wagging)/Stretching C-C
E		1377		1377		1377		1377	Bending CH ₃ symetrical/CH ₂
E			1329		1329				Bending CH ₂ (Wagging)
E	1301		1303		1303		1301		Bending CH ₂ (Twisting)
E			1282		1282				Bending CH ₃ symetrical/CH (Twisting)
E	1265		1265		1265		1265		Bending =CH
F		1238, 1157		1232, 1157		1234, 1157		1230, 1157	Stretching C-C
G		1118		1118		1118		1118	Stretching C-O
F	1079, etc.	1097, etc.	1079, etc.	1097, etc.	1079, etc.	1097, etc.	1079, etc.	1097, etc.	Stretching C-C
F				832		832			Bending C-C (Rocking)

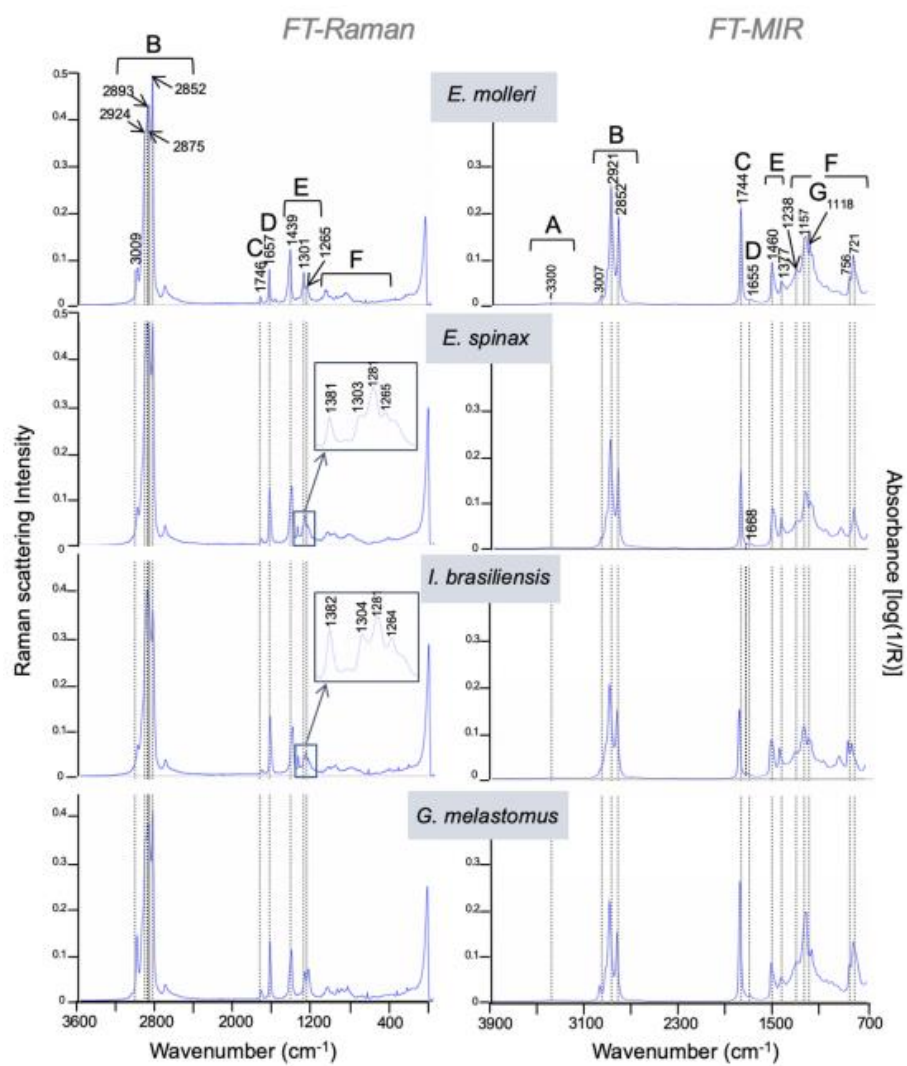


Fig 1 Mean spectra of the four studied species obtained with FT-Raman and IR. The letters (A, B, C, D, E, F, G) separate the spectral region of interest.

Qualitative analyses

PCA models have been developed on FT-Raman and FT-MIR spectral data of studied species without application of any chemometric pre-treatment. In FT-Raman, a PCA realized on the spectral ranges containing the peaks specific to squalene allowed a reliable differentiation between the squalene content of the several studied species (Fig. 2AB). The analysis of these results shows that the species that are richer in squalene are *Isistius brasiliensis* and *Etmopterus spinax*. More specifically, the direct observation of the peaks associated to squalene (1669 , 1382 and 1282cm^{-1}) pre-treated as first and second derivative showed that these two former species are the only out of the four studied species containing squalene. The results obtained in FT-MIR, by selecting the three peaks characterizing DAGEs and TAGs (Fig. 2CD) show that *Galeus melastomus* contains more TAGs (1743cm^{-1} , C=O stretching vibrations) and that *Etmopterus molleri* contains more DAGEs (1118cm^{-1} , O-H stretching vibrations that is absent in TAGs and in the main fatty acids found in DAGE and TAGs in animal oils (O'Connor et al., 1955; Sargent et al., 1972; Deprez et al., 1990; Bakes and Nichols, 1995; Davidson et al., 2002; Pethybridge et al., 2010; Czamara et al., 2014).

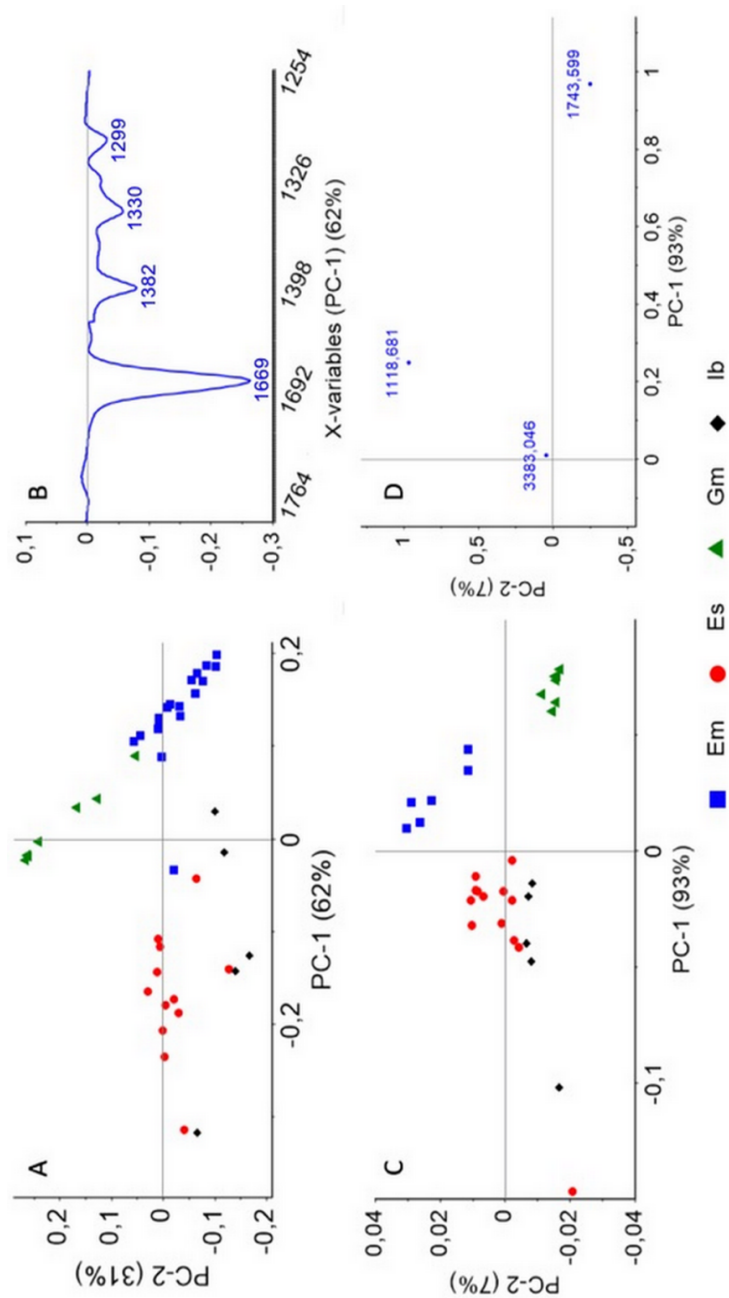


Fig. 2 Scores (PCA) and loadings obtained (**A,B**) by selecting the FT-Raman spectral ranges containing the peaks of squalene (from 1765.16 to 1628.2 cm^{-1} and 1404.5 to 1233.8 cm^{-1}); (**C,D**) by selecting the FT-MIR spectral peaks (3383.5, 1743.8 and 1118.8 cm^{-1}). Each dot represents one individual. *Em*, *Etmopterus mollerii*; *Es*, *Etmopterus spinax*; *Gm*, *Galeus melastomus*; *lb*, *Isistius brasiliensis*.

Quantitative analyses

PLS-R was based on FT-Raman spectra of six different squalene samples with known concentrations. The model was then applied on studied species to quantify the squalene concentration in the liver oil samples (Fig. 3B). No pre-treatment has been applied and only spectral regions characteristic of squalene were considered ($1692\text{-}1638\text{cm}^{-1}$ (peak at 1667cm^{-1}), $1392\text{-}1368\text{cm}^{-1}$ (peak at 1382cm^{-1}) and $1291\text{-}1272\text{cm}^{-1}$ (peak at 1282cm^{-1}). Developed regression model showed a very high coefficient of determination ($R^2 = 0.99$) and a root mean square error of cross-validation RMSECV equal to 2.3. Results showed that *Isistius brasiliensis* contains ~41.9% of squalene in its liver oil, followed by *Etmopterus spinax* with 36.9% then by both *Etmopterus molleri* and *Galeus melastomus* which showed values close to 0% (Fig. 3A).

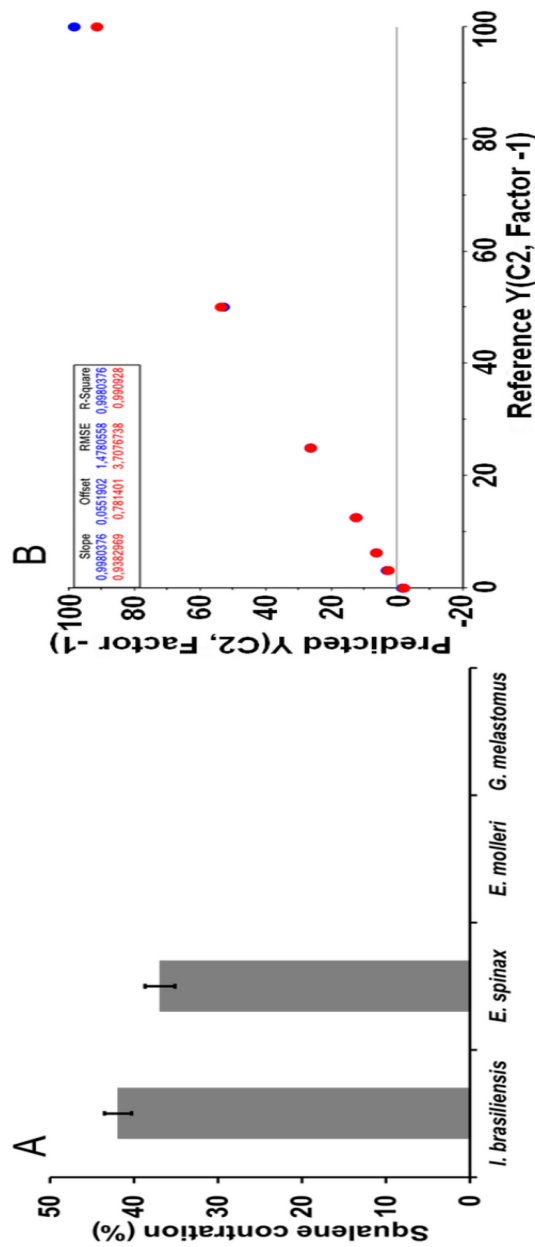


Fig. 3 (A) Squalene concentration of the liver oil lipid fraction of each shark species studied. Quantification was possible through PLS-R; **(B)** PLS regression on spectral ranges containing the peaks of squalene (1667 cm^{-1} : from 1692 to 1638 cm^{-1} ; 1382 cm^{-1} : from 1392 to 1368 cm^{-1} ; 1282 cm^{-1} : from 1291 to 1272 cm^{-1}) from six different squalene concentration samples (100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.125 %). In blue, the calibration dots; in red, the cross-validation dots. RMSE, Root Mean Square Error.

Contribution of the liver hydrostatic force according to the Median Depth of Occurrence (MDO)

Statistical analysis of the contribution of the liver to the hydrostatic force reveals a strong difference between deep-sea sharks (e.g. living mostly under 200 meters depth) and shallow-water sharks (e.g. living mostly above 200 meters depth). Deep-sea sharks have a higher mean value (22.16 ± 2.97 g per kg of shark) than mean value of shallow-water counterparts (5.12 ± 1.09 g per kg of shark) (Student's t test, t_{13} , $P < 0.001$) (Fig. 4A). One deep-sea species, *E. molleri*, is clearly an outlier with a lower value than other deep-sea species (Fig. 4A). The linear regression between the hydrostatic force provided by the liver and the MDO is significant (Linear regression, $r^2 = 0.59$, $F_{1, 13} = 1524.53$, $P < 0.05$) (Fig. 4B). The following equation, $LHF = -2.09 + (0.02 \times MDO)$, shows a linear increase with depth.

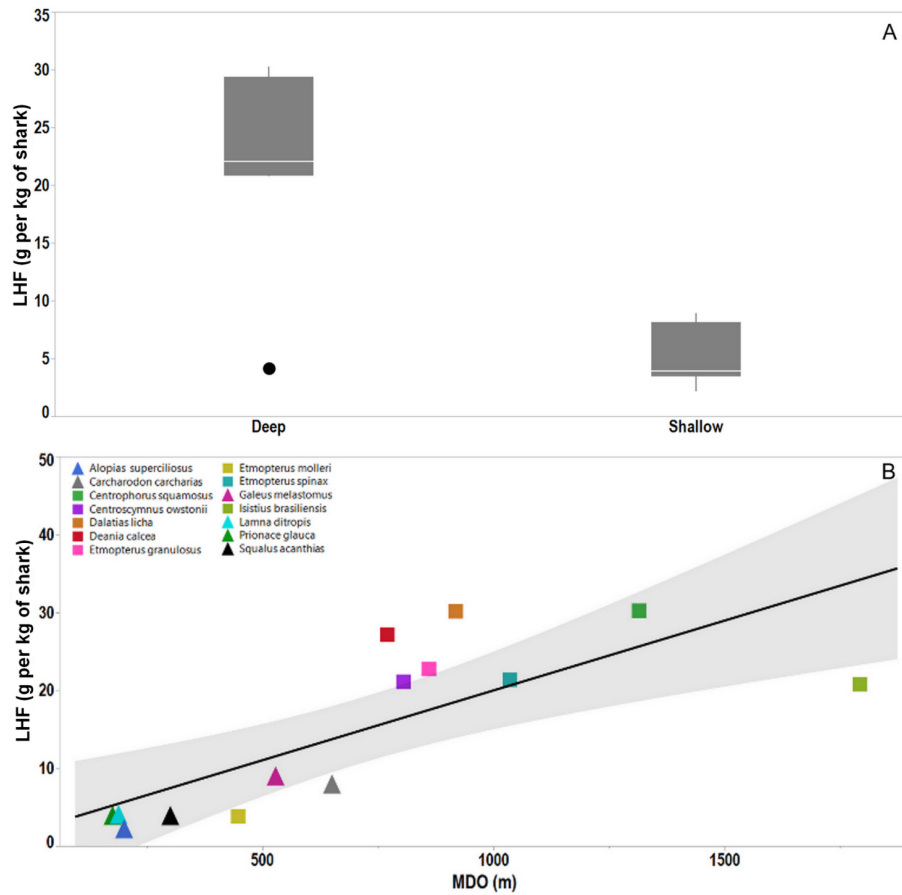


Fig. 4 (A) Boxplot representation of the liver hydrostatic force (LHF – g kg⁻¹ of shark) according to their habitat; “Deep” corresponds to deep-sea sharks (e.g. living mainly under 200 meters depth: *C.squamosus*, *C. owstonii*, *D. licha*, *D. calcea*, *E. granulosus*, *E. molleri*, *E. spinax*, *I. brasiliensis*), “Surface” correspond to shallow-water sharks (e.g. living mainly above 200 meters depth: *A. superciliosus*, *C. carcharias*, *G. melastomus*, *L. ditropis*, *P. glauca*, *S. acanthias*) ; (B) Linear regression of the liver hydrostatic force (LHF – g kg⁻¹ of shark) according to the median depth of occurrence (MDO) of 14 different shark species (cfrs. graph legend), “MDO” corresponds to the mean between the maximal and the minimal depth recorded for each shark species and is expressed in meters ; ▲ corresponds to shallow-water sharks and ■ corresponds to deep-sea sharks. Dark grey region corresponds to the confidence region (0.95) for the fitted line.

Relationship between the liver hydrostatic force and the overall tissues density

The regression between the liver hydrostatic force and the overall tissues density was significant (Linear regression, $r^2=0.84$, $F_{1, 10} = 2912.84$, $P < 0.001$). Using Fig.5, it is easy to see which species are close to neutrality in seawater. Indeed, the average density of seawater is 1025Kg/m³, many deep-sea species (*D. calcea*, *E. pinceps*, *C. coelopsis*, *C. squamosus*, and *D. licha*) and a surface species (*C. maximus*) approach this value, while the other surface species (*C. falciformis*, *C. plumbeus*, *P. glauca*, *S. canicula*, and *S. acanthias*) have higher values and therefore have negative buoyancy.

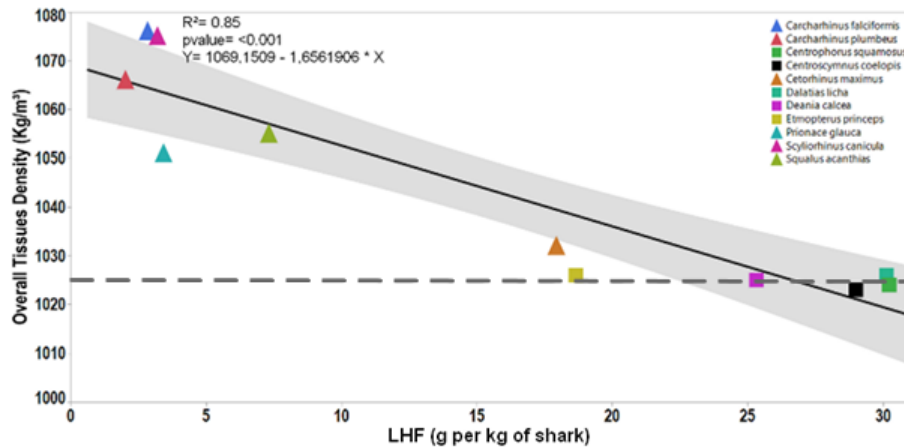


Fig. 5 Linear regression of the Liver Hydrostatic Force (LHF – g kg⁻¹ of shark) according to the overall tissues density (kg/m³) of 11 different shark species (see graph legend). ▲ corresponds to shallow-water sharks and ■ corresponds to deep-sea sharks. Dark grey region represents the confidence region (0.95) for the fitted line; Dotted gray line corresponds to sea water density.

Red aerobic muscle distribution and red aerobic muscle mass

All sharks display a posterior lateral (PL) distribution of their red aerobic muscles. *E. mollerii*, *E. granulosus*, *C. squamosus*, *D. calcea*, *D. licha*, *C. owstonii* and *S. acanthias* display a similar red aerobic muscle distribution pattern, with contents increasing along the fork length to reach their maximal values at 55%, 65%, 70%, 65%, 60%, 70% and 65%, respectively (Fig.5 A-G for deep-sea species and H for shallow-water one). For all these species, the red aerobic muscle proportion rapidly declines to zero at fork length values between 85% and 90%. While *Isistius brasiliensis* displays a red aerobic muscle distribution slightly different, red aerobic muscle proportions reach a maximum around 85% of the fork length with a small proportion remaining at the end of the fork. We therefore characterized this distribution as highly posterior lateral (HPL). Mean values of red aerobic muscles proportions are summarized in Table 2; mean value analysis shows a significant difference between deep-sea sharks displaying fewer red aerobic muscles ($1.71 \pm 0.69\%$ RM) than their shallow-water counterparts ($2.4 \pm 0.25\%$ RM) (Student's t test, t_{10} , $P < 0.001$). Finally, two of the eight deep-sea shark species, *E. mollerii* and *C. owstonii*, show a red aerobic muscle mass that is more characteristic of shallow-water species ($2.64 \pm 0.67\%$ RM and $2.47 \pm 0.21\%$ RM, respectively).

Contribution of the liver hydrostatic force according to the red aerobic muscle proportion

The linear regression between the hydrostatic force of the liver and the red aerobic muscle proportion is significant (Linear regression, $r^2 = 0.59$, $F_{1, 13} = 4.79$, $P < 0.05$). The following equation, $LHF = 2.62 - (0.04 \times \text{red aerobic muscle proportion})$, reveals a linear decrease with the red aerobic muscle proportion (Fig. 6).

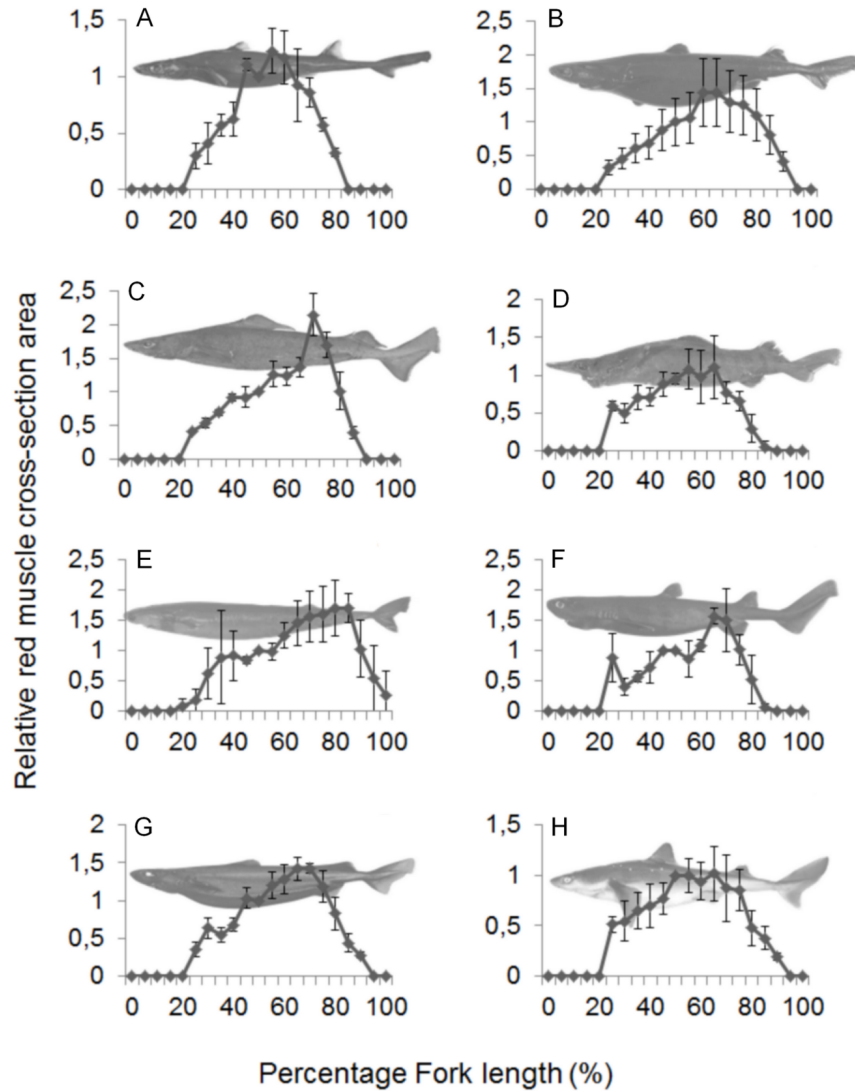


Fig. 5. Red muscle (RM) distribution pattern in seven deep-sea shark species **(A-G)** and on shallow-water shark species **(H)**. All sharks have posterior and lateral distribution of their RM (PA): **(A)** *Etmopterus mollerii* ; **(B)** *Etmopterus granulosus* ; **(C)** *Centrophorus squamosus* ; **(D)** *Deania calcea* ; **(E)** *Isistius brasiliensis* ; **(F)** *Dalatias licha* ; **(G)** *Centroscyrnus owstonii* ; **(H)** *Squalus acanthias*. The relative amounts of RM in the different positions along the body are expressed as a proportion of the RM cross-sectional area equal to 1 at 50 % fork length. Values are means $\pm$ S.E.M. Shark illustrations are modified from fishes of Australia.

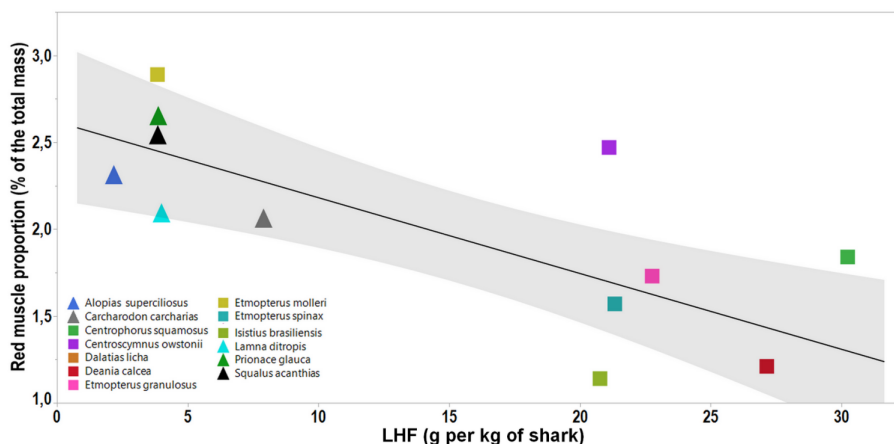


Fig. 6 Linear regression of the liver hydrostatic force (LHF – g kg⁻¹ of shark) according to the red muscle proportion of 13 different shark species (see graph legend). ▲ corresponds to shallow-water sharks and ■ corresponds to deep-sea sharks. Dark grey region represents the confidence region (0.95) for the fitted line.

DISCUSSION

Our study highlights the benefits of spectroscopic techniques for shark liver oil analysis, as a rapid, non-destructive, reliable and less laborious technique than conventional ones (Abbas et al., 2009; McGoverin et al., 2009; Chiu et al., 2017; Tiryaki et al., 2017). Previous studies on shark liver oil focused on squalene determination (Chun et al., 2013; Hall et al., 2016) but ours allows to complete the previous spectral analysis by the characterization of the absorption bands specific to squalene (FT-Raman), DAGEs and TAGs (FT-MIR), also highlighting the complementarity of these two techniques. However, quantification was only possible for squalene (PLS-R) while FT-MIR spectroscopy only allowed a semi-quantitative determination of TAGs and DAGEs, emphasizing the need to identify other specific absorption bands for these lipid classes through further spectral analyses or through the use of traditional lipid approaches in parallel.

