

Ana Magny

**Development and improvement of
gamete (cryo)preservation protocols
for elasmobranchs**



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Ana Magny

Development and improvement of gamete (cryo)preservation protocols for elasmobranchs

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Trabalho efetuado sob a orientação de:

Scientific Supervisor & UPV Tutor:

Doutor. Víctor Gallego; Universitat Politècnica de Valencia (Spain)

UALg Tutor:

Doutora. Zélia Velez; CCMAR, Universidade do Algarve (Portugal)



Faculdade de Ciências e Tecnologia

2024

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Development and improvement of gamete (cryo)preservation protocols for elasmobranchs

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Abstract

Elasmobranchs are among the most threatened groups of vertebrates on Earth, and primary threats to their survival are overfishing and habitat destruction. To address this, both in-situ conservation efforts and ex-situ conservation programs are essential. Implementing assisted reproductive technologies, though not widely developed for elasmobranchs, is a crucial aspect of these ex-situ measures. This study aimed to improve the current gamete preservation protocols in elasmobranch using new storage conditions, new cryoprotectants, and new biodegradable vials.

These trials used small-spotted catshark (*Scyliorhinus canicula*) as the model species, with sperm collected on the day of each experiment, and samples showing more than 70% motility were selected for the experiments. For the short-term storage trials, our data showed that the best short term storage method is at 4°C and pH 6.5, maintaining 40% motility for up to 14 days. About the study aimed at improving methods for "sending gamete samples", the best option was to store the sperm sample at room temperature with ice in it without cryoprotectants, achieving motilities close to 40-50% after 1 week.

On the other hand, and regarding cryopreservation trials, the best result was obtained with the combination of 5% methanol and 5% DMSO, reaching post thawed motilities of 25-30%. In addition, biodegradable capsules were tested as an ecologic alternative to plastic vials in elasmobranchs, and first attempts showed that traditional vials, such as cryotubes, showed better results (30-35% of post-thawing motility) than biodegradable vials (15-20% after cryopreservation process).

This study significantly advances gamete handling, evaluation, and cryopreservation protocols for elasmobranchs. Several objectives have been achieved, both by attempting to adapt and apply several reproductive techniques and tools previously used in other fish species, and by carrying out the current cryopreservation protocols developed on elasmobranch species.

Key words:

Sharks, Sperm; Cryobank; Reproduction; Cryobiology

Resumo

Os elasmobrânquios estão entre os grupos de vertebrados mais ameaçados da Terra e as principais ameaças à sua sobrevivência são a sobrepesca e a destruição do habitat. Para fazer face a esta situação, são essenciais tanto os esforços de conservação *in-situ* como os programas de conservação *ex-situ*. Para garantir a conservação efetiva dos elasmobrânquios, é essencial compreender os diferentes fatores que influenciam o sucesso da reprodução assistida. Embora a tecnologia para criopreservação de esperma tenha avançado consideravelmente em outras espécies aquáticas, os elasmobrânquios apresentam desafios únicos devido à sua biologia reprodutiva distinta e à complexidade dos seus espermatozoides. A baixa disponibilidade de dados e de estudos relacionados a estas espécies reforça a importância de pesquisas contínuas, que visam otimizar técnicas de reprodução assistida e, em última análise, garantir a preservação de populações. Por tanto, este estudo teve como objetivo melhorar os actuais protocolos de preservação de gâmetas de elasmobrânquios, utilizando novas condições de armazenamento, novos crioprotectores e novos frascos biodegradáveis.

A espécie modelo utilizada nestes ensaios foi o pata-roxa de manchas pequenas (*Scyliorhinus canicula*), que é uma espécie frequentemente utilizada como organismo modelo para os elasmobrânquios. Esta espécie pode ser regularmente encontrados como parte do *bycatch* da pesca, onde alguns animais foram recolhidos e transportados para as instalações de aquicultura da Universitat Politècnica de València (UPV). Os animais são mantidos separadamente em seis tanques IRTAMAR de 500L com água do mar em recirculação (temperatura: 16-18 °C; salinidade: 38 ‰). Eles são alimentados uma vez por dia com arenques, lulas e algumas vitaminas. O esperma foi recolhido no dia da experiência e foi diluído 1:10 para avaliar a sua qualidade inicial.

Para os ensaios de armazenamento de curto prazo, foram testados dois pHs diferentes (6.5 e 7.8) e duas temperaturas diferentes (4 e 20 °C). As amostras foram observadas com um microscópio e os vídeos de cada amostra foram depois analisados manualmente, tendo sido registada a percentagem de espermatozóides móveis. Em geral, os dados mostraram que o melhor método para armazenar espermatozóides por um período de curto prazo é manter as amostras a 4°C em pH de 6,5, sendo possível atingir 40% de motilidade até 14 dias de armazenamento. Em contrapartida, as amostras incubadas a 20 °C mostraram diferenças estatísticas em relação ao grupo de controlo ao fim de 1 dia de armazenamento (motilidade de 50-55%, independentemente do pH). Além disso, a motilidade dos espermatozóides era inferior a 10% aos 3 dias de armazenamento a esta temperatura de incubação. As amostras de esperma armazenadas a 4 °C não mostraram diferenças estatísticas em relação ao grupo de controlo (dia 0) até ao tempo de armazenamento de 3 dias, mostrando valores entre 55-60% em ambos pHs. A partir desse ponto, a motilidade das amostras de esperma armazenadas a 4 °C diminuiu para 45-50% após 7 dias, e para menos de 40% após 14 dias (sem diferenças significativas entre pHs). Por outro lado, e relativamente ao estudo que visava melhorar os métodos de “transporte de gâmetas”, a melhor opção foi armazenar a amostra de esperma à temperatura ambiente com gelo no seu interior sem crioprotectores, conseguindo motilidades próximas de 40-50% após 1 semana.

Em relação à criopreservação, os gametas também foram recolhido no dia da experiência, tendo sido selecionadas para as experiências amostras com mais de 60% de motilidade. No primeiro ensaio, utilizámos uma série de combinações diferentes de crioprotectores (metanol, MET; dimetil-sulfóxido, DMSO; glicerol, GLY; e etileno-glicol, ETG) e duas concentrações finais (10 e 20%) de acordo com 6 protocolos diferentes: MD-10: 5% MET + 5% DMSO; MD-20: 10% MET + 10% DMSO; GLY-10: 10% GLY; GLY-20: 20% GLY; ETG-10: 10% ETG; e ETG-20: 20% ETG. As amostras de esperma foram observadas antes e depois da criopreservação utilizando um microscópio, e uma câmara de vídeo da amostra foram depois analisados manualmente. Em geral, os resultados mostraram que as amostras pós-descongelamento apresentaram valores de motilidade significativamente mais baixos do que as amostras frescas (que tinham cerca de 60-65%). Os melhores resultados foram obtidos pela combinação MD-10, com 25-30% de espermatozóides móveis pós-descongelamento, seguida pela combinação MD-20, com valores de motilidade pós-descongelamento de 20-25%. Por outro lado, os outros crioprotectores (GLY e ETG) não produziram bons resultados, provavelmente devido à elevada toxicidade destes crioprotectores. Isto foi mais pronunciado na utilização de GLY, quando a amostra de esperma imediatamente antes do processo de criopreservação (após o tempo de incubação), mostrou uma diminuição dramática de 60 a 30% durante um período de 15 minutos.

Para o processo de criopreservação, foram testados dois crioviais diferentes: criotubos de 2 ml (CT) e cápsulas biodegradáveis de 1 ml (BC). A motilidade dos espermatozóides foi avaliada também antes e depois do processo de criopreservação. As amostras criopreservadas apresentaram valores de motilidade pós-descongelamento significativamente mais baixos do que as amostras frescas (que tinham cerca de 60-70%). No entanto, encontrámos diferenças entre os diferentes crioviais testados: O CT apresentou melhores valores de motilidade pós-descongelamento (30-35%) do que o BC (15-20%). Outros estudos que utilizem estes recipientes biodegradáveis devem abordar o tema das taxas de congelação, o que poderia melhorar a motilidade final dos gametas. No entanto, a nossa investigação pode potencialmente ajudar no desenvolvimento de projectos de inseminação artificial em elasmobrânquios, nos quais estas cápsulas biodegradáveis poderiam ser introduzidas nas fêmeas para obter sucesso na fertilização.

Em conclusão, este estudo contribui significativamente para o avanço do conhecimento na área de preservação de gametas de elasmobrânquios, oferecendo novas percepções sobre os melhores métodos de armazenamento e as potenciais melhorias tecnológicas que podem ser implementadas para aumentar a viabilidade das populações em cativeiro. A implementação dessas tecnologias, juntamente com a continuidade das pesquisas e a aplicação de práticas de conservação sustentáveis, é essencial para garantir a sobrevivência de espécies de elasmobrânquios ameaçadas e para preservar a integridade dos ecossistemas marinhos que dependem delas.

