DEVELOPMENT AND APPLICATION OF TECHNIQUES FOR THE CONTROL OF CAPTIVE BREEDING IN ELASMOBRANCHS

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Author: **Pablo García-Salinas**
Directors: **Juan F. Asturiano · Víctor Gallego**

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEVELOPMENT AND APPLICATION OF TECHNIQUES FOR THE CONTROL OF CAPTIVE BREEDING IN ELASMOBRANCHS

Pablo García Salinas

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THESIS SUPERVISORS

Dr. Juan F. Asturiano Nemesio

Dr. Víctor Gallego Albiach

Grupo de Acuicultura y Biodiversidad

Instituto de Ciencia y Tecnología Animal

Universitat Politècnica de València

A ti, que me has hecho reír y me has visto llorar

"Sharks have everything a scientist dream of"

— Peter Benchley

"You're gonna need a bigger boat"

— Chief Brody

***Jaws* (1975)**

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I remember starting to write this thesis thinking about the day I would have to face the acknowledgements section. At first my idea was to be as concise and impersonal as possible. Just one sentence, not necessarily mine, to move on and avoid the monumental task of not forgetting someone by mistake. And I remember the moment I realize that was not going to happen. Not the fact of leaving someone unacknowledged; that is inevitable, and I apologize in advance for not naming you. But the fact that just to say thanks is not fair. This thesis is a collaborative work of people who are not listed as authors of the chapters, but who are just as essential. People who have kept me from feeling an outsider: the only one talking about assisted reproduction at shark conferences and the only one talking about sharks at assisted reproduction conferences. Now is the time for sweet words and sentimentality, so to the objective and detached reader I recommend skipping this part. Part that is in my home language, as I find it easier to get to the essence this way.

En primer lugar, he de agradecer la guía, el apoyo y toda la paciencia que conmigo han tenido mis directores de tesis. Tanto Johnny, como Víctor (como Luz fugazmente), han estado a mi lado en los buenos y los malos momentos, brindándome ayuda y orientando cada uno de los pasos que daba. Los tres acogieron a un loco que sólo hablaba de tiburones, y confiaron y se arriesgaron abriendo una vía llena de incertidumbre. Testigo de todo esto han sido mis increíbles compañeras: Marina, Leonor y Marta, con las que he compartido viajes por el mundo, tiempo, y anguilas. A los seis, no sabéis lo que agradezco que me hayáis haber hecho tan ligero este tiempo.

Más allá de una dimensión académica, existe una dimensión personal que permea toda la tesis. He de destacar especialmente que no podría haber llegado a este punto sin mi familia. Y esto es tanto literal, como figurado. Mi familia me ha proporcionado una red de seguridad intangible, pero siempre presente, que me ha permitido el privilegio de poder arriesgar al tomar decisiones. Si miro hacia detrás veo un camino claro y lógico de mi progresión hasta alcanzar el punto en que me encuentro. Pero soy consciente de que esto no es más que una ilusión construida a posteriori. Visto desde arriba, desde donde miran las madres y los padres, el camino ha sido tortuoso,

incierto e incomprensible muchas veces. Por todo esto, gracias por haber estado siempre, respetando formas, tiempos y distancia, incluso cuando os he cerrado la puerta en las narices.

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A lo largo de este camino he ido encontrando gente que me ha servido de guía y de referente, tanto de las cosas que quería ser, como de las cosas que no. Sin duda, un elemento clave durante mi formación y definición del futuro fue encontrarme con la asociación de estudiantes de biología marina de la Universitat de València, *Bioblau*. Gracias a Joan, Laia y Alicia, el mundo marino tiene un puñado más de investigadores y profesionales trabajando en su protección. Aquellos años en el laboratorio de Biología Marina con Romana, Borja, Mustapha y tantas otras personas, asentaron bases sin las cuales hoy no estaría escribiendo estas palabras.

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Puedo decir con total franqueza que la dimensión profesional, en mi caso, está totalmente mezclada con la dimensión personal. Tengo la inmensa fortuna de poder trabajar en un puesto y un lugar que me apasiona, con gente que ha trascendido la categoría de compañero para convertirse en amigos. Mil gracias a Pepa, Nacher, Pedro, Alicias, Mario, Jose Luís, Daniel,

Marta, Carlos y a tantas otras que debería nombrar. Nuestra misión es muy sencilla, sólo pretendemos cambiar el mundo. Y tal vez esto parecería imposible, pero basta conocerlas para saber que solo necesitamos tiempo.

Por último, quiero agradecer una decisión casual. Hace tres años (justo hoy que escribo estas líneas), giré a la derecha en vez de seguir recto. Esta decisión, tan aparentemente trivial, ha terminado poniendo mi vida del revés. Nunca imaginé que era así como siempre debería haber estado. No puedo dejar de agradecer el instante que me llevó a conocerte y empezamos a construir un futuro juntos. El momento en que me di cuenta de que estaba en Brasil. El momento en que me di cuenta de que tenía un hogar. *Obrigado Danilo, eu amo você*.

Finally, just as this thesis has been a collaborative effort, so is science and its progress. Or should be. Just as a coral reef is built by little polyps expanding their surroundings, science is built thanks to individuals expanding their little patch of knowledge. This community of expanding knowledge has allowed me, allowed us, to continue in this shared process that started when the first ape asked the first question. In science, our discoveries are not our own, we are bound to others, past, present, and future. For this reason, I would like to thank all the authors listed in the references section. To all the Fowlers, Dulvys, Compagnos, Dalys, Penfolds, Hamletts, Wyffels, Musicks, Conraths, and all the others who I quote again and again, who started asking questions before me. To all of them, thank you, I will try to keep expanding my little patch too.

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SUMMARY

Appearing 420 million years ago, sharks and rays constitute an ancient and ecologically diverse group of aquatic vertebrates known as elasmobranchs. This diverse group possesses life strategies that make them highly vulnerable to rapid environmental changes, such as those resulting from anthropogenic actions. Despite being key elements in regulating the ecosystems in which they live, they are currently one of the most threatened vertebrate groups on the planet.

In parallel with *in situ* conservation, *ex situ* conservation programs can be used to improve the situation of some sensitive species. Among these programs, captive breeding plans would increase the sustainability of public aquariums and research centres, as well as allow the development of conservation strategies in the wild. However, to be effective, these plans should include the use of assisted reproductive control techniques. Unfortunately, these techniques have been scarcely developed in the past, so their usefulness has not been adequately proven.

Faced with this situation, the present thesis aims to fill certain gaps in knowledge regarding the use of these techniques in various elasmobranch species, focusing especially on the collection, handling, maintenance, and preservation of sperm.

Although our research initially focused on two model species, the small-spotted dogfish *Scyliorhinus canicula* and the rough skate *Raja radula*, gaining access to other chondrichthyan species made us aware of the great variability in the arrangement of their reproductive system structures. Not considering this diversity can hinder the application of assisted reproductive techniques, either by reducing the efficiency of the procedures, the quality of the samples, or by unintentionally harming the animals. For this reason, Chapters 1 and 2 focused on cataloguing and describing the structures of different species, covering a wide taxonomic spectrum, and focusing on the best techniques for obtaining gametes and performing artificial insemination.

Sperm conservation in the short, medium, and long term plays a key role in developing efficient *ex situ* conservation plans. The availability of good-quality sperm could avoid the transport of males between institutions, minimize problems derived from the lack of synchronicity between breeding adults, reduce conflicts during courtship periods, and decrease inbreeding. On the other hand, it allows for a constant supply of sperm to develop research projects, without continuous extractions from males kept in a controlled environment or the wild.

The third chapter focuses on the development and assessment of different formulas to achieve the maintenance of fresh sperm. Finally, a medium was obtained capable of keeping diluted alive sperm for more than 30 days. Subsequently, by adding various cryoprotectants (egg yolk, dimethyl sulfoxide, and methanol) in different concentrations, the chapter explains how the cryopreservation of sperm from several skate species and, for the first time, sharks, was achieved.

One of the first obstacles faced when working with spermatozoa during the techniques discussed in Chapter 3 was due to the morphology of these cells compared to that of other aquatic species. The head of chondrichthyan spermatozoa, particularly elasmobranchs, has a helical shape, with a variable number of turns depending on the species. In addition, in many cases, the cells do not appear free in the seminal fluid; instead, they form structured aggregations called spermatozeugma. Throughout Chapter 4, we explore how these two features evolve as a result of internal fertilization and the mechanical characteristics of the medium in which they perform their function. For the first time, it is possible to observe how cells react to media with different properties, highlighting the importance of viscosity when applying assisted reproduction techniques.

Finally, the Discussion compares the efficacy of the methods used and explores new avenues of action arising from having access to more individuals of different species. This includes the possibility of detailed descriptions using image analysis techniques, the evaluation of the importance of viscosity and aggregates in preservation processes, and the possibility of artificial inseminations.

RESUMEN

Los tiburones y rayas aparecieron hace 420 millones de años, conformando el antiguo y ecológicamente diverso, grupo de vertebrados acuáticos conocido como elasmobranquios. Este variado grupo posee unas estrategias vitales que los hace muy vulnerables a los cambios rápidos del entorno, como los derivados de la acción antrópica. Pese a ser elementos clave en la regulación de los ecosistemas en los que habitan, en la actualidad, se trata de uno de los grupos vertebrados más amenazados del planeta.

Paralelamente a la conservación *in situ*, los programas de conservación *ex situ* se pueden utilizar para mejorar la situación de algunas especies sensibles. Entre estos programas, los planes de cría en cautividad aumentarían la sostenibilidad de acuarios públicos y centros de investigación, además de permitir el desarrollo de estrategias de conservación en estado salvaje. Sin embargo, para que sean efectivos, estos planes deberían incluir el uso de técnicas de reproducción asistida. Desafortunadamente, estas técnicas apenas se han desarrollado en el pasado, por lo que su utilidad no se ha demostrado adecuadamente.

Ante esta situación, la presente tesis pretende llenar ciertas lagunas en el conocimiento acerca del uso de estas técnicas en diversas especies de elasmobranquios, centrándose especialmente en la obtención, manipulación, mantenimiento y preservación del esperma.

Si bien nuestra investigación comenzó centrándose especialmente en dos especies modelo, la pintarroja *Scyliorhinus canicula* y la raya áspera *Raja radula*, a lo largo del estudio se logró tener acceso a otras especies de tiburones, rayas e incluso de una especie de quimera. Este acceso nos permitió ser conscientes de la gran variabilidad en la disposición de las estructuras del sistema reproductor. Esta diversidad puede dificultar la aplicación de técnicas de reproducción asistida, bien reduciendo la eficacia de los procedimientos, la calidad de las muestras, o dañando a los animales. Si bien en la literatura hay descripciones generales y, en ocasiones, en detalle de los sistemas reproductores de ciertas especies, dichas descripciones no se han centrado nunca en la aplicación práctica de las técnicas de reproducción asistida. Por este motivo, los capítulos 1 y 2 se centraron en la catalogación

y descripción de las distintas estructuras de especies cubriendo un amplio espectro taxonómico, centrándose en las mejores técnicas de obtención de gametos y de inseminación artificial.

La conservación del esperma a corto, medio y largo plazo juega un papel clave para poder desarrollar planes de conservación eficientes en condiciones *ex situ*. Disponer de esperma de calidad permite limitar el transporte de machos entre instituciones, minimizar problemas derivados de la falta de sincronicidad entre adultos reproductores, reducir conflictos durante épocas de cortejo y reducir la endogamia. Por otra parte, permite disponer de muestras para desarrollar proyectos de investigación sin necesidad de tener que extraer continuamente esperma de machos mantenidos en medio controlado. El tercer capítulo se centra en cómo se desarrollaron y probaron diferentes fórmulas para lograr el mantenimiento en fresco del esperma, hasta lograr obtener un médium en el que diluir el esperma capaz de mantenerlo con vida durante más de 30 días. Posteriormente, mediante la adición de varios crioprotectores (Yema de huevo, DMSO y Metanol) en diversas concentraciones, explica cómo se logró la criopreservación del esperma de varias especies de raya y, por primera vez, la criopreservación del esperma de varias especies de tiburones.

Uno de los primeros obstáculos que se detectaron a la hora de trabajar con los espermatozoides durante las técnicas planteadas en el capítulo 3 se debió a la particular morfología de estas células comparada con la de otras especies acuáticas. La cabeza de los espermatozoides de condrictios, y de elasmobranquios en particular, presentan una forma helicoidal, con un número variable de giros en función de la especie. Además, en muchas ocasiones las células no aparecen libres en el fluido seminal, formando en su lugar agregaciones estructuradas denominadas espermatozeugma. A lo largo del capítulo 4 se explora cómo estas dos características surgen como resultado de la fecundación interna y las características mecánicas del medio en el que han de realizar su función. Por primera vez, se puede observar cómo las células reaccionan ante medios con diferentes propiedades, poniendo de relevancia la importancia de la viscosidad a la hora de aplicar técnicas de reproducción asistida.

Por último, en la discusión, se compara la eficacia de los métodos empleados y se exploran nuevas vías de actuación surgidas raíz de tener acceso a más individuos de diferentes especies. Esto incluye la posibilidad de realizar descripciones detalladas empleando técnicas de análisis de imagen, evaluar la importancia de la viscosidad y los agregados en los procesos de preservación y la posibilidad de realizar inseminaciones artificiales.

RESUM

Els taurons i les ratjades van aparèixer fa 420 milions d'anys, conformant l'antic i ecològicament divers grup de vertebrats aquàtics conegut com elasmobranquis. Aquest variat grup posseeix unes estratègies vitals que els fa molt vulnerables als canvis ràpids de l'entorn, com els derivats de l'acció antròpica. Malgrat ser elements clau en la regulació dels ecosistemes en els quals habiten, en l'actualitat, es tracta d'un dels grups vertebrats més amenaçats del planeta.

En paral·lel a les accions de conservació *in situ*, els programes de conservació *ex situ* poden ser emprats per a millorar la situació d'algunes espècies sensibles. Entre aquests programes, els plans de cria en captivitat permetrien augmentar la sostenibilitat d'aquaris públics i centres d'investigació, a més de permetre la creació d'estratègies de propagació d'individus en el medi natural. No obstant això, per a ser efectius, aquests plans haurien de comptar amb l'ús de tècniques de control assistit de la reproducció. Per desgràcia, aquestes tècniques a penes han sigut desenvolupades en el passat, per la qual cosa la seua utilitat no ha sigut degudament constatada.

Davant aquesta situació, la present tesi pretén omplir unes certes llacunes en el coneixement sobre l'ús d'aquestes tècniques en diverses espècies de elasmobranquis, centrant-se especialment en l'obtenció, manipulació, manteniment i preservació de l'esperma.

Si bé el capítol anterior se centra especialment en dues espècies model, el tauró gat *Scyliorhinus canicula* i la ratjada peluda *Raja radula*, al llarg de l'estudi es va aconseguir tindre accés a altres espècies de taurons, ratjades i fins i tot d'una espècie de quimera. Aquest accés ens va permetre ser conscients de la gran variabilitat en la disposició de les estructures del sistema reproductor. Aquesta diversitat pot dificultar l'aplicació de tècniques de reproducció assistida, bé reduint l'eficàcia dels procediments, la qualitat de les mostres, o danyant als animals. Si bé en la literatura hi ha descripcions generals i, a vegades, detalladament dels sistemes reproductors d'unes certes espècies, aquestes descripcions no s'han centrat mai en l'aplicació pràctica de les tècniques de reproducció assistida. Per aquest motiu, els capítols 1 i 2 es van centrar en la catalogació i descripció de les diferents

estructures d'espècies cobrint un ampli espectre taxonòmic, centrant-se en les millors tècniques d'obtenció de gàmetes i d'inseminació artificial.

La conservació de l'esperma a curt, mitjà i llarg termini juga un paper clau per a poder desenvolupar plans de conservació eficients en condicions *ex situ*. Disposar d'esperma de qualitat permet limitar el transport de mascles entre institucions, minimitzar problemes derivats de la falta de sincronicitat entre adults reproductors, reduir conflictes durant èpoques de festeig i reduir l'endogàmia. D'altra banda, permet disposar de mostres per a desenvolupar projectes d'investigació sense necessitat d'haver d'extraure contínuament esperma de mascles mantinguts al mig controlat. El tercer capítol es centra en com es van desenvolupar i van provar diferents fórmules per a aconseguir el manteniment en fresc de l'esperma, fins a aconseguir obtindre un medi en el qual diluir l'esperma capaç de mantindre'l amb vida durant més de 30 dies. Posteriorment, mitjançant l'addició de diversos crioprotectors (Rovell d'ou, DMSO i Metanol) en diverses concentracions, explica com es va aconseguir la criopreservació de l'esperma de diverses espècies de ratlla i, per primera vegada, la criopreservació de l'esperma de diverses espècies de taurons.

Un dels primers obstacles que es van detectar a l'hora de treballar amb els espermatozoides durant les tècniques plantejades en el capítol 3 es va deure a la particular morfologia d'aquestes cèl·lules comparada amb la d'altres espècies aquàtiques. El cap dels espermatozoides de condrictis, i de elasmobranquis especialment, presenten una forma helicoidal, amb un nombre variable de girs en funció de l'espècie. A més, en moltes ocasions les cèl·lules no apareixen lliures en el fluid seminal, formant en el seu lloc agregacions estructurades denominades espermatozeugma. Al llarg del capítol 4 s'explora com aquestes dues característiques sorgeixen com a resultat de la fecundació interna i les característiques mecàniques del mitjà en el qual han de realitzar la seua funció. Per primera vegada, es pot observar com les cèl·lules reaccionen davant mitjans amb diferents propietats, posant de rellevància la importància de la viscositat a l'hora d'aplicar tècniques de reproducció assistida.

Finalment, en la discussió, es compara l'eficàcia dels mètodes emprats i s'exploren noves vies d'actuació sorgides arrel de tindre accés a més

individus de diferents espècies. Això inclou la possibilitat de realitzar descripcions detallades emprant tècniques d'anàlisis d'imatge, avaluar la importància de la viscositat i els agregats en els processos de preservació i la possibilitat de realitzar inseminacions artificials.

GENERAL INTRODUCTION

1. The chondrichthyans

The class Chondrichthyes is a lineage of aquatic vertebrates whose origin can be traced back to the boundary between the Silurian and Devonian periods, approximately 430 million years ago (Amaral et al., 2018). Today, the class is one of the oldest and most ecologically diverse groups of vertebrates, with representatives thriving in oceans, seas and inland waters all over the world (Dulvy et al., 2014; Weigmann, 2016).

Chondrichthyans are a monophyletic group characterized by two distinctive synapomorphies: their skeletal mineralization and male copulatory structures (Schaeffer, 1981; Nelson et al., 2016). The term Chondrichthyes, literally meaning cartilaginous fish (from the classical Greek *khóndros*, cartilage and *ikhthús*, fish), refers to their aquatic nature and cartilaginous endoskeleton, as opposed to the bony endoskeleton of most vertebrates (Hall, 2005). How this skeleton is mineralized is, precisely, the defining synapomorphy of the group, with polygonal calcified structures called tesserae reinforcing the endoskeleton, giving it unique mechanical qualities (Lund and Grogan, 1997; Seidel et al., 2016).

The second synapomorphy refers to the mating system. In all male chondrichthyans intromittent organs located in the pelvic fins can be observed. These structures, called myxopterygia, or more commonly claspers, are the result of modifications of the endoskeleton of the pelvic girdle and serve as a mechanism for sperm transport during mating (Gilbert and Heath, 1972; Hamlett, 1999a; Jones et al., 2005; Grogan et al., 2012).

The class is subdivided into two subclasses (Figure 1): the elasmobranchs (subclass Elasmobranchii), commonly referred to as sharks and rays, and the holocephalans (subclass Holocephalii), commonly referred to as chimaeras, separated approximately 421 million years ago (Renz et al., 2013). This colloquial grouping under the general common terms masks the actual diversity of the groups, with approximately 1477 species and remarkable evolutionary distances between them. Elasmobranchs make up the majority of these species, with almost 96% of them, while holocephalans are a less diverse group, with 57 species (Amaral et al., 2018; Fricke et al., 2022).

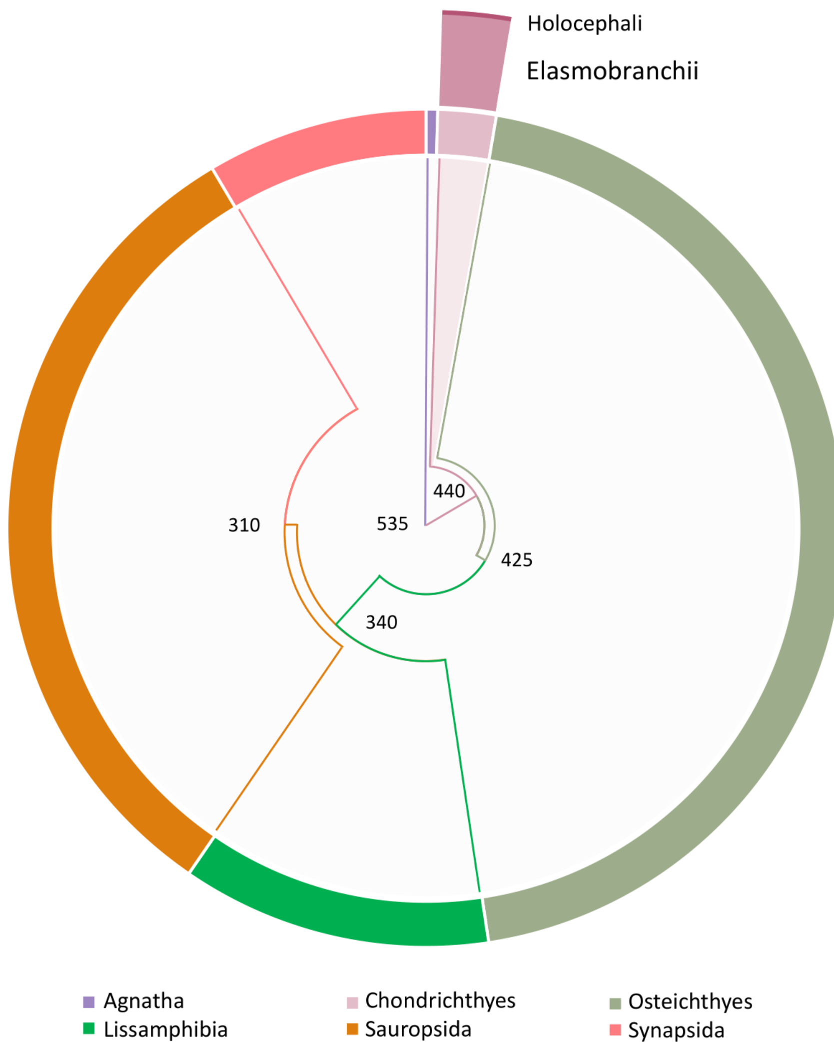

Figure 1. Relative abundance in the number of species among the various taxa of present-day vertebrates. The numbers represent, in millions of years, the time of radiation among the distinct groups. The group corresponding to the chondrichthyans is highlighted, also showing the difference in number of species between elasmobranchs (sharks and rays) and holocephalans (chimaeras). Adapted from Boryshpolets et al. (2023).

As previously mentioned, elasmobranchs can be further classified into sharks (or superorder Selaciomorpha) and rays (or superorder Batoidea). Batoidea is the larger group (around 800 species) with a wider variety of habitats exploited. This diversity is evident in the various body patterns and morphologies observed within this group. The term "ray" serves as an

umbrella term encompassing a variety of species such as skates, mantas, guitarfishes, sawfishes, and stingrays, among others. On the other hand, the superorder Selaciomorpha encompasses all shark species (approximately 600), ranging from large basking sharks to sawsharks, angelsharks and lanternsharks (Ebert et al., 2021; Fricke et al., 2022). It is important to highlight that both groups are superorders, which are taxonomic categories with a wide range of species. Using the common terms "sharks" and "rays" may oversimplify and overlook the actual diversity within these groups. As an example, Laurasiatheria is also a superorder, but of placental mammals, encompassing species as different as bats, shrews, whales, and giraffes (Zhou et al., 2012). This same variety should be expected in elasmobranchs.

Sharks, rays, and chimaeras have played a key role in the ecosystems they inhabit for millions of years. Their function as apex predators is notable for certain large species, but their impact extends beyond top-down control. Juvenile and small to medium-sized species act as mesopredators, connecting various elements of food webs as both predator and prey. Many species may act as regular or opportunistic scavengers, both in the deep ocean and in coastal ecosystems. While other species, especially batoids, contribute as bioturbators by stirring up sediment, promoting nutrient resuspension and creating new niches for colonization by other organisms (Heithaus et al., 2010; Heupel et al., 2014; Hussey et al., 2015; Martins et al., 2018). Regardless of the exact mechanism, it has been shown that the disappearance of certain species from ecosystems can lead to significant alterations and direct consequences for human interests (Myers et al., 2007; Baum and Worm, 2009; Heithaus et al., 2008, 2012; Ruppert et al., 2013).

Sharks, rays, and chimaeras have not only played a key ecological role but also hold significant cultural and socio-economic value for humans (Castro, 2014). Nowadays shark fins, and meat, consumed fresh, dried or transformed into fish meal, are in demand (Ketchen, 1986; Clarke et al., 2007; Dulvy et al., 2017). While some species' meat holds commercial value, it often serves as a low-cost protein source for coastal communities, particularly in developing regions. Other products are also traded, notably oil from their livers, vertebrae, skin, and teeth (Lack et al., 2014; Dent, 2015; Davidson et al., 2022). Additionally, recent years have seen an unprecedented growth in ecotourism associated to these animals, with a rising economic impact (Healy et al., 2020).

These animals have an extensive distribution, so that these ecological and socioeconomical functions occur in a wide diversity of habitats. Some species have specific habitat requirements, such as coral reefs or seagrass meadows, while others have a global distribution or undertake large-scale migrations (Weigmann, 2016; Ebert et al., 2021). Furthermore, their presence extends beyond the marine environment. Certain species, such as sawfishes of the genus *Pristis*, or the bull shark *Carcharias leucas* migrate to inland waters such as rivers, lakes, and floodplains during specific life stages. Others may live in estuarine areas, such as sharks of the genus *Glyphis*, or be fully adapted to freshwater life, such as all the rays of the family Potamotrygonidae and some species of the family Dasyatidae (Ballantyne and Fraser, 2012; Ebert et al., 2021; Kyne and Lucifora, 2022).

Despite the above-mentioned relevance for the proper functioning of ecosystems, and their social and economic importance, chondrichthyans are disappearing at an alarming rate (Figure 2). At present, with one third their species threatened, they are the second most endangered group of vertebrates in the world (Dulvy et al., 2021). In fact, Chondrichthyans are the group of marine vertebrates with the lowest percentage of species listed as "Least Concern" by the IUCN (Davidson et al., 2022). Overfishing, both targeted and due to by-catch, and habitat destruction, are the drivers behind the decline of their populations (Worm et al., 2013; Dulvy et al., 2014, 2016a; Pacoureau et al., 2021; Yan et al., 2021). However, other stressing factors, such as exposure to pollutants (Gelsleichter and Walker, 2010; Alves et al., 2016) and climate change (Rosa et al., 2017; Osgood et al., 2021; Rummer et al., 2022) may have been overshadowed by overfishing, but are growing in importance. To understand the reasons behind this disappearance, and why these stressors have such a severe impact on these animals, it is necessary to understand their life histories.

When discussing the life histories of chondrichthyans, generalizations are inevitable. Also, sharks tend to receive more attention, leading to a tendency to assign their attributes to other batoids and chimaeras for illustrative purposes. Additionally, there is a lack of sufficient information on attributes like longevity, growth rate, and fecundity for many species. However, where data is available, the diversity within this group is astonishing (Dulvy and Forrest, 2010; Dulvy et al., 2017). As an example, size ranges from 0.20 to 12 meters, and lifespans vary from 5 to over 300 years. Reproductive diversity

is even more pronounced, with gestation periods ranging from 2 to 42 months, fecundity from 1 to 400 offspring, offspring size from 0.05 to 1.8 meters, age at sexual maturity from 1.5 to over 35 years, and a range of reproductive modes encompassing egg deposition to placental viviparity, as further explained below (Compagno, 1990; Cortés, 2000; Dulvy and Forrest, 2010). Considering these ranges and the aforementioned limitations, chondrichthyans generally exhibit low productivity due to low fertility rates, slow growth, late sexual maturation, and some of the highest levels of maternal investment in the animal kingdom in terms of gestation duration and embryo nutrition (Cortés, 2000; Simpfendorfer et al., 2011). For this reason, chondrichthyans are very susceptible to rapid changes in their environment, as the results of fishing or habitat destruction (Cortés, 2000; Cortés, 2002; García et al., 2008; Dulvy and Forrest, 2010; Dulvy et al., 2014).

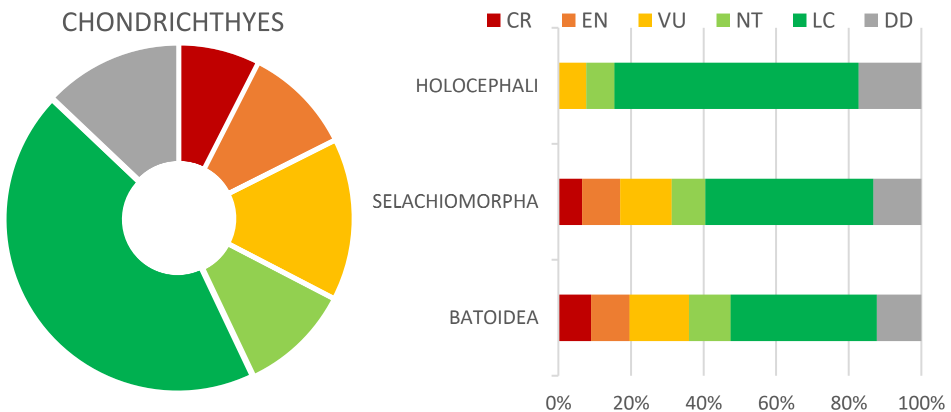

Figure 2. Conservation status according to the criteria of the International Union for Conservation of Nature (IUCN). The circle represents the 1199 species assessed and their status. The bars represent the percentage of species of each category per taxonomic group. The abbreviations and color codes are: Red/CR, *Critically Endangered*; Orange/EN, *Endangered*; Yellow/VU, *Vulnerable*; Light Green/NT, *Near Threatened*; Dark Green/LC, *Least Concern*; Gray/DD: *Data Deficient*. Information obtained from Dulvy et al., 2021.

A detailed understanding of the life histories of these animals is crucial for establishing effective science-based fisheries management regulations. Therefore, studies focusing on age, growth rates and reproductive biology are more important than ever.

2. Reproductive biology

Elasmobranch reproductive biology has long been a subject of interest, and in fact, one of the earliest recorded observations we owe to Aristotle. The polymath made cryptic yet insightful descriptions of oviparous and viviparous species, including comparisons to mammalian placentas. He also noted reproductive structures, copulation, reproductive periods, and what we now recognize as nursery areas (Aristotle, trans. Pallí Bonet, 1992). The history of the successive loss and recovery of knowledge about chondrichthyan reproductive biology is rich and fascinating, summarised in John P. Wourms' work of 1977. For the development of the present thesis, suffice it to say that 2300 years after Aristotle, the reproductive biology of these animals remains one of the most prominent topics of study of this group (Awruch et al., 2019; Shiffman et al., 2020; Coelho et al., 2021).

Chondrichthyans are dioecious animals, with a clear sexual dimorphism evidenced by the presence of the claspers (Figure 3). Claspers are tube-shaped paired structures that allow sperm to flow through an internal groove. While barely developed in immature males, in adult animals are fully calcified and articulated at their base to facilitate their insertion. In all chondrichthyan species, fertilisation is internal, a feature maintained throughout the evolutionary history of the group (Gilbert and Heath, 1972; Wourms, 1977; Musick and Ellis, 2005; Conrath and Musick, 2012; Walker, 2020). During the mating process, males bite females profusely to retain them, while inserting one or both claspers into the cloaca. In chimeras, retention is accomplished by paired spatulate-like structures called prepelvic tenacula (or prepelvic claspers) and a frontal tenaculum, a club-like appendage with denticles on the head of males which they use to grasp females (Dean, 1906; Didier, 2004; Whitney et al., 2004; Barnett et al., 2009).

Although the anatomy of females and males will be described in more detail in Chapters 1 and 2, several key points can be highlighted as an introduction. In females, the reproductive tract begins with paired ovaries (or sometimes a single ovary) located in the cranial portion of the abdominal cavity (Figure 4). Mature oocytes are released into the abdominal cavity and transported via cilia to the ostium, a funnel-shaped structure located in the anterior part of the oviducts. During their passage through the oviducts, the oocytes encounter the oviductal gland, where fertilisation and encapsulation within the egg capsule occurs.

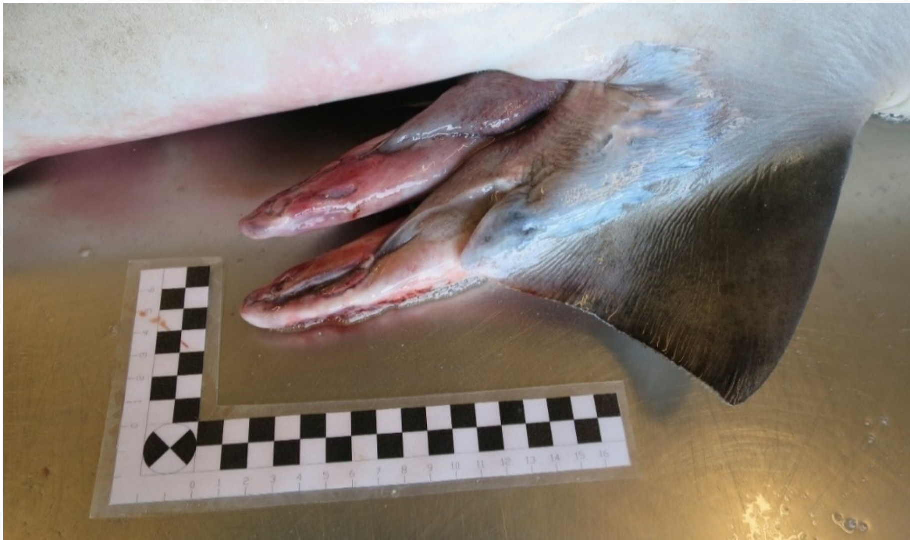

Figure 3. Detail of the right pelvic fin and the two claspers of a 2.72m mature blue shark *Prionace glauca*, stranded in València, Spain. White and black squares represent 1cm.

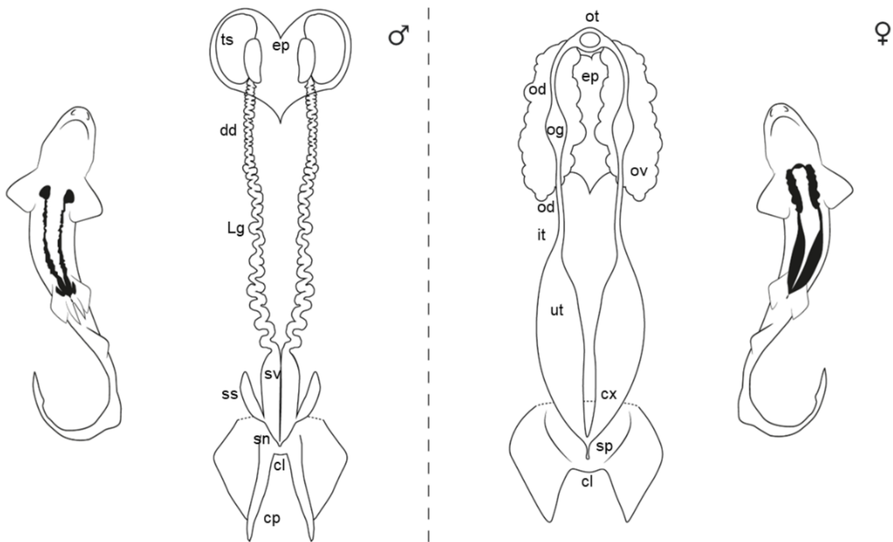

Figure 4. General scheme of the reproductive system of a generic chondrichthyan. The arrangement of the reproductive system is highlighted in the silhouette of the animals, male on the left side and female on the right side. In the male, from top to bottom, the abbreviations indicate: (ts) testis, (ep) epigonal organ, (dd) ductus deferens, (Lg) Leydig's gland, (sv) seminal vesicle, (ss) sperm sac, (sn) sinus, (cl) cloaca, (cp) clasper. In the female, from top to bottom, the abbreviations indicate: (ot) ostium, (ep) epigonal organ, (od) oviduct, (og) oviductal gland, (ov) ovary, (ov) ovary, (it) isthmus, (ut) uterus, (cx) cervix, (sp) sphincter, (cl) cloaca.

Finally, the encapsulated embryos pass through an isthmus into the uterus, where they remain isolated from the outside environment by the cervix. In males, immature spermatozoa are produced by a pair of testes located in the cranial part of the abdominal cavity. From there, via the efferent ducts, the immature spermatozoa pass into the convoluted epididymis, and then on to the *vas deferens* (or *ductus deferens*) to end up in seminal vesicle at the caudal end of the abdominal cavity (Wourms, 1977; Hamlett, 1999a, 2005; Hamlett and Koob, 1999; Jones et al., 2005; Lutton, 2011)

Secretions from the Leydig's gland, closely associated with in the vas deferens, allows the passing of the sperm through the successive ducts. Throughout this, the immature spermatozoa mature into motile spermatozoa (Hamlett, 1999a; Engel and Callard, 2011). It is at this point that the phenomenon of sperm aggregate formation occurs. Depending on the species (Figure 5), the spermatozoa in the lumen of the *vas deferens* and seminal vesicle may appear in various states: free, encapsulated by a matrix (spermatophores), or embedded in a matrix but not encapsulated (spermatozeugma).

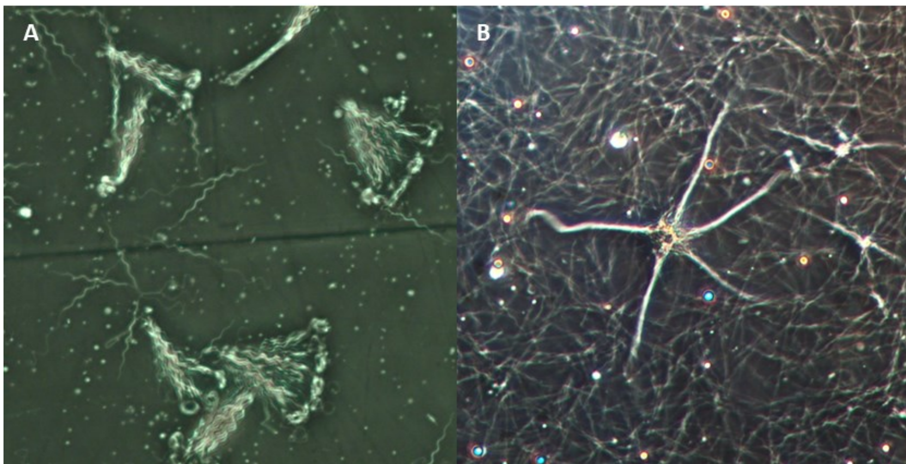

Figure 5. Diverse types of sperm aggregations according to the different species observed under an optical microscope. (A) common stingray *Dasyatis Pastinaca.* (B) rabbitfish *Chimaera monstrosa*.

These aggregations are maintained during transit to the female. (Henningsen, 1994; Pratt and Tanaka, 1994; Hamlett, 1999a; Jamieson and Hamlett, 2005). Although its exact function is unclear, there are several theories to justify the existence of these aggregates. They could protect the

spermatozoa in transit to the female, they could displace rival spermatozoa from previous matings, they could serve to improve progress inside the uterus, or allow better storage in the oviductal gland. The importance of these aggregates will be discussed in more detail in chapter 4 (Pratt and Tanaka, 1994; Tanaka, 1995; Hamlett, 1999a, 1999b; Luer et al., 2008; Storrie et al., 2008).

Depending on the group, sperm transfer from males to females is driven by a different process. Male sharks have sacs (or siphons) located ventrally under the skin at the base of the pelvic fins. This sac, which has an opening at its distal end called an apopyle, is aligned with the internal clasper groove. During copulation, the sacs pump water through muscular contractions, propelling the sperm that enters the clasper groove and flows into the female's uterus (Gilbert and Heath, 1972; Jones et al., 2005). While some male batoids have a structure at the base of the pelvic fins, called the clasper gland. Although its function is still uncertain, it secretes lubricating fluids that may allow more efficient transfer of sperm from males to females. A gland with a similar function has been described in chimaeras (Lacy, 2005; Piercy et al., 2006; Walker, 2020).

Reaching sexual maturity triggers important changes in chondrichthyans, including shifts in distribution patterns, social behaviour (sexual segregation), and developmental biology (sexual dimorphism in body size) (Compagno, 1984; Sims, 2006; Orlov et al., 2010; Nunes et al., 2015; Colonello et al., 2020). Thus, defining maturation stages in these species is crucial. However, there is no universal criterion, varying among authors and species. Potential criteria for females include animal length, size of ovarian follicles, oviductal gland diameter, and uterus size and shape. Obvious indicators include trophonemata, embryos, eggs in the uterus, or sperm in the oviductal gland (Wourms, 1977; Stehmann, 2002; Conrath and Musick, 2012; Follesa and Carbonara, 2019; Walker, 2020). Some species may have a hymen, lost during the first copulation, though this criterion is questionable due to premature copulation and natural membrane disintegration (Sumpter and Dodd, 1979; Carrier et al., 2004; Francis and Duffy, 2005). In terms of males, clasper calcification degree is a commonly used criterion, but swelling and varying stiffness between species can lead to misinterpretations. Other criteria include epididymis sinuosity, testes size, and reproductive hormone concentration. Presence of spermatozoa in seminal vesicles can indicate

maturity but should be used with caution, as it has been found in juveniles without the potential to copulate (Stehmann, 2002; Moura et al., 2011; Conrath and Musick, 2012; Follesa and Carbonara, 2019; Walker, 2020).

As previously mentioned, female chondrichthyans can store spermatozoa in their oviductal gland. This gland, also known as shell gland or nidamental gland, is located in the cranial region of the oviduct. The gland is divided into four specialised sections: club, papillary, baffle, and terminal. The club and papillary sections secrete protective gelatine layers for embryos and eggs, while the baffle section produces the tertiary envelope. The terminal zone is responsible for storing, nourishing, and protecting sperm before fertilization occurs. Females can accumulate sperm from multiple copulations, leading to litters with multiple paternities (polyandry). Sperm storage can last several months (up to 45), enabling consecutive pregnancies without the need for further matings. In addition to avoiding the unwanted effects of matings (a traumatic and sometimes lethal experience), facilitating post-copulatory selection, and allowing reproduction in isolated situations (Pratt, 1993; Hamlett et al., 1998; Hamlett, 2005; Pratt and Carrier, 2005; Storrie et al., 2008; Daly-Engel et al., 2010; Lamarca et al., 2020; Jordan et al., 2021; der Merwe et al., 2022).

Parthenogenesis, an asexual reproductive strategy in which embryos develop in the absence of fertilisation, has also been documented in several elasmobranch species. Initially the occurrence of gravid females in aquaria without males was attributed to sperm storage, so the parthenogenesis was overlooked for a time (Chapman et al., 2007, 2008; Harmon et al., 2016; Feldheim et al., 2017a, 2023; Wyffels et al., 2021; Adams et al., 2022). The fact that individuals have been found in the wild born as a result of this strategy (Feldheim et al., 2017b), and individuals capable of alternating between sexual and asexual reproduction (Dudgeon et al., 2017), exemplifies the reproductive plasticity of these animals under different environmental conditions.

Chondrichthyans exhibit diverse reproductive strategies (Figure 6), particularly regarding maternal involvement in the nutrition of embryos. Two broad groups can be distinguished: *lecithotrophy* and *matrotrophy*. Lecithotrophic species are those which solely rely on yolk reserves as the source of nutrition for embryos. Matrotrophic species, on the other hand, receive an extra nutrient input in addition to embryonic yolk reserves

(Wourms, 1977; Hamlett et al., 2011b; Conrath and Musick, 2012). It's worth noting that some viviparous elasmobranchs utilize a combination of yolk and maternal resources during different stages of embryonic development, highlighting the non-exclusive nature of these strategies (Cotton et al., 2015; Sato et al., 2016; Buddle et al., 2019).

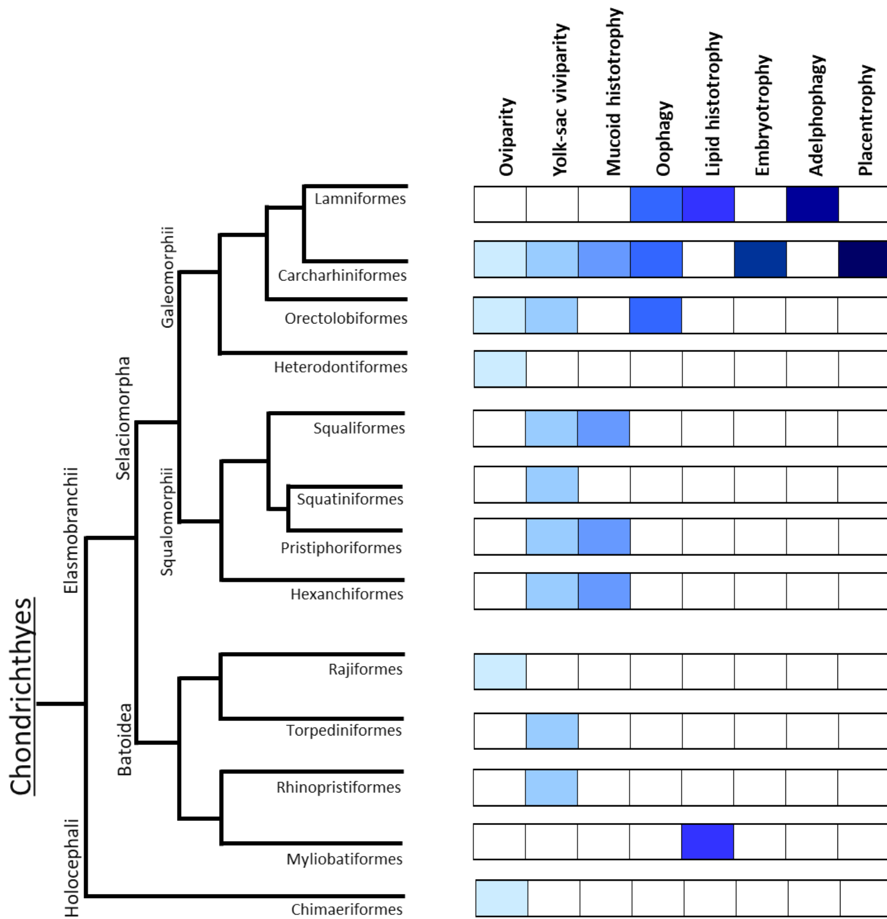

Figure 6. Phylogenetic relationship between the various orders of chondrichthyans and their different reproductive modes. Adapted from Penfold and Wyffels, 2019.

2.1. Lecithotrophy

Oviparity: this strategy is observed in all current chimaera species, all skates (order Rajiformes) and some sharks. *Single oviparity* refers to the presence of one egg per uterus, while *multiple oviparity* involves multiple eggs. *Singular sustained oviparity* has recently been observed, where eggs are retained in the uterus until embryos reach a certain level of development (Nakaya et al., 2020). Eggs have collagen external capsules with tendril-like structures and may be attached to various surfaces or deposited in crevices (Hunt, 1985; Knupp and Squire, 1998; Hamlett, 2005).

Yolk sac viviparity/ Lecithotrophic viviparity/ Ovoviviparity: fertilized eggs are retained in the uterus, and embryos develop internally using yolk reserves for both structural and nutritional purposes. This mode of reproduction is widespread occurring in species in all orders except Heterodontiformes and Lamniformes (Musick and Ellis, 2005; Hamlett et al., 2011).

2.2. Matrotrophy

Limited histotrophy/ Mucoid histotrophy: the uterus secretes a mucus that is absorbed by the embryo to supplement their insufficient yolk sac reserves. The lack of obvious secretory structures makes it difficult to detect this subtle form of embryo nourishment. However, it is assumed that any full-term embryo weighing more than 80% of a mature oocyte's weight (dry/ash weight) is being nourished in this way (Wourms, 1977; Musick and Ellis, 2005; Conrath and Musick, 2012).