Previous studies have suggested that sharks may be either negatively, neutrally (Corner, 1969; Wetherbee and Nichols, 2000) or even positively (Nakamura et al., 2015) buoyant. The buoyancy capability of sharks seems to be one of the major constrain explaining their actual vertical distribution. Indeed, fewer shark species are found in the deep-sea cold environment because of the metabolic costs associated with large livers (Priede et al. 2006; Treberg and Speers-Roesch, 2016). Due to the cold and oligotrophic environment of the deep sea, shark metabolism is reduced, as observed for most species thriving in this environment (Treberg et al., 2003; Condon et al., 2012; Treberg and Speers-Roesch, 2016). Their swimming capabilities are altered by this constrain and buoyancy close to the neutrality may act as a way to reduce the energetic cost of lowered swimming capabilities. Indeed, negative buoyancy was shown to be favorable for fast moving species (Alexander, 1990; Gleiss et al. 2017) whereas less negative buoyancy becomes increasingly efficient as routine swimming speeds declines (Gleiss et al. 2017). Our study provides data in favor of this approach, through the characterization of the liver oil composition of three new species of deep-sea sharks and the comparison with a shallow-water counterpart. More specifically, our results showed that *G. melastomus* (shallow-water species) displays a low HSI, a liver rich in oil content that has no squalene and is more characterized by TAGs, the lipid class providing a low hydrostatic force. In contrary, deep-sea species (*I. brasiliensis* and *E. spinax*) display a high HSI, with livers rich in oil containing squalene as a predominant lipid class (providing more hydrostatic force). However, *E. molleri* shows unusual liver features for a deep-sea species: low HSI and low liver oil content mainly composed of DAGEs, suggesting this shark to have buoyancy characteristics closer to shallow-water species than deep-sea ones. Some studies have shown that several deep-sea sharks do not have squalene (Pethybridge et al., 2010) and tend to accumulate DAGEs in higher concentrations

as compensation (Treberg and Speers-Roesch, 2016). However, even if *E. molleri* liver was composed of a 100% of DAGEs, it would not be sufficient to get a similar LHF value than deep-sea sharks, according to the equation. The LHF calculated in this study is well correlated with the overall tissues density of sharks. When this overall tissues density is close to 1025Kg/m³ (see water density), we can affirm that shark is neutrally buoyant and its LHF is between 18 and 30 g per Kg of shark, while the overall tissues density is superior (1050 to 1080Kg/m³ here), shark display probably a negative buoyancy and its LHF is between 1 and 7 g per Kg of shark.

In our study, the red aerobic muscle distributions of most deep-sea shark species were similar with the ones found in most shallow-water shark species (Bernal et al., 2003). The red aerobic muscles were mainly located in the half posterior of the body, in lateral position just below the skin. These red aerobic muscle fibers were connected with the skin as well as white muscle fibers located just underneath (Krivy et al., 1977). Red fibers are powered by aerobic metabolism during cruise swimming mode (i.e. usual fish swimming mode) (Treberg et al., 2003). Only *I. brasiliensis* displayed a slightly different red aerobic muscle distribution with most of the red aerobic muscles located close to the caudal fin. This result could explain the thunniform swimming mode observed for this species. Indeed, red aerobic muscle concentration next to the caudal region gathers all red muscles contraction around this location but mechanistical studies should be performed to understand better this particularity and its use. Thanks to the red aerobic muscles distribution, we were able to calculate the red aerobic muscle proportion corresponding to the red aerobic muscle mass divided by the total body mass. Results showed that deep-sea sharks have less red aerobic muscles than their shallow-water counterparts with the exception of *E. molleri*, which exhibited a red aerobic muscle proportion similar to shallow-water sharks.

Finally, it has been proposed that sharks with neutral buoyancy would need less red aerobic muscle fibers than those with negative buoyancy as it is an advantage for slow swimming mode (Bone et al., 1966). Our results show a strong relationship between the liver hydrostatic force and the red aerobic muscle proportions which supports Bone's hypothesis. Indeed, as deep-sea sharks have a lower metabolism, their tail beat is thus slowed and their swimming speed reduced. As fewer works are performed in the caudal region, less red aerobic fibers are needed. To compensate this lowered thrust, sharks require higher hydrostatic force.

While numerous studies have investigated the buoyancy of marine organisms through density measurements, modeling of some features involving the buoyancy, our study use the liver to examine a part of shark buoyancy and its relation with the muscular composition. To reflect correctly the buoyancy of shark, we need to incorporate all parameters which can have impacts on it: temperature, osmolytes composition, other tissue densities, etc. However, the main role of liver in the hydrostatic buoyancy and the change of its features through species make it a good proxy for buoyancy estimation. Also the impact of pregnancy state on shark liver composition should be more detailed to take it into account in future studies.

CONCLUSION

The FT-Raman and FT-MIR spectroscopy have allowed the identification of the different lipid classes found in shark liver oil and to quantify its squalene composition. Further spectral analyses should allow to quantify the two main other lipid classes (DAGEs and TAGs). Our results combined with data from the literature suggest that most deep-sea shark species studied have livers that provide higher hydrostatic force than shallow-water sharks, according to the LHF equation. They also highlight an increase of the buoyancy provided by the liver as a function of the median depth of occurrence of the shark. Finally,

the 1966 Bone's hypothesis is supported by showing a relation between the red aerobic muscle proportion and the buoyancy provided by the liver. However, a complete modelling of the buoyancy of sharks would be essential for further research on this topic.

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We really thank Quentin Arnould from CRA-W for the time he spent to explain and help us about the FT-Raman and FT-MIR spectroscopy. We also thank Laurent Duchatelet from the marine biology laboratory of UCL for the help provided during the *Etmopterus spinax*, *Etmopterus molleri* and *Galeus melastomus* liver sampling. Finally, we thank Emma Jones from NIWA Auckland to collect and deliver the deep-sea sharks from the Hoki Survey to the physiological lab of NIWA in Wellington, New Zealand.

AUTHOR CONTRIBUTION

N.P., J.M. & M.G. collected data and contributed equally to the data analyses and the article preparation. O.A. and V.B. provided all the technical spectroscopy support and helped during the spectral data analyses. All authors reviewed the final version and agree to final article submission.

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ETHICAL ISSUES

Etmopterus spinax, *Galeus melastomus* were collected in Norway under the “experimental fish care permit” number 12/14048. *Etmopterus molleri* were collected and handled according to Churaumi aquarium husbandry and veterinary rules for fish experimentations. All sharks were euthanized by a knock on the chondrocranium followed by an incision at the level of the spinal cord, following the local rules for experimental fish care and the European regulation for research animal handling. *Isistius brasiliensis* was fished as by-catch specie in La Reunion Island. *Centroscymnus owstonii*, *Centrophorus squamosus*, *Deania calcea*, *Dalatias licha*, *Etmopterus granulosus*, *Squalus acanthias* were collected as by-catch species during the Hoki survey around the Chatham Rise in New Zealand. These species are not in the CITES list.

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Supplementary data

Supplementary data 1: sex and maturity effect (only for females) on the HSI and the liver squalene concentration of *E. spinax*.

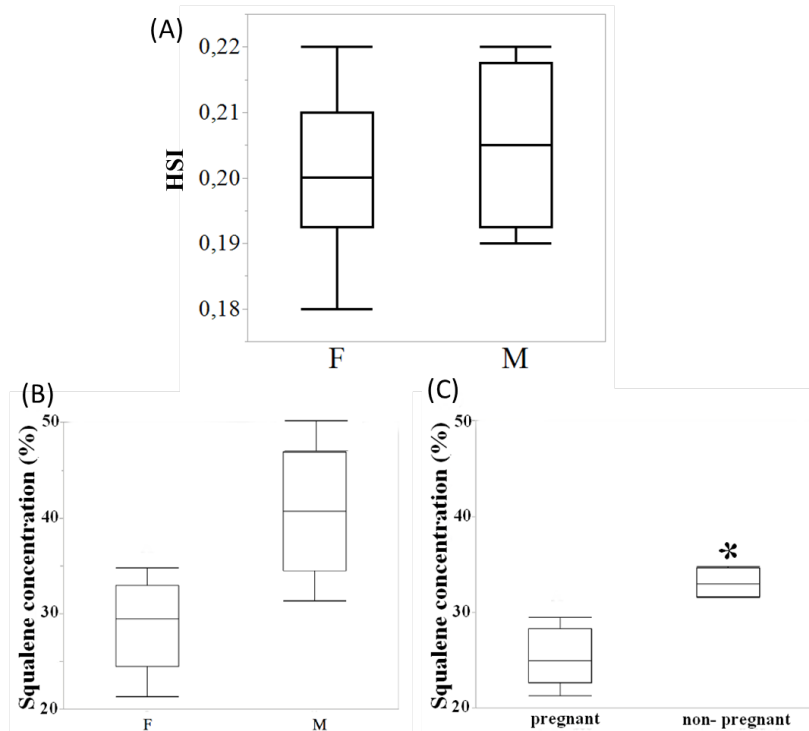


Fig. 1 (A) sex effect on the HSI of *E. spinax*, F= female (N=9), M= male (N=4); (B) sex effect on liver squalene concentration of *E. spinax*, F= female (N=9), M= male (N=4); (C) pregnancy effect on the liver squalene concentration of *E. spinax* females, pregnant (N=5), non-pregnant (N=4). *correspond to significant difference.

The male/female comparison in *E. spinax* showed that there was no significant difference in HSI (Anova, $p > 0.05$) and in squalene concentration in the liver (Anova, $p > 0.05$). However, if we look only at females, the squalene concentration is significantly lower in pregnant females than in non-pregnant females (Anova, $p < 0.05$).

Supplementary data 2: complete data to calculate the Liver Hydrostatic Force (LHF) g per Kg of shark for 14 species of sharks.

Table 1 Summary of data collected from this study and the literature on the 14 species of sharks; median depth occurrence (MDO) corresponds to the mean between the maximum and the minimum depth where shark was observed (m), Hepatosomatic index (HSI % $\pm$ SD), Oil content correspond to the percentage of lipid g wet mass⁻¹ (OC % $\pm$ SD), Percentage composition of main lipid classes in shark liver: TAG = Triacylglyceride, DAGE= Diacylglyceride, HC= Hydrocarbon, mainly squalene and WE= Wax Ester (% $\pm$ SD), Liver lipid (g.Kg of shark⁻¹), Liver lipid density (g.ml⁻¹), Liver Hydrostatic Force (g per kg of shark) (LHF), Ref corresponding to the literature where come from data: ¹ Wetherbee and Nichols, 2000; ² Pethybridge et al., 2010; ³ Jayasinghe et al., 2003a; Jayasinghe et al., 2003b; ⁵ Treberg and Speers-Roesch 2016; ⁶ Mull et al. 2013.

Order/species	habitat	MDO (m)	HSI (%)	OC (%)	percentage composition				liver lipid (g.kg of shark ⁻¹)	Liver lipid density (g ml ⁻¹)	LHF (g per kg of shark)	Ref
					TAG	DAGE	HC	WE				
<i>Isistius brasiliensis</i>	deep	1793	18.8	71.5	0.435	0.145	0.419	0.01	134.42	0.90254	20.7624442	Present study
<i>Dalatias licha</i>	deep	918	20.9	79	0.02	0.18	0.79	0.01	165.11	0.8668	30.1342893	1,2
<i>Etmopterus spinax</i>	deep	1035	20.2	69.7	0.155	0.365	0.369	0.01	140.794	0.79494	21.3308905	Present study
<i>Etmopterus granulosus</i>	deep	860	18.7	72	0.09	0.35	0.55	0.01	134.64	0.8768	22.757354	1,2

<i>Etmopterus malleri</i>	deep	447	5.88	51.2	0.25	0.75	0.01	0.01	30.1056	0.9172	3.84112649	Present study
<i>Centroscyminus owstonii</i>	deep	805	20.08	65	0.22	0.23	0.54	0.01	130.52	0.8823	21.1098311	1,2
<i>Deania calcea</i>	deep	770	19.3	81	0.09	0.24	0.67	0.01	156.33	0.8821	27.1419788	1,2
<i>Centrophorus squamosus</i>	deep	1315	21.4	83	0.18	0.11	0.7	0.01	177.62	0.8759	30.2353488	1,2
<i>Squalus acanthias</i>	shallow	300	8.7	41.2 ₉	0.747	0.157	0.01	0.01	35.9223	0.85164	3.85357819	1,2
<i>Galeus melastomus</i>	shallow	528	10.02	69.9	0.75	0.25	0.01	0.01	70.0398	0.9372	8.9362687	Present study
<i>Prionace glauca</i>	shallow	175	6.7	49.9	0.723	0.027	0.1095	0.01	33.433	0.79919	3.87211862	3
<i>Lamna ditropis</i>	shallow	188	7.3	48.2 ₈	0.804	0.0283	0.044	0.01	35.2444	0.819347	3.99624395	4
<i>Alopias superciliosus</i>	shallow	200	5.35	36	0.8065	0.0105	0.0495	0.01	19.26	0.81056	2.17273786	3
<i>Carcharodon carcharias</i>	shallow	650	12.7	63	0.963	0	0	0	80.01	0.89559	7.89835092	5,6

**Supplementary data 3: Data allowing the validation of
Liver Hydrostatic Force (LHF – g kg⁻¹ of shark)
according to the overall tissue density and the liver
density for 12 sharks from the literature.**

Table 2 Summary of data collected from this study and the literature on the 14 species of sharks; median depth occurrence (MDO) corresponds to the mean between the maximum and the minimum depth where shark was observed (m), Hepatosomatic index (HSI % $\pm$ SD), Oil content correspond to the percentage of lipid g wet mass⁻¹ (OC % $\pm$ SD), Percentage composition of main lipid classes in shark liver: TAG = Triacylglyceride, DAGE= Diacylglyceride, HC= Hydrocarbon, mainly squalene and WE= Wax Ester (% $\pm$ SD), Liver lipid (g.kg of shark⁻¹), Liver lipid density (g.ml⁻¹), Liver Hydrostatic Force (g kg⁻¹ of shark) (LHF), Ref corresponding to the literature where come from data: ¹ Treberg and Speers-Roesch 2016; ² Gleiss et al. 2017.^a, due to the lack of data regarding TAG and DAGE composition, we assume a 50/50 composition of these lipid classes;^b due to the lack of data regarding DAGE and HC composition, we assume a 50/50 composition of these lipid classes

Species	Habitat	MDO (m)	HSI (%)	OC (%)	Percentage composition				Liver lipid (g.kg of shark ⁻¹)	Liver lipid density (g.ml ⁻¹)	LHF (g.kg ⁻¹ of shark)	Overall Tissues Density (kg/m ³)	Liver Density (kg/m ³)	Ref
					TAG	DAGE	HC	WE						
<i>Dalatias licha</i>	deep	918	20.9	79	0.02	0.18	0.79	0.01	165.11	0.87	30.13	1026	905	1.2
<i>Deania calcea</i>	deep	770	19.3	81	0.09	0.24	0.67	0.01	156.33	0.88	25.33	1025	890	1.2
<i>Centrophorus squamosus</i>	deep	1315	21.4	83	0.18	0.11	0.7	0.01	177.62	0.88	30.24	1024	890	1.2

<i>Squalus acanthias</i>	shallow	300	8.7	41.29	0.747	0.157	0.01	0.01	35.9223	0.98	1.69	1055	975	1.2
<i>Prionace glauca</i>	shallow	175	6.7	49.9	0.723	0.027	0.1095	0.01	33.433	0.91	3.88	1051	988	1.2
<i>Carcharhinus falciformis</i>	shallow	250	5.5	48.9	0.69	0.05	0	0	26.895	0.93	2.83	1076	1010	1.2
<i>Carcharhinus plumbeus</i>	shallow	141	12.4	15.9	0.83	0	0	0	19.716	0.93	2.01	1066	956	1.2
<i>Centroscyrius coelopsis</i>	deep	1902	24.9	69.1	0.2	0.23	0.48	0	172.059	0.88	28.99	1023	906	1.2
<i>Cetorhinus maximus</i> ^a	shallow	452	17.3	74.3	0.39	0.38	0.21	0	128.539	0.90	17.94	1032	939	1.2
<i>Etmopterus princeps</i> ^a	deep	2534	17.1	70.3	0.27	0.28	0.45	0	120.213	0.89	18.66	1026	891	1.2
<i>Scyllorhinus canicula</i> ^b	shallow	55	6.5	44.7	0.88	0.06	0.06	0	29.055	0.92	3.20	1075	975	1.2

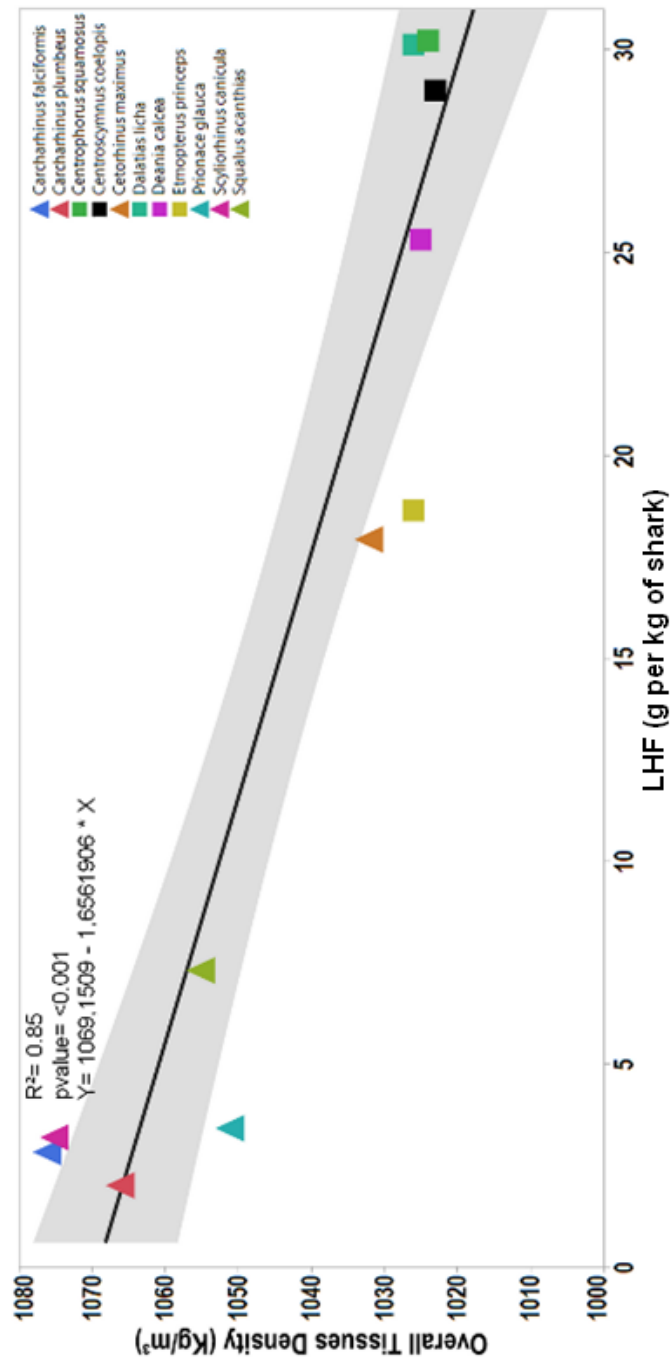


Fig. 1 Linear regression of the Liver Hydrostatic Force (LHF – g kg⁻¹ of shark) according to the overall tissues density (kg/m³) of 11 different shark species (see graph legend). ▲ corresponds to shallow-water sharks and ■ corresponds to deep-sea sharks. Dark grey region represents the confidence region (0.95) for the fitted line.

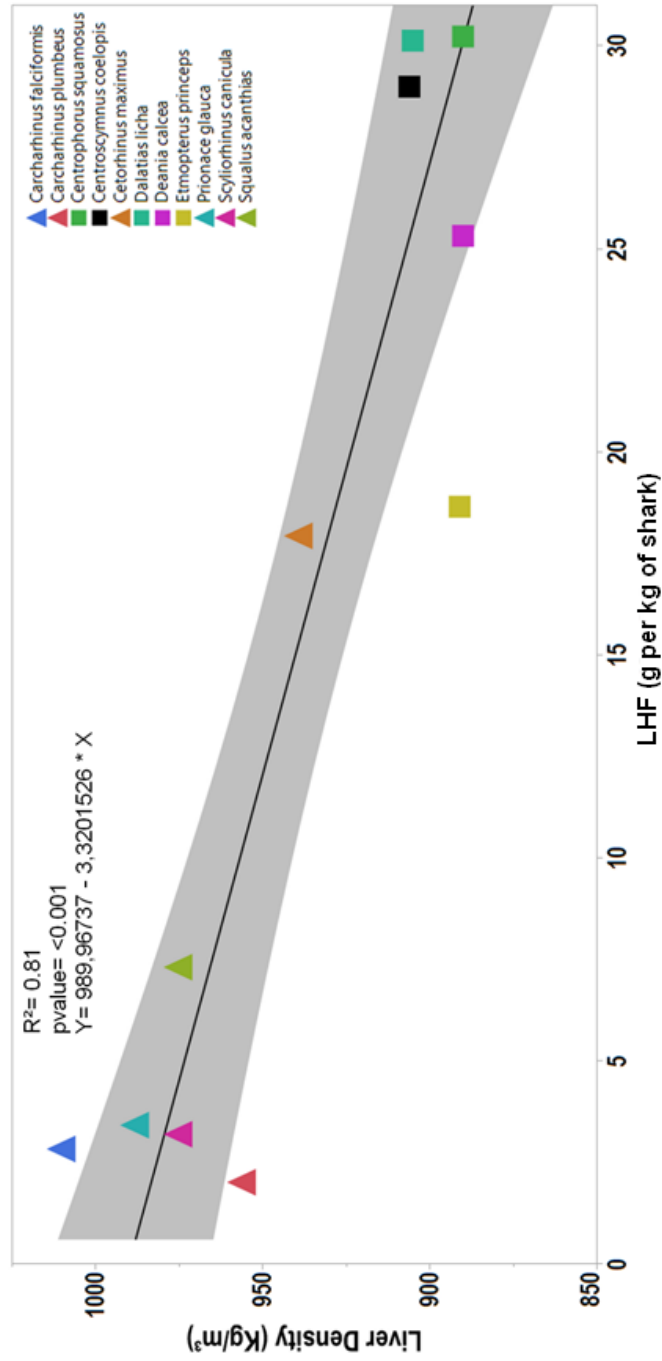


Fig. 2 Linear regression of the Liver Hydrostatic Force (LHF – g kg⁻¹ of shark) according to the liver density (kg/m³) of 11 different shark species (see graph legend). ▲ corresponds to shallow-water sharks and ■ corresponds to deep-sea sharks. Dark gray region represents the confidence region (0.95) for the fitted line.

Chapter III:

Morphological adaptations relative to
deep-sea shark swimming capabilities

Previous chapters have revealed differences in cruise swimming speed between deep-sea shark species. The red muscles proportion and enzyme of aerobic metabolism are highly correlated with cruise swimming speed (**Paper 1**). For the majority of deep-sea sharks, the proportion of red muscles is lower compared to their shallow-water counterparts (**Paper 2**) and it is correlated with their liver providing more lift (**Paper 3**). Morphological adaptations are the last features related to velocity needed be analyzed. For shallow-water sharks, several features have already been proven to have an impact on the velocity such as the caudal fin shape, the body shape, distance between fins, denticles, and also dorsal fin spines. However, deep-sea sharks' multivariate analyses of morphology were never performed.

The last chapter of this thesis will study morphological features of nine deep-sea sharks (**Paper 4**) with the aim in showing if there are correlations between certain morphological features and the cruise swimming speed previously measured. Pictures of sharks were taken digitally and measurements were done using ImageJ software.

Paper 4:

Study of morphological features
(body, fin and spine shape) of eight
deep-sea shark species and one
shallow-water one (Squaliformes)
according to their swimming
capabilities.

Nicolas Pinte, Elliot Finet, Emma Jones, Bruno Frédérich and
Jérôme Mallefet

In preparation

Study of morphological features (body, fin and spine shape) of eight deep-sea shark species and one shallow-water one (Squaliformes) according to their swimming capabilities.

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ABSTRACT

Sharks have been present in oceans for more than 400 millions of years and have survived several mass extinctions. Although morphological features are well preserved between all shark species, they show some differences according to their different lifestyles. Morphological features of shallow-water sharks were well studied and allowed a classification of species in three different morphotype while squaloid deep-sea species are all grouped in the fourth morphotype without any deep research. Moreover, some morphological variables such as the caudal fin shape, the body shape, the rostrum shape, the spine shape and the denticles' shape have been proved or suggested to have an impact on swimming capabilities. Recent study revealed that deep-sea sharks display different cruise swimming speed with Etmopteridae species displaying the highest values of velocity, non-luminous deep-sea counterparts an intermediate value and *Dalatias licha* the slowest speed never recorded for a shark. In this study, we measured 26 linear and area morphological

measurements, three ratios and also we use geometric morphometric of caudal fin and dorsal fin spines to evaluate the potential morphological differences which can occur in squaloid deep-sea sharks. These measurements were done on eight deep-sea shark species and one shallow-water counterparts in order to reveal the shape similarity between this species. Result revealed that deep-sea species from squaliformes order present some significant shape differences. Interestingly, Etmopteridae species do not display efficient body for a high velocity except regarding the spines' shape but mechanistic studies are still required to define the impact on velocity. Non luminous deep-sea shark species display the most efficient morphology for good swimming capabilities while *D. licha* display the worst. Indeed, the *D. licha* caudal fin is even described with the ventral and dorsal lobe but the fork is reduced to its minimum which could be less efficient than heterocercal caudal fin but future studies should be performed to conclude the potential impact of this uncommon caudal fin shape.

KEYWORDS Squaliformes, caudal fin, spines, deep-sea, sharks, swimming capabilities

INTRODUCTION

Evolutionary and functional biology mainly aim to understand the diversity observed between organisms and relate it to their ecology and life history. In that context, morphological studies are good indicators to figure out how an organism evolved in its environment (Wainwright & Reilly, 1994; Lauder, 1996; Irschick & Henningsen, 2009; Losos, 2011; Irschick & Higham, 2016; Irschick et al., 2017; Biewener and Patek, 2018). Among organisms sharks are certainly the most diversified group of predators (Little and Bamis, 2004; Compagno et al., 2005; Ebert et al., 2013). Even if they display highly varied biology, ecology and behaviors, their general body form is well

conserved. Indeed, most shark have a fusiform body with several fins (pectoral, pelvic, dorsals, caudal and sometimes anal), five to seven gill slits are observable and their skin is covered by small teeth-like structures called placoid scales (Ebert et al., 2013).

However, morphological differences exist between species: for example regarding the size, from the smallest shark (*Etmopterus perryi*) reaching 15-20 cm of total length to the whale shark (*Rhincodon typus*) reaching more than 12 meters of total length (Ebert et al., 2013). Other differences also occur with the: (i) pectoral fins bigger and stiffer for some species while others display small and soft ones (Thomson and Simanek, 1977; Wilga and Lauder, 2001); (ii) caudal fin which can be homo/isocercal (i.e. almost same length for the upper and lower lobe of the caudal fin) or heterocercal (i.e. dorsal lobe longer than the ventral one) (Thompson, 1976; Ferry & Lauder, 1996; Wilga and Lauder, 2002); (iii) the body shape with sharks flattened and other more fusiform (Thomson and Simanek, 1977; Lebrun, 2001); (iv) the head dimension which can be from streamlined and narrow snouts to flattened and rounded heads (Lebrun, 2001). In fact, Thomson and Simanek in 1977 have described four morphotypes of sharks based on these differences. The first one is characterized by a caudal ratio which is near to one indicating homocercal caudal. The body is thick, the head is conical and pelvic, anal and the second dorsal fins are reduced. This allows a faster swim but reduces maneuverability and is characteristic of fast pelagic sharks like Lamnidae or Carcharhinidae. The second morphotype is similar to the first apart from the caudal fin which is more heterocercal and presents sometimes a peduncle on the dorsal lobe. The body is less thick and the pelvic, anal and second dorsal fins are more developed what increases maneuverability. It's the typical shape of generalist pelagic sharks. The third is significantly different of the two others with its dorsoventral flattening more pronounced and its rounded rostrum. The tail is

heterocercal with a ventral lobe reduced. The pelvic fins are in a more anterior position and the first dorsal fin is more posterior. It is the typical shape of benthic sharks that swim on the floor. Finally, the fourth morphotype is those of the Squaloid sharks including the majority of known deep-sea sharks. The caudal fin is also heterocercal and anal fins are absent. The two dorsal fins are well developed and are often topped with a dorsal spine while the pectoral and pelvic fins are much reduced. This last group covers a large diversity of shape in sharks and should be clarified. A fifth group exists for rays, chimeras or extremely flattened sharks but is not useful in this paper.