Palavras-chave

Tubarões, Esperma; Criobanco; Reprodução; Criobiologia

Table of contents

| | |
|---|-----------|
| Declaration of Authorship / Declaração de Autoria | i |
| Acknowledgements / Agradecimentos | iii |
| Abstract..... | v |
| Resumo | vi |
| Table of contents..... | viii |
| List of abbreviations | x |
| Index of figures..... | xi |
| Index of tables | xii |
| 1. INTRODUCTION | 1 |
| 1.1 The chondrichthyans | 1 |
| 1.2 Reproductive biology | 3 |
| 1.3 Aquaculture from a conservation perspective | 4 |
| 1.4 Use of reproductive technologies in elasmobranchs | 5 |
| ▶ Using gametes for preserving biodiversity | 7 |
| 1.5 Projects and grants involved in this master's Thesis..... | 8 |
| 2. OBJECTIVES | 8 |
| 3. MATERIAL AND METHODS | 9 |
| 3.1 Model species: <i>Scyliorhinus canicula</i> | 9 |
| 3.2 Maintenance of the fish..... | 9 |
| 3.3 Sperm collection | 10 |
| 3.4 Sperm motility assessment | 11 |
| 3.5 Viability assessment | 11 |
| 3.6 Short-Term storage trials | 12 |
| 3.7 Cryopreservation trials..... | 12 |
| 3.8 Comet assay | 14 |
| 3.9 Data treatment and statistical analysis | 14 |
| 4. RESULTS | 15 |
| 4.1 Short-Term storage..... | 15 |
| 4.2 Cryopreservation | 17 |
| ▶ Testing different cryo-protocols (cryoprotectants and concentrations) | 17 |
| ▶ Testing different cryovials..... | 20 |
| 4.3 Comet assay | 21 |
| 5. DISCUSSION | 22 |
| 5.1 Short term storage | 22 |

| | |
|--|-----------|
| 5.2 Gamete cryopreservation..... | 24 |
| ▶ The use of different cryoprotocols for shark sperm..... | 24 |
| ▶ The use of different vials to cryopreserve shark sperm | 26 |
| 5.3 Comet assay | 28 |
| 5.3 Future perspectives in elasmobranch’s gametes..... | 29 |
| 6. CONCLUSIONS..... | 31 |
| 7. REFERENCES..... | 32 |
| 8. ANNEXES | 35 |
| Annex 1. Congress posters | 35 |

List of abbreviations

ART – Artificial Reproduction Technique
BC – Biodegradable capsules
CaCl₂-2H₂O – Calcium Chloride Dihydrate
CASA – Computer assisted semen analysis
CT – Cryotubes
DMSO – Dimethyl sulfoxide
EE – Elasmobranch extender
EEV – Elasmobranch extender variant
EG – Ethylene glycol
EG-10 – Ethylene glycol 10%
EG-20 – Ethylene glycol 20%
FS – Fresh sample
GLY – Glycerol
GLY-10 – Glycerol 10%
GLY-20 – Glycerol 20%
HPMC – Hydroxypropyl methylcellulose
IVF – In vitro fertilization
KCl – Potassium chloride
LN – Liquid Nitrogen
MD-10 – Methanol 5% + DMSO 5%
MD-20 – Methanol 10% + DMSO 10%
MET – Methanol
MgSO₄ – Magnesium sulfate
Na₂SO₄ – Sodium sulfate
NaCl – Sodium chloride
NaHCO₃ – Sodium bicarbonate
PI – Propidium iodide
TMAO – Trimethylamine N-oxide

Index of figures

Figure 1.1. Graphic built according to the IUCN data (International Union for Conservation of Nature). The colors represent the percentage of each category per taxonomic group. CR (Red): Critically Endangered; EN (Orange): Endangered; VU (Yellow): Vulnerable; NT (Light Green): Near Threatened; LC (Green): Least concern; DD (Grey): Data Deficient. [Original draw from Garcia-Salinas *et al.*, 2023].

Figure 1.2. General scheme of the reproductive system of a generic chondrichthyan. 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, (it) isthmus, (ut) uterus, (cx) cervix, (sp) sphincter, (cl) cloaca. [Original draw from Garcia-Salinas *et al.*, 2023].

Figure 3.1. (A) Photo of *S. canicula* in the Oceanographic Aquarium in Valencia, Spain. (B) Photo of the embryonic stage of *S. canicula* in the egg capsule.

Figure 3.2. Pictures showing: (A) The bycatch of the animals at the port of Valencia. The fishermen collected some sharks, and we went to the port to bring them back to the UPV; (B) 3 tanks of the IRTAMAR system filled with around 400L of sea water; and (C) some small spotted catshark resting on the tank and waiting for the feeding moment.

Figure 3.3. (A) *S. canicula* was maintained in the tonic immobility to collect the sperm. To do that we were at least three people: one to maintain the shark, one to make a pression on the abdominal area and the last one collected the sperm. (B) Collection of the sperm samples avoiding any contamination in this area. The sharks were not eating one day before the collection

Figure 3.4. (A) The different vials tested during this experiment: on the left two cryotubes of 2mL filled with the cryopreservation solution and on the right four bio capsules: the biggest ones are made of vegetables, and the smallest ones are the animals made of gelatine. (B) Placement of cryotubes in the cryobox to start the cryopreservation process. (C) The system used to analyze the samples under the microscope and being observable on the computer to calculate the motility.

Figure 4.1. Graph showing changes in motility with time in short-term preservation experiments. The artificial seminal plasma extender variant (EEV) was used to dilute *Scyliorhinus canicula* sperm 1:9 (sperm: dilution medium) and stored at different temperatures: 4°C and 20°C and pH: 6.5 and 7.8. Motility was measured on different days (0, 1, 2, 3, 4, 7, 11, 14, 18, 21, 25, 28, and 3) after initial dilution. Different letters mean significant differences between storage groups.

Figure 4.2. Graph showing changes in motility variation over time in short-term preservation trials. *Scyliorhinus canicula* sperm was diluted 1:9 with artificial seminal plasma extender variant (EEV) and stored in different ways: some samples were kept at room temperature inside of a box full of ice and others were kept in the fridge also in a

box full of ice. Motility was assessed on different days (0, 1, 3, 5, 7) after initial dilution. Different letters mean significant differences between storage groups; and arrows indicate the first significant difference respect to the control group (motility at time 0).

Figure 4.3. Motility results for the different cryopreservation protocols in *S. canicula* (n=10). Sperm was diluted with artificial elasmobranch seminal plasma extender variant (EEV) and frozen using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET), ethylene glycol (EG) and glycerol (GLY) under different concentrations: 10% and 20%. Fresh sperm represents the control group, showing the motility on fresh sperm samples prior to freezing. Different letters mean significant differences between control and experimental groups (protocols).

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Figure 4.5. Sample of 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. Samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10× magnification.

Figure 4.6. Motility results for the different cryopreservation vials in small spotted catshark (n=10). The sperm from *Scyliorhinus canicula* was frozen in cryotubes and two types of biodegradable capsules: vegetal capsules and gelatin capsules. after being diluted with the variant of the artificial elasmobranch seminal plasma extender (EEV). Fresh sperm represents the control group, showing the motility on fresh sperm samples prior to freezing. Different letters mean significant differences between groups (vials).

Figure 4.7. Spermatozoa of the *S. canicula* after using the comet assay technique. (A) Fresh sperm under the microscope after the comet assay process; (B) Sperm under the microscope after the use of 250µL of H₂O₂ in the comet assay process; (C) Sperm under the microscope after the use of 350µL of H₂O₂ in the comet assay process; (D) Sperm under the microscope after the use of 450µL of H₂O₂ in the comet assay process; and (E) Sperm under the microscope after the use of 550µL of H₂O₂ in the comet assay process.

Index of tables

Table 3.1. 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, variant of the artificial elasmobranch seminal plasma extender (EEV) and different cryoprotectants: methanol (MET), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glycerol (GLY).

1. INTRODUCTION

1.1 The chondrichthyans

Sharks and rays are members of the ancient and biologically diversified class of aquatic vertebrates known as elasmobranchs, which first appeared 420 million years ago. Today, the class is one of the oldest and most ecologically diverse vertebrate groups, with representatives thriving in oceans, seas, and inland waters all over the world (Dulvy *et al.*, 2014). This diverse group is highly susceptible to rapid environmental changes, especially those induced by human activities. Despite playing a crucial role in controlling the ecosystems in which they exist, they are among the vertebrate groups that are most endangered on earth. For millions of years, chimaeras, sharks, and rays have been an integral part of their respective ecosystems (Garcia-Salinas, 2023).

Some elasmobranchs are critically endangered according to the IUCN red list. The main reasons for that are overfishing and their habitat destruction due to climate change. The increase of the temperature and the acidification of the oceans will have negative effects on the reproduction of many aquatic species (Dulvy *et al.*, 2021). In this sense, establishing breeding programs in captivity, often with assisted reproduction techniques, is one of the ex-situ conservation projects' primary goals. Both aquatic and terrestrial endangered species, mostly fish that are interesting for aquaculture or aquarium hobbies—as well as several endangered terrestrial species—like lynxes, tortoises, and others—have received help from the effective use of these approaches. Elasmobranchs, on the other hand, are seldom used.

For some large species, their role as apex predators is significant, but their influence goes beyond top-down management. Macropredators, or young, small- to medium-sized animals, serve as both predators and prey, bridging different food components. Many species can act as habitual or accidental scavengers in both coastal and deepwater environments. However, some species —particularly batoids— contribute as bioturbators by agitating the sediment, encouraging the resuspension of nutrients, and generating new niches that can be occupied by other animals (Heithaus *et al.*, 2010).