Lipid histotrophy: This mode is observed only in Myliobatiformes rays. Secretory extensions in the uterine epithelium, named trophonemata, produce a milk-like lipid and protein-rich substance to nourish the embryo during their development. Unlike the previous mode, this abundant nourishment significantly increases embryo size and it is very obvious (Musick and Ellis, 2005; Hamlett et al., 2011a).

Embryotrophy: This mode has only been observed in the tiger shark *Galeocerdo cuvier.* The embryo develops inside a capsule filled with a large quantity of clear nutrient-rich liquid, which is absorbed through the digestive system, presumably through gastric eversion (Castro et al., 2016).

Oophagy: in this mode some oocytes are ingested by the embryo as additional nutrition, either encapsulated along with the embryo (family

Pseudotriakidae) or released in the uterus (order Lamniformes) (Yano, 1992, 1993). Adelphophagy, is the most extreme form of this and only occurs in the species *Carcharias taurus*. In this strategy, the developing embryos prey on each other as a source of nutrition, until only one per uterus remains (Springer, 1948; Gilmore and Dodrill, 2005; Musick and Ellis, 2005).

Placentotrophy/ Placental viviparity: is a common method found in 5 families of Carcharhiniformes. It involves the use of a placenta, analogous (not homologous) to that of placental mammals. After a previous stage using the yolk reserves, the yolk sac attaches to the uterine epithelium and the short yolk stalk elongates into an umbilical cord. The embryonic tissues do not directly contact the maternal bloodstream, and the embryo is further nourished through histotrophy secreted by maternal tissues (Wourms, 1981; Musick and Ellis, 2005; Hamlett et al., 2011; Buddle et al., 2019).

3. Use of reproductive technologies in chondrichthyans

The reproductive characteristics, life history strategies, and low market value of their products have discouraged captive breeding programs using aquaculture techniques. Despite the growing demand for certain products like oil, corneas, fins, and meat, these programs remain unprofitable. Consequently, knowledge on species handling and husbandry relies on lessons from public aquaria and research center (Penfold and Wyffels, 2019; Walker, 2020).

Public aquaria have featured chondrichthyans, particularly sharks, since the mid-19th century. In fact, one of the earliest descriptions of the reproductive biology of sharks comes from the Bristol Aquarium in 1873. There, the observation of the oviposition behavior of a nursehound *Scyliorhinus stellaris*, settled a debate about how their eggs appeared entangled in all kinds of algae, corals, rocks or shells (Koob, 2004). Since then, public aquaria have been changing the focus of their collections, adopting more conservation and research-oriented approaches. Aquaria are able to house animals that are difficult to care maintain in traditional research facilities and academic facilities. In addition, aquaria are able to monitor the same animals over long periods of time, which is especially important when trying to fill in certain gaps in their life histories (Daly and Jones, 2017; Penfold and Wyffels, 2019; Feldheim et al., 2022).

These opportunities have advanced the study of chondrichthyan physiology, reproductive cycles, courtship, mating behaviors, gestation length, and offspring ontogeny. Additional observations have expanded knowledge on growth rates, longevity, captivity-related aspects (pathologies, social behavior, nutrition, bioenergetics) (Henningsen et al., 2004b; Smale et al., 2004). Moreover, techniques such as tagging, handling, and sampling, initially tested in aquaria, have been applied in the wild to gather data from wild populations (Smale et al., 2004; Daly and Jones, 2017; Penfold and Wyffels, 2019; Feldheim et al., 2022).

The use of non-invasive diagnostic processes, such as hormone analysis or imaging techniques have made it possible to monitor follicular and testicular stages, embryo development, and even embryo movement between uteri (Whittamore et al., 2010; Nozu et al., 2018; Tomita et al., 2018). These advancements, coupled with non-lethal information-gathering procedures, are reducing the impact on studied animals, even in the field. Being beyond the scope of this thesis a detailed review of all these applications can be found in the works of Penfold and Wyffels (2019), Mylniczenko et al. (2017), and Feldheim et al. (2022). Unfortunately, one of the areas of study that has not received much attention has been the use of reproductive breeding technologies.

The source of shark, ray and chimaera populations housed in controlled environments is the natural environment. Breeding programs in aquaria have traditionally relied on natural mating, even for endangered species. While spontaneous successful reproduction has been achieved for some species, it has been challenging for many others (Daly and Jones, 2017; Janse et al., 2017). Aquaria should be considered naturalized (not natural) environments, with abiotic factors, biomass, species composition, and population structures that are not necessarily optimal. Nonetheless, the need for breeding programs for chondrichthyans has grown, especially since many displayed species are threatened, and limitations exist regarding capturing from the wild (Buckley et al., 2018).

To improve these controlled environment breeding programs reproductive techniques can be used. However, these techniques, widely used in other terrestrial and aquatic species, have hardly been used in sharks, rays, and chimaeras thus far.

4. Artificial insemination

Artificial insemination (AI) is gaining attention as a technique for captive elasmobranchs. While widely used in other animals for decades (Vishwanath, 2003; Foote, 2010), its application in elasmobranchs has only recently been explored (Daly and Jones, 2017; Feldheim et al., 2022). Despite being in the preliminary stages of development, promising results demonstrate its potential as a vital tool in breeding programs. AI offers several advantages, such as reducing male aggression and female damage, eliminating the need to transfer animals for breeding between institutions, introducing new genetic diversity, overcoming synchronized reproductive cycles, preventing dominance of a single male, and limiting inbreeding. Moreover, it provides greater control for zoological collection managers in determining optimal reproductive timing (Morrell, 2011; Daly and Jones, 2017; Feldheim et al., 2022).

Despite these advantages, AI has had limited documented success, and has only been attempted in a few species: cloudy catshark *Scyliorhinus torazame* (Masuda et al., 2003); clearnose skate *Raja eglanteria* (Luer et al., 2008); whitespotted bamboo shark *Chiloscyllium plagiosum* (Masuda et al., 2005; Wyffels et al., 2021); brownbanded bamboo shark *Chiloscyllium punctatum* (Daly and Jones, 2017); ocellate river stingray *Potamotrygon motoro* (Daochai et al., 2020); zebra shark *Stegostoma tigrinum* (Adams et al., 2022); sand tiger shark *Carcharias taurus* (Ripley's Aquarium, 2022, unpublished). However, there have been few unsuccessful attempts, with limited records in the literature (Daly and Jones, 2017; Watson and Janse, 2017). It's worth noting that the target species thus far have been mostly oviparous, except for *Potamotrygon motoro* (lipid histotrophy) (Thorson et al., 1983) and *C. taurus* (adelphophagy) (Springer, 1948), leaving the challenge of AI in placentotrophic species remaining.

Successful artificial insemination requires a thorough understanding of the animal's reproductive biology. Factors such as sperm storage capacity, reproductive cycle seasonality, optimal sperm dosage, and genetic compatibility are crucial considerations (Feldheim et al., 2022). Detailed knowledge of internal anatomy is also essential, as the site of sperm deposition (cloaca, uterus, or oviductal gland) affects insemination success and risks potential damage to delicate internal structures (Luer et al., 2008; Penfold and Wyffels, 2019). Additionally, the availability of high-quality

sperm and proper storage methods pose challenges for artificial insemination procedures regardless of the target species.

5. Sperm management

Sperm from sharks, rays, and chimeras can be obtained from live animals through methods like abdominal massage (Masuda et al., 2003, 2005; Daly and Jones, 2017; Dzyuba et al., 2019a; Wyffels et al., 2020a; Padilha et al., 2021; Adams et al., 2022) or catheter insertion through the urogenital papilla (Minamikawa and Morisawa, 1996; Watson and Janse, 2017). However, the diverse morphology of their reproductive systems requires careful consideration to avoid harm and ensure sample quality (Figure 7). Improper insertion or pressure can cause internal injuries or spoil the samples. Additionally, factors such as reproductive seasonality and variability in the quantity and quality of stored sperm must be considered (Penfold and Wyffels, 2019).

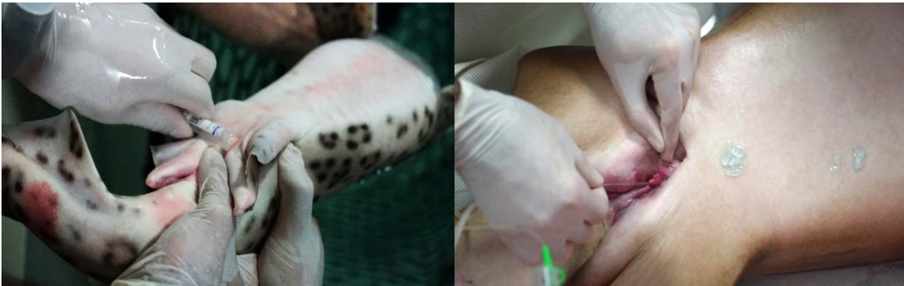

Figure 7. Left: abdominal massage technique for obtaining sperm from a nursehound *Scyliorhinus stellaris*, in tonic immobility position. Right: annulation process to obtain sperm from a nurse shark *Ginglymostoma cirratum* in tonic immobility.

Sperm quality has traditionally been assessed evaluating motility and plasma membrane integrity (Daly and Jones, 2017). However, due to high sperm concentration, dilution is necessary for proper observation. Dilution has been achieved by mixing sperm with media that mimic the physicochemical conditions of elasmobranch blood and tissues. This is typically accomplished by adding urea, trimethylamine oxide (TMAO), or NaCl to increase the osmotic pressure to values close to 900-1000 mOsm kg^{-1} (Jones et al., 1984; Minamikawa and Morisawa, 1996; Luer et al., 2008; Daly et al., 2011; Daly and Jones, 2017). However, seawater has also been used for dilution (Wyffels

et al., 2020a, 2021; Adams et al., 2022), or even centrifuged seminal plasma (Dzyuba et al., 2019a, 2019b).

Motility assessment involves observing and counting the percentage of motile spermatozoa, or simply indicating whether motile sperm is observed (Minamikawa and Morisawa, 1996; Daly et al., 2011; Daly and Jones, 2017; Dzyuba et al., 2019a; Padilha et al., 2021; Adams et al., 2022). Alternative methods use a subjective scale from 0 to 5, or 0 to 4, to rate sperm progression (Jones et al., 1984; Wyffels et al., 2020a, 2021; Gillis et al., 2021a; Padilha et al., 2021), a criterion used previously in land and aquatic mammals (Howard, 1986; O'Brien and Robeck, 2010). CASA (Computer-assisted sperm analysis) is a system commonly used for in multitude of animal species (Gallego and Asturiano, 2019; Valverde Abarca et al., 2019; Singh et al., 2021). The system measures sperm motility percentage and kinetic parameters, categorizing them based on cell velocity. However, until very recently (Montano et al., 2023), the system could not be used on chondrichthyans, so alternative image analysis systems were used to obtain this information (Dzyuba et al., 2019a, 2019b).

The second system for assessing sperm quality is the plasma membrane integrity, or viability, using hypoosmotic swelling test or specific stains (Daly and Jones, 2017; Penfold and Wyffels, 2019). Typically, the staining method combines two stains, one membrane-permeant labels intact cells while the other membrane-impermeant binds to DNA only when cell membrane is damaged. Live-Dead staining employs the fluorescent dyes SYBR Green and propidium iodide for this purpose and has been used frequently in chondrichthyans (Daly et al., 2011; Daly and Jones, 2017; Gillis et al., 2021a, 2021b; Muñoz-Baquero et al., 2021; Wyffels et al., 2021; Adams et al., 2022).

A reliable supply of sperm is crucial for successful artificial insemination, and freezing sperm stored in cryobanks is the common method used to ensure this supply (Asturiano et al., 2017; Martínez-Páramo et al., 2017). Cryobanks store sperm samples in containers surrounded by liquid nitrogen, preserving them at ultra-low temperatures (-196 °C) to minimize physiological activity (Horváth and Urbányi, 2020). However, freezing can cause damage to the sample, either through ice crystal formation or abrupt changes in pH and osmolality. To mitigate this, cryoprotectants (acting both within and outside the cell) are used to reduce damage (Liu et al., 2021). However, these substances may be toxic or harmful to the biological material. Therefore,

cryoprotectant concentration, exposure time of the samples to cryoprotectants (equilibration time), and the speed of the freezing and thawing processes are critical in the overall success (Daly and Jones, 2017; Martínez-Páramo et al., 2017; Horváth and Urbányi, 2020).

Cryopreservation is a technique commonly employed in both terrestrial and aquatic species), either for commercial purposes (Yánez-Ortiz et al., 2022; Yang and Huo, 2022) or for the conservation of threatened fauna (Fickel et al., 2007, Clulow and Clulow, 2016). However, cryopreservation trials in elasmobranchs have been limited. The first attempts focused on brain tissue cell lines, not sperm (Poyer and Hartmann, 1992), and the first successful cryopreservation of sperm occurred in 2011 with the sparsely spotted stingaree *Urolophus paucimaculatus*, using egg yolk and glycerol or DMSO as cryoprotectants (Daly et al., 2011). Glycerol was later successfully used for the Australian bull ray *Myliobatis australis*, but render unsuccessful results for the brownbanded bamboo shark *Chiloscyllium punctatum*, hinting a possible variable tolerance between species (Daly and Jones, 2017). At this point, it is worth noting Penfold and Wyffels' observation: “Cryopreservation of elasmobranch sperm is not widely practiced and barriers such as membrane phase transitions at critical temperatures is currently hindering development of protocols for freezing shark sperm” (Penfold and Wyffels, 2019), showing the unsuccessful progress in this field. It is in this context that one of the key points of this thesis began to be developed, trying to expand the knowledge on chondrichthyan cryopreservation, especially on the cryopreservation of shark sperm.

Sperm cryopreservation ensures long-term sample preservation, but freezing can affect sample quality. In addition, lack of specific equipment and adequate knowledge, or logistical problems, may make cryopreservation unfeasible. In these cases, short-term preservation protocols, such as cold storage at 4 °C, can maintain sperm functionality for a limited time (Bobe and Labbé, 2008; Peñaranda et al., 2008; Shaliutina et al., 2013; Contreras et al., 2020). Spermatozoa from elasmobranchs (even in tropical species) have been found to be cold-resistant and can withstand these conditions for some time. This has allowed samples to be shipped between aquaria, although so far only within the United States (Penfold and Wyffels, 2019; Wyffels et al., 2021).

The routine application of these techniques is necessary to achieve more efficient and sustainable management of zoological collections. But these techniques have the potential to be used also in wild populations (Pizzutto et al., 2021). Especially in the management of small populations, where it is necessary to introduce new alleles or to avoid losing the reproductive potential of a deceased animal. This transfer of knowledge, technique and human capacity is an example of what the *ex situ* conservation model can contribute to the survival of these animals.

6. Projects, grants and companies involved in this Thesis.

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In order to obtain some samples, the collaboration of several fishermen from Castelló, València and Xàbia was essential. Likewise, the collaboration of personnel from the Marine Zoloogy Unit of the Cavanilles Institute of Evolutionary Biology, belonging to the University of València and the Stranding Network of the Valencian Community was also essential.

OBJECTIVES

The overall objective of the thesis was to adapt and test the feasibility of various assisted reproductive techniques, especially sperm management, in different species of elasmobranchs. In doing so, the aim was to improve the control of the reproduction of these animals in a controlled environment. The specific objectives include:

- To expand the basic knowledge on the reproductive biology of a sensitive group of species.
- To standardize protocols for obtaining viable gametes under different conditions and in different species.
- To describe and catalog the existing differences between species, which are relevant when implementing assisted reproduction techniques.
- To develop short-term sperm preservation techniques by using an extender and temperatures above 0 ºC.
- To develop long-term sperm maintenance and preservation techniques through cryopreservation using an extender supplemented with different cryoprotectants.
- To study and understand the physiological and dynamic processes that occur in sperm in order to optimize the above processes.

CHAPTER 1

Reproductive Anatomy of Chondrichthyans: Notes on Specimen Handling and Sperm Extraction. I. Rays and Skates

P. García-Salinas[1,2], V. Gallego[1], J.F. Asturiano[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, València, Spain.

[2]Associació LAMNA per a l'estudi dels Elasmobranquis a la Comunitat Valenciana, Fraules 10, 13, 46020 València, Spain

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Abstract

The superorder Batoidea (rays, skates, and relatives), constitutes one of the most threatened group of vertebrates. Strengthening *ex situ* conservation programs developed in research centers and public aquaria could be a way of addressing this situation. However, captive breeding programs must be improved to prevent the capture of wild animals and to develop proper *in situ* reintroduction strategies. Sperm extraction and artificial insemination are two techniques commonly used in other threatened species, which could also be used in rays and the like. However, the different reproductive morphologies present within this group of animals may hamper both processes. Here, we present a comparison of the reproductive anatomies of 11 distinct batoid species, emphasizing the important differences between the species when performing sperm extraction or artificial insemination. Both male and female animals, belonging to the Rajidae, Dasyatidae, Torpedinidae and Myliobatidae families, from the Mediterranean Sea were studied. In addition, we describe the procedure to extract sperm using both cannulation and abdominal massage, either from live or dead batoids Finally, the obtention of motile sperm recovered from the oviducal gland of females is described. These techniques generate a new range of possibilities for the conservation of these threatened species.

1. Introduction

Appearing almost 400 million years ago, Chondrichthyan fishes are an old and ecologically diverse group with a key role in the regulation of the ecosystems they inhabit (Compagno, 1990; Stevens, 2000). The class is comprised of 1472 species (Fricke et al. 2021), classically divided into the Holocephalans, commonly named chimaeras, and the Elasmobranchs, which include sharks and rays. This last group, rays and their relatives (skates, guitarfishes, sawfishes and the like), is the most diverse group among Chondrichthyan fishes, with 816 species accepted under the formal name of Batoidea (Fowler and Cavanagh, 2005; Last et al., 2016; Weigmann, 2016). Like all other elasmobranchs, batoids possess life histories that make this group sensitive to elevated anthropic pressures (García et al., 2008; Dulvy and Forrest, 2010), and in fact, overfishing and habitat destruction are the main drivers for the global decline of their populations (Dulvy et al., 2014; Pacoureau et al., 2021).

In order to understand the current global situation of the batoid populations, it is essential to know their life histories and reproductive strategies. Rays and their relatives are characterized by large body sizes, late sexual maturity, long gestation periods, high maternal investment, and reduced offspring (Cortés, 2000; Pacoureau et al., 2021). Batoid reproductive strategies are highly diverse and can be categorized based on the nutrition method of the embryos. Lecithotrophic methods include oviparity (as in the case of Rajiformes) and yolk sac viviparity (as in the case of Torpediniformes), where the only nourishment comes from their yolk sack, while matrotrophic methods include an additional source of nourishment at some point in the embryo development, in the form of lipid histotrophy, also known as uterine milk (as in the case of Myliobatiformes) (Musick and Ellis, 2005; Conrath and Musick, 2012; Walker, 2020). Like all other Chondrichthyans, fertilization in batoids is internal, with males having intromittent appendages called claspers as a modification of their pelvic fins (Wourms, 1977)

All these reproductive factors and complex life histories have discouraged captive breeding programs in aquaculture industries (Walker, 2020) but not in aquaria facilities, neither public nor private. The reproduction of batoids in aquaria has been reported for some species (Henningsen et al., 2004b;

Janse et al., 2017), but breeding programs in aquaria have traditionally relied on natural mating rather than the use of reproductive techniques, such as artificial insemination (Daly and Jones, 2017). This technique has been receiving increasing attention, but to ensure the success of this technique, a reliable supply of sperm is required, especially in the case of endangered species (Luer et al., 2008; Daly and Jones, 2017; Penfold and Wyffels, 2019; Daochai et al., 2020). Sperm can be obtained from dead or live animals, and although there has been success in obtaining sperm from several batoids (Daly et al., 2011; Daly and Jones, 2017; Dzyuba et al., 2019a; Morales-Gamba et al., 2019), the procedures of extraction may vary between the different species. Cannulation and abdominal pressure are the traditional procedures used (Penfold and Wyffels, 2019), but with these techniques, the location and morphology of the species-specific reproductive structures (for example seminal vesicles and urogenital papilla) need to be considered. The main objective of this study is to provide a useful guide of the anatomy of the reproductive system of batoids, with a particular focus on sperm procurement procedures, and to propose preliminary indications in the female anatomy to be considered during artificial insemination.

2. Materials and Methods

2.1. Origins of the Specimens

Males and females belonging to 11 batoid species of the order Rajiformes (n = 20), Myliobatiformes (n = 14) and Torpediniformes (n = 4) were studied (Table 1). Some of the species (rough skate *Raja radula*, Mediterranean starry skate *Raja asterias*, thornback skate *Raja clavata*, spotted skate *Raja montagui* were available in fish markets or from commercial fishing vessels' bycatch (longnosed skate *Dipturus oxyrinchus*, spiny butterfly ray *Gymnura altavela*, common stingray *Dasyatis pastinaca*, common eagle ray *Myliobatis aquila*, bull ray *Aetomylaeus bovinus*, marbled electric ray *Torpedo marmorata*). Others were part of the zoological collection of a public aquarium (Oceanogràfic, València). In this case, the fish (Mediterranean starry skate *Raja asterias*, undulate skate *Raja undulata*) were kept separately in two 8000 L tanks with recirculating sea water (temperature: 16–18 °C; salinity: 35–37‰) and fed twice a day with herring, squid, and

shrimps. Maturity of all the specimens was determined by gonad development, clasper calcification and their size according to bibliography (Ebert and Dando, 2020).

Table 1. Species in the study. Number of males (NM) and females (NF) from each species, size range of the specimens used and their origin and conservation status according to IUCN (International Union for Conservation of Nature) criteria for the Mediterranean (Ebert and Dando, 2020): least concern (LC), near threatened (NT), vulnerable (VU), endangered (EN) and critically endangered (CR). Animals from commercial fisheries were captured by gill net or bottom trawler and were available in fish markets (FM) or discarded as bycatch (BC). Animals from aquaria were part of the Oceanogràfic zoological collection (AQ).

Common name	Scientific name	NM	NF	IUCN	Source	Range (cm)
Rough skate	*Raja radula*	3	3	EN	FM	47-63
Spotted skate	*Raja montagui*	2	1	LC	FM	55-67
Mediterranean skate	*Raja asterias*	2	4	NT	AQ/FM	61-68
Thornback skate	*Raja clavata*	1	1	NT	FM	68-72
Undulate skate	*Raja undulata*	1		NT	AQ	86
Longnosed skate	*Dipturus oxyrinchus*	1	1	NT	BC	104/112
Spiny butterfly ray	*Gymnura altavela*	1		CR	BC	104
Common stingray	*Dasyatis pastinaca*	2	3	VU	BC	52-56
Common eagle ray	*Myliobatis aquila*	2	2	VU	BC	54-63
Bull ray	*Aetomylaeus bovinus*	2	2	CR	BC	89-103
Marbled electric ray	*Torpedo marmorata*	2	2	LC	BC	22-51

2.2. Dissection Procedure

A dissection was performed on each individual to gain access to the reproductive system of the animals. Dissection focused only on the reproductive system of the specimens, adapting the dissection protocols used by other authors for sharks (King and Custance, 1982; Crow and Brock, 2004; De Iuliis and Pulerà, 2019). Anatomical terminology used in the present study was adapted from De Iuliis and Pulerà (2019). The specimens were flipped dorsally, exposing their ventral surface (Figure 1A). Using scissors, a small incision was made over the coracoid bar of the pectoral girdle (arrowhead in Figure 1A), large enough to allow the introduction of a scalpel blade. Next, with the help of forceps to keep the abdominal wall (skin, muscle, parietal peritoneum) elevated, a cut was made along the scapular

process contour towards the pelvic girdle (dotted line in Figure 1A). Thus, the tissue was removed to leave the pleuroperitoneal cavity exposed. To access the reproductive system, the liver (arrowhead in Figure 1B) and the oesophagus, stomach, spiral valve, and rectum (arrowhead in Figure 1C) were removed. Hepatic lobes were drawn forward and removed by cutting through their cranial attachments (hepatic ducts and falciform ligament) (dotted line Figure 1B). Special care was taken throughout this process so as not to damage the hepatic lobes or gallbladder, and to avoid the leakage of the oil and bile present in these organs. The stomach and intestine were removed by making an incision through the oesophagus and a cut through the rectum, close to the rectal gland (dotted lines in Figure 1C). To remove the digestive tract, the mesogaster and caudal mesenteries were carefully cut to avoid damage to the mesorchium. In this way, easy access to the urogenital system was achieved (Figure 1D). To clearly expose the caudal part of the urogenital system (seminal vesicle, urogenital sinus, urogenital papilla in males, or uterine sphincters and urinary papilla in females), an incision through the puboischiadic bar was made (dotted line in Figure 1E). Furthermore, in some species, the removal of the cloacal lips to access the urogenital/urinary papillae was necessary. For a clear view of the lower portion of the ductus deferens and seminal vesicle, the parietal peritoneum (arrowhead in Figure 1F) should carefully be removed.

2.3. Description of Reproductive Structures

A macro lens was used to take detailed photographs of each species throughout the dissection procedure of the specimens, and illustrated notes were also taken. The main objective of each dissection was to: (i) determine how to gain easy access to the urogenital papilla, (ii) observe the number and disposition of urogenital pores, (iii) observe urogenital sinus morphology, (iv) access the seminal vesicle/uterus. Plastic tubes with different gauges (0.5–2 mm in diameter) were used as probes during the dissection to confirm the access and connections from the external part of the reproductive system (urogenital/urinary pores and papilla) to the internal part (urogenital sinus, seminal vesicle, uterus). Additionally, the information obtained was

combined and used to propose a general morphological scheme for an ideal batoid, both for females and males.

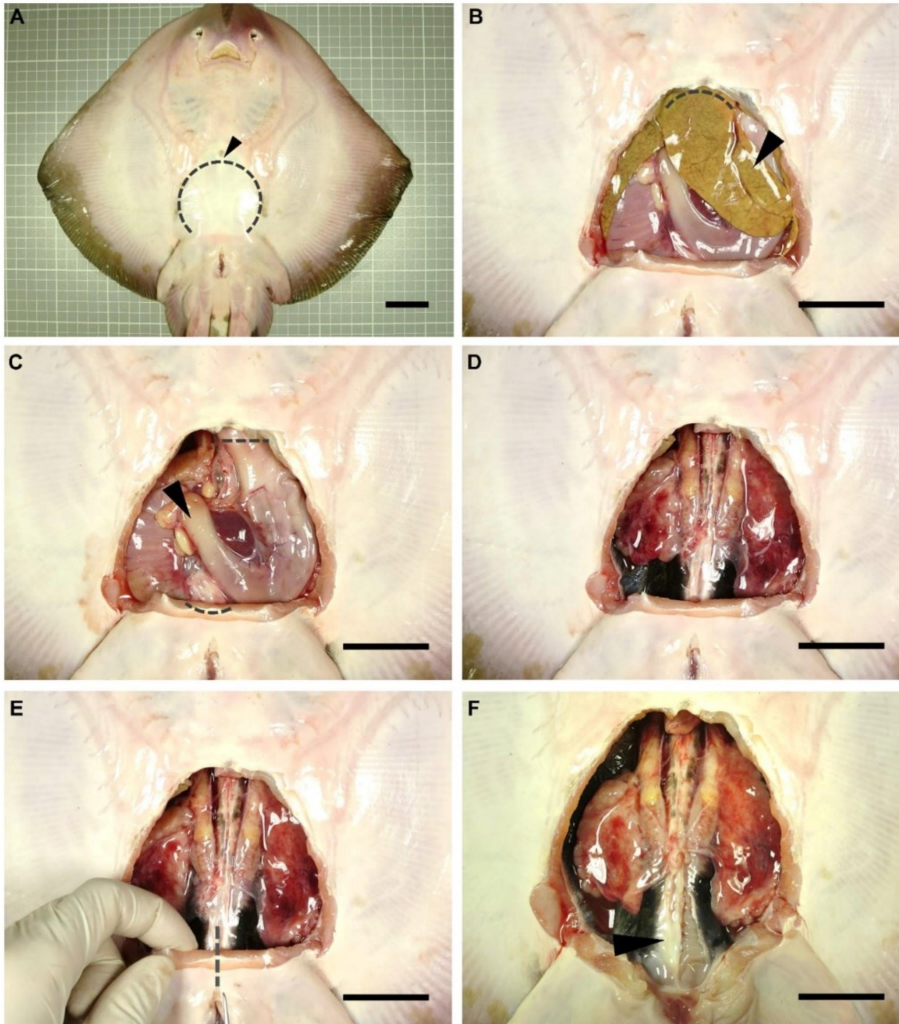

Figure 1. Dissection procedure. Relevant steps in the dissection procedure of a rough skate (*Raja radula*) male to reach the reproductive system (**A**). Removal of the abdominal wall (skin, muscle, parietal peritoneum) to gain access to the pleuroperitoneal cavity (**B**). Extraction of the liver (**C**) and digestive system (**D**). Incision of the puboischiadic bar (**E**) to fully expose the cloaca and the reproductive system (**F**). Arrowhead (**A**): coracoid bar; (**B**): liver; (**C**): digestive system; (**F**): parietal peritoneum. Dotted lines mark incision areas. Dotted line (**A**): abdominal wall; (**B**): cranial liver attachments; (**C**): oesophagus and rectum; (**E**): puboischiadic bar. Scale bar indicates 4 cm.

2.4. *Sperm Collection*

2.4.1. *In Vivo Sperm Extraction*

Tonic immobility was induced prior to sperm extraction to minimize struggling and reducing the stress during handling (Henningsen, 1994; Smith et al., 2004; Kessel and Hussey, 2015), by placing the animals in an upside-down position with their mouth, spiracles and gill slits submerged, while gentle pressure was applied in their snouts. Having the cloaca emerged above water, gentle pressure was applied to the abdominal area over the location of the seminal vesicle, to make the sperm flow through the urogenital papilla. The sperm was immediately collected using a sterile syringe or pipette and transferred to sterile tubes after collection.

2.4.2. *Post-Mortem Sperm Extraction*

Animals were cleaned using marine water to remove mucus and other biological remains such as blood and fishery residues (mud and remains of other organisms which may be found in animals obtained from fisheries). Three different methods were used to obtain sperm form dead animals: (i) abdominal massage on the ventral region immediately anterior to the pelvic girdle, or by pressing around the urogenital papillae in the cloacal cavity with curved pincers; (ii) accessing the internal cavity through dissection and stripping directly on the seminal vesicle (in both cases, sperm flowing from the urogenital papilla was immediately collected using a sterile syringe or a pipette); and (iii) introducing a polyurethane cat catheter (BUSTER cat catheter, 1.0 x 130 mm, Kruuse. Langeskov, Denmark) or a PVC nasogastric tube (Feeding Probe L/RX CH-05 2.67-50 mm2, JMEDIS. Cádiz, Spain) through the appropriate pore on the urogenital papilla. A sterile lubricating jelly with antiseptic (Optilube ActiveTM, Optimum Medical. Leeds, UK) was used to facilitate the introduction of the tube. Tubes and catheters were continuously rotated while inside the seminal vesicle to avoid clogging. Oviducal glands from common stingray *D. pastinaca*, rough skate *R. radula*, Mediterranean starry skate *R. asterias*, longnosed skate *D. oxyrinchus* and marbled electric ray *T. marmorata* females were obtained (Figure 2A) during dissection by cutting through the oviduct on both sides of the gland. The organ was carefully cleaned with artificial sea water to remove blood stains from the extraction procedure (Figure 2B). Care was taken to avoid sea water

flowing into the gland through the oviducts. The glands were split along the longitudinal axis, exposing the lumen (Figure 2C). Using the blunt edge of a scalpel blade, the surface of the luminal epithelium (Figure 2D) was scraped to collect a pearly mucus. The mucus was diluted in an artificial seminal plasma extender described by García-Salinas et al. (2021a). The pH and osmolality of the main components of the extender (in mM; 433 Urea, 376 NaCl, 120 Trimethylamine N-oxide (TMAO), 8.4 KCl, 50 Glucose, 7 $CaCl_2$-$2H_2O$, 3.5 $NaHCO_3$, 0.08 Na_2SO_4, 1.4 $MgSO_4$) were adjusted to 6.5 and 1000 mOsm/kg respectively, in order to match the levels of the physiological fluids.

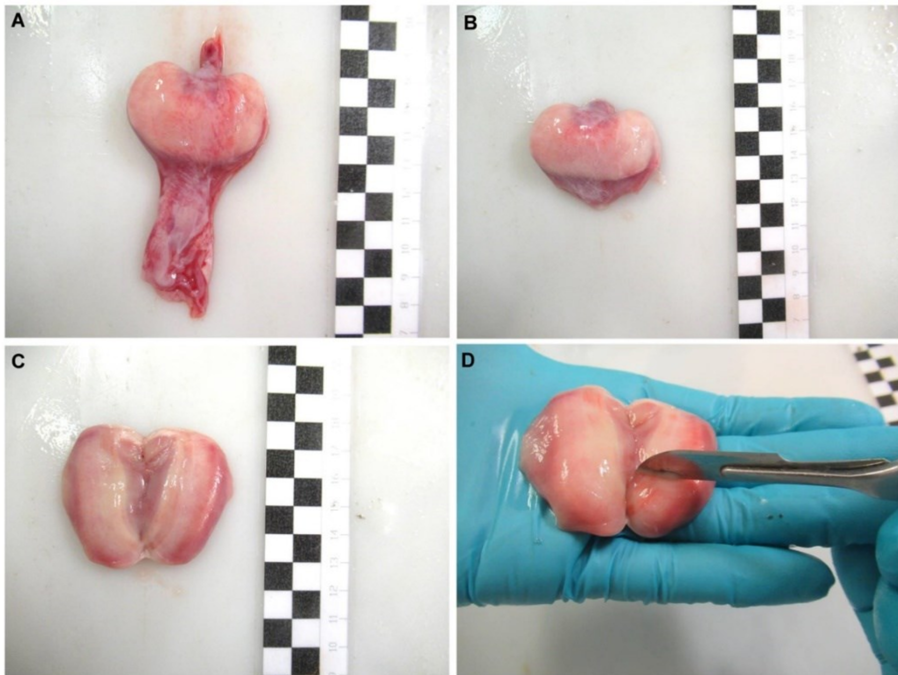

Figure 2. Sperm recovery from the oviducal gland. Oviducal gland from longnosed skate *Dipturus oxyrinchus* (**A**) removed from the oviduct. The gland was cleaned (**B)** and split through its longitudinal axis, exposing its lumen (**C**). A scraping was done over its luminal epithelium using the blunt edge of a scalpel (**D**) to collect a pearly mucus containing sperm. Each black and white square of the scale bar indicates 1 cm.

3. Results and Discussions

3.1. Female General Anatomy

Although there are a wide variety of anatomical differences between the species studied, this study aims to highlight the differences in the reproductive system that may be relevant to the use of specific techniques, such as sperm extraction and artificial insemination. Other possible differences not related to this topic have not been considered. Even though abdominal pores are not part of the reproductive system, its misidentification may cause errors during cannulation, so its description is offered.

Unlike males (Figure 3), batoid females do not possess intromittent structures on their pelvic fins. The cloaca is located between the pelvic fins, and in some species is enveloped by two cloacal lips on either side, covering the anus and the urinary papilla. As the only pores in the papilla are those found in the urinary system, the term urogenital papilla should be limited to males, and not used for females. Depending on the species, one or two pores can be located on the papilla. In the cranial section of the cloaca, the apertures of the uteri can be found. In some species, there are two separated orifices (one for each uterus) easily accessible from the cloaca, whereas in other species, the orifices converge in a common cervix formed by the union of the cervices (or cervixes) of each uterus. The two abdominal pores (also known as celomic pores) are located near the cloaca closed by a sphincter. These pores connect the pleuroperitoneal cavity with the exterior and may allow the removal of fluid from the inner cavity (De Iuliis and Pulerà, 2019).

Internally, females have one or two functional uteri (Walker, 2020) (depending on the species) either where the embryos develop or where the eggs are kept until they are laid. The oviducts are connected to the cephalic portion of the uteri. The oviducal gland (also known as the shell gland or nidamental gland) can be found on these narrow tracts. The functions of this gland are the storage of sperm, oocyte fertilization, and the formation of the egg case (Hamlett et al., 1998). Thus, in oviparous species, the shell gland is bigger than in ovoviviparous or viviparous species. In some species, the gland also supposes the terminological division of the oviduct in posterior or

anterior oviduct. A sphincter-like structure, the isthmus, can seal the oviducal gland from the posterior oviduct and the uterus in some species. The anterior oviducts converge in the ostium, a funnel-like structure through which the unfertilized oocytes coming from the ovaries pass into the uterine system. Depending on the species, the ovaries can be paired; fused in a single ovary; or vestigial, with only one ovary developed.

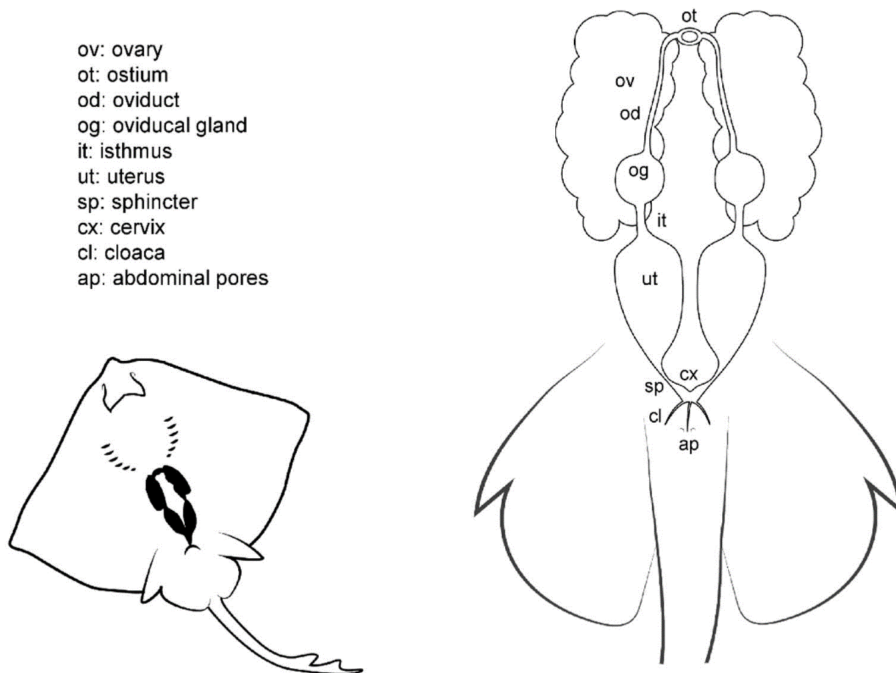

Figure 3. Female general anatomy. Morphological scheme of an ideal batoid female, showing the main reproductive structures: ovaries, oviducts, ostium, oviducal glands, isthmus, uteri, uterine sphincter, cervix, cloaca, and abdominal pores.

3.2. Female Comparative Anatomy

Overall, the general morphological structure of the female reproductive system is well maintained across all the species studied (Figure 4). This anatomical resemblance was particularly remarkable between skates (species from the genus *Raja*) (Figure 4A) and the marbled electric ray *T. marmorata* (Figure 4E), probably because of the close evolutionary relationship between these two plesiomorphic groups (Villalobos-Segura and

Underwood, 2020). Both groups have the same disposition of the basic anatomical structures, but with a clear divergence in the size of the oviducal gland. Members of the Rajiformes order (where genera *Raja* and *Dipturus* are assigned) are oviparous (Musick and Ellis, 2005), and the oviducal gland is therefore well developed in order to produce a hard egg case (Hamlett et al., 1998), while members of the order Torpediniformes are ovoviviparous (also called yolk-sac viviparous) (Musick and Ellis, 2005), and therefore, their gland is not so well developed. The gland is also not well developed in the common stingray *D. pastinaca* or the other two species of Myliobatiformes (common stingray *M. aquila*, and bull ray *A. bovinus*). Not only are they evolutionarily close, but they share the same reproductive method (lipid histotrophy) and do not produce hard egg cases [although the fertilized eggs are grouped together and encapsulated in one thin egg case at the beginning of the development (Capapé et al., 2007; Saadaoui et al., 2015)].

Other differences can be seen in the shape of the cloaca and the position of the abdominal pores, particularly in the common stingray *D. pastinaca* (Figure 4C) and common eagle ray *M. aquila* (Figure 4B), where the abdominal pores are placed on the cloacal lips instead of on the caudal part of the cloaca. As mentioned before, the pores may help with the regulation of liquid in the celomic cavity and should not be confused with uterine sphincters or other structures related to the reproductive system. Also, in this species, the urinary papilla is larger than that of the other species observed and has two independent urinary pores instead of one. Access to the uteri is closed by a sphincter, which is easy to reach with the help of tweezers when the cloacal lips are removed or separated. In the longnosed skate *D. oxyrinchus* (Figure 4F), the paired cervices (or cervixes) are fused in a wide common cervix, with the urinary papilla in one extreme and the cloaca in the other. The dimensions of this cervix can be explained by the large size of the eggs of this species [up to 14 cm in length and 10 cm in width (Ebert and Dando, 2020)]. In the other viviparous species examined (genus *Raja*, Figure 4A), a smaller cervix compared with that of *D. oxyrinchus* could be found. Animals from this genus possess considerably smaller eggs than those of the genus *Dipturus*, despite belonging to the same family Rajidae (Ebert and Dando, 2020).

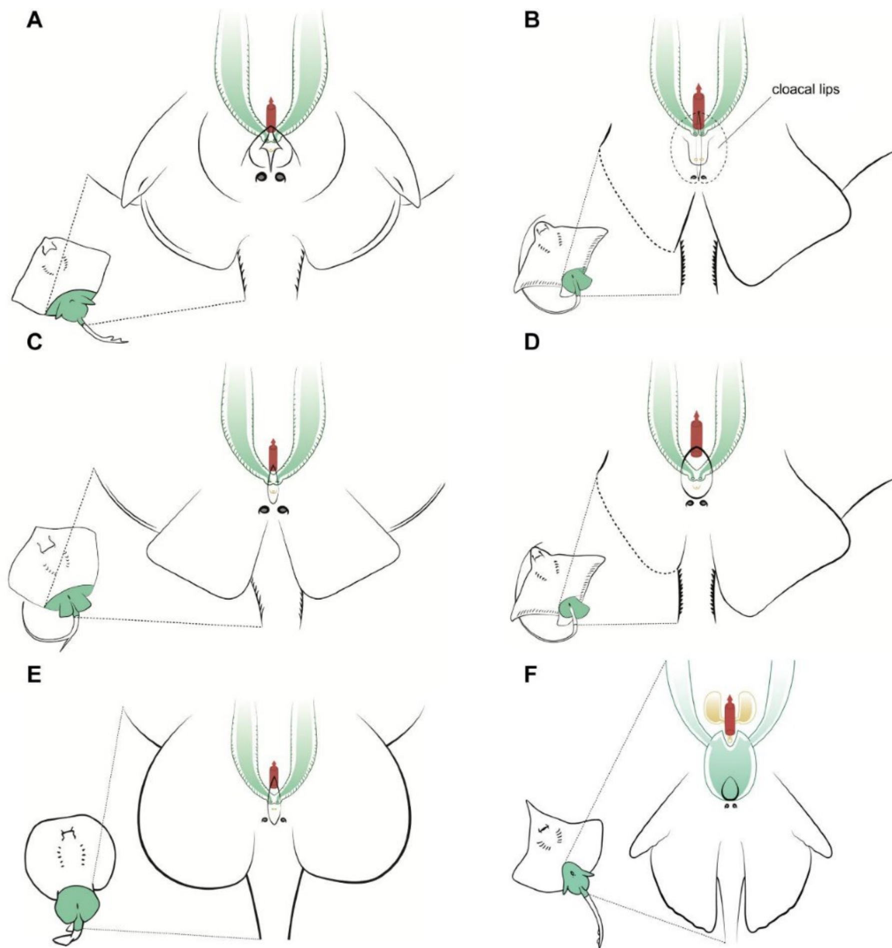

Figure 4. Specific female anatomy. Species-specific morphologies in female batoids that could be relevant when performing artificial insemination. Reproductive, excretory, and digestive systems are marked with different colors. Green: the uteri; yellow: the excretory system; red: access to the digestive system. The grey circles are the abdominal pores that communicate the pleuroperitoneal cavity with the exterior. (**A**) model for skates, genus *Raja*; (**B**) common eagle ray *Myliobatis aquila*; (**C**) model observed in common stingray *Dasyatis pastinaca*; (**D**) bull ray *Aetomylaeus bovinus*; (**E**) marbled electric ray *Torpedo marmorata*; (**F**) longnosed skate *Dipturus oxyrinchus*.

It should be noted that despite the females presenting two uteri, both are not always functional. This consideration is of special interest if an artificial insemination is intended, as mentioned below. Some species, such the common stingray *D. pastinaca*, the common eagle ray *M. aquila*, and the bull ray *A. bovinus*, showed developmental differences between the right and left

ovaries. This phenomenon has also been described in other members of the same families (Snelson et al., 1988; Henningsen et al., 2004a; Araújo et al., 2016; Swider et al., 2017) and could result in a non-functional right uterus. Nevertheless, in this study, both uteri of both *M. aquila* females were shown to be functional, despite the ovarian size difference, as previously described for the species (Capapé et al., 2007).

3.3. Anatomic Notes for Artificial Insemination

To date, there are only two published cases of artificial insemination in batoids: in the clearnose skate *R. eglanteria* (Luer et al., 2008) and recently in the ocellate river stingray *Potamotrygon motoro* (Daochai et al., 2020). This technique has also been considered for the spotted eagle ray *Aetobatus narinari* (Swider et al., 2017). In short, insemination is achieved by inserting a catheter either through the cloaca to deliver sperm into the cervix or through the uterus and into the oviducal gland, although it has been proposed that intrauterine (or oviducal) insemination results in higher fertilization rates than cervix insemination (Luer et al., 2008; Daly and Jones, 2017). To be able to deposit a sperm sample in the correct oviduct, the catheter needs to be inserted through different structures (such as cloaca, uterine sphincters or cervix), which can hamper the overall process (Daochai et al., 2020). Moreover, other factors such as the functionality of the uterus mentioned above should be considered. Thus, to perform artificial insemination protocols, the female reproductive system must be known in the same way as the morphology of the male reproductive system when extracting sperm.

3.4. Male General Anatomy

Externally, male batoids have paired elongated claspers (also called myxopterygia) at the base of the pelvic fins, used as intromittent organs for internal fertilization (Figure 5). Claspers are in fact a rolled-up prolongation of the pelvic fins, forming a tube-like structure with a ventral groove known as the hypopyle. Through this groove, sperm is transported from the male urogenital papilla to the female cloaca and then into the uterine sphincter, although alternate sperm ducts may ejaculate semen, coating the claspers which are then inserted (Walker, 2020). The clasper gland is located in the

proximal part of the clasper. The function of these ovoid glands is uncertain, although it seems to be related to the process of sperm propulsion through the hypopyle and the secretion of substances to protect or activate the sperm (Lacy, 2005; Piercy et al., 2006). As in females, two abdominal pores are located near the cloaca closed by a sphincter, connecting the pleuroperitoneal cavity to the exterior (De Iuliis and Pulerà, 2019).

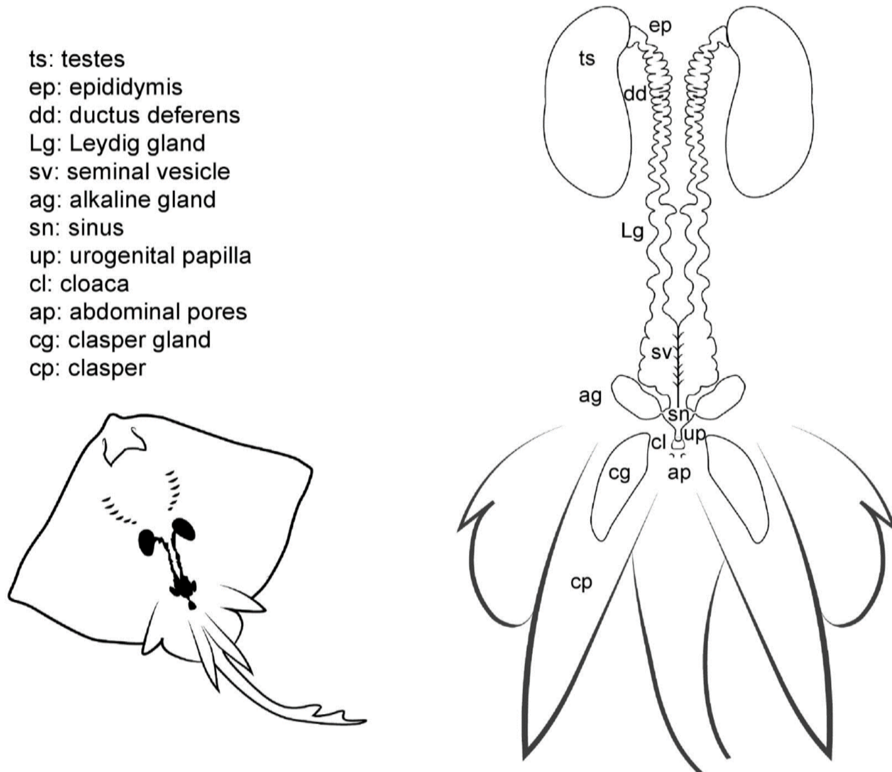

Figure 5. Male general anatomy. Morphological scheme of an ideal batoid male, showing the main reproductive structures: testes, epididymis, ductus deferens, Leydig seminal vesicles, alkaline glands, sinus, urogenital papilla, cloaca, abdominal pores, and clasper glands.