Each morphotype have been related to general life-style but also to specific locomotor capabilities. Indeed, several studies have shown or suggested that several morphological adaptations can improve or diminish the swimming velocity or also maneuverability (Carrier et al., 2012; Ebert et al., 2013; Klimley et al., 2013; Maia and Wilga, 2013; Iosilevskii and Papastamatiou, 2016; Irschick et al., 2017). Homocercal caudal fin allows a better swimming efficiency because more energy is involved in thrust and less is spent in the lift. In contrast, heterocercal caudal fin creates a dorsoventrally water flow generating both an upward force and a forward force according to the classical model resulting in less thrust. It could be explained by the wider wave of the upper lobe (more rigid) compared to the lower lobe (more flexible) which forces the caudal fin to be tilted most of the time with an angle nearly horizontal (Ferry & Lauder, 1996). However, Thompson had previously developed in 1976 an antagonistic model in which the water is pushed backward and upward allowing the shark to be propelled forward but downward. It is based on the fact that the upper lobe of the tail has a larger area than the lower lobe, and so, moves slightly slower. The consequence is that the caudal fin has a nearly horizontal move but with water pushed upward (Thompson, 1976; Ferry & Lauder, 1996). Further kinematics models are more nuanced (Ferry & Lauder, 1996), the lower

lobe seems to move first but at the end of the movement it is the upper lobe which is farther to either side (right and left), corroborating the classical model. Wilga and Lauder in 2002 confirmed this theory by analyzing vorticity around the caudal fin and deducting a jet angle of -45° and -42° backward and downward for leopard shark (*Triakis semifasciata*) and bamboo shark (*Chiloscyllium plagiosum*).

It has been also suggested that the dorso-caudal fin distance have a role in the shark propulsion. These fins are also able to generate thrust by accelerating water posteriorly and creating a Venturi effect (Wilga and Lauder, 2000), the vortex of water created by the dorsal fin can then interact with the water flow incident to the caudal fin in order to let the tail beat easier (Lauder & Santo, 2016). The body shape has also been proved to have an impact on locomotor capabilities: fusiform shark display a better water penetration than flattened sharks (Lebrun, 2001). Then, the length of the shark is inversely correlated with the swimming speed (Graham et al., 1990; Sambilay, 1990). Small sharks swim faster because the cardiac and muscles investment required is too significant for bigger species (Graham et al., 1990). However, recent study precised that the size does not act alone and it is more a species-specific effect (Ryan et al., 2015). All parameters such as the species life-style and specific other morphological features should take into account.

Finally, it has also shown that placoid scales can also, depending of their shape, improve the swimming efficiency of shark (Raschi and Elsom, 1986). They generate little vortices reducing the flow and then the drag of water, it is called the Ribblet effect (Fletcher et al., 2014). The keels also passively control flow by limiting the lateral transfer of force, training the vortices in the direction of the flow (Raschi and Elsom, 1986; Fletcher et al., 2014). Shark's dorsal spines are located ahead of the two dorsal fins for the majority of squaloid sharks. These

spines have often been regarded as a defense attribute, especially for juveniles, but have little been considered as a locomotor function. However, Maisey in 1979 suggested its role as a “cutwater”. The angle of spine insertion is probably related to the speed and habitat of the shark, like at bottom-dwelling species for which finspines stand nearly at right-angles of the vertebral column (Maisey, 1979). Another theory is that the presence of a spine in the anterior position of the dorsal fin limits the creation of a stronger wave vorticity by binding with muscles of the fin and reducing the amplitudes of kinematic oscillations of this fin (Maia et al., 2017).

All these morphological features have been related to shallow-water sharks' velocity while deep-sea sharks have been believed as slow and listless swimmers without any morphological evidences. However, recent study has shown differences of cruise swimming speed between several Squaloid deep-sea shark species (Pinte et al., 2019). In this study, *Dalatias licha* display the slowest cruise swimming speed observed for sharks, other non-luminous deep-sea sharks swim at a intermediate value and luminous deep-sea shark from Etmopteridae family and *Centrophorus harrissoni* display higher velocity value, similar to benthic shallow-water sharks (Pinte et al., 2019). It has been suggested by analyzing the precaudal length and the total length that some of fast deep-sea shark could reach this velocity thanks to morphological adaptations but morphological studies are still missed and needed (Pinte et al., 2019).

While the morphological features of shallow-water sharks are documented deep-sea sharks are still grouped in the same morphotype even if they present several morphological differences. In this study we described the morphology of eight deep-sea species (*Centroscymnus owstonii*, *Centrophorus squamosus*, *Dalatias licha*, *Deania calcea*, *Etmopterus granulosus*, *Etmopterus lucifer*, *Etmopterus molleri*, and

Scymnodon plunketi) and one shallow-water control species (*Squalus acanthias*) using 26 morphological measurements and the geometric morphometric of caudal fin and dorsal fin spines. The first aim was to better assess the morphological difference occurring in Squaliformes order. Secondly, morphological variables will be analyzed according to the respective cruise swimming speed previously recorded in order to see if morphological adaptations could explain the difference of velocity observed between these sharks.

MATERIALS AND METHODS

Sampling

Individuals of *Etmopterus molleri* were caught in East Chinese Sea off the coast of Okinawa Island, Japan, in November 2017 (Fig. 1A) (26° 28' 94" N; 127° 41' 22"E). Sharks were fished using a longline at 500 meters depth. Sharks were placed in a plastic bag filled with fresh water saturated in oxygen and brought back to the lab in a thermic dark box. In the research center of the Churaumi Okinawa Aquarium, sharks' pictures were taken on lateral and dorsal side.

Majority of data comes from the Hoki survey which took place on the Chatham Rise (East New-Zealand) between January and February 2018 aboard the RV *Tangaroa* research ship (Fig. 1B). Sharks were fished at depth ranges from 200 to 1000 using mesopelagic trawling. The last seven deep-sea species (*Centroscymnus owstonii*, *Centrophorus squamosus*, *Deania Calcea*, *Dalatias licha*, *Etmopterus granulosus*, *Etmopterus lucifer* and *Scymnodon plukenti*) and one shallow-water species (*Squalus acanthias*) were collected during this expedition. Sharks' pictures were taken onboard.

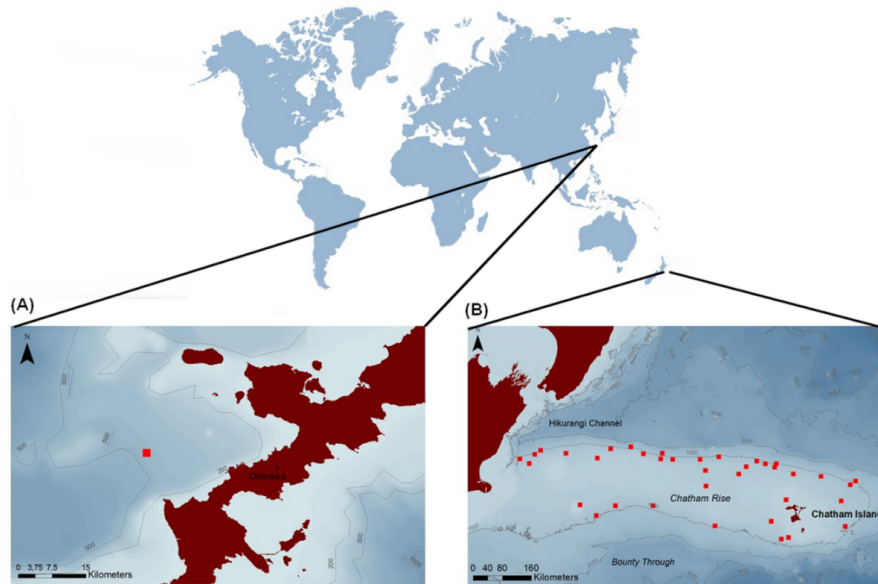


Fig. 1 Maps showing sampling location in Okinawa, Japan (A) and Chatham rise, New Zealand (B).

In total, eight deep-sea shark species and one shallow-water counterpart from Squaliformes order were studied. The minimum of individuals by species was 8 and the maximum was 18, a total of 130 sharks were measured along this study.

Morphological variables collection

The 26 morphological variables concern linear and area measurements (Fig. 2A and B). Most of these variables are inspired and selected from previous morphological studies focusing shallow-water sharks (Irschick et al., 2017; Thompson & Simanek, 1977). They are carried out from the pictures using ImageJ software (Schneider et al., 2012). Each picture includes a measuring instrument to set the scale and then obtaining the correct value of each morphological variable. Abbreviations corresponding to each variable are resumed in the figure 2C.

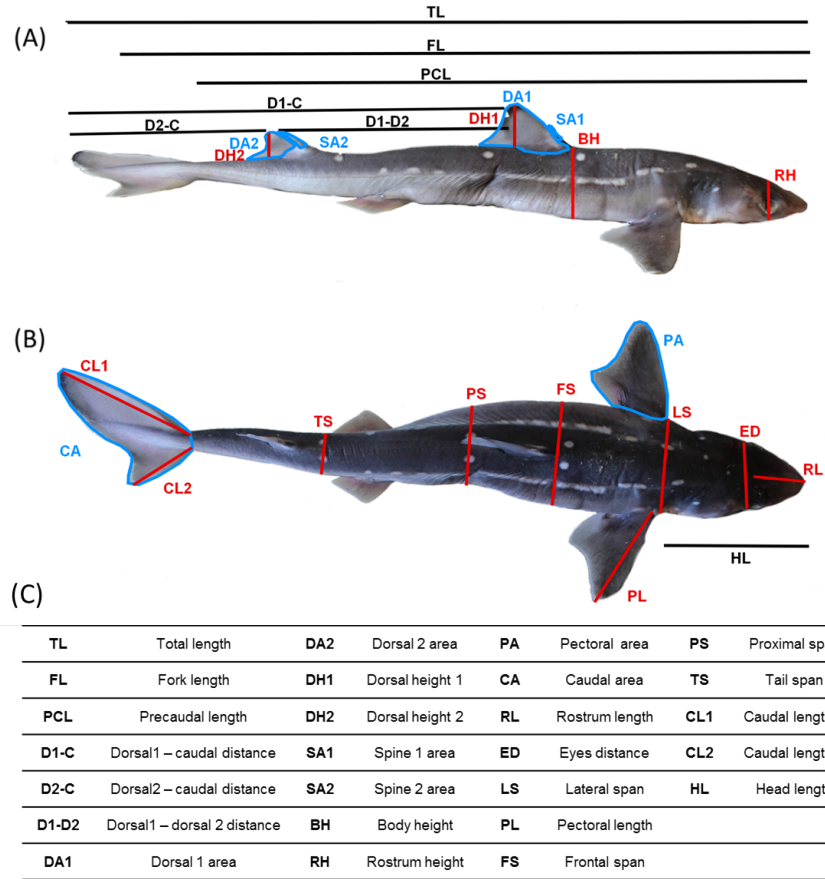


Fig. 2 The 27 morphological variables measured using ImageJ software. (A) shark lateral side; (B) shark dorsal side; (C) morphological variable abbreviations; black and red line correspond to linear measurements and blue surface correspond to area measurements.

In addition to linear and area measurements, four morphological ratios are performed using the different morphological variables collected: The first is the caudal ratio (CR, equation 1), a higher caudal ratio is linked with a more homocercal fin and play a role in swimming performance (Ferry & Lauder, 1996; Thompson, 1976; Wilga & Lauder, 2002). Then, there are rostral (RR, equation 2) and corporal ratio (BR, equation 3) explaining a more fusiform shape of the head and

the body respectively (Lebrun, 2001). More the RR is high more the head is rounded. For the BR, more the value is high less the shark body is flattened (Lebrun, 2001).

$$(1) CR = CL2/CL1$$

$$(2) RR = RH/RL$$

$$(3) BR = BH/TL$$

Caudal fin and spines geometric morphometric collection

Geometric morphometric are a quantitative way of addressing shape comparison between individuals for a body part of interest (Rohlf and Bookstein, 1990). By opposition with traditional morphological values previously recorded, geometric morphometrics ease data visualization, interpretation, communication of results (Leah Zelditch *et al.*, 2012) and, more generally, to extract information otherwise difficult to obtain (Viscosi and Cardini, 2012). Therefore, selected body parts are analyzed by a landmark-based approach to visualize shape variation through landmarks and semi-landmarks. Landmarks are anatomical markers often termed as “homologous points” because they can be recognized as same points in all specimens of the study (Leah Zelditch *et al.*, 2012). They describe less shape variation than semi-landmarks used to maximize shape variation between individuals. Semi-landmarks position are more arbitrary selected and are traditionally plotted along the curves we want to describe. They are viewed as a set of markers adding information on “non-homologous” shape variation between individuals (Leah Zelditch *et al.*, 2012).

Using the geomorphological software “tpsDig232” (Rohlf, 2006). Landmarks and semi-landmarks have been fixed on three body parts of interest. We choose to focus on the spines in front of the two dorsal fins because their role has been relatively

unexamined but may be relevant to understand the swimming kinematics of sharks (Maia *et al.*, 2017; Maisey, 1979). The last body part studied is the caudal fin because its major role in fish locomotion has often been described (Ferry and Lauder, 1996; Thompson, 1976; Wilga and Lauder, 2002).

The spine of the first dorsal fin was set with three landmarks and four semi-landmarks. Landmarks were fixed at the front of the basis, back of the basis and top of the spine from lateral view. Semi landmarks were distributed at equal distance along the front and back curve of the spine. The spine of the second dorsal fin was set with three landmarks (the same than for the first spine) and six semi-landmarks, also distributed along the spine. Then, the caudal fin was set with six landmarks fixed at locations homologous to all individuals (top and down of the basis, top and down of the fin, end of the caudal peduncle and at the fork). Nineteen semi-landmarks were plotted at equal distance on the trailing edge of the fin because of the necessity to fit the shape of the fin as much as possible.

Statistical analyses

All statistical analyses and graphs were performed with JMP pro 14 (JMP®, Version <14>. SAS Institute Inc., Cary, NC, 1989-2007).

Firstly data were analyzed thanks to PCA and Pearson's test correlation matrix to reveal the possible correlation and allometry processes which often occurs with morphological variables. The allometry was observed and characterized as isometric with the total length. To delete this tendency, all morphological variables of one individual were divided with its total length to obtain relative values that better represent morphological differences between sharks of different sizes. This process is called an isometric transformation because it

preserves distances between original variables. Except ANOVAs, all future analyses are based on these relative morphological measurements. the phylogenetic correction was not used in analyses mainly to avoid the overestimation of degree of freedom.

The second analysis aimed to see morphological differences between studied species. PCA was carried out to reveal it. For this PCA, clusters have been shaped to show out the impact of each morphological trait on the shark individual dispersion. Indeed, PCA maximize variance explained by the variables selected. Because the PCA analysis does not provide p-value, we also performed univariate ANOVAs. These ANOVAs is carried out on all log10- transformed 25 variables (without the total length) using three terms in each model: (i) the total length; (ii) the species; (iii) and the interaction between species and total length. Because we integrated the total length in the model, ANOVAs was done using absolute values of morphological variables. The key term of interest is the interaction between species and the total length, which informs us that for a given body size, there is heterogeneity among the species in the variable. These test are based on fisher test, therefore, F-value are significant when they move away to “1”.

The second objective was to see if morphological adaptations can explain the differences of cruise swimming speed previously observed between deep-sea sharks. Indeed, *Etmopterus granulosus* and *molleri* (Group C) present significant higher speed than the second group (Group B) (*Centroscymnus owstonii*, *Scymnodon plunketi*, *Centrophorus squamosus*, and *Deania calcea*). Finally, *Dalatias licha* displays the slowest cruise swimming speed value observed (Group A). For that purpose PCA was carried out based and cluster of velocity group have been shaped. Finally, traditional ratios (caudal ratio, rostrum ratio, and body ratio) were analyzed with univariate test according to the group of velocity. The Gaussian

distribution was not obtained and therefore nonparametric equivalent tests (Kruskal-Wallis test and Steel-Dwass post hoc test) were used to revealed possible differences.

RESULTS

Data transformation

A total of 3770 classical morphological measurements and ratios are summarized in the table 1. The first PCA carried out shows high correlation level between all 26 morphological variables despite a slightly deviation for the “Spine2.area” variable (Fig. 3A). The correlation matrix confirmed the result observed in the PCA pointing out high correlation level (Pearson's test, $r > 0.7$, $p\text{-value} < 0.05$) between all variables (Fig. 3B).

The high correlation can be explained by a isometry grow rate according to the total length and therefore after the isometric transformation (i.e. all variable was related to the total length), a second PCA was carried out from these relative values showing more dispersive variables even though some of them are still correlated together (Fig. 3C). Finally, the second correlation matrix, based on relative values, revealed also a decrease of correlation between variable with a maximum “ r ” reaching 0.75 between the body height (BH) and the caudal area “CA” while the lowest “ r ” value is close to 0 (Fig. 3D).

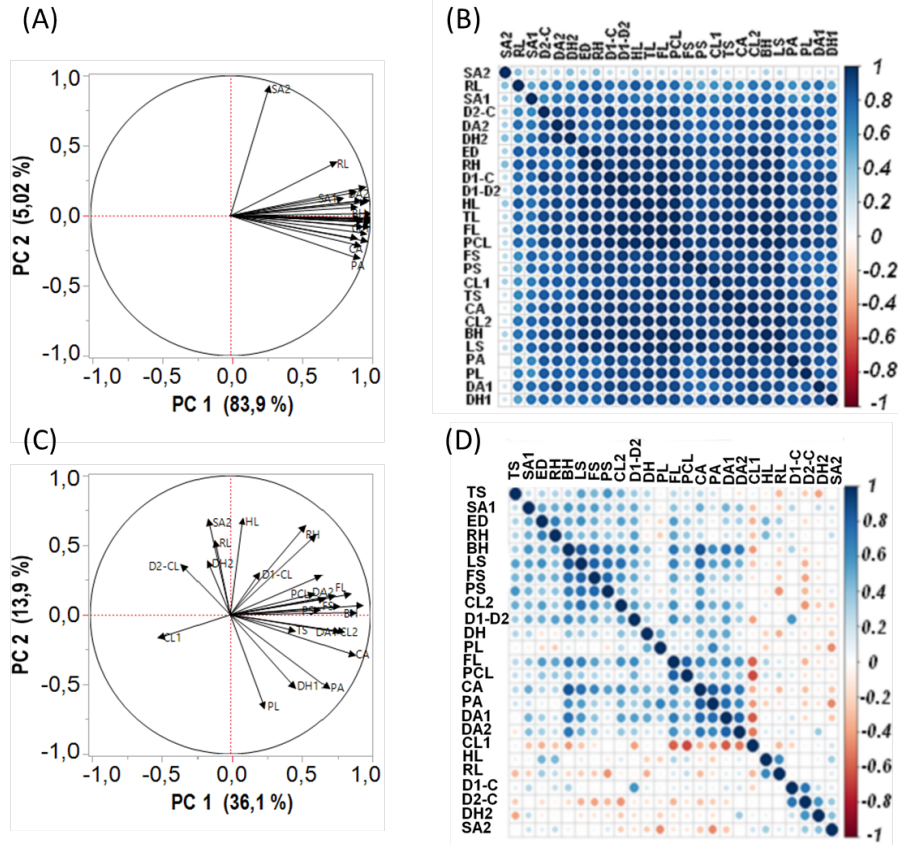


Fig. 3 Graph analyzing the correlation between all 26 morphological variables (A) PCA gathering absolute values; (B) Correlation matrix gathering absolute values, red color indicates positive correlation while red color indicates negative correlation; (C) PCA gathering relative values; (D) Correlation matrix gathering relative values, blue color indicates positive correlation while red color indicates negative correlation.

Table 1 Summary of data collected from this study on eight deep-sea sharks species (*Centroscyrnus owstonii*, *Centropristis squamosus*, *Dalatias licha*, *Deania calcea*, *Etmopterus granulosus*, *Etmopterus lucifer*, *Etmopterus molleri*, and *Scymnodon plunketi*) and one shallow-water one (*Squalus acanthias*); number of replicates (N); Group of velocity are based on Pinte et al., 2019, A correspond to slowest sharks, B intermediate value and C highest value of cruise swimming speed; Morphological variable abbreviations are found in the Fig. 2C; For ratios, CR correspond to caudal ratio, RR to rostrum ratio and BR to the body ratio. All values are means $\pm$ the standard deviation.

Group of velocity	B	B	B	C	C	C	C	B	B	A	/
Morphological variables											
BH (cm)	20.61 $\pm$ 4.62	12.71 $\pm$ 2.20	11.68 $\pm$ 2.29	3.01 $\pm$ 0.59	4.47 $\pm$ 0.10	18.11 $\pm$ 4.35	21.31 $\pm$ 4.08	14.87 $\pm$ 8.06	8.92 $\pm$ 2.61		
CA (cm ²)	147.94 $\pm$ 46.54	81.51 $\pm$ 17.97	61.13 $\pm$ 13.66	11.72 $\pm$ 3.07	15.30 $\pm$ 2.91	111.89 $\pm$ 37.39	202.36 $\pm$ 43.97	119.34 $\pm$ 86.18	61.70 $\pm$ 14.57		
CL1 (cm)	15.68 $\pm$ 2.33	13.82 $\pm$ 1.50	12.68 $\pm$ 1.73	6.52 $\pm$ 0.83	8.12 $\pm$ 0.97	15.63 $\pm$ 2.31	20.50 $\pm$ 2.97	16.29 $\pm$ 4.47	12.99 $\pm$ 1.57		
CL2 (cm)	12.16 $\pm$ 2.59	8.08 $\pm$ 0.94	7.17 $\pm$ 0.93	3.12 $\pm$ 0.51	3.23 $\pm$ 0.34	9.36 $\pm$ 1.52	13.59 $\pm$ 1.32	8.13 $\pm$ 3.00	7.13 $\pm$ 0.97		
DA1 (cm ²)	51.22 $\pm$ 23.31	33.83 $\pm$ 12.34	9.48 $\pm$ 2.95	1.30 $\pm$ 0.40	2.53 $\pm$ 0.65	16.21 $\pm$ 7.61	47.06 $\pm$ 8.22	22.28 $\pm$ 16.09	15.09 $\pm$ 5.95		
DA2 (cm ²)	32.47 $\pm$ 11.26	30.00 $\pm$ 10.71	11.49 $\pm$ 3.26	2.48 $\pm$ 0.58	5.54 $\pm$ 1.31	19.25 $\pm$ 6.13	35.86 $\pm$ 5.91	22.50 $\pm$ 17.97	6.77 $\pm$ 2.16		
D1-C (cm)	48.66 $\pm$ 12.28	37.84 $\pm$ 5.82	29.40 $\pm$ 3.67	12.83 $\pm$ 2.20	20.99 $\pm$ 1.72	35.80 $\pm$ 10.21	52.02 $\pm$ 5.40	35.82 $\pm$ 14.76	29.63 $\pm$ 6.45		
D2-C (cm)	14.41 $\pm$ 3.36	12.82 $\pm$ 2.30	9.43 $\pm$ 1.52	3.76 $\pm$ 0.81	8.99 $\pm$ 1.04	11.03 $\pm$ 1.95	15.95 $\pm$ 3.12	11.57 $\pm$ 4.68	8.88 $\pm$ 2.55		
D1-D2 (cm)	38.85 $\pm$ 7.12	24.24 $\pm$ 3.23	20.21 $\pm$ 2.72	9.02 $\pm$ 1.52	11.96 $\pm$ 1.01	31.34 $\pm$ 5.48	34.75 $\pm$ 5.41	24.23 $\pm$ 10.51	20.77 $\pm$ 4.10		
DH1 (cm)	4.39 $\pm$ 1.07	2.71 $\pm$ 0.56	2.16 $\pm$ 0.50	0.81 $\pm$ 0.15	1.28 $\pm$ 0.21	2.22 $\pm$ 0.45	4.44 $\pm$ 0.68	3.68 $\pm$ 1.33	3.58 $\pm$ 0.73		
DH2 (cm)	4.45 $\pm$ 0.95	4.19 $\pm$ 0.77	2.75 $\pm$ 0.53	1.28 $\pm$ 0.17	2.5 $\pm$ 0.40	3.44 $\pm$ 0.47	4.94 $\pm$ 0.47	4.30 $\pm$ 1.78	2.10 $\pm$ 0.35		
ED (cm)	12.04 $\pm$ 2.36	8.58 $\pm$ 1.04	7.54 $\pm$ 0.90	3.09 $\pm$ 0.44	3.73 $\pm$ 0.28	10.18 $\pm$ 1.54	11.85 $\pm$ 1.23	6.60 $\pm$ 2.08	5.91 $\pm$ 0.85		

FL (cm)	96.36 ± 20.76	78.23 ± 7.85	59.55 ± 7.27	28.78 ± 4.14	37.14 ± 2.91	85.11 ± 15.09	106.15 ± 11.40	73.33 ± 28.16	61.94 ± 7.98
FS (cm)	17.65 ± 4.71	10.16 ± 1.60	11.30 ± 2.07	3.94 ± 0.81	4.46 ± 0.93	14.86 ± 3.73	16.84 ± 6.31	13.30 ± 6.51	9.13 ± 1.79
HL (cm)	21.88 ± 4.30	19.46 ± 1.96	14.81 ± 2.09	6.88 ± 1.05	8.67 ± 0.65	18.72 ± 2.39	22.59 ± 2.67	16.53 ± 5.34	13.62 ± 2.22
LS (cm)	16.39 ± 4.07	10.46 ± 1.69	10.10 ± 1.76	3.79 ± 0.66	4.43 ± 0.53	13.06 ± 2.52	18.08 ± 2.67	11.87 ± 4.71	8.80 ± 1.23
PA (cm²)	55.99 ± 22.45	39.44 ± 11.34	13.88 ± 4.09	4.86 ± 1.30	6.09 ± 1.80	37.75 ± 10.51	95.62 ± 27.86	41.38 ± 33.30	34.64 ± 8.25
PCL (cm)	85.00 ± 18.71	69.75 ± 7.21	52.71 ± 6.75	25.87 ± 3.95	34.04 ± 2.76	75.68 ± 13.23	94.02 ± 10.08	64.47 ± 25.08	56.11 ± 7.35
PL (cm)	10.85 ± 2.28	9.91 ± 1.34	5.29 ± 0.88	3.01 ± 0.37	4.12 ± 0.66	10.64 ± 1.83	15.73 ± 2.10	9.37 ± 3.51	9.83 ± 1.51
PS (cm)	13.26 ± 3.93	7.15 ± 1.12	9.85 ± 1.93	3.62 ± 0.82	4.08 ± 0.93	13.08 ± 3.47	15.92 ± 2.49	11.27 ± 5.94	7.57 ± 1.71
RH (cm)	6.03 ± 1.42	4.41 ± 0.50	4.07 ± 0.73	1.71 ± 0.32	2.16 ± 0.27	5.60 ± 1.19	5.98 ± 0.36	3.37 ± 0.67	3.17 ± 0.67
RL (cm)	7.73 ± 1.32	9.67 ± 1.06	4.91 ± 0.84	2.71 ± 0.39	3.09 ± 0.32	6.91 ± 1.18	6.84 ± 0.65	3.10 ± 0.81	4.71 ± 0.69
SA1 (cm²)	0.84 ± 0.35	0.27 ± 0.13	0.38 ± 0.13	0.08 ± 0.03	0.20 ± 0.08	/	0.69 ± 0.34	/	0.23 ± 0.07
SA2 (cm²)	0.55 ± 0.29	1.05 ± 0.22	1.32 ± 0.40	0.23 ± 0.07	0.59 ± 0.17	/	0.45 ± 0.14	/	0.46 ± 0.11
TL (cm)	102.28 ± 21.07	84.94 ± 8.27	65.08 ± 7.40	32.40 ± 4.55	42.43 ± 3.51	92.20 ± 15.76	114.89 ± 13.27	81.01 ± 29.68	68.34 ± 8.40
TS (cm)	5.54 ± 1.14	3.58 ± 0.54	3.89 ± 0.58	1.62 ± 0.30	1.98 ± 0.21	4.63 ± 0.79	6.66 ± 1.21	4.01 ± 1.59	3.83 ± 0.43

Ratio									
CR	0.77 ± 0.10	0.59 ± 0.06	0.57 ± 0.04	0.48 ± 0.03	0.40 ± 0.04	0.60 ± 0.06	0.67 ± 0.06	0.50 ± 0.04	0.55 ± 0.03
RR	0.79 ± 0.09	0.45 ± 0.04	0.82 ± 0.08	0.63 ± 0.08	0.70 ± 0.09	0.81 ± 0.08	0.91 ± 0.09	1.23 ± 0.06	0.67 ± 0.8
BR	0.19 ± 0.01	0.15 ± 0.01	0.18 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.03	0.13 ± 0.23

Species discrimination

Based on these 25 relative morphological measurements, PCA was carried out to reveal the individual dispersion. As a reminder, all morphological variables were divided by the total length of each individual. The first two axes of the PCA explained 49.6% of all species dispersion (35.1% for the PC1 and 14.5% for the PC2) (Fig. 4A). The other PCs after does not explain so much variation (PC3 1.2%; PC 4 0.89%) and thus were not taken into account. Morphological variables explaining the PC1 separation are mainly the pectoral area, the distance between the first and the second dorsal, the frontal span, the caudal length 2, the first dorsal area, the fork length, the lateral span, the caudal area, and the body height gradually which reach more than 70% of PCA weight (Fig. 4B). Morphological variables participating to the PC2 separation are mainly based on rostrum measurement (head length and rostrum length) and the spine area 2 which weight more than 60% of the PC2 (Fig 4B).