Even though chondrichthyans are crucial to the well operation of ecosystems and have significant social and economic value, they are disappearing at an alarming rate. At

present, with one third of their species threatened, they are the second most endangered group of vertebrates in the world (Dulvy *et al.*, 2021; Figure 1.1). 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). Their populations are declining because of habitat degradation and overfishing, both intentional and accidental bycatch. Overfishing may have obscured other stressors, but pollution exposure and climate change are becoming increasingly significant. It is important to comprehend these species' life cycles to comprehend the causes of their disappearance and the reasons that these stressors affect them so severely.

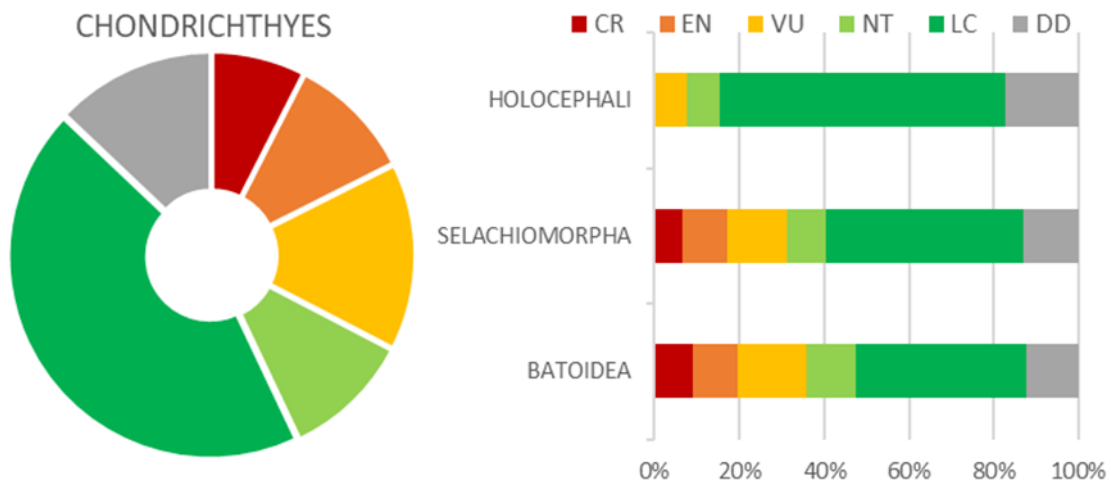


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To overcome this issue *in situ* and *ex situ* conservation programs can be used. Plans for captive breeding would be one of these initiatives that would improve the sustainability of research facilities and public aquariums while also enabling the creation of wild conservation tactics. But for these plans to work, assisted reproductive control methods must be used and overall, it is necessary to understand their reproductive biology (Garcia-Salinas, 2023).

1.2 Reproductive biology

The presence of claspers on chondrichthyan animals clearly indicates that they are dioecious. Claspers are paired structures that resemble tubes and through which sperm can pass through an interior groove (Figure 1.2). In adult animals, they are fully calcified and articulated at their base to assist insertion, whereas in young males, they are little developed. In all chondrichthyan species, fertilization is internal, a feature maintained throughout the evolutionary history of the group (Walker, 2020). Males stab females repeatedly to hold them in place while they pierce the cloaca with one or both claspers during mating. Prepelvic tenacula, also known as prepelvic claspers, are paired spatulate-like features that help retain chimeras. A frontal tenaculum, on the other hand, is a club-like appendage on males' heads that has denticles that they use to hold females (Barnett *et al.*, 2009).

The cranial part of the abdominal cavity contains the paired (or occasionally single) ovaries that form the beginning of the reproductive canal in females (Figure 1.2). The ostium, a funnel-shaped structure in the anterior region of the oviducts, receives mature oocytes that are released into the abdominal cavity and are carried there by cilia. Ovum encounters the oviductal gland during its journey through the oviducts, where it undergoes fertilization and becomes encapsulated in an egg capsule. Ultimately, the encapsulated embryos go via an isthmus and enter the uterus, where the cervix keeps them sealed off from the outside. In males, the two testes situated in the cranial region of the abdominal cavity produce immature spermatozoa. 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 (Hamlett, 2005).

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, 2005). The occurrence of sperm aggregation formation takes place at this point. The spermatozoa in the seminal vesicle and *vas deferens* lumen can appear in three different states, depending on the species: free, encapsulated in a matrix (spermatophores), or embedded in a matrix but not encapsulated (spermatozeugma).

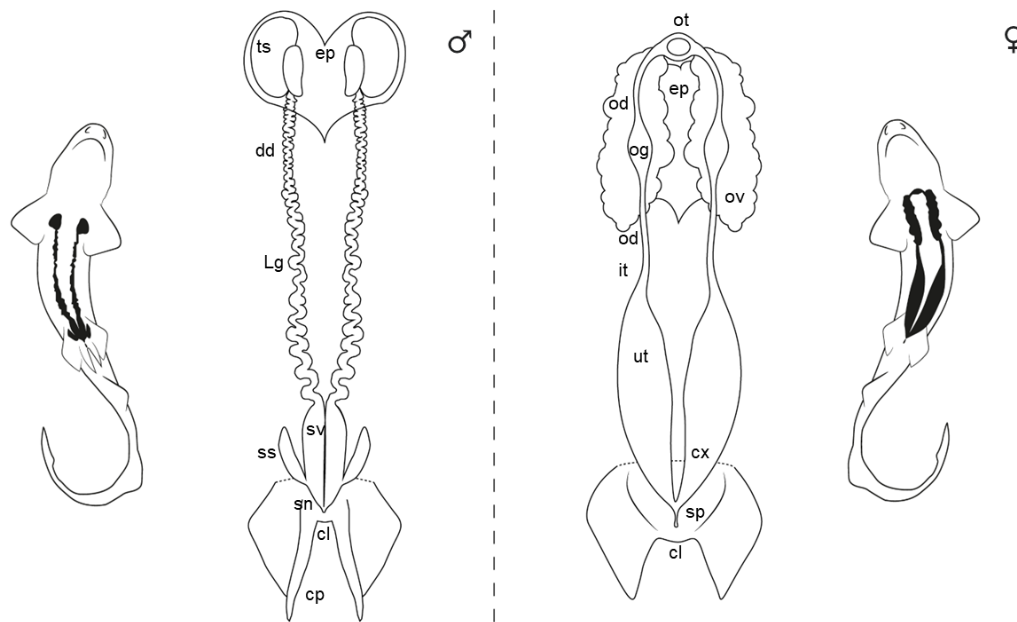


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Depending on the group, sperm transfer from males to females is driven by a different process. Male sharks have sacs (or siphons) located ventrally under the skin at the base of the pelvic fins. This sac, which has an opening at its distal end called an apophyle, 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 (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 (Walker, 2020).

1.3 Aquaculture from a conservation perspective

As marine aquaculture expands around the world, there is increased concern for protected species. Documented cases of marine mammal, sea turtle, seabird, and shark interactions with aquaculture installations do exist but are challenging to find (Bath *et al.*, 2023). Around the globe, aquaculture is the food production method with the

quickest rate of growth (Mizuta, *et al.*, 2023). Diverse strategies for reducing the ecological and social effects of aquaculture production methods have emerged because of industry's growth (Mizuta, *et al.*, 2023). At the same time, aquaculture is being used more and more for conservation, including habitat restoration, species recovery, and mitigating the effects of wild capture on harvested species (Mizuta, *et al.*, 2023).

Conservation aquaculture defined by Froehlich as human cultivation of an aquatic organism for the planned management and protection of a natural resource is a tool with enormous potential to benefit both nature and people, by simultaneously supporting populations of marine species and providing economic and social benefits to human communities (Ridlon *et al.*, 2021). Conservation aquaculture emphasizes the importance of ecologically responsible methods to implement, and scientifically rigorous methods to evaluate, the use of aquaculture techniques that purposefully align with conservation goals. Its techniques also specifically seek to minimize the risks sometimes associated with conventional aquaculture (Ridlon *et al.*, 2021).

More extensive conservation initiatives might be possible by improving the knowledge about shark physiology and behavior as well as the aquaculture technologies. Controlled breeding environments intended to look like natural habitats and artificial insemination are viable approaches to reintroducing endangered shark species (Shiffman *et al.*, 2021). Even though aquaculture is still a relatively new approach to shark conservation, it can be helpful in promoting population recovery, advancing scientific studies, and raising awareness of the importance of protecting these essential marine predators.

1.4 Use of reproductive technologies in elasmobranchs

Many aquaculture fish species (such as salmon, basses, soles, etc.) are often reproduced in captivity using zoo technological and biotechnological procedures developed over the years (Mayer, 2019). In that sense, artificial insemination, cryopreservation of gametes (sperm and eggs), and in vitro fertilization (IVF) techniques are examples of artificial reproductive technologies (ART). However, the intricate internal fertilization mechanisms and reproductive physiology of chondrichthyans pose difficulties for the use of ART, thus the development of universal reproductive technologies for

chondrichthyans is further complicated by the diversity of reproductive modes among this group, which ranges from oviparity to viviparity.