The cloaca is located between the pelvic fins, usually near the origin of the claspers. In some species, the cloaca is enveloped by two cloacal lips at either side, covering the anus and the urogenital papilla. Depending on the species, between one and four pores can be located on the urogenital papilla, connecting with the urinary and reproductive systems. In members of the genus *Raja*, a special sphincter resembling two small lips closes the ducts leading to the urogenital system.

Internally, the male reproductive system is composed of paired testes embedded in the epigonal organ located near the cephalic region of the animal, and as well as two genital tracts that can also be divided into different sections: the proximal convoluted part, which is called the epididymis; the ductus deferens, also called Wolffian duct or vas deferens (Walker, 2020); and the distal widened portion of the ductus deferens, called seminal vesicle or ampulla (Wourms, 1977), where the sperm is stored. The Leydig gland is adjacent to the ductus deferens onto which it empties its contents, as well as onto the epididymis. The gland, formed by the cranial part of the mesonephros, has a secretory function by producing seminal fluid and the matrix where the spermatozeugmata or the spermatophores are formed (Wourms, 1977; Jamieson and Hamlett, 2005; Musick and Ellis, 2005). Another gland related to reproduction, and unique to batoids, is the alkaline gland. The gland is located in the caudal part of the body cavity and empties its content into the urogenital sinus or directly into the last portion of the seminal vesicle or ductus deferens (Lacy, 2005). Although the main function of the gland is still being discussed, it is known that its secretions have a positive effect on the activation of the sperm, thus increasing spermatozoa motility (Lacy, 2005; Luer et al., 2008).

3.5. Male Comparative Anatomy

Some important differences were observed in the urogenital papilla morphology between the species studied that can affect the cannulation and sperm extraction procedures. Firstly, the members of the genus *Raja* studied have a sphincter similar to two small muscular lips that can block the entrance of an extraction catheter (Figure 6A). Once the sphincter is passed, the seminal vesicles converge in a sinus of small size where a scarce amount of sperm can be found, and so to perform the sperm extraction, the catheter must be diverted slightly to one side or the other to access the seminal vesicles. In this genus, seminal vesicles are not pouch-shaped, but a sinuous widening of the end of the vas deferens. The structure is fragile, and the catheter should not be inserted more than 2 cm, or else there is a risk of damage. The same sinuous morphology can be observed in *D. oxyrinchus*, but in this species, there is no sphincter, and a single pore at the tip of the urogenital papilla can be found.

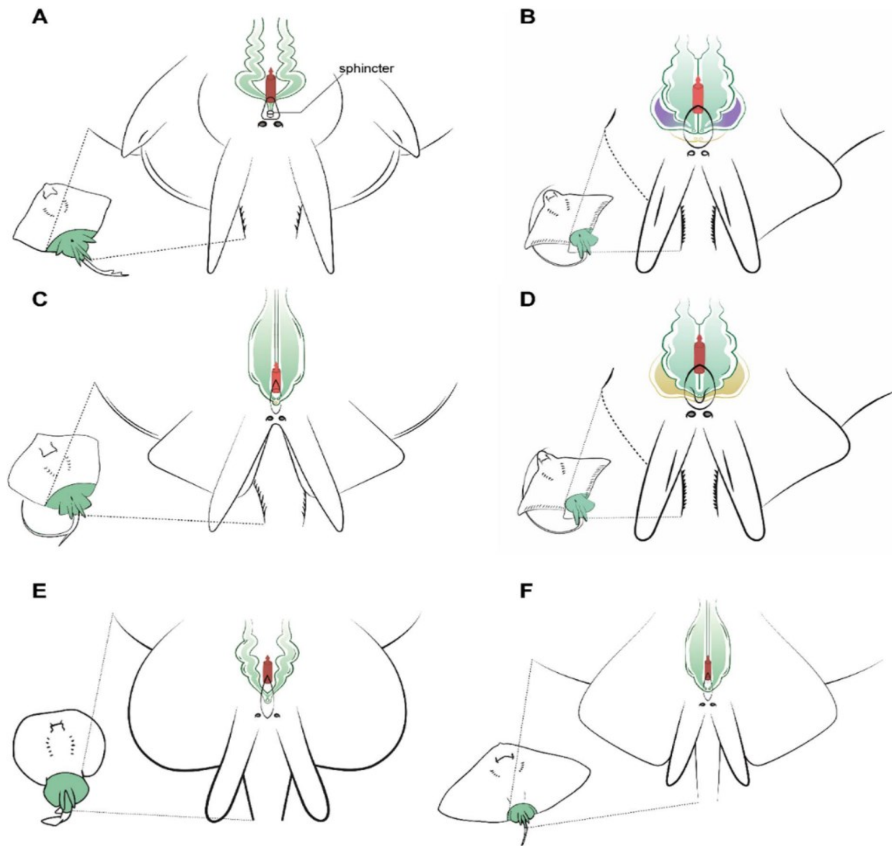

Figure 6. Specific male anatomy. Species-specific morphologies in batoids with relevance to performing sperm extraction, such as the pores leading to the seminal vesicles. Reproductive, excretory, and digestive systems are marked with different colors. Green: the seminal vesicle and ductus deferens; purple: the alkaline gland; yellow: the excretory system; red: the access to the digestive system. The grey circles are the abdominal pores that facilitate communication between the pleuroperitoneal cavity and the exterior. (**A**) model observed in genus *Raja*, with a unique sphincter on its urogenital papilla; (**B**) model observed in common stingray *Dasyatis pastinaca*, with two pores leading to the seminal vesicle and one pore to the urinary system; (**C**) model observed in common eagle ray *Myliobatis aquila* showing four different pores, with the two upper pores facilitating access to the seminal vesicle; (**D**) in bull ray *Aetomylaeus bovinus*, there are only two pores: one in the cranial part of the urogenital papilla leading to the seminal vesicle and one leading to the urinary system; (**E**) model observed in the marbled electric ray *Torpedo marmorata*, where there are two pores to access the seminal vesicles which are a widening of the ductus deferens; (**F**) the spiny butterfly ray *Gymnura altavela* reproductive system is similar to that of *D. pastinaca*, with two different pores to access the reproductive system.

Unlike in the previous species, the common stingray *D. pastinaca* has three pores on the tip of its urinary papilla (Figure 6B). Two sit alongside one another and are the access to the seminal vesicles, while the third pore in the centre of the papilla leads to the urinary system. When using a catheter to perform sperm extraction, the tube should be introduced through the genital pores to empty each seminal vesicle's contents, as the seminal vesicles are independent. Seminal vesicles in *D. pastinaca* are less sinuous than in *Raja*, thus the catheter can be inserted easily. Similar morphology can be observed in the reproductive system of *G. altavela*, but it must be noted that the only specimen examined was an underdeveloped male, and some changes are possible during ontogeny.

Although being closed from an evolutionary perspective (Villalobos-Segura and Underwood, 2020), some variations exist in the morphology of the urogenital system of the common eagle ray *M. aquila* (Figure 6C) and the bull ray *A. bovinus* (Figure 6D). *Myliobatis aquila* possess four different pores in the urogenital papilla, with the upper two (slightly bigger) leading to the seminal vesicles. As in *D. pastinaca*, both seminal vesicles are independent, and the catheter for obtaining sperm must be inserted in a straight line, otherwise it may swerve and end up in the alkaline gland. On the other hand, *A. bovinus* has only two pores arranged longitudinally on the tip of the urogenital papilla. The pore located above is the access to the reproductive system (seminal vesicle and alkaline gland), while the other pore leads to the excretory system. Both seminal vesicles converge in a small sinus in the urogenital papilla, but for sperm extraction purposes, they can be considered to be two independent seminal vesicles. Cannulation through the genital pore should be done using a slight angle to the left or to the right in order to reach both vesicles. Although all the males from all the species possess alkaline glands (albeit in some cases small in size, as in the case of *T. marmorata*), only those of the bull ray *A. bovinus* have been depicted, because in the other species, the presence of this gland does not suppose any obstruction during sperm collection. However, in this species, the size and location of the efferent duct from the gland resulted in some failed cannulation attempts before finally reaching the seminal vesicle.

Torpedo marmorata seminal vesicles converge in a sinus in the urogenital papilla with a single pore (Figure 6E). The overall position is similar to that of *Raja* or *D. oxyrinchus*, but the vas deferens and seminal vesicle are less sinuous. The urogenital papilla does not have a sphincter as in the case of *Raja*, but the cannulation to obtain sperm can be complicated because of the small size of the male electric rays compared to the females.

3.6. Anatomic Notes for Sperm Extraction

Abdominal massage in flat batoids (Rajiformes, Myliobatiformes, Torpediniformes) for sperm extraction can sometimes be difficult because of the shape and position of the seminal vesicles. In sharks, abdominal massage can be performed by applying pressure to the pelvic region and the sides of the body; however, the seminal vesicle of flat batoids cannot be reached laterally due the presence of pectoral fins, which hampers extraction. Thus, obtaining sperm was only possible in mature males with a large quantity of sperm in their seminal vesicles by pressing on the area around the urogenital papilla. It should be noted that when pressure is applied to the abdominal region, some of the contents of the digestive system may come out, spoiling the sample. Furthermore, the flow of sperm from the urogenital papilla to the cloaca can easily expose the samples to microbial contamination, compromising its preservation if a medium-term preservation (days or weeks) of the samples is desired.

On the other hand, cannulation is a better technique for obtaining sperm samples when the amount of sperm in the seminal vesicle is scarce, or a clean sample is required. The use of a lubricating jelly with antiseptic can improve the insertion of the cannulation tubes, reducing damage to the surrounding tissue and reducing microbial contamination. Most batoids have morphologically complex seminal vesicles and care must be taken when inserting the cannulation tubes so as not to damage the structure. Perforations in the seminal vesicle can be produced easily, resulting in the catheter accessing the retroperitoneal space and the kidney. If that is the case, blood will be easily seen inside the catheter. While performing a cannulation, the suction of the syringe must be gentle to avoid clogging the distal apertures of the cannula. Also, to prevent clogging, the cannula should be rotated constantly. The angle of insertion of the catheter should also be

considered to avoid the catheterization of the alkaline gland instead of the seminal vesicle. For this reason, an angle of 0–5° in relation to the longitudinal axis is advised.

Sperm storage in the oviducal gland has been reported for several batoids, sharks and even chimaeras (for a brief review, see Marongiu et al., 2015), and is considered to be an evolutionary mechanism conserved to ensure fertilization in nomadic species or those with low population densities (Pratt and Tanaka, 1994). The storage of the sperm occurs in the gland regions called baffle and terminal zones (Hamlett et al., 1998; Marongiu et al., 2015), and Marongiu et al. state that it is possible to find sperm in other zones, perhaps as a result of recent mating events. After gentle scraping of the luminal epithelium of the oviducal glands from *D. pastinaca*, *D. oxyrinchus*, *R. radula*, *R. asterias* and *T. marmorata*, a pearly mucus was obtained and diluted in an extender solution. Aliquots from this dilution were observed under a microscope, revealing the presence of ciliated epithelial cells, cellular fragments (including spermatozoa remains) and, in four species (*D. oxyrinchus*, *R. radula*, *R. asterias* and *T. marmorata*), motile sperm were found. Although the presence of motile cells could be an indicator of recent mating and not long-term stored sperm, it should be noted that in at least one case, (*D. oxyrinchus*) developed eggs were found in the uteri, so mating could not have been very recent. Although the small concentrations of motile spermatozoa in these samples perhaps limits the use of this technique as a way to source sperm to perform artificial insemination, other studies focusing on genetics, spermatozoa morphology or motility patterns can still be developed.

Public aquaria play an important role in batoid conservation, through *ex situ* conservation programs (IUCN/SSC, 2014) based on public outreach initiatives and the establishment of an emotional connection between the visitor and the animal (Grassmann et al., 2017), by supporting research (including reproductive methods) of different species, and by training professional staff for animal handling and sampling (Penfold and Wyffels, 2019). But more steps towards real sustainability should be made, especially when threatened species are involved (Janse et al., 2017; Buckley et al., 2018). Public aquaria should start specific breeding programs and cease relying on

the spontaneous reproduction of the animals in their collections or on captures from the wild. Reproductive breeding programs using reproductive technologies, such as sperm extraction and artificial insemination, could not only aid these steps towards aquaria sustainability, but could also be the key to developing reintroduction programs back to the wild for threatened species of elasmobranchs. It should be noted that among the batoids, we can find some of the most threatened marine animals on the planet: the sawfishes, guitarfishes and wedgfishes (Dulvy et al., 2016b; Moore, 2017; Kyne et al., 2020). In fact, “responsible husbandry” is one of the objectives of the global strategy for the conservation of sawfishes (Harrison and Dulvy, 2014). Although more research on batoid reproduction must be carried out, the goal of this study was to offer a useful guide with protocols that can serve as tools for the conservation of these and other species.

4. Conclusions

Many threatened rays and related species could benefit from breeding programs using specific reproductive techniques. These programs could provide specimens with which to carry out conservation, either through education, research or even reintroduction into the wild. The techniques developed in this study are intended to be useful for carrying out these programs. Although abdominal massage is the simplest technique for obtaining sperm, it is not the most effective in the case of batoids, due to the peculiar morphology of their body and seminal vesicles, which are often reduced in size. Although cannulation is a more complex technique, it allows the sampler to obtain sperm in a more precise and clean way if the anatomy of the animal is known. Finally, obtaining active sperm from the oviducal gland in females opens new research opportunities that should be exploited in the future. Much work remains to be done in the development and application of reproductive techniques in batoids, but these first steps may be crucial for the future conservation of these animals.

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Institutional Review Board Statement

Manipulation of animals kept in aquarium condition was authorized by the Ethic Committee of Fundación Oceanogràfic (Project reference: OCE-16-19) and the sperm extraction process was conducted under the supervision of their veterinary team.

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CHAPTER 2

Reproductive Anatomy of Chondrichthyans: Notes on Specimen Handling and Sperm Extraction. II. Sharks and Chimaeras

P. García-Salinas[1,2], V. Gallego[1], J.F. Asturiano[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia, Spain.

[2]Associació LAMNA per a l'estudi dels Elasmobranquis a la Comunitat Valenciana, Fraules 10, 13, 46020 València, Spain

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Abstract

The chondrichthyan fishes, which comprise sharks, rays, and chimaeras, are one of the most threatened groups of vertebrates on the planet. Given this situation, an additional strategy for the protection of these species could be the *ex situ* conservation projects developed in public aquaria and research centers. Nevertheless, to increase sustainability and to develop properly *in situ* reintroduction strategies, captive breeding techniques, such as sperm extraction and artificial insemination, should be developed. These techniques are commonly used in other threatened species and could be also used in chondrichthyans. However, the different reproductive morphologies found in this group can complicate both processes. Therefore, a comparison of the reproductive anatomy of eight distinct chondrichthyans, with an emphasis on those important differences when performing sperm extraction or artificial insemination, is carried out herein. Sharks and chimaeras belonging to the Scyliorhinidae, Carcharhinidae, Centrophoridae, Etmopteridae, Hexanchidae, and Chimaeridae families were obtained from commercial fisheries, public aquaria, and stranding events. In addition, the process of obtaining viable sperm samples through cannulation, abdominal massage, and oviducal gland extraction is described in detail for both living and dead animals.

1. Introduction

The Chondrichthyes are a group of vertebrates that appeared more than 400 million years ago. Nowadays, this group is an ecologically diverse group with great importance in the regulation of the ecosystems where these animals inhabit (Compagno, 1990; Stevens, 2000). The class Chondrichthyes comprises 1472 species classically divided into the holocephalans (subclass Holocephali), which are commonly named chimaeras, and the elasmobranchs (subclass Elasmobranchii), commonly named sharks and rays (Fricke et al., 2021).The chimaeras are the smallest of these three divisions in terms of the number of extant species, and currently there are 57 species described (Fricke et al., 2021). All chimaeras are marine animals, and very few species inhabit shallow waters, most species relegated to the deep waters (>200 m) despite their global distribution in the past (Didier, 2004). Chimaeras have significantly different features compared to the elasmobranchs, such as the fusion of the lower jaw to the cranium (hence their name Holocephalans, “complete heads”) and non-replaceable tooth plates as teeth (Didier, 2004). In general, holocephalans are a less studied group than their relatives, the rays and sharks. This last group, the sharks, is perhaps the most recognizable group among Chondrichthyans despite not being as numerous in terms of the number of species as the group formed by the rays and skates (Fowler and Cavanagh, 2005; Weigmann, 2016).

Regarding their conservation status, Chondrichthyes possess life histories that make them sensitive to elevated anthropic pressure, threatening their populations (García et al., 2008; Dulvy and Forrest, 2010). In fact, chondrichthyan extinction risk is higher than for most other vertebrates, and only one-third of the species assessed are considered safe according to IUCN red list criteria (Dulvy et al., 2014). The situation is particularly sensitive in places such as the Mediterranean Sea, a key hotspot of extinction risk, where half the species of rays and sharks face an elevated risk of extinction (Dulvy et al., 2016a). Among the drivers for the global decline of its populations, overfishing (intentional or incidental) and habitat destruction are the main causes (Dulvy et al., 2014, 2016a; Pacoureau et al., 2021).

As mentioned above, to understand the current global situation of chondrichthyan populations, their reproductive strategies and life histories

should be noted. Sharks and their relatives show larger body sizes, slower sexual maturity, longer gestation periods, higher maternal investment, and fewer offspring than other fishes (Cortés, 2000; Dulvy et al., 2021b). Chondrichthyan species reproduction modes are diverse and can be divided according to the nutrition of the embryos. Lecithotrophic modes include oviparity (such as the catsharks of the family Scyliorhinidae, or the entire subclass Holocephali) and yolk sac viviparity (such as Hexanchiformes), where the only nourishment comes from their yolk sack. Matrotrophic modes include an additional nourishment source at some point of the embryo development, in the form of lipid histotrophy, unfertilized eggs (or fertilized eggs in the extreme case of the sand tiger shark *Carcharias taurus*), or the formation of a placenta (such as some Carcharhiniformes) (Musick and Ellis, 2005; Conrath and Musick, 2012; Walker, 2020).

In aquaculture industries, the reproductive factors and complex life histories mentioned above have discouraged captive breeding programs (Walker, 2020), but not in aquaria facilities, either public or private. The reproduction in captivity of elasmobranchs and chimaeras has been reported for some species (Henningsen et al., 2004b; Barnett et al., 2009; Janse et al., 2017), but these events have traditionally relied more on natural mating rather than on the use of reproductive techniques (Daly and Jones, 2017). A potentially useful technique in breeding programs is the artificial insemination of females, but to ensure its success, a reliable supply of sperm is required, especially in the case of endangered species (Masuda et al., 2003, 2005; Luer et al., 2008; Daly and Jones, 2017; Penfold and Wyffels, 2019; Daochai et al., 2020; Wyffels et al., 2021). Although the obtention of sperm has been previously achieved for several shark and chimaera species (Pratt and Tanaka, 1994; Minamikawa and Morisawa, 1996; Masuda et al., 2003, 2005; Daly and Jones, 2017; Wyffels et al., 2020a; García-Salinas et al., 2021a), the procedures of extraction may vary between the different groups. In live animals, the most common procedures for sperm obtention have been cannulation and abdominal massage (Daly and Jones, 2017; Penfold and Wyffels, 2019; García-Salinas et al., 2021a), but these techniques should consider the morphology and location of the reproductive structures, such as the seminal vesicles and urogenital papillae, to be truly effective.

Due to their position as one of the oldest groups of vertebrates (Wourms, 1977), Chondrichthyes have been previously used as animal models for physiological and morphological studies (Luer, 1989; Coolen et al., 2008; Lauriano et al., 2017; Luer and Walsh, 2018). Moreover, certain aspects of their reproductive morphology, such as the form and function of their intromittent organs (Jungersen, 1899; Leigh-Sharpe, 1922; Gilbert and Heath, 1972; Jones et al., 2005) or gonads (Dean, 1906; Chen et al., 1973; Dodd, 1983; Pratt, 1988; Davenport et al., 2011; Lutton, 2011; del Mar Pedreros-Sierra et al., 2016), have received attention from researchers and are well studied. However, some details about the morphology of certain reproductive structures, which are important during the use of reproductive techniques, have not been previously considered for sharks and chimaeras. Thus, the aim of this study is to offer an anatomical guide intended to be useful during sperm obtention procedures, as well as propose preliminary indications to be considered during artificial insemination. This practical guide complements previous work focused on the anatomy of batoids (rays, skates, and close species) and the use of reproductive techniques on them (García-Salinas et al., 2021b). The tools presented in both studies are intended to delve into the development of reproductive techniques, such as sperm cryopreservation (García-Salinas et al., 2021a) or artificial insemination (Wyffels et al., 2021), some of the important steps toward successful and sustainable breeding programs.

2. Materials and Methods

2.1. Origins of the Specimens

Males and females of eight Chondrichthyan species belonging to the orders Carcharhiniformes (*n* = 35), Hexanchiformes (*n* = 1), Squaliformes (*n* = 5), and Chimaeriformes (*n* = 4) were studied.

Other animals were held alive in captivity as part of the zoological collection of a public aquarium (Oceanogràfic, València). Both nursehound *Scyliorhinus stellaris* and small-spotted catsharks *Scyliorhinus canicula* were kept separately in two 8000 L tanks with recirculating seawater (temperature: 16–18 °C; salinity: 35–37‰), and fed twice a day with herring, squid, and shrimps. The handling of the specimens was authorized by the Ethic

Committee of Fundación Oceanogràfic (project reference: OCE-16-7777719), and the sperm extraction process was carried out under the supervision of their veterinary team. The maturity of all the specimens was determined by their gonad development, grade of their clasper calcification and development of secondary copulatory organs (in *C. monstrosa*), and their size, according to the literature (Ebert and Dando, 2020).

Table 1. Some of the species, such as the small-spotted catshark *Scyliorhinus canicula* and blackmouth catshark *Galeus melastomus*, were available in fish markets and from commercial fishing vessels' by-catch. The rabbitfish *Chimaera monstrosa*, little gulper shark *Centrophorus uyato*, and velvet belly lanternshark *Etmopterus spinax* specimens were obtained from the bottom trawling by-catch. The blue sharks *Prionace glauca* and the bluntnose sixgill shark *Hexanchus griseus* specimens appeared stranded on beaches and were recovered by the Comunitat Valenciana Stranding Network (Valencian Community, Valencia, Spain).

Common name	Scientific name	NM	NF	IUCN	Source	Range (cm)
Small-spotted catshark	*Scyliorhinus canicula*	7	5	LC	AQ/FM/BC	38-57
Nursehound	*Scyliorhinus stellaris*	7	3	NT	AQ	75-144
Blackmouth catshark	*Galeus melastomus*	4	6	LC	BC/FM	48-69
Blue shark	*Prionace glauca*	2	1	CR	ST	290-297
Velvet belly lanternshark	*Etmopterus spinax*		4	LC	BC	33-40
Little gulper shark	*Centrophorus uyato*	1		NE	BC	86
Bluntnose sixgill shark	*Hexanchus griseus*	1	1	LC	ST	250
Rabbitfish	*Chimaera monstrosa*	2	2	NT	BC	104-112

2.2. Dissection Procedure

On dead specimens, a dissection was performed to specifically gain access to the reproductive system (Figure 1). The procedure focused only on the reproductive structures, following and adapting the dissection procedures and terminology used by other authors (King and Custance, 1982; Crow and Brock, 2004; De Iuliis and Pulerà, 2019). In the case of medium-size and small sharks, the animals were flipped dorsally, exposing their ventral surface. A small incision was made over the coracoid bar of the pectoral girdle and a

longitudinal cut was made along the ventral midline toward the pelvic girdle, over the cloaca (Figure 1A). If needed, a cut made transversely to the midline, over the pectoral girdle, and another cut over the pelvic girdle, allowed us to fully expose the pleuroperitoneal cavity (Figure 1B).

In large sharks and chimaeras, where it was not possible to fully expose the ventral part of the animal due to its size or the morphology of its dorsal fins, the process was slightly different. First, a small incision with scissors was made at one side of the ventral midline, near the pectoral fin base, over the coracoid bar. Through this incision and with the help of a scalpel blade, a curved cut was made toward the pelvic girdle. The cut followed a slight curve from the ventral midline to the lateral and then back again to the ventral midline, to create a kind of flap. Note that to avoid damage to the inner organs, forceps were used to keep the abdominal wall (skin, muscle, parietal peritoneum) elevated. The pleuroperitoneal cavity was exposed by pulling out the flap to one side.

To access the reproductive system, the hepatic lobes were drawn forward and removed by cutting through their cranial attachments (hepatic ducts and falciform ligament) (Figure 1C), trying to avoid damage to this organ and to avoid leakage of the oil and bile present in the liver (Figure 1D). A cut close to the rectal gland was made on the rectum to separate the intestine (spiral valve), and close to the pericardiac cavity, through the oesophagus, to separate the stomach (Figure 1E). Then, the mesogaster and caudal mesenteries were cut to completely remove the internal parts of the digestive tract. Special care was taken to not damage the mesorchium holding the ovaries and the testes.

In sharks, a cut through the cloaca (Figure 1F) and puboischiadic bar was made to expose the caudal part of the urogenital system (seminal vesicle, urogenital sinus, urogenital papilla in males, or uterine sphincters and urinary papilla in females). In rabbitfish, both urogenital papilla and uterine sphincters are external, so there was no need for this procedure. Moreover, in some species the removal of cloacal lips to access the urogenital/urinary papilla was necessary.

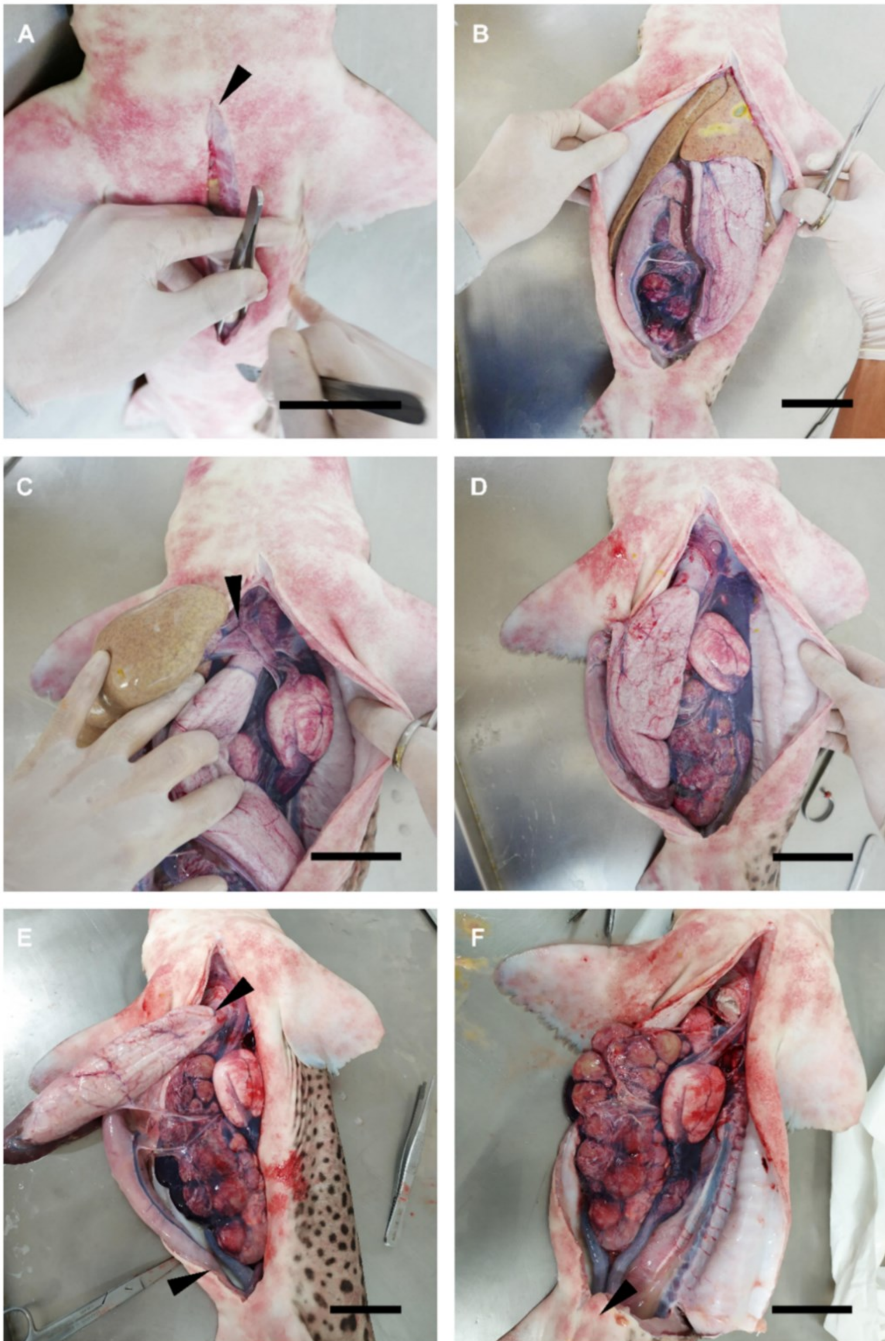


Figure 1. Dissection procedure. Relevant steps in the dissection procedure of a female nursehound *Scyliorhinus stellaris* to reach the reproductive system. (**A**) Longitudinal incision over the pectoral girdle of the coracoid bar following the ventral midline. Notice the use of

forceps to elevate the abdominal wall. (**B**) General vision of the pleuroperitoneal cavity. (**C**) Lateral displacement of the liver, esophagus, and stomach to reach the falciform ligament. (**D**) General vision of the digestive and reproductive systems. (**E**) Lateral displacement of the esophagus, stomach, spiral valve, and rectum and cut on the esophagus and rectum. (**F**) General vision of the reproductive system. Arrowheads mark incision areas: (**A**) coracoid bar, (**C**) falciform ligament, and (**E**) esophagus (superior) and rectum (inferior); (**F**) puboischiadic bar. The scale bar indicates 6 cm.

2.3. Description of Reproductive Structures

Detailed photographs were taken with a macro lens camera throughout the dissection procedure of every species and illustrated notes were taken. The focus of each dissection was on (i) determining how to gain easy access to the urogenital papilla, (ii) observing the number and disposition of urogenital pores, (iii) observing the urogenital sinus morphology, and (iv) accessing the seminal vesicle/uterus. Plastic tubes with different gauges (0.5–2 mm in diameter) were used as probes during the dissection to confirm the access and connections from the external part of the reproductive system (urogenital/urinary pores and papilla) to the internal part (urogenital sinus, seminal vesicle, and uterus). The information obtained was combined and used to propose a general morphological scheme of an ideal male and female shark.

2.4. Sperm Collection

2.4.1. In Vivo Sperm Extraction

Before sperm extraction, tonic immobility was induced in a small-spotted catshark *S. canicula* and a nursehound *S. stellaris* to minimize struggling and reduce stress during handling (Henningsen, 1994; Kessel and Hussey, 2015). The animals were held in an upside-down position (with their ventral region exposed) with their mouth and gill slits submerged, while gentle pressure was exerted on their snouts. Then, with the cloaca emerged, gentle pressure on the abdominal area (over the location of the seminal vesicle on the sides of the animal) was exerted to make sperm flow through the urogenital papilla. Then, sperm was immediately collected using a sterile syringe or pipette and transferred to sterile tubes after collection.

2.4.2. Postmortem Sperm Extraction

The cloacal area in sharks, and the posterior portion of the body in chimaeras, was cleaned of mucus and other biological remains (such as blood, mud, and the remains of other organisms, which may be found in animals obtained from fisheries) using marine water. Three different methods were used to obtain sperm from dead males in every species: (i) abdominal massage on the ventral region immediately anterior to the pelvic girdle, or by pressing around the urogenital papilla in the cloacal cavity with the fingers or with curved pincers (only in sharks); (ii) accessing by dissection and stripping directly on the seminal vesicle. In both cases, the sperm flowing from the urogenital papilla was immediately collected using a sterile syringe, plastic tube, or a pipette; and (iii) through cannulation, by introducing a polyurethane cat catheter (BUSTER cat catheter, 1.0 × 130 mm, Kruuse, Langeskov, Denmark) or a PVC nasogastric tube (Feeding Probe L/RX CH-05 2.67 × 50 mm, JMEDIS, Cádiz, Spain) through the appropriate pore on the urogenital papilla. A sterile lubricating jelly with antiseptic (Optilube ActiveTM, Optimum Medical, Leeds, UK) was used to facilitate the insertion of the tube. Tubes and catheters were continuously rotated inside the seminal vesicle to avoid the clogging of their orifices.

The nidamental glands from small-spotted catshark *S. canicula*, nursehound *S. stellaris*, blue shark *P. glauca*, and rabbitfish *C. monstrosa* females were obtained (Figure 2A). The organs were carefully removed during the dissection by cutting through the anterior and posterior oviduct. Then the glands were externally cleaned with artificial seawater to remove the blood and biological remains (Figure 2B). Care was taken during the cleansing process to avoid the passing of seawater into the lumen of the glands. The organs were split along its cranial–caudal axis to expose its lumen (Figure 2C). A gentle scrape was done over its luminal epithelium using a scalpel blade (Figure 2D). Then, a pearly mucus was collected over the edge of the blade. The mucus was diluted in an artificial seminal plasma extender described by García-Salinas et al. (2021). To summarize, the main components of the extender (in mM; 433 urea, 376 NaCl, 120 trimethylamine N-oxide (TMAO), 8.4 KCl, 50 glucose, 7 $CaCl_2.2H_2O$, 3.5 $NaHCO_3$, 0.08 Na_2SO_4, 1.4 $MgSO_4$) were kept in balance with physiological fluids by adjusting pH to 6.5 and the osmolality to 1000 mOsm/kg.

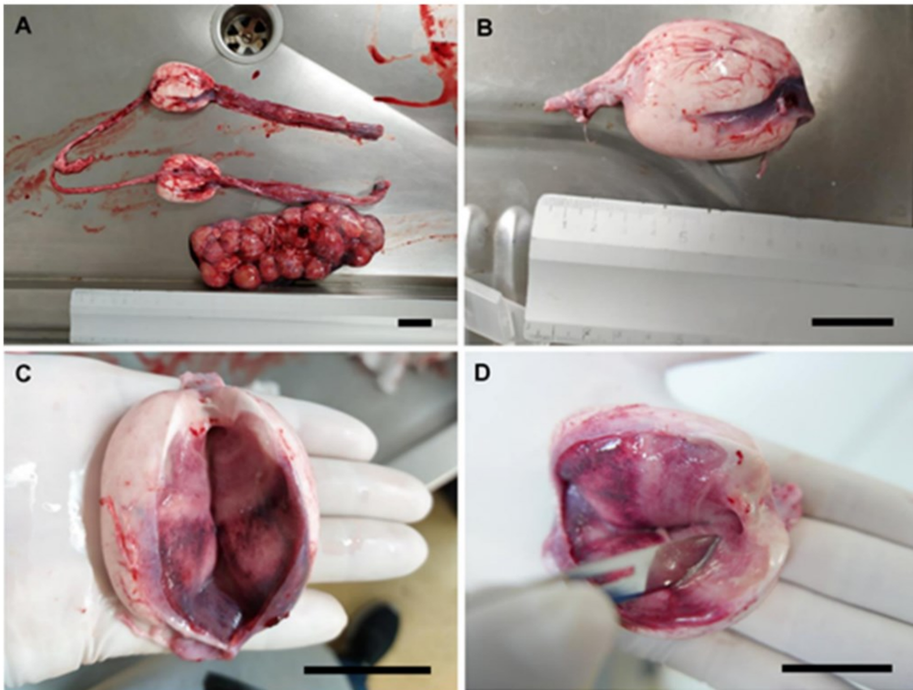


Figure 2. Sperm recovery from nidamental gland. Nidamental glands from a nursehound *Scyliorhinus stellaris* (**A**), united by the oviducts and ostium. The gland was removed and cleaned (**B**) and split through its cranial–caudal axis, exposing its lumen (**C**). Scraping was done over its luminal epithelium using the edge of a scalpel (**D**) to collect a pearly mucus containing sperm. The scale bar indicates 3 cm.

3. Results and Discussion

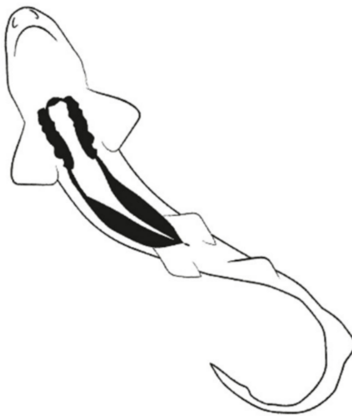
3.1. Female General Anatomy: Sharks

It should be noticed that there is a wide variety of anatomical differences between the species studied, but this study aims to highlight only those that may be important while using specific techniques, such as sperm extraction and artificial insemination. Thus, other anatomical differences have not been considered. However, even though the abdominal pores are not part of the reproductive system, its misidentification may cause errors during cannulation, so its description is offered.

Externally, shark females possess a cloaca located between the pelvic fins, where the urinary, reproductive, and digestive system converge (Figure 3). Next to the caudal margin of the cloaca, the abdominal (or celomic) pores

are found. These small orifices closed by a sphincter connect the pleuroperitoneal cavity with the exterior and may allow the removal of fluid from the inner cavity (De Iuliis and Pulerà, 2019). In females, the urinary system ends in the urinary papilla (the term urogenital papilla should be limited to males). Inside the cloaca, the caudal end of the uteri can be independent or converge and fuse in a common cervix (or urogenital sinus). The cranial orifice inside the cloaca is the caudal section of the digestive system: the rectum and the anus.

ov: ovary
ot: ostium
od: oviduct
og: oviducal gland
it: isthmus
ut: uterus
sp: sphincter
cx: cervix
cl: cloaca



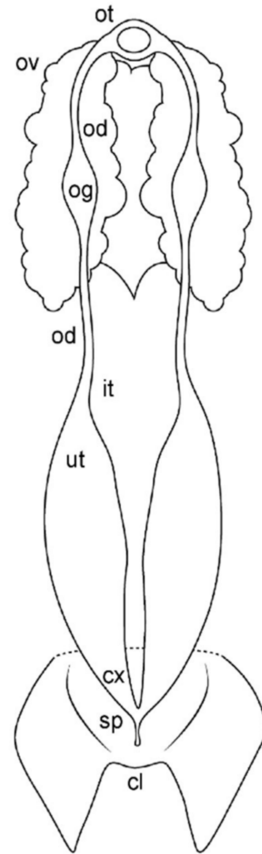


Figure 3. Female general anatomy: sharks. Morphological scheme of an ideal female shark, showing the main reproductive structures: ovary (ov), oviducts (od), ostium (ot), oviducal glands (og), isthmus (it), uterus (ut), sphincter (sp), cervix (cx), and cloaca (cl).

Internally, females possess a pair of reproductive tracts distributed along with the entire pleuroperitoneal space. The ovaries are embedded in the epigonal gland and located on the cranial part of the pleuroperitoneal cavity. Depending on the species, the ovaries can be paired, fused in a single ovary or vestigial, with only one ovary developed (Jones et al., 2005; Conrath and Musick, 2012; Walker, 2020). The oogenesis is produced in the ovaries, and the mature oocytes are released into the celoma and transported to the ostium by the action of cilia. The ostium is a funnel-like structure to allow the passage of the unfertilized oocytes into the anterior part of the oviduct and then, to the oviducal gland (the terms shell gland and nidamental gland are commonly used, although in sharks, eggshells or embryo envelopes are not always produced).

That gland is responsible for the storage of the sperm, the oocyte fertilization, and the formation of the egg case (Hamlett et al., 1998). Thus, in oviparous species, the shell gland is proportionally bigger than in ovoviviparous or viviparous species. The gland also supposes the terminological division of the oviduct in the anterior (pre-oviducal gland) and posterior (post-oviducal gland) oviduct. In some species, a sphincter-like structure, the isthmus, can seal the oviducal gland from the uterus. Internally, females have one or two functional uteri depending on the species (Jones et al., 2005; Conrath and Musick, 2012; Walker, 2020), where the embryos develop, or the eggs are kept until they are laid.

3.2. Female Anatomy: Chimaera monstrosa

Externally, *C. monstrosa* does not possess a common chamber (cloaca) where the digestive, urinary, and reproductive systems converge, but a single opening for the caudal portion of the digestive system, located between the pelvic fins (Figure 4) (Dean, 1906). Two small pores closed by a sphincter sit along with this orifice, the abdominal (or celomic) pores that allowed an exchange of fluids between the exterior and the pleuroperitoneal cavity (De Iuliis and Pulerà, 2019). The entrances to the uteri are two independent orifices closed by a sphincter located at the base of the pectoral fins. In juvenile females, the orifices are narrow and hard to find, while in mature females, they are easier to locate.

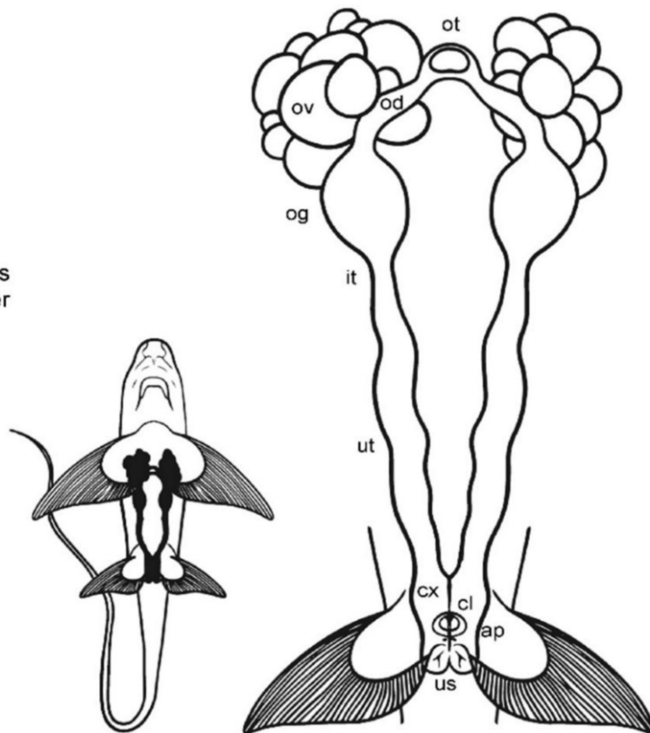


Figure 4. Female general anatomy: *Chimaera monstrosa*. Morphological scheme of *C. monstrosa* main female reproductive structures: ovary (ov), ostium (ot), oviduct (od), oviducal gland (og), isthmus (it), uterus (ut), cervix (cs), cloaca (cl), abdominal pores (ap), and uterine sphincter (us).

Internally, their reproductive system is composed of paired structures arranged longitudinally, in the same way as in the general model for sharks and rays. Following the longitudinal axis, two ovaries, a single ostium, two oviducts (divided into anterior and posterior) with an oviducal gland each, two isthmus, two functional uteri ended by the cervix, and a sphincter in the posterior section can be found. As in the rest of oviparous chondrichthyans (Hamlett and Koob, 1999), the oviducal gland of *C. monstrosa* is well developed, as at present, all chimaera species lay hard egg cases, despite their other past reproductive strategies (Musick, 2010).

3.3. Female Comparative Anatomy

In sharks, there are no great differences between the species studied (Figure 5) regarding the general morphological structure of the female reproductive

system. The overall morphology of the reproductive system in *Scyliorhinus canicula*, *S. stellaris*, and *Galeus melastomus* (Figure 5A) is similar, and closely resembles that of the ideal model proposed for sharks (Figure 3). However, in the two *Scyliorhinus* species, the abdominal pores lay over the metapterygium of the pelvic fins instead of inside the cloaca, while in blackmouth catshark *G. melastomus* the pores are located inside the cloaca.

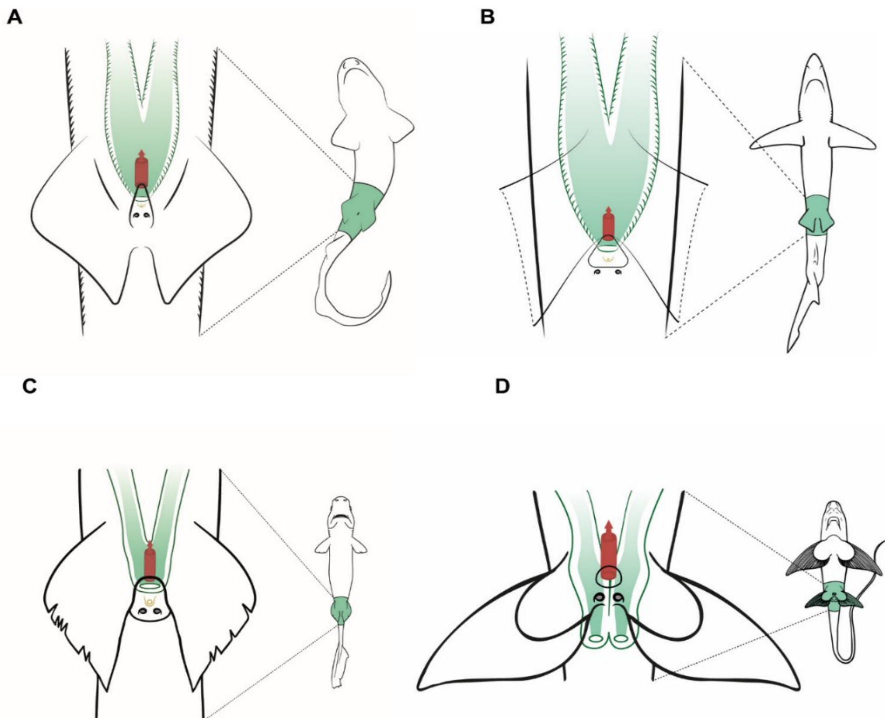


Figure 5. Specific female anatomy. Species-specific morphologies in three female sharks and one chimaera species that can be relevant to perform artificial insemination. The reproductive, excretory, and digestive systems are marked with different colors. The uteri are shown in green, the excretory system in yellow, and the access to the digestive system in red. The grey circles are the abdominal pores that connect the pleuroperitoneal cavity with the exterior. (**A**) Model for catsharks, family Scyliorhinidae. (**B**) Blue shark *Prionace glauca*. (**C**) Model observed in velvet lanternshark *Etmopterus spinax*. (**D**) Rabbitfish *Chimaera monstrosa.*

All three species belong to the family Scyliorhinidae, one of the six shark families where oviparous sharks have been described (Musick and Ellis, 2005). As happens with oviparous sharks, their oviducal glands are well developed, especially in *G. melastomus* where multiple (or retained) oviparity occurs (Costa et al., 2005).

In the blue shark *P. glauca*, three papillae are located in the cloaca (Figure 5B). The central one is the urinary papilla and it connects with the urinary system. The two others are located on both sides of the cloaca and contain the abdominal pores (or abdominal papillae in this case). Unlike the rest of the species observed, only the right ovary is developed and functional in blue sharks. The oocytes pass into the heart-shaped oviducal gland, where they are fertilized and encapsulated in a thin, soft capsule before passing into the uterus. *Prionace glauca* is a species where placental viviparity has been described; thus, the oviducal gland and the oocytes are proportionally smaller than in other species (Pratt, 1979). Lastly, the two entrances to the uterus fuse into a large cervix, where the pups pass momentarily before birth.