As for the data transformation section, univariate ANOVAs tests show that the main axis of variation between species is the total length with a least a p-value < 0.01 (Table 2). There are also significant differences for all variables between species but without taking into account the total length, we can assume that those differences are explained by the length differences between species (Table 2), indeed, for this analysis variables were not divided by the total length. In contrast for data regarding species total length crossed, only five variables are significantly different (BH, D2-C, FS, PA, SA2) with at least p-value < 0.05 . These last results indicate that, once the body size is accounted for, there are differences between squaloid deep-sea shark species for these five variables (Table 2).

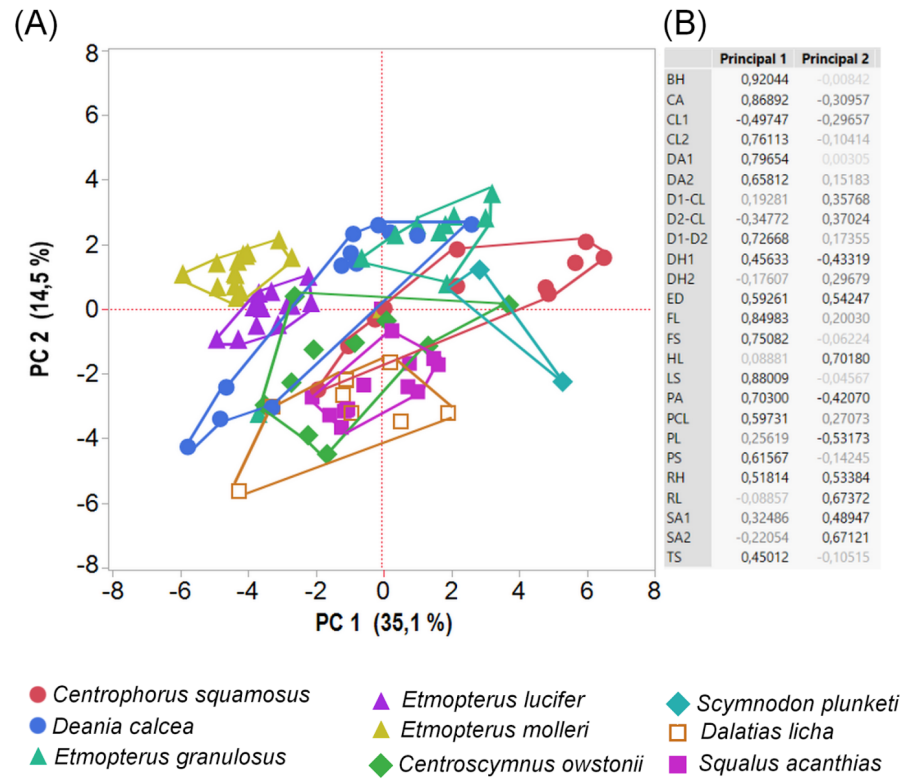


Fig. 4 (A) PCA clustering species according to morphological variables selected with confidence interval of ellipses ; (B) Principal component (PC) loading from an analysis of 25 variables in nine shark species.

Table 2 Result from univariate ANOVAs comparing nine different squaloid shark species for a variety of morphological variables (log10-transformed) using three terms: total length, species, and the interaction between both. Value are F-value of fisher test, * p-value < 0.05, ** p-value < 0.01, *** p-value <0.001.

Variables	Total length	Species	Total length * species	Variables	Total length	Species	Total length * species
BH	334.74***	11.02***	2.44*	FS	61.82***	3.86***	2.30*
CA	367.31***	6.08***	0.95	HL	424.72***	7.34***	1.93
CL1	204.47***	3.46**	0.40	LS	592.15***	11.76***	0.91
CL2	188.56***	3.74**	0.70	PA	273.10***	23.06***	2.90**
DA1	207.46***	10.16***	1.28	PCL	4432.13***	4.16***	1.02
DA2	161.48***	20.08***	0.97	PL	147.91***	29.12***	1.20
D1-CL	216.67***	2.28*	1.93	PS	364.92***	15.54***	1.25
D2-C	69.89***	2.07*	2.47*	RH	98.24***	7.53***	1.32
D1-D2	251.74***	2.86**	1.26	RL	155.17***	61.50***	1.94
DH1	88.07***	12.13***	0.17	SA1	9.60***	4.78***	1.26
DH2	76.51***	15.30***	1.23	SA2	10.25**	12.01***	2.94*
ED	332.59***	40.97***	0.88	TS	387.96***	12.42***	1.03
FL	5170.70***	3.05**	0.90				

Velocity discrimination

As for the species discrimination, a PCA was carried out to analyze how velocity group are dispersed based on the 25 relative morphological variables. As a reminder, in this analysis, all morphological variables were divided by the total length of each individual. In contrast with the species discrimination PCA, only species with known cruise swimming speed values are clustered in the next analysis (Group A: *Dalatias licha*; Group B: *Centroscyrnus owstonii*, *Scymnodon plunketi*, *Centrophorus*

squamosus, and *Deania calcea*; Group C: *Etmopterus granulosus* and *molleri*). The first two axes of the PCA explained 55.3% of all species dispersion (37.8% for the PC1 and 15.7% for the PC2) (Fig. 5A) while next axes explain less than 2.8 % of the variation and thus were not taken into account. Morphological variables explaining the PC1 separation are mainly the first dorsal area, the distance between the second dorsal and the caudal fin, the caudal length 2, the fork length, the caudal area, the lateral span, and the body height gradually which reach more than 70% of PCA weight (Fig. 5B). Morphological variables participating to the PC2 separation are mainly based on head measurement (eyes distance, head length, rostrum height and rostrum length) and the spine area 1 and 2 which weight more than 55% of the PC2 (Fig 5B).

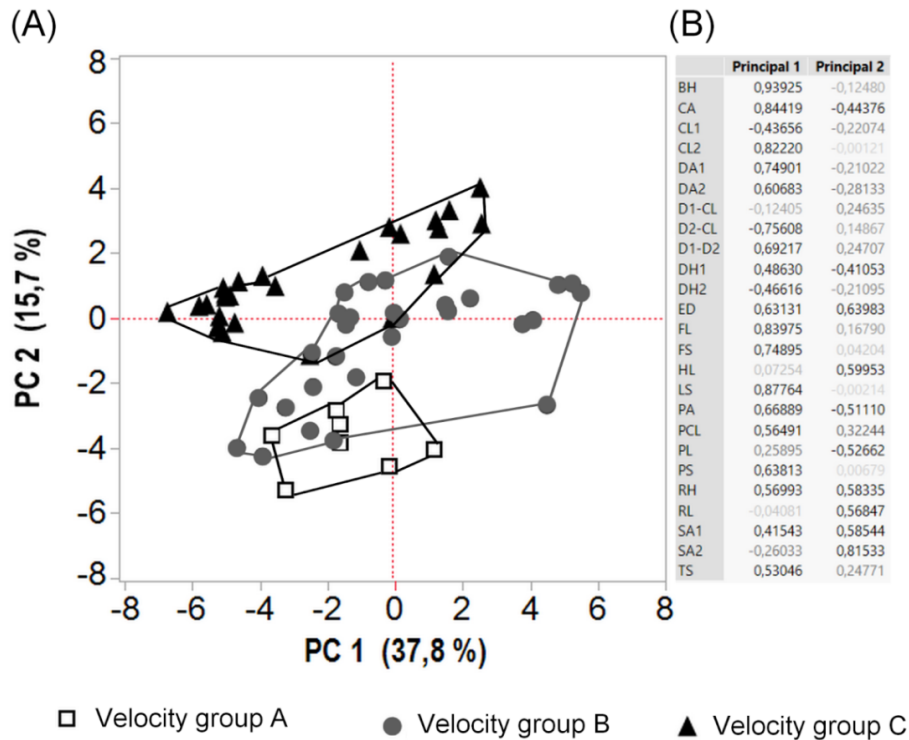


Fig. 5 (A) PCA clustering species according to morphological variables selected with confidence interval of ellipses ; (B) Principal component (PC) loading from an analysis of 25 variables in seven shark species grouped by cruise swimming speed; Velocity group A includes *Dalatias licha*, the velocity group B is represented by *Centroscyrnus owstonii*, *Scymnodon plunketi*, *Centrophorus squamosus*, and *Deania calcea*; The velocity group C includes *Etmopterus granulosus* and *molleri*.

Traditional ratios linked with the swimming speed were tested between the velocity groups. The caudal ratio was significantly higher for sharks from the velocity group B (Kruskal-wallis and Steel-Dwass test, p -value < 0.05) (Fig. 6A), the rostrum ratio was significantly higher for sharks from the velocity group A (Kruskal-wallis and Steel-Dwass test, p -value < 0.05) (Fig. 6B), and the body ratio was significantly lower for sharks from the velocity group C (Kruskal-wallis and Steel-Dwass test, p -value < 0.05) (Fig. 6C).

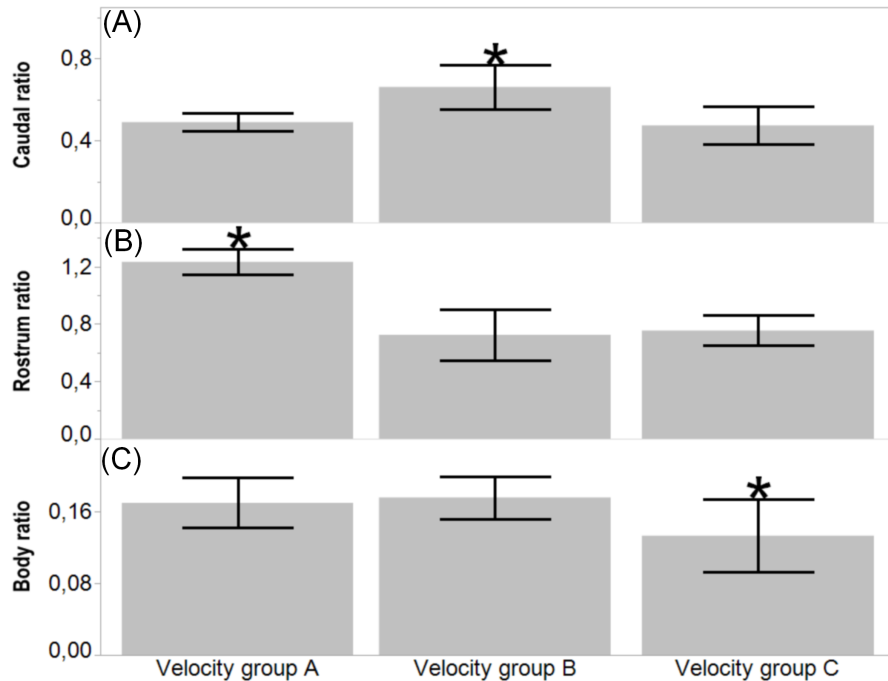


Fig. 6 (A) PCA clustering species according to morphological variables selected with confidence interval of ellipses ; (B) Principal component (PC) loading from an analysis of 25 variables in seven shark species grouped by cruise swimming speed; Velocity group A includes *Dalatias licha*, the velocity group B is represented by *Centroscyrnus owstonii*, *Scymnodon plunketi*, *Centrophorus squamosus*, and *Deania calcea*; The velocity group C includes *Etmopterus granulosus* and *molleri*.

Geometric morphometric discrimination

Only seven species were geomorphologically analyzed for the first and the second spine because two collected species (*D. licha* and *C. owstonii*) do not display any spines. The first PCA (Fig. 7A) illustrates variation between species for the first spine shape. Variation explained by the first principal component (PC1) is illustrated by two opposite typical shapes (upper left and bottom right). Shape on the left is smaller and wider while shape on the right is longer and narrower. All individuals are

widely dispersed on this graph except for them of *E. molleri* clustered on the upper right side.

In the same way, Fig. 7B illustrates differences on the second spine shape between species. Species clustered on the right of the first axis (PC1) have longer and narrower spines while species on the left have smaller and wider spine (also explained by the narrowing of the squares associated to the shape). Species distributed mainly along PC1 and that seem to have a longer second spine are the species *E. lucifer*, *E. granulatus*, *E. molleri*, and *D. calcea*. Two others species (*C. squamosus* and *S. plunketi*) are dispersed equally between PC1 and PC2 and seem to have smaller and wider first spine.

Regarding the geomorphological analysis of the caudal fin, all the nine species collected were illustrated on a PCA (Fig. 8A). The principal axis describes variation of caudal fin shape mainly by the spacing between the semi-landmarks, illustrated by the two typical shapes on the sides. Species clustered on the left side (*E. molleri* and *E. lucifer*) display longer upper caudal lobe and more detailed lower and upper lobes. This tendency is emphasized by three dimensional effects of the squares which are correlated with longer proportions. Conversely, species on the right side of PC1 display restricted caudal fin with less detailed lobes (*C. owstonii*, *S. plunketi* and *S. acanthias*). Another notable tendency is that *D. licha* individuals are clustered on top of PC2 axis. Investigating shape variation explained by this axis is interesting to discover which geomorphological factors of the caudal fin are more represented for *D. licha* individuals.

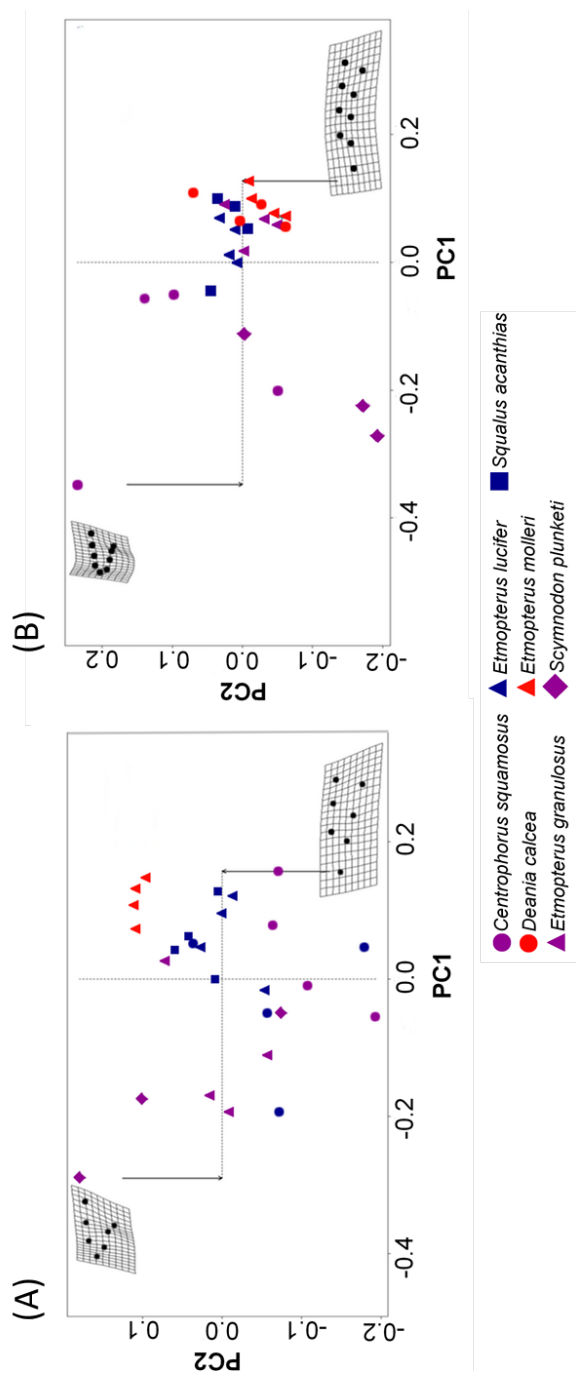


Fig. 7 PCA illustrating distribution of individuals from 7 different species for the shape of their spines. (A) correspond to the first dorsal fin spine, variation explained by the first principal component (PC1) is illustrated with two opposite typical shapes (upper left and bottom right), variance explained by the two axes is from 90.56%; (B) correspond to the second dorsal fin spine, variation explained by the first principal component (PC1) is illustrated with two opposite typical shapes (upper left and bottom right), variance explained by the two axes is from 89.06%.

To explain the shape variation along PC2, another PCA has been carried out (Fig. 8B) by inverting the two principal components and selecting PC2 as main axis. The shape variation on this axis is very different than shape variation on PC1 (Fig. 8A). Species clustered on the left show out a caudal fin with detailed lobes and with a caudal fork while species clustered on the right (like *D. licha*) display a particular caudal fin shape with a with a pronounced upper lobe, an almost non-existent fork and a reduced ventral lobe. Therefore, there is no lower lobe to this kind of caudal fin.

DISCUSSION

This first PCA study of morphological features of eight squaloid deep-sea sharks and one shallow-water species reveals several finding. Firstly, as it was already observed on shallow-water shark species, all morphological variables displayed a strong correlation with the total length of each individual which is called isometry (Irschick et al., 2017). Only the second spine area seemed less correlated with the total length but it is mainly explained because two deep-sea shark species (*Dalatias licha* and *Centroscymnus owstonii*) does not display spines on their dorsal fins. This result is confirmed with all test including absolute values of morphological variables (PCA, Pearson's test, and univariate ANOVAs). However, even if the size is the main factor explaining the variation between species, the univariate ANOVAs using Species-total length crossed terms revealed significant differences in the body shapes of these nine squaloid sharks. Indeed, the body height (BH), the distance between the second dorsal fin and the caudal fin (D2-C), the frontal span (FS), the pectoral area (PA), and the second spine area (SA2) differed between species. In the PCA regarding the species discrimination, these entire variables weighted also relatively strongly in the axis one and two. While deep-sea sharks from squaloid order are often grouped in the

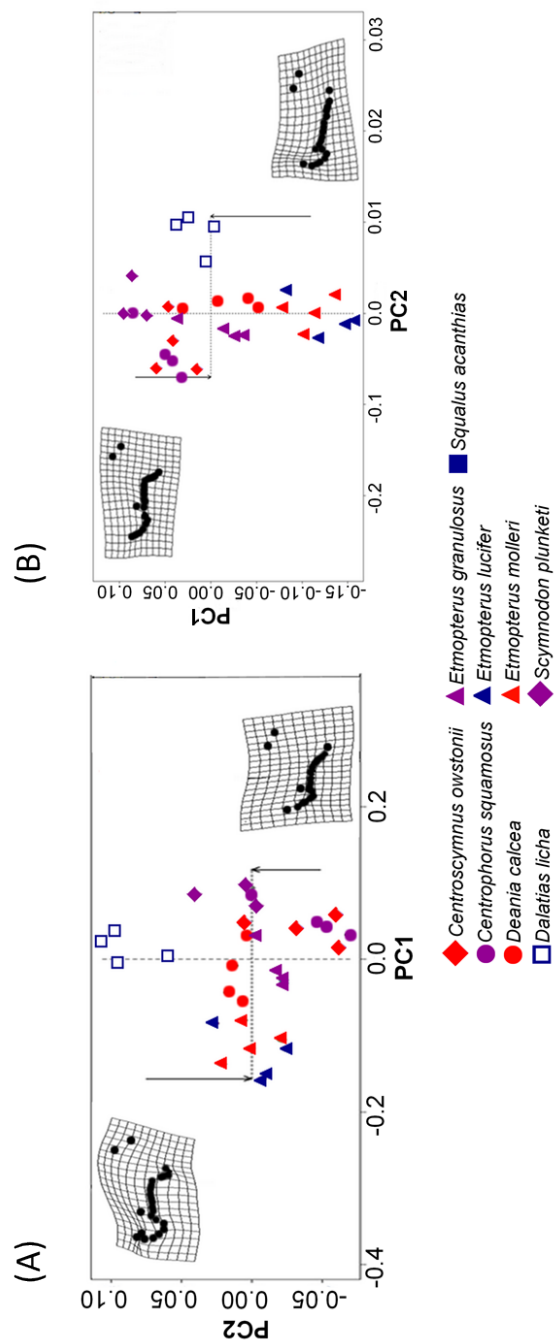


Fig. 8 PCA illustrating distribution of individuals from 9 different species for the shape of the caudal fin. (A) Variation explained by the first principal component (PC1) is illustrated with two opposite typical shapes (upper left and bottom right), variance explained by the two axes is from 68.34%; (B) variation explained by the second

same morphotype (morphotype 4; Thomson and Simanek, 1977), this study present some significant differences which could precise this previous morphotype to create sub-classes inside it, especially for *Etmopterus molleri* which display a body shape separated to others as seen in the species discrimination PCA.

Regarding the impact of body shape differences on velocity groups, the PCA revealed a clear gradient of velocity according to its second axis. Indeed the Group A displaying the lowest cruise swimming speed reached the inferior part of the graph, the Group B the intermediate part and the Group C which display the highest cruise swimming speed reached the superior part. The second axis of the PCA can be explained by several morphological variables but one of the most important is the spines areas, especially the one of the second dorsal fin spine. It revealed that faster sharks display a relative bigger spine than slower species. This result was also confirmed by the geometric spines morphometric. For the first dorsal fin spine the difference is less obvious but for the second dorsal fin spine, Etmopteridae sharks were all clustered in the right side of the graph corresponding to long and sharp spine. It has been suggested that longer and wider spine could create a water vortex, enabling the caudal fin to beat easier as the same effect than described for the second dorsal fin (Lauder & Santo, 2016; Maia et al., 2017). A sharper second spine could also confine the turbulent flow behind the spine enabling the shark to swim with rhythmic undulations (Maisey, 1979). However, these effects of the second spine on the speed are only theoretical and need to be tested mechanically by observing whether the interaction of the water flow with spine could improve the speed. Nevertheless, it is still difficult to achieve it with living deep-sea sharks; modeling studies are therefore the best way. Moreover, the geometric morphology analysis of the second spine gives only information on the lateral shape of the fin but

not on the anterior shape. Analyses of the anterior part of the spine could bring further information on the width of the spine and if the spine is sharper, which are major variables explaining the amount of water deviate by the second spine (Maisey, 1979). Finally, the longer and sharper spines for Etmopteridae could also be explained by the fact that these sharks are the smallest of this study and therefore more sensitive to predation (Compagno, 1984; Ebert, 2013; Nelson, 2006).

It has already been proved that spines on Etmopteridae act as defensive tools and they even illuminate them using their luminescence as aposematic signal (Duchatelet et al., 2019).

The second morphological variables weighting in the second axis are linked with the head shape. Faster deep-sea sharks displayed a more elongated head than slower ones which displayed a more rounded head especially for *Dalatias licha*. This result was also confirmed by the rostrum ratio analysis which revealed that deep-sea sharks from the velocity group A exhibit a rounded head. This result agree with what it was previously said in the literature regarding head shape effect on the water penetration (Lebrun, 2001).

Regarding the caudal fin shape, the main propeller of sharks, our results are in contradiction with the literature. Indeed, faster deep-sea sharks (Group C - Etmopteridae) displayed the more heterocercal caudal fin and the lowest caudal fin area than other deep-sea sharks. In the literature, it has been proved that homo/isocercal caudal fin provides more thrust than heterocercal one (Thompson, 1976; Ferry & Lauder, 1996; Wilga and Lauder, 2002). However, the ratio differences between species observed in this study is lower than the differences between fast swimmers such as mako sharks and benthic sharks. Moreover, the geometric caudal fin morphometric analysis revealed that *D. licha*, the slowest shark,

is an outlier regarding the PC 2. The analysis of PC 2 variation revealed that this species display almost non-existent fork in its caudal fin and a reduced ventral lobe. It could suggest the importance of the fork in the thrust generation. Indeed, another shark presenting a slow cruise displacement but fast burst capabilities is the frilled shark (*Chlamydoselachus anguineus*); this shark displays also a caudal fin with almost non-existent fork and a reduced ventral lobe (Ebert et al., 2013). Nevertheless, mechanistic studies of fork importance in thrust generation would allow to better understanding if this morphological feature has a real impact on the velocity. Finally the small caudal fin area of Etmopteridae species could also explained why Etmopteridae species can reach a higher tail beat than other squaloid deep-sea species. Indeed, recent study has revealed that the tail beat of these lantern shark reach more than 1Hz during the cruise swimming speed while other deep-sea species reach 0.7Hz at the maximum. These results agree with other study revealing a high aerobic metabolism in Etmopteridae species (Pinte et al., 2019).

In the same way than the caudal fin shape, the link between the body shape of deep-sea shark and their velocity is in contradiction with what it was said in the literature. Regarding the body ratio, Etmopteridae display the more flattened body which is usually related with slower sharks. However, as for the caudal fin ratio, it is important to mention that the differences with the two other groups of velocity are weak in comparison with faster swimmer sharks. Indeed, all deep-sea sharks display a body shape relatively flattened.

CONCLUSION

This study was the first which measures morphological variables on fresh squaloid deep-sea sharks and compare them

through PCA and other multivariate statistical analyses. It appeared that these sharks were grouped as morphologically similar in the fourth group created by Thomson and Simanek in 1977 but some differences occurs and sub-division of this group could be realize with future analyses. Regarding the link between the body shape and the swimming capabilities of deep-sharks, dorsal fin spines and head shape seem to act mainly while caudal fin shape and body shape seem to be in contradiction with the literature. However the differences inside deep-sea species are less obvious than the comparison between benthic and fast swimmer sharks previously studied. Moreover the study of the caudal fin of deep-sea shark highlighted a uncommon caudal fin shape which is the caudal fin with almost non-existent fork and a reduced ventral lobe, as discovered on *Dalatias licha*. It is suggested that the fork could have a real impact on the swimming capabilities but as for the spines potential impact; future mechanistic analyses should be done to confirm it.

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General discussion and perspectives

GENERAL DISCUSSION AND PERSPECTIVES

General discussion

Studies regarding shark swimming capabilities have been instigated since a long time in shallow-water species. They have allowed to characterize numerous differences with some sharks displaying higher velocity, lower maneuverability, etc. than others which could be related with their specific life-style (Shadwick and Goldbogen 2012; Klimley, 2013; Schadwick et al. 2016). Others studies have revealed relations between swimming capabilities and specific adaptations that can be sorted as physiological, anatomical, morphological and behavioral adaptations (Thomson and Simanek, 1977; Sambilay, 1990; Treberg et al., 2003; Low and Chong, 2010; Condon et al., 2012; Shadwick et al., 2012; Lauder & Santo, 2016; Treberg and Speers-Roesch, 2016). Information regarding deep-sea sharks swimming capabilities is still poorly documented. Indeed, only the speed of the Greenland shark (*Somniosus microcephalus*) was measured using satellite telemetry and revealed the slowest velocity recorded for a shark (Watanabe et al., 2012). Others studies have focused on proxy of the locomotor muscle metabolism and have shown that anaerobic metabolism of deep-sea species is lower than their shallow-water counterparts. In contrast, aerobic metabolism does not show significant differences (Treberg et al, 2003; Condon et al., 2012). Therefore, it has been commonly acknowledged that deep-sea sharks should have lower swimming capabilities than their shallow-water counterparts. This conclusion is mainly based on two hypothesis: (i) the effect of the cold environment decreasing muscle activities (Treberg et al., 2003; Watanabe et al., 2012) and (ii) the visual interaction hypothesis which suggests that the swimming capabilities of an organism decreases when it lives in a darker environment (Condon et al., 2012). However, the very low

number of deep-sea shark species studied in these studies does not allow in drawing a general conclusion.