Among the ARTs, artificial insemination is emerging as a prominent reproductive technique in addressing the challenges at hand, with increasing attention from researchers (Daly and Jones, 2017; Daochai *et al.*, 2020). However, the success of this method hinges on the availability of a dependable sperm supply, particularly concerning endangered species. The utilization of cryopreserved sperm in artificial insemination serves to mitigate the risks of inbreeding and the depletion of genetic diversity in both aquaculture enterprises and public aquaria.

Although sperm cryopreservation is a well-established practice in various aquatic species (Martínez-Páramo *et al.*, 2017), information pertaining to elasmobranch sperm cryopreservation is notably scarce, confined to merely three scientific publications (García-Salinas *et al.*, 2021; Montano *et al.*, 2023; Gillis *et al.*, 2021). Among these publications, it is important to highlight the PhD, carried out by Salinas *et al.* (2023), in which they achieved for the first time the establishment of successful cryopreservation protocols for elasmobranch sperm. In those studies, they formulated a sperm extender that can be used for different elasmobranch species, capable of maintaining sperm motility for several weeks and, additionally, they also achieved the cryopreservation of sperm by previously diluting it in an elasmobranch extender and supplementing it with different combinations of cryoprotectants. However, these novel insights in elasmobranchs should be considered the starting point for a powerful branch of research, in which new techniques should be developed to complement this work.

To enhance protocols, it is beneficial to explore various elements through testing, such as new cryoprotectants, vials, and antioxidants. By assessing novel combinations and toxicity tolerances across species to take interspecies diversity into account, the range of cryoprotectants can be expanded. Recently, there has been a suggestion to investigate combinations like trehalose with DMSO or glycerol, incorporating both external and internal cryoprotectants. This approach holds promise based on prior findings with these compounds (Montano *et al.*, 2023). Furthermore, the examination of additional substances like antioxidants, as proposed by Len *et al.* (2019), could be considered to enhance survival post-freezing.

► ***Using gametes for preserving biodiversity***

Recent decades have witnessed dramatic population declines for many fish species, together with a loss in overall fish biodiversity. Globally, fish biodiversity is being threatened by a multitude of anthropogenic impacts including overfishing, habitat loss, pollution, loss of river connectivity, climate change and the impact of alien species (Mayer *et al.*, 2024). The preservation of genetic resources through the cryobanking of reproductive cells and tissues, collectively known as germplasm, will be a valuable tool in the conservation of fish biodiversity (Mayer *et al.*, 2024). With the help of a range of emerging reproductive technologies, frozen germplasm will play a key role in the future *in situ* and *ex situ* conservation initiatives (Mayer *et al.*, 2024). For species in which natural mating is challenging due to limited population size or behavior, cryopreserved sperm can be used in artificial insemination.

Cryopreservation is one of the most effective ways to store gametes for decades without genetic degradation. Cryopreservation, as a cutting-edge scientific technique, plays a pivotal role in the conservation and preservation of biodiversity within the realm of fish species. This process involves the preservation of sperm at ultra-low temperatures (-196°C) to minimize physiological activity (Horváth and Urbányi, 2020). However, to avoid damage from freezing, cryoprotectants (acting both within and outside the cell) are used (Liu *et al.*, 2021). Cryopreservation contributes to the conservation of rare and endangered fish species by establishing genetic repositories that can be utilized for artificial reproduction programs (Asturiano *et al.*, 2017; Martínez-Páramo *et al.*, 2017).

As fish populations face increasing threats from anthropogenic activities and environmental changes, having a diverse genetic pool preserved through cryopreservation becomes a crucial tool for enhancing the resilience and adaptive potential of these species (Martínez-Páramo *et al.*, 2017). Cryopreservation is a proactive approach to preserving genetic diversity that is crucial for maintaining the health and resilience of fish populations in the face of evolving environmental challenges and contribute to the sustainable management of aquaculture and fisheries. In this sense, gamete preservation offers a unique approach to conserving biodiversity, allowing us to safeguard genetic material, enhance genetic diversity and provide a means of population recovery for endangered species.

1.5 Projects and grants involved in this master's Thesis.

All the work of thesis was partially funded by the by the Spanish Ministry of Science and Innovation through the project ELASMOREP “Development of assisted reproduction techniques for the conservation of sharks and rays” (PID2022-138847-I00). This national project has a duration of 3 years and during this thesis part of the goals proposed in the project have been covered. On the other hand, Ana Magny received an Erasmus grant from the European Union; and the supervisor Victor Gallego has a contract Beatriz Galindo Junior (BG22/00024) funded by the Ministerio de Universidades (Spain).

2. OBJECTIVES

The general goal of these was focused on the development and improvement of gamete (cryo)preservation protocols for elasmobranchs. Due to this topic involves several fields, tools and techniques, the specific objectives of the project were:

I. *To improve the short-term storage methods for elasmobranchs gametes.*

This goal was focused on improving the short-term storage methods in *Scyliorhinus canicula* sperm testing different temperatures, pHs, and storage methods to keep sperm motility for a long time.

II. *To improve the long-term storage methods for elasmobranchs gametes.*

This goal was focused on improving the cryopreservation protocols in elasmobranch sperm using a battery of cryoprotectants and concentrations and, in addition, assessing the use of new biodegradable vials.

III. *To test and set up new techniques for elasmobranchs gametes assessment.*

Because the shape of the shark's sperm is elongated, this goal was focused on developing new tools like the comet assay process to analyze the DNA damage of the sperm before cryopreservation.

3. MATERIAL & METHODS

3.1 The model species: *Scyliorhinus canicula*

The small spotted catshark (*Scyliorhinus canicula*) is the most abundant catshark in European inshore waters and one of the most abundant cartilaginous fish in the central Mediterranean Sea (Ragonese *et al.*, 2013). Characterized as a small-sized demersal shark (Figure 3.1A), its geographical distribution extends from the Senegalese coast to the west coast of Norway (Ellis & Shackley, 1997). *S. canicula*, like the majority of Scyliorhinidae, is an oviparous species and lays its eggs in a protective egg case which are deposited on seaweed throughout the year (Figure 3.1B). It is a small, shallow-water shark inhabiting waters of depths ranging from a few meters down to 400 meters (Mancia., *et al.* 2020). Due to its small size and benthic habits, this shark species offers special possibilities as a model organism, and it is easy to maintain in aquaculture (Mancia., *et al.* 2020). They are caught primarily as shark bycatch or secondary targeted catch in various artisanal and industrial fisheries, especially in the Mediterranean (Mancia., *et al.* 2020).

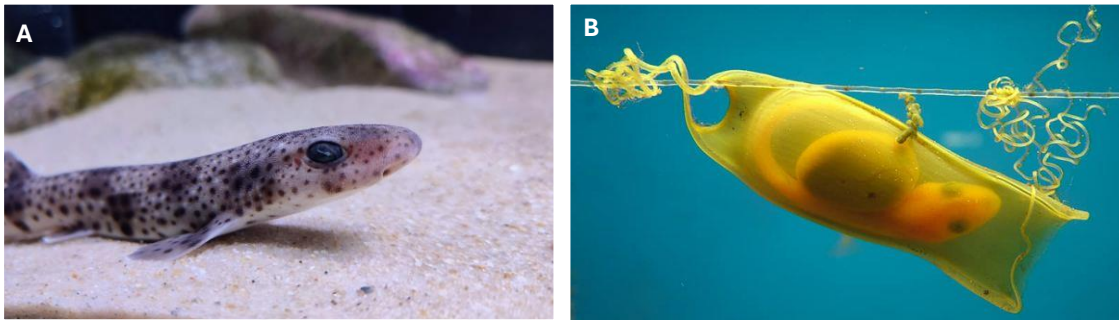


Figure 3.1. (A) Photo of *S. canicula* in the Oceanographic Aquarium in Valencia, Spain. (B) Photo of the embryo of *S. canicula* in the egg capsule in an aquarium.

3.2 Maintenance of fish

To set up different techniques, we will use as model species the small-spotted catshark (*Scyliorhinus canicula*), that can be regularly found as part of the fishery by-catch (Figure 3.2A). Some animals were collected from fishermen in Valencia's harbor and transported to the aquaculture facilities in the Universitat Politècnica de València (UPV). Animals are kept separately in six tanks of 500L with recirculating seawater

(temperature: 16-18 °C; salinity: 38-40‰). The animals are selected between males and females and mature and immature females. At the end we had 4 tanks of females and 2 tanks for the males (Figure 3.2B & C). They are fed once a day with herrings, squids, and some vitamins. Around an hour after they eat the leftover food is taken away to avoid the increase in the bacteria level. To help the good control of the animals, twice a week the water parameters were checked: pH, temperature, oxygen, level of nitrite and nitrate. In case of a rare result the water was changed for half and the parameters were checked then every day until having good results.