In the velvet belly lanternshark *Etmopterus spinax*, two functional ovaries and uteri can be found. The uteri converge in a small common cervix located in the cranial part of the cloaca, next to the urinary papilla. Two small abdominal pores are located at the end of the cloaca, covered by the pelvic fins. Unlike the previous species, *E. spinax* is an ovoviviparous species (also called viviparous lecithotrophic or aplacental viviparous) (Capae et al., 2001) and does not produce any case covering the fertilized ova; thus, their oviducal gland is reduced and scarcely developed with no lateral expansions (Porcu et al., 2013).

The rabbitfish *Chimera monstrosa* shares the same anatomic features as the oviparous sharks examined, such as well-developed paired ovaries and relatively big oviducal glands. However, instead of uteri converging inside the cloaca, the uterine openings of *C. monstrosa* are located behind the pectoral fins. The absence of cloaca is described for most Holocephali (Dean, 1906; Grasse, 1958; Bell, 2012), but some species such as the elephant fish *Callorhynchus milli* (Chierichetti et al., 2017), the cockfish *Callorhynchus callorhynchus* (Chierichetti et al., 2017), and the Eastern Pacific black ghost shark *Hydrolagus melanophasma* (Márquez-Farías and Lara-Mendoza, 2014) possess a common cloaca where the anus and the uterine openings are found. Moreover, a sperm pouch for sperm storage can be found on these species, but it is absent in *C. monstrosa*. Another reproductive trait, the prepelvic clasper pouch (present in female *Callorhynchus* species) (Bell,

2012; Márquez-Farías and Lara-Mendoza, 2014; Chierichetti et al., 2017) is not present in *C. monstrosa* females.

3.4. Anatomic Notes for Artificial Insemination

Currently, artificial insemination is not a widespread technique in sharks, and there is not any report about the use of this technique in chimaeras. To date, artificial insemination for shark reproduction has been used in the brown banded bamboo sharks *Chiloscyllium punctatum*, the zebra shark *Stegostoma tigrinum*, the cloudy catshark *Scyliorhinus torazame*, and the white-spotted bamboo shark *Chiloscyllium plagiosum* (Masuda et al., 2003, 2005; Penfold and Wyffels, 2019; Wyffels et al., 2021), although the technique has also tried in other species such the sand tiger shark *Carcharias taurus* and the broadnose sevengill shark *Notorynchus cepedianus* (Daly and Jones, 2017).

The process involves depositing viable sperm into the female reproductive tract by inserting a catheter through the uterine sphincter into the cervix or into the oviducal gland or the posterior oviduct (intrauterine). However, it has been proposed that intrauterine or oviducal insemination results in higher fertilization rates than cervix insemination (Daly and Jones, 2017; Penfold and Wyffels, 2019). The catheter with the sperm sample should be inserted through different anatomical structures (such as cloacal morphology, cervix, or uterine sphincters) that can hamper the overall insemination procedure (Daochai et al., 2020). Thus, to perform artificial insemination protocols, the female reproductive system must be well known, and in the same way, the morphology of the male reproductive system when extracting sperm.

3.5. Male General Anatomy: Sharks

Externally, male sharks have paired prolongations of the pelvic fins called claspers (or myxopterygium) used as intromittent organs for internal fertilization (Jungersen, 1899; Leigh-Sharpe, 1922; Gilbert and Heath, 1972; Luer et al., 2008; Walker, 2020)(Figure 6). Claspers are located at the inner margin of the pelvic fins, forming a tube-like structure with a ventral groove known as hypopyle. The sperm flows through this groove from the male urogenital papilla to the female cloaca, and then into the uterine sphincter.

To impulse the sperm, most sharks possess paired muscular structures at the base of the claspers, under the ventral skin, called siphon sacs (Mann and Prosser, 1963; Gilbert and Heath, 1972). The siphon sacs secrete serotonin (5-hydroxytryptamine), capable of producing muscle contractions in the uterus of females, which could favour the intrauterine transport of sperm (Mann and Prosser, 1963). Because multiple mating in sharks is well known, it has also been proposed that siphon sacs may play a role in sperm competition by washing rival sperm from the cloaca of females (Whitney et al., 2004; Pratt and Carrier, 2005).

ts: testes
ep: epididymis
dd: ductus deferens
Lg: Leydig gland
sv: seminal vesicle
ss: sperm sac
sn: sinus
up: urogenital papilla
cl: cloaca
cp: clasper

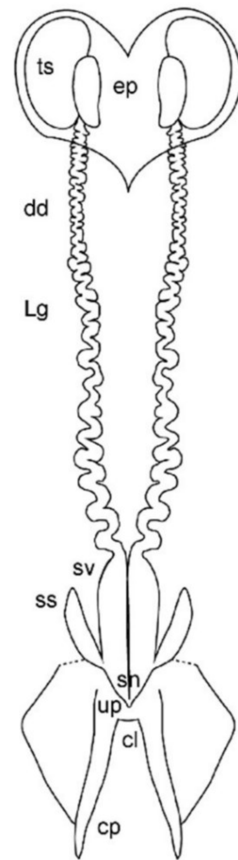


Figure 6. Male general anatomy: sharks. Morphological scheme of an ideal shark male, showing the main reproductive structures: testes (ts), epididymis (ep), ductus deferens (dd), Leydig gland (Lg), seminal vesicle (sv), sperm sac (ss), sinus (sn), urogenital papilla (up), cloaca (cl), and clasper (cp).

The cloaca is located between the pelvic fins. Typically, it is covered by the inner margins of the pelvic fins (the metapterygia), or in some species by two cloacal lips. Inside the cloaca are located the anus (caudal portion of the digestive system) and the urogenital papilla. Depending on the species, on the tip of the urogenital papilla, there are one or two pores through which the sperm and urine flow. Lastly, two abdominal pores connecting the pleuroperitoneal cavity with the exterior are located inside or near the cloaca, closed by a sphincter (De Iuliis and Pulerà, 2019).

Internally, the male reproductive system is located along the pleuroperitoneal cavity. The paired testes, embedded in the epigonal organ, are located at the cranial end of the reproductive tract. The testes are connected via efferent ductules (or *ductuli efferentes*) with two genital tracts composed of the cranial convoluted part called epididymis, the ductus deferens (also called the Wolffian duct or vas deferens) (Walker, 2020), and the caudal part called seminal vesicle (or ampulla) where the sperm is stored. The Leydig gland is adjacent to the ductus deferens and empties its content on it and on the epididymis. The gland is formed by the cranial part of the mesonephros and produces the seminal fluid and the matrix where the spermatozeugmata, or the spermatophores, are formed (Gilbert and Heath, 1972; Jamieson and Hamlett, 2005; Jones et al., 2005; Musick and Ellis, 2005). In some species, a pouch-like structure can be found on the ventral surface of the seminal vesicle, the sperm sac, where mature sperm is stored until mating (Chen et al., 1973).

3.6. Male Anatomy: Chimaera monstrosa

The internal male reproductive system in *C. monstrosa* (Figure 7) is similar to that of sharks in many aspects (Bell, 2012). Two paired testes located in the cranial region of the pleuroperitoneal cavity are connected with two genital tracts (which includes the epididymis, the ductus deferens with the Leydig gland and the seminal vesicle) by efferent ductules (or *ductuli efferentes*). The two seminal vesicles [or green glands (Márquez-Farías and Lara-Mendoza, 2014)] are divided by an isthmus into a cranial section, first whitish and opaque and then greenish and translucent, and a greyish posterior section. The greenish coloration of the middle part of the seminal vesicle is a trait present in Chimaeriformes (Dean, 1906; Malagrino et al., 1981;

Márquez-Farías and Lara-Mendoza, 2014). These caudal sections converge in a common sinus before forming the urogenital papilla in the exterior of the body.

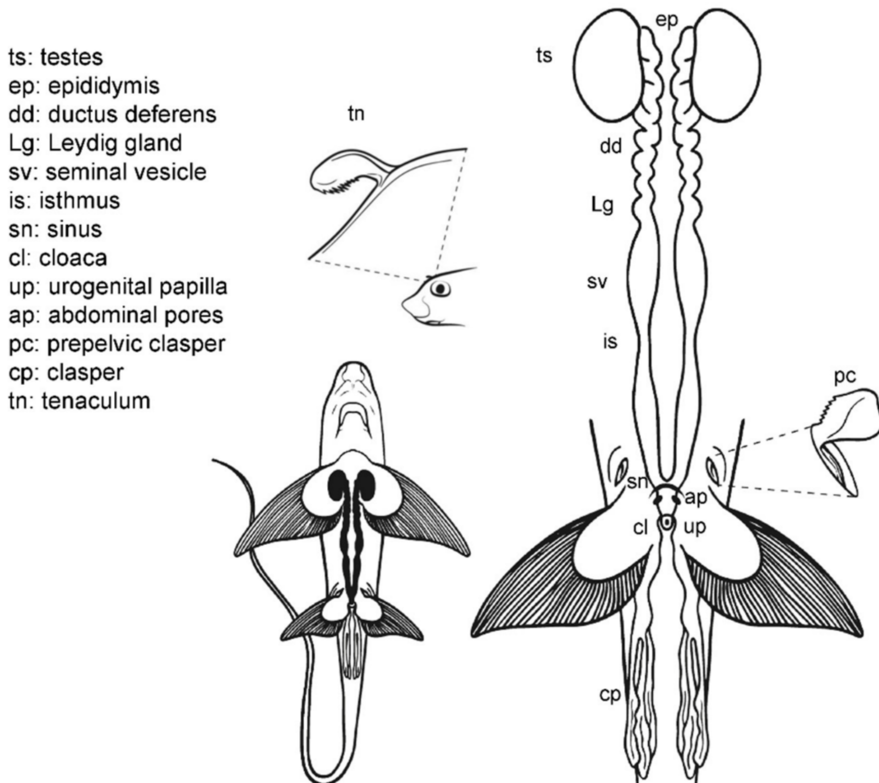


Figure 7. Male general anatomy: *Chimaera monstrosa*. Morphological scheme of the main reproductive male structures of *C. monstrosa*: testes (ts), epididymis (ep), ductus deferens (dd), Leydig gland (Lg), seminal vesicle (sv), isthmus (is), sinus (sn), cloaca (cl), urogenital papilla (up), abdominal pores (ap) and claspers [cephalic or tenaculum (tn), prepelvic (pc) and pelvic (cl)].

The external reproductive traits are multiple in *C. monstrosa*. First, the claspers are located at the base of the pelvic fins and receive the sperm from the external urogenital papilla. The claspers are three-lobed, with dermal denticles along its terminal region. Moreover, in *C. monstrosa* males, there are two slits located in the cranial part of the pelvic fins. In juvenile males, the slits are small and almost closed, but in adult males, the slits house prepelvic claspers, two serrated blade-like structures that emerge to grab the female during mating (Dean, 1906; Barnett et al., 2009). Another

reproductive external trait is the cranial tenaculum. The tenaculum is a single mallet-like structure in the forehead of male chimaeras, with dermal denticles in its extreme. As in the case of prepelvic claspers, the tenaculum is used during reproduction to grab the females (Barnett et al., 2009).

3.7. Male Comparative Anatomy

The overall morphology of the structures which can be relevant during sperm extraction is well preserved in all the species studied (Figure 8). However, there are some significant differences. Members of the family Scyliorhinidae studied (*S. canicula*, *S. stellaris*, *G melastomus*) are quite similar (Figure 8A). The three species have two independent seminal vesicles converging in a single sinus and urogenital papilla, but both *Scyliorhinus* species possess abdominal pores near the inner margin of the pelvic fins, while the abdominal pores in *G. melastomus* are slightly more centred near the cloaca. The siphon sacs in the three species are also visible under the skin cranial to the pelvic fins. In *P. glauca* the siphon sacs are also easily visible and are clearly associated with each clasper, but there are several morphological differences with the previous group. In this species (Figure 8B) the abdominal pore takes the form of a papilla (abdominal papilla) located outside the cloaca. Inside, the two caudal parts of the deferent ducts converge in a wide common urogenital sinus along the ureters of the urinary system. Both the urogenital sinus and the deferens ducts are capable of storing a large amount of sperm. The urogenital sinus opens to the exterior through the urogenital papilla located in the middle of the cloaca. Even with these differences, the species are close relatives and belong to the same order Carcharhiniformes.

In the case of the little gulper shark *Centrophorus uyato*, a deep-water shark that belongs to the order Squaliformes, the internal reproductive structures (Figure 8C) closely resemble those of the previous models observed: two paired testes, reproductive ducts, and independent seminal vesicles converging in a urogenital sinus. The external reproductive traits, however, showed several differences. The cloaca is partially covered by two cloacal lips, and only the tip of the urogenital papilla is visible if the lips are not separated. Claspers are proportionally shorter and thinner, with internal spines that unfold inside the female tract during mating. Moreover, siphon sacs are not visible in the ventral region, as in the previous species, but there

are two folds under the ventral surface of the pelvic fins (also called siphons) with the same function. The absence of ventral siphon sacs also occurs in the bluntnose sixgill shark *Hexanchus griseus*, another deep-water species belonging to the order Hexanchiformes (Figure 8D). In this species, a sac-like structure along the clasper (called clasper sac) can be found. This structure, a unique feature of Hexanchiformes, can inflate and function like the siphon sac of other elasmobranchs. Moreover, in this species the claspers lie in the inner rear margin of the pelvic fins which form a scroll, absent in females (Ebert, 2002). As in *C. uyato*, the abdominal pores in *H. griseus* are located at the base of the cloaca, but in this species as two abdominal papillae. Unlike the rest of the species studied, the ductus deferens and seminal vesicles do not converge in a common urogenital sinus. Instead, the two reproductive tracts are independent even in the urogenital papilla, where two different pores are located, one for each tract. This difference has also been described in the broadnose sevengill shark *Notorynchus cepedianus* (Daly, 2016), another member of the Hexanchiformes, suggesting that it could be a common trait for this order.

Chimaera monstrosa is the only holocephalan studied, but with some exceptions, the overall internal morphology of the reproductive system is shared with the rest of the species observed (Figure 8E). The epigonal organ, present in elasmobranchs, is absent or cannot be easily identified in *C. monstrosa* and probably in the rest of holocephalans (Dean, 1906; Stanley, 1963; Jamieson and Hamlett, 2005; Márquez-Farías and Lara-Mendoza, 2014). The division of the seminal vesicle into two sections separated by an isthmus does not occur in elasmobranchs but appears in other holocephalans such as the spotted ratfish *Hydrolagus colliei* (Stanley, 1963) and *H. melanophasma* (Márquez-Farías and Lara-Mendoza, 2014), though neither in *C. callorhynchus* (Chierichetti et al., 2017) nor *Chimaera phantasma* (Malagrino et al., 1981). The greatest difference between *C. monstrosa* and the rest of species studied appears when observing their external reproductive traits such as the clasper morphology, the absence of cloaca, and the presence of prepelvic claspers and tenaculum.

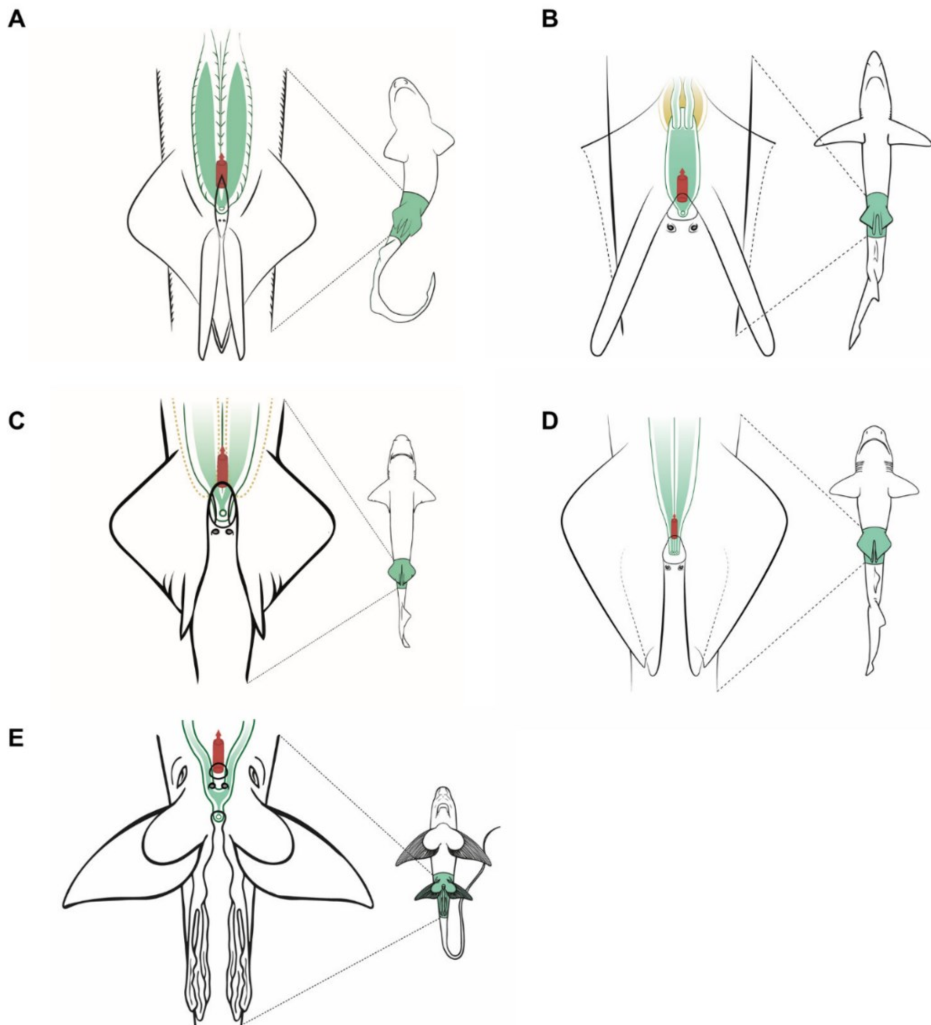


Figure 8. Specific male anatomy. Species-specific morphologies in sharks and *C. monstrosa* with relevance in sperm extraction procedures. The reproductive, excretory, and digestive systems are marked with different colors: the seminal vesicle and ductus deferens in green, the excretory system in yellow, and the access to the digestive system in red. The dotted lines in *Centrophorus uyato* represent the position of the hepatic lobes. The grey circles are the abdominal pores that connect the pleuroperitoneal cavity with the exterior. (**A**) Model observed in catsharks, family Scyliorhinidae. (**B**) Model for blue shark *Prionace glauca*. (**C**) Model observed in little gulper shark *Centrophorus uyato*. (**D**) Bluntnose sixgill shark *Hexanchus griseus*. (**E**) Rabbitfish *Chimaera monstrosa*.

3.8. Anatomic Notes for Sperm Extraction

In mature sharks, the abdominal massage is easily performed by pressing in the pelvic region and the lateral of the body, especially in small and medium-size animals. The idea behind this procedure is to be able to put pressure on the seminal vesicles or the area around them. The technique is useful in mature males that have abundant sperm stored in their seminal vesicles. In animals outside of the peak of the reproductive season, or in immature males, this technique is less useful. In bigger animals, where the fingers can be inserted in the cloaca, the sperm extraction can be carried out by pressing around the urogenital papilla with the tip of the fingers or, in smaller animals, with the tip of curved pincers (Figure 9A). One of the advantages of the technique is that it allows to obtain a large amount of sperm in a small amount of time, reducing the level of stress of the animal. However, with this technique it is not possible to obtain the full volume of sperm stored in the seminal vesicle of live animals. Some anatomic features can hamper the use of the abdominal massage, such as the position of the pelvic girdle, or other organs. For example, in *C. monstrosa*, part of the digestive system covers the section of the seminal vesicle where the sperm bundles are more abundant, while in *C. uyato*, the hepatic lobes were large enough to completely cover the seminal vesicles near the pelvic fins, limiting the effectiveness of abdominal massage.

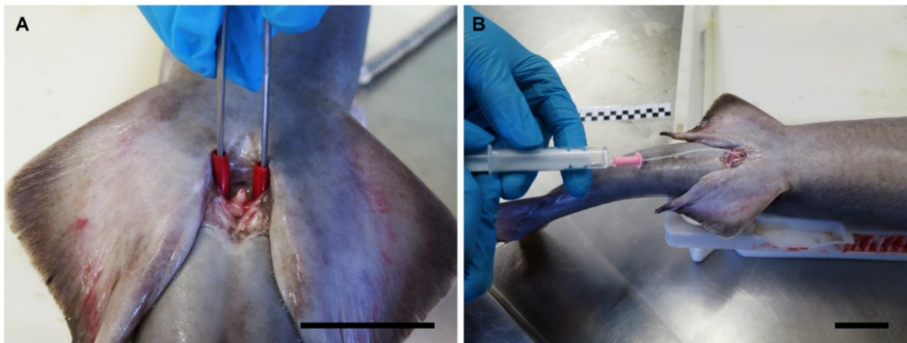


Figure 9. Different sperm extraction procedures in the little gulper shark *Centrophorus uyato*. (**A**) Massage over the urogenital sinus using forceps with rubber tips in species where abdominal massage is inefficient in this case, because of the hepatic lobes covering the seminal vesicles. (**B**) Cannulation through the urogenital papilla using a catheter. The scale bar indicates 3 cm.

Due to the pressure on the abdominal region, the sperm sample can be spoiled with urine or the contents of the digestive system. Microbial contamination can easily occur if this is the case. Therefore, if the sperm is intended to be fresh stored [medium-term preservation (García-Salinas et al., 2021a)] abdominal massage is not advised. It must be noted that mere passage from the urogenital papilla to the cloaca can also contaminate the samples. Finally, the amount of pressure applied over the reproductive structures should be considered carefully. The entire area surrounding the urogenital papilla is irrigated by capillaries that can be easily damaged, and the internal ducts and structures are fragile. Obviously, when dissection and direct access to the seminal vesicle is possible, all these limitations are easily avoided.

When the amount of sperm in the seminal vesicle is scarce (in immature animals or outside of their reproductive season) or a clean sperm sample is required, cannulation is a better technique to obtain sperm samples. This technique allows the extraction of all the sperm stored in the seminal vesicle. In addition, it allows samples not to be contaminated by microorganisms from the cloaca or the digestive system. However, it is a technique that requires more time than abdominal massage. On the other hand, it requires specialized instruments and can damage the tissue if the anatomy is not well known. Cannulation involves the insertion of a thin tube into the animal body through an orifice, in this case, the urogenital pore (Figure 9B). The diameter of the tube is important as it must be adjusted to the pore size to avoid tissue damage during insertion while keeping a vacuum during the suction. The use of a lubricating jelly with antiseptic can improve the insertion of the tubes while reducing the damage to the tissue and decreasing microbial contamination. The gauge of the cannula should also be selected considering the degree of fluidity or thickness of the sperm. In this study, *H. griseus* and *C. uyato* sperms were extremely dense and the gauge of the cannula had to be increased to get a good sample. In *P. glauca* and *S. canicula*, the sperm fluidity was higher. In *C. monstrosa*, the sperm was so dense (due to the concentration of sperm bundles) in the cranial section of the seminal vesicle that a scarce amount was recovered, and only in the caudal section of the organ was some sperm available.

To make proper cannulation, the position and morphology of the urogenital sinus, seminal vesicle, and ductus deferens must be well known. Otherwise, tissue damage can occur, as the reproductive ducts are very delicate and easy to damage. Perforations in the seminal vesicle can be produced easily, and the catheter can be inserted into the retroperitoneal space and the kidney. If that is the case, blood will be easily seen inside the catheter and the procedure must be stopped immediately. In some species, such as the basking shark *Cetorhinus maximus* and the silver chimaera *Chimaera phantasma* (Matthews, 1950; Malagrino et al., 1981) the seminal vesicles have transverse folds inside, which can be damaged during the insertion or hamper the extraction procedure. The angle of insertion of the catheter should also be considered and a horizontal angle of 5–10° and a vertical angle of 30° in relation to the axis are advised. For example, in *P. glauca*, there is the possibility of inserting the cannula into the ureter if the cannula deviates. Moreover, in this species, the sperm can be obtained from the urogenital sinus or from the paired ductus deferens if the cannula is inserted deeper.

Once the cannula is inserted, a gentle suction should be enough to make the sperm flow into the syringe. If too much suction is required and there is no advance of sperm through the tube, the cannula could be clogged, or the gauge could be smaller than needed. To prevent the cannula holes from becoming clogged, it is recommended to rotate the cannula gently while the suction is conducted. Moreover, the recommended cannulas are those with lateral perforations and the blunt tip.

The sperm storage in the oviducal gland has been reported in Chondrichthyes [for a brief review, see Marongiu et al. (2015)], being an evolutionarily conserved mechanism to ensure fertilization in nomadic or low population density species (Pratt, 1993). The storage of the sperm occurs in the gland regions called baffle and terminal zone (Hamlett and Koob, 1999), but it has been noted that it is possible to find sperm in other zones, perhaps as a result of recent mating events (Marongiu et al., 2015). A pearly mucus was obtained after scraping of the luminal epithelium of the nidamental glands from *S. canicula*, *S. stellaris*, *P. glauca*, and *C. monstrosa*. The mucus was diluted in an extender solution and aliquots from this dilution were observed under the microscope. The observation revealed the presence of ciliated

epithelial cells (moving their cilia), cellular fragments (including spermatozoa remains), and motile sperm in the four species. Although the presence of motile cells could be an indicator of recent mating and not long-term stored sperm, it should be noted that *C. monstrosa* were in their spawning season and in mature phase [stage 3a according to MEDITS category (Follesa and Carbonara, 2019)], with no body marks of recent mating (no scrapes or cuts in fins or the body) and with a prolapse in the uteri suggesting recent egg laying. Thus, the mating could hardly be recent. The same can be said for the blue sharks, where motile sperm in the oviducal gland was found in one of the females, only in the left uterus, where also were 25 pups at early stages of development. *P. glauca* males insert just one clasper into the female during mating (Pratt, 1979), thus finding only sperm in one of the oviducal glands is plausible. Although the small concentration of motile spermatozoa in the samples could limit the use of this technique as a sperm source to perform artificial insemination, other studies focusing on genetics, spermatozoa morphology, or motility patterns can still be developed.

Chondrichthyan fishes have a higher intrinsic risk of extinction compared to other fish groups (Field et al., 2009), which leads to their being one of the most threatened groups on the planet nowadays (Dulvy et al., 2016a). Public aquaria play an important role in the conservation of these animals, through *ex situ* conservation programs (IUCN/SSC, 2014). These programs are based on public outreach initiatives and the establishment of an emotional connection between the visitor and the animal (Grassmann et al., 2017), by supporting research (including reproductive methods) of different species, and by training professional staff for animal handling and sampling (Penfold and Wyffels, 2019). However, real sustainability of these programs is still far, especially when threatened species are involved (Janse et al., 2017; Buckley et al., 2018). Nowadays, public aquaria still rely on captures in the wild or in the spontaneous reproduction of the animals under their care to sustain their zoological collections. Reproductive breeding programs using reproductive techniques, such as sperm extraction and artificial insemination, could allow the advance of public aquaria toward sustainability, but can also be the key to develop reintroduction programs in the wild for threatened species of elasmobranchs. Some shark species have never been able to reproduce in captivity or have done so anecdotally (Janse et al., 2017). The captive

breeding of one of the most emblematic sharks, the sand tiger shark *Carcharias taurus*, has only been possible after years of effort and dedication of multiple institutions and researchers, even when this shark species has been kept in aquarium facilities for more than a century (Wyffels et al., 2020b).

4. Conclusions

This work is intended to be a useful guide for veterinarians, aquarists, and researchers who wish to delve into aspects related to the reproduction of these animals. Knowing this specific anatomy in detail is crucial to developing successful protocols for artificial insemination and sperm extraction.

Regarding this technique, although abdominal massage is the simplest technique for obtaining a considerable amount of sperm, it is not the most effective in animals not fully mature or if clean samples are required. Cannulation allows to obtain sperm in a more precise and clean way if the anatomy of the animal is known. Care should be taken during the sample extraction procedure to avoid damage to internal tissues. The dissection of specimens allows samples to be obtained directly from the sperm storage structures, such as seminal vesicles, sperm sacs, and deferent ducts. Finally, obtaining active sperm from the oviducal gland in females opens new research opportunities that should be exploited in the future. Much work remains to be done on the development and application of reproductive techniques in Chondrichthyes, but these first steps can be crucial for the future conservation of these animals.

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Institutional Review Board Statement

Manipulation of animals kept in aquarium conditions was authorized by the Ethic Committee of Fundación Oceanogràfic (project reference: OCE-16-19) and the sperm extraction process was conducted under the supervision of their veterinary team.

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CHAPTER 3

Development of sperm cryopreservation protocols for sharks and rays: new tools for elasmobranch conservation.

P. García-Salinas[1,2], V. Gallego[1], J.F. Asturiano[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia, Spain.

[2]Associació LAMNA per a l'estudi dels Elasmobranquis a la Comunitat Valenciana, Fraules 10, 13, 46020 València, Spain

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Abstract

Elasmobranchs are one of the most endangered vertebrate groups on the planet, but despite this situation the use of reproductive techniques in elasmobranch conservation strategies has been scarce. Among these techniques, sperm preservation is a potential tool for *ex situ* conservation and aquaria sustainability. However, there are no widespread preservation protocols for elasmobranch sperm, and shark sperm cryopreservation has never been achieved before. Here we present the establishment of successful cryopreservation protocols for elasmobranch sperm, tested in several species. We have formulated a sperm extender that can be used for different elasmobranch species, capable of maintaining sperm motility for several weeks. Additionally, we achieved the cryopreservation of sperm by previously diluting it in our extender and supplementing it with different combinations of cryoprotectants. The effects of methanol and dimethyl sulfoxide as permeating cryoprotectants were evaluated, as well egg yolk as a non-permeating cryoprotectant. Sperm quality was assessed by studying the motility and membrane integrity post-thawing, demonstrating its effectiveness in the 10 species tested, including two which are considered Critically Endangered. This is the first time that shark sperm cryopreservation has been reported, broadening our knowledge of the reproductive techniques that can be applied to elasmobranchs and laying the foundations for the first cryobanks for shark and ray sperm. Outcomes from this study will be useful for *ex situ* conservation efforts developed by public aquaria. A regular supply of frozen sperm will reduce the problems that result from the transport of specimens, inbreeding or lack of synchronized reproductive cycles in captivity.

1. Introduction

Appearing almost 400 million years ago, elasmobranchs are nowadays an ecologically diverse vertebrate group that plays a key role in the regulation of the ecosystems they inhabit (Compagno, 1990; Stevens, 2000). But the extreme life histories of sharks (Superorder Selachiomorpha) and rays (Superorder Batoidea), with some species exhibiting long gestation periods, high maternal investment, and slow sexual maturity (Cortés, 2000) make this group extremely sensitive to elevated mortality from fishing (García et al., 2008; Dulvy and Forrest, 2010). Overfishing and habitat destruction are the main drivers for the decline in elasmobranch populations, and almost one quarter of the elasmobranch species are considered threatened according to the criteria of the IUCN (Dulvy et al., 2014).

Given this situation, the use of *ex situ* conservation breeding programs could be a strategy worthy of consideration in elasmobranch conservation plans. *Ex situ* conservation involves the protection of organisms, or their progeny, extracting or isolating them from their natural environment and keeping them in artificial conditions (Frankel and Soulé, 1981; IUCN/SSC, 2014). In that sense, *in situ* and *ex situ* conservation are two complementary and non-exclusive techniques that should benefit one another and increase the effectivity of each (Pritchard et al., 2012; Braverman, 2014).

However, to successfully develop this type of project, an essential step is to develop successful breeding programs in aquaria using reproductive techniques. These programs are common for a multitude of aquatic species, but not for elasmobranchs. In fact, until now, most public aquaria tend to rely on natural spontaneous mating events or captured wild specimens for the maintenance of their populations (Henningsen et al., 2004; Buckley et al., 2018). Nevertheless, some factors need to be considered when looking at reproduction in captivity of elasmobranchs, including suboptimal population structures and environmental factors (e.g., temperature and lighting cycles). Indeed, these factors have limited the success of the breeding programs in aquaria to date (Daly and Jones, 2017).

Among the reproductive techniques that can be used to deal with this situation, artificial insemination is one that is receiving increasing attention

(Luer et al., 2008; Daly and Jones, 2017; Daochai et al., 2020). Nevertheless, to ensure the success of this technique a reliable supply of sperm is required, especially in the case of endangered species. At present, cryobanking is the most common way to guarantee the availability of viable sperm samples when necessary (Asturiano et al., 2017; Martínez-Páramo et al., 2017). The use of cryopreserved sperm for artificial insemination can avoid inbreeding and reduction of genetic variation in aquaculture companies and public aquaria, as well as solving the problem resultant of needing males and females with a synchronized reproductive cycle, and the need to transport sires from one aquarium to another for reproductive purposes.

While sperm cryopreservation is a well-established procedure used in other aquatic species (Martínez-Páramo et al., 2017; Beirão et al., 2019; Cabrita et al., 2021) information on elasmobranch sperm cryopreservation is very scarce, being limited to just two scientific publications. These publications offered information regarding three marine species: sparsely spotted stingaree *Urolophus paucimaculatus* (Daly et al., 2011), the Australian bull ray *Myliobatis australis* and the bambooshark *Chiloscyllium punctatum* (Daly and Jones, 2017). While the authors reported successful results in the cryopreservation of both batoids (*U. paucimaculatus* and *M. australis*), no conclusive information was given about the bambooshark (*C. punctatum*) besides the toxic effect of the cryoprotectant used on the sperm. To date there is no reported information on successful shark sperm cryopreservation.

It must be noted that sperm cryopreservation results in a reduction of sperm quality because of the effects induced in the cell by the freezing conditions and the potential toxicity effects of the cryoprotectants (Horváth and Urbányi, 2020). To avoid these problems and improve sperm quality, the researchers studying cryobiology have been working for years with various procedures, technologies, cryoprotectants and specific supplements, adapted to different species (Martínez-Páramo et al., 2017; Horváth and Urbányi, 2020). But in the case of elasmobranchs research is still in its initial steps (Penfold and Wyffels, 2019). In addition, sperm cryopreservation requires the use of special equipment not always affordable for researchers, as well as supplies such as liquid nitrogen and cryoprotectants, which are not necessarily available when working in the field.

When cryopreservation is not possible, the alternative is to use techniques for the short-term storage of the sperm. Short-term storage methods are designed to preserve the integrity and quality of sperm for several days or weeks. In general, diluents are used to keep the sperm during storage at low temperatures (close to 0 °C) in physio-chemical conditions like those in the seminal plasma, with the intention of maintaining high values of sperm viability (Tan-Fermin et al., 1999; Muñoz Gutiérrez, 2011) and even increase fertilization rates for some species (Tambasen-Cheong et al., 1995; Ohta and Izawa, 1996). In the few studies carried out on elasmobranchs to date, ionic solutions based on their biological fluids have been used to work with sperm, though not with the specific goal of using those solutions as short-term storage media. Jones et al. (1984) showed that sperm from the Port Jackson shark *Heterodontus portusjacksoni* was active after being kept in a phosphate buffer solution based on the ionic composition of the blood, and Minamikawa and Morisawa (1996) showed that sperm from the banded houndshark *Triakis scyllium* maintain motility in solutions based on blood and uterine conditions. Luer et al. (2008) used an extender based on a poultry semen extender modified to elasmobranch conditions through the addition of urea, TMAO and NaCl while working with the clearnose skate *Raja eglanteria*, and Daly et al. (2011) used a modified Ringer's solution adapted to elasmobranchs. According to Daly and Jones (2017) the exact ionic composition of these solutions is not as important as the osmolality in which the sperm samples are kept. In fact, Wyffels et al. (2020) showed that the sperm of sand tiger shark (*Carcharhinus taurus*) remained active while diluted in sea water.

The sperm in elasmobranchs consists of a head with acrosome, a midpiece covered by a cytoplasmic sleeve and a slender flagellum. The external features of the head and midpiece show some variations depending on the species. Typically, the head is helical with a variable number of gyres (from 3 to 24) and longer (from 30 up to 93 µm) than the midpiece (from 6 to 21 µm). Spermatozoa in the seminal vesicle are grouped in sperm bundles, called spermatophores if are formed by encapsulated sperm, and spermatozeugmata if are formed by unencapsulated masses of naked sperm cells (Tanaka, 1995; Penfold and Wyffels, 2019).

As occurs with cryopreservation, in fish the main application of short-term storage for the sperm cells is in aquaculture and aquaria, to improve breeding management by synchronizing the production of the gametes of males and females (Gallego and Asturiano, 2019) while avoiding the complexity and cost of cryopreservation procedures.

The aim of the study was to advance in the development of strategies for *ex situ* conservation programs for elasmobranchs, through the use of short- and long-term conservation of their sperm. For this reason, the objectives were: (i) the development of a dilution medium (extender) to perform short-term sperm preservation in elasmobranchs, and (ii) the development of sperm cryopreservation protocols to be used as the foundations for the creation of the first cryobank for shark and ray sperm (Figure 1).

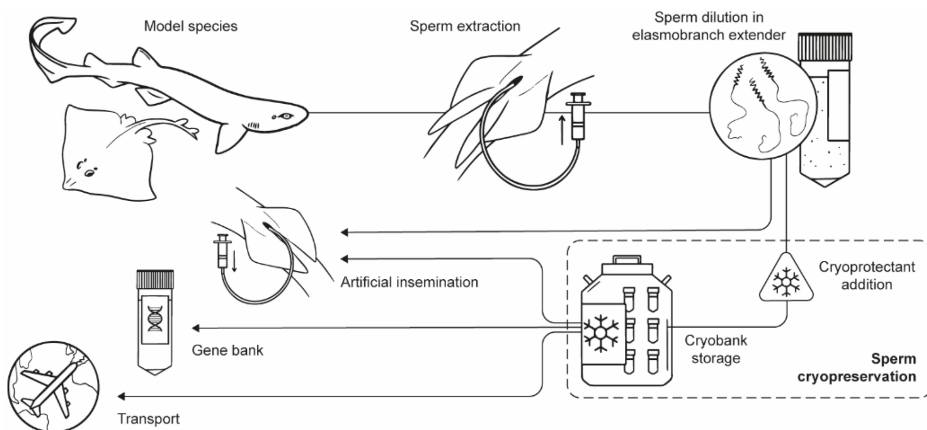

Figure 1. Experimental steps and potential applications. Scheme of the main experimental steps of the process (obtention of the specimens, sperm extraction, sperm dilution, cryopreservation, and storage) and potential uses derived from the outcomes of the study in *ex situ* conservation programs (sperm worldwide transport, gene bank, artificial insemination).

2. Materials and methods

2.1. Origin of Samples

Sperm samples were obtained from either dead and alive mature individuals belonging to 10 different elasmobranch species with various conservation

status (Table 1); 4 sharks (Selachimorpha) and 6 rays (Batoidea). Some of the species were regularly available in fish markets, while the availability of others was fortuitous. Maturity of the specimens was determined by their size according to bibliography, gonad development and clasper calcification (Ebert and Dando, 2020).

Table 1. Species in the study. Number (N) of animals from each species, size range (cm), weight range (kg) and their origin and conservation status according to IUCN criteria for the Mediterranean: least concern (LC), near threatened (NT), vulnerable (VU), endangered (EN) and critically endangered (CR). Animals in aquaria were part of the Oceanogràfic zoological collection (AQ). Animals from commercial fisheries were captured by gill net and shelled in fish markets (FM) or discarded as by-catch (BC). Stranded animals appeared dead on beaches of the Comunitat Valenciana (ST).

Common name	Scientific name	N	IUCN	Source	Length cm	Weight kg
Small-spotted catshark	*Scyliorhinus canicula*	11	LC	AQ	43-62	0.2-0.6
Nursehound	*Scyliorhinus stellaris*	1	NT	AQ	96	2.2
Blue shark	*Prionace glauca*	1	CR	ST	297	96
Bluntnose sixgill shark	*Hexanchus griseus*	1	LC	BC	250	72
Rough ray	*Raja radula*	6	EN	FM	47-63	1.2-1.7
Spotted ray	*Raja montagui*	2	LC	FM	55-67	1.4-1.9
Starry ray	*Raja asterias*	2	NT	FM	61-68	1.7-2.0
Thornback ray	*Raja clavata*	1	NT	BC	68	2.1
Common stingray	*Dasyatis pastinaca*	2	VU	BC	52-56	1.7-1.8
Bull ray	*Aetomylaeus bovinus*	1	CR	ST	103	23.3

Dead specimens were obtained from fish markets, commercial fishing vessels that catch in the Gulf of Valencia, or from the Comunitat Valenciana Stranding Network. The animals from the fish markets were caught and kept in ice approximately 20 h before their acquisition. Animals from commercial fishing by-catch and stranded animals were dead for approximately 2 h when they were obtained. In all the situations, sperm was recovered immediately after the obtention of the specimen. Alive animals were obtained from a public aquarium (Oceanogràfic, València). In the latter case, 11 small spotted catsharks (*Scyliorhinus canicula*) and 5 nursehounds (*Scyliorhinus stellaris*) were kept separately in two 8,000 L tanks with recirculating sea water (temperature: 16–18°C; salinity: 35–37‰) and fed twice a day with herring,

squid, and shrimps. Manipulation of animals kept in aquarium condition was authorized by the Ethic Committee of Fundación Oceanogràfic (Project reference: OCE-16–19) and the sperm extraction process was carried out under the supervision of their veterinary team.

2.2. In vivo Sperm Extraction

Tonic immobility was induced prior to sperm extraction to minimize struggling and reduce stress during handling, as this technique carries fewer risks to animal health than the application of anesthetics (Henningsen, 1994; Kessel and Hussey, 2015). The procedure was carried out by holding the animals in an upside-down position while gentle pressure was applied to its snout. With the cloaca emerged, pressure on the abdominal area over the seminal vesicle was enough to make sperm flow through the urogenital papillae (Figures 2A, B). Sperm flowing form the urogenital papillae was immediately collected using a sterile syringe or pipette. Sperm was transferred to sterile tubes after collection.

2.3. Post-mortem Sperm Extraction

The cloacal region of the dead animals was cleaned using sea water to remove mucus excess and other biological remains, such as blood or fishery residues. Sperm samples were obtained through the application of pressure to the abdominal area, over the seminal vesicle.

In fish where the amount of sperm was scarce, samples were obtained through cannulation with a polyurethane cat catheter (BUSTER cat catheter, 1.0 × 130 mm, Kruuse) or a PVC nasogastric tube (Feeding Probe L/RX CH-05 2.67 × 50 mm) (Figures 2C,D). Sperm was obtained after necropsy through stripping directly over the seminal vesicle in the rough ray *Raja radula* (Figures 2E,F), the bull ray *Aetomylaeus bovinus*, and the bluntnose sixgill shark *Hexanchus griseus* (Figures 2G,H).

In both processes, the sperm flowing form the urogenital papillae was immediately collected using a sterile syringe, a steel medical spatula, or a pipette. After collection, the sperm was transferred to sterile tubes.

Figure 2. Sperm extraction procedures. Sperm obtained from: (A,B) live animals (*Scyliorhinus canicula*) by abdominal massage. (C,D) Stranded animals (*Prionace glauca*) by cannulation through the urogenital papilla. (E,F) Animals from commercial fisheries (*Raja radula*) by necropsy, and (G,H) animals obtained from by-catch (*Hexanchus griseus*) by necropsy.

2.4. Media Composition.

An artificial elasmobranch seminal plasma extender (EE) was formulated to be similar in composition (solutes, pH, and osmolality) to the inner fluids of marine elasmobranchs. An aliquot of sperm extracted *in vivo* from small spotted catshark (*S. canicula*), and post-mortem from rough skate (*R. radula*),

common stingray (*Dasyatis pastinaca*), blue shark (*Prionace glauca*) and bull ray (*A. bovinus*) was centrifuged to obtain the seminal plasma and evaluate its pH and osmolality. EE solutes composition was concluded following Daly and Jones (2017), Minamikawa and Morisawa (1996), Robertson (1989), and Yancey (2015).

The components of the extender [in mM; 433 Urea, 376 NaCl, 120 Trimethylamine N-oxide (TMAO), 8.4 KCl, 50 Glucose, 7 $CaCl_2$-$2H_2O$, 3.5 $NaHCO_3$, 0.08 Na_2SO_4, 1.4 $MgSO_4$ (all the solutes were purchased from Sigma-Aldrich)] were kept in balance with physiological fluids by adjusting pH to 6.5 and the osmolality to 1,000 mOsm/kg. These values were obtained from the literature and confirmed by our own pH and osmolality measures. TMAO can be considered a limiting factor due to its price or availability in some laboratories. Thus, an EE variant (EEV) was formulated without TMAO, and it was used to dilute sperm samples and to test its usefulness as short-term medium. In this case EEV osmolality was adjusted to 1,000 mOsm/kg by increasing the amount of NaCl and urea (for a final concentration in mM of; 500 Urea, 435 NaCl) to compensate the osmolality loss because of TMAO removal. Artificial sea water (SW) was elaborated following manufacturer's instructions (Tropic marine REEF salt) for a final osmolality of 1,000 mOsm/kg. Artificial sea water (SW) was elaborated following manufacturer's instructions (Tropic marine REEF salt) for a final osmolality of 1,000 mOsm/kg (in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4, 28.2, KCl 9.4, in distilled water).

2.5. *Short-Term Preservation Trial*

To test the effectiveness of different short-term preservation media, *S. canicula* sperm samples (n = 33) were diluted with EE, EEV, or SW (dilution ratio 1:9; sperm:dilution medium). Polypropylene 2 mL vials were filled with the different mixtures and stored closed in a refrigerator at 4°C. Vials were assessed after 5, 12, 19, 28, and 36 days. During each assessment, an aliquot of each treatment was obtained from the refrigerated samples and the percentage of sperm motility was evaluated twice (see Sperm motility evaluation below). The assessment was done at room temperature. Only sperm samples with an initial motility higher than 60% were considered for the trials.

2.6. *Cryopreservation Protocols*

A series of different combinations of cryoprotectants were tested according to 7 different protocols (Table 2). Three protocols used only permeating cryoprotectants [methanol (MET), dimethyl sulfoxide (DMSO) or the combination of both] for a final cryoprotectant concentration of 10%. Other three protocols used a mixture of the permeating cryoprotectants with the addition of a non-permeating cryoprotectant [fresh egg yolk (EGG)] for a final concentration of 20%. One last protocol only used the non-permeating cryoprotectant (EGG) on a final concentration of 20%. Methanol was obtained from VWR International Eurolab S.L, dimethyl sulfoxide was obtained from PanReac Química SLU.

Fresh sperm obtained from the specimens was diluted with EE at 4°C and the corresponding cryoprotectant (Table 2) inside 2 mL internal threaded polypropylene cryotubes. In all 7 protocols the final volume of mixture in each cryotube was 1.5 mL. The cryopreservation mixture was gently shaken and left for an equilibration period of 15 min at 4°C to ensure the correct performance of the cryoprotectant. Duplicate sperm samples were tested for each protocol in order to minimize the experimental error. A cryotube filled with 1.5 mL of fresh sperm diluted with EE (dilution ratio 1:9; sperm:EE) was used as a control.

Table 2. Cryopreservation media composition. Percentage of the different components on the final volume of the cryopreservation mixture. Our reference volume was 1.5 ml and the mixture contained fresh sperm, artificial elasmobranch seminal plasma extender (EE) and different cryoprotectants: methanol (MET), dimethyl sulfoxide (DMSO) and egg yolk. The mixture was incubated for 15 minutes at 4 °C before freezing.

Protocol	Sperm	EE	MET	DMSO	Egg Yolk
Control	10%	90%	-	-	-
DMSO+EGG	10%	70%	-	10%	10%
MET+EGG	10%	70%	10%	-	10%
DMSO+MET+EGG	10%	70%	5%	5%	10%
EGG	10%	70%	-	-	20%
DMSO	10%	70%	-	10%	-
MET	10%	80%	10%	-	-
DMSO+MET	10%	80%	5%	5%	-

Due to the small amount of sperm obtained from skates (*Raja* spp.) two protocols (DMSO + MET + EGG and EGG) were not tested on these species. Only sperm samples with an initial motility higher than 60% were considered for the trials.

Freezing and thawing process:
A styrofoam box was partially filled with liquid nitrogen (LN) and kept closed until the temperature of the container was stable (the LN stopped boiling). After the equilibration process, cryotubes were placed over a metal mesh platform floating 1 cm over the LN, for a period of 15 min. Cryotubes were completely submerged into the LN after that time, where they remained for 5–10 min. For the thawing process, cryotubes were extracted from the LN and submerged for 75 s in a water bath at 70°C.