In that context, my thesis appears to be the first multidisciplinary study of deep-sea sharks' swimming capabilities. Moreover, it is also the first study based on eleven squaloid deep-sea species. From this work several major discoveries were made regarding deep-sea sharks biology:

- Not all deep-sea sharks swim slowly, some of them, such as the luminous sharks from Etmopteridae family and *Centrophorus harrissoni*, even display cruise swimming speed values similar to neritic shallow-water counterparts.
- Higher cruise swimming speed for Etmopteridae luminous deep-sea sharks can be advantageous based on the isolume followers' hypothesis (moving rapidly in the water column to stay at the depth where light equal their ventral luminescence for the counter-illumination function)
- The Greenland shark (*Somniosus microcephalus*) was previously described as the slowest shark in the world. Our data have shown that the kitefin shark, *Dalatias licha*, displays an even slower cruising speed and thus, becomes the new slowest shark of the world.
- Aerobic metabolism and the red muscle proportions are related with the cruise swimming speed measured, suggesting physiological adaptations of deep-sea species for an efficient cruise swim.
- Anaerobic metabolism and white muscle proportions were higher for species displaying a slow cruise swimming speed, which suggests better burst capabilities allowing for short and fast attacks during hunting.

- Our results confirm that deep-sea sharks display a buoyancy closer to the neutrality than their shallow-water counterparts (that are negatively buoyant).
- Bone, in 1966, suggested that the buoyancy of sharks is related to red muscle proportions. According to him, sharks that are closer to the neutral buoyancy should display less red muscle fiber. While this hypothesis has been cited many times, our study is the first one to confirm it with measured values.
- The first PCA morphological studies of Squaliformes sharks were performed revealing some differences in this group, while it was previously considered by Thomson and Simanek in 1977 to be part of only one morphotype.
- The possible role of the dorsal fin spines in the swimming capabilities has been highlight but future mechanistic studies still need to be carried out.

To synthesize our data, we summarized the main results obtained in table 1. In this table the values of each species are compared with the average values of the relevant parameter for deep-water sharks. From this average, the values are estimated similar (=) if it does not exceed one alpha error; they are considered lower (-) or higher (+) if they exceed one alpha error and they are considered much lower (--) or much higher (++) if they exceed two alpha errors. Morphological characteristics have not been taken into account in the summary table because future studies are needed to fully understand their impacts. From these results, we were able to describe five different groups:

- (i) The first, underlined in blue (Table 1), includes *Squalus acanthias*, the shallow-water squaloid species. This shark displays high values in each feature related to the swimming capabilities. Even if its cruise swimming speed is unknown, as a shallow-water species, we can presume that its speed (cruise

and burst) is higher than other deep-sea squaloid species. This is supported by all anatomical, physiological and morphological features observed (**Paper II, III, and IV**, p 121, 141, 177). Its buoyancy is negative, this being commonly found in other shallow-water species, but its large pectoral fin might provide more hydrodynamic lift (**Paper III**, p 141; Wilga and Lauder, 2001; Treberg and Speers-Roesch, 2016).

- (ii) The second group, highlighted in green (Table 1), is represented by sharks from the Etmopteridae family. For two of these sharks (*E. granulosus* and *E. molleri*), cruise swimming speed values obtained are the highest swimming speed values ever observed for deep-sea sharks (**Paper I**, p 81). Data regarding red muscle proportions and aerobic metabolism were highly related to this high cruise swimming speed. This situation was observed for two other Etmopteridae species (*E. spinax* and *E. lucifer*), however speed values were not measured (**Paper II**, p 121). Regarding white muscle and aerobic metabolism, data revealed the lowest range observed, suggesting low burst capabilities which could explain why these sharks are often found in stomach contents of bigger species (**Paper II**, p 121). Except for *E. molleri* that displays negative buoyancy similar to shallow-water species, other Etmopteridae species display higher hydrostatic lift provided by their liver (**Paper III**, p 141). The negative buoyancy of *E. molleri* can be counteracted by its high level of red muscle which can provide better swimming to participate to its buoyancy (Bones and Robert, 1969; Shadwick and Goldbogen, 2012).
- (iii) Sharks from the third group, colored in purple (Table 1), are the non-luminous deep-sea species (*C. squamosus*, *D. calcea*, *C. owstonii*, and *S. plunketi*). In comparison with all deep-sea sharks studied these

sharks displayed intermediate values of cruise swimming speed (**Paper I**, p 81), which has also been related with their red muscle proportion and their aerobic muscle metabolism (**Paper II**, p 121). Regarding the white muscle proportion and the anaerobic muscle metabolism, they displayed intermediate values (except for *D. calcea*), suggesting better burst capabilities than sharks from the previous group (**Paper II**, p 121). These sharks could therefore hunt Etmopteridae using really fast and short attacks. Furthermore, they appear to be more generalist. These sharks, as the majority of deep-sea sharks, displayed more hydrostatic lift than shallow-water counterparts which can be related with the lack of red muscle (**Paper III** – p 171).

- (iv) The fourth group (in orange, Table 1) is represented by *Dalatias licha*. As previously said, this shark swims at the slowest speed ever measured shark (**Paper I**, p 81). Its slow cruise swimming speed is also correlated with a low proportion of red muscle and aerobic muscle metabolism (**Paper II** – p 121). This low proportion of red muscle could also be counteracted by the highest lift provided by the liver for this studied species (**Paper III**, p 141). As for sharks from the third group, its proportion of white muscle and anaerobic muscle metabolism is higher than Etmopteridae and therefore suggests better burst capabilities, which could explain the findings of lantern sharks in its stomach (**Paper II**, p 121).
- (v) The fifth group, (in red, Table 1) characterizes sharks without enough data preventing us from drawing accurate conclusions (*G. melastomus*, *I. brasiliensis*, and *C. harrisoni*). Data regarding these species are missing mainly due to the low number of specimens available (3, 3 and 0 specimens collected respectively).

Table 1 Synthesized table of all data collected for eleven deep sea species and two shallow-water species, discussed across the different chapters of this thesis. The values of each species are compared with the average value of the relevant parameter of deep-water sharks. From this average, the values are estimated similar (=) if it does not exceed an alpha error (0.05); they are considered lower (-) or higher (+) if they exceed an alpha error and they are considered much lower (--) or much higher (++) if they exceed two alpha errors; % of white muscle and anaerobic metabolism results.

	Cruise swimming capabilities	% red muscle	% white muscle	Aerobic metabolism	Anaerobic metabolism	Burst swimming capabilities*	Buoyancy
Mean value measure for deep-sea species	0.36 ms ⁻¹	1.73%	23.45%	54 UE	71 UE	22.15 g.kg ⁻¹ of shark	
<i>Squalus acanthias</i>	++	++	+	=	+	++*	--
<i>Etmopterus spinax</i>		=	-	++	-	--*	=
<i>Etmopterus molleri</i>	++	++	-	++	=	-*	--
<i>Etmopterus granulosus</i>	++	=	-	+	--	--*	=
<i>Etmopterus lucifer</i>		=	--	+	--	--*	
<i>Centrophorus squamosus</i>	=	=	+	=	=	++*	++
<i>Deania calcea</i>	=	-	+	=	--	=*	+
<i>Centroscyllium owstonii</i>	-	++	+	=	++	++*	=
<i>Scymnodon plunketi</i>	=				++	++*	
<i>Dalatias licha</i>	--	--	+	-	=	++*	++
<i>Galeus melastomus</i>							--
<i>Isistius brasiliensis</i>		=					=
<i>Centrophorus harrisoni</i>	++						

Because the present thesis mainly focuses on swimming capabilities of deep-sea shark species, sharks from the first and fifth group will not be taken into account for the rest of the discussion since they do not provide enough data to draw a strong conclusion.

As previously indicated in the introduction section, the swimming capabilities can provide a lot of different information regarding the ecology, life range and distribution of marine organisms (Sainte-Marie and Hargrave 1987; Wilson et al. 2005; Watanabe et al. 2012; Shadwick et al. 2016). From our own data, we can thus draw some potential hypothesis regarding the life-style and home-range of sharks.

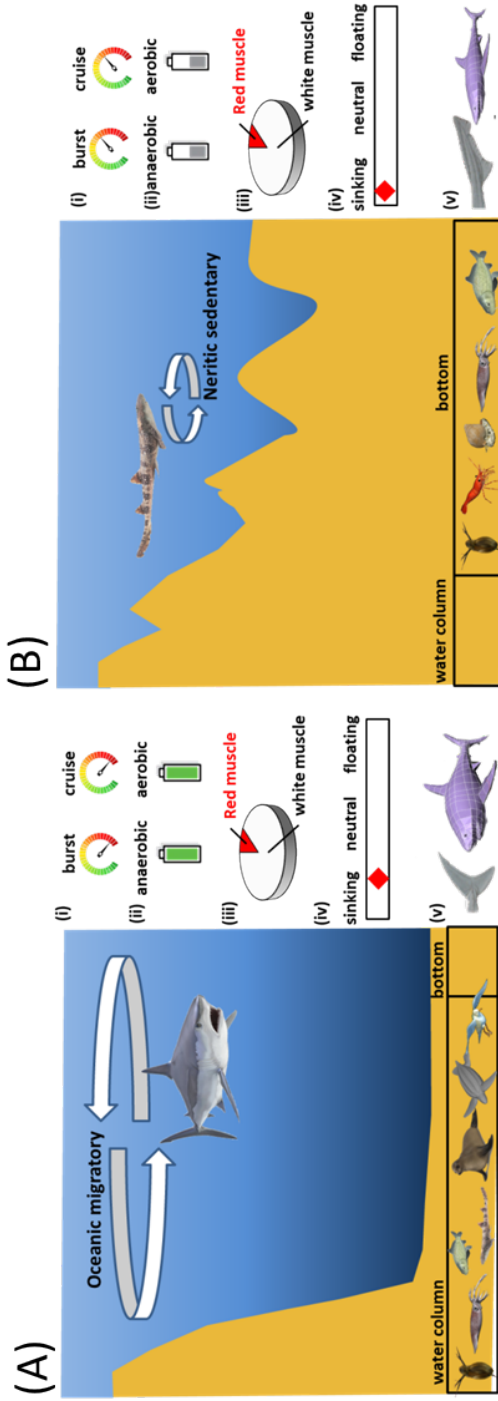
Through former studies, shallow-water sharks can be placed into two main categories: oceanic (Fig. 1A) and neritic (Fig. 1B) sharks.

- The first are species that migrate mainly horizontally over long periods of time. As a result, the swimming speed of these sharks is relatively high, both in cruise and in burst modes. These sharks have a wide range of adaptations related to swimming: (i) a developed musculature (Bernal et al., 2003; Shadwick and Goldbogen, 2012), (ii) an efficient aerobic and anaerobic metabolism (Condon et al. 2012), (iii) an excellent hydrodynamism (Thompson and Simanek, 1977, Ebert et al., 2013), (iv) and even for some, a system that allows the body to be warmer than the surrounding water (regionalized endotherm) (Dickson and Graham, 2004). Thanks to all these adaptations, pelagic sharks have a wide range of different prey, from plankton for whale sharks to seals for great white sharks. Reptile, fish and even

some seabirds, are all preys that mainly live in the water column in the open sea (Cortes, 1999).

- The second category includes benthic sharks. They are mainly found on the bottom near shores in shallow water (benthic neritic species). These species are more sedentary and therefore the pressure for effective swimming speed is reduced in favor of maneuverability allowing them to zigzag on the substrate (Shadwick and Goldbogen, 2012). Therefore, adaptations regarding swimming capabilities are quite different: (i) they also have a developed musculature which is more flexible than powerful (Gemballa et al., 2003; Gemballa et al., 2006; Shadwick and Goldbogen, 2012), (ii) aerobic metabolism is lower than pelagic sharks while anaerobic metabolism is quite similar for some species (Condon et al. 2012), (iii) their morphology is more flattened and designed to move near the bottom without stirring the substrate and injuring themselves (Thompson and Simanek, 1977; Ebert et al., 2013). They feed mainly on organisms such as crustacean, gastropods, bivalves, fish and cephalopods located close to the bottom (Cortes, 1999).
- For the two categories described, the buoyancy provided by the liver remains negative, thus they sink. They compensate this through morphological adaptations (heterocercal caudal fin, large pectoral fins) and by expending more energy to stay in the water column.

Shallow-water



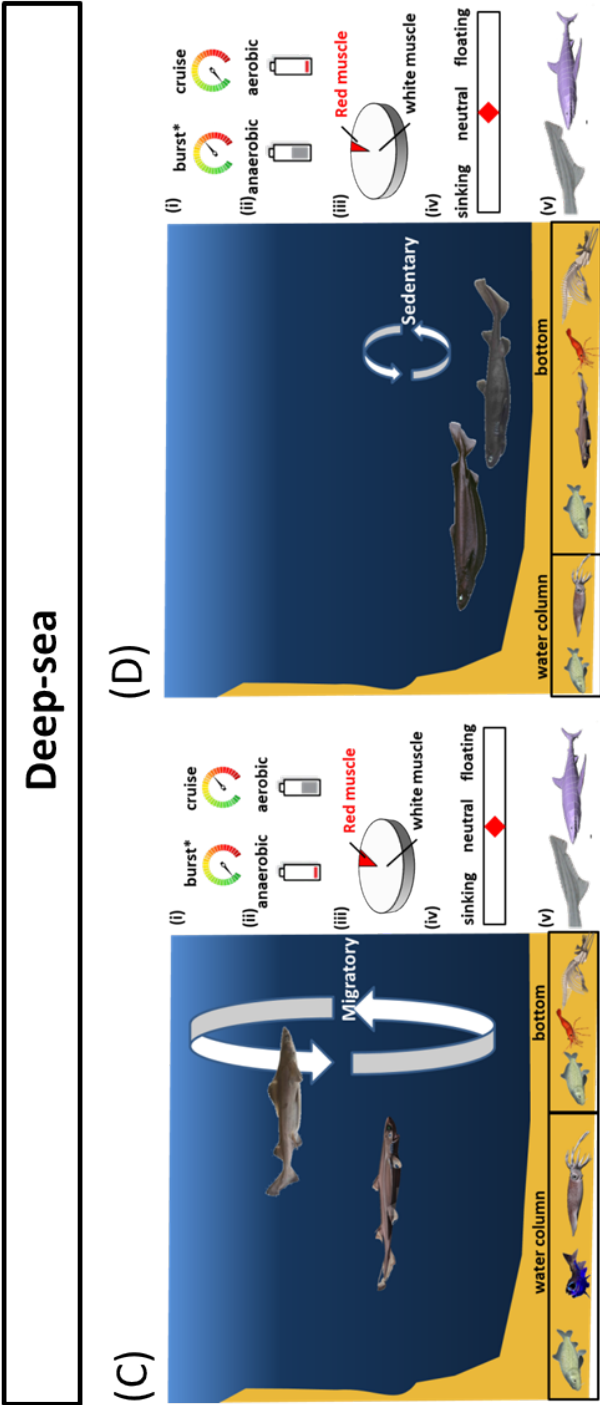


Fig. 1 Simplified schemes of the four ecological categories of sharks: shallow-water oceanic (A) and neritic sharks (B) and deep-water migratory (C) and sedentary sharks (D); each illustration corresponds to (i) the speed data, (ii) the metabolism data, (iii) the white muscle red muscle ratio in the body, (iv) the general buoyancy of sharks, (v) their morphology; the diet of each category is represented below as well as the prey location.

Now regarding deep-sea sharks, it has already been shown in the literature that some deep-sea sharks perform circadian vertical migrations such as bluntnose sixgills shark (*Hexanchus griseus*), the velvet belly lantern shark (*E. spinax*) (Mallefet, personal comm.) and other squaliformes (Finucci and Jones, personal comm.). These sharks are found lower in the water column during the day and move up into shallower water at night to feed. This information was obtained either through telemetric studies or fisheries data. Conversely, fisheries data have also shown that some species are not fished at different depths between day and night, suggesting more sedentary behavior. From our results, deep-sea sharks could also be divided into two ecological categories: migratory (Fig.1C) and more sedentary (Fig. 1D) sharks.

- The first will correspond to sharks with higher cruise swimming speed such as Etmopteridae species, *C. harrisoni* and possibly other species in the intermediate speed group. These sharks will be characterized by a higher cruise swimming speed and associated adaptations: (i) efficient aerobic metabolism while the anaerobic one is weaker (ii) more red muscles (>1.73% of the total body mass) than other deep-sea sharks and thus less white muscle fibers (<23.45% of the total body mass), (iii) regarding the morphology, the presence of dorsal fin spine can be an advantage but futures mechanistic studies should be done. They move in the water column as it was described in the literature, from deep layers during the day to shallow ones during the night. This behavior is partly confirmed by the analysis of stomach contents. Indeed, it has been shown for some deep-sea shark species, notably Etmopteridae, that their diet was mainly based on prey found closer to the surface in the water column (Daley et al.,

2002). Another hypothesis which can be related to this vertical displacement is the “isolume follower”. Indeed, as already described, Etmopteridae species produce a ventral blue light aiming to mimic the environmental downwelling light of their environment to make their silhouette disappears from the sight of underneath predators by a camouflage called counter-illumination (Claes and Mallefet, 2008; Claes et al., 2010; Claes et al., 2014a and 2014b). However, to be a good counterilluminator, they need to change quickly their light ventral intensity what is not the case since lanternsharks harbour a slow hormonal control of their luminescence (Latz 1995; Case et al. 1977; Young and Roper 1976; Clarke, 1963; Claes and Mallefet, 2011; Claes et al., 2011a; Claes et al., 2012; Claes and Mallefet, 2015; Duchatelet et al., 2019). Therefore, to overcome this slow control, shark should swim constantly up and down in the water column to find the right surrounding light level matching they own luminescence. In the same matter, the light decrease and increase, at dusk and dawn respectively, could be taken into account for luminous shark vertical migration.

- The second group will correspond to sharks with slower cruise swimming speed such as *D. licha* and possibly other species in the intermediate speed group (*C. owstonii*). These sharks will be more sedentary and more associated with the bottom than deep-sea sharks described in the previous group. Adaptations related with their velocity are: (i) efficient anaerobic metabolism while the aerobic one is weaker, (ii) less red muscle (<1.73% of the total body mass) than other deep-sea sharks and therefore more white muscles (>23.45% of the total body mass), (iii) regarding the morphology, no presence of any structure/features

improving the swimming capabilities. The stomach contents of these sharks are mainly composed of benthic prey (fish, crustaceans). This reinforces the idea that these sharks move very little in the water column. Surprisingly, some of the smaller but faster sharks from the previous group (Etmopteridae) are also found in these stomach contents. How can a slower predator eat faster prey? One possible explanation comes from the higher proportion of white muscle and anaerobic metabolism in sedentary deep-sea sharks. One can assume a slow approach followed by a rapid attack by these sedentary sharks, as it has been highlighted for *D. licha* by Zintzen et al. in 2012. The first category of deep-sea sharks would therefore correspond to smaller species that are active tracker and hunter, while the second would correspond to less active, opportunistic species with good acceleration. Regarding *D. licha* luminescence, it is unlikely that this shark will use its bioluminescence as Etmopteridae. Indeed, apart from the presence of ventral photophores, nothing is known about its luminescence. However, it seems unlikely that this large shark with very few predators uses its bioluminescence to hide from below predators (counterillumination); most plausible hypothesis could be that it might shed light on the bottom to spot and identify potential prey.

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Isolume followers' hypothesis proof

Through telemetry study it has already highlighted that some deep-sea sharks perform vertical circadian migration. These data collected on the *Hexanchus griseus* (Comfort et al., 2015) and *Centrophorus harrissoni* (Rodriguez and Sanchez 2014; Rodriguez-Cabello et al. 2016) showed a circadian migration of these two sharks very dependent on light conditions: they wait until

the end of the dusk to start their ascent and they start their descent before dawn arrives.

In this work, the main explanation why Etmopteridae species have a higher cruise swimming speed leans on the “isolume followers’ hypothesis”. According to the “isolume followers’ hypothesis”, luminous sharks would be continuously placed at the isolume layer that corresponds to their ventral light emission. Therefore, when the changes of light take place at dawn and dusk, they will begin their descent and ascend at the same time as these changes rather than wait for them to pass. The upward and downward swim in the water column might also occur during sudden changes in light intensity, for example as the cloud cover thickens. The change in light with the seasons might also have an impact and these migrations leading probably to different patterns between summer and winter for example. In this context, downwelling solar light could have played a strong selective pressure leading to the development of a high swimming speed in deep-sea Etmopteridae.

However, even if the high velocity might be considered as a support of this hypothesis, it does not prove it. To analyze that purpose, the acoustic telemetry appears to be the best way. Therefore, we have submitted a project with the Norwegians colleagues that still waiting ethical approvals. In this research project we plan to study the vertical displacement of *E. spinax*, within the Raunefjord. The Espegrend Marine Research Field Station (University of Bergen, Norway) is a perfect place to develop this project since it is very close to the Raunefjord where lantern sharks live to the shallower water (max 250m) (Claes and Mallefet, 2011b) than everywhere else (500-700m) (Coelho and Erzini, 2008). The study area is restricted (+/- 1km²), and fjords are relatively quieter than other marine place, which is a benefit in acoustic telemetry (high background noise causes interferences in data collection).

The receiver deployments will be done in the deep area of the fjord (250m) where we used to fish lantern sharks. The specific place of each receiver will be determined after the range test that will allow estimating the tag optimal range in this environment. Vemco designed new small tags: the V7TP (22 mm, 1.7 g in air) and the V9TP (37.5 mm, 4.9 g in air). These new tags are crafted specifically for small and juvenile specimens, therefore suitable for lanternsharks. They are equipped with depth and temperature sensor allowing to obtain supplementary to the position (by triangulation), three kinds of information: the ID of tag, the temperature and the depth of the tagged animal. The pressure sensor of the tag could measure the deep until 290 meters based on the fjord topography (Allen, personal comm.).

These tags are internal tags needing a shark surgery. Therefore, sharks will be collected at dawn by longlines set for one hour at 220 meter depths. Then, they will be placed in surgery seawater-filled table. There, sharks will be measured, weighed and anesthetized by a 3 min bath of clove oil extract (20mg/liter) (Cunha and Rosa, 2006), they will undergo a quick surgery (a ventral incision that will be stitched back) to place the transmitter tag into the peritoneal cavity. After the surgery, sharks will be released after few minutes of observation. The initial study will occur during the first three months (winter period) collecting a minimum of one data transmitted per minute.

Sharks' displacements will be correlated with several physical factors (salinity, temperature but also chlorophyll and oxygen concentration) obtained using a CTD and underwater ECO-PAR (for light intensity) sits lowered down to the bottom of the fjord during the whole study period. CTD parameters will be taken regularly (once a week) while ECO-PAR sit will record light for the whole duration of the project. The ambient light intensity of all the

individual swimming places will be calculated from the surface irradiance continuous measurements and downwelling irradiance attenuation of the water column measurements.

Our zero hypothesis is that the individuals will not change their depth position according to changes in the ambient downwelling irradiance (not isolume-follower). Thus for each individual, we will test whether their depth positioning is unrelated to changes in incoming sunlight. This will be done by linear regression analysis between all depth registrations of an individual (y-variable) versus the simultaneous surface irradiance (x-variable) logarithm. If the recorded depth of individual increases linearly with the log irradiance, the zero hypothesis can be rejected and the result is consistent with the alternative hypothesis that *E.spinax* regulate their depth according to changes in ambient light (isolume-follower).

Moreover, using acoustic telemetry on such small shark will be first and future studies on other deep-sea shark species would represent an interesting challenge, especially to characterize their vertical displacements still poorly documented.

Stereo-video analyses and perspectives

The use of video analyses remains one of the best non-invasive ways to study deep-sea sharks' behaviors. Since stereo-video has appeared, the number of study deploying this method has considerably increased (Mallet and Pelletier, 2014). Our script created with MatLab has as advantages that with the open source code, it can be adapted to relatively every purpose that can be imagined. However, working with a script is still difficult to handling and code lines are not intuitive at all. Therefore, the creation of graphical open source software will still be a breakout while current similar commercial softwares are still expensive.

Moreover, even if the use of a cube allowed a better estimation of the depth and inherent camera parameters compared to the classical board use with Rstudio software (Harvey and Shortis, 1995; Boutros *et al.*, 2015). The analysis of data has revealed some limits especially in the corner of images. Indeed, to better assess distortions' parameters we need to cover the entire images provided by the left and the right cameras (**Paper I** - supplementary data II, p 113). As a consequence, a better calibration of cameras cannot be done with a cube but could probably be realized with the grid of the tiles presents around the cube in the swimming pool.

Metabolism analyses and perspectives

Results from the study of deep-sea shark muscle aerobic and anaerobic metabolism have provided new insights. Firstly, it has been proven that aerobic metabolism can be related to the cruise swimming speed as it was already demonstrated for shallow-water counterparts (Carrier *et al.*, 2012; Klimley, 2013). Regarding the anaerobic metabolism, it can be only suggested since we miss burst values for many species. Measurements of burst behaviors were linked with the anaerobic metabolism of shallow-water species and extrapolation to deep-sea can be accepted but burst value of these deep-sea species will be an attractive feature. However, it is difficult to catch this moment with video footages and moreover it is almost impossible to determine if the velocity values are the maximum that one species can reach; at most, it can be stated that observed values are the highest ever recorded.

The high aerobic metabolism of Etmopteridae brought us some new questions related to the oxygen consumption of these species. Indeed, aerobic metabolism required oxygen to work and the majority of currently known luminous reactions need oxygen.

Therefore, it is essential for the future to know how Etmopteridae species supplied their high oxygen demands. For that purpose, the best way is to measure the metabolism by respirometry. However, this method is still impracticable since we cannot keep deep-sea shark species more than one week alive; moreover, the potential stress caused by the fishing and the captivity remains a major bias for this kind of analyses. Another possible way consists to measure the potential oxygen uptake that a shark can display according to gills' morphology. For that, it is necessary to determine the gills' surface using the following equation (equation 1) (Hughes, 1966; Muir and Hughes, 1969):

$$(7) A = L_{fil} \times 2n_{lam} \times A_{lam}$$

Where= A is the total gills' surface

L_{fil} is the total length of all the filaments

n_{lam} is the lamellar frequency, that is the number of lamellae per unit length on one side of a filament (hence the multiplication by two to take into account the lamellae on both side of the filament)

A_{lam} is the mean bilateral surface area of a lamella.

When the gills' surface is known, it is possible to measure the oxygen uptake by measuring the thickness of lamellae epithelium corresponding to the diffusion distance (equation 2) (Wegner, 2011 and 2016):

$$(2) V_{O_2} = (K \times A \times \Delta P_{O_2})/t$$

Where: V_{O_2} is the rate of oxygen uptake [mgO₂.min⁻¹].

K is the diffusion (or Krogh) coefficient through a specific material [mgO₂.cm⁻².mmHg⁻¹.min⁻¹].

A is the respiratory surface of the gills [cm²].

ΔP_{O_2} is the mean difference in the partial pressure oxygen between the blood and water [mmHg]

t is the diffusion distance between the water and the blood [μ m].

This work is actually in progress on several deep-sea sharks (non-luminous and luminous) and shallow-water counterparts. For that purpose, shark gills are firstly analyzing with optical microscopy and SEM to determine the gill's surface and in a second time TEM will be done to determine the thickness of lamellae epithelium.

This research will allow determining if deep-sea shark species display physiological adaptation to extract more oxygen from their poorly oxygenated (half concentration than shallow-water) environment. The result will also allow seeing if Etmopteridae luminous species have a better oxygen uptake than non-luminous deep-sea counterparts.