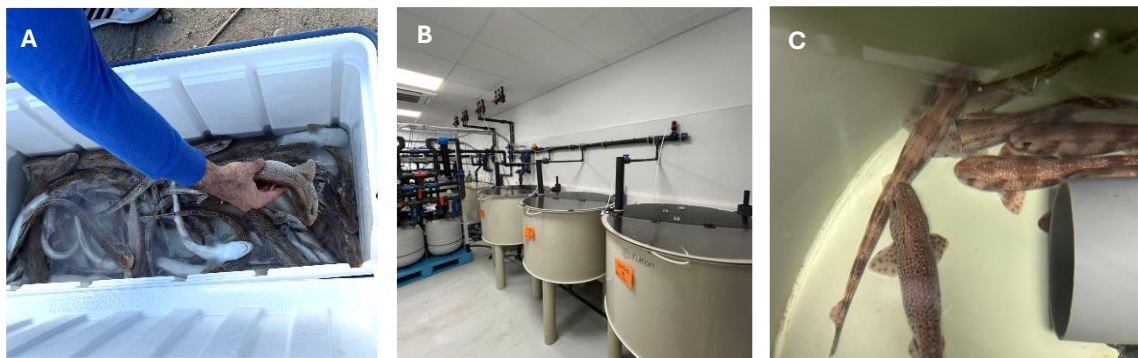


Figure 3.2. Pictures showing: (A) The bycatch of the animals at the port of Valencia. The fishermen collected some sharks, and we went to the port to bring them back to the UPV; (B) 3 tanks of the IRTAMAR system filled with around 400L of sea water; and (C) some small spotted catshark resting on the tank and waiting for the feeding moment.

3.3 Sperm collection

For “*in vivo*” collection, tonic immobility will be induced prior to *in vivo* sperm extraction to minimize struggling and reduce stress during handling (Figure 3.3A), as this technique carries fewer risks to animal health than the application of anesthetics. The procedure is carried out by holding the animals in an upside-down position while gentle pressure is applied to its snout (Figure 3.3B). With the cloaca emerged, pressure on the abdominal area over the seminal vesicle is enough to make sperm flow through the urogenital papillae (García-Salinas *et al.*, 2021). Sperm flowing from the urogenital papillae will be immediately collected using a sterile syringe or pipette and transferred to sterile tubes after collection.



Figure 3.3. (A) *S. canicula* was maintained in the tonic immobility to collect the sperm. To do that we were at least three people: one to maintain the shark, one to make a pression on the abdominal area and the last one collected the sperm. (B) Collection of the sperm samples avoiding any contamination in this area. The sharks were not eating one day before the collection.

3.4 Sperm motility assessment

Before assessing the sperm motility, fresh sperm will be diluted in artificial elasmobranch seminal plasma extender, called EE, and reported by Salinas *et al.* (2021). The components of the extender [in mM; 433 Urea, 376 NaCl, 120 Trimethylamine N-oxide (TMAO), 8.4 KCl, 50 Glucose, 7 CaCl₂·2H₂O, 3.5 NaHCO₃, 0.08 Na₂SO₄, 1.4 MgSO₄ (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.

To assess the spermatozoa motility, a 0.5 µL of diluted sperm will be collected from each sample and added to 4.5 µL of EE placed on a counting chamber ISAS Spermtrack 10 (Proiser R + D, S.L., Spain). Samples will be observed using a microscope with a 10x magnification lens (Nikon Eclipse 80i) and videos of the sample will be recorded by a camera (Nikon Digital Sight DS-5M). The videos are then analyzed subjectively (by human eye), and the percentage of motile (displacing, rotating, or actively beating flagella) and non-motile spermatozoa will be recorded. A mean of 80–100 spermatozoa were individually counted per sample. During this thesis we also explored the synergy between the CASA system and ImageJ, an open-source image processing software, as a powerful combination for comprehensive sperm analysis.

3.5 Viability assessment

Viability analysis was conducted for the cryopreservation experiment, in every fresh and thawed sample with flow cytometry using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher) containing SYBR 14, that stains in green the nuclei of living cells, and

propidium iodide (PI) that stains in red the nuclei of dead cells. For each sample, SYBR 14 (final concentration 100 nM) and PI (final concentration 12 μ M) will be added to fresh or thawed sperm samples and incubated at room temperature and darkness for 10 min (Garcia-Salinas *et al.*, 2021). Thereafter, the samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10 \times magnification and pictures were taken. Pictures were analyzed to count the number of green and red sperm heads.

3.6 Short-term storage trials

For the short-term storage, *S. canicula* sperm samples were initially diluted at a 1:9 ratio, with 100 μ L of fresh sperm combined with 900 μ L of the variant extender (EEV). Only samples with initial motility exceeding 60% were included in the trials.

For the first trial (n=12), and to identify optimal preservation conditions, samples were stored at different temperatures (4°C and 20°C) and pHs (6.5 and 7.8) making up 4 experimental groups. Sperm motility was assessed after initial dilution on days 1, 2, 3, 4, 7, 11, 14, 18, 21, 25, 28, and 31. For the second trial (n=7), and in order to find a easy and cheap shipment method to send shark gametes, some storage methods were kept at different temperatures putting the sperm in a Styrofoam box full of ice with or without cryoprotectants. Motility was assessed on different days (0, 1, 3, 5, 7) after initial dilution.

3.7 Cryopreservation trials

Regarding cryopreservation trials, sperm samples with motility greater than 60% will be selected for cryopreservation assays. In general, 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). 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. After the incubation time the solution was analyzed to see the sperm motility. After the equilibration process, vials were placed over a metal mesh platform. Vials were completely submerged into the LN after that time, where they remained for 5–10 min. For the thawing process, vials were extracted from the LN and submerged for a time in a water bath. Duplicate sperm samples will be tested for each protocol to minimize the experimental error.

For the first trial, sperm samples were frozen using 6 cryoprotocols based on different concentrations (from 10 to 20%) and different internal cryoprotectants: methanol (MET); dimethyl sulfoxide (DMSO); glycerol (GLY); see Table 3.1. In this trial, the 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.

Table 3.1. 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, variant of the artificial elasmobranch seminal plasma extender (EEV) and different cryoprotectants: methanol (MET), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glycerol (GLY).

| Protocol | Sperm | EEV | DMSO | MET | EG | GLY |
|----------------|-------|-----|------|-----|-----|-----|
| Control | 10% | 90% | - | - | - | - |
| DM-10 | 10% | 80% | 5% | 5% | - | - |
| DM-20 | 10% | 70% | 10% | 10% | - | - |
| EG-10 | 10% | 80% | - | - | 10% | - |
| EG-20 | 10% | 70% | - | - | 20% | - |
| GLY-10 | 10% | 80% | - | - | - | 10% |
| GLY-20 | 10% | 70% | - | - | - | 20% |

For the second trial, we aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers to plastic cryotubes. For that, sperm samples were packed both in cryotubes of 2mL and in some new biodegradable capsules of 1-mL tested previously by França *et al.* (2023) in other teleost species (Figure 3.4A). Those biodegradable capsules come from plant and animal basis and contained only cryoprotectants at a concentration of 10%. In this trial, capsules were placed over a metal mesh platform floating 2 cm over the LN, for a period of 10 min. Biodegradable capsules were completely submerged into the LN after that time, where they remained for 5–10 min. For the thawing process, biodegradable capsules were extracted from the LN and submerged for 15 seconds at 25°C. The capsules exploded in the tube and placed in a vortex for 30 seconds.

In both trials, and for assessing the effect of the cryopreservation process, both fresh and cryopreserved sperm will be analyzed using the techniques set up (Figure 3.4C) and developed during the thesis described in the last steps (i.e. CASA analysis).

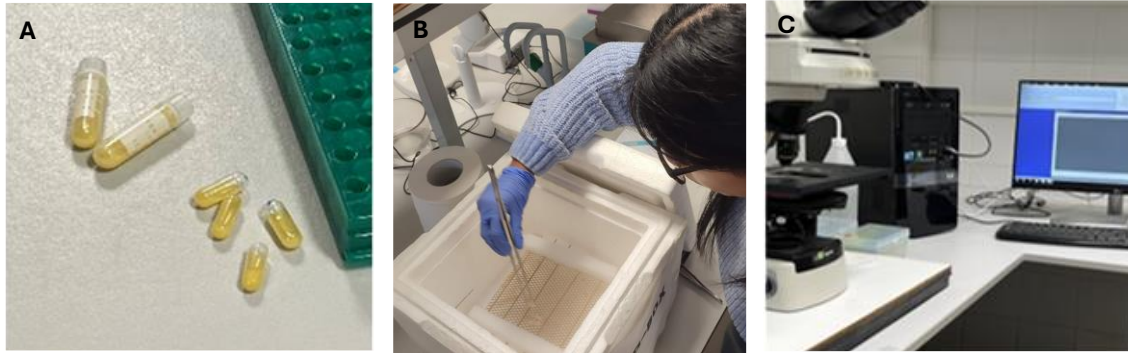


Figure 3.4. (A) The different vials tested during this experiment: on the left two cryotubes of 2mL filled with the cryopreservation solution and on the right four bio capsules; (B) Placement of cryotubes in the cryobox to start the cryopreservation process. (C) The system used to analyze the samples under the microscope and being observable on the computer to calculate the motility.