2.7. Sperm Motility Evaluation

To evaluate spermatozoa motility, a 0.5 µL aliquot was collected from each sample and diluted in 4.5 µL of EE placed on a counting chamber ISAS Spermtrack 10 (Proiser R + D, S.L., Spain). The assessment of motility was done at room temperature. Samples were observed using a microscope with a 10x magnification lens (Nikon Eclipse 80i) and videos of the sample were recorded by a camera (Nikon Digital Sight DS-5M). The videos were then analyzed manually, and the percentage of motile (displacing, rotating, or actively beating flagella) and non-motile spermatozoa were recorded. A mean of 80–100 spermatozoa were individually counted per sample. Fresh (non-frozen) samples were also analyzed as a control.

2.8. Sperm Viability

To study the viability of the thawed spermatozoa their plasma membrane integrity was assessed using a fluorescence LIVE/DEAD Sperm Viability Kit (Thermo Fisher Scientific, MA, United States). The kit uses SYBR-14, which stains intact cells green, and propidium iodide (PI) that stains damaged cells red. For each thawed sample, a 50 µL aliquot was obtained and mixed with 1 µL PI (final concentration 12 µM) and 1.5 µL of SBYR-14 (final concentration 100 nM). After an incubation period of 10 min in darkness and at room temperature, the samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10× magnification and pictures were taken. Pictures

were analyzed to count the number of green and red spermatozoa heads. An average of 135 spermatozoa per sample were identified as live or dead. As mentioned above for the motility study, fresh (non-frozen) samples were also processed and analyzed.

2.9. Statistical Analysis

Two species were selected as model species to perform the first preservation protocols: S. canicula and R. radula. Regarding the genus *Raja*, preliminary analysis showed that there were no significant differences in the motility and viability values of the species belonging to this genus (the Mediterranean starry ray *Raja asterias*, the thornback ray *Raja clavata*, the spotted ray *Raja montagui* and *R. radula*), thus a common group was established for statistical analysis.

Shapiro-Wilk and Levene tests were used to check data normality and variance homogeneity, respectively. To analyze the EE as a short-term preservation medium over time, a univariate General Linear Model (GLM) was used. A One-factor ANOVA was used to compare the different cryopreservation protocols as well the difference between EE, sea water and EEV. A Student-Newman-Keuls (SNK) post hoc test was used in all cases. All statistical analyses were carried out using the IBM SPSS Statistics 24 statistical software.

3. Results

3.1. Sperm Collection

Although sperm extraction was achieved for 39 animals, the obtention of high-quality sperm was not feasible in all the specimens. This was particularly the case with adult skates obtained in fish markets (some of which possess a low sperm volume) and most of the *S. stellaris* kept in aquaria (which exhibited low sperm motility). Table 1 shows the species and the number of specimens (n = 28) whose sperm had enough motility (>60%) to be used in the trials. As an exception, bull ray (*A. bovinus*) sperm was used despite its low motility value (45%) because of its Critically Endangered conservation status.

The sperm obtained had a creamy white coloration and viscous consistency. Under the microscope, a mass of helical heads, typical of elasmobranchs, was clearly visible. The sperm mass disaggregated when the sample was diluted, allowing the spermatozoa to be seen individually. The spermatozoa concentration of the samples ranged from 2.27×10^8 to 4.22×10^8 cells per mL and the average pH value was 6.46 (SD: 0.15).

3.2. Short-Term Preservation

Short-term preservation was evaluated by analyzing the sperm motility of samples diluted with different solutions (EE, sea water, EEV) over time. The motility of the samples was checked on days 0, 5, 12, 19, 28, and 36. The results represented by Figure 3 (mean motility of each treatment after different times) showed that there was a continuous decrease in motility in all the samples over time.

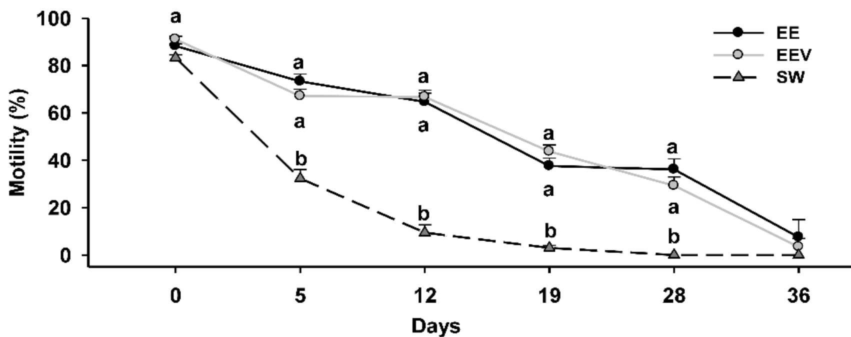

Figure 3. Short-term preservation. Motility variation over time in short-term preservation trials. *Scyliorhinus canicula* sperm was diluted 1:9 (sperm: dilution medium) with artificial elasmobranch seminal plasma extender (EE), artificial seminal plasma extender variant (EEV), or seawater (SW). Motility was assessed on different days (0, 5, 12, 19, 28, 36) after initial dilution. Lowercase letters represent statistical differences between treatments.

This reduction in motility was drastic in SW, while for EE and EEV it was more gradual and with no significant differences between the two treatments. Starting from an initial motility value of 85–90% for the three treatments, the motility of sperm samples diluted in EE (Figure 4A) and EEV (Figure 4B) decreased to values of 60–65% after 12 days, and 40% after 28 days. Then the sperm motility values of both treatments (EE and EEV) showed a progressive reduction until day 36 day, reaching values close to 10%.

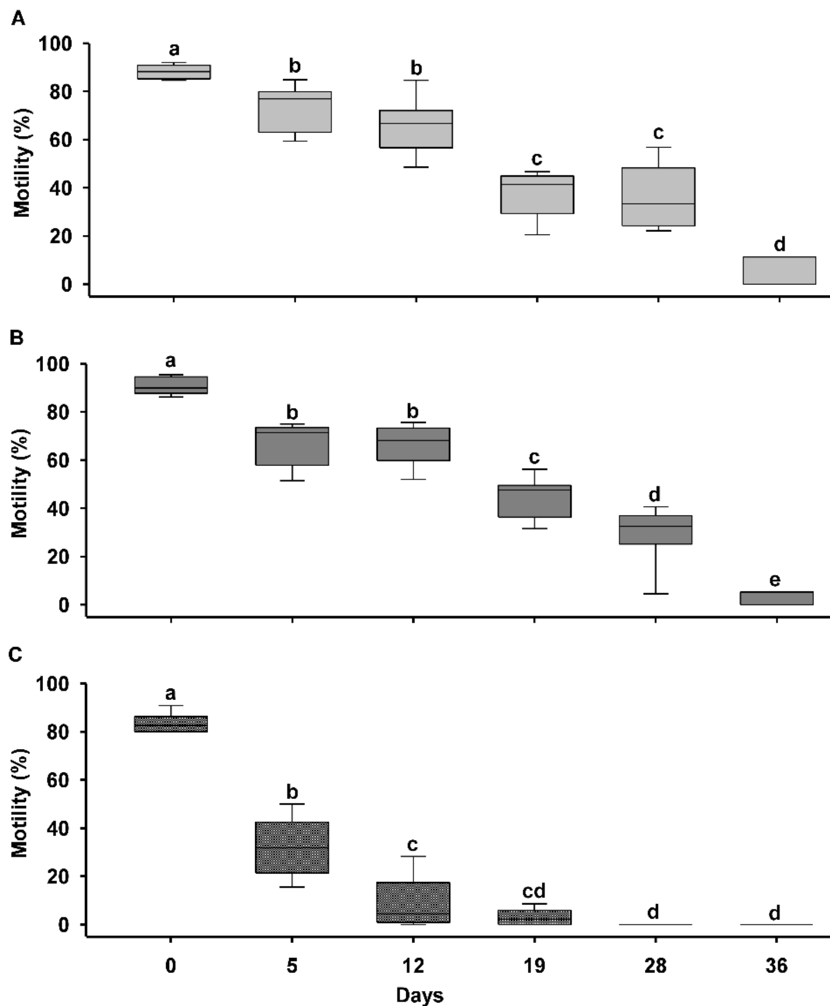

Figure 4. Media comparison for short-term preservation. Short-term preservation results after diluting 1:9 (sperm: dilution medium) *Scyliorhinus canicula* sperm with three diluents. (A) Artificial elasmobranch seminal plasma extender (EE); (B) artificial seminal plasma extender variant (EEV); (C) sea water (SW). Motility was assessed on different days after initial dilution (CONTROL). Different letters mean significant motility differences between days into every treatment.

Overall, there were no significant differences between the two treatments (EE and EEV) in terms of motility. In contrast, sperm samples diluted in SW (Figure 4C) showed a significant decrease in the motility values after just 5 days, reaching values close to 30%. The sperm motility values were close to 0 on day 19 for sperm samples diluted in SW.

3.3. Cryopreservation Protocols

Cryopreserved samples showed significantly lower motility and viability values than the fresh samples, irrespective of the protocol used or the species from which the sperm came. In general, skates (*Raja* spp., Order Rajiformes) showed higher motility and viability results than sharks (*Scyliorhinus* spp. And *P. glauca*, Order Carcharhiniformes, and *H. griseus*, Order Hexanchiformes, which in turn showed higher values than stingrays (*A. bovinus* and *D. pastinaca*, Order Myliobatiformes).

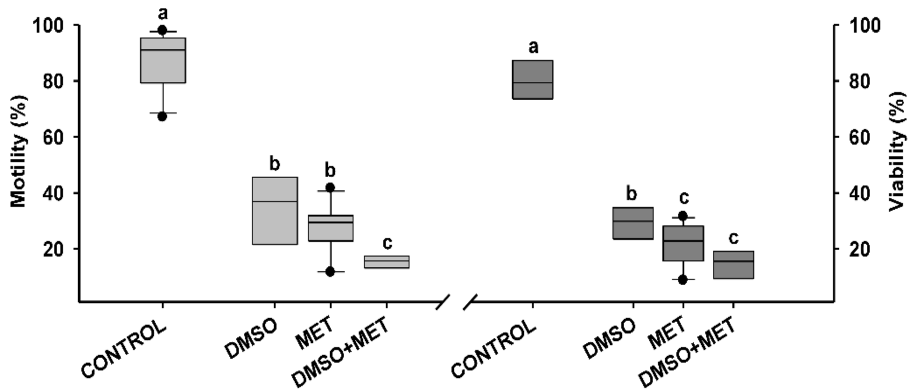

Figure 5. Cryopreservation results in rays. Motility and viability results for sperm cryopreservation protocols in skates (n = 11). Sperm obtained from *Raja asterias*, *Raja clavata*, *Raja montagui*, and *Raja radula* were diluted with artificial elasmobranch seminal plasma extender (EE) and frozen using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET) or DMSO + MET. FRESH represents the motility and viability values in fresh sperm samples prior to freezing. Different letters mean significant differences between viability or motility values from each protocol.

In skates (Figure 5) the use of both 10% DMSO and 10% MET rendered motility values higher than 40%. However, the use of MET as a cryoprotectant caused a higher variability. The protocol combining of 5% DMSO plus 5% MET, obtained significantly lower results than the other protocols. The viability results were like those of motility, showing that the protocol using 10% DMSO produced higher values than the protocols which used 10% MET and the combination of MET plus DMSO.

In the case of the cryopreserved shark sperm samples (Figure 6 and Table 3) the best motility values were seen with protocols using 5% DMSO plus 5% MET and 10% egg yolk, or using 5% DMSO plus 5% MET, which induced average values close to 35%.

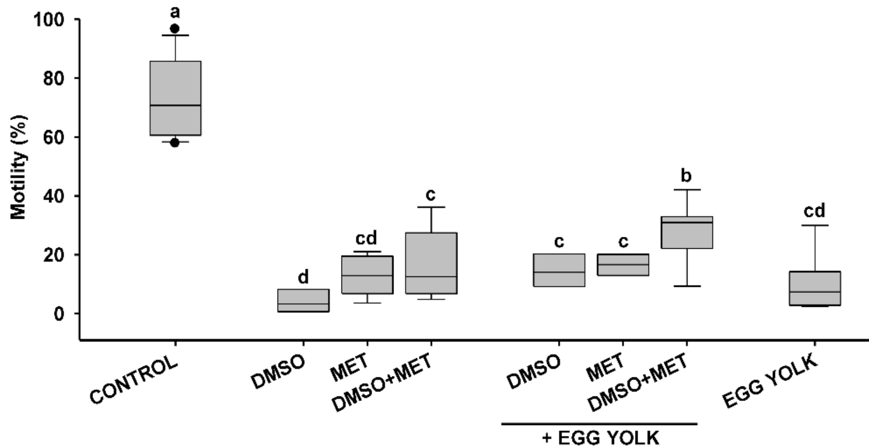

Figure 6. Motility after cryopreservation in sharks. Motility results for cryopreservation protocols in small spotted catshark (n = 11). Sperm obtained from *Scyliorhinus canicula* was diluted with artificial elasmobranch seminal plasma extender (EE) and frozen using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET) and fresh egg yolk. FRESH represents the motility and viability values in fresh sperm samples prior to freezing. Different letters mean significant differences between motility values from each protocol.

Overall, the addition of egg yolk as non-permeating cryoprotectant increased the post-thawing motility values, by up to 42.1% in samples with initial motility values of 70%. As was the case of skates, in terms of viability sharks showed similar patterns to those found in motility (Figures 7A,B). Although after cryopreservation samples did not reach values higher than 40%, protocols using the combination of DMSO, MET, and EGG obtained significantly higher viability values (around 30%) than those obtained without this combination (10–20%). As a side note, the best motility and viability values after cryopreservation were obtained for *H. griseus* samples, reaching values of 74.3 and 73.2%, respectively, using the combination of 5% DMSO plus 5% MET and 10% egg yolk.

Table 3. **Results for non-model species**. Motility and viability results for cryopreservation protocols in five different species *Prionace glauca* (n=1), *Aetomylaeus bovinus* (n=1), *Scyliorhinus stellaris* (n=1), *Dasyatis pastinaca* (n=2), *Hexanchus griseus* (n=1), where no sufficient data allowed for proper statistical analysis. Between parentheses standard deviation (SD) is shown. Sperm obtained was diluted with artificial elasmobranch seminal plasma extender (EE) and different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET) and fresh egg yolk (EGG). FRESH represents the initial motility and viability values prior to freezing.

Motility (%)								
Species	FRESH	DMSO	MET	DMSO +MET	DMSO +EGG	MET +EGG	DMSO +MET +EGG	EGG
P. glauca	65	41.7	5.9	27.8	31.8	5	36.7	15.2
A. bovinus	45	0	1.3	2.8	4.5	16.9	9.3	0.4
S. stellaris	86.7	-	-	30.1	-	-	42.9	1.2
D. pastinaca	67.4 (6.45)	4.3 (1.3)	6.9 (2.9)	-	-	-	-	-
H. griseus	87.4	53.6	22.2	38.8	71.9	32.3	74.3	40.2
Viability (%)								
P. glauca	56	17.7	7.9	17.9	22.2	11.1	21.5	8.8
A. bovinus	45.8	5.2	15.8	5.9	11.1	21.6	10.1	2.2
H. griseus	79.8	55.6	5.8	16.4	70.2	14.9	73.2	-

Only three stingrays were obtained from by-catch and stranding, two *D. pastinaca* and one *A. bovinus*. Although the number of animals was too small to perform a proper statistical analysis, some useful information was obtained (Table 3). For the common stingray (*D. pastinaca*) only protocols using 10% DMSO and 10% MET were used, because of the small volume of sperm obtained. The protocols used on *D. pastinaca* were not nearly as successful as those used in the other species tested, causing a maximum motility value post-thawing of 6.9%.

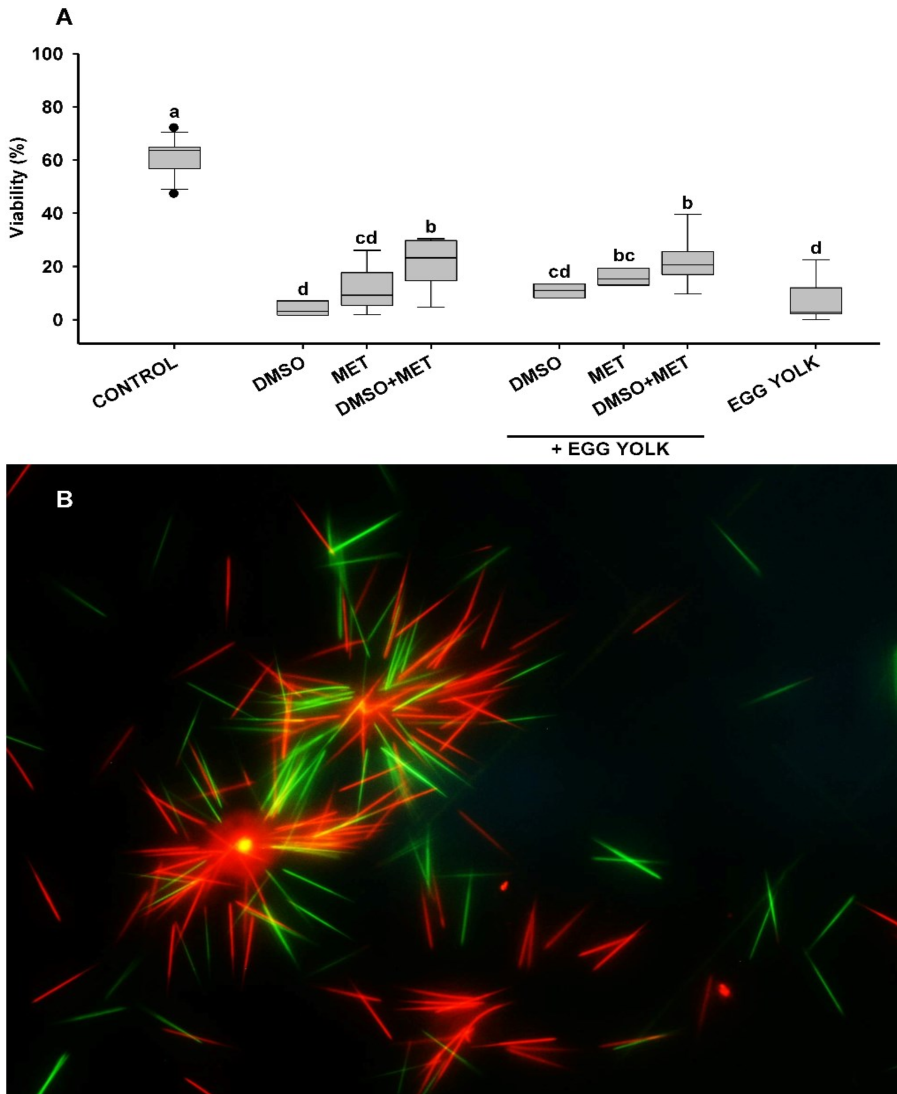

Fig 7. Viability after cryopreservation in sharks. (A) Viability results for cryopreservation protocols in small spotted catshark (n = 11). Sperm obtained from *Scyliorhinus canicula* was diluted with artificial elasmobranch seminal plasma extender (EE) and frozen using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET) and fresh egg yolk. FRESH represents the motility and viability values in fresh sperm samples prior to freezing. Different letters mean significant differences between viability or motility values from each protocol. (B) Stained sperm to study the viability of the thawed spermatozoa by assessing their plasma membrane integrity. SYBR-14 stains intact cells green, and propidium iodide (PI) stains damaged cells red. Stained samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10× magnification.

Unlike in the other species tested, the bull ray *A. bovinus* motility values were lower than their viability values. MET supplemented with egg yolk induced better results than the other protocols in both motility (17%) and viability (21.6%), from an initial motility of 45%.

In the present study, all the samples belonging to the control protocol (sperm diluted but no cryoprotectant added) showed motility and viability values lower than 0.5%.

4. Discussion

Elasmobranch breeding attempts in public aquaria, to improve the sustainability of their zoological collections, or to carry out conservation programs are not as developed as those of other species. However, captive breeding programs are gaining importance, especially for threatened species (Daly and Jones, 2017). There are a wide variety of reproductive techniques that could be used on elasmobranchs, such as the monitoring of reproductive cycles, hormonal treatments, gamete collection, sperm cryopreservation and artificial insemination. These techniques have been well documented in many mammals, reptiles, birds, and bony fish, but their application in elasmobranchs has been anecdotal (Daly and Jones, 2017; Penfold and Wyffels, 2019). This study presents new contributions in this field, with the intention of serving as a basis for developing affordable preservation methods for elasmobranch sperm, without the need of expensive or complex equipment.

Short-term storage methods are designed to preserve sperm quality, by diluting the cells in an extender with physio-chemical conditions like those found in the seminal plasma. In the development of our dilution medium (EE), we considered the comments of Daly and Jones (2017) who proposed that while is true that osmotic pressure might be more important than ionic composition, most authors adapted their dilution media to the physiological characteristics of elasmobranchs. Thus, they included components present in this group such urea and NaCl. For this reason, we considered Minamikawa and Morisawa (1996) work on banded houndshark *T. scyllium* because of the description they provided on the ionic composition of the different fluids, and especially because of their comments on hexose. They observed that the

duration of sperm movement was longer in a hexose-rich uterine fluid-like solution than in seawater, suggesting hexose plays an important role in maintaining sperm cell motility in the female reproductive tract. Thus, our proposed dilution medium has a high concentration of hexose in the form of glucose as a possible energy source for sperm motility. We also considered the work on osmotic constituents in *S. canicula* developed by Robertson (1989) and the review of Yancey (2015) about organic solutes in elasmobranchs, especially regarding the balance between the proportions of urea and TMAO used.

Our results show that samples diluted with EE maintained a motility > 70% for up to 12 days of storage, and up to 40% 28 days after dilution. It was from day 36 when the sperm motility drastically decreased. In contrast, the motility of samples stored after dilution in SW decreased rapidly: from an initial 80% to approximately 30% after 5 days of storage and falling to values under 10% after 12 days of storage. Samples diluted in EEV showed a similar motility pattern during the first few days to that of samples diluted in EE. Thus, for a up to 36 days storage there would be no need to include TMAO in the medium if the required osmolality is maintained. However, we chose EE as the extender for our cryopreservation trials for two reasons: (i) we wanted to use an extender with a composition like seminal plasma, and (ii) some authors considered that TMAO plays a key role in the physiology of marine animals by enhancing the binding ability and structural stability of proteins, thereby counteracting the negative effects of high urea and NaCl concentrations (Yancey et al., 2002). Additionally, these authors pointed out that it could play a role in protecting the tissues against other stressors, such as pressure and temperature. For these reasons, we believe that TMAO should not be discarded as a key element in the ionic composition of media for the short-term storage and the cryopreservation of elasmobranch sperm.

Sperm cryopreservation involves its dilution in a medium containing one or more cryoprotectants, which help to protect sperm from damage produced in the freezing and thawing processes. Cryoprotectant effectiveness depends on its toxicity to the spermatozoa, its concentration, the equilibration duration time, and the diluents used. DMSO is the most used cryoprotectant for the sperm of aquatic species such as cyprinids (Lujić et al., 2017), while

MET, dimethylacetamide or glycerol are more effective in other groups such as salmonids (Labbé et al., 2013). In elasmobranchs, two successful cryopreservation trials with sparsely spotted stingaree *U. paucimaculatus* (Daly et al., 2011) and Australian bull ray *M. australis* (Daly and Jones, 2017) used DMSO or glycerol supplemented with egg yolk as non-permeating cryoprotectant. Non-permeating cryoprotectants in addition to permeating cryoprotectants can significantly increase the percentage of viable and motile spermatozoa post-thawing in different species (Gallego et al., 2017). In our study the best results for shark cryopreservation were obtained when adding egg yolk to the permeating cryoprotectants, suggesting the importance of including a non-permeating cryoprotectant in the freezing medium. The use of egg yolk for cryopreservation is common for teleost species reared in aquaculture, as it has a thermo-protective action, which is exercised by the lecithin and cephaline of its lipid fraction and a preservative action, given by the lipoproteic fraction (Robles et al., 2005). In fact, egg yolk has been used as an additive in many cryopreservation studies, and although positive effects have been observed in many cases, it may not be applicable in some species. For example, the addition of egg yolk increases fertilization times in common trout *Salmo trutta* after cryopreservation (Piironen, 1993) but has a negative effect on the sperm cryopreservation of asp *Leuciscus aspius* (Babiak et al., 1998).

In the present study, the cryopreserved sperm samples of sharks and skates showed post-thawing motility values of 30–40% depending on the species, with the notable exception of the bluntnose sixgill shark *H. griseus*, with a motility value of 70%. This species can be found in depths up to 2,500 m where is adapted to cold deep-sea conditions (Ebert and Dando, 2020). There might be a connection between the high cryosurvival values and these adaptations (such as high levels of TMAO), that should be considered in future studies. The effects of DMSO also demonstrated the existence of species-dependent reactions to cryoprotectants. While 10% DMSO worked best for skates, it compromised post-thawed motility and viability of sharks and stingrays due to possible toxicity after prolonged exposure. Although not conclusive, 10% MET was the most effective protocol in *A. bovinus* sperm samples, with a post-thawing motility of 17%.

Even though the use of DMSO is highly recommended for the cryopreservation of fish sperm due to its rapid penetration into the cells, it can have negative effects by changing the osmolality of the medium (Garzón et al., 2007; Peñaranda et al., 2008). Also, DMSO can have a species-specific toxic effect (Arciniegas et al., 2005). Other studies, such as that carried out by Daly and Jones (2017), described the toxic effect of glycerol on brownbanded bambooshark *C. punctatum* sperm despite its protective effects in sparsely spotted stingaree *U. paucimaculatus* and Australian bull ray *M. australis* sperm. Thus, further research should be conducted to learn about the toxic effects of cryoprotectants in different species and whether certain aspects of the protocols should be specifically adjusted.

The present study expands the list of elasmobranch species in which cryopreserved sperm has been achieved. The use of two model species (the small spotted catshark *Scyliorhinus canicula* and the rough ray *Raja radula*) allowed us to refine protocols before testing them on other species. It is important to note that the source of the sperm samples covers almost every possible origin of elasmobranch gametes: aquaria collection, by-catch, fish markets and stranded animals, confirming the possible application of these protocols in all kinds of samples.

The protocols described here for short-term and long-term sperm preservation can be considered a complement to *ex situ* conservation projects for sharks and rays. In addition, to our knowledge this is the first time that cryopreserved shark sperm has been achieved, not only in our model species *S. canicula* but in a Nearly Threatened species, the nursehound *Scyliorhinus stellaris*, a Critically Endangered species, the blue shark *Prionace glauca* and a deep-sea shark species, the bluntnose sixgill shark *Hexanchus griseus*. Skate sperm has also been cryopreserved for the first time in four different species, including two Nearly Threatened species, the thornback ray *Raja clavata* and the Mediterranean starry ray *Raja asterias* and an Endangered endemism *Raja radula*. Other threatened species such as *D. pastinaca* and the Critically Endangered *A. bovinus* expand the Myliobatiformes list where cryopreservation has been previously achieved. However, we were not able to design a universal protocol for elasmobranch sperm cryopreservation, so more research needs to be carried out to assess

the different responses of different species. Our research can potentially help with the development of artificial insemination projects in elasmobranchs and has expanded our knowledge on the reproductive biology of this group of species.

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Institutional Review Board Statement

Manipulation of animals kept in aquarium condition was authorized by the Ethic Committee of Fundación Oceanogràfic (Project reference: OCE-16-19) and the sperm extraction process was carried out under the supervision of their veterinary team.

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CHAPTER 4

The ancient and chiral architecture of Elasmobranchii's spermatozoa enables sperm penetration in viscous environments.

Serhii Boryshpolets[1], Borys Dzyuba[1], Pablo García-Salinas[2-3], Hermes Bloomfield-Gadêlha[1], Victor Gallego[2], Anatolii Sotnikov[1] and Juan F. Asturiano[2]

[1]Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, Vodňany 389 25, Czech Republic.

[2]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia, Spain.

[3]Associació LAMNA per a l'estudi dels Elasmobranquis a la Comunitat Valenciana, Fraules 10, 13, 46020 València, Spain

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Abstract

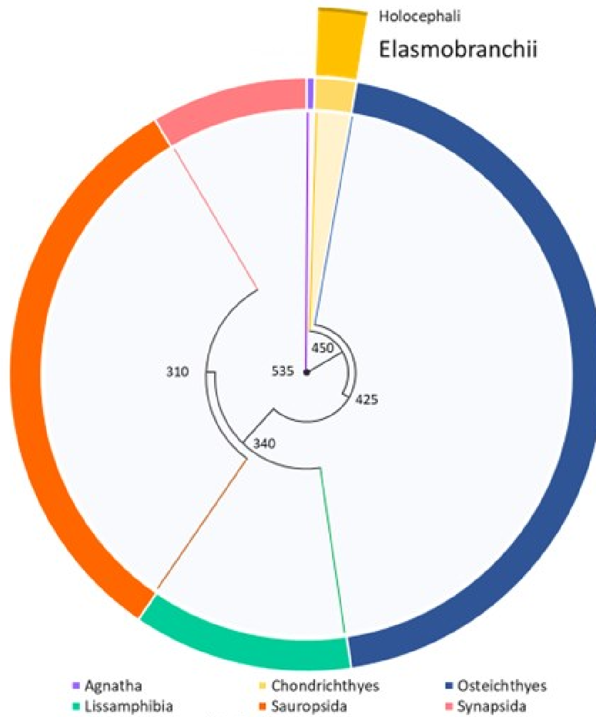
Subclass Elasmobranchii belongs to an old evolutionary class of Chondrichthyes that diverged 450 mya, presenting a wide diversity of reproductive strategies while preserving the ancient mode of internal fertilization. These species possess relatively big spermatozoa (compared to bony fishes) with an elongated spiral shape head and tail similar to one currently existing (but later diverged) in birds and reptiles. These structures may be associated with the necessity to penetrate viscous ovarian fluid or the jelly layer of eggs, suggesting environmental viscosity as the driving pressure shaping large-sized sperm heads into spiral shapes through evolution. We investigated the effect of surrounding media viscosity in three Elasmobranchii species on spermatozoa motility parameters. Our observations suggest the surrounding viscosity is critical to allow the progression and regulation of spermatozoa motility with unique chiral head-to-flagellum architecture. As such, it may be a key element in controlling sperm performance during fertilization in the Elasmobranchii species.

1. Introduction

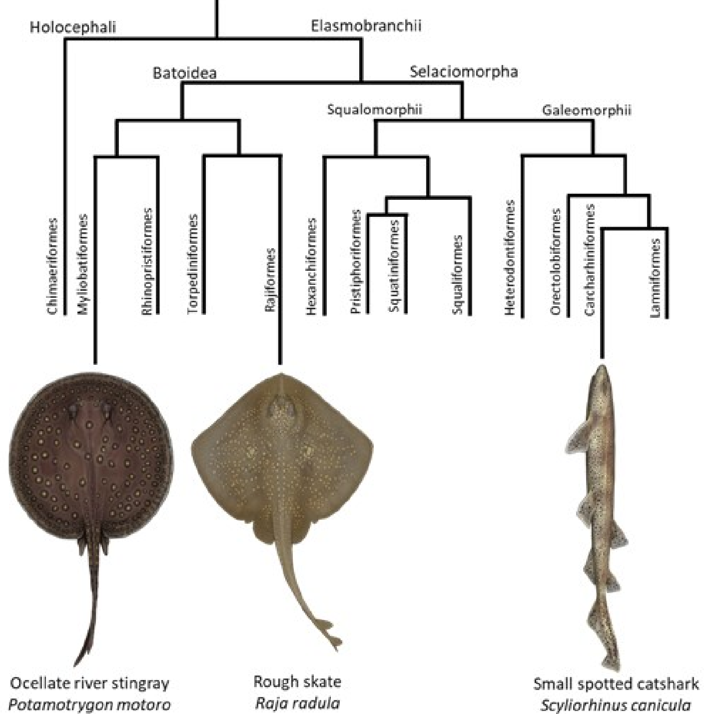
Chondrichthyans (cartilaginous fishes, including Elasmobranchii) are an old evolutionary class of aquatic vertebrates that diverged 450 mya (Fig. 1) (Amaral et al., 2018). Modern cartilaginous fishes are characterized by various reproductive strategies (Walker, 2020) while still being unique since they preserved an ancient mode of internal fertilization in contrast to bony fishes, in which internal fertilization appeared evolutionary later from externally fertilizing ancestors (Fitzpatrick, 2020). As such, the cartilaginous, particularly Elasmobranchii fish (most commonly known as sharks and rays), are critically important for reproductive biology studies, from its spermatology, physiology, and biophysics since their reproduction strategy remained almost unchanged in these species for millions of years.

Spermatozoa are one of our planet's most diverse eukaryotic cell types (Pitnick et al., 2009). While highly specific for reproduction purposes (its primary function is to deliver the male genetic information to the female ova), spermatozoa are taxa–specific and have morphed as a response to different fertilization and environmental pressures. This is the fundamental hypothesis of Darwinian sex evolution. It is predicted that due to gamete competition, males start to produce larger amounts of smaller size cells, which compete for fertilizing ova produced by females, which provides a safe environment for the nourishment of the future embryo and its development.

Several studies have demonstrated that different modes of fertilization could drive the overall length of the spermatozoa: longer cells are correlated with internally fertilizing species and shorter cells with externally fertilizing ones (Kahrl et al., 2021). As internal fertilization can be considered an evolutionary primitive feature for all gnathostomes (Long et al., 2015), the evolutionary studies of early diverged taxa, such as cartilaginous fishes, are of primary interest for understanding the fundamental pathways leading to the diversity of reproduction modes in vertebrates and taxa specificity of spermatozoa morphology. Interestingly, some evolutionary later diverged birds (Støstad et al., 2018), reptiles (Simon et al., 1996), monotremes (Carrick and Hughes, 1982; Ecroyd et al., 2009; Nixon et al., 2016), and amphibians (Scheltinga and Jamieson, 2003) share a similar sperm morphology to that of cartilaginous fish, characterized by elongated and spiral-shaped heads.



Holocephali
Elasmobranchii
450
425
535
310
340
Agnatha
Chondrichthyes
Osteichthyes
Lissamphibia
Sauropsida
Synapsida



Chondrichthyes
Holocephali
Elasmobranchii
Batoidea
Selaciomorpha
Squalomorphii
Galeomorphii
Chimaeriformes
Myliobatiformes
Rhinopristiformes
Torpediniformes
Rajiformes
Hexanchiformes
Pristiphoriformes
Squatiniformes
Squaliformes
Heterodontiformes
Orectolobiformes
Carcharhiniformes
Lamniformes
Ocellate river stingray
Potamotrygon motoro
Rough skate
Raja radula
Small spotted catshark
Scyliorhinus canicula

Figure 1. Cladogram showing the relationship between present-day vertebrates. The length of the outer ring segments shows the number of extant species. The number next to the nodes refers to the last common ancestor in millions of years. The yellow portion represents the chondrichthyans, consisting of the *Elasmobranchii* and the *Holocephali*, whose proportional representation is based on their number of extant species. The phylogenetic tree is adapted from Amaral et al., 2018. The three species illustrated, *Potamotrygon motoro*, *Raja radula,* and *Scyliorhinus canicula* are placed next to the order to which they belong.

This similarity hints that chondrichthyan fishes' spermatozoa shape is evolutionarily ancient (plesiomorphic for vertebrates). Their distinctive features, such as sperm head morphology and flagellar structures, could be a specific adaptation to allow sperm to penetrate the highly viscous environment of the ovarian fluid and the jelly layer that coats the eggs. If this is the case, the environmental fluid viscosity could be the main driving force while shaping large-sized spermatozoa heads into slender spiral shaped-heads during evolution.

Generally, hydrodynamically effects associated with body shape and size are well known to influence the swimming kinematics of microorganisms (Lisicki et al., 2019). The sperm head shape was recently found to be essential also for sperm swimming progression and rotation movement in two cartilaginous fishes, rays- *Rhinoptera javanica* and *Taeniura meyeni*, possessing the head spiral-shape with four turns (Wang et al., 2021). This head shape was experimentally and computationally found to be essential for adaptation to a viscous environment. However, authors observed low progression of spermatozoa in high viscosity conditions. Thus, future studies of viscosity's biological relevance and its effect on reproduction are still highly interesting.

Moreover, it is unclear whether these conclusions can be transferred to all elasmobranchs and other species with chiral spermatozoa architecture, with a big diversity of spermatozoon shape and size (Tanaka, 1995). Other studies suggest that sperm competition associated with postcopulatory sperm selection also affects the sperm flagellum length in sharks, though the reasons for diversity in spermatozoon head and midpiece shape and length remain unclear (Rowley et al., 2019). Understanding the spermatozoon structure and principles of sperm motility and physiology is an essential step toward better predicting the different pressures driving their evolutionary

adaptations. Also, from a more pragmatic point of view, this knowledge may be indispensable for developing the next generation of assisted reproductive techniques in Elasmobranchii, as many of them face extinction (Penfold and Wyffels, 2019).

In the present study, we investigate the environmental pressures of the surrounding fluid viscosity on spermatozoa motility traits and its ability to swim progressively in different viscous environments. To gain a better understanding of the role of viscosity across different species, we investigated the sperm motility in three Elasmobranchii*,* known as differing in level of spermatozoon dimensions associated with sperm head shape: (1) Small-spotted catshark -*Scyliorhinus canicula,* (2) Mediterranean starry skate *Raja asterias* and (3) the South American stingray - *Potamotrygon motoro* as a representative of freshwater later diverged Elasmobranchii species (Fig. 1). Our results shed light on the fundamental principles of sperm progression in Elasmobranchii*.* Their unique helical head morphology and three-dimensional flagellar beating enable sperm cells to penetrate a highly viscous environment that would otherwise not be possible. We show that in low viscosity, sperm cells are nonprogressive while gaining the ability to move progressively in an environment with high viscosity. High viscosity was required during spermatozeugmata dissociation, allowing spermatozoa to unbundle from the others and move progressively and straightforwardly. Increased viscosity also creates an environment under which elongated helical heads of spermatozoa can buckle during motion, thus being able to change direction very efficiently. In contrast, in low viscosity, it is not possible at all. Altogether, our observations demonstrate that high viscosity is a critical environment pressure to which these cells are specialized.

2. Experimental Model

Three Elasmobranchii species were used in this study: *Scyliorhinus canicula* (n = 4)*, Raja asterias* (n = 4)*, and Potamotrygon motoro* (n = 3)*.* The small-spotted catsharks *Scyliorhinus canicula* were randomly selected from a sample of 11 male individuals sharing their aquarium with no other species. The sharks were kept in an 8,000 L aquarium with recirculating seawater (temperature: 16–18 °C; salinity: 35–37‰) and fed twice daily with herring,

squid, and shrimps. All animals were adult males of an estimated age of 5 years. Maturity status could be determined by the degree of calcification of the claspers and by having been previously used in other sperm quality studies. Manipulation of animals was approved by the Oceanogràfic Animal Care & Welfare Committee at the Fundación Oceanogràfic Valencia, Spain (Project reference: OCE-16–19).

The Mediterranean starry skate *Raja asterias* specimens came from commercial fisheries. The animals were obtained fresh from the fish market approximately five hours after capture and kept on ice until pick-up. The animals came from artisanal trammel net fisheries in the Gulf of Valencia, Spain. All animals were adult males of undetermined age. Maturity status could be determined by the degree of calcification of the claspers and the development of testes and epididymis. The ocellate river stingray (*Potamotrygon motoro*) was kept in experimental facilities at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice. These facilities are certified by the Ministry of Agriculture of the Czech Republic for breeding potamotrygonids and using them as experimental animals (reference numbers: 56665/2016-MZE-17214 and 55187/2016-MZE-17214). The stingrays were housed in a 900 L aquarium with recirculating freshwater, maintained at 25–26°C and 100% oxygen saturation, with a pH range of 7.2-7.4. They were fed twice daily with dry pellets and frozen forage fish. All the animals used in the study were adult males aged between 5 and 7 years. All animal manipulation procedures were conducted in accordance with the Animal Research Committee of the Faculty of Fisheries and Protection of Waters, following the principles based on the EU-harmonized animal welfare act of the Czech Republic and the principles of laboratory animal care in compliance with the national law (Act No. 246/1992 on the protection of animals against cruelty).

2.1. Method Details

2.1.1. *Sample collection and sperm motility video recordings*

Sperm samples were collected from a shark - *Scyliorhinus canicula* (n = 4), skate - *Raja asterias* (n = 4), and ray species - *Potamotrygon motoro* (n = 3) males after abdomen massage according to recommendations for cartilaginous fishes (García-Salinas et al., 2021b, c) and stored at 4 °C before

motility recordings. One aliquot of sperm (0.1-0.3 µl approximately) was deposited in 40 µl of artificial seminal fluid (ASF) on a glass slide using the tip of an injection needle. The composition of the ASF varied according to the target species and was formulated to mimic its natural seminal fluid osmolality and ionic composition. For the freshwater species, *P. motoro,* ASF consisted of: NaCl 130 mM, KCl 8 mM, $CaCl_2$ 0.6 mM, Glucose 0.4 mM, Tris 10 mM, pH 7.84, osmolarity 295 mOsm modified after Dzyuba et al., 2019. For the marine species, *S. canicula* and *R. asterias*, ASF consisted of urea 433 mM, NaCl 376 mM, Trimethylamine N-oxide (TMAO) 120 mM, KCl 8.4 mM, Glucose 50 mM, $CaCl_2$ 7 mM, $NaHCO_3$ 3.5 mM, Na_2SO_4 0.08 mM, 1.4 mM $MgSO_4$, adjusted to pH 6.5 and osmolarity 1000 mOsm (García-Salinas et al., 2021a). To vary the viscosity of ASF and cover the physiological isotonicity of internal fluids, the concentrations of 0, 0.125, 0.5, 0.75, 1, and 2% of Methylcellulose (MC; Sigma-Aldrich, M0512) were added (Ivic et al., 2002). These solutios have resulted in viscosity of 0, 15, 50, 200, and 4000 mPa×s correspondingly (according to Methylcellulose M0512 manufacturer product information).

The motility of all three species (11 males in total) was recorded under room temperature conditions (22-24 °C) with a digital video camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) set to 193 FPS (800 × 600) and a high-speed video camera (Olympus i-speed TR, Tokyo, Japan, providing 848 × 688 pixels spatial resolution) at 2000 FPS mounted on a phase-contrast microscope and x20 or x100 (200 or 1000 total magnification) objectives for several minutes (depends on experimental conditions). Motility records were stored in AVI format before analyses.

2.1.2. *Analysis of sperm motility and flagella characteristics*

Digital frame-to-frame analyses of our video-microscopy recording were performed to measure linear swimming velocity, flagellar waveform amplitude, wavelength and frequency of wave oscillation, and sperm head-rotational speed frequency in ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). An example of estimated parameters is shown in Fig. S1, where the sperm head- rotational speed frequency is measured directly from the rotation motion of the spiral-shaped head, which travels along the head axis, similarly to a propagating wave.

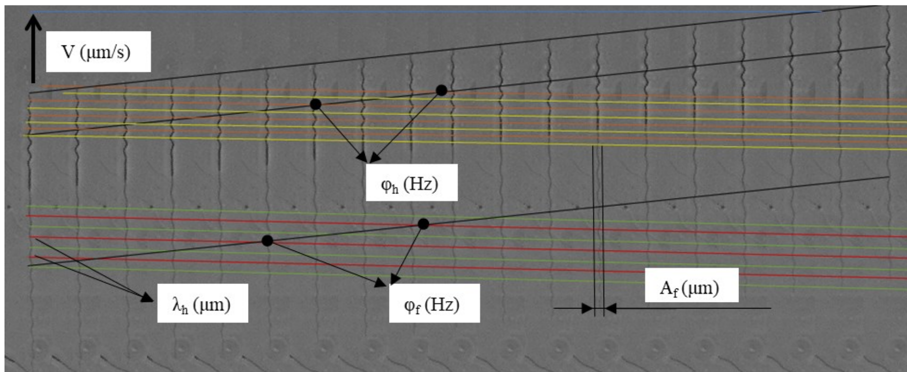


Figure S1. Example of skate flagellar and head motility analysis. The black line corresponds to the line connecting the tips of the head on each frame. The yellow and orange lines connect the corresponding "waves" on the spermatozoa head, and the green and red connect waves on the tails. By calculating intervals (number of frames) of the line of the same color crossing the black line, we can estimate the time needed for one complete rotation or beat cycle (0.05 s between each frame). The number of such cycles per second will be expressed as frequency (Hz). The distance between the green and red lines provides information about the length of the flagellar wave (µm). Recalculating the distance spermatozoa tip traveled during one second will give us info about sperm velocity V (µm/s). The amplitude of the flagella wave is the distance between two parallel lines connecting the waves of the flagellum from both sides, A (µm). Related to STAR Methods section.

2.1.3. Analysis of spermatozoa head morphology

Frames obtained from video records were processed to measure head length, head helix amplitude, and wavelenght, as presented in Fig. S2. The number of helices is calculated as the total number of all crests on both sides of the head divided by 2.

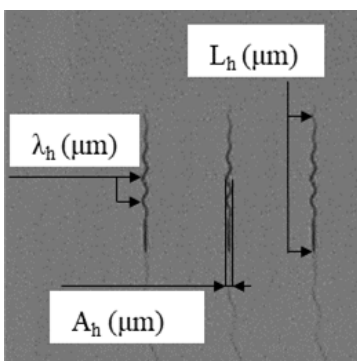


Figure S2. Example of skate head morphology measurements. The distance from the tip of the head to the beginning of the flagellar is measured as head length (including midpiece). The distance between the two parallel lines connecting the waves of the head helix from both sides is the amplitude of the head helix. The length of the head helix is measured as the average distance between two wave crests on one side of the head. The number of helices is calculated as the total number of all crests on both sides of the head divided by 2. Related to STAR Methods section.

2.1.4. Electron microscopy

Sperm samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for two days at 4 °C. For Transmission Electron Microscopy (TEM), samples were post-fixed in osmium tetroxide for 2 h at 4 °C, washed, dehydrated through an acetone series, and embedded in resin (Poly/Bed 812; Polysciences, Inc., Warrington, USA). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) after being counterstained with uranyl acetate and lead citrate and examined in a TEM JEOL 1010. The fixed samples for the Scanning Electron Microscope (SEM) were step-wise dehydrated in an acetone concentration, dried using a critical point dryer PELCO CPD 2 (Ted Pella Inc., California, USA), and coated with gold in vacuum SEM Coating Unit E5100 (Polaron Equipment Ltd., California, USA). Samples were examined with an SEM JSM 401-F or SEM JEOL 6300 (JEOL Ltd., Tokyo, Japan) equipped with a Sony CCD camera.

2.2. Quantification and Statistical Analysis

The parameters of the sperm head are presented as mean ± standard deviation from data obtained from 18 spermatozoa of rays (3 males), 15 spermatozoa of skates (4 males) and 23 spermatozoa of sharks (4 males).

The low sample size of sperm motility and flagellum characteristics, non-normally distributed values inside of these experimental groups ($p < 0.05$ in Kolmogorov-Smirnov test), and no homogeneous variances ($p < 0.05$, Levene's test) suggested application of nonparametric statistical analysis. Thus the analogue of one-way ANOVA using the Brunner-Dette-Munk test followed by pairwise comparisons with Bonferroni adjustments of p-values were performed by R- software environment with the "asbio"-package version 1.7 (MacFarland, 2012) Statistically significant differences in values inside the same species are marked by capital letters, while differences inside one viscosity concentration are marked by lowercase letters in corresponding figures.

Correlation analysis was performed using Statistica (version 13, TIBCO Software Inc., 2017, Palo Alto, CA, USA) to estimate Pearson Correlation (r) and to determine a linear regression equation presented in the figures.

3. Results

3.1. Spermatozoa structure

The Elasmobranchii spermatozoa structure is distinctive: it possesses a long and helical head, an elongated midpiece, and a flagellum supplemented with additional ultrastructural components to its axoneme. The head shape of the ray and skate spermatozoa observed are helices turning toward the left (counterclockwise helices), similar to a corkscrew, with a large non-monotonic modulated pitch and diameter along its length, increasing to a maximum before decreasing again at the connecting piece (Fig. 2A). Skate spermatozoa had a length head of 48.16±1.31 µm, helix amplitude of 4.48±0.35 µm, and wavelength of 11.33±0.75 µm possessed 4.5 helix were similar in all 4 males. Ray spermatozoa had a slightly larger head, with a length of 54.21±1.09 µm, helix amplitude of 5.32±0.17 µm, and wavelength of 10.63±0.6 µm possessed 5,5 helix and also were similar in all 3 males. The shark (*Scyliorhinus canicula*) spermatozoa observed, on the other hand, is a "screw-shaped" helix turning toward the left (counterclockwise helices) with approximately 30-35 turns, accurate measurement of which was not possible due to a tiny diameter (3.28±0.66 µm) and pitch (2.23±0.27 µm) that remains almost constant along its length 59.36±1.51 µm (slightly decreasing towards the tip of the heads; Fig. 2B).