Buoyancy analyses and perspectives

The buoyancy of shark through the hydrostatic lift provided by livers was first studied by a new methodology based on measurement of lipid using Raman and MIR spectroscopy (Hall et al., 2016). This methodology was efficient for squalene and wax esters quantification but not for DAGE and TAG where only semi-quantitative data were collected. The DAGE and TAG interpretation was complicated due to the fact that their spectrum

peaks were too large and possibly covered other chemical links. To improve the quantification of DAGE and TAG, work with purified extract of shark DAGE and TAG are required to highlight the specific peaks of these lipids with the spectroscopy. Indeed, in this thesis, only one or two DAGE and TAG were used for the calibration but this is not enough because sharks can display in their liver more than 22 different fatty acids associated with DAGE and TAG (Malin et al., 1965; Bakes and Nichols, 1995; Guil-Guerrero et al., 2011). Traditional method to extract sharks' DAGE and TAG such as thin layer chromatography or iatroscan should be used before spectroscopy analysis.

Based on the liver lipids content, we were able to estimate the hydrostatic lift provided by this organ. However, the buoyancy of shark depends on several other factors such as (i) density of other tissues (muscle, stomach, brain,...) (Bone and Moore, 2008), (ii) the osmolyte composition (Urea and TMAO) (Treberg and Speers-Roesch, 2016), (iii) the morphological impact providing hydrodynamic lift (Wilga and Lauder, 2001), (iv) the temperature (Carrier et al., 2012) and (v) the pressure. Currently, no modeling integrates the complete set of variables but it appears to be important to try to perform this before drawing any conclusion on sharks' buoyancy. Nevertheless, even if a general modeling will provide essential information, the physiological processes occurring in sharks' body can change quickly over the time; the season, the fitness and therefore the modeling should take into account all these changes which are still-not yet characterized.

An example of particular physiological process change, is the change of TAG to DAGE inside the liver. It has been shown on *S. acanthias* that if sharks are weighted down, TAGs to DAGEs conversion occurred in the liver providing more hydrostatic lift; this change being detected after about 10 hours. It would be interesting

to study this phenomenon in deep-sea sharks performing vertical migration to reveal if they use this physiological process to change their buoyancy with a minimum energy expenditure. However, to be efficient this process should be faster in deep-sea species. One way to study this could be based on a similar protocol as the one on *S. acanthias*; we can sort deep-sea sharks in two groups: one unweighted and one weighted down. After different fixed period of time from 1 to 4 hours, livers will be sampled and the proportion of DAGE and TAG will be quantified. If a change of proportion is detected between the two experimental groups (weighted and unweighted) within such a short time, we could suggest that such process occurs *in situ* when shark goes up in the water column. Because sharks are suggested to display neutral buoyancy in depth, the change from DAGE to TAG will avoid also being positively buoyant. Moreover, TAG will be a reserve of energy for foraging. In opposite, after the night when deep-shark needs to reach deeper layers, the DAGE could be changed in TAG to initiate the descent. In addition to that the sea water changes occurring with the depth should be taking into account. In addition of this methodology, studies of this metabolism between deep-sea species and shallow-water counterparts may highlight higher enzyme activities for deep-sea species than shallow-water ones. The transformation of these two different lipid classes is done in two steps: (i) the first one removes the ether group from the diacylglycerol ether (DAGE) to transform them into diacylglycerol (DAG), one of the enzymes catalyzing this reaction is in particular Diacylglycerol cholinephosphotransferase (Fig. 2). Then (ii), the DAG-TAG transformation can be performed by three different enzymes: The diacylglycerol acyltransferase (DGAT), the phospholipid diacylglycerol acyltransferase (PDAT), and the diacylglycerol transacylase (DGTA) (Fig. 3).

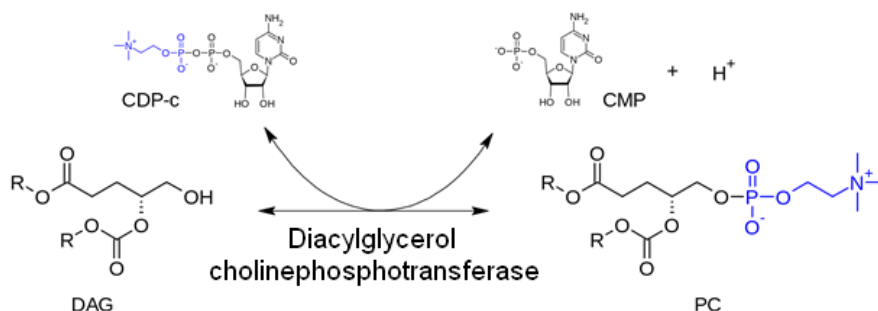


Fig. 2 Reaction catalyzing the transformation diacyldlycerol (DAG) to diacylglycerol ether (in this case phosphatidylcholine - PC) to - and inversely. CDP-c corresponds to cytidine diphosphate-choline; CMP corresponds to cytidine monophosphate.

For the first reaction catalyzing the DAGE to DAG transformation, quantification by ELISA method can be done but there are several other transformation possible of these two lipids which should be study in shark metabolism before quantification (Roney and Wai-Lee 1976). However regarding the transformation of DAG to TAG, it is possible to characterize the enzyme activities using fluorescent assay or radiolabeled fatty acids for DGAT and DGAT (McPie and Stone, 2011); for PDAT, quantification can be done through gene expression analyses (Liu et al., 2016).

Morphological analyses and perspectives

Our data regarding the morphological features of squaloid deep-sea sharks are the first which are compared with multivariate analyses. Analysis has revealed significant difference of morphology between deep-sea sharks but future researches on more species are needed to further characterize the morphology of deep-sea sharks. However, data analysis has revealed the potential effect of dorsal fin associated spines on swimming capabilities which was already suggested for shallow-water sharks.

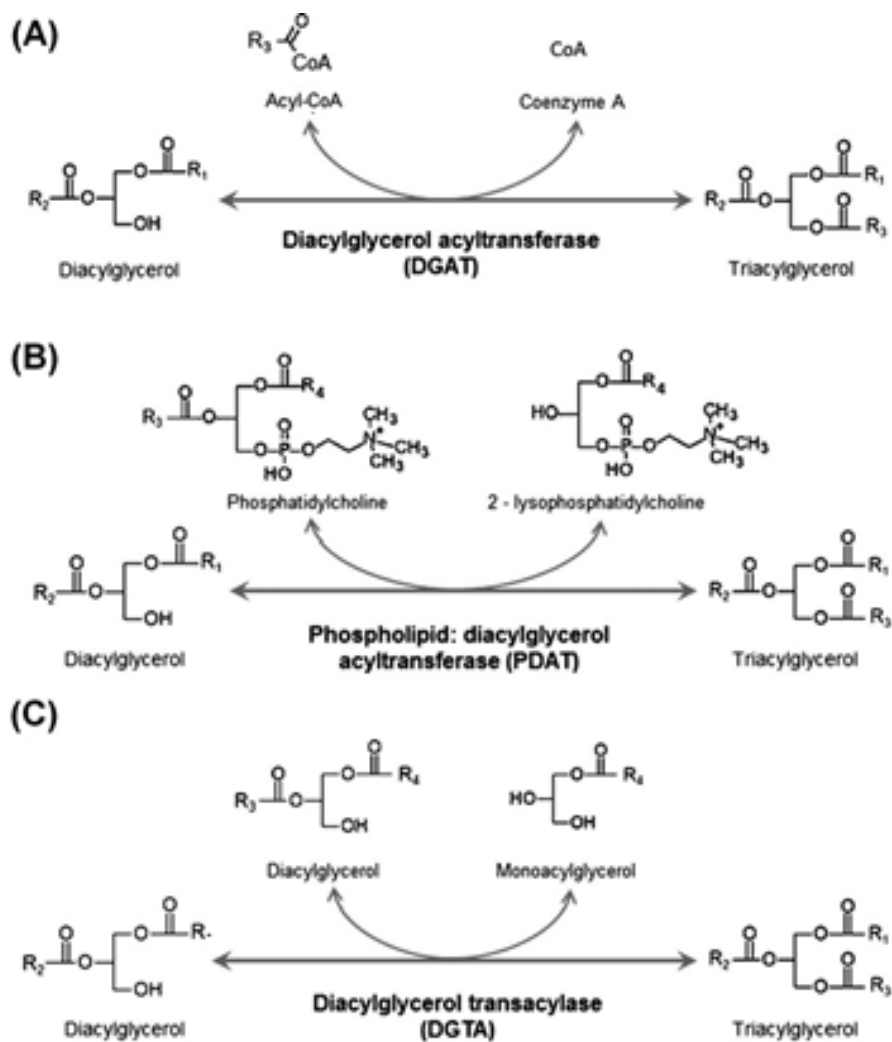


Fig. 3 Reactions catalyzing the transformation of DAG to TAG and inversely. (A) diacylglycerol acyltransferase (DGAT) reaction; (B) phospholipid diacylglycerol acyltransferase (PDAT) reaction; and (C) diacylglycerol transacylase (DGTA) reaction.

Indeed, longer and wider spine could create a water vortex, enabling the caudal fin to beat easier similarly to what was described for the second dorsal fin (Lauder & Santo, 2016; Maia et

al., 2017). A sharper second spine could also confine the turbulent flow behind the spine enabling the shark to swim with rhythmic undulations (Maisey, 1979). However, these effects on the speed are only theoretical and need to be tested mechanically by observing whether the interaction of the water flow with spine could improve the speed what is very difficult to achieve with living deep-sea sharks; modeling studies are still the best way to test this hypothesis.

Analysis reveals a new shape of caudal fin for *D. licha*. Indeed, this shark displaying the slowest velocity ever observed has a caudal fin without any fork and thus no clear separation delimiting a lower lobe. This new shape can be also found in another slow shark, the frilled shark (*Chlamydoselachus anguineus*) and even if no speed values are available for this species, videos suggest a slow swimmer (Ebert et al., 2013). According to the results, a ranking from iso/homocercal caudal fin (the best shape for fish propulsion), followed by heterocercal fin (intermediate propulsion value) and finally caudal fin with no clear lower lobe (weakest propulsion) could be suggested. However, as for the spine potential effect on speed, mechanistic studies would allow to better understanding if this morphological feature has a real impact on the velocity.

Final words

This thesis sets up as the first multidisciplinary approach regarding the swimming capabilities of deep-sea sharks. It revealed that in opposition with what was suggested and commonly admitted, not all deep-sea sharks are slow and listless swimmers. Some of them, especially, luminous sharks from Etmopteridae family display relatively high cruise swimming speed which could be linked to the isolume followers' hypothesis. However, future study

such as telemetry work should be done to confirm this hypothesis. Other information regarding speed value allowed us to suggest hypothesis regarding home range and hunting behaviors of other deep-sea sharks.

Also we highlight the relation between the velocity observed and physiological, anatomical and morphological adaptations such as it was observed in shallow-water sharks. All these analyses brought us new questions regarding other possible adaptations and future researches and projects are currently in progress to still better understand the swimming ecology of these fascinating animals.

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Annexes

LIST OF ANNEXES

This section includes side project publications, communication abstracts presented in scientific conferences made during this PhD thesis mandate as well as information of the sharks collected during the 4 years granted and a final curriculum vitae.

Annex 1 Publication list

Annex 2 Oral presentation list

Annex 3 Poster presentation list

Annex 4 **Duchatelet, L., Pinte, N., Tomita, T., Sato, K. and Mallefet, J. (2019). Etmopteridae bioluminescence: dorsal pattern specificity and aposematic use. *Zoological Letters*. 5: 9.**

Annex 5 **Duchatelet, L., Delroisse, J., Pinte, N., Sato, K., Ho, H. C., & Mallefet, J. (2019). Adrenocorticotrophic Hormone and Cyclic Adenosine Monophosphate are Involved in the Control of Shark Bioluminescence. *Photochemistry and photobiology*.**

Annex 1: Publication list

2019:

Pinte, N., Godefroid, M., Abbas, O., Baeten, V., Mallefet, J. (2019). Deep-sea sharks: Relation between the liver's buoyancy and red aerobic muscle volumes, a new approach. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 236, 110520.

Duchatelet, L., Pinte, N., Tomita, T., Sato, K., Mallefet, J. (2019). Etmopteridae bioluminescence: dorsal pattern specificity and aposematic use. *Zoological Letters*. 5: 9.

Duchatelet, L., Delroisse, J., Pinte, N., Sato, K., Ho, H. C., Mallefet, J. (2019). Adrenocorticotrophic Hormone and Cyclic Adenosine Monophosphate are Involved in the Control of Shark Bioluminescence. *Photochemistry and photobiology*.

Pinte, N., Parisot, P., Martin, U., DeVleeschouwer, C., Zintzen, V., Robert, C., Mallefet, G. (2019a). Swimming capabilities of deep-sea sharks from New Zealand: New insight for the isolume-followers' hypothesis. *Deep-sea Research: Part A* (submitted).

Pinte, N., Jones, E., Coubris, C., Mallefet, J. (2019). Enzymatic activities in the aerobic and anaerobic metabolism of mesopelagic sharks and impact on their swimming capabilities. *Journal of Fish Biology* (submitted).

Pinte, N., Finet, E., Jones, E., Frédéricich, B., Mallefet, J. (2019). Study of morphological features (body, fin and spine shape) of eight deep-sea shark species and one shallow-water one

(Squaliformes) according to their swimming capabilities. In preparation.

2015:

Pinte, N. (2015) Etude de la vitesse de nage des requins d'eaux profondes déduite de l'analyse des images video de cameras appâtées. Master thesis, Université catholique de Louvain.

Annex 2: Oral presentation list

2018:

N. Pinte, P. Parisot, U. Martin, V. Zintzen, C. Devleschouwer, L. Duchatelet, J.. Are luminous deep-water sharks faster swimmers?. 20th International Symposium on Bioluminescence & Chemiluminescence, Nantes, France

N. Pinte, M. Godefroid, V. Baeten, O. Abbas, L. Duchatelet, J. Mallefet. Relation between red muscle volume and buoyancy of several deep-water sharks. 4th Sharks International Conference, Joao Pessoa, Brasil

N. Pinte, C. Coubris, E. Jones, L. Duchatelet, J. Mallefet. Aerobic and anaerobic metabolism could be related to swimming capabilities of deep-sea sharks?. 22th European Elasmobranch Association, Peniche, Portugal

E. Finet, N. Pinte, B. Frédéricich, E. Jones, J. Mallefet. Are morphological traits explaining swimming performances of several deep-sea sharks from New Zealand?. 25th Benelux Congress of Zoology, Antwerpen, Belgium

2017:

L. Duchatelet, N. Pinte, J. Delroisse, J. Mallefet. Shark bioluminescence control possibly evolved/co-opted from vertebrate skin pigmentation control. 21th European Elasmobranch Association, Amsterdam, The Netherlands

M. Godefroid, N. Pinte, L. Duchatelet, V. Baeten, O. Abbas, J. Mallefet. Characterization of the buoyancy of three species of deep-sea sharks. 21th European Elasmobranch Association, Amsterdam, The Netherlands

U. Martin, N. Pinte, L. Duchatelet, P. Parisot, V. Zintzen, J. Mallefet. Three-dimensional speed analysis of deep-sea sharks. 21th European Elasmobranch Association, Amsterdam, The Netherlands

N. Pinte, P. Parisot, U. Martin, L. Duchatelet, V. Zintzen, J. Mallefet. Ecological features of several deep-water sharks species from New-Zealand infer by stereo video footage analyses. 21th European Elasmobranch Association, Amsterdam, The Netherlands

L. Duchatelet, N. Pinte, T. Tomita, J. Mallefet. Species-specific dorsal luminous pattern in Etmopterids with an emphasis on the dorsal spine associated photophores. 33rd American Elasmobranch Society, Austin, Texas, USA

N. Pinte, V. Zintzen, L. Duchatelet, U. Martin, J. Mallefet. Swimming speed of deep-water sharks inferred by stereo footage analyses. 33rd American Elasmobranch Society, Austin, Texas, USA

M. Godefroid, N. Pinte, L. Duchatelet, V. Baeten, O. Abbas, J. Mallefet. Swimming capability: Physiological evidences from buoyancy and muscle distribution of three deep-sea sharks. 24th Benelux Congress of Zoology, Wageningen, The Netherlands

U. Martin, N. Pinte, P. Parisot, L. Duchatelet, C. De Vleeschouwer, V. Zintzen, J. Mallefet. Swimming capabilities of deep-water sharks from New-Zealand inferred by stereo-video footages analyses. 24th Benelux Congress of Zoology, Wageningen, The Netherlands

2016:

N. Pinte, L. Duchatelet, J. Mallefet. Study of swimming capabilities of *Etmopterus spinax*, the velvet belly lantern shark, inferred by muscle enzyme activities. 20th European Elasmobranch Association, Bristol, UK

Annex 3: Poster presentation list

2014:

J. Mallefet, N. Pinte, V. Sintzen, J.M. Claes. Swimming speed from deep-sea sharks inferred from video footage analysis. 21st Benelux Congress of Zoology, Liège, Belgium

Annex 4:

Duchatelet, L., Pinte, N., Tomita, T., Sato, K. and Mallefet, J. (2019). Etmopteridae bioluminescence: dorsal pattern specificity and aposematic use. *Zoological Letters*. **5**: 9.

RESEARCH ARTICLE

Open Access

Etmopteridae bioluminescence: dorsal pattern specificity and aposematic use



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Abstract

Background: In the darkness of the ocean, an impressive number of taxa have evolved the capability to emit light. Many mesopelagic organisms emit a dim ventral glow that matches with the residual environmental light in order to camouflage themselves (counterillumination function). Sharks use their luminescence mainly for this purpose. Specific lateral marks have been observed in Etmopteridae sharks (one of the two known luminous shark families) suggesting an inter/intraspecific recognition. Conversely, dorsal luminescence patterns are rare within these deep-sea organisms.

Results: Here we report evidence that *Etmopterus spinax*, *Etmopterus molleri* and *Etmopterus splendidus* have dorsal luminescence patterns. These dorsal patterns consist of specific lines of luminous organs, called photophores, on the rostrum, dorsal area and at periphery of the spine. This dorsal light seems to be in contrast with the counterilluminating role of ventral photophores. However, skin photophores surrounding the defensive dorsal spines show a precise pattern supporting an aposematism function for this bioluminescence. Using in situ imaging, morphological and histological analysis, we reconstructed the dorsal light emission pattern on these species, with an emphasis on the photogenic skin associated with the spine. Analyses of video footage validated, for the first time, the defensive function of the dorsal spines. Finally, we did not find evidence that Etmopteridae possess venomous spine-associated glands, present in Squalidae and Heterodontidae, via MRI and CT scans.

Conclusion: This work highlights for the first time a species-specific luminous dorsal pattern in three deep-sea lanternsharks. We suggest an aposematic use of luminescence to reveal the presence of the dorsal spines. Despite the absence of venom apparatus, the defensive use of spines is documented for the first time in situ by video recordings.

Keywords: Etmopteridae, Dorsal pattern, Bioluminescence, Intraspecific recognition, Aposematism, Spines

Background

Deep in the ocean, a great many taxa have evolved the capability to emit light [1, 2]. This phenomenon, called bioluminescence, is a mechanism whereby organisms emit visible light by biochemical reactions [1, 3, 4]. Functions of bioluminescence are mainly divided into three categories: predation, avoid predation (interspecific) and intraspecific communication [1, 3–6]. Among these organisms, sharks are the first vertebrate to utilize this phenomenon [1, 7, 8]. Currently, there are two families of luminous deep-sea sharks (Etmopteridae and Dalatiidae) which are capable of emitting a blue-green light (from 460 to 486 nm according to the species) thanks to thousands of tiny luminous

organs, called photophores, mainly present on the ventral skin epidermis [7, 9–11]. The photophore structure, conserved in the genus *Etmopterus*, is composed of a “deep” pigmented sheet of embedded cells responsible for the light emission, called photocytes, surmounted by an iris-like structure topped externally by one or two lens cells [12–14]. Shark luminescence has been suggested to have several ecological roles. Firstly, like a large number of mesopelagic organisms emitting a continuous ventral glow similar to the down-welling light, lanternsharks use their ventral light to disrupt their silhouette and avoid being seen by predators swimming below; this is the counterillumination mechanism [1, 6, 10, 15]. Secondly, interspecific and intraspecific communication have been suggested: (i) the presence of species-specific lateral flank marks may provide a way to facilitate reproductive isolation hence the high species richness in *Etmopterus* genus [8, 16], while (ii)

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sexual dimorphism is observed in the Etmopteridae species [12]. Aposematism is also suggested, provided by the spine associated photophore luminescence [17].

Previous studies have demonstrated that shark spines fulfill numerous functions, these include improving hydrodynamics of the organism and serving as a mechanism for defense. The presence of venomous glands associated with the posterior side of the spine in Heterodontidae and Squalidae are evidence for this function [18–22]. However, there is now in situ evidence for a defensive function of the dorsal spine in Etmopteridae. In contrast to ventral luminescence, dorsal luminescence is rare in the ocean and has received less interest, probably because it is easily detectable in contrast to the darker background from the deeper waters underneath the organism. This dorsal pattern is usually utilized for predation [1, 5], as indicated by the dorsal lure located above the jaws in anglerfish species [23] or for an anti-predatory function, where the light acts as an aposematic warning signal [24–26].

In this study, we investigated dorsal light emission in three deep-sea shark species from the Etmopteridae family: the velvet belly lanternshark, *Etmopterus spinax* (Linnaeus, 1758); the slendertail lanternshark, *Etmopterus molleri* (Whitley, 1939); and the splendid shark, *Etmopterus splendidus* (Yano, 1988). These three species are small deep-sea sharks (see Table 1) occurring at depths ranging from 200 to 500 m, where sunlight dimly penetrates the water column [27–29]. Due to their small sizes, these sharks are preyed upon by larger organisms, such as other elasmobranch species including *Dalatias licha*, *Echinorhinus cookei* and large Hexanchiformes species ([30–33], JM personal communication). Lanternsharks are characterized by mineralized spines in front of dorsal fins, a subterminal notch on the dorsal part of the caudal fin and a specific pattern of light organs on the ventral and lateral body surface [11–13, 17]. A recent study highlighted the specialization of the visual system in luminous deep sea sharks compared to non-luminous species: (i) longer rod outer segments, higher rod densities and larger eye:body size ratio, these are in favor of high light sensitivity; (ii) maximal absorption wavelengths in visual pigments (484–491 nm) for perception of bioluminescent emissions (476 nm *E. splendidus*, 486 nm *E. spinax* and 488 nm *E. molleri*) and downwelling light; (iii) the presence of a secondary dorsal arch with a high density of rods in the retina which

facilitates the detection of moving objects in the inferior visual field [34].

Our results reveal the presence of a dim blue-green light from the dorsal epidermis for these sharks. We report a species-specific dorsal pattern of photophores that may be utilized for species recognition, schooling, and other intraspecific communication. We also provide details of the specific luminous pattern associated with the spine in different Etmopteridae species. In this study, the first evidence of dorsal spine in a defensive use, is recorded by in situ video footages of Etmopteridae sharks. While CT and MRI scan images indicate that Etmopteridae sharks do not show the presence of a venomous gland associated with their spines.

Materials and methods

Etmopteridae sampling

The three elasmobranch species come from two different regions. *E. spinax*, is mainly found in the north-east part of the Atlantic, and were collected in the Raunefjord (60°15' 54" N; 05°07'46" E) next to Bergen in Norway during winter 2017. A total of 31 specimens were sampled during this field session. They were caught using a deep-long line at a depth ranging from 180 to 250 m. Specimens were transferred in a dark cold tank (4 °C) and brought to the Espesgrend marine field station where they are kept alive in a seawater tank placed in a cold dark room (4 °C) until manipulations.

E. molleri and *E. splendidus* were collected in the East China Sea (26°28'94" N; 127°41'20" E) near the coast of Okinawa Island (Japan). They were fished using a bottom hook-and-line method at a depth ranging from 480 to 510 m. Data on *E. splendidus* and *E. molleri* were collected during the fishing seasons winter 2011 and winter 2016, respectively. Three specimens of *E. splendidus* and 24 specimens of *E. molleri* were collected. All specimens were transferred to oxygen saturated plastic bags filled with seawater and transferred in a refrigerated box to the Okinawa Churaumi Aquarium where they were kept alive in a cold dark tank filled with seawater (13 °C) until manipulations.

Data on the collected specimens are summarized in Table 1.

Table 1 Mean morphological values. N: number of specimens; ♀: female; ♂: male

Species	N	Total length (cm)	Fork length (cm)	Pre-caudal length (cm)	Weight (g)
<i>Etmopterus spinax</i>	25 ♀	46.3 ± 1.1	39.6 ± 1.1	35.6 ± 1.1	416.1 ± 23.8
	6 ♂	40.0 ± 1.3	35.2 ± 1.3	31.3 ± 1.3	248.1 ± 34.2
<i>Etmopterus molleri</i>	15 ♀	42.7 ± 2.1	36.7 ± 1.9	33.9 ± 1.4	217.6 ± 43.1
	9 ♂	40.1 ± 0.9	33.8 ± 0.9	31.6 ± 0.7	165.6 ± 17.2
<i>Etmopterus splendidus</i>	3 ♂	21.9 ± 1.6	19.6 ± 1.2	17.5 ± 0.9	/

Dorsal luminescence pattern analysis

Dorsal photos of luminous and living shark were taken with a Sony alpha 7S II camera (Sony Corporation, Japan), these images were analyzed, digital noise was removed using Photoshop software (Adobe; San Jose, CA, USA). Close-up images of the photogenic structure associated with the dorsal spines was also completed with the same software.

Spine-associated luminous structure analysis

Since photophores located on the dorsal fin (SAP) highlighting the spine were documented in *E. spinax*, we analyzed the structure and orientation of photophores located around the spines in different Etmopteridae species with the aim to compare arrangements among species.

Captive sharks were euthanized by a knock on the chondrocranium followed by an incision at the level of the spinal cord. The local rules for experimental fish care and the European regulation for research animal handling were followed. Shark dorsal skin, spine and fin were dissected and directly stored in 4% paraformaldehyde phosphate buffer saline (PBS) for 12 h at 4 °C, and stored in PBS until further use. A 1.5 cm diameter skin patch around the spine was removed and separated from the spine. For histological analyses, dorsal skin, fin epidermis and skin patches were bath in PBS with increasing sucrose concentrations (10% for 1 h, 20% for 1 h and finally, overnight in 30% sucrose), embedded in O.C.T. compound (Tissue-Tek, The Netherlands) and finally, rapidly frozen at -80 °C. Thin sections (10 µm) were cut with CM3050 S. Leica cryostat microtome (Germany) and were laid on Superfrost-coated slides (Thermo Scientific, Waltham, MA, USA) and left overnight to dry. Slides were analyzed using an epifluorescence microscope and a light microscope (Leitz Diaplan, Germany) equipped with a Nikon Coolpix 950 camera (Nikon, Japan). General morphology of the photophore, distance and the inclination angle (α) measured in relation to the spine location were all described. The photophore inclination angle (light pathway) was evaluated by taking the difference between the perpendicular to the iris opening as the reference axis and the line passing through the central point of the largest lens. Photophore distance was measured from the center of the light organ to the base of the spine. These two measurements were taken on pictures via ImageJ software [35]. Statistical analyses were performed with JMP® software (JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989–2007.). The Gaussian distribution respected an ANOVA followed by a Tukey-test to reveal significant differences.

Computed tomography and MRI analyses

Knowing that spine associated venom glands were detected as soft tissue located at the posterior side of the

spine [18–20, 22], magnetic resonance imaging (MRI) data of the *E. spinax* spine and the associated tissues were obtained thanks to a Bruker Biospec 11,7 T (Bruker BioSpin, Ettlingen, Germany) in order to visualize the presence/absence of a specific venomous structure. A bird-cage coil with an internal diameter of 40 mm was used in emission/reception mode. The run sequence was Flash type with the following parameters: TE: 3.2 ms; TR: 320 ms; FA: 25°; matrix size: 396 × 396; field of vision of 30 × 30 mm²; ten non-continuous slides separated from 350 µm (center to center); resolution: 76 × 76 × 250 µm³; number of repetitions: 700. Computed tomography (CT) data of *E. spinax* were acquired using a cone beam micro-CT scanner (NanoSPECT/CT, Bioscan inc., Washington D.C., USA) with the following characteristics: spatial resolution: 48 µm; X-ray tube voltage: 45 kVp; number of projections: 360; exposure time: 1000 ms. The CT projections were reconstructed with a voxel size of 0.111 × 0.111 × 0.11 mm³ by ray-tracing based filtered back projection.