3.8 Comet assay

The protocol was adapted from the original developed by Cabrita *et al.* (2005) with some modifications to fit the target species. Agarose coated slides were used to support 50 μ l of cells previously embedded in low-melting agarose. Different parameters were tested as the hydrogen peroxide (H₂O₂) at different volumes to use it as the positive control of the comet assay. After incubation in a lysis solution and electrophoresis, the samples were incubated in a neutralization solution and finally fixed with ethanol. Once dried and fixed, the samples were stained with PI and observed with a 40x objective on a fluorescence microscope. Photographs were taken with Wave-Image software and then cell damage was analyzed. The parameter analyzed was the percentage of DNA in the tail (% DNAt). The comet assay scale was used to quantify DNA damage based on the appearance and distribution of DNA in the comet. This scale is often used to grade the extent of damage by comparing the size and intensity of the comet tail relative to the head.

3.9 Data treatment and statistical analysis

Shapiro-Wilk and Levene tests are used to check data normality and variance homogeneity, respectively. To analyze the differences between the short-term preservation trials over time, a univariate General Linear Model (GLM) was used. On the contrary, a One-factor ANOVA was used to compare the different cryopreservation vials and protocols. Model results of p-values <0.05 were considered significant; and a Tukey post hoc test was used in all cases. All analyses were conducted in the R-environment.

4. RESULTS

4.1 Short-Term storage

For the first trial, samples were stored at different temperatures (4°C and 20°C) and pHs (6.5 and 7.8) making up 4 experimental groups, and assessing the sperm motility over time on days 0, 1, 2, 3, 4, 7, 11, 14, 18, 21, 25, 28, and 31. The findings, which can be seen in Figure 4.1, showed that in general there was a continuous decrease in motility over time, with significant differences between treatments.

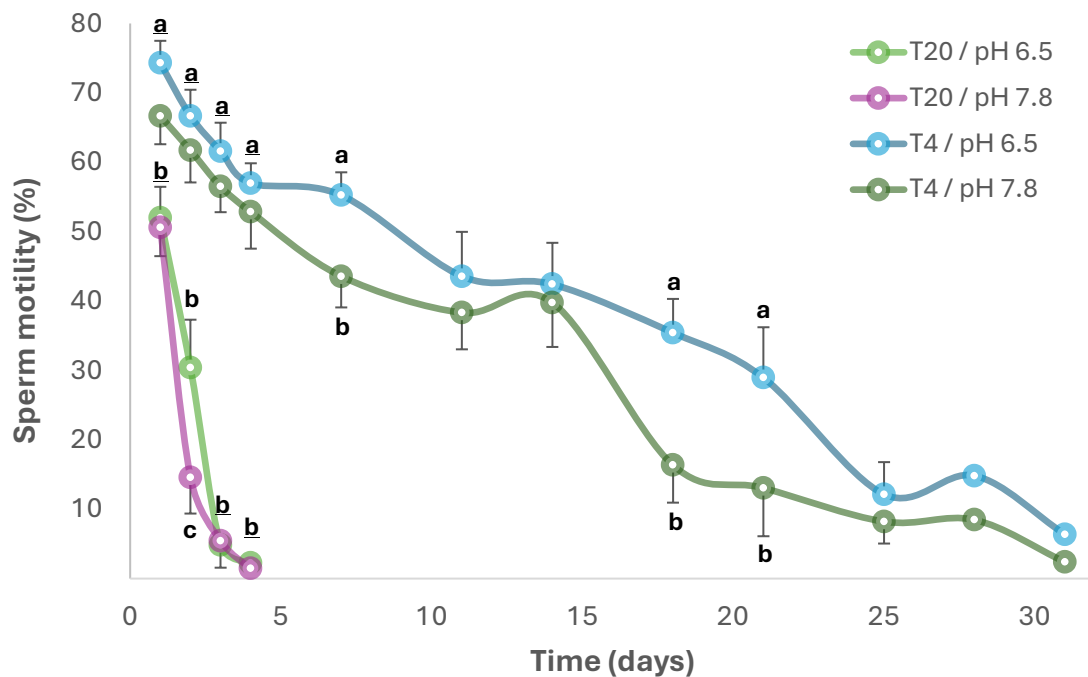


Figure 4.1. Graph showing changes in sperm motility over time in short-term preservation trials (n= 12). The artificial seminal plasma extender variant (EEV) was used to dilute *Scyliorhinus canicula* sperm 1:9 (sperm: dilution medium) and stored at different temperatures: 4°C and 20°C and pH: 6.5 and 7.8. Motility was measured on different days (0, 1, 2, 3, 4, 7, 11, 14, 18, 21, 25, 28, and 31) after initial dilution. Different letters mean significant differences between storage groups.

Sperm motility in control group (day 0) was 70-75% (data not shown), and it was checked over one month. In general, sperm samples storage at 4°C (independently of pH incubated), showed higher motility than samples incubated at 20°C in every checking point. Samples stored at 20°C displayed significant differences from the first day compared to control samples (65-75% versus 50-55%, independently of the pH). In addition, sperm motility was below 10% at 3-day storage at room temperature. On the other hand, sperm samples stored at 4°C did not show statistical differences respect to

the control group (day 0) until 3-day storage time, showing values between 55-65% at both pHs. From that point, the motility of sperm samples stored at 4°C decreased to 45–50% after 7 days, and below 40% after 14 days (without significant differences between pHs). From 2 weeks of storage, sperm motility samples showed a progressive reduction until day 30, with significantly higher motility values of samples incubated at pH 6.5.

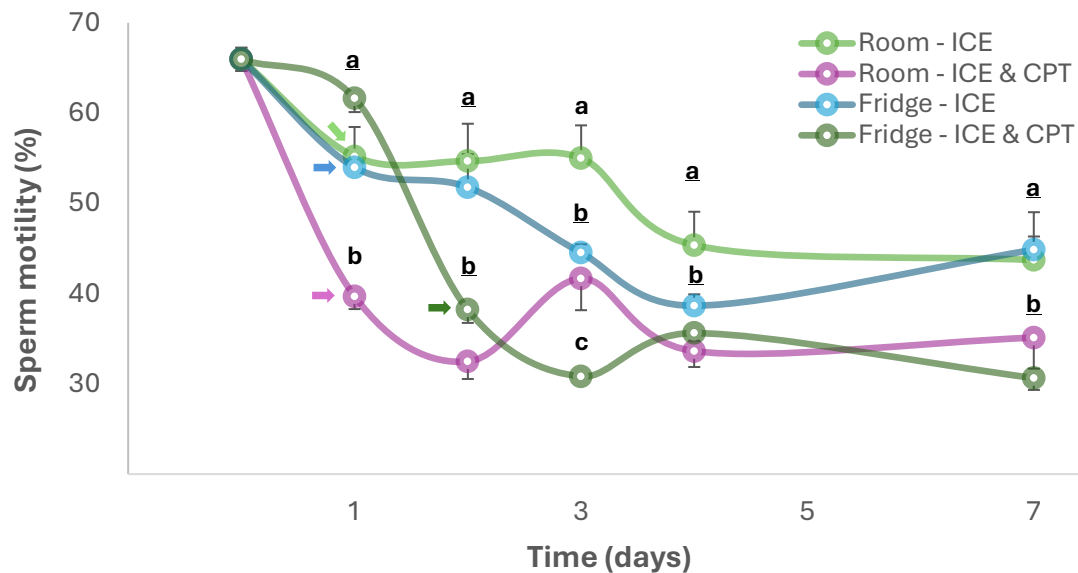


Figure 4.2. Graph showing changes in sperm motility over time in short-term preservation trials (n = 7). *S. canicula* sperm was diluted 1:9 with artificial seminal plasma extender variant (EEV) and stored in different ways crossing temperatures and the addition of cryoprotectants. Motility was assessed on different days (1, 3, 5, 7) after initial dilution. Different letters mean significant differences between storage groups; and arrows indicate the first significant difference respect to the control group (motility at time 0).

On the other hand, Figure 4.2 illustrates the variation in sperm motility over time for *Scyliorhinus canicula* under different short-term preservation conditions (shipment trials). Sperm was diluted at 1:9 ratio with an artificial seminal plasma extender (EEV), and four protocols were tested: i) samples were either kept at room temperature with ice, ii) at room temperature with ice and a cryoprotectant treatment, iii) in a refrigerator with ice, and iv) in a refrigerator with ice and a cryoprotectants. Motility assessments after day 0 reveal that sperm motility generally declined over time, but the rate of decline and stabilization varied by protocol. Samples stored at room temperature with ice showed a decrease in motility by day 1 but stabilizing between day 1 and 3 at around 55% motility and showing significant differences respect to the rest of treatments. The use of cryoprotectants led to an immediate decrease in motility in samples incubated at room temperature, while the samples at 4°C did not show this sudden drop.

Comparing all these protocols, the best one is the one for samples stored at room temperature with ice inside without cryoprotectants, as it shows less gradual variation over time. Samples stored in the refrigerator also performed well, with both protocols achieving motility close to 50% after one week.

4.2 Cryopreservation

Testing different cryo-protocols (cryoprotectants and concentrations)

In general, results showed (Figure 4.3) that post-thawed samples have significantly lower motility values than the fresh samples (which were around 70%). The best results were obtained by the DMSO (5%) and Methanol 5%) combination, with 25% of motile spermatozoa after post-thawing process, followed by the MD-20 combination, with post-thawing motility values of 15-20%. On the other hand, the other cryoprotectants (GLY and ETG) did not produce good results, probably due to the high toxicity of these cryoprotectants. This was more pronounced in the use of GLY, when sperm sample just before cryopreservation process (after incubation time), showed a dramatic decrease from 60 to 30% for a period of 15 min.