In all species, the flagellar ultrastructure is reinforced by two accessory axonemal columns that sandwich the axoneme in the middle (Fig. 2C). These columns rotate clockwise relative to the 9+2 axoneme, following the same handedness of their helical heads. Thus, they are twisted around the axoneme in a counterclockwise helical fashion (Fig. 2E). Similar twisting is observed at the midpiece (Fig. 2D). Interestingly, the counterclockwise helical chirality of head-to-flagellum structures is conserved for all three species, despite the dramatic changes in head morphology between rays/skates and sharks.

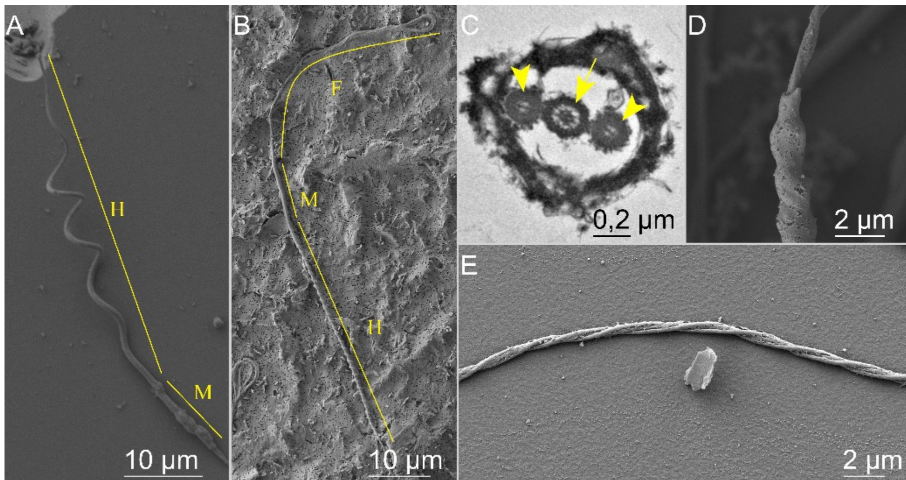


Figure 2. Electron micrographs of spermatozoon structures. A) - ray *Potamotrygon motoro* spermatozoon head (H) and midpiece (M), B) - shark *Scyliorhinus canicula*, SEM of the whole spermatozoon: head (H), midpiece (M) and flagellum (F). C) – element of the flagellum in ray *Potamotrygon motoro*, transverse section of the flagellum, TEM, arrowheads - longitudinal columns, arrow – 9+2 structure axoneme. D) - Electron micrographs of midpiece in ray *Potamotrygon motoro*. E) - Electron micrographs of the flagellum in ray *Potamotrygon motoro*.

3.2. Spermatozeugmata: collective beating sperm bundles in large sperm clusters

Raw sperm samples carefully placed in artificial seminal fluid (ASF) without mixing (preserving in this way the natural physiological condition by which sperm is found in ejaculate) contained several clusters of high-density entangled sperm agglomerations with different shapes and sizes for all three species (Fig. 3). We observed spermatozeugmata for all three species, sperm not encapsulated and tails of peripheral sperm protruding in all cases.

The cluster shape of ray and skate spermatozoa are relatively similar, but their arrangement within the spermatozeugmata is very distinct (Fig. 3A, C). Spermatozeugmata in rays contain randomly distributed sperm, forming a mesh-like structure similar to porous-like materials (Fig. 3A). Skate sperm clusters ranged from dozens to hundreds of sperm cells (Fig. 3C). Spermatozoa were all braided and coiled together by the helical heads, aligned in parallel, in such a way that all heads seemed to work as one unit. The parallel alignment permitted the self-organized formation of large flagellar bundles that could beat in collective synchrony with high levels of

order, with traveling waves propagating in the bundle from head to tail. Shark spermatozoa were connected by the tip of the heads and at the midpiece region, forming a symmetric arrangement of their cluster towards a common centre. As such, sperm heads tended to be directed to the centre while their moving flagellum formed a large-scale beating flagellar bundle in the outer direction (Fig. 3B), similar to flagellar-like beating of actin bundles (Pochitaloff et al., 2022).

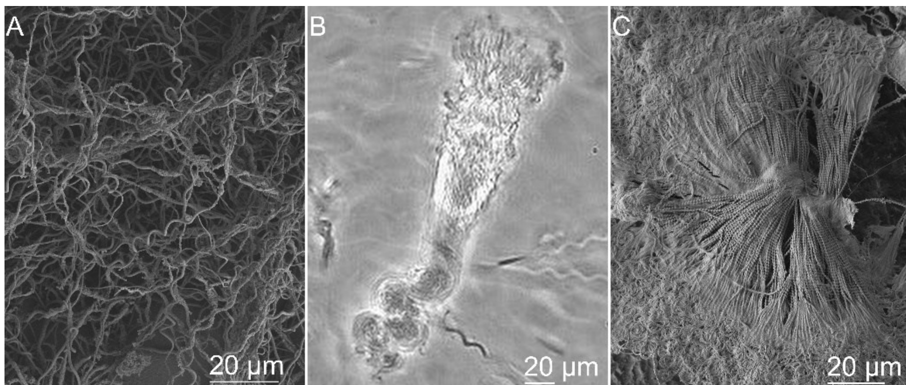


Figure 3. Agglomeration of sperm samples observed (A) by electron microscopy in ray *Potamotrygon motoro*, demonstrating randomly distributed spermatozoa inside of spermatozeugmata; (B) by phase-contrast microscopy in skate *Raja asterias* spermatozeugmata, demonstrating coiled together spermatozoa heads with synchronously moving flagella; (C) by electron microscopy in shark *Scyliorhinus canicular*, demonstrating spermatozoa connected by the tip of the heads and at the midpiece region. See also Video S1.

3.3. Sperm release from spermatozeugmata clusters

Spermatozeugmata in all three species were observed to possess intensively beating active flagellum within the high-density clusters of spermatozoa. In a low-viscosity medium (LVM, 0% MC in ASF), very few sperm cells escaped the cluster entanglement, causing a very slow release of spermatozoa from clusters. The few sperm cells that managed to break free from clusters, however, did not manage to swim far from it. After 20 min of observation, the spermatozeugmata conserved their structure, with most spermatozoa residing inside the cluster despite the vigorous motion of individual cells and their collective beating bundles. This strongly indicates that low-viscosity environments suppress sperm progression and release from spermatozeugmata in these species. This scenario dramatically changes

under high-viscosity fluid environments (HVM, 1-2% MC in ASF). In this case, spermatozoa release from spermatozeugmata is fast, effective, and efficient, with 100% of all spermatozoa successfully released in a few minutes. After 20 min of observation, no remaining structure of spermatozeugmata could be found in high viscosity media (2% MC) in all three species.

The randomly arranged spermatozeugmata of rays (Fig. 3A) was observed to fully release sperm from its entangled clusters in HVM (Video S1a) but remained in the cluster in LVM. In sharks, the radial configuration of spermatozeugmata (Fig. 3B) switched into a "spikey shape" arrangement in HVM due to the progressive collective motility of the spermatozoa bundle. The connection between head tips was broken, and spermatozoa sheared relative to each other towards the centre, ending with all heads pointing outwards while still stacked at the midpiece region, with the beating flagellum in the inner core, forming a perfect monopole configuration with sperm cells aligned radially (Oriola et al., 2018) (Video S1b). The rate of shape changes of the sperm cluster from a symmetrical circular arrangement to a monopole configuration also depended on the viscosity of the media. LVM considerably suppressed any cluster rearrangement for extended periods compared with HVM. In 2% MC, spermatozeugmata was broken in the first few seconds after contact with the medium, and the sperm spiked monopole "explodes" with the sperm concentrated at the centre released and swimming in all directions, like fireworks (Video S1c).

Skate spermatozeugmata are made of braided sperm, coiled together by their large-diameter spiral heads whose flagellar bundle self-organizes into a collective motion with waves traveling from head to flagellum tip. Despite its collective motion, spermatozeugmata with motile flagella are nonprogressive and tumble around the same region. Interestingly, sperm reverse their flagellar beat with waves traveling from tip to head, forcing the head to spin in the opposite direction, thus effectively unentangling all sperm spirals from the cluster. The flagellar bundle stops entirely before the reversal in wave progression, thus provoking a backward movement. The backward motion and reversal in the rotating direction cause the sperm spiral heads to "unscrew" from the dense spermatozeugmata cluster (Video S1d). Once spermatozoa are released from nearby cells, the flagellar wave

progression switches to regular beating, and all spermatozoa swim efficiently away from the cluster. Interestingly, we observed that this switch in wave-propagation could be equally utilized to unscrew spiral sperm from dense structures and reverse steering to avoid obstacles, making them highly adaptative and autonomous swimmers in a variety of environments (Video S1e).

3.4. Cytoplasmic sleeve

Another specific structure of Elasmobranchii sperm is the so-called "cytoplasmic sleeve." Before sperm activation, the sleeve covers the midpiece (Fig. 2D), while it is not typically present after activation and in free-swimming sperm. However, at the very beginning of sperm activation, some motile spermatozoa still carry their sleeves. In some cases, it is even possible to observe the progressive release of the sleeve as it slides and corkscrew down the flagellum while the sperm carefully swims away due to hydrodynamic friction acting on the sleeve (Video S2a). When the spermatozoa leave the spermatozeugmata by their own motion, most do not have this sleeve. The sleeves remain in the cluster "exit" area. An example of such a phenomenon can be observed in Video S2b, where disaggregation of skate spermatozeugmata was slow due to the relatively low viscosity of the ASF (0.125% MC), and many sleeves can be observed surrounding the cluster of beating spermatozoa.

3.5. Sperm motility and progression are specific for the highly viscous environment.

After dilution in ASF, spermatozoa were released from spermatozeugmata, forming a dilute suspension in which active motility was maintained for 30 to 40 min. As expected from the sperm morphology, the flagellar beating was helical. The flagellum rotated around its swimming axis, causing the helical head to spin as sperm swam freely in the fluid. During such movement, parts of the flagellum and head were observed to go in and out of focus, demonstrating the 3D nature of swimming behaviour (see Video S3a for a skate in 2% HVM). Interestingly, the spermatozoa of all three species in the LVM were almost nonprogressive, tumbling and rotating around with high frequency, though ineffectively, and with highly asymmetric flagellar waveforms containing coiled parts on the distal ends of their flagella (see ray

sperm exemplar in LVM in Video S3b). The sperm progression efficiency increased with increasing viscosity (Fig. 4). At the same time, at such conditions, the linear velocity is strongly connected with the sperm's ability to spin around its longitudinal axis, effectively "corkscrewing" the sperm into the medium. The highest progressive velocity for shark sperm was observed at 0.75% MC (Video S3c). In comparison, 1% MC instigated higher speeds in ray and skate sperm (Video S3d, e).

Links for the videos on this Chapter:
Video S1: https://youtu.be/BWZuVzZH7FE
Video S2: https://youtu.be/7HXSrjCNL6A
Video S3: https://youtu.be/TRdzVQAjJaI
Video S4: https://youtu.be/Cj5HqVLsCmw

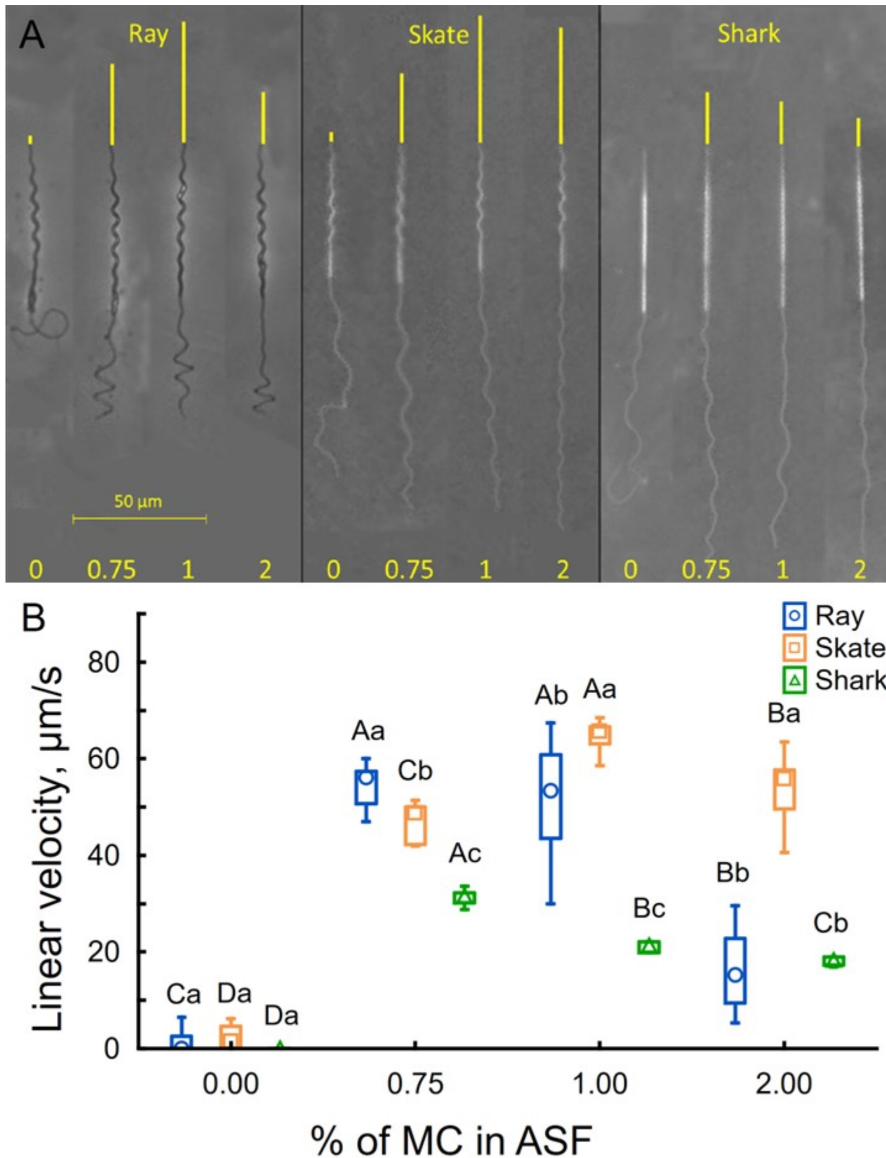


Figure 4. The velocity of ray *Potamotrygon motoro*, skate *Raja asterias*, and shark *Scyliorhinus canicula* spermatozoa at different viscosities (0, 0.75, 1, 2% MC present in ASF). A) - example of flagellar shape and passed the distance of spermatozoa; B) – linear velocity. Each experimental group has an average of 8 (3-15) measured spermatozoa. Significant differences inside the same species are marked by capital letters, and differences inside one viscosity concentration are marked by lowercase letters (Brunner-Dette-Munk test, followed by pairwise comparisons with Bonferroni adjustments, $p < 0.05$). See also Video S3.

3.6. *Flagellar wave adaptation to viscosity*

As seen in Fig. 4A, the flagellar shape is highly regulated by viscosity. The increased viscosity of the fluid limits the propagation of flagella beating from side to side, decreasing its overall amplitude and wavelength. Exceptionally, the amplitude of ray sperm flagella seemed less affected by the increased viscosity. However, bending waves shifted axially due to the wave compression phenomenon (Gadêlha and Gaffney, 2019), slightly decreasing wavelength without a visible effect on flagellar amplitude (Fig. 4A; Fig. 5A, C).

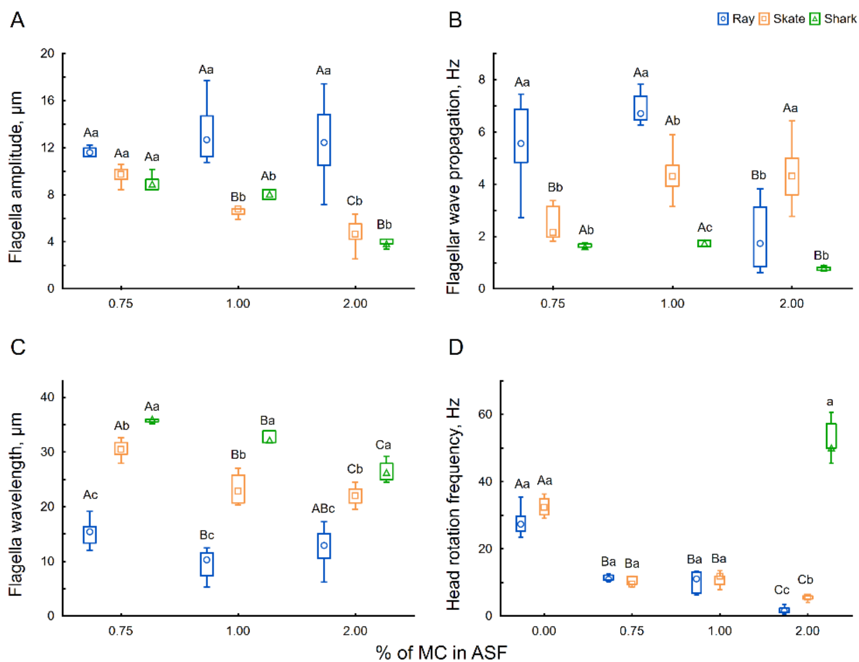


Figure 5. Parameters of spermatozoa and flagella motility of ray *Potamotrygon motoro*, skate *Raja asterias*, and shark *Scyliorhinus canicula* spermatozoa at different viscosities (0, 0.75, 1, 2% MC present in ASF): A) – flagella amplitude; B) – flagellar wave propagation frequency; C) – flagellar wavelength; D) – head rotation frequency. Data are presented as median, 75, and 25 percentiles (box), min, and max values (whiskers). Each experimental group has an average of 8 (3-15) measured spermatozoa. Significant differences inside the same species are marked by capital letters, and differences inside one viscosity concentration are marked by lowercase letters (Brunner-Dette-Munk test, followed by pairwise comparisons with Bonferroni adjustments, $p < 0.05$).

The correlation between wavelength and tail amplitude at different viscosity is presented for all three species in Fig. 6. Moreover, a slight wave compression was also observed in skate and shark spermatozoa when they were mortorquee compressed and shifted to the distal end of the flagellum. The part of the flagellum proximate to the midpiece remains active during such motion and oscillates with extremely high frequency and almost undetectable amplitude (Video S3f).

Surprisingly, the sperm penetration in high viscosity was not hindered by the fact the amplitude of the flagellar waves was very small. It may be attributed to the induced head rotation by the flagellum. This suggests that sperm penetration in high viscosity can be achieved with two distinct modes in Elasmobranchii: the classical way in which large amplitude bending waves propel the large head forwards and a novel mechanism in which bending waves are insufficient to produce cell propulsion but sufficient to induce torque on the helical head that subsequently corkscrew into the medium.

3.7. Helical head rotation

When the flagellum propagates helically, the whole spermatozoon spins around its longitudinal axis. Thus, the rotation frequency could be related to sperm velocity, especially given their chiral sperm architecture. In our study, the observed frequency of flagellar propagation was low and sometimes almost identical to rotation frequency, up to 6 Hz for all three species and studied viscosities (Fig. 5B). In the condition of LVM, the frequency of head rotation was very high, reaching rates higher than 50 Hz; but usually with the frequency between 20 and 35 Hz for skate and ray. Increasing media viscosity stabilized rotation frequency at approximately 10 Hz for skate and ray spermatozoa (Fig. 5D). The correlation of velocity and total frequency of a head rotation and flagellar wave progression under different viscosity is presented in Fig. 7. The detection of rotation in shark sperm was challenging due to the small radius of the head helix being partly out of focus in lower viscosities, also restricting the number of measurements for shark spermatozoa in 1 and 0.75% MC to 4 and 3 correspondingly. The head-rotational speed frequency was only possible to measure at 2% MC at x100 magnification, demonstrating exceptionally high rates, around 50 Hz (Fig. 5D).

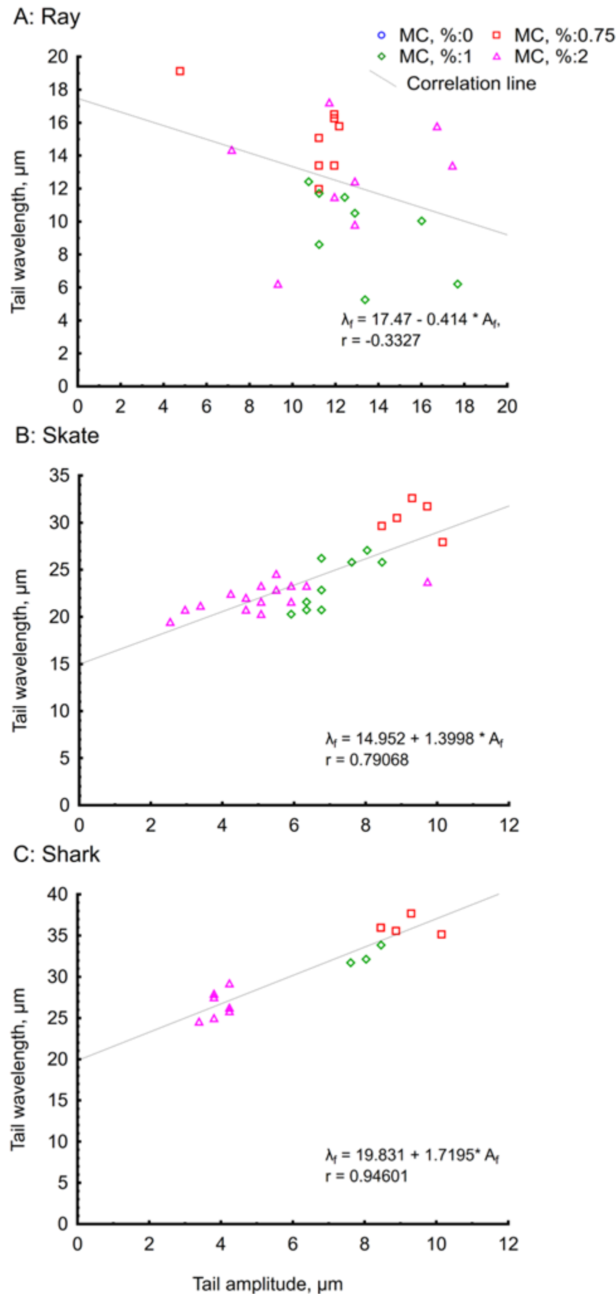


Figure 6. Correlations between tail wavelength and amplitude under conditions of different viscosity (0, 0.75, 1, 2% MC present in ASF) for A: ray *Potamotrygon motoro*; B: skate *Raja asterias*, and C: shark *Scyliorhinus canicula* spermatozoa. Each individual point on a graph represents measurements of a single spermatozoon.

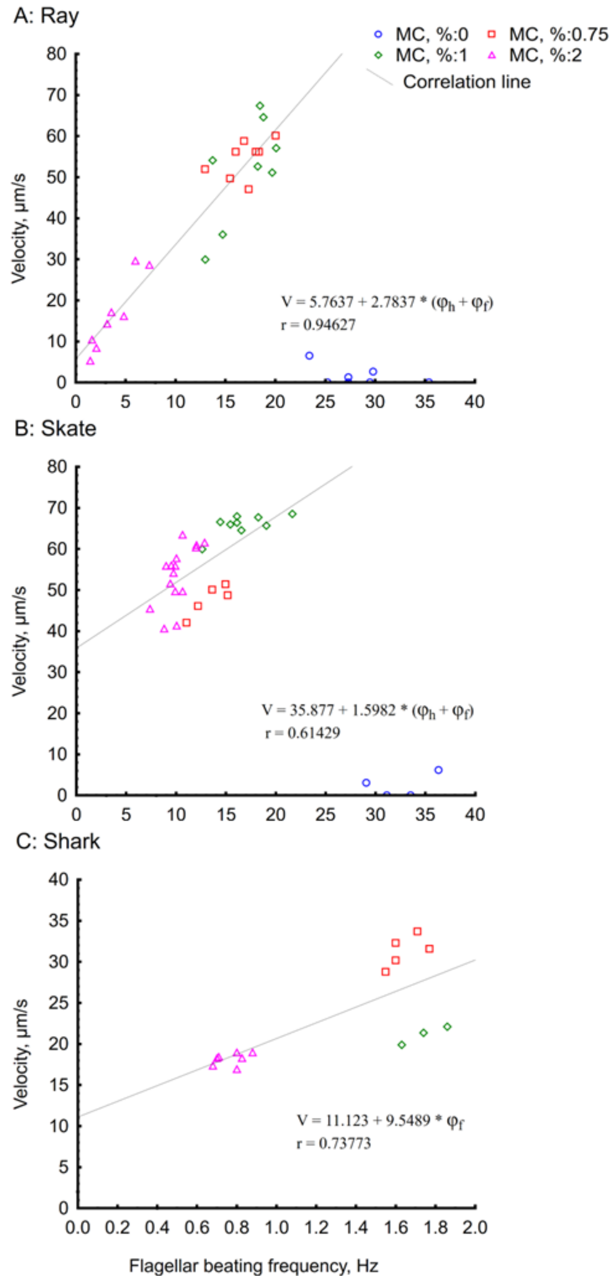


Figure 7. Correlations between velocity and total frequency of a head rotation and flagellar wave progression under conditions of different viscosity (0, 0.75, 1, 2% MC present in ASF) for A: ray *Potamotrygon motoro*; B: skate *Raja asterias*, and C: shark *Scyliorhinus canicula* spermatozoa (in case of shark only the frequency of flagellar wave propagation is present). Each individual point on a graph represents measurements of a single spermatozoon.

3.8. *Steering long sperm chiral bodies in high viscosity*

During the regular motion of such a long, rotational helical head, Elasmobranchii spermatozoa usually swim in a straight line. However, we observed that steering their long helical heads was possible in high-viscosity media through forced buckling of the elastic head, which deformed and bent due to buckling instability (Gadêlha et al., 2010; Jawed et al., 2015; Gadêlha and Gaffney, 2019). Depending on the level of helical buckling, the swimming trajectories ranged from smooth turns (Video S4a) to sharp bends ending in large directional changes (Fig. 8 and Video S4b).

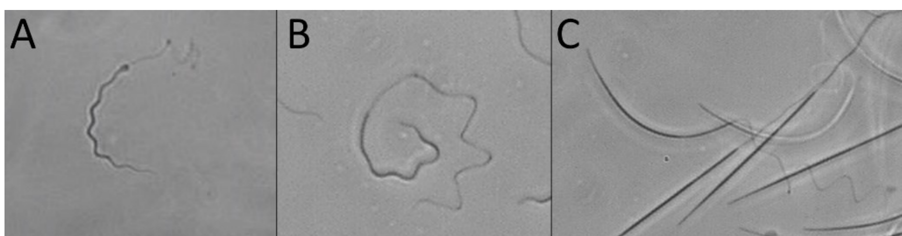


Figure 8. Pictures of motile spermatozoa in 2% MC demonstrates buckling of the head leading to a sperm turning, A: ray *Potamotrygon motoro*; B: skate *Raja asterias*, and C: shark *Scyliorhinus canicular*. See also Video S4.

This helical buckling is unique because while it occurs, the helical head continues to rotate around its longitudinal axis with a high rotational speed frequency and a conserved spinning direction (Video S4b). The head buckling could also be transient, leading to fast and sharp temporal directional changes (Video S4c). No head helical buckling could be observed in LVM. Even when instigated due to the higher friction from the flagellar entangled, the helical head rapidly returned to its unstrained straight, helical configuration (Video S4d). The backward motion of spermatozoa, which could be used to disentangle sperm from spermatozeugmata, was also utilized to change the swimming direction and steer spermatozoa. This swimming reversal re-aligns the long helical sperm head to avoid obstacles or penetrate a more viscous environment (Video S4e).

In our experiments, we also deposited sperm in the centre of the ASF drop under the microscope and observed spermatozoa swimming for several minutes. This allowed us to track spermatozoa motility from the moment sperm were released from the spermatozeugmata until it reached the

boundary of the drop. After release from spermatozeugmata, the spermatozoa moved linearly away from the centre until they reached the edges of the viscous drop, where the properties of media differ (due to evaporation, surface tension, etc.). Precisely at this moment, near the drop boundary, the swimming direction of most of the cells changed, and the process of sperm steering was observed. An example of the different stages of sperm release, the linear progression, and the sperm steering as the drop boundaries are approached can be found in Video S4f.

4. Discussion

The spermatozoa of Elasmobranchii species are bigger than most internal and external fertilizers (Kahrl et al., 2022). This leads to very distinct elastohydrodynamic effects compared to micro-swimmers with smaller sizes. The elastohydrodynamic sperm number parameter (Gadêlha et al., 2010; Gadêlha and Gaffney, 2019), $Sp = L\left(\frac{\eta\omega}{E_b}\right)^{1/4}$, contrasts flagellar bending and viscous forces acting on the tail and is proportional to the flagellar length L, where the viscosity of the fluid is denoted by η, frequency of the beat is ω, and the bending stiffness of the flagellum is E_b. Low Sp values are associated with effectively "stiff" tails, while large Sp is linked with effectively "floppy" behaviour of the flagellum. Having large Sp, due to a very long flagellum or high viscosity of the fluid, poses difficult challenges to be overcome by the sperm, as it makes it harder to propagate waves due to the large elastohydrodynamic dissipation continually acting along the flagellum.

Large Sp also makes the flagellum prone to flagellar buckling (Gadêlha and Gaffney, 2019) and ineffective swimming (Woolley, 2007). Interestingly, mammalian species overcome these difficulties by reinforcing the flagellum with large ultrastructural elements that taper along the flagellum to prevent flagellar buckling and promote cell progression in high Sp regime (Gadêlha and Gaffney, 2019; Leung et al., 2021). Elasmobranchii species, however, retain the longitudinal columns but distribute them helically along the flagellum. These force the flagellum into a helical shape during the beating. Hence, instead of pushing the long head by the beating flagellum, the flagellum rotates around the swimming axis to induce torque around the

elongated chiral head – the helical shape stabilizes the rotation around the head axis. Ultimately, this induces the exquisite corkscrewing motion of the chiral head into the thick fluid environment. However, this mechanism was ineffective in our experiments under low-viscosity environments when Sp is low. Overall, this avoids flagellar buckling and transfers most of the motion in the direction which seems more effective for this size and shape of cell: the rotation of the chiral head. Altogether, the body-to-flagellum chiral architectures allow a matched helical motion, with rotation of the flagellum inducing the corkscrewing of the large-sized head into the media, making swimming more effective in highly viscous fluids. Indeed, our results demonstrated that despite the specific shape and large sizes of the Elasmobranchii spermatozoa, they could reach swimming speeds similar to the much smaller mammalian spermatozoa in a similar medium, as an example of human spermatozoa with an average of 62 µm/s of progressive velocity (Smith et al., 2009).

The above-described physical background for differences in spermatozoa swimming with differentially shaped heads is essential for understanding fundamental relationships between spermatozoon shape and size evolution and its biological performances (Kahrl et al., 2022). Moreover, it is suggested that spermatozoa physiology is a complex subject that should be studied, considering the balance between reproduction strategy, specific physiological environment (conditions of fertilization), and the ability of sperm to adapt, progress, and react to the changes under this environment. Several studies attempted to correlate the sperm size and motility in different species, including fish, mammals, birds, etc. (Briskie and Montgomerie, 1992; Fitzpatrick et al., 2009; Lüpold et al., 2009; Immler et al., 2011; Tourmente et al., 2011), including cartilaginous fish (Kahrl et al., 2021; Rowley et al., 2019), sometimes reporting no or even negative correlation (Stockley et al., 1997; Gage et al., 2002; Gage and Freckleton, 2003; Langen et al., 2019). Most of the studies simplified spermatozoa size to a single parameter, such as flagellar length or whole spermatozoa length, without considering the head size or shape or not optimizing the swimming environment, which could explain the high heterogeneity of results and sometimes contradictory conclusions.

This study attempted to test that the head-to-flagellum shape of Elasmobranchii species is specific to high viscosity, as found in physiological conditions. For this, we explored the spermatozoa performance of three Elasmobranchii species, covering different physiological situations: the spermatozeugmata releasing, progression, and motility regulation. We demonstrated that in all studied species, spermatozoa head-to-flagellum chirality is specific to promote propulsion in high viscosity media that otherwise would not be possible.

4.1. Activation of spermatozoa

All Elasmobranchii species are internal fertilizers. At the same time, the fertilization process itself is not fully understood in these species. Males deposit spermatozoa through specific grooves on claspers (extended pelvic fins) during mating (Walker, 2020). However, these channels are not fully closed, allowing contact with seawater. Also, the sperm is propelled into the female by the accumulation and subsequent release of water from the siphonal sacs in sharks (Gilbert and Heath, 1972; Whitney et al., 2004) or, presumably, by secretions from claspers glands, in rays and skates (Piercy et al., 2016). Thus, sperm motility could be activated by external factors similar to external fertilizers (Cosson, 2004). However, our observation indicates that it is not the case. The spermatozoa flagellum was actively beating in seminal fluid (in undiluted samples) and ASF in all three species, even when it was still compacted inside the spermatozeugmata. If the LVM was used, spermatozoa were not progressive, remained tumbling in the same place, and had difficulties leaving spermatozeugmata (if it was not destroyed mechanically during mixing with ASF).

4.2. Spermatozeugmata and spermatophores

Similar aggregations of spermatozoa, when spermatozoa connected without surrounding matrix (unencapsulated), were observed in different Elasmobranchii species and called spermatozeugmata (Girard et al., 2000; Jones and Hamlett, 2002; Del Mar Pedreros-Sierra, 2015; McClusky, 2015). Clumped spermatozoa were also observed surrounded by additional structures (matrix), thus being embedded inside capsules, so-called

spermatophores, and observed in several other Elasmobranchii species (Wyffels et al., 2019; Pratt and Tanaka, 1994).

Several authors suggest that Elasmobranchii species may store spermatozoa after ejaculation in the terminal zone of the oviducal gland, ensuring a supply of sperm for successive fertilization of ova released during ovulation over a period of several weeks or months (Storrie et al., 2008; Moura et al., 2011). Thus, the presence of spermatozeugmata or spermatophores in ejaculation could have several biological functions. It can keep spermatozoa aggregated during transport along the female reproductive tract if the water flow from the siphonal sacs carries sperm close to the fertilization site (presumably, the oviducal gland). In this case, the aggregations would not disintegrate, as the surrounding medium (water) would be low viscosity. Another possible role can be associated with enhanced motility of the entire group of spermatozoa. This has been suggested for the clusters of spermatozoa that appear in some monotremes and apparently have a similarity with rays (Nixon et al., 2016). Last, these aggregations may be linked to sperm competition between males in polyandrous species where multiple matings have been described, such as elasmobranchs (Lamarca et al., 2020). In this case, the formation of sperm bundles has been considered an adaptive mechanism to improve the reproductive fitness of males due to selective pressure to optimize their sperm delivery. In this way, sperm can arrive more quickly at the storage area or more easily displace sperm from other males (Pearcy et al., 2014; Nixon et al., 2016).

Spermatozeugmata and spermatophores could be stored safely inside the female body for a long time, waiting for a signal to release and start a free active movement to ensure fertilization at the right time. That mechanism could be similar to the synchronization mechanism in external fertilizers when sperm is activated only after ejaculation and contact with water (Dzyuba and Cosson, 2014). Still, the elements of such a signalling cascade should be further investigated. In some mammals, fertilization synchronization is realized through the capacitation process when only a small part of the sperm population is capacitated and ready to fertilize during the ovulation period, thus prolonging the time window for successful fertilization (Perez-Cerezales et al., 2015), but for fishes, sperm capacitation

was described in only a few species (Dzyuba et al., 2019; Pérez, 2020). For many Chondrichthyan species, synchronization may be needed to ensure fertilization immediately after ovulation and before egg encapsulation (Walker, 2020). At the same time, the process of spermatozoa releasing from agglomerations and capsulation of the eggs in Elasmobranchii has not been studied in detail. In our study, the process of sperm unbundling from spermatozeugmata was significantly accelerated when the HVM was used for sperm dilution, resulting in a burst of spermatozoa motility in all three studied species. Thus, the increased viscosity may play a role in the trigger of sperm unbundle in these species.

Moreover, we observed cytoplasmic sleeves in all of the studied species. From our results, we cannot conclude the exact function of these sleeves and if they are involved in spermatogenesis, playing the Hermes body's role, as suggested before (Wyffels et al., 2019). The Hermes body, or the cytoplasmic droplet, is a component of the sperm flagellum unique to epididymal spermatozoa. Its role and function are unclear, but several authors suppose it might be associated with the maturation process and acquisition of sperm motility (Au et al., 2015; Hermo et al., 2018). Our results confirm that the sleeves are not required for proper motility of spermatozoa. Instead, it could be associated with the unbundling process since, spermatozoa remove them while exiting the spermatozeugmata. Thus, we suggest that the cytoplasmic sleeves connect spermatozoa inside spermatozeugmata. In that case, the spermatozoon can rotate around its axis inside the sleeve (like inside a tube) while still connecting to other spermatozoa inside the spermatozeugmata.

It should be mentioned that some of the authors reported sperm storage at the oviducal gland involving only individual spermatozoa, which are basically stacked inside the gland tissue in a terminal zone (Hamlett et al., 2002). Currently, there is no direct indication of exactly how the storage of sperm is maintained. Many questions still exist and require further study and deeper consideration: Do the spermatophores or spermatozeugmata preserve their structure during storage inside the female reproductive tract? What are the exact triggers of spermatozoa activity and unbinding process in species with different sperm aggregation structures? Are the cytoplasmic sleeves involved in organizing spermatozoa inside spermatozeugmata or spermatophores,

and what is their exact function? How do spermatozoa aggregates impact sperm competition and cryptic female choice in different species? All of those questions are fundamental for a general understanding of the reproduction strategy. They should be further considered considering specific spermatozoa physiology and their ability to progress and adapt to specific environmental viscosity.

4.3. Sperm progression

After being released from spermatozeugmata, nearly 100% of spermatozoa moved straightforwardly in HVM. However, spermatozoa stayed almost nonprogressive in LWM, even though the flagellum is active and head rotation frequency is high. In this situation, the rotating spermatozoa head could move the liquid around without effective propulsion, while flagellar waves did not produce enough forces to push forward cells with such a large chiral head. When the medium viscosity increased, the situation changed. Instead of rotation without propulsion, the spermatozoon head began to screw into the media with the help of the helical shape head and the helical flagellar wave, thus progressing forward. We could observe high viscosity effects on the flagellum: more symmetrical flagella wave, decreasing wave amplitude and wavelength as described previously (Smith et al., 2009). Consequently, spermatozoa began to swim more straightforwardly since the side-to-side movement of the head and flagellum was limited by increased viscosity. Nevertheless, Elasmobranchii spermatozoa (particularly ray spermatozoa) were prone to wave compression (Woolley and Vernon, 2001; Woolley, 2007). The lateral to midpiece part of the flagellum remained highly active, producing high-frequency shallow amplitude waves. This behaviour suggests that different parts of the flagellum could be regulated differently, depending on the environment, leading to the appearance of varying shapes of waves along the flagellum. During such motion, the specific helical shape of the head contributes to the progression since the spermatozoon is screwing into the medium. It is possible only due to the functioning of the flagellum, which in this respect not only pushes spermatozoa forward but also generates the rotation of the whole spermatozoa.

The recent publication of Wang et al. suggested that head shape could contribute up to 31% efficiency of forces used for spermatozoon

propagation/movement. The authors tested the Heterogeneous Dual Helical Propulsion Mechanism, explaining the high adaptability of ray spermatozoa to different viscosities. In many aspects, the observed spermatozoa behaviour that they described, including the rotational basis of sperm progression, rotation frequency, and velocity of spermatozoa, are very consistent with our observation, except for the effect of viscosity on spermatozoa progression. In its case, the most efficient progression of spermatozoa was observed in LVM. Our study observed the highest velocity for shark sperm at 0.75% MC, at 1% MC for ray and skate, and a slight decrease in higher viscosities. This difference could be related to species-specific differences in motility regulation or the differences in the chemical effect of compounds used to create viscosity (MC in our study and alginate in Wang et al. study) and will require further investigations. Interestingly, Wang et al. observed similar tail deformation in HVM due to the curling of the flagellar tip and entangling of the flagellum on the helical head, a typical observation in our study for ray spermatozoa in LVM. Critically, in HVM we found that close to 100% of observed spermatozoa were progressive, showing efficient forward movement with highly symmetrical flagellar waves.

As discussed above, the properties of spermatozoa observed in our study for Elasmobranchii could be easily applied to explain sperm motility in any other species with similar shaped spermatozoa as an adaptation to high viscosity during fertilization, for example birds and amphibians (Muto and Kubota, 2009; Schmoll et al., 2020). This suggests that medium viscosity is a highly overlooked parameter affecting sperm behaviour in many species. It potentially provokes adaptation by elongating the head and applying a helical structure for the rotational mode of progression in large-sized evolutionarily conserved sperm types and/or decreasing size and simplification of structure in later diverged taxa.

4.4. Sperm navigation/directional changes

After being released and finding proper conditions for cell progression, all spermatozoa continue to move straightforwardly with the primary goal of locating and fertilizing the egg. Recently, the common hypothesis that fertilization is random has been replaced with the guidance hypothesis

(Eisenbach and Giojalas, 2066). According to this hypothesis, spermatozoa sense the changes in the environment and adapt their motility accordingly, thus increasing the chance of encountering/meeting the egg. If the cells can control motility dynamically (time) and spatially (navigation), the opportunities for fertilization will be highly improved. Thus, the ability of spermatozoa to react to environmental changes may predetermine the fertilization success and increase the value of such males, providing additional selection criteria. Our study showed the sperm's ability to steer and change direction successfully despite the large sizes and complex motility mechanism. This is done by uniquely exploiting the head buckling in high viscosity. Simpler buckling phenomena have been explored by bacterial hook flagellum to induce directional changes in navigation (Son et al., 2013).

In our study, in addition to the already observed and discussed phenomenon of the backward motion of spermatozoa (Minamikawa and Morisawa, 1996; Wyffels et al., 2019), we also demonstrated that this is involved in sperm release from the bundle. Moreover, we observed that these long helical spermatozoa could exploit a helical buckling instability of their heads to change swimming direction in an advanced strategy utilized by other microorganisms in simpler, smaller parts of their body (Son et al., 2013), as opposed to the large-scale buckling observed here. This mechanism is probably passive and may appear in a situation when high forces coming from the flagellum (relatively high flagellar amplitude) push the spermatozoon head forward while rotational speed slows down. The same spermatozoon can quickly change the bending/turning direction and thus could respond rapidly to changes in properties of the surrounding medium. Interestingly, the media cannot keep the bent head's elastic forces in LVM conditions due to the fast relaxation time associated with an effectively “stiff” head (Coy and Gadêlha, 2017), similar to a low Sp regime, as discussed above. In such situations of LVM, we only observed the head bending after the tail entangled the spermatozoa head during backward wave propagation in ray spermatozoa (since it could not propagate and rotated in one place). During this process, the spermatozoa head was bent, and after a while, the flagellum was released, leaving the head free again. When the head was not held by the flagellum, its shape immediately returned to its unstressed straight configuration, indicating the head shape did not possess intrinsic

curvatures. Apart from forming its helical shape, it responds elastically as a passive structure.

5. Conclusions

We investigated the spermatozoa behaviour of three Elasmobranchii species, the presence of spermatozeugmata, the bundle formation and unbundling processes, their progressive motility, and directional changes in navigation. Environmental viscosity was key in all aspects of spermatozoa motion and specific set of features to allow motion in high viscosity conditions. Our results suggest that these cells perform optimally in high-viscosity media and should be considered for future spermatological studies and possible artificial reproduction in these important species. The observed chiral head-to-flagellum architecture in these spermatozoa may suggest that high viscosity is one of the main environmental conditions affecting and shaping the spermatozoa and their performance during evolution to the state in which we observe them now.

Limitations of the study

The study's main limitations are connected with the sample size used for observation during experiments, which is linked to the accessibility of mature males in these species. Moreover, the specific helical structure of spermatozoa (the helical motion occurs in 3D) restricts our measurements to manual estimations of each individual spermatozoon motion characteristics, which in turn requires more time and effort spent for the acquisition of reliable quality records and future analyses per sample in a specific experimental condition. At the same time, our results and conclusions are based not only on observing individual spermatozoa but also on their collective behaviour (when nearly 100% of spermatozoa responded), which was highly repeatable between replicates and consistent between samples received from different males in different species.

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GENERAL DISCUSSION

1. Reproductive Anatomy: notes on sperm extraction

Chondrichthyans constitute an ancient lineage of cartilaginous fishes that dates back over 400 million years. Throughout their evolutionary history, a multitude of species have emerged, with over 1400 species existing today. This significant diversity is reflected in their life histories, behaviours, body plans, and subtle anatomical variations, which can pose challenges when applying assisted reproductive techniques such as artificial insemination (AI) and sperm extraction.

Both are promising techniques in management and development of breeding plans (Penfold and Wyffels, 2019; Wyffels et al., 2021a; Adams et al., 2022). However, to properly develop the procedures, knowledge of the reproductive anatomy is crucial. Due to the invasive nature of some of these techniques, and the disposition of certain delicate structures, damage may occur that could have been avoided. In addition, the outcome of both procedures can be greatly influenced by knowledge of the anatomy. In AI the site of sperm deposition is key to a successful outcome, while in sperm extraction the effectiveness of different procedures may vary (Luer et al., 2008; Daly and Jones, 2017; Daochai et al., 2020).

Abdominal massage compresses the seminal vesicles (or analogous structures involved in sperm storage) for sperm collection, without requiring extensive anatomical knowledge or specific materials. The procedure yields a substantial volume of sperm flowing through the urogenital papilla and can be easily collected. However, there are several limitations to be considered, such as the amount of sperm in the sperm storage structures, the presence of certain anatomical features, and contamination risks from urine, feces, or the environment. Despite drawbacks, it is a quick and relatively easy technique, providing samples of good quality and volume unless aseptic conditions are required.

Cannulation is the optimal technique for obtaining specific, uncontaminated samples, especially when the sperm quantity is limited. By inserting a cannula through the urogenital papilla, high-quality samples can be obtained, extracting most of the stored sperm. However, it has limitations: the animal must remain in tonic immobility or be anesthetized, and a thorough knowledge of species anatomy is necessary to avoid internal damage. However, ultrasound guidance can compensate for this limited anatomical

knowledge. With adequate time, resources, and knowledge, cannulation yields excellent-quality sperm.

The techniques discussed so far allow the obtention of the sperm from terminal areas of the reproductive system. While cannulation allows access to the final part of the *vas deferens*, it is not recommended to collect sperm from upstream areas due to the ducts' fragility. To obtain sperm from these regions, dissection of deceased animals is commonly performed, while micropuncture can be also done on live sharks (Jones et al., 1984). Dissection offers direct access to the sperm storage structures, enabling retrieval of all stored sperm in a clean and aseptic manner, even from animals with limited sperm volume. In addition to males, female animals can also have sperm stored in the oviductal gland, which can be obtained by scraping the internal lumen. Although the amount of recovered sperm in females is typically limited, it suffices for morphology, motility, integrity, and potential insemination studies. Internal reproductive structures maintain their integrity after death for some time, and spermatozoa exhibit remarkable stress tolerance (Montano et al., 2023). Under favorable conditions (especially low environmental temperature or refrigeration), sperm can be recovered in good condition even days after death. This capability helps to conserve the reproductive potential of adult individuals, even after their death, providing another tool for their conservation.