Video footage

During the field session in November 2016, video footage of *E. molleri* was collected at one location in the oriental China Sea (26°34'94" N; 127°45'20" E). Two deployments occurred at depths of 500 and 540 m. Each video device was on the seabed during a period of 2 h. The underwater video system was designed by ourselves. Video footage was taken by a GoPro Hero 4 (GoPro, Inc., San Mateo, CA, USA) placed in a special underwater case, benthic 2 (Group B Distribution Inc., Jensen Beach, FL, USA) and fixed on the metal frame. The bait consisted of 1 kg of cephalopod and mackerel in a metal cage fixed to the frame by a steel bar. Lighting was provided by LED light in a housing, GPH-1750 M (Group B Distribution Inc.) and provided us a clear view until four meters and did not appear to disturb shark behaviors. The depth was recording using the sonar system of the boat.

Results

Dorsal luminescence pattern

Analysis of different dorsal luminous pictures allowed us to build schematic drawings of the dorsal side of each species (Fig. 1). Analyses of these patterns for each species revealed some luminous arrangements common to all species and others species-specific patterns: (i) the three luminous longitudinal lines from the back of the head to the beginning of the caudal fin were common for the three species studied (Fig. 1a–f), even if the median line of *E. splendidus* was denser than median line of the two other species; (ii) a relatively brighter luminous mark located on the ventral side of the caudal fin dorsal lobe was observed; (iii) on the rostrum dorsal side, despite many different patterns, the photophore aggregation

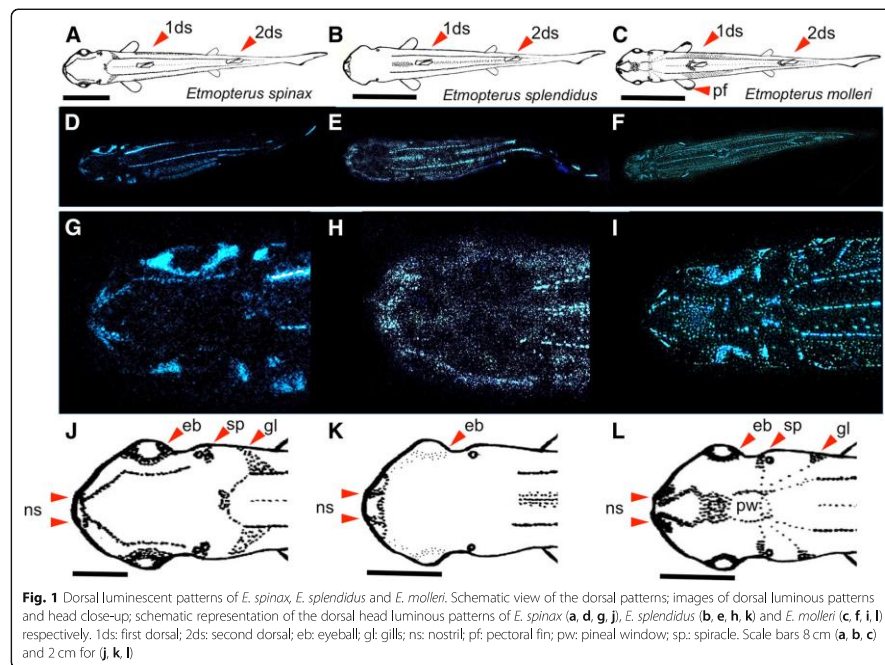


Fig. 1 Dorsal luminescent patterns of *E. spinax*, *E. splendidus* and *E. molleri*. Schematic view of the dorsal patterns; images of dorsal luminous patterns and head close-up; schematic representation of the dorsal head luminous patterns of *E. spinax* (a, d, g, j), *E. splendidus* (b, e, h, k) and *E. molleri* (c, f, i, l) respectively. 1ds: first dorsal; 2ds: second dorsal; eb: eyeball; gl: gills; ns: nostril; pf: pectoral fin; pw: pineal window; sp: spiracle. Scale bars 8 cm (a, b, c) and 2 cm for (j, k, l)

around nostrils was a common feature (Fig. 1g-l); (iv) a brighter luminous spot appeared next to the dorsal spines in these three species.

In addition to these similarities, many different luminous arrangements occurred between these three species, mainly focused on the rostrum and pectoral fins. Indeed, we saw that *E. spinax* and *E. molleri* show aggregation of the luminous organs around the eyes, next to spiracles and at the edge of gill slits. A luminous impala horn shape pattern was observed on the *E. spinax* head (Fig. 1g, j) and a particularly luminous shape arrangement was visible on *E. molleri* head (Fig. 1i, l). The pineal window surrounded by a circle of luminous dots from which radiating lines connecting spiracle, gills slits, and dorsal lines were visible. Between this window and the nostril, a luminous V shape and blotch were observed on the rostrum. Moreover, *E. molleri* shows a specific luminous zone on pectoral fins (Fig. 1c, f).

In contrast to these two species, *E. splendidus* possessed no specific luminous zones on the rostrum (Fig. 1h, k).

The dorsal light emission is about one order of magnitude dimmer than ventral light emission. We did not

distinguish any sexual differences during our observations, although we observed that transferring the shark from a captivity tank to an aquarium induces a transient increase of bioluminescence lasting around 10 min.

Spine-associated luminous structure

Close-up observations of the dorsal fin body region were performed to analyze spine-associated luminous structure for the three studied species (Fig. 2). Dissected spines with the 1.5 cm skin-associated allowed us to characterize two different spine-associated photogenic cluster patterns. Claes et al., (2013) describes the patterns in *E. spinax*, which consists of spine associated photophore, called SAP. These photophores are precisely localized along the fin anterior ridge facing the spine (Fig. 2a, d). This pattern was found and can be visualized through the thin dark strip on the antero-dorsal part of the fin (Fig. 2a, d). At this location, the photophores are oriented directly towards the spine and illuminate it (Fig. 2g). *E. molleri* did not show any photophores on the dorsal fin ridge (Fig. 2c, f, i) but careful examination of dorsal skin revealed the presence of luminous structures on the front and on each side of the spine base,

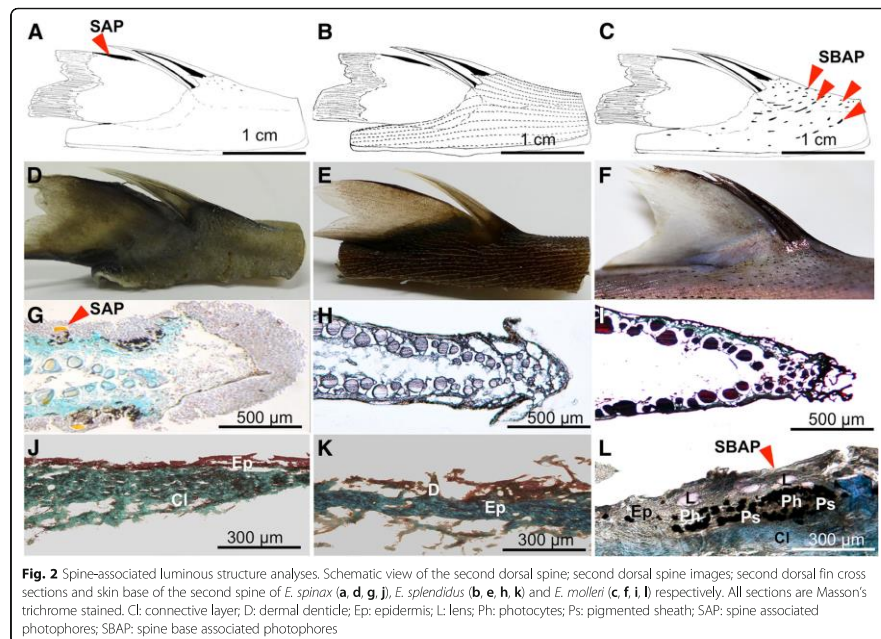


Fig. 2 Spine-associated luminous structure analyses. Schematic view of the second dorsal spine; second dorsal spine images; second dorsal fin cross sections and skin base of the second spine of *E. spinax* (a, d, g, j), *E. splendidus* (b, e, h, k) and *E. molleri* (c, f, i, l) respectively. All sections are Masson's trichrome stained. Cl: connective layer; D: dermal denticle; Ep: epidermis; L: lens; Ph: photocytes; Ps: pigmented sheath; SAP: spine associated photophores; SBAP: spine base associated photophores

these photogenic structures were named spine base associated photophore (SBAP) (Fig. 2c, l). These photophores group together in small lines all around the spine with a rostro-caudal orientation (Fig. 2c, f). The size of SBAP clusters in front of the spine measured $775 \mu\text{m} \pm 322$ and side clusters were estimated as $443 \mu\text{m} \pm 543$ (Fig. 3a). Longitudinal and transversal sections across SBAP allowed us to describe this new photophore type (Fig. 3b, e). SBAP is mainly composed of numerous photocytes forming an antero-posterior elongated tube shape, surrounded by pigmented cells and surmounted by at least five lens cells (Fig. 3a, b, e). These SBAPs are localized at a mean distance of 4.75 ± 0.74 mm from the spine base ($n = 210$ SBAP observed). To find out if these photophore lines were able to illuminate the spines, the inclination angles (α) between the SBAP (Fig. 3d, e) and ventral photophores (Fig. 3c, d) were measured. Ventral photophores showed an angle of $1.6^\circ \pm 0.8$ ($n = 42$) significantly different from right side SBAP presenting a value of $29.1^\circ \pm 7.2$ ($n = 36$) and left side, a value of $39.4^\circ \pm 9.1$ ($n = 50$) (Fig. 4; Tukey $p < .001$). Regarding *E. splendidus*, no specific spine associated photophores (SAP/SBAP) could be highlighted neither on the dorsal fin nor at the level of the spine base skin (Fig. 2b, e, h, k).

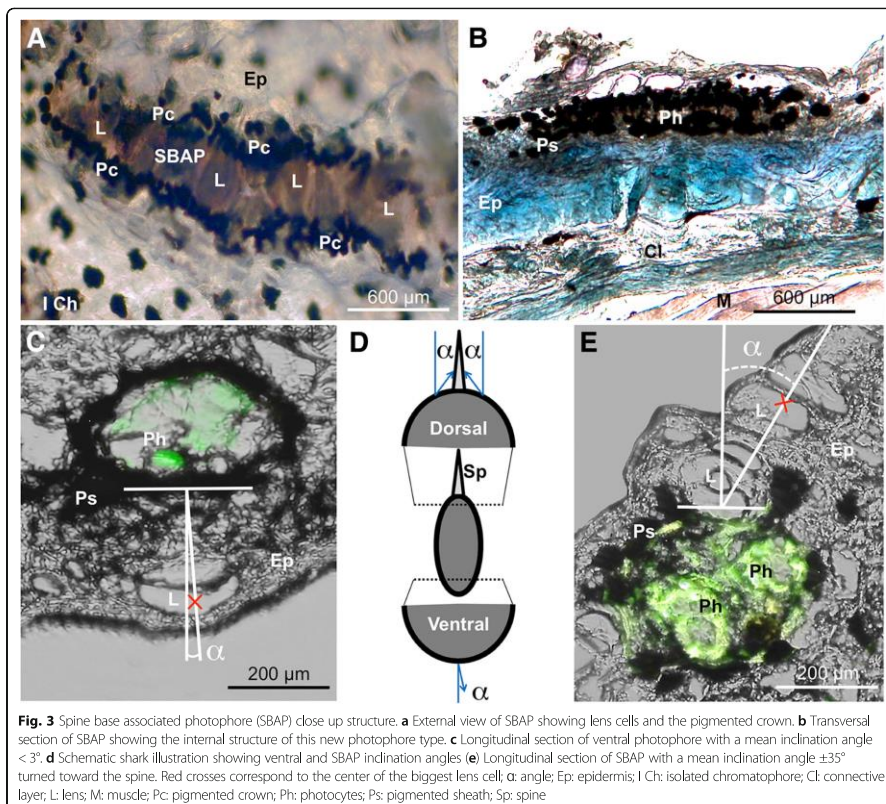
Spine structure and associated putative gland

To visualize a putative venom apparatus in Etmopteridae, MRI analysis was performed on fixed specimens; image analyses do not show any evidence of a soft tissue between the posterior side of the spine and the dorsal fin (Fig. 5a; Additional file 1). CT scan analyses did not allow to highlight any canal/duct on the anterior side of the spine that can be used for venom injection into a potential predator (Fig. 5b; Additional file 2).

Video footage

The video footage filmed in Okinawa allowed us to highlight the defensive function of the dorsal spine in Etmopteridae (Fig. 6). The video shows a sharpnose sevengill shark, *Heptanchias perlo* (Bonnaterre, 1788), catching an *Etmopterus splendidus* (Fig. 6a), attempting to bite it twice (Fig. 6b, c, d), the shark then opened its jaws widely (Fig. 6e, f, g) letting the *Etmopterus* escape (Fig. 6h, i). The original recording is provided as Additional file 3.

We also observed sequences where a sevengill shark attempts to catch a lanternshark by the tail avoiding the spine sting, as shown on the Additional file 4. During field collections, caught lanternsharks were observed on



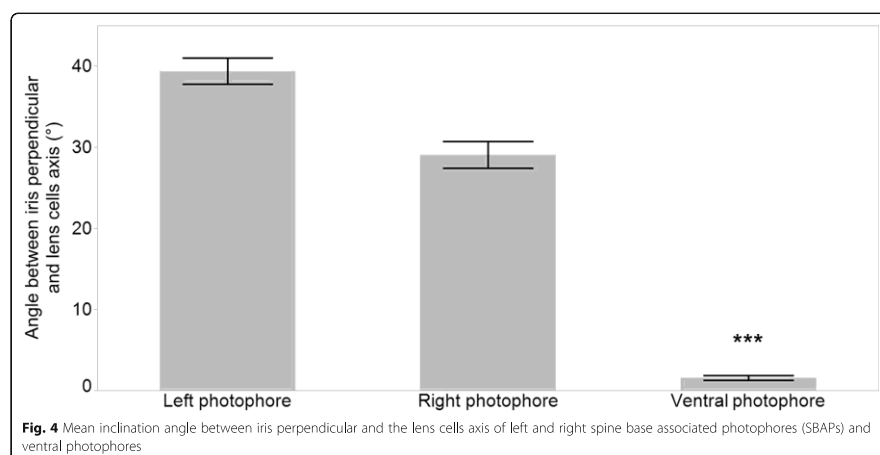
the hook with the tail cut off or the belly showing bite signs (Additional file 5).

Discussion

Among luminous organisms of the mesopelagic zone, dorsal luminescence is rare, as it seems counter-intuitive to produce a dorsal luminous signal making the emitter highly visible against the darkness of the deeper waters, while most organisms produce a ventral light to counter-illuminate and escape from the sight of predators [6, 36–38]. We observed specific rostrum and dorsal light patterns, which may be utilized for intraspecific communication (schooling, mating), similarly to the dorsal caudal gland of certain Myctophidae fishes [39]. Assuming this intraspecific function, we suggest that this dorsal luminous pattern, like the specific flank marks of Etmopteridae,

may have contributed to the large evolutionary radiation and speciation occurring in the *Etmopterus* genus [8, 16]. The dorsal lines already described in daylight by taxonomists were never referred as bioluminescent lines [40–42]. The species-specific patterns could be a useful feature for the Etmopteridae species determination/taxonomy, and therefore used as a new morphological phylogeny criterion.

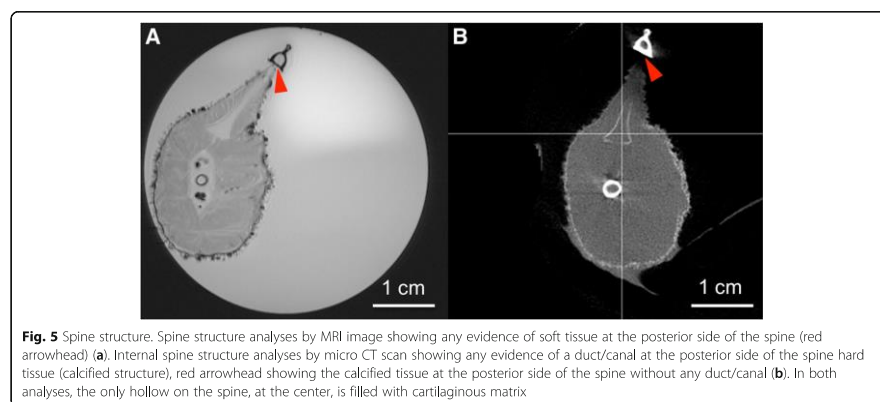
However, Claes et al. (2013) have suggested the dorsal aposematic function of light for *E. spinax*, where the light from SAP (spine associated photophore) is strongly transmitted by the dorsal spine making it visible for potential predators. Our results show that handling the lanternshark species induces an increase of bioluminescence and the presence of a brighter spot of luminescence surrounding the spine areas, these findings agree with this aposematic function. However, in *E. molleri* we

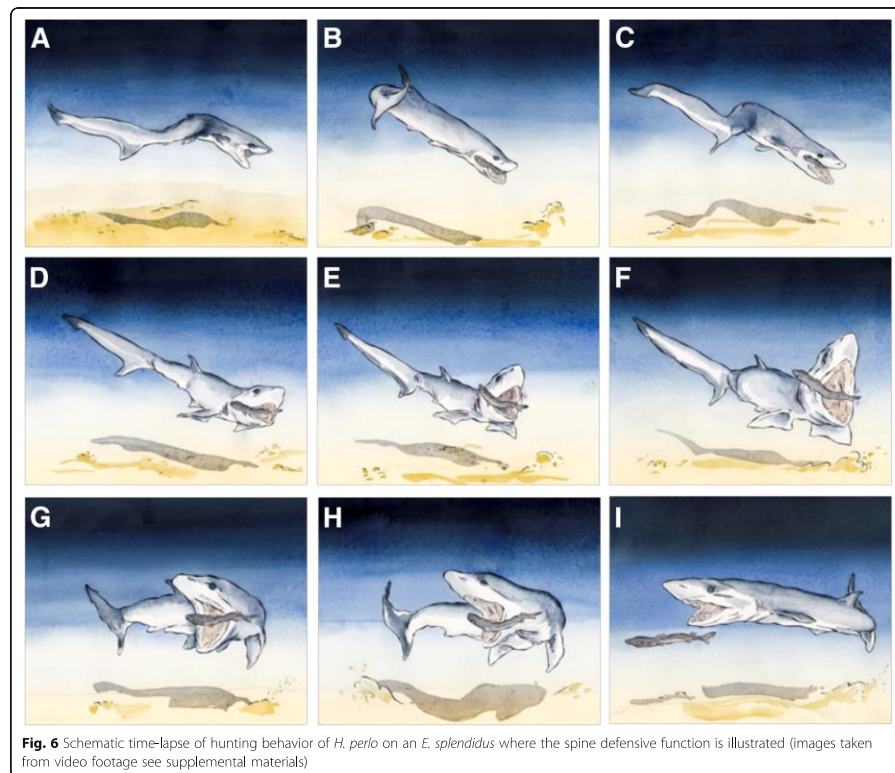


found a new elongated photophore type with numerous lenses whose orientation points towards the dorsal spine, we call these spine base associated photophore or SBAP. These are much larger than the photophore commonly described for Etmopteridae [7, 14, 43], and may represent a cluster of numerous photophores aiming to light up the spine. Consistent with the Squaliformes phylogeny, our results reveal a morphologically divergent evolution of photophore (SAP/SBAP) within Etmopteridae in order to reach a convergent functionality, aposematism. The primary homology hypothesis seems unlikely due to the positioning of the three studied

species [44]. The use of conspicuous signals to warn predators of unprofitability, aposematism [45–48], has been suggested for bioluminescent organisms in terrestrial and oceanic environments [17, 24–26, 49–51].

The presence of a venomous gland at the spine base in two shark families (Squalidae and Heterodontidae) was considered proof of a defensive function of this spine [18–20, 22]. Despite that no evidence of such gland was shown by MRI and CT scan analysis at the level of the dorsal spines in Etmopteridae, our video recordings and images are the first in situ validation of a defensive use of the dorsal spines. Attacks by predators at the level of





the belly and tail of Etmopteridae seem to indicate that learning behavior has led predators to specifically avoid dorsal spines during predation attempts.

Conclusion

This work highlights for the first time a species-specific luminous dorsal pattern in three deep-sea lanternsharks. New photophore assemblages were described and their arrangement suggests an aposematic use of luminescence to reveal the presence of the dorsal spines. In Etmopteridae, a morphological divergence might be involved in a convergent function, aposematism. Despite the absence of venomous apparatus, the defensive use of spines is documented for the first time by in situ video recordings. Development of highly sensitive underwater video recording devices could allow footage of bioluminescence to be recorded, revealing the use of living light by deep-sea sharks during encounters with conspecifics or predators.

Additional files

Additional file 1: Animated GIF of MRI transversal section of *Etmopterus spinax* at the level of spine base, going from the tip to the base of the spine. (GIF 557 kb)

Additional file 2: Animated GIF of CT scan sagittal section of *Etmopterus spinax* dorsal spine and fin, starting from the body (1) till the end of the spine (4) and backward. (GIF 74 kb)

Additional file 3: Video recording of *Heptanchias perlo* attack on an *Etmopterus splendidus* body. (MOV 6040 kb)

Additional file 4: Video recording of *Heptanchias perlo* attack on an *Etmopterus molleri* tail. (MOV 2448 kb)

Additional file 5: Pictures of injured *E. molleri* collected by deep sea rod fishing (A) Tail cut; (B) ventral side open; (C) closer view of B; (D) another occurrence of ventral bite. Scale bar A = 3 cm, B–C–D = 4 cm. (TIF 19094 kb)

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

L.D., N. P., T.T. and J.M. collected data, L.D., N. P. and J.M. contributed to data analysis and article preparation, T.T. and K.S. provided access to the field and revised the manuscript. All authors reviewed the final version and agree to final article submission.

Authors' information

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Ethics approval and consent to participate

Etmopterus spinax were collected in Norway under the "experimental fish care permit" number 12/14048. *Etmopterus molleri* and *splendidus* were collected and handled according to Churaumi aquarium husbandry and veterinary rules for fish experimentations. All sharks were euthanized by a knock on the chondrocranium followed by an incision at the level of the spinal cord, following the local rules for experimental fish care and the European regulation for research animal handling. These species are not in the CITES list.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Annex 5:

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Adrenocorticotrophic Hormone and Cyclic Adenosine Monophosphate are Involved in the Control of Shark Bioluminescence

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ABSTRACT

Among Etmopteridae and Dalatiidae, luminous species use hormonal control to regulate bioluminescence. Melatonin (MT) triggers light emission and, conversely, alpha melanocyte-stimulating hormone (α -MSH) actively reduces ongoing luminescence. Prolactin (PRL) acts differentially, triggering light emission in Etmopteridae and inhibiting it in Dalatiidae. Interestingly, these hormones are also known as regulators of skin pigment movements in vertebrates. One other hormone, the adrenocorticotrophic hormone (ACTH), also members of the skin pigmentation regulators, is here pharmacologically tested on the light emission. Results show that ACTH inhibits luminescence in both families. Moreover, as MT and α -MSH/ACTH receptors are members of the G-protein coupled receptor (GPCR) family, we investigated the effect of hormonal treatments on the cAMP level of photophores through specific cAMP assays. Our results highlight the involvement of ACTH and cAMP in the control of light emission in sharks and suggest a functional similarity between skin pigment migration and luminescence control, this latter being mediated by pigment movements in the light organ-associated iris-like structure cells.

INTRODUCTION

The lanternsharks *Etmopterus spinax* (Linnaeus, 1758) and *Etmopterus molleri* (Whitley, 1939), two common etmopterid deep-sea sharks, are known to emit an intrinsic blue-green light by using thousands of tiny photophores mainly distributed in the ventral epidermis ((1–4); Fig. 1A). Photophores are composed of a cup-shaped pigmented cell layer, which form a dark granular sheath, enclosing the emitting cells, the photocytes, capped by one or several lens cells. A multilayer cell zone called the iris-like structure (ILS) acting as a shutter of the light emission is present between the lens cells and the photocytes ((1,3); Fig. 1B). Various functions of bioluminescence have been suggested in lanternsharks such as counterillumination, aposematism or intraspecific communication (1,5–8).

To achieve these specific biological functions, photophore light emission has to be under precise control. While bony fish mainly regulate their luminescence with a fast-nervous control mechanism (9–12), sharks are the only known vertebrates to make use of a slow hormonal bioluminescent control (13). Melatonin (MT) and prolactin (PRL) trigger a low long-lasting and a strong fast light emission, respectively, while α -melanocyte-stimulating hormone (α -MSH) inhibits luminescence in lanternsharks ((13); Fig. 1B,C). Moreover, some neurotransmitters like nitric oxide (NO) and γ -aminobutyric acid (GABA) are able to modulate etmopterid light emission ((14–16); Fig. 1B,C).

The smallest known dalatiid species, *Squaliolus aliae* (Teng, 1959) is the smallest known dalatiid species. Individuals of *S. aliae* harbor thousands of tiny ventral photophores that are smaller and of a simpler structure than in etmopterids, that is one single photocyte surrounded by a pigmented sheath cup and capped by lens cells ((1,17); Fig. 1B). A role in counterillumination has been suggested for pygmy shark bioluminescence since a bluish ventral luminescence matching downwelling light has been observed ((18,19); Fig. 1A). MT and α -MSH have, respectively, the same stimulatory and inhibitory effect on the light emission while PRL inhibits luminescence in *S. aliae* ((19); Fig. 1B,C). Taken together, these observations suggest a common mechanism for the regulation of bioluminescence in luminous sharks in which the opening or closing of the ILS, through pigment movements, is assumed to be involved in altering the light output (13,16,19).

In vertebrates, physiological pigment movement is mainly promoted by bidirectional translocation of dark melanin granules (melanosome) within pigment cells (21–24). PRL, MT, α -MSH, adrenocorticotrophic hormone (ACTH), melanin-concentrating hormone (MCH) and catecholamines are the most intensively studied vertebrate pigment cell regulators (25–31). Most of these hormones (α -MSH, ACTH, MCH and MT) interact with a G-protein coupled receptor (GPCR) modulating adenylate cyclase activity, which controls the intracellular levels of cyclic adenosine monophosphate (cAMP) (21,23,25,32,33). Visconti and Castucci (1993) demonstrated that α -MSH and PRL hormones are both involved in the darkening of the stingray (*Potamotrygon reticulatus*) skin by modulating cAMP levels (34).

To further understand hormone-mediated light emission control in luminous elasmobranchs, the effects of ACTH were

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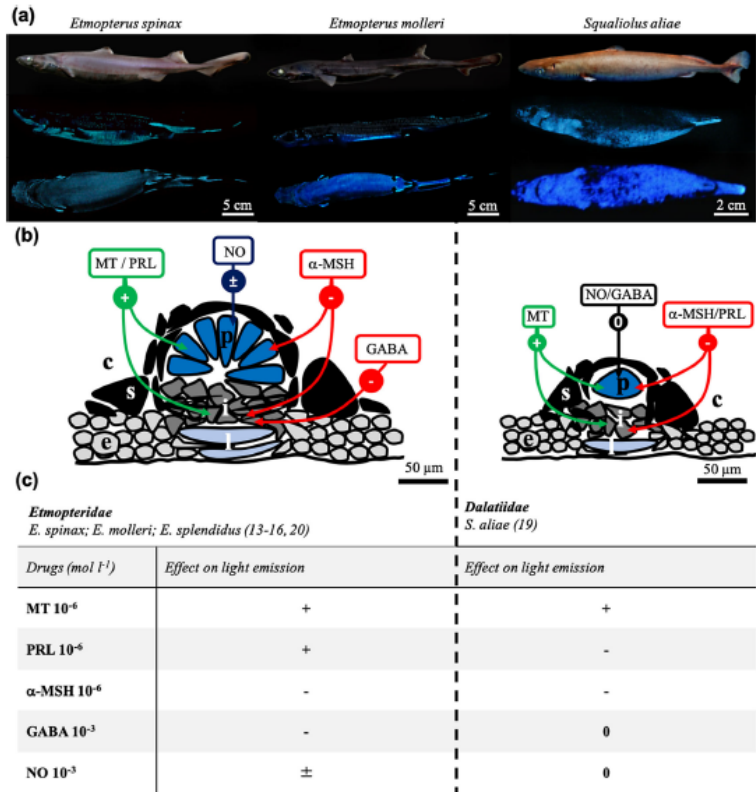


Figure 1. Comparative aspects of light emission between Etmopterid and Dalatiid. (a) lateral view (upper), bioluminescent lateral (middle) and ventral view (lower) of the three studied species, *E. spinax* (left), *E. molleri* (middle) and *S. aliae* (right). (b) Schematic representation of the photophore (transversal section) with an emphasis on bioluminescence control in Etmopterid (left) and Dalatiid (right). Colored arrows indicate the targets of the different substances involved in the control of Etmopterid and Dalatiid. Symbols in color circles indicate the effects of these substances on shark bioluminescence. (c) Table resuming the hormonal and neuromodulator control of the light emission in luminous sharks according to literature (13–16,19,20). Effects are symbolized by: +, activatory; -, inhibitory; ±, modulatory; 0, no effects. Substances are abbreviated: GABA, γ-aminobutyric acid; α-MSH, α-melanocyte-stimulating hormone; MT, melatonin; NO, nitric oxide; PRL, prolactin. In photophore structure: c, connective tissue; e, epidermis; i, iris-like structure cells; l, lens cell; p, photocyte; s, pigmented sheath.

investigated in parallel on luminescence and on ILS pigment movement. Based on the assumption that all targeted hormone-GPCRs act through cAMP modulation, this work investigated the intracellular cAMP concentration during the hormonal modulation of light emission.