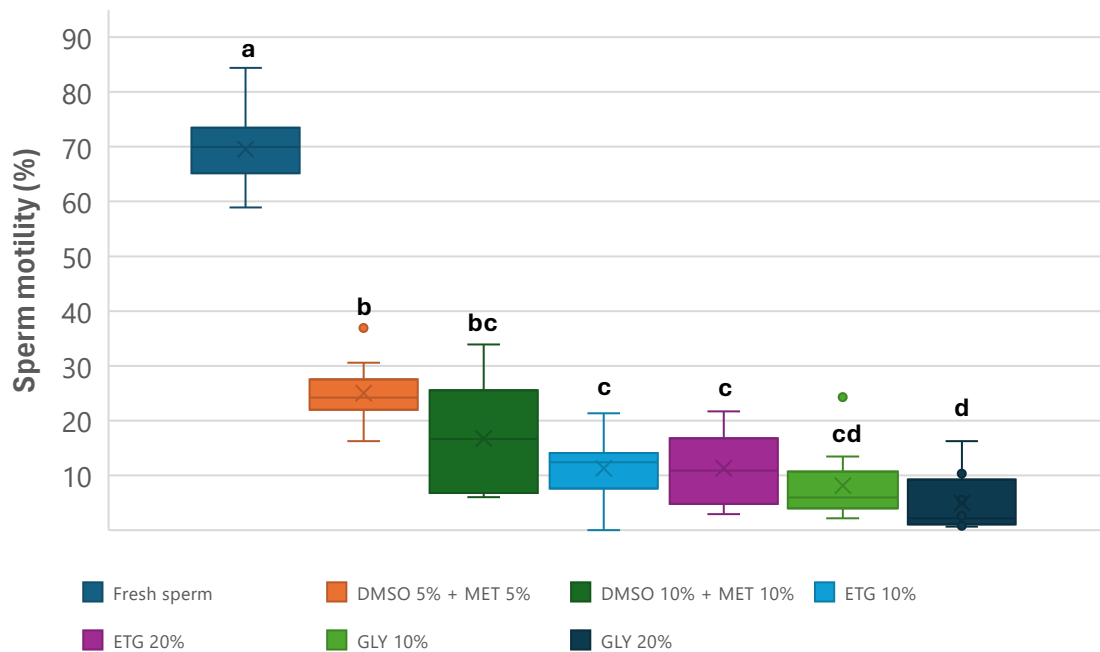


Figure 4.3. Motility results for the different cryopreservation protocols in *S. canicula* (n = 10-12). Sperm was cryopreserved using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET), ethylene glycol (ETG) and glycerol (GLY) under different concentrations: 10% and 20%. Fresh sperm represents the control group, showing the motility on fresh sperm samples prior to freezing. Different letters mean significant differences between control and experimental groups (protocols).

Regarding viability, Figure 4.4 shows sperm viability (%) across different cryoprotectant treatments compared to fresh sperm as a control. Fresh sperm shows the highest viability, with a median value near 60%. In contrast, all treated samples, regardless of the cryoprotectant used, Cell viability decreased significantly.

Among the cryoprotectant treatments, samples treated with MD-10 displayed reasonable viability between 5 at 25%. This suggests that the combination of DMSO at 5% and MET at 5% can be effective in maintaining sperm viability in some trials. On the other hand, a higher concentration of DMSO and MET, specifically 10% each showed an increase in viability with a median closer to 20%, though still significantly lower than fresh sperm. The increased DMSO and MET concentrations offer some additional protection, but as we reported in the last section, also can reduce the spermatozoa motility.

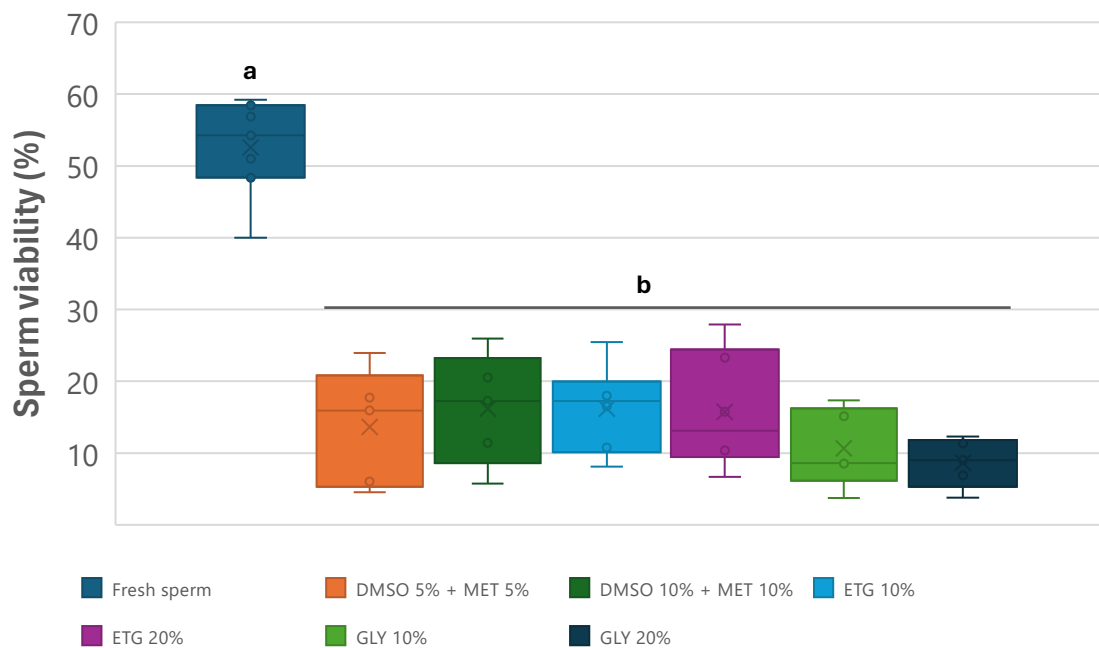


Fig 4.4. Viability results for the different cryopreservation protocols in *S. canicula* (n=10-12). Sperm was diluted with artificial elasmobranch seminal plasma extender variant (EEV) and frozen using different cryoprotectant combinations: dimethyl sulfoxide (DMSO), methanol (MET), ethylene glycol (ETG) and glycerol (GLY) under different concentrations: 10% and 20%. Fresh sperm represents the control group, showing the motility on fresh sperm samples prior to freezing. Different letters mean significant differences between control and experimental groups (protocols).

ETG treatments, at both 10% and 20% concentrations, have mixed results. The 10% ETG treatment displayed a median viability closer to 20%, with a moderate range. In contrast, the 20% EG treatment showed slightly lower viability around 15%. Treatments using

glycerol at 10% and 20% also demonstrated low viability outcomes. The 10% glycerol treatment has a median viability around 10%, while the 20% glycerol treatment produced a similar median but slightly under 10% of viability, indicating consistency but no improvement in sperm survival. This suggests that glycerol at these concentrations is more toxic than the other cryoprotectants and does not perform as well as fresh sperm or even the higher DMSO and MET combination.

The live and dead kit to stain the sperm was also used. Figure 4.5 shows the live and dead staining that employs the fluorescent dyes SYBR Green (live cells) and propidium iodide (PI) (dead cells) that have been used frequently in Chondrichthyans. The viability of the sample is around 15% according to the final count.

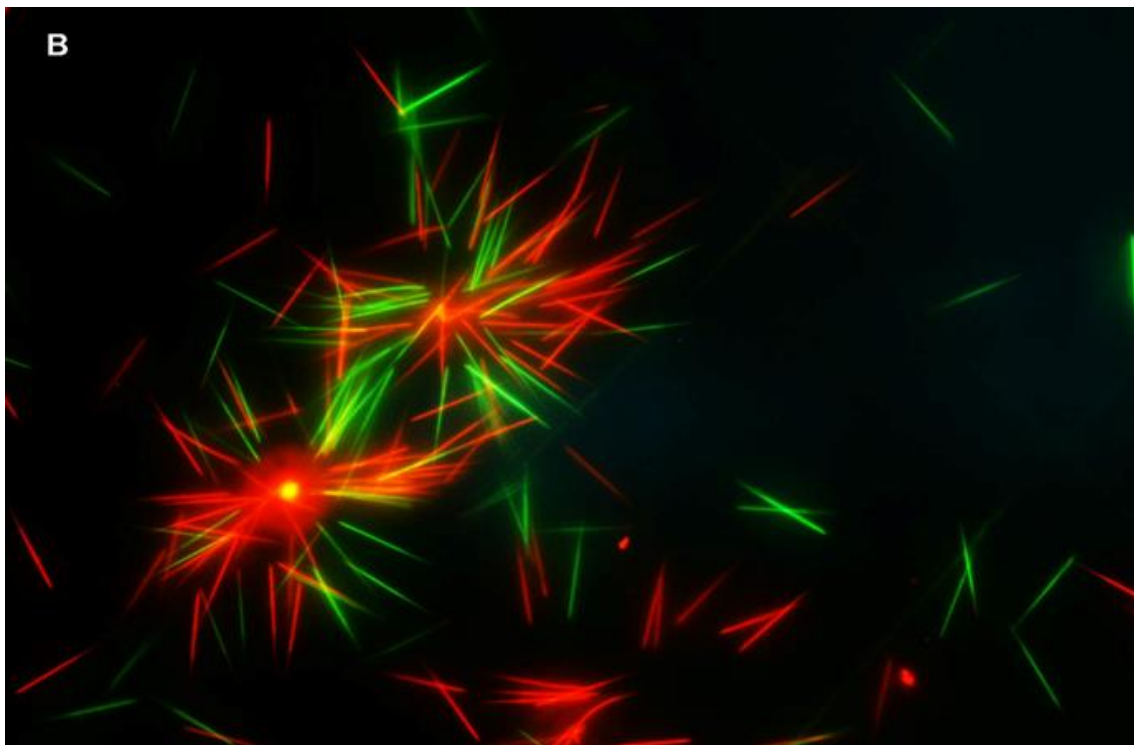


Figure 4.5. Sample of stained sperm to study the viability of the thawed spermatozoa by assessing their plasma membrane integrity. SYBR-14 stains intact cells green, and propidium iodide (PI) stains damaged cells red. Samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10× magnification.