2. Development of sperm preservation protocols

Many elasmobranch species currently face an uncertain future due to anthropic pressures on nature. In such circumstances, all possible options available to help conserve the populations of these species should be evaluated. Captive breeding of organisms using assisted reproduction techniques has made it possible to carry out conservation strategies (both *ex situ* and *in situ*) in species that were on the verge of extinction. Unfortunately, in sharks, rays and chimaeras, the application of assisted reproductive techniques has only recently begun to be considered. This is especially true in the case of sperm preservation, where, despite the promising results with Myliobatiformes (Daly et al., 2011; Daly and Jones, 2017), cryopreservation of shark sperm was an elusive goal.

it was not until the work explained throughout chapter 3 of this thesis that the cryopreservation of small-spotted catshark *Scyliorhinus canicula,* and the rough skate *Raja radula* sperm was achieved for the first time, using methanol, DMSO and fresh egg yolk as cryoprotectants (García-Salinas et al., 2021a). This accomplishment represented the first-ever cryopreservation of shark sperm. By using these species as model organisms, protocols for sperm collection, handling, and preservation were refined, providing valuable insights for future conservation efforts involving threatened species such as the bull ray *Aetomylaeus bovinus* (globally Critically Endangered) (Fig.8) or the blue shark *Prionace glauca* (Critically Endangered in the Mediterranean Sea) (Fig.9).

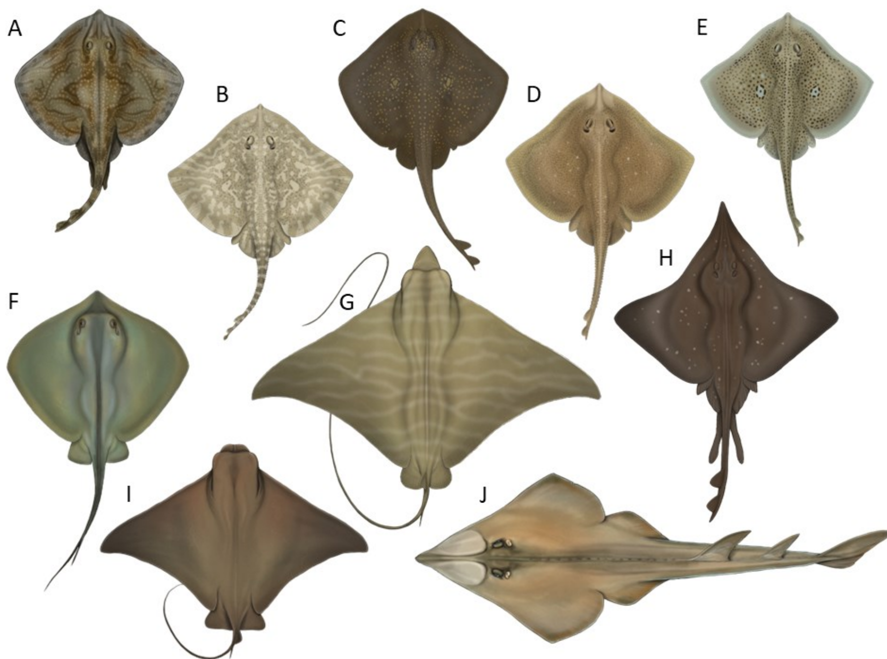

Figure 8. Batoid species used in our sperm cryopreservation trials: A-*Raja undulata*, B-*R. clavata*, C-*R. radula*, D-*R. asterias*, E-*R. montagui*, F-*Dasyatis pastinaca*, G-*Aetomylaeus bovinus*, H-*Dipturus oxyrinchus*, I-*Myliobatis aquila*, J-*Glaucostegus cemiculus*.

Afterwards, other authors were able to cryopreserve sperm from the southern stingray *Hypanus americanus* (Gillis et al., 2021a), and the white spotted bambooshark *Chiloscyllium plagiosum* (Gillis et al., 2021b), using egg yolk with glycerol, DMSO or the combination of glycerol with N-methyl formamide. Later, the cryopreservation of the first non-elasmobranch

chondrichthyan, the rabbit fish *Chimaera monstrosa*, was achieved (García-Salinas et al., 2022). Last, the sperm of the small gulper shark *Centrophorus uyato*, and the longnose skate *Dipturus oxyrinchus*, both deep-sea species, was preserved. Notably, the critically endangered blackchin guitarfish *Glaucostegus cemiculus*, one of the most endangered ray species globally, also had its sperm cryopreserved (García-Salinas et al., 2023).

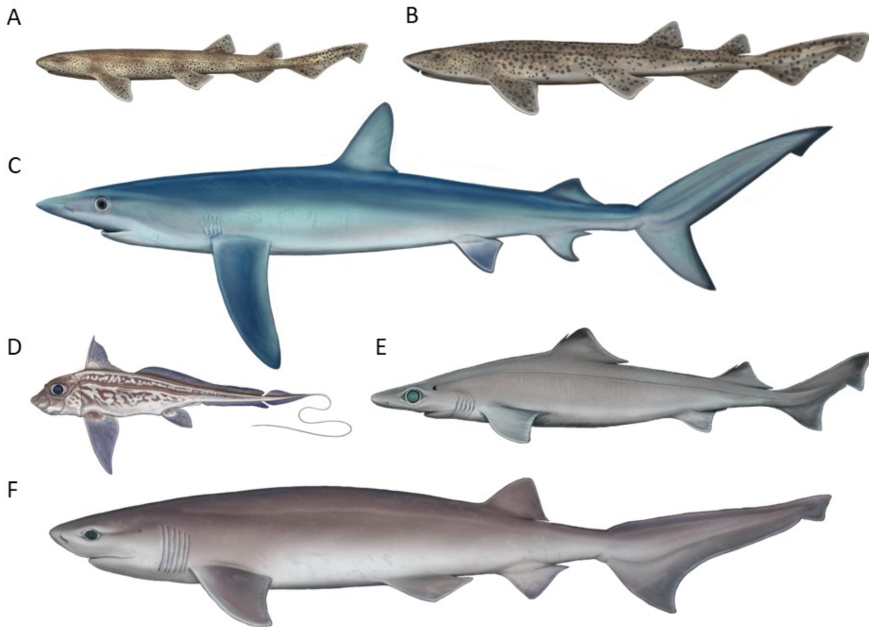

Figure 9. Selachian and holocephalan species used in our sperm cryopreservation trials: A-*Scyliorhinus canicula*, B-*S. stellaris*, C-*Prionace glauca*, D-*Chimaera monstrosa*, E-*Centrophorus uyato*, F-*Hexanchus griseus.*

Despite these results, standardization of procedures for sperm preservation in elasmobranchs remains a challenge. Different authors have varied approaches for sample collection, short-term storage, and freezing methods, resulting in a lack of common protocols (Penfold and Wyffels, 2019). Also, comparing methodologies is particularly difficult due to the inherent variability among species. Until now, sperm cryopreservation has been successfully achieved in 19 chondrichthyan species (Table 1).

Table 1. This table shows the 19 chondrichthyan species in which sperm cryopreservation has been successfully performed to date (12 batoids, 6 selachians and one holocephalan).

Common name	Scientific name	Reference
Sparsely Spotted Stingaree	*Urolophus paucimaculatus*	Daly et al., 2011
Australian Bull Ray	*Myliobatis australis*	Daly and Jones, 2017
Small-Spotted Catshark	*Scyliorhinus canicula*	García-Salinas et al., 2021a
Nursehound	*Scyliorhinus stellaris*	García-Salinas et al., 2021a
Blue Shark	*Prionace glauca*	García-Salinas et al., 2021a
Bluntnose Sixgill Shark	*Hexanchus griseus*	García-Salinas et al., 2021a
Rough Skate	*Raja radula*	García-Salinas et al., 2021a
Spotted Skate	*Raja montagui*	García-Salinas et al., 2021a
Mediterranean Starry Skate	*Raja asterias*	García-Salinas et al., 2021a
Thornback Skate	*Raja clavata*	García-Salinas et al., 2021a
Common Stingray	*Dasyatis pastinaca*	García-Salinas et al., 2021a
Bull Ray	*Aetomylaeus bovinus*	García-Salinas et al., 2021a
Southern Stingray	*Hypanus americanus*	Gillis et al., 2021a
White Spotted Bamboo Shark	*Chiloscyllium plagiosum*	Gillis et al., 2021b
Rabbit Fish	*Chimaera monstrosa*	Garcia-Salinas et al., 2022
Gulper Shark	*Centrophorus uyato*	García-Salinas et al., 2023
Longnose Skate	*Dipturus oxyrinchus*	García-Salinas et al., 2023
Blackchin Guitarfish	*Glaucostegus cemiculus*	García-Salinas et al., 2023
Undulate skate	*Raja undulata*	García-Salinas et al., 2023

To begin with, there is no standardization when it comes to choosing the medium for diluting elasmobranch spermatozoa post-extraction. In our study, we developed an artificial seminal plasma extender that outperformed existing formulations in terms of the capacity to maintain sperm over time. This allowed us to work with the sperm for several days and see its evolution over time. Usually, to achieve short- or medium-term preservation, commercial formulations modified to suit the peculiarities of elasmobranchs are generally used. Although they receive common names, such as Hanks' solution adapted to elasmobranchs (Gillis et al., 2021a, 2021b), or elasmobranch ringer (Daly et al., 2011; Daly and Jones, 2017), in practice there are considerable differences in the ionic composition between studies (Luer et al., 2008; Wyffels et al., 2021a; Montano et al., 2023).

Differences are particularly notable in trimethylamine oxide (TMAO) concentration, calcium levels, and energy sources (glucose, fructose, or sucrose) for cells. Similarly, variations exist in the composition of artificial water (due different commercial brands) among the studies. The consistent motility and membrane integrity responses of cells in solutions with diverse ionic compositions suggest that osmolarity plays a more significant role than solute ratio (Daly and Jones, 2017; Montano et al., 2023). However, consensus should be established regarding the essential elements in the medium to distinguish between experimental and interspecific variability.

Interspecific variability is clearly exemplified in cryopreservation protocols. In our study, sharks, stingrays (Myliobatiformes), and skates (Rajiformes) showed significant differences in freezing response. Notably, *Hexanchus griseus* demonstrates exceptional cryopreservation tolerance (corroborated in a second individual after the original publication), warranting further research. Cryoprotectant effectiveness depends on concentration, equilibration time and potential toxic effect on cells. As an example, glycerol proves ineffective (Gillis et al., 2021b) and probably toxic (Daly and Jones, 2017) for the white spotted bamboo shark *Chiloscyllium plagiosum,* while has been effective in several species of rays (Daly et al., 2011; Daly and Jones, 2017; Gillis et al., 2021a). Another cryoprotectant, DMSO, used in other species of sharks, rays and a chimera (Daly et al., 2011; García-Salinas et al., 2021a, 2022; Gillis et al., 2021a) hardly has a positive effect on *C. plagiosum*, or on the common stingray *Dasyatis pastinaca* and *A. bovinus* (García-Salinas et al., 2021a; Gillis et al., 2021b). These two ray species are Myliobatiformes, as are the ray species where glycerol has been shown to be useful, so it would be appropriate to include this compound in future tests with both species. The only compound that appears to be useful in all species to date is egg yolk. However, its use should be standardized, either diluted and centrifuged (Daly et al., 2011; Gillis et al., 2021a), freeze-dried and hydrated, or fresh from the egg (García-Salinas et al., 2021a).

Just as cryoprotectants could have an interspecific effect, so could the freezing process speed. Good outcomes obtained by Daly and Jones (2017) in the Australian bull ray *Myliobatis australis* were attributed to a high freezing speed (>10 °C/min), especially when comparing with a previous study with the sparsely spotted stingaree *Urolophus paucimaculatus* (3 °C/min) (Daly et al., 2011). Opposed to this Gillis et al., 2021 showed an

improvement in cryopreservation processes employing a slow (2 °C/min) versus a fast (~45 °C/min) freezing method, both in the case of the southern stingray *Hypanus americanus*, and in the case of *C. plagiosum.* The container used for freezing samples also plays a role to be considered. Some studies have used 0.25 ml straws (Daly et al., 2011; Gillis et al., 2021a) or 0.5 ml straws (Gillis et al., 2021b). Others, on the other hand, have preferred to use 1.5 ml cryotubes, achieving lower freezing speeds (García-Salinas et al., 2021a, 2022, 2023).

The lack of standardization, and their interspecific variations, means that it will probably take a long time, if ever, to find a single cryopreservation method for all chondrichthyan species. However, ongoing research offers hope for establishing assisted breeding programmes in a variety of threatened species. We are still in the early stages of knowledge and technique development, but in the future, these methods could enable *ex situ* and *in situ* conservation strategies. Whether in aquaria, monitored areas and research centres, or reinforcing wild populations with new recruits, new alleles or triggering reproductive events. For sensitive endangered species, these techniques may provide a unique opportunity to avoid extinction.

3. Viscosity, morphology, and its importance

When studying the sperm of this group of animals, three distinct characteristics stand out. Firstly, the spermatozoa are relatively large (142 µm on average) compared to the sperm of other fish (Tanaka, 1995). Secondly, their heads possess a helical shape with varying numbers of turns depending on the species (Tanaka, 1995). Thirdly, the spermatozoa are grouped in complex structures, both spermatozeugmas and spermatophores, which vary according to the group. The larger size of the sperm can be attributed to the internal fertilization, since species with this strategy have longer sperm than those species with external fertilization (Kahrl et al., 2021). The exact purpose of these sperm aggregations is yet to be fully understood. It may serve as a mechanism to protect and facilitate sperm transport during ejaculation or enable synchronized swimming (Nixon et al., 2016). It could also be a strategy for sperm competition in polyandrous species, allowing males to deliver genetic packets and displace sperm from previous matings (Pearcy et al., 2014; Nixon et al., 2016; Lamarca et al., 2020). Chapter 4 of this study aimed to explain the helical shape of the sperm

as an adaptive response to the more viscous environment of the uterus compared to seawater.

Morphology has a direct impact on the hydrodynamic characteristics of microorganisms immersed in a liquid environment (Lisicki et al., 2019), so the peculiar shape and size of the spermatozoa must be linked to their displacement. A parameter that relates displacement to the morphological characteristics of spermatozoa and their environment is the elastohydrodynamic sperm number (Sp) (Gadêlha and Gaffney, 2019):

$$(Sp): Sp = L(\eta\omega/E_b)^\wedge(1/4)$$

In summary, lower Sp values are related to better swimming performance, and higher values to worse performance. Thus, exceptionally long flagella (L), high viscosity media (η) or low flagellum stiffness (E_b) led to worst performances by hindering the propagation of waves along the axis of displacement. However, elasmobranch flagella compensate for this by having structures resembling longitudinal columns, which force the beating filament to adopt a helical shape. This helical beating promotes forward movement of the spermatozoa and causes the head to rotate along its axis. Then, the helical head contributes to forward movement by screwing into the medium, maintaining rotational stability and preventing bending. Consequently, elasmobranch spermatozoa demonstrate efficient displacement in viscous media but less effectiveness in low-viscosity media.

Assessing the motility of chondrichthyan sperm in an aqueous solution proved challenging during the third chapter of our study. And, despite exhibiting high flagellar movement (rotating on their axis, coiling their flagella, or beating) the cells demonstrated limited forward movement. However, we observed a significant improvement when we realized the role of medium viscosity. By increasing viscosity using methylcellulose (MC) in the diluent, the cells began to exhibit excellent longitudinal axis movement. Specifically, a concentration of 1% MC in skates and stingrays, and 0.75% in sharks, resulted in optimal displacement.

Increasing the viscosity revealed several specific behaviours of the spermatozoa. Instead of moving in a straight line, the cells exhibited the ability to change direction, bend their trajectories, and even move backwards before redirecting. These behaviours may serve as a guidance system to locate and fertilize the egg or reach the oviductal gland for storage. For other

species the guidance hypothesis proposes that spermatozoa are able to respond to environmental changes to achieve their objective (Eisenbach and Giojalas, 2006). It would be suggestive that in chondrichthyans, instead of active chemotaxis towards a given compound, attraction could be due to passive chemotaxis, following a viscosity gradient that promotes displacement.

Possibly the most unexpected and visually striking findings of this research concerned the sperm aggregates. Altering the viscosity of the medium resulted in the disintegration and immediate release of the spermatozoa from these structures. Although the species studied exhibited different aggregate morphologies, it is likely that these aggregates encounter an environment with the appropriate viscosity in the female's uterus.

The insights gained from the study presented in chapter 4 hold significant importance when developing and modifying assisted reproductive techniques. Understanding the effect of the environment on spermatozoa helps us comprehend the mechanisms of cell storage in females, and how to improve the chances of fertilization during artificial insemination. Similarly, in the context of short- and long-term preservation, it is crucial to understand the behavior of the cells. For instance, during spermatozoa storage, the cells tend to settle quickly, resulting in concentration disparities between the medium and the cells.

Using a medium with a higher viscosity would allow a more homogeneous distribution within the storage tubes, which is particularly vital in cryopreservation processes. The cryoprotectants need to reach the cells at planned concentrations, and this is challenging if the cells cannot distribute evenly throughout the diluent. Moreover, during cryopreservation, it has been observed that cell mortality rate within the aggregates is almost absolute. The survival of spermatozoa could be hindered due to difficulty in being permeated by the cryoprotectants, temperature differences, high sedimentation rate, or other variables. Given that viscosity rapidly disaggregates these structures, future cryopreservation media should be designed to increase its viscosity.

4. Future perspectives

When it comes to the development and application of techniques for the control of elasmobranch reproduction we are still in our infancy. To make progress, it is essential to expand our understanding of the biology, anatomy, and ecology of various species, from cellular level to the entire organism. The more comprehensive our knowledge becomes, the better equipped we will be to address new challenges working with different groups.

Future work should continue with:

Detailed descriptions of reproductive systems: The list of species should be further expanded, as phylogenetically separated from each other as possible. Descriptions should consider the direct applicability to the procedures described throughout the thesis. The use of technologies such as ultrasound or computed tomography scans allows to obtain three-dimensional models in anatomical position of seminal vesicles, sperm sacs and other structures of interest, so their use in these descriptions should be encouraged.

New cryoprotectants: The list of cryoprotectants should be further expanded, testing new combinations and toxicity tolerances in different species for interspecies variation. Recently, the use of combinations of trehalose and DMSO or glycerol has been suggested, a combination of external and internal cryoprotectants that may be interesting in view of previous results with these compounds (Montano et al., 2023). Similarly, the use of additional substances, such as antioxidants, could be assessed to improve survival after freezing (Len et al., 2019).

Check for damage to genetic material: Assessment of morphology, motility, and membrane integrity have now been used as indicators of cell damage after freezing (Daly et al., 2011; Daly and Jones, 2017; García-Salinas et al., 2021a, 2022, 2023; Gillis et al., 2021a, b). However, damage to the genetic material is possible, but none of these three procedures shows it directly. The comet assay technique should be tried to observe the degradation of genetic material, as is done for other species after freezing (Balamurugan et al., 2019; Lee et al., 2021).

Experiment with different freezing speeds: The speed of freezing could be of critical importance for the successful cryopreservation of some species (Daly et al., 2011; Gillis et al., 2021b). For this reason, future cryopreservation

experiments could use controlled cooling rates (using biofreezers) to observe possible differential responses in freezing tolerance as a function of the rate of temperature change.

Standardize the use of CASA: Until recently, the use of CASA (computer-assisted sperm analysis) has not been possible due to the size and shape of the sperm cells. Technical modifications developed by Montano et al. (2023) seem to have solved this setback and should be implemented in future studies. In addition to the formulation of diluents that allow the free movement of sperm, other parameters such as linear or curvilinear velocity could be accurately assessed.

Artificial inseminations using cryopreserved sperm: There would be no point in developing cryopreservation procedures for sperm if it could not realistically be used for artificial insemination. The technique of insemination with fresh sperm has been tested in elasmobranchs with varying degrees of success (Masuda et al., 2003; Luer et al., 2008; Daochai et al., 2020; Wyffels et al., 2021a; Adams et al., 2022), but it has not yet been combined with the use of cryopreserved sperm, so this line of work should be a priority. The use of the technique in aquaria would allow monitoring of gestation in females (or embryonic development in the egg) and monitoring of the ontogeny of the neonates to ensure their correct development.

We are beginning to develop techniques and procedures that could be the ultimate lifeline for a multitude of threatened species. However, there is still an overwhelming amount of knowledge yet to be gained. Dealing with the vast variability and diversity within this group of species presents a significant challenge. Nevertheless, similar challenges have been faced in the past with other animal groups, from birds to molluscs, and the situation has been reversed. Initiatives such as the stAR project (https://www.reshark.org/project-star) are attempting to demonstrate that breeding and reintroducing sharks into the wild, it is feasible, by using a network of aquaria and assisted reproduction specialists around the world. It just takes effort, time, and resources. Not all species may require these techniques, and some may present practical limitations. But it is certainly better to have the techniques ready when they are needed than not to have them at all. There is still a long journey ahead, with much to learn and evaluate, but we must act swiftly and boldly because, for many of these species, time is running out.

CONCLUSIONS

Throughout the successive chapters presented in this document, the aim has been to expand knowledge about the use and application of assisted reproductive techniques in elasmobranchs. This relatively unexplored field of knowledge has the potential to become a key tool for management and conservation plans for these species in the near future. Among the most relevant achievements of this thesis, the following stand out:

i. The anatomy of the reproductive system of 18 species of elasmobranchs, and one holocephalan has been described, having a special focus on those structures that should be considered when performing sperm extraction and artificial insemination procedures: urogenital pores, sperm storage structures, uteri openings, and specific glands.

ii. Four methods of obtaining sperm from chondrichthyans have been described to obtain quality samples considering the purpose of the samples and the condition of the animals. Abdominal massage allows medium quality samples to be obtained with minimal resources and handling. Cannulation allows better quality samples to be obtained but requires more knowledge of the species and specific equipment. Dissection allows the recovery of excellent quality samples in males and females from areas that cannot be reached by other techniques. Dissection of the oviductal gland yields stored sperm, which, although motile and of good quality, is very scarce.

iii. An artificial seminal plasma capable of maintaining, at 4°C, elasmobranch sperm motility at values of 60-65% after 12 days and 40% after 28 days, for initial motility of 85-90%, has been developed.

iv. By adding various cryoprotectants (methanol, dimethyl sulfoxide, and fresh egg yolk), sperm from 15 species of chondrichthyans have been cryopreserved. For the first time, cryopreservation of sperm from sharks, skates or holocephalans has been achieved.

v. The best results for sharks were obtained when sperm were diluted 1:9 with the combination of 5% methanol, 5% DMSO and 10% egg yolk, in artificial seminal plasma, with motility and post-freezing membrane integrity values close to 35%. In the case of rays, the best results (close to 30%) were obtained using 10% DMSO.

vi. The study of spermatozoa behaviour in three species of elasmobranchs, using high-speed video microscopy, has allowed us to examine the kinetics of flagellum and head movement, the process of spermatozeugma disintegration and changes in cell navigation. Spermatozoa displacement is due to the combined action of head rotation and flagellum beating in the medium.

vii. Our results suggest that these cells perform optimally in high viscosity media. Optimal displacement and spermatozeugma disintegration were achieved at % MC concentration in skates and stingrays, and 0.75% MC concentration in sharks. Possibly this feature is one of the main environmental conditions that have driven the evolution of spermatozoa to improve their performance during reproduction.

REFERENCES

Adams, L., Lyons, K., Larkin, E., Leier, N., Monday, J., Plante, C., et al. (2022). Artificial Insemination and Parthenogenesis in the Zebra Shark *Stegostoma tigrinum*. *Front. Mar. Sci.* 9, 886616. doi:10.3389/fmars.2022.886616.

Alves, L. M. F., Nunes, M., Marchand, P., Le Bizec, B., Mendes, S., Correia, J. P. S., et al. (2016). Blue sharks (*Prionace glauca*) as bioindicators of pollution and health in the Atlantic Ocean: Contamination levels and biochemical stress responses. *Sci. Total Environ.* 563–564, 282–292. doi:10.1016/j.scitotenv.2016.04.085.

Amaral, C. R. L., Pereira, F., Silva, D. A., Amorim, A., and de Carvalho, E. F. (2018). The mitogenomic phylogeny of the Elasmobranchii (Chondrichthyes). *Mitochondrial DNA Part A* 29, 867–878. doi:10.1080/24701394.2017.1376052.

Araújo, P. R. V., Oddone, M. C., and Velasco, G. (2016). Reproductive biology of the stingrays, *Myliobatis goodei* and *Myliobatis ridens* (Chondrichthyes: Myliobatidae), in southern Brazil. *J. Fish Biol.* 89, 1043–1067. doi:10.1111/jfb.13015.

Arciniegas, S. P., Rodríguez, J. M., and Casallas, P. E. (2005). Ensayos preliminares sobre crioconservación de semen de bagre rayado/ Preliminary essays on crioconservation of semen of catfish “lined bagre”. *Orinoquía* 9, 28–37.

Aristotle (1992). *Investigación sobre los animales, Historia animalium (J. Pallí Bonet, Trans)*. Gredos, Biblioteca Clásica: Barcelona.

Asturiano, J. F., Cabrita, E., and Horváth, Á. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. *Gen. Comp. Endocrinol.* 245, 69–76. doi:10.1016/j.ygcen.2016.06.019.

Au, C. E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Kearney, R. E., et al. (2015). Compartmentalization of membrane trafficking, glucose transport, glycolysis, actin, tubulin and the proteasome in the cytoplasmic droplet/Hermes body of epididymal sperm. *Open Biol.* 5, 150080. doi:10.1098/rsob.150080.

Awruch, C. A., Somoza, G. M., and Baldock, C. (2019). Chondrichthyan research in South America: Endocrinology overview and research trends over 50 years (1967–2016) compared to the rest of the world. *Gen. Comp. Endocrinol.* 273, 118–133. doi:10.1016/j.ygcen.2018.06.005.

Babiak, I., Glogowski, J., Kujawa, R., Kucharczyk, D., and Mamcarz, A. (1998). Cryopreservation of Sperm from Asp *Aspius aspius*. *Progress. Fish-Culturist* 60, 146–148. doi: 10.1577/1548-86401998060<0146:COSFAA<2.0.CO;2.

Balamurugan, R., Prapaporn, W., and Munuswamy, N. (2019). Sperm activation and effects of cryopreservation on motility, ultrastructure and DNA integrity in Grey mullet *Mugil cephalus*. *Aquac. Reports* 14, 100204. doi:10.1016/j.aqrep.2019.100204.

Ballantyne, J. S., and Fraser, D. I. (2012). “Euryhaline Elasmobranchs,” in *Fish Physiology*, (Academic Press), eds. S. D. McCormick, A. P. Farrell, and C. J. Brauner, 125–198. doi:10.1016/B978-0-12-396951-4.00004-9.

Barnett, L. A. K., Earley, R. L., Ebert, D. A., and Cailliet, G. M. (2009). Maturity, fecundity, and reproductive cycle of the spotted ratfish, *Hydrolagus colliei*. *Mar. Biol.* 156, 301–316. doi:10.1007/s00227-008-1084-y.

Baum, J. K., and Worm, B. (2009). Cascading top-down effects of changing oceanic predator abundances. *J. Anim. Ecol.* 78, 699–714. doi:10.1111/j.1365-2656.2009.01531.x.

Beirão, J., Boulais, M., Gallego, V., O’Brien, J. K., Peixoto, S., Robeck, T. R., et al. (2019). Sperm handling in aquatic animals for artificial reproduction. *Theriogenology* 133, 161–178. doi:10.1016/j.theriogenology.2019.05.004.

Bell, J. D. (2012). Reproduction and ageing of Australian holocephalans and white-fin swell shark. Ph.D. Thesis, Deakin University, Melbourne, VIC, Australia, 2012.

Bobe, J., and Labbe, C. (2008). “Chilled Storage of Sperm and Eggs,” in *Methods in Reproductive Aquaculture* (CRC Press: Boca Raton, FL), 219–235. doi:10.1201/9780849380549.sec4.

Braverman, I. (2014). Conservation without nature: the trouble with *in situ* versus *ex situ* conservation. *Geoforum* 51, 47–57. doi:10.1016/j.geoforum.2013.09.018.

Briskie, J. V., and Montgomerie, R. (1992). Sperm size and sperm competition in birds. *Proc. R. Soc. London. Ser. B Biol. Sci.* 247, 89–95. doi:10.1098/rspb.1992.0013.

Buckley, K. A., Crook, D. A., Pillans, R. D., Smith, L., and Kyne, P. M. (2018). Sustainability of threatened species displayed in public aquaria, with a case study of Australian sharks and rays. *Rev. Fish Biol. Fish.* 28, 137–151. doi:10.1007/s11160-017-9501-2.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A., and Whittington, C. M. (2019). Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Mar. Freshw. Res.* 70, 908. doi:10.1071/MF18076.

Cabrita, E., Horváth, A., Marinović, Z., and Asturiano, J. F. (2021). "Technologies and strategies for *ex situ* conservation of aquatic organisms: the role of cryopreservation in long-term management," in *Cellular And Molecular Approaches In Fish Biology* (Springer: Berlin), eds I. Monzón and J. Fernandes, 1-48.

Capapé, C., Bradai, M. N., Seck, A. A., Diata, Y., Tomasin, J. A., and Quignard, J. P. (2001). Aspects of the Reproductive Biology of the Velvet Belly, *Etmopterus Spinax* (Elasmobranchii : Squalidae). *Bull. Inst. Natn. Scien. Tech. Mer Salammbô* 28, 55–63.

Capapé, C., Guélorget, O., Vergne, Y., and Quignard, J. P. (2007). Reproductive biology of the common eagle ray *Myliobatis aquila* (Chondrichthyes: Myliobatidae) from the coast of Languedoc (southern France, northern Mediterranean). *Vie Milieu* 57, 125–130.

Carrick, F. N., and Hughes, R. L. (1982). Aspects of the structure and development of monotreme spermatozoa and their relevance to the evolution of mammalian sperm morphology. *Cell Tissue Res.* 222. doi:10.1007/BF00218293.

Carrier, J., Pratt, H., and Castro, J. (2004). “Reproductive Biology of Elasmobranchs,” in *Biology of sharks and their relatives* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 269–286. doi:10.1201/9780203491317.ch10.

Castro, J. I. (2014). Historical Knowledge of Sharks: Ancient Science, Earliest American Encounters and American Science, Fisheries, and Utilization. *Mar. Fish. Rev.* 75, 1–26. doi:10.7755/MFR.75.4.1.

Castro, J. I., Sato, K., and Bodine, A. B. (2016). A novel mode of embryonic nutrition in the tiger shark, *Galeocerdo cuvier*. *Mar. Biol. Res.* 12, 200–205. doi:10.1080/17451000.2015.1099677.

Chapman, D. D., Firchau, B., and Shivji, M. S. (2008). Parthenogenesis in a large-bodied requiem shark, the blacktip *Carcharhinus limbatus*. *J. Fish Biol.* 73, 1473–1477. doi:10.1111/j.1095-8649.2008.02018.x.

Chapman, D. D., Shivji, M. S., Louis, E., Sommer, J., Fletcher, H., and Prodöhl, P. A. (2007). Virgin birth in a hammerhead shark. *Biol. Lett.* 3, 425–427. doi:10.1098/rsbl.2007.0189.

Chen, C.-T., Teshima, K., and Mizue, K. (1973). Studies on Sharks—IV Testes and Spermatogeneses in Selachians. *Bull. Fac. Fish. Nagasaki Univ.* 35, 53–65.

Chierichetti, M. A., Scenna, L. B., Giácomo, E. E. Di, Ondarza, P. M., Figueroa, D. E., and Miglioranza, K. S. B. (2017). Reproductive biology of the cockfish, *Callorhinchus callorynchus* (Chondrichthyes: Callorhinchidae), in coastal waters of the northern Argentinean Sea. *Neotrop. Ichthyol.* 15, 531–539. doi:10.1590/1982-0224-20160137.

Clarke, S., Milner-Gulland, E. J., and BjørndaL, T. (2007). Social, Economic, and Regulatory Drivers of the Shark Fin Trade. *Mar. Resour. Econ.* 22, 305–327. doi:10.1086/mre.22.3.42629561.

Clulow, J., and Clulow, S. (2016). Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians

and reptiles: bringing the ARTs up to speed. *Reprod. Fertil. Dev.* 28, 1116. doi:10.1071/RD15466.

Coelho, K. K. F., Lima, F. S., Wosnick, N., Nunes, A. R. O. P., Silva, A. P. C., Gava, T. T., et al. (2021). Research trends on elasmobranchs from the Brazilian Amazon Coast: a four-decade review. *Biota Neotrop.* 21. doi:10.1590/1676-0611-bn-2021-1218.

Colonello, J. H., Cortés, F., and Belleggia, M. (2020). Male-biased sexual size dimorphism in sharks: the narrowmouth catshark *Schroederichthys bivius* as case study. *Hydrobiologia* 847, 1873–1886. doi:10.1007/s10750-020-04219-9.

Compagno, J. V. L. (1984). *FAO species catalogue Vol. 4 part 1 sharks of the world: An annotated and illustrated catalogue of shark species known to date*. Food and Agriculture Organization of the United Nations: Rome.

Compagno, L. J. V. (1990). Alternative life-history styles of cartilaginous fishes in time and space. *Environ. Biol. Fishes* 28, 33–75. doi:10.1007/BF00751027.

Conrath, C. L., and Musick, J. A. (2012). "Reproductive biology of elasmobranchs," in *Biology of sharks and their relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 291–311.

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J. G., et al. (2019). Effects of short-term storage on sperm function in fish semen: a review. *Rev. Aquac.* 12, raq.12387. doi:10.1111/raq.12387.

Coolen, M., Menuet, A., Chassoux, D., Compagnucci, C., Henry, S., Lévèque, L., et al. (2008). The Dogfish *Scyliorhinus canicula:* A Reference in Jawed Vertebrates. *Cold Spring Harb. Protoc.* 2008, pdb.emo111. doi:10.1101/pdb.emo111.

Cortés, E. (2000). Life History Patterns and Correlations in Sharks. *Rev. Fish. Sci.* 8, 299–344. doi:10.1080/10408340308951115.

Cortés, E. (2002). Incorporating Uncertainty into Demographic Modeling: Application to Shark Populations and Their Conservation. *Conserv. Biol.* 16, 1048–1062. doi:10.1046/j.1523-1739.2002.00423.x.

Cosson, J. (2004). The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa. *Aquac. Int.* 12, 69–85. doi:10.1023/B:AQUI.0000017189.44263.bc.

Costa, M. E., Erzini, K., and Borges, T. C. (2005). reproductive biology of the blackmouth catshark, *Galeus melastomus* (Chondrichthyes: Scyliorhinidae) off the South coast of Portugal. *J. Mar. Biol. Assoc. United Kingdom* 85, 1173–1183. doi:10.1017/S0025315405012270.

Cotton, C. F., Dean Grubbs, R., Dyb, J. E., Fossen, I., and Musick, J. A. (2015). Reproduction and embryonic development in two species of squaliform sharks, *Centrophorus granulosus* and *Etmopterus princeps*: Evidence of matrotrophy? *Deep Sea Res. Part II Top. Stud. Oceanogr.* 115, 41–54. doi:10.1016/j.dsr2.2014.10.009.

Coy, R., and Gadêlha, H. (2017). The counterbend dynamics of cross-linked filament bundles and flagella. *J. R. Soc. Interface* 14, 20170065. doi:10.1098/rsif.2017.0065.

Crow, G. L., and Brock, J. A. (2004). “Necropsy Methods and Procedures for Elasmobranchs,” in *Elasmobranch Husbandry Manual* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney, and R. Heuter, 467-478.

Daly-Engel, T., Grubbs, R., Feldheim, K., Bowen, B., and Toonen, R. (2010). Is multiple mating beneficial or unavoidable? Low multiple paternity and genetic diversity in the shortspine spurdog Squalus mitsukurii. *Mar. Ecol. Prog. Ser.* 403, 255–267. doi:10.3354/meps08417.

Daly, J. (2016). Clinical studies. Available at: https://www.theaquariumvet.com/wp-content/uploads/2016/03/Jon-Daly-PhD-Clinical-Studies.pdf. (accessed on 12 April 2021)

Daly, J., Holland, M. K., and Galloway, D. B. (2011). “Preliminary investigations on sperm cryopreservation of a stingray, the sparsely spotted stingaree,” in *Cryopreservation in Aquatic Species* (World Aquaculture Society: Baton Rouge, LA), eds. T. R. Tiersch, and P. M. Mazik, 337–344.

Daly, J., and Jones, R. (2017). “The use of reproductive technologies in breeding programs for elasmobranchs in aquaria,” in *The Elasmobranch Husbandry Manual II: Recent Advances in the Care of Sharks, Rays and their Relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney, R. Heuter, M. Murray and J. Ezcurra, 363–374.

Daochai, C., Keschumras, N., Chansue, N., and Haetrakul, T. (2020). Preliminary of intra-vagina artificial insemination using fresh semen in Ocellate river stingray (*Potamotrygon motoro*). *Thai. J. Vet. Med.* 50, 383–385.

Davenport, I. R., Weaver, A. L., and Wourms, J. P. (2011). A novel set of structures within the elasmobranch, ovarian follicle. *J. Morphol.* 272, 557–565. doi:10.1002/jmor.10932.

Davidson, L. N. K., Jaiteh, V. F., Chin, A., and Jabado, R. W. (2022). "Conservation Science for Sharks and Rays," in *Biology of Sharks and Their Relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 657–688.

De Iuliis, G., and Pulerà, D. (2019). *The dissection of vertebrates*. Academic press: London, UK.

Dean, B. (1906). *Chimæeroid fishes and their development*. Carnegie Institution of Washington: Washington. doi:10.5962/bhl.title.29471.

del Mar Pedreros-Sierra, T., Arrieta-Prieto, D. M., and Mejía-Falla, P. A. (2016). Reproductive system of females of the Magdalena River endemic stingray *Potamotrygon magdalenae* : Anatomical and functional aspects. *J. Morphol.* 277, 680–697. doi:10.1002/jmor.20527.

Dent, F. (2015). State of the global market for shark products. *FAO Fisheries and Aquaculture technical paper*, 590, Rome, Italy.

der Merwe, A. B., Lyons, K., Kacev, D., and Feldheim, K. (2022). "Elasmobranch Mating Systems," in *Biology of Sharks and Their Relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 203–229. doi:10.1201/9781003262190-7.

Didier, D. A. (2004). "Phylogeny and classification of extant Holocephali," in *Biology of sharks and their relatives*, (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 115–138.

Dodd, J. M. (1983). "Reproduction in Cartilaginous Fishes (Chondrichthyes)," in *Reproduction*, (Academic Press), eds. W. S. Hoar, D. J. Randall, and E. M. B. T.-F. P. Donaldson, 31–95. doi:10.1016/S1546-5098(08)60286-1.

Dudgeon, C. L., Coulton, L., Bone, R., Ovenden, J. R., and Thomas, S. (2017). Switch from sexual to parthenogenetic reproduction in a zebra shark. *Sci. Rep.* 7, 40537. doi:10.1038/srep40537.

Dulvy, N., and Forrest, R. (2010). "Life Histories, Population Dynamics, and Extinction Risks in Chondrichthyans," in *Biology of Sharks and their Relatives*, (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 639–679. doi:10.1201/9781420080483-c17.

Dulvy, N. K., Allen, D. J., Ralph, G. M., and Walls, R. H. L. (2016a). The conservation status of sharks, rays and chimaeras in the Mediterranean Sea.

Dulvy, N. K., Davidson, L. N. K., Kyne, P. M., Simpfendorfer, C. A., Harrison, L. R., Carlson, J. K., et al. (2016b). Ghosts of the coast: global extinction risk and conservation of sawfishes. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 26, 134–153. doi:10.1002/aqc.2525.

Dulvy, N. K., Fowler, S. L., Musick, J. A., Cavanagh, R. D., Kyne, P. M., Harrison, L. R., et al. (2014). Extinction risk and conservation of the world's sharks and rays. *Elife* 3, 1–34. doi:10.7554/eLife.00590.

Dulvy, N. K., Pacoureau, N., Rigby, C. L., Pollom, R. A., Jabado, R. W., Ebert, D. A., et al. (2021). Overfishing drives over one-third of all sharks and rays toward a global extinction crisis. *Curr. Biol.* 31, 4773-4787.e8. doi:10.1016/j.cub.2021.08.062.

Dulvy, N. K., Simpfendorfer, C. A., Davidson, L. N. K., Fordham, S. V., Bräutigam, A., Sant, G., et al. (2017). Challenges and Priorities in Shark and Ray Conservation. *Curr. Biol.* 27, 565–572. doi:10.1016/j.cub.2017.04.038.

Dzyuba, V., and Cosson, J. (2014). Motility of fish spermatozoa: from external signaling to flagella response. *Reprod. Biol.* 14, 165–175. doi:10.1016/j.repbio.2013.12.005.

Dzyuba, V., Ninhaus-Silveira, A., Kahanec, M., Veríssimo-Silveira, R., Rodina, M., Holt, W. V., et al. (2019a). Sperm motility in ocellate river stingrays: evidence for post-testicular sperm maturation and capacitation in Chondrichthyes. *J. Zool.* 307, 9–16. doi:10.1111/jzo.12610.

Dzyuba, V., Sampels, S., Ninhaus-Silveira, A., Kahanec, M., Veríssimo-Silveira, R., Rodina, M., et al. (2019b). Sperm motility and lipid composition in internally fertilizing ocellate river stingray *Potamotrygon motoro*. *Theriogenology* 130, 26–35. doi:10.1016/j.theriogenology.2019.02.029.

Ebert, D. A. (2002). Some observations on the reproductive biology of The sixgill shark *Hexanchus griseus* (Bonnaterre, 1788) from southern African waters. *South African J. Mar. Sci.* 24, 359–363. doi:10.2989/025776102784528439.

Ebert, D. A., and Dando, M. (2020). *Field Guide to Sharks, Rays and Chimaeras of Europe and the Mediterranean*. Princeton University Press doi:10.2307/j.ctv12sdwkk.

Ebert, D. A., Dando, M., and Fowler, S. (2021). *Sharks of the world: a complete guide*. Princeton University Press.

Ecroyd, H., Nixon, B., Dacheux, J.-L., and Jones, R. C. (2009). Testicular descent, sperm maturation and capacitation. Lessons from our most distant relatives, the monotremes. *Reprod. Fertil. Dev.* 21, 992. doi:10.1071/RD09081

Eisenbach, M., and Giojalas, L. C. (2006). Sperm guidance in mammals — an unpaved road to the egg. *Nat. Rev. Mol. Cell Biol.* 7, 276–285. doi:10.1038/nrm1893.

Engel, K. B., and Callard, G. V. (2011). “The testis and spermatogenesis” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (CRC Press: Boca Raton, FL), ed. W. C. Hamlett, 181–183.

Feldheim, K. A., Clews, A., Henningsen, A., Todorov, L., McDermott, C., Meyers, M., et al. (2017a). Multiple births by a captive swellshark *Cephaloscyllium ventriosum via* facultative parthenogenesis. *J. Fish Biol.* 90, 1047–1053. doi:10.1111/jfb.13202.

Feldheim, K. A., Dubach, J., and Watson, L. (2023). Parthenogenesis in an elasmobranch in the presence of conspecific males. *J. Fish Biol.* 102, 525–527. doi:10.1111/jfb.15268.

Feldheim, K. A., Wyffels, J. T., and Lyons, K. (2022). The role of aquaria in the advancement of elasmobranch reproductive biology. *Front. Mar. Sci.* 9, 1–13. doi:10.3389/fmars.2022.963542.

Feldheim, K., Fields, A., Chapman, D., Scharer, R., and Poulakis, G. (2017b). Insights into reproduction and behavior of the smalltooth sawfish *Pristis pectinata*. *Endanger. Species Res.* 34, 463–471. doi:10.3354/esr00868.

Fickel, J., Wagener, A., and Ludwig, A. (2007). Semen cryopreservation and the conservation of endangered species. *Eur. J. Wildl. Res.* 53, 81–89. doi:10.1007/s10344-007-0089-z.

Fitzpatrick, J. L., Montgomerie, R., Desjardins, J. K., Stiver, K. A., Kolm, N., and Balshine, S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proc. Natl. Acad. Sci.* 106, 1128–1132. doi:10.1073/pnas.0809990106.

Fitzpatrick, J. L. (2020). Sperm competition and fertilization mode in fishes. *Philos. Trans. R. Soc. B Biol. Sci.* 375, 20200074. doi:10.1098/rstb.2020.0074.

Follesa, M. C., and Carbonara, P. (2019). Atlas of the maturity stages of Mediterranean fishery resources. *Gen. Fish. Comm. Mediterr. Stud. Rev.*, I–259.

Foote, R. H. (2010). The history of artificial insemination: Selected notes and notables. *J. Anim. Sci* 80, 1–10.

Fowler, S. L., and Cavanagh, R. D. (2005). *Sharks, rays and chimaeras: the status of the Chondrichthyan fishes: status survey*. IUCN.

Francis, M. P., and Duffy, C. (2005). Length at maturity in three pelagic sharks (*Lamna nasus*, *Isurus oxyrinchus*, and *Prionace glauca*) from New Zealand. *Fish. Bull.* 103, 489–500.

Fricke, R., Eschmeyer, W. N., and Van der Laan, R. C. (2021) - Eschmeyer's Catalog of Fishes: Available at: https://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp [Accessed April 7, 2021].

Fricke, R., Eschmeyer, W. N., and Van der Laan, R. (2022). Eschmeyer's Catalog of Fishes: Genera, Species, References. Available at: https://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp [Accessed June 27, 2023].

Gadêlha, H., and Gaffney, E. A. (2019). Flagellar ultrastructure suppresses buckling instabilities and enables mammalian sperm navigation in high-viscosity media. *J. R. Soc. Interface* 16, 20180668. doi:10.1098/rsif.2018.0668.

Gage, M. J. G., Macfarlane, C., Yeates, S., Shackleton, R., and Parker, G. A. (2002). Relationships between sperm morphometry and sperm motility in the Atlantic salmon. *J. Fish Biol.* 61, 1528–1539. doi:10.1111/j.1095-8649.2002.tb02495.x.

Gage, M. J. G., and Freckleton, R. P. (2003). Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. *Proc. R. Soc. London. Ser. B Biol. Sci.* 270, 625–632. doi:10.1098/rspb.2002.2258.

Gallego, V., Cavalcante, S. S., Fujimoto, R. Y., Carneiro, P. C. F., Azevedo, H. C., and Maria, A. N. (2017). Fish sperm subpopulations: changes after cryopreservation process and relationship with fertilization success in

tambaqui (*Colossoma macropomum*). *Theriogenology* 87, 16–24. doi: 10.1016/j.theriogenology.2016.08.001.

Gallego, V., and Asturiano, J. F. (2019). Fish sperm motility assessment as a tool for aquaculture research: a historical approach. *Rev. Aquac.* 11, 697–724. doi:10.1111/raq.12253.

García-Salinas, P., Gallego, V., and Asturiano, J. F. (2021a). Development of Sperm Cryopreservation Protocols for Sharks and Rays: New Tools for Elasmobranch Conservation. *Front. Mar. Sci.* 8, 941. doi:10.3389/fmars.2021.689089.

García-Salinas, P., Gallego, V., and Asturiano, J. F. (2021b). Reproductive Anatomy of Chondrichthyans: Notes on Specimen Handling and Sperm Extraction. I. Rays and Skates. *Animals* 11, 1888. doi:10.3390/ani11071888.

García-Salinas, P., Gallego, V., and Asturiano, J. F. (2021c). Reproductive Anatomy of Chondrichthyans: Notes on Specimen Handling and Sperm Extraction. I. Sharks and Chimaeras. *Animals* 11, 1888. doi:10.3390/ ani11082191.

García-Salinas, P., Gallego, V., and Asturiano, J. F. (2022). Another Tool for Chondrichthyan *Ex Situ* Conservation: First-Time *Chimaera monstrosa* Sperm Cryopreservation. in *SIBIC 2022* (Basel Switzerland: MDPI), 9. doi:10.3390/blsf2022013009.

García-Salinas, P., Gallego, V., and Asturiano, J. F. (2023). Cryopreservation protocols for chondrichthyan sperm cryobanking: new *ex situ* conservation tools for sharks, rays, and chimaeras. in *12th International Symposium on the Reproductive Physiology of Fish* (Hersonissos, Crete), 234.

García, V. B., Lucifora, L. O., and Myers, R. A. (2008). The importance of habitat and life history to extinction risk in sharks, skates, rays and chimaeras. *Proc. R. Soc. B Biol. Sci.* 275, 83–89. doi:10.1098/rspb.2007.1295.

Garzón, D. L., Peñaranda, D. S., Pérez, L., Marco-Jiménez, F., Espert, X., Müller, T., et al. (2007). Effects of pH, sodium bicarbonate, cryoprotectants and fetal bovine serum on the cryopreservation of European eel

sperm. *Reprod. Domest. Anim.* 43, 99–105. doi: 10.1111/j.1439-0531.2007.00861.x.