Using pharmacological assays and luminometry recordings, we demonstrated (1) the inhibitory effect of ACTH on the bioluminescence of two lanternsharks and one pygmy shark species and (2) the modulation of cAMP levels following MT and α-MSH treatments in Etmopteridae.

Interestingly, these observations, combined with previous studies (35), are consistent with the first steps of transduction pathways involved in skin pigment movement, that is GPCRs of MT/ACTH/ α -MSH regulating cAMP intracellular levels through modulation of adenylyl cyclase activity. This suggests a potential functional shift from general skin color change for counter-shading *via* skin pigment migration, to specific modulation of ILS cell pigmentation regulating light emitted by photocytes in elasmobranch and elasmobranch species.

MATERIALS AND METHODS

Experimental animals. Thirty-one adults of *E. spinax* [41–51 cm total length (TL)] were captured during a field collection in January 2017 by longlines lowered at 250 m depth in the Raunefjord, Norway (60°15'54" N; 05°07'46" E). Living specimens were kept in a 1 m³ tank filled with running cold seawater (6°C) and placed in a dark cold room at Bergen University Marine Station (Espeland, Norway). These sharks were collected in Norway under the "experimental fish care permit" number 12/14048.

A total of 35 adults of *E. mollerii* [37–48 cm TL] were collected during field collection in November 2016 and 2017 in the East China Sea near the coast of Sesoko Island, Okinawa, Japan (26°28'94"N; 127°41'20"E). They were captured using a fishing rod equipped with an electric reel at depths ranging from 480 to 510 m. All specimens were transferred to oxygen saturated plastic bags filled with seawater and brought in a refrigerated box to the Okinawa Churaumi Aquarium where they were kept alive in a dark tank filled with running seawater (13°C) until manipulations. Sharks were handled according to Churaumi Aquarium husbandry and veterinary rules for animal experimentations.

A total of 11 adults of *S. aliae* [15.5–22 cm TL] were sampled during a field session in November 2018 in Donggang Harbor, Taiwan (22°24'46"N; 120°24'11"E). They were collected alive by midwater trawls operating at 50–150 m depth as bycatch by local fishermen. Living specimens were brought to the National Museum of Marine Biology and Aquarium (NMMBA; Pingtung, Southern Taiwan) and placed in dark tank filled with seawater (18°C). Sharks were handled following the NMMBA rules for animal experimentations.

All sharks were euthanized by a blow to the head followed by a full incision of the spinal cord at the level of the first vertebrae, following the local rules for experimental vertebrate care. Animals were treated according to the European regulation for animal research handling. Sharks were sexed, measured and weighed (Table 1) before experimentation took place.

Skin tissues were dissected from the ventral luminous zone of each shark. Round shape skin patches were sampled from the skin using a metal cap drill [6 mm diameter] as described in Claes and Mallefet, 2009 (13). These ventral skin patches were washed and kept in a shark saline (5.36) in the dark at 4°C before being used.

Pharmacological studies. In order to document the effect of ACTH drug applications on the shark bioluminescence, ventral skin patches were placed into wells of 96-well plate allowing to run multiple treatments simultaneously.

Application of MT served as control of the light emission induction since this hormone is known to trigger shark bioluminescence (13). Since α -MSH is an inhibitor of the light emission (13), its application with melatonin served as model of light inhibition. Application of ACTH was made to test the effect of this hormone alone on light emission, shark saline (5.36) being used as nontreatment control. Finally, MT followed by an application of ACTH showed the potential role of the latter hormone on light emission. All drugs were obtained at Sigma, St. Louis, Missouri.

Experiments were first conducted on 16 *E. spinax* specimens. A 96-well plate was filled with 200 μ L of either MT, ACTH at 10^{-6} mol L⁻¹ or shark saline alone as controls, skin patches were immersed during 60 min. In a second series of experiments, a 96-well plate was filled with 100 μ L of MT 10^{-6} mol L⁻¹ as the first hormone, then, after 20 min, 100 μ L of the second hormone: MT 10^{-6} mol L⁻¹, α -MSH 10^{-6} mol L⁻¹ or ACTH was added. In order to perform a dose-dependent response curve, various concentrations of ACTH, ranging from 10^{-8} to 10^{-5} mol L⁻¹, were used. Luminescence of ventral skin patches subjected to the various treatments was measured using a microplate

Table 1. Experimental specimens.

Species	N	Mean total length (cm)	Mean weight (g)
<i>Etmopterus spinax</i>	25 ♀	46.3 ± 1.1	416.1 ± 23.8
	6 ♂	40.0 ± 1.3	248.1 ± 34.2
<i>Etmopterus mollerii</i>	29 ♀	43.8 ± 2.3	242.0 ± 40.0
	6 ♂	40.4 ± 0.8	173.8 ± 15.2
<i>Squaliolus aliae</i>	9 ♀	20.5 ± 1	53.8 ± 9.8
	2 ♂	16.7 ± 1.3	28.5 ± 4.5

N, number of specimens; ♀, female; ♂, male. Measures are expressed with SEM.

luminometer (Berthold MPL12/Orion; Pforzheim, Germany) calibrated using a standard 470 nm light source (Beta light; Saunders Technology, Hayes, UK). Light outputs were recorded using Simplicity software (Berthold, Pforzheim, Germany) during 65 min with a measurement every minute.

For comparative purposes, treatments giving relevant results on *E. spinax* were tested on 13 *E. mollerii* specimens (ACTH 10^{-5} and 10^{-6} mol L⁻¹) and 11 *S. aliae* specimens (ACTH 10^{-5} mol L⁻¹).

Luminescence responses were characterized according to Claes and Mallefet, 2009 (13), namely: the maximum intensity of light emission [L_{max}, in megaquanta per second (Mq s⁻¹)], the total amount of light emitted during this experimentation [L_{tot}, in Gigaquanta per hour (Gq h⁻¹)] and the time to reach the maximum of light intensity [TL_{max}, in seconds (s)]. Inhibitory action of α -MSH and ACTH was measured as of total amount of light emitted after second drug application [L_{tot,app}, in Gq h⁻¹]. All these light parameters were standardized according to the skin patch surface area (in cm²). The second treatment was added to the first one when the light intensity plateau was reached with the MT application, each timing being species-specific. Results of luminescence decrease were expressed as a percentage of the maximal luminescence value (i.e. plateau MT) measured before the second application.

To illustrate the *in vivo* action of ACTH on the light organ, experiments on *E. mollerii* were repeated under a video microscope (Keyence VHX200, Osaka, Japan). Pictures and time-lapse movies were recorded during 20 min after the second drug application.

cAMP assay. Variations of cAMP concentration were calculated according to different treatments for the two lanternshark species (*E. spinax* and *E. mollerii*). Ventral skin patches (n = 4 specimens for each treatment and both species) were dissected and placed in a small chamber filled with different solutions: saline (control), MT 10^{-6} mol L⁻¹, α -MSH 10^{-6} mol L⁻¹ during 20 min. Skin patches were then directly frozen at -80°C. Each skin patch was homogenized according to the manufacturer's instructions, and cAMP concentration was measured using the cAMP-Glo™ Assay kit (V1501, Promega, Madison, WI). Light output was recorded using a Berthold microplate luminometer (MPL12 Orion; Pforzheim, Germany) with simplicity software. cAMP concentrations were calculated using the standard linear regression of cAMP-Glo™ Assay kit, results were then standardized in cAMP concentration per square centimeter (μ M cm⁻²).

Statistical analyses. All analyses [ANOVA, *posthoc* Tukey tests] were performed with the software JMP pro v.14 (SAS Institute Inc., Cary, NC, 1989–2007). The Gaussian distribution condition was obtained for all analyses after logarithm transformation allowing the use of parametric

Table 2. Hormone-induced luminescence parameters (mean maximal light intensity: L_{max}; time to reach the L_{max}: TL_{max}) after melatonin (MT) treatments for the three investigated species.

Species	N	Treatment	L _{max} (Mq s ⁻¹ cm ⁻²)	TL _{max} (s)
<i>Etmopterus spinax</i>	16	MT	0.85 ± 0.03	1241 ± 156
<i>Etmopterus mollerii</i>	13	MT	0.98 ± 0.14	312 ± 170
<i>Squaliolus aliae</i>	11	MT	2.67 ± 0.28	332 ± 166

N, number of specimens, data for L_{max} and TL_{max} are means ± SEM.

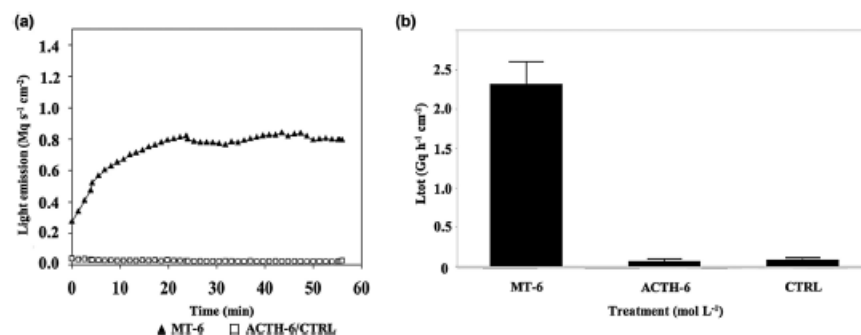


Figure 2. Effects of MT, ACTH and control application on *E. spinax* luminescence. (a) Time course of the mean light emissions (Mq s⁻¹ cm⁻²) from ventral skin patches of velvet belly lanternshark ($n = 16$). (b) Total amount of light produced (Gq h⁻¹ cm⁻²) under hormonal treatments expressed in 10^{-6} mol L⁻¹. CTRL, shark saline, $n = 16$, error bars correspond to s.e.m.

tests. ANOVA was used to show significant difference between treatments while *posthoc* Tukey tests allowed the differentiation of clusters.

RESULTS

Pharmacological studies

The effects of ACTH on the ventral skin patches were first tested on *E. spinax*. As shown in the Fig. 2A,B, ACTH alone did not induce a significant light production at all concentrations tested similar to saline controls (P -value < 0.05; Table S1; MT application at 10^{-6} mol L⁻¹ triggered a long-lasting luminescence (Fig. 2A,B; Table S1).

Table 2 summarizes the observed maximum intensity of light (L_{max}) and time to reach the maximum intensity (TL_{max}) for the three investigated species.

A dose-dependent inhibitory effect of ACTH on the light emission of *E. spinax* was demonstrated (Fig. 3). At lower concentrations (10^{-6} to 10^{-8} mol L⁻¹), ACTH did not have any effects on MT-induced luminescence while a strong inhibition of MT-induced luminescence was observed at a concentration of 10^{-5} mol L⁻¹ (P -value < 0.05), whereas it was observed at 10^{-6} mol L⁻¹ with α -MSH (Fig. 3A,B; Table S2).

In *E. mulleri*, MT-induced a long-lasting luminescence while ACTH alone failed to trigger a significant light emission compared with shark saline controls (Table S1). Applications of ACTH after the MT pretreatment induced a rapid decrease of light production (Fig. 4A,B). ACTH 10^{-5} and 10^{-6} mol L⁻¹ inhibited light production within <5 min as did α -MSH 10^{-6} mol L⁻¹ (Fig. 4A). After MT-induced luminescence, L₀-t_{app} values of α -MSH 10^{-6} , ACTH 10^{-6} and ACTH 10^{-5} mol L⁻¹ presented statistical differences compared with

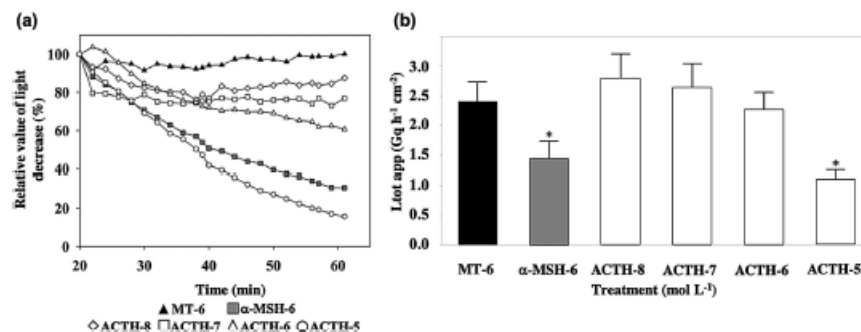


Figure 3. Effects of ACTH and α -MSH on luminescence induced by MT in *E. spinax*. (a) Time courses of the light produced by ventral skin patches; light emission decline was expressed as percentage of maximal MT control value. (b) Effects of α -MSH and ACTH on total amount of light (Gq h⁻¹ cm⁻²) produced after 30-minute MT pretreatment. * corresponds to significant statistical difference from MT-6 control. $n = 16$, error bars correspond to s.e.m.

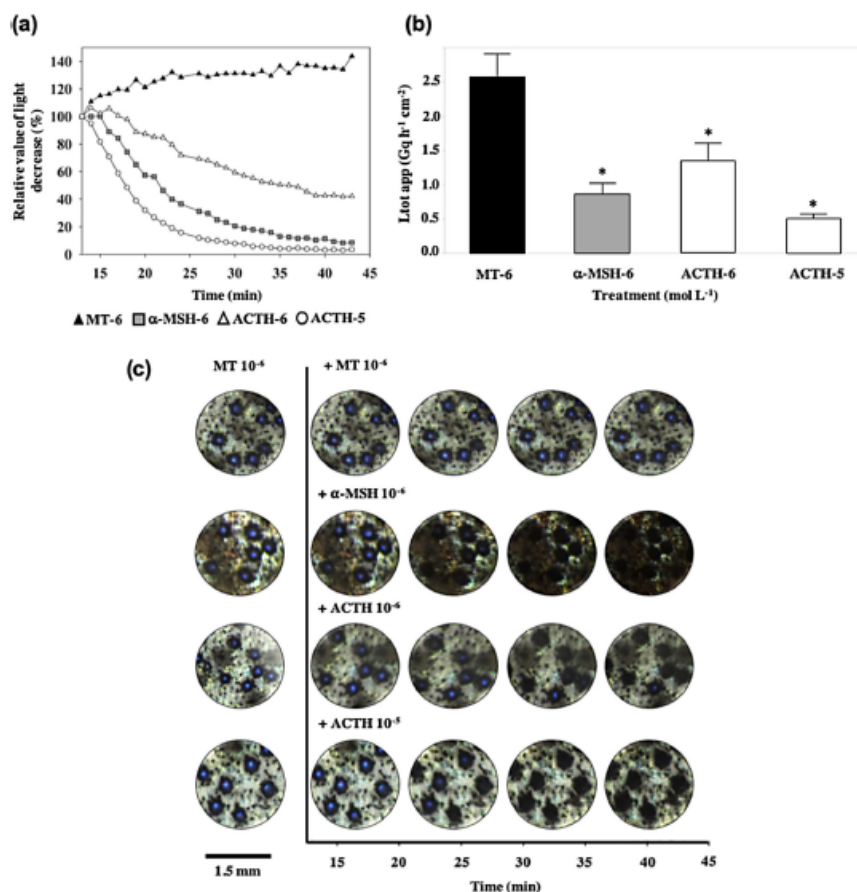


Figure 4. Inhibitory effect of ACTH and α -MSH on luminescence induced by MT in *E. melleri*. (a) Time course of light produced from ventral skin patches of slendertail lanternshark. Results are expressed as percentage of maximal light value recorded for MT pretreatment. (b) Effects of α -MSH and ACTH on the total amount of light ($\text{Cq h}^{-1} \text{cm}^{-2}$) produced after 13 minute pretreatment with MT. * corresponds to significant statistical difference from MT control. $n = 13$, error bars correspond to s.e.m. (c) Time-lapse of pigment movements in skin patches of *E. melleri* pretreated with MT $10^{-6} \text{ mol L}^{-1}$; second application: MT $10^{-6} \text{ mol L}^{-1}$ (ctrl); α -MSH $10^{-6} \text{ mol L}^{-1}$; ACTH 10^{-6} and $10^{-5} \text{ mol L}^{-1}$.

MT $10^{-6} \text{ mol L}^{-1}$ controls (Fig. 4B; P -value < 0.05 ; Table S2). Additionally, time-lapse images of skin patches clearly showed that after 13 min, all preparations exhibited a bluish reflection resulting from photophore-associated ILS opening triggered by MT pretreatment (Fig. 4C, Movie S1). A second application of α -MSH or ACTH induced a closure of the ILS, as the photophores became very dark within 20 min (Fig. 4C, Movie S1). Furthermore, the whole skin patch appeared nearly black in response to α -MSH (Fig. 4C).

ACTH $10^{-5} \text{ mol L}^{-1}$ was the only concentration tested in *S. albae*, at this concentration, ACTH alone did not induce light emission since the total amount of light remained significantly below the control level (*i.e.* spontaneous light occurring in shark saline; P -value < 0.05 ; Table S1). Time courses of controls and total light after treatment are presented in Fig. 5A,B. ACTH $10^{-5} \text{ mol L}^{-1}$ rapidly inhibited MT-induced light emission within 4 min, faster than the inhibition observed with α -MSH $10^{-6} \text{ mol L}^{-1}$. Measured Ltot app for α -MSH 10^{-6} and ACTH

10^{-5} mol L $^{-1}$ were statistically different from MT 10^{-6} mol L $^{-1}$ controls (Fig. 5B; P -value < 0.05; Table S2).

cAMP concentration assay

In the two etmopterids, *E. spinax* and *E. molleri*, the cAMP assay revealed concentration changes within the photogenic epidermis after treatment. As shown in Fig. 6, ventral skin patches of both species treated with 10^{-6} mol L $^{-1}$ MT showed a significant decrease in cAMP levels compared with untreated controls (P -value < 0.05). In *E. spinax*, the mean cAMP concentration was 0.053 ± 0.009 μ M cm $^{-2}$ and 0.020 ± 0.003 μ M cm $^{-2}$ for the untreated and the MT-treated skin patches, respectively (Fig. 6A). In *E. molleri*, the mean concentration was 0.112 ± 0.006 μ M cm $^{-2}$ and 0.075 ± 0.010 μ M cm $^{-2}$ for untreated and MT treatments, respectively (Fig. 6B). In contrast, α -MSH has no effect on cAMP levels in *E. spinax* (0.055 ± 0.007 μ M cm $^{-2}$) but it triggered a small increase of cAMP concentration in *E. molleri* (0.132 ± 0.006 μ M cm $^{-2}$) (Fig. 6). However, α -MSH treatments did not produce any significant difference compared with controls (P -value > 0.05) for both shark species.

DISCUSSION

Investigations of various luminous shark species strongly suggest that shark bioluminescence is under hormonal control. While a common regulation mechanism core is present in Etmopteridae and Dalatiidae, these two families are characterized by opposite PRL effects on bioluminescence (13,19). Luminescence appears to be mediated by pigment movement within the ILS cells of the light organ (13,19,37).

Among the numerous hormones, MT, PRL, α -MSH and ACTH are known to control pigment migration in vertebrates through the modulation of cAMP concentrations (25–31,38). Melanocortins (*i.e.* MSH and ACTH) are main initiators of the pigment dispersion pathway. According to current understanding of vertebrate pigment dispersion, pathway is thought to unfold as follows: (1) activation of Gs-linked GPCR through a specific ligand (*e.g.* ACTH, α -MSH) which (2) stimulates adenylate cyclase activity leading to (3) a rise of intracellular cAMP levels and, consequently, (4) the activation of the catalytic subunit of cAMP-dependent protein kinase A (PKA) (5) and finally the phosphorylation of a specific protein allowing plus-end cellular motor kinesin to ferry out pigment toward the melanophore digit extensions (39–

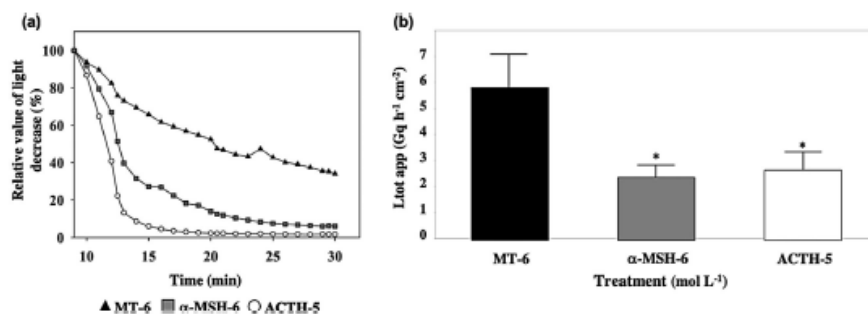


Figure 5. Inhibitory effect of ACTH and α -MSH on luminescence induced by MT in *S. aliae*. (a) Time course of light produced from ventral skin patches of smalleye pygmy shark. Results are expressed as percentage of maximal light value recorded for MT pretreatment. (b) Effects of α -MSH and ACTH on the total amount of light ($Gq\ h^{-1}\ cm^{-2}$) produced after 8-minute pretreatment with MT. * corresponds to significant statistical difference from MT control. $n = 11$, error bars correspond to s.e.m.

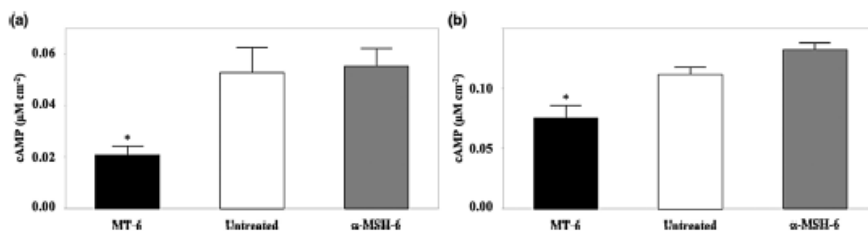


Figure 6. Effect of hormonal treatments on cAMP concentrations in skin patches of *E. spinax* and *E. molleri*. Mean cAMP concentrations in *E. spinax* (a) and *E. molleri* (b); MT at 10^{-6} mol L $^{-1}$; untreated, shark saline (control); α -MSH at 10^{-6} mol L $^{-1}$. $n = 4$ for each treatment, error bars correspond to s.e.m. * corresponds to significant statistical difference from shark saline control.

45). Conversely, the MT pathway leads to the inhibition of adenylyl cyclase activity and the downregulation of the aforementioned kinesin cellular motor activity through receptor-linked Gi protein (25,46). cAMP intracellular concentration plays a central role on the pigment dispersion movements. Furthermore, other studies highlight the implication of the MT in the pigment aggregation pathway (47,48). Some studies have also demonstrated the role of opsins in the light sensitivity of melanophores; this local response to light can also impact the movements of pigmented granules (49,50). Photoreception of the light emitted [i.e. through the encephalopsin (51,52)] could, therefore, act on ILS pigment movements, and thereby, photoreceptive pigment cells could play a role in bioluminescence feedback regulation.

Here, pharmacological tests demonstrated that ACTH, like α -MSH (13,16,19), has a kinetic inhibitory effect on shark ventral luminescence. These two phylogenetically related proteins seem to play an analogous role in luminescence inhibition, but whether they act synergistically or additionally remains to be determined. In *E. molleri*, at the tested concentrations, α -MSH darkens both skin and ILS melanophores, while ACTH acts only at the ILS melanophore level. This observation suggests a differential expression of the melanocortin receptors (i.e. MC1-5R) or/and a differential specific affinity for the melanocortin peptides (i.e. α -, β -, γ -MSH or ACTH) to one receptor. Further studies are suggested to decipher the exact involvement of each melanocortin on the ILS skin melanophores.

All these hormones (MT, α -MSH, ACTH, except PRL) bind to GPCRs which activate the release of a G-protein that modulates adenylyl cyclase activity (32,33). To evaluate hormonal actions on cAMP levels in lanternshark, cAMP concentration assays were performed. MT treatment induced a decrease in cAMP concentration leading to light triggering and ILS cell pigment aggregation in photophores (13). These results are consistent with the coupling of the MT receptor with a Gi protein that inhibits adenylyl cyclase activity (25,46). This cellular pathway exists both in the mechanical control of light emission by ILS cells in bioluminescent sharks and in the modulation of skin pigment movement in shallow-water sharks (53). As skin pigment motion regulation pathways generally involve other molecules such as IP_3 , Ca^{2+} and PKA signaling in vertebrates (42,54–58), the role of intracellular IP_3 and Ca^{2+} in ILS cell modulation could be tested to reveal a putative effect on both light emission and pigment motility. In the same perspective, testing cAMP-dependent PKA activation could also be considered.

The pharmacological studies and the cAMP concentration assays suggest the existence of a functional switch between the control of skin pigment movement and the luminescence control mechanism via regulation of ILS cell pigment movement. Luminous counterilluminating sharks, as aforementioned, need to finely control their bioluminescence in order to camouflage themselves in the oceanic twilight zone. Further studies need to be conducted to precisely determine the transduction pathways involved in light emission control. In particular, it involves an examination of the intracellular motor carrying melanin granule (kinesin and dynein) and/or skin photoreceptive pathways (encephalopsin). Since *E. spinax*, *E. molleri* and *S. aliae* display specific melanophore cells (ILS cells) overlying photocytes, a specific control of pigment movements could represent an essential step in counterillumination control that is crucial for lanternshark survival.

This work combined with previous studies lead to assume that two interconnected controls exist in the luminous shark light

organ: (1) The first control, operating directly at the photocyte level, triggers light emission through an unknown biochemical reaction (i.e. unknown luciferin/luciferase system or photoprotein) and (2) The second, the mechanical control of the ILS-associated melanophore pigment motion to regulate the amount of light transmitted. Hormones, neuromodulators and even extraocular opsins being able to act separately or simultaneously on both controls (13–16,19,20,51,52).

The ancestral mechanism of skin color change through skin pigment migrations, involved in countershading to camouflage the body and mimic the background in shallow-water sharks, may have been adapted to specifically regulate ILS cell pigment movements, thus creating favorable conditions for the development of counterillumination mechanisms in deep-sea living velvet belly lanternshark, slendertail lanternshark, small-eye pigmy shark and possibly luminous sharks in general.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Total amount of light emitted (Li_{tot}) for each treatment in *Etmopterus spinax* ($n = 16$), *E. molleri* ($n = 13$) and *Squalius aliae* ($n = 11$) integrated on 65, 45 and 30 min, respectively.

Table S2. Total amount of light emitted after the second drug application (Li_{2nd app}) for each treatment in *Etmopterus spinax* ($n = 16$), *E. molleri* ($n = 13$) and *Squalius aliae* ($n = 11$) integrated on 45, 32 and 22 min, respectively.

Movie S1. Video recording (accelerated 80 times) of photophore closure after ACTH application on a skin patch of *E. molleri* pretreated with MT.

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