► **Testing different cryovials**

For cryopreservation process, two different cryovials were tested: 2-mL cryotubes (CT) and 1-mL biodegradable capsules (BC). Sperm motility was assessed both before and after the cryopreservation process. Cryopreserved samples showed (Figure 4.6) significantly lower post-thawing motility values than the fresh samples (which had around 70%). However, we found differences between the different cryovials tested: CT showed better post-thawing motility values (30-35%) than BC (15-20%). Further studies using these biodegradable containers should address the topic of freezing rates, which could improve the final motility of gametes. However, our research can potentially help with the development of artificial insemination projects in elasmobranchs, in which these biodegradable capsules could be introduced directly into females to achieve fertilization success.

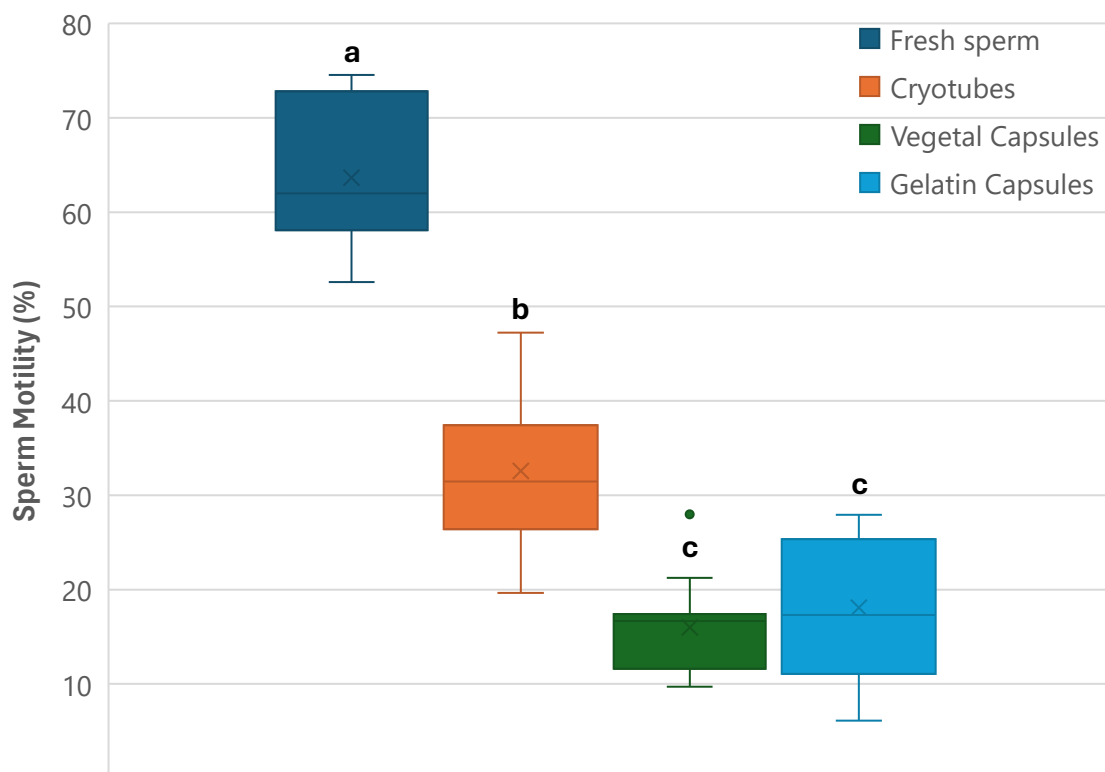


Figure 4.6. Motility results in the different cryopreservation vials in small spotted catshark (n=12). The sperm from *Scyliorhinus canicula* was frozen in cryotubes and two types of biodegradable capsules: vegetal capsules and gelatin capsules. after being diluted with the variant of the artificial elasmobranch seminal plasma extender (EEV). Fresh sperm represents the control group, showing the motility on fresh sperm samples prior to freezing. Different letters mean significant differences between control and experimental groups (vials).

4.3 Comet assay

Few experiments were carried out to assess the comet assay technique on elasmobranch spermatozoa, and Figure 4.7 showed the results. The protocol used is the one described in the material and method part adapted from the one used with the eels. This comet assay is commonly used to assess DNA damage at the cellular level.

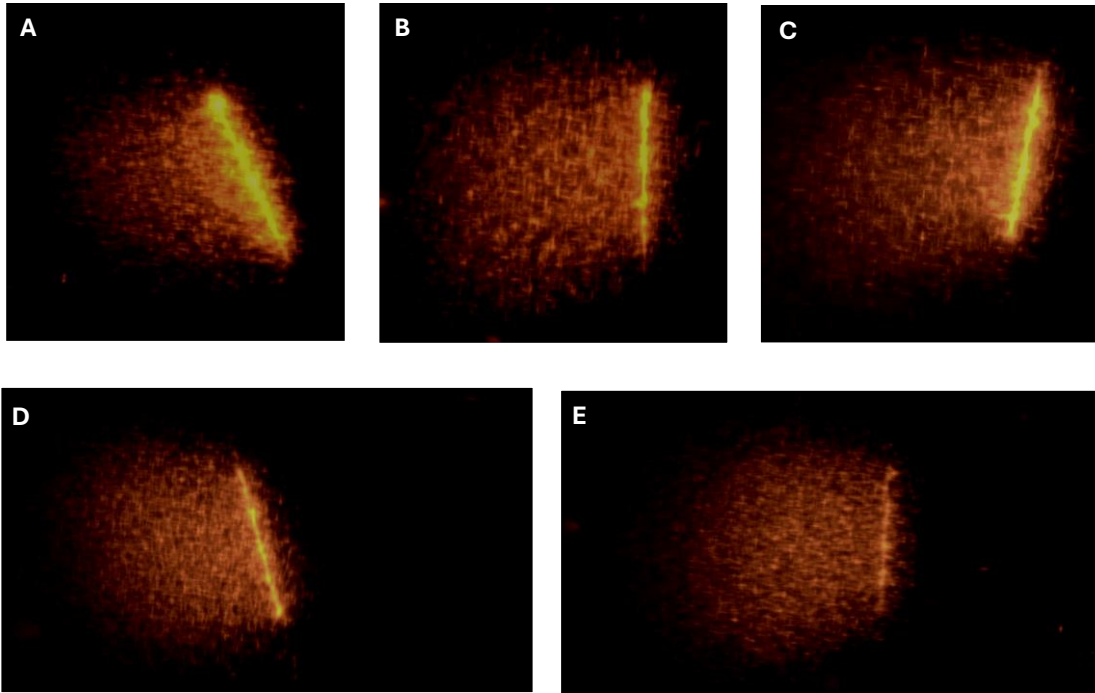


Figure 4.7. Spermatozoa of the *S. canicula* after using the comet assay technique. (A) Fresh sperm under the microscope after the comet assay process; (B) Sperm under the microscope after the use of 250μL of H₂O₂ in the comet assay process; (C) Sperm under the microscope after the use of 350μL of H₂O₂ in the comet assay process; (D) Sperm under the microscope after the use of 450μL of H₂O₂ in the comet assay process; and (E) Sperm under the microscope after the use of 550μL of H₂O₂ in the comet assay process.

In the image presented we can see the head of the spermatozoa, which is the brightest area which is a concentrated area at one end of the comet. The intensity of this area suggests that a portion of the DNA has not experienced significant breakage. For the tail the trailing smear of light extending from the head indicates fragmented DNA, with smaller DNA fragments migrating further away from the head during electrophoresis.

5. DISCUSSION

Attempts to breed elasmobranchs in public aquariums to improve the sustainability of their zoological collections or to implement conservation programs are not as developed as for other species. However, captive breeding programs are becoming increasingly important, particularly for threatened species (Daly and Jones, 2017). There is a wide range of reproductive techniques that could be used for elasmobranchs, such as reproductive cycle monitoring, hormone treatments, gamete collection, sperm cryopreservation and artificial insemination. These techniques are well documented in many mammals, reptiles, birds and bony fishes, but their use in elasmobranchs has been anecdotal (Daly and Jones, 2017; Penfold