Gelsleichter, J., and Walker, C. (2010). "Pollutant Exposure and Effects in Sharks and Their Relatives," in *Sharks and their relatives II: Biodiversity, Adaptive Physiology, and Conservation* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 491–537. doi:10.1201/9781420080483-c12.

Gilbert, P. W., and Heath, G. W. (1972). The clasper-siphon sac mechanism in *Squalus acanthias* and *Mustelus canis*. *Comp. Biochem. Physiol. Part A Physiol.* 42, 97–119. doi:10.1016/0300-9629(72)90371-4.

Gillis, J. D., Penfold, L. M., and Mylniczenko, N. D. (2021a). Initial Characterization of Male Southern Stingray (*Hypanus americanus*) Reproductive Parameters and Preliminary Investigation of Sperm Cryopreservation. *Animals* 11, 2716. doi:10.3390/ani11092716.

Gillis, J., Montano, G., and Penfold, L. (2021b). Comparison of conventional and controlled-rate freezing methods to cryopreserve white spotted bamboo shark (*Chiloscyllium plagiosum*) sperm: implications for elasmobranch biobanking efforts. *Reprod. Fertil. Dev.* 34, 245–245. doi:10.1071/RDv34n2Ab22.

Gilmore, R. G., and Dodrill, J. W. (2005). “Oophagy, intrauterine cannibalism and reproductive strategy in lamnoid sharks,” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 435–462.

Girard, M., Rivalan, P., and Sinquin, G. (2000). Testis and sperm morphology in two deep-water squaloid sharks, *Centroscymnus coelolepis* and *Centrophorus squamosus*. *J. Fish Biol.* 57, 1575–1589. doi:10.1111/j.1095-8649.2000.tb02233.x.

Grasse, P. P. (1958). Super-order des holocephali: anatomie, ichtyologie, systematique. *Trait. Zool. Anat. Syst. Biol.* 13, 2060–2067.

Grassmann, M., McNeil, B., and Wharton, J. (2017). Sharks in Captivity: The

Role of Husbandry, Breeding, Education, and Citizen Science in Shark Conservation. *Adv. Mar. Biol.* 78, 89–119. doi:10.1016/bs.amb.2017.08.002.

Grogan, E. D., Lund, R., and Greenfest-Allen, E. (2012). “The origin and relationships of early chondrichthyans,” in *Biology of sharks and their relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 3–29.

Hall, B. K. (2005). *Bones and Cartilage*. Elsevier doi:10.1016/B978-0-12-319060-4.X5000-3.

Hamlett, W. C., and Koob, T. J. (1999a). "Male reproductive system", in *Sharks, Skates and Rays: The biology of elasmobranch fishes’* (The Johns Hopkins University Press: Baltimore, Maryland), ed. W. C. Hamlett, 444–471.

Hamlett, W. C. (1999b). "Sperm aggregation and spermatozeugmata formation in the male genital ducts in the clearnose skate, *Raja eglanteria*", in *Proceedings, 5th Indo-Pacific Fish Conference, Noumea, 1999* (Societe Francaise d’Ichthyologie. OSRAM).

Hamlett, W. C. (2005). “Oviducal glands in chondrichthyans,” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 311–346.

Hamlett, W. C., Knight, D. P., Koob, T. J., Jezior, M., Luong, T., Rozycki, T., et al. (1998). Survey of oviducal gland structure and function in elasmobranchs. *J. Exp. Zool.* 282, 399–420. doi:10.1002/(SICI)1097-010X(199811/12)282:4/5<399::AID-JEZ2>3.0.CO;2-6.

Hamlett, W. C., and Koob, T. J. (1999). Female reproductive system, in *Sharks, Skates and Rays: The biology of elasmobranch fishes’*, (Baltimore, Maryland, USA: The Johns Hopkins University Press), ed. W. C. Hamlett, 398–443.

Hamlett, W. C., Musick, J. A., Hysell, C. K., and Sever, D. M. (2002). Uterine epithelial-sperm interaction, endometrial cycle and sperm storage in the terminal zone of the oviducal gland in the placental smoothhound, *Mustelus canis. J. Exp. Zool.* 292, 129–144. doi:10.1002/jez.1149.

Hamlett, W. C., Kormanik, G., Storrie, M., Stevens, B., and Walker, T. I. (2011b). "Chondrichthyan parity, lecithotrophy and matrotrophy," in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (CRC Press: Boca Raton, FL), ed. W. C. Hamlett, 405–421.

Harmon, T. S., Kamerman, T. Y., Corwin, A. L., and Sellas, A. B. (2016). Consecutive parthenogenetic births in a spotted eagle ray *Aetobatus narinari*. *J. Fish Biol.* 88, 741–745. doi:10.1111/jfb.12819.

Harrison, L. R., and Dulvy, N. K. (2014). Sawfish: A Global Strategy for Conservation. doi:DOI:10.6084/M9.FIGSHARE.1084458.

Healy, T. J., Hill, N. J., Barnett, A., and Chin, A. (2020). A global review of elasmobranch tourism activities, management and risk. *Mar. Policy* 118, 103964. doi:10.1016/j.marpol.2020.103964.

Heithaus, M., Frid, A., Vaudo, J., Worm, B., and Wirsing, A. (2010). "Unraveling the Ecological Importance of Elasmobranchs," in *Sharks and their relatives II: Biodiversity, Adaptive Physiology, and Conservation* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 611–637. doi:10.1201/9781420080483-c16.

Heithaus, M. R., and Dill, L. M. (2002). Food availability and tiger shark predation risk influence bottlenose dolphin habitat use. *Ecology* 83, 480–491. doi:hDOI:10.1890/0012-9658(2002)083[0480:FAATSP]2.0.CO;2.

Heithaus, M. R., Frid, A., Wirsing, A. J., and Worm, B. (2008). Predicting ecological consequences of marine top predator declines. *Trends Ecol. Evol.* 23, 202–210. doi:10.1016/j.tree.2008.01.003.

Heithaus, M. R., Wirsing, A. J., and Dill, L. M. (2012). The ecological importance of intact top-predator populations: a synthesis of 15 years of research in a seagrass ecosystem. *Mar. Freshw. Res.* 63, 1039–1050. doi:DOI:10.1071/MF12024.

Henningsen, A. D. (1994). Tonic immobility in 12 elasmobranchs: Use as an aid in captive husbandry. *Zoo Biol.* 13, 325–332. doi:10.1002/zoo.1430130406.

Henningsen, A. D., Smale, M., Garner, R., and Kinnunen, N. (2004a). "Reproduction, embryonic development, and reproductive physiology of elasmobranchs," in *The elasmobranch husbandry manual: captive care of sharks, rays and their relatives.* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney and R. Heuter. 227–236.

Henningsen, A. D., Smale, M. J., Gordon, I., Garner, R., Marin-Osorno, R., and Kinnunen, N. (2004b). "Captive breeding and sexual conflict in elasmobranchs," in *The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney and R. Heuter. pp, 237–248.

Hermo, L., Oliveira, R. L., Smith, C. E., and Bergeron, J. J. M. (2018). "Inherent Sperm Maturation: A Role for the Hermes Body (Cytoplasmic Droplet) of Sperm," in *Encyclopedia of Reproduction* (Elsevier), 72–84. doi:10.1016/B978-0-12-801238-3.66122-6.

Heupel, M., Knip, D., Simpfendorfer, C., and Dulvy, N. (2014). Sizing up the ecological role of sharks as predators. *Mar. Ecol. Prog. Ser.* 495, 291–298. doi:10.3354/meps10597.

Horváth, Á., and Urbányi, B. (2020). "Sperm Cryopreservation of Aquatic Species," in Reproduction in aquatic animals (Springer: Singapore), eds. M. Yoshida and J. F. Asturiano, 321–334. doi:10.1007/978-981-15-2290-1_16.

Howard, J. G. (1986). "Semen collection, analysis and cryopreservation in nondomestic mammals," in *Current therapy in theriogenology* (Saunders: Philadelphia, PA), ed. D.A. Marrow, 1047–1053.

Hunt, S. (1985). "The Selachian Egg Case Collagen," in *Biology of Invertebrate and Lower Vertebrate Collagens* (Boston, MA: Springer US), 409–434. doi:10.1007/978-1-4684-7636-1_36.

Hussey, N. E., MacNeil, M. A., Siple, M. C., Popp, B. N., Dudley, S. F. J., and Fisk, A. T. (2015). Expanded trophic complexity among large sharks. *Food Webs* 4, 1–7. doi:10.1016/j.fooweb.2015.04.002.

Immler, S., Pitnick, S., Parker, G. A., Durrant, K. L., Lüpold, S., Calhim, S., et al. (2011). Resolving variation in the reproductive tradeoff between sperm size and number. *Proc. Natl. Acad. Sci.* 108, 5325–5330. doi:10.1073/pnas.1009059108.

IUCN/SSC (2014). *IUCN Species Survival Commission Guidelines on the Use of Ex Situ Management for Species Conservation. Version 2.* Available at: https://portals.iucn.org/library/sites/library/files/documents/2014-064.pdf.

Ivic, A., Onyeaka, H., Girling, A., Brewis, I.A., Ola, B., Hammadieh, N., Papaioannou, S., and Barratt, C.L.R. (2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Hum. Reprod*. 17, 143-149. doi:10.1093/humrep/17.1.143.

Jamieson, B. G. M., and Hamlett, W. C. (2005). Chondrichthyan spermatozoa and phylogeny. *Reprod. Biol. Phylogeny Chondrichthyes Sharks, Batoids Chimaeras. Sci. Publ. Inc*, 201–236.

Janse, M., Zimmerman, B., Geerlings, L., Brown, C., and Nagelkerke, L. A. J. (2017). Sustainable species management of the elasmobranch populations within European aquariums: a conservation challenge. *J. Zoo Aquarium Res.* 5, 172–181. doi:https://doi.org/10.19227/jzar.v5i4.313.

Jawed, M. K., Khouri, N. K., Da, F., Grinspun, E., and Reis, P. M. (2015). Propulsion and Instability of a Flexible Helical Rod Rotating in a Viscous Fluid. *Phys. Rev. Lett.* 115, 168101. doi:10.1103/PhysRevLett.115.168101.

Jones, C. J. P., Walker, T. I., Bell, J. D., Reardon, M. B., Ambrosio, C. E., Almeida, A., et al. (2005). "Male genital ducts and copulatory appendages in chondrichthyans," in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 361–393.

Jones, C. J. P., and Hamlett, W. C. (2002). Glycosylation of the Male Genital Ducts and Spermatozeugmata Formation in the Clearnose Skate *Raja eglanteria*. *Histochem. J.* 34, 601–615. doi:10.1023/A:1026093902502.

Jones, R. C., Jones, N., and Djakiew, D. (1984). Luminal composition and maturation of spermatozoa in the male genital ducts of the Port Jackson shark, *Heterodontus portusjacksoni*. *J. Exp. Zool.* 230, 417–426. doi:10.1002/jez.1402300311.

Jordan, R. P., Graham, C. T., Minto, C., and Henderson, A. C. (2021). Assessment of sperm storage across different reproductive modes in the elasmobranch fishes. *Environ. Biol. Fishes* 104, 27–39. doi:10.1007/s10641-020-01051-x.

Jungersen, H. F. E. (1899). *On the appendices genitales in the Greenland shark, Somniosus microcephalus (Bl. Schn.), and other selachians / by Hector F. E. Jungersen ; translated by Torben Lundbeck.* Printer to the Court: Copenhagen. doi:10.5962/bhl.title.14334.

Kahrl, A. F., Snook, R. R., and Fitzpatrick, J. L. (2021). Fertilization mode drives sperm length evolution across the animal tree of life. *Nat. Ecol. Evol.* 5, 1153–1164. doi:10.1038/s41559-021-01488-y.

Kahrl, A. F., Snook, R. R., and Fitzpatrick, J. L. (2022). Fertilization mode differentially impacts the evolution of vertebrate sperm components. *Nat. Commun.* 13, 6809. doi:10.1038/s41467-022-34609-7.

Kessel, S. T., and Hussey, N. E. (2015). Tonic immobility as an anaesthetic for elasmobranchs during surgical implantation procedures. *Can. J. Fish. Aquat. Sci.* 72, 1287–1291. doi:10.1139/cjfas-2015-0136.

Ketchen, K. S. (1986). The spiny dogfish (*Squalus acanthias*) in the northeast Pacific and a history of its utilization.

King, M. G, and Custance, D. R. N. (1982). *Colour Atlas of Vetebrate Anatomy: Integrated Text and Dissection Guide.* Blackwell Scientific Publications.

Knupp, C., and Squire, J. (1998). X–ray diffraction analysis of the 3D organization of collagen fibrils in the wall of the dogfish egg case. *Proc. R. Soc. London. Ser. B Biol. Sci.* 265, 2177–2186. doi:10.1098/rspb.1998.0556.

Koob, T. J. (2004). “Elasmobranchs in the Public Aquarium: 1860 to 1930,” in *The elasmobranch husbandry manual: captive care of sharks, rays and their relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney and R. Heuter, 1–14.

Kyne, P. M., Jabado, R. W., Rigby, C. L., Dharmadi, Gore, M. A., Pollock, C. M., et al. (2020). The thin edge of the wedge: Extremely high extinction risk in wedgefishes and giant guitarfishes. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 30, 1337–1361. doi:10.1002/aqc.3331.

Kyne, P. M., and Lucifora, L. O. (2022). “Freshwater and euryhaline elasmobranchs,” in *Biology of sharks and their relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 567–602.

Labbé, C., Robles, V., and Herráez, M. P. (2013). “Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation,” in *Advances in Aquaculture Hatchery Technology* (Woodhead Publishing Series: Amsterdam), eds G. Burnell and G. Allan, 76–116. doi: 10.1533/9780857097460.1.76.

Lack, M., Sant, G., Burgener, M., and Okes, N. (2014). Development of a rapid management-risk assessment method for fish species through its application to sharks: framework and results. *WCPFC Scientific Committee 10th Regular Session, EB-IP-12a*, Majuro, Republic of the Marshall Islands.

Lacy, E. R. (2005). “Alkaline glands and clasper glands of batoids,” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 336–360.

Langen, K., Thünken, T., Klemm, J., Sandmann, S., and Bakker, T. C. M. (2019). Sperm size is negatively related to relative testis size in West African riverine cichlid fishes. *Sci. Nat.* 106, 30. doi:10.1007/s00114-019-1622-0.

Lamarca, F., Carvalho, P. H., Vilasboa, A., Netto-Ferreira, A. L., and Vianna, M. (2020). Is multiple paternity in elasmobranchs a plesiomorphic characteristic? *Environ. Biol. Fishes* 103, 1463–1470. doi:10.1007/s10641-020-01034-y.

Last, P., Naylor, G., Séret, B., White, W., de Carvalho, M., and Stehmann, M. (2016). *Rays of the World*. CSIRO Publishing doi:10.1071/9780643109148.

Lauriano, E. R., Pergolizzi, S., Gangemi, J., Kuciel, M., Capillo, G., Aragona, M., et al. (2017). Immunohistochemical colocalization of G protein alpha subunits and 5-HT in the rectal gland of the cartilaginous fish *Scyliorhinus canicula*. *Microsc. Res. Tech.* 80, 1018–1027. doi:10.1002/jemt.22896.

Lee, Y. H., Park, J. Y., Lee, I. Y., Zidni, I., and Lim, H. K. (2021). Effects of cryoprotective agents and treatment methods on sperm cryopreservation of stone flounder, Kareius bicoloratus. *Aquaculture* 531, 735969. doi:10.1016/j.aquaculture.2020.735969.

Leigh-Sharpe, W. H. (1922). The comparative morphology of the secondary sexual characters of holocephali and elasmobranch fishes. the claspers, clasper siphons, and clasper glands. *J. Morphol.* 36, 199–220. doi:10.1002/jmor.1050360204.

Len, J. S., Koh, W. S. D., and Tan, S.-X. (2019). The roles of reactive oxygen species and antioxidants in cryopreservation. *Biosci. Rep.* 39. doi:10.1042/BSR20191601.

Leung, M. R., Roelofs, M. C., Ravi, R. T., Maitan, P., Henning, H., Zhang, M., et al. (2021). The multi-scale architecture of mammalian sperm flagella and implications for ciliary motility. *EMBO J.* 40. doi:10.15252/embj.2020107410.

Lisicki, M., Velho Rodrigues, M. F., Goldstein, R. E., and Lauga, E. (2019). Swimming eukaryotic microorganisms exhibit a universal speed distribution. *Elife* 8, e44907. doi:10.7554/eLife.44907.

Liu, X., Pan, Y., Liu, F., He, Y., Zhu, Q., Liu, Z., et al. (2021). A Review of the Material Characteristics, Antifreeze Mechanisms, and Applications of Cryoprotectants (CPAs). *J. Nanomater.* 2021, 1–14. doi:10.1155/2021/9990709.

Long, J. A., Mark-Kurik, E., Johanson, Z., Lee, M. S. Y., Young, G. C., Min, Z., et al. (2015). Copulation in antiarch placoderms and the origin of gnathostome internal fertilization. *Nature* 517, 196–199. doi:10.1038/nature13825.

Luer, C. A. (1989). “Elasmobranchs (sharks, skates, and rays) as animal models for biomedical research,” in *Nonmammalian Animal Models for Biomedical Research* (CRC Press: Boca Raton, FL), ed. A.D. Woodhead, 121-148.

Luer, C. A., Walsh, C. J., Bodine, A. B., and Wyffels, J. T. (2008). “Normal embryonic development in the clearnose skate, *Raja eglanteria*, with experimental observations on artificial insemination,” in *Biology of Skates* (Springer Netherlands: Dordrecht), eds. D. A. Ebert, J. A. Sulikowski, 133–149. doi:10.1007/978-1-4020-9703-4_9.

Luer, C., and Walsh, C. (2018). Potential Human Health Applications from Marine Biomedical Research with Elasmobranch Fishes. *Fishes* 3, 47. doi:10.3390/fishes3040047.

Lujić, J., Bernáth, G., Marinović, Z., Radojković, N., Simić, V., Ćirković, M., et al. (2017). Fertilizing capacity and motility of tench *Tinca tinca* (L., 1758) sperm following cryopreservation. *Aquac. Res.* 48, 102–110. doi: 10.1111/are.12865.

Lund, R., and Grogan, E. D. (1997). Relationships of the Chimaeriformes and the basal radiation of the Chondrichthyes. *Rev. Fish Biol. Fish.* 7, 65–123. doi:DOI:10.1023/A:1018471324332.

Lüpold, S., Calhim, S., Immler, S., and Birkhead, T. R. (2009). Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. B Biol. Sci.* 276, 1175–1181. doi:10.1098/rspb.2008.1645.

Lutton, B. V (2011). “The elasmobranch ovary,” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (CRC Press: Boca Raton, FL), ed. W. C. Hamlett, 247–276.

Morrell, J. M. (2011). “Artificial Insemination: Current and Future Trends” in *Artificial Insemination in Farm Animals* (InTech Open: London), ed. M. Malafi, 1–14. doi:10.5772/17943.

MacFarland, T. W. (2012). *Two-Way Analysis of Variance Statistical Tests and Graphics Using R, 2012th edition*. Springer Science & Business Media.

Malagrino, G., Takemura, A., and Mizue, K. (1981). Studies on Holocephali—II On the Reproduction of *Chimaera phantasma* Jordan *et* Snyder Caught in the Coastal Waters of Nagasaki. *Bull. Fac. Fish. Nagasaki Univ.*, 1–7.

Mann, T., and Prosser, L. C. (1963). Uterine response to 5-Hydroxytryptamine in clasper-siphon secretion of spiny dogfish, *Squalus acanthias*. in *Biological Bulletin* (Marine Biological Laboratory: Woods Hole, MA), 384.

Marongiu, M. F., Porcu, C., Bellodi, A., Cuccu, D., Mulas, A., and Follesa, M. C. (2015). Oviducal gland microstructure of *Raja miraletus* and *Dipturus oxyrinchus* (Elasmobranchii, Rajidae). *J. Morphol.* 276, 1392–1403. doi:10.1002/jmor.20426.

Márquez-Farías, J. F., and Lara-Mendoza, R. E. (2014). Notas sobre la morfología del aparato reproductor de la quimera, *Hydrolagus melanophasma* (Chondrichthyes, Holocephali), de la costa oeste de Baja California, México. *Hidrobiológica* 24, 151–158. Available at: https://hidrobiologica.izt.uam.mx/index.php/revHidro/article/view/572 [Accessed June 27, 2023].

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., et al. (2017). Cryobanking of aquatic species. *Aquaculture* 472, 156–177. doi:10.1016/j.aquaculture.2016.05.042.

Martins, A., Heupel, M., Chin, A., and Simpfendorfer, C. (2018). Batoid nurseries: definition, use and importance. *Mar. Ecol. Prog. Ser.* 595, 253–267. doi:10.3354/meps12545.

Masuda, M., Izawa, Y., Kametuta, S., Ikuta, H., and Isogai, T. (2003). Artificial insemination of the cloudy catshark. *J. Jpn. Assoc. Zool. Gard. Aquariums* 44, 39–43.

Masuda, M., Izawa, Y., Kametsuta, S., Ikuta, H., and Isogai, T. (2005). Artificial insemination of the white-spotted bamboo shark, *Chiloscyllium plagiosum*. *J. Jpn. Assoc. Zool. Gard. Aquariums* 46, 91–96.

Matthews, L. H. (1950). Reproduction in the basking shark, *Cetorhinus maximus* (Gunner). *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 234, 247–316. doi:10.1098/rstb.1950.0003.

McClusky, L. M. (2015). Sperm of *Galeorhinus galeus* (Elasmobranchii, Triakidae) Traverse an Excurrent Duct System Characterized by Pronounced Regionalization: A Scanning Electron and Light Microscopy Study. *Anat. Rec.* 298, 1938–1949. doi:10.1002/ar.23204.

Minamikawa, S., and Morisawa, M. (1996). Acquisition, initiation and maintenance of sperm motility in the shark, *Triakis scylli*a. *Comp. Biochem. Physiol. Part A Physiol.* 113, 387–392. doi:10.1016/0300-9629(95)02080-2.

Montano, G. A., Steinman, K. J., McDonnell, A., and Robeck, T. R. (2023). Development of a diluent storage media and effect of cryoprotectants on semen collected from white spotted bamboo sharks (*Chiloscyllium plagiosum*) and cooled to 5 °C. *Theriogenology Wild* 2, 100017. doi:10.1016/j.therwi.2023.100017.

Moore, A. (2017). Are guitarfishes the next sawfishes? Extinction risk and an urgent call for conservation action. *Endanger. Species Res.* 34, 75–88. doi:10.3354/esr00830.

Morales-Gamba, R. D., Caldas, J. S., Godoy, L., and Marcon, J. L. (2019). Sperm characterization of the Amazonian freshwater cururu stingray *Potamotrygon wallacei* (Potamotryogonidae): basic knowledge for reproduction and conservation plans. *Zygote* 27, 259–261. doi:10.1017/S096719941900039X.

Moura, T., Serra-Pereira, B., Gordo, L. S., and Figueiredo, I. (2011). Sperm storage in males and females of the deepwater shark Portuguese dogfish with notes on oviducal gland microscopic organization. *J. Zool.* 283, 210–219. doi:10.1111/j.1469-7998.2010.00775.x.

Muñoz-Baquero, M., Marco-Jiménez, F., García-Domínguez, X., Ros-Santaella, J. L., Pintus, E., Jiménez-Movilla, M., et al. (2021). Comparative Study of Semen Parameters and Hormone Profile in Small-Spotted Catshark (*Scyliorhinus canicula*): Aquarium-Housed vs. Wild-Captured. *Animals* 11, 2884. doi:10.3390/ani11102884.

Muñoz Gutiérrez, M. E. (2011). Biotecnología aplicada en la reproducción de peces. *Inf. Técnico* 75, 66–72. doi: 10.23850/22565035.21.

Musick, J. A. (2010). "Chondrichthyan Reproduction," in *Reproduction and Sexuality in Marine Fishes: Patterns and Processes* (University of California Press), ed. K. S. Cole, 2–19. doi:10.1525/california/9780520264335.003.0001.

Musick, J. A., and Ellis, J. K. (2005). "Reproductive evolution of chondrichthyans," in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 45–71.

Muto, K., and Kubota, H.Y. (2009). A novel mechanism of sperm motility in a viscous environment: Corkscrew-shaped spermatozoa cruise by spinning. *Cell motil. cytoskelet.* 66, 281-291. doi: 10.1002/cm.20358.

Myers, R. A., Baum, J. K., Shepherd, T. D., Powers, S. P., and Peterson, C. H. (2007). Cascading Effects of the Loss of Apex Predatory Sharks from a Coastal Ocean. *Science* 315, 1846–1850. doi:10.1126/science.1138657.

Mylniczenko, N. D., Culpepper, E. E., and Clauss, T. (2017). "Diagnostic imaging of elasmobranchs: updates and case examples," in *The Elasmobranch Husbandry Manual II: Recent Advances in the Care of Sharks, Rays and Their Relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney, R. Heuter, M. Murray and J. Ezcurra, 303–327.

Nakaya, K., White, W. T., and Ho, H.-C. (2020). Discovery of a new mode of oviparous reproduction in sharks and its evolutionary implications. *Sci. Rep.* 10, 12280. doi:10.1038/s41598-020-68923-1.

Nelson, J. S., Grande, T. C., and Wilson, M. V. H. (2016). *Fishes of the World*. Hoboken, NJ, USA: John Wiley & Sons, Inc doi:10.1002/9781119174844.

Nixon, B., Ecroyd, H., Dacheux, J.-L., Dacheux, F., Labas, V., Johnston, S. D., et al. (2016). Formation and Dissociation of Sperm Bundles in Monotremes. *Biol. Reprod.* 95, 91–91. doi:10.1095/biolreprod.116.140491.

Nozu, R., Murakumo, K., Yano, N., Furuyama, R., Matsumoto, R., Yanagisawa, M., et al. (2018). Changes in sex steroid hormone levels reflect the reproductive status of captive female zebra sharks (*Stegostoma fasciatum*). *Gen. Comp. Endocrinol.* 265, 174–179. doi:10.1016/j.ygcen.2018.03.006.

O'Brien, J. K., and Robeck, T. R. (2010). Preservation of beluga (*Delphinapterus leucas*) spermatozoa using a trehalose-based cryodiluent and directional freezing technology. *Reprod. Fertil. Dev.* 22, 653. doi:10.1071/RD09176.

Ohta, H., and Izawa, T. (1996). Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. *Aquaculture* 142, 107–118. doi: 10.1016/0044-8486(95)01246-X.

Oriola, D., Needleman, D. J., and Brugués, J. (2018). The Physics of the Metaphase Spindle. *Annu. Rev. Biophys.* 47, 655–673. doi:10.1146/annurev-biophys-060414-034107.

Orlov, A. M., Cotton, C. F., and Shevernitsky, D. A. (2010). Sexual dimorphism of external morphological characters in some deepwater skates (Rajidae, Rajiformes, Chondrichthyes) of the North Atlantic. *Moscow Univ. Biol. Sci. Bull.* 65, 40–44. doi:10.3103/S0096392510010086.

Osgood, G. J., White, E. R., and Baum, J. K. (2021). Effects of climate-change-driven gradual and acute temperature changes on shark and ray species. *J. Anim. Ecol.* 90, 2547–2559. doi:10.1111/1365-2656.13560.

Pacoureau, N., Rigby, C. L., Kyne, P. M., Sherley, R. B., Winker, H., Carlson, J. K., et al. (2021). Half a century of global decline in oceanic sharks and rays. *Nature* 589, 567–571. doi:10.1038/s41586-020-03173-9.

Padilha, F. L. A., Colbachini, H., Ramos, S. D., Reisfeld, L. C., Henrique, P. C., Leite, R. F., et al. (2021). Validation of manual semen collection methods and sperm evaluation in living freshwater stingrays (*Potamotrygon falkneri*) kept in ex situ conditions. *Environ. Biol. Fishes* 104, 463–469. doi:10.1007/s10641-021-01086-8.

Pearcy, M., Delescaille, N., Lybaert, P., and Aron, S. (2014). Team swimming in ant spermatozoa. *Biol. Lett.* 10, 20140308. doi:10.1098/rsbl.2014.0308.

Peñaranda, D., Pérez, L., Gallego, V., Barrera, R., Jover, M., and Asturiano, J. (2008). European Eel Sperm Diluent for Short-term Storage. *Reprod. Domest. Anim.* 45, 407–415. doi:10.1111/j.1439-0531.2008.01206.x.

Penfold, L. M., and Wyffels, J. T. (2019). “Reproductive Science in Sharks and Rays,” in *Reproductive Sciences in Animal Conservation* (Springer International Publishing), eds. P. Comizzoli, J.L. Brown, W.V. Holt, 465–488. doi:10.1007/978-3-030-23633-5_15.

Pérez, L.M. (2020). "Fish Sperm Maturation, Capacitation, and Motility Activation" in *Reproduction in Aquatic Animals* (Springer Nature: Singapore), eds. M. Yoshida, and J.F. Asturiano, 47-68.

Perez-Cerezales, S., Boryshpolets, S., and Eisenbach, M. (2015). Behavioral mechanisms of mammalian sperm guidance. *Asian J. Androl.* 17, 628. doi:10.4103/1008-682X.154308.

Piercy, A., Gelsleichter, J., and Snelson, F. F. (2006). Morphological changes in the clasper gland of the Atlantic stingray, *Dasyatis sabina*, associated with the seasonal reproductive cycle. *J. Morphol.* 267, 109–114. doi:10.1002/jmor.10389.

Piironen, J. (1993). Cryopreservation of sperm from brown trout (*Salmo trutta m. lacustris* L.) and Arctic charr (*Salvelinus alpinus* L.). *Aquaculture* 116, 275–285. doi: 10.1016/0044-8486(93)90014-P.

Pitnick, S., Hosken, D. J., and Birkhead, T. R. (2009). “Sperm morphological diversity,” in *Sperm Biology* (Academic Press), eds. S. S. Pitnick, D. J. Hosken, T. Birkhead, 69–149. doi:10.1016/B978-0-12-372568-4.00003-3.

Pizzutto, C. S., Colbachini, H., and Jorge-Neto, P. N. (2021). One Conservation: the integrated view of biodiversity conservation. *Anim. Reprod.* 18. doi:10.1590/1984-3143-ar2021-0024.

Pochitaloff, M., Miranda, M., Richard, M., Chaiyasitdhi, A., Takagi, Y., Cao, W., et al. (2022). Flagella-like beating of actin bundles driven by self-organized myosin waves. *Nat. Phys.* 18, 1240–1247. doi:10.1038/s41567-022-01688-8.

Porcu, C., Marongiu, M. F., Follesa, M. C., Bellodi, A., Mulas, A., Pesci, P., et al. (2013). Reproductive aspects of the velvet belly lantern shark *Etmopterus spinax* (Condrichthyes: Etmopteridae), from the central western Mediterranean sea. Notes on gametogenesis and oviducal gland microstructure. *Mediterr. Mar. Sci.* 15, 313. doi:10.12681/mms.559.

Poyer, J. C., and Hartmann, J. X. (1992). Establishment of a cell line from brain tissue of the silky shark, *Carcharhinus falciformis*. *Vitr. Cell. Dev. Biol. - Anim.* 28, 682–684. doi:10.1007/BF02631047.

Pratt, H. L. (1979). Reproduction in the blue shark, *Prionace glauca*. *Fish. Bull.* 77, 445–470.

Pratt, H. L. (1988). Elasmobranch Gonad Structure: a Description and survey. *Copeia* 1988, 719. doi:10.2307/1445394.

Pratt, H. L. (1993). The storage of spermatozoa in the oviducal glands of western North Atlantic sharks. *Environ. Biol. Fishes* 38, 139–149. doi:10.1007/BF00842910.

Pratt, H. L., and Carrier, J. C. (2005). “Elasmobranch Courtship and Mating Behavior,” in *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimaeras* (Science Publishers: Enfield, NH), ed. W. C. Hamlett, 129–169.

Pratt, H. L., and Tanaka, S. (1994). Sperm storage in male elasmobranchs: A description and survey. *J. Morphol.* 219, 297–308. doi:10.1002/jmor.1052190309.

Pritchard, D. J., Fa, J. E., Oldfield, S., and Harrop, S. R. (2012). Bring the captive closer to the wild: redefining the role of *ex situ* conservation. *Oryx* 46, 18–23. doi:10.1017/S0030605310001766.

Renz, A. J., Meyer, A., and Kuraku, S. (2013). Revealing less derived nature of cartilaginous fish genomes with their evolutionary time scale inferred with nuclear genes. *PLoS One* 8, e66400. doi:10.1371/journal.pone.0066400.

Robles, V. M. M., Santamaría, Y. M. V., and Casallas, P. E. C. (2005). Aspectos generales de la crioconservación espermática en peces teleósteos. *Rev. Colomb. Ciencias Pecu.* 18, 34–48.

Rosa, R., Rummer, J. L., and Munday, P. L. (2017). Biological responses of sharks to ocean acidification. *Biol. Lett.* 13, 20160796. doi:10.1098/rsbl.2016.0796.

Rowley, A., Locatello, L., Kahrl, A., Rego, M., Boussard, A., Garza-Gisholt, E., et al. (2019). Sexual selection and the evolution of sperm morphology in sharks. *J. Evol. Biol.* 32, 1027–1035. doi:10.1111/jeb.13501.

Rummer, J. L., Bouyoucos, I. A., Wheeler, C. R., Santos, C. P., and Rosa, R. (2022). "Climate change and sharks," in *Biology of sharks and their relatives,* (CRC Press: Boca Raton, FL), eds. M. R. Heithaus, J. A. Musick, and J. C. Carrier, 767–793.

Ruppert, J. L. W., Travers, M. J., Smith, L. L., Fortin, M.-J., and Meekan, M. G. (2013). Caught in the Middle: Combined Impacts of Shark Removal and Coral Loss on the Fish Communities of Coral Reefs. *PLoS One* 8, e74648. doi:10.1371/journal.pone.0074648.

Saadaoui, A., Saidi, B., Enajjar, S., and Bradai, M. N. (2015). Reproductive biology of the common stingray *Dasyatis pastinaca* (Linnaeus, 1758) off the Gulf of Gabès (Central Mediterranean Sea). *Cah. Biol. Mar.* 56, 389–396.

Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K., and Nozu, R. (2016). How great white sharks nourish their embryos to a large size: evidence of lipid histotrophy in lamnoid shark reproduction. *Biol. Open* 5, 1211–1215. doi:10.1242/bio.017939.

Schaeffer, B. (1981). The xenacanth shark neurocranium, with comments on elasmobranch monophyly. *Bull. Am. Museum Nat. Hist.* 169, 1-66.

Scheltinga, and Jamieson (2003). "Spermatogenesis and the mature spermatozoon: Form, function and phylogenetic implications" in *Reproductive Biology and Phylogeny of Anura* (CRC Press: Boca Raton, FL), ed. B. G. M. Jamieson, pp. 119-217. 10.1201/9781482280074.

Schmoll, T., Rudolfsen, G., Schielzeth, H., and Kleven, O. (2020). Sperm velocity in a promiscuous bird across experimental media of different viscosities. *Proc. R. Soc.* B 287, 20201031. doi:10.1098/rspb.2020.1031.

Seidel, R., Lyons, K., Blumer, M., Zaslansky, P., Fratzl, P., Weaver, J. C., et al. (2016). Ultrastructural and developmental features of the tessellated endoskeleton of elasmobranchs (sharks and rays). *J. Anat.* 229, 681–702. doi:10.1111/joa.12508.

Shaliutina, A., Hulak, M., Gazo, I., Linhartova, P., and Linhart, O. (2013). Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. *Anim. Reprod. Sci.* 139, 127–135. doi:10.1016/j.anireprosci.2013.03.006.

Shiffman, D. S., Ajemian, M. J., Carrier, J. C., Daly-Engel, T. S., Davis, M. M., Dulvy, N. K., et al. (2020). Trends in Chondrichthyan Research: An Analysis of Three Decades of Conference Abstracts. *Copeia* 108, 122-131. doi:10.1643/OT-19-179R.

Silva Nunes, J. L., Barbosa Martins, A. P., Da Silva Filho, E., Manir Feitosa, L., Nunes e Silva, L. P., and Da Silva de Almeida, Z. (2015). Sexual dimorphism of sharks from the amazonian equatorial coast. *Univ. Sci.* 20, 297. doi:10.11144/Javeriana.SC20-3.sdos.

Simon, C.O., Barrie, G.M.J., and David, M.S. (1996). The Ultrastructure of spermatozoa of Squamata. II. Agamidae, Varanidae, Colubridae, Elapidae, and Boidae (Reptilia). *Herpetologica* 52, 216-241.

Simpfendorfer, C. A., Heupel, M. R., White, W. T., and Dulvy, N. K. (2011). The importance of research and public opinion to conservation management of sharks and rays: a synthesis. *Mar. Freshw. Res.* 62, 518. doi:10.1071/MF11086.

Sims, D. W. (2006). “Differences in habitat selection and reproductive strategies of male and female sharks,” in *Sexual Segregation in Vertebrates* (Cambridge University Press), eds. K. Ruckstuhl, P. Neuhaus, 127–147. doi:10.1017/CBO9780511525629.009.

Singh, A. K., Kumar, A., and Bisla, A. (2021). Computer-assisted sperm analysis (CASA) in veterinary science: A review. *Indian J. Anim. Sci.* 91. doi:10.56093/ijans.v91i6.115435.

Smale, M. J., Jones, R. T., Correia, J. P., Henningsen, A. D., Crow, G. L., and Garner, R. (2004). “Research on elasmobranchs in public aquariums,” in *The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney and R. Heuter, 533–541.

Smith, D. J., Gaffney, E. A., Gadêlha, H., Kapur, N., and Kirkman-Brown, J. C. (2009). Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. *Cell Motil. Cytoskeleton* 66, 220–236. doi:10.1002/cm.20345.

Snelson, F. F., Williams-Hooper, S. E., and Schmid, T. H. (1988). Reproduction and Ecology of the Atlantic Stingray, *Dasyatis sabina*, in Florida Coastal Lagoons. *Copeia* 1988, 729. doi:10.2307/1445395.

Son, K., Guasto, J. S., and Stocker, R. (2013). Bacteria can exploit a flagellar buckling instability to change direction. *Nat. Phys.* 9, 494–498. doi:10.1038/nphys2676.

Springer, S. (1948). Oviphagous Embryos of the Sand Shark, *Carcharias taurus*. *Copeia* 1948 (3), 153-157. doi:10.2307/1438449.

Stanley, H. P. (1963). Urogenital morphology in the chimaeroid fish *Hydrolagus colliei* (Lay and Bennett). *J. Morphol.* 112, 99–127. doi:10.1002/jmor.1051120202.

Stehmann, M. F. W. (2002). Proposal of a maturity stages scale for oviparous and viviparous cartilaginous fishes (Pisces, Chondrichthyes). *Arch. Fish. Mar. Res.* 50, 23–48.

Stevens, J. (2000). The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems. *ICES J. Mar. Sci.* 57, 476–494. doi:10.1006/jmsc.2000.0724.

Stockley, P., Gage, M. J. G., Parker, G. A., and Møller, A. P. (1997). Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics. *Am. Nat.* 149, 933–954. doi:10.1086/286031.

Storrie, M. T., Walker, T. I., Laurenson, L. J., and Hamlett, W. C. (2008). Microscopic organization of the sperm storage tubules in the oviducal gland of the female gummy shark (*Mustelus antarcticus*), with observations on sperm distribution and storage. *J. Morphol.* 269, 1308–1324. doi:10.1002/jmor.10646.

Støstad, H. N., Johnsen, A., Lifjeld, J. T., and Rowe, M. (2018). Sperm head morphology is associated with sperm swimming speed: A comparative study of songbirds using electron microscopy. *Evolution (N. Y).* 72, 1918–1932. doi:10.1111/evo.13555.

Sumpter, J. P., and Dodd, J. M. (1979). The annual reproductive cycle of the female lesser spotted dogfish, *Scyliorhinus canicula* L., and its endocrine control. *J. Fish Biol.* 15, 687–695. doi:10.1111/j.1095-8649.1979.tb03678.x.

Swider, D. A., Corwin, A. L., Kamerman, T. Y., Zimmerman, S. L., Violetta, G. C., Davis, J., et al. (2017). "Reproduction of spotted eagle rays, *Aetobatus narinari,* in aquaria," in *The Elasmobranch Husbandry Manual II: Recent Advances in the Care of Sharks, Rays and their Relatives* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney, R. Heuter, M. Murray and J. Ezcurra, 422–442.

Tambasen-Cheong, M. V. P., Tan-Fermin, J. D., Garcia, L. M. B., and Baldevarona, R. B. (1995). Milt-egg ratio in artificial fertilization of the Asian freshwater catfish, *Clarias macrocephalus*, injected salmon gonadotropin-releasing hormone analogue and domperidone. *Aquat. Living Resour.* 8, 303–307. doi: 10.1051/alr:1995031.

Tan-Fermin, J. D., Miura, T., Adachi, S., and Yamauchi, K. (1999). Seminal plasma composition, sperm motility, and milt dilution in the Asian

catfish *Clarias macrocephalus* (Gunther). *Aquaculture* 171, 323–338. doi: 10.1016/S0044-8486(98)00402-5.

Tanaka, S. (1995). "Comparative morphology of the sperm in chondrichthyan fishes," in *Advances in spermatozoal phylogeny and taxonomy* (Memoires du Museum National d'Histoire Naturelle: Paris), eds. B. G. M. Jamieson, J. Ausio, and L. Justine 313–320.

Thorson, T. B., Langhammer, J. K., and Oetinger, M. I. (1983). Reproduction and development of the South American freshwater stingrays, *Potamotrygon circularis* and *P. motoro*. *Environ. Biol. Fishes* 9, 3–24. doi:10.1007/BF00001055.

Tomita, T., Murakumo, K., Ueda, K., Ashida, H., and Furuyama, R. (2018). Locomotion is not a privilege after birth: Ultrasound images of viviparous shark embryos swimming from one uterus to the other. *Ethology* 125, eth.12828. doi:10.1111/eth.12828.

Tourmente, M., Gomendio, M., and Roldan, E. R. (2011). Sperm competition and the evolution of sperm design in mammals. *BMC Evol. Biol.* 11, 12. doi:10.1186/1471-2148-11-12.

Valverde Abarca, A., Castro-Morales, O., and Madrigal-Valverde, M. (2019). Sperm kinematics and morphometric subpopulations analysis with CASA systems: a review. *Rev. Biol. Trop.* 67, 1473–1487. doi:10.15517/rbt.v67i6.35151.

Villalobos-Segura, E., and Underwood, C. J. (2020). Radiation and Divergence Times of Batoidea. *J. Vertebr. Paleontol.* 40, e1777147. doi:10.1080/02724634.2020.1777147.

Vishwanath, R. (2003). Artificial insemination: the state of the art. *Theriogenology* 59, 571–584. doi:10.1016/S0093-691X(02)01241-4.

Walker, T. I. (2020). "Reproduction of Chondrichthyans," in *Reproduction in aquatic animals* (Springer: Singapore), eds. M. Yoshida and J. F. Asturiano, 193–223.

Wang, P., Al Azad, M. A. R., Yang, X., Martelli, P. R., Cheung, K. Y., Shi, J., et al. (2021). Self-adaptive and efficient propulsion of Ray sperms at different viscosities enabled by heterogeneous dual helixes. *Proc. Natl. Acad. Sci.* 118. doi:10.1073/pnas.2024329118.

Watson, L., and Janse, M. (2017). "Reproduction and husbandry of zebra sharks, *Stegostoma fasciatum*, in aquaria," in *The Elasmobranch Husbandry Manual II* (Ohio Biological Survey: Columbus, OH), eds. M. Smith, D. Warmolts, D. Thorney, R. Heuter, M. Murray and J. Ezcurra, 421-432.

Weigmann, S. (2016). Annotated checklist of the living sharks, batoids and chimaeras (Chondrichthyes) of the world, with a focus on biogeographical diversity. *J. Fish Biol.* 88, 837–1037. doi:10.1111/jfb.12874.

Whitney, N. M., Pratt, H. L., and Carrier, J. C. (2004). Group courtship, mating behaviour and siphon sac function in the whitetip reef shark, *Triaenodon obesus*. *Anim. Behav.* 68, 1435–1442. doi:10.1016/j.anbehav.2004.02.018.

Whittamore, J. M., Bloomer, C., Hanna, G. M., and McCarthy, I. D. (2010). Evaluating ultrasonography as a non-lethal method for the assessment of maturity in oviparous elasmobranchs. *Mar. Biol.* 157, 2613–2624. doi:10.1007/s00227-010-1523-4.

Worm, B., Davis, B., Kettemer, L., Ward-Paige, C. A., Chapman, D., Heithaus, M. R., et al. (2013). Global catches, exploitation rates, and rebuilding options for sharks. *Mar. Policy* 40, 194–204. doi:10.1016/j.marpol.2012.12.034.

Wourms, J. P. (1977). Reproduction and Development in Chondrichthyan Fishes. *Am. Zool.* 17, 379–410. doi:10.1093/icb/17.2.379.

Wourms, J. P. (1981). Viviparity: The Maternal-Fetal Relationship in Fishes. *Am. Zool.* 21, 473–515. doi:10.1093/icb/21.2.473.

Wyffels, J. T., George, R., Adams, L., Adams, C., Clauss, T., Newton, A., et al. (2020a). Testosterone and semen seasonality for the sand tiger shark *Carcharias taurus*†. *Biol. Reprod.* 102, 876–887. doi:10.1093/biolre/ioz221.

Wyffels, J., Coco, C., Schreiber, C., Palmer, D., Clauss, T., Bulman, F., et al. (2020b). Natural environmental conditions and collaborative efforts provide the secret to success for sand tiger shark *Carcharias taurus* reproduction in aquaria. *Zoo Biol.* 39, 355–363. doi:10.1002/zoo.21558.

Wyffels, J. T., Adams, L. M., Bulman, F., Fustukjian, A., Hyatt, M. W., Feldheim, K. A., et al. (2021). Artificial insemination and parthenogenesis in the whitespotted bamboo shark *Chiloscyllium plagiosum*. *Sci. Rep.* 11, 9966. doi:10.1038/s41598-021-88568-y.

Woolley, D. M. (2007). A novel motility pattern in quail spermatozoa with implications for the mechanism of flagellar beating. *Biol. Cell* 99, 663–675. doi:10.1042/BC20070050.

Woolley, D. M., and Vernon, G. G. (2001). A study of helical and planar waves on sea urchin sperm flagella, with a theory of how they are generated. *J. Exp. Biol.* 204, 1333–1345. doi:10.1242/jeb.204.7.1333.

Yan, H. F., Kyne, P. M., Jabado, R. W., Leeney, R. H., Davidson, L. N. K., Derrick, D. H., et al. (2021). Overfishing and habitat loss drive range contraction of iconic marine fishes to near extinction. *Sci. Adv.* 7, 1–11. doi:10.1126/sciadv.abb6026.

Yancey, P. H. (2015). Organic osmolytes in elasmobranchs. *Fish Physiol.* 34, 221–277. doi: 10.1016/B978-0-12-801286-4.00004-6.

Yancey, P. H., Blake, W. R., and Conley, J. (2002). Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 133, 667–676. doi: 10.1016/S1095-6433(02)00182-4.

Yánez-Ortiz, I., Catalán, J., Rodríguez-Gil, J. E., Miró, J., and Yeste, M. (2022). Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Anim. Reprod. Sci.* 246, 106904. doi:10.1016/j.anireprosci.2021.106904.

Yang, H., and Huo, Y. (2022). Review of molluscan larval cryopreservation and application to germplasm cryobanking and commercial seed production. *Aquaculture* 547, 737491. doi:10.1016/j.aquaculture.2021.737491.

Yano, K. (1992). Comments on the Reproductive Mode of the False Cat Shark *Pseudotriakis microdon*. *Copeia* 1992, 460. doi:10.2307/1446205.

Yano, K. (1993). Reproductive biology of the slender smoothhound, *Gollum attenuatus*, collected from New Zealand waters. *Environ. Biol. Fishes* 38, 59–71. doi:10.1007/BF00842904.

Zhou, X., Xu, S., Xu, J., Chen, B., Zhou, K., and Yang, G. (2012). Phylogenomic Analysis Resolves the Interordinal Relationships and Rapid Diversification of the Laurasiatherian Mammals. *Syst. Biol.* 61, 150. doi:10.1093/sysbio/syr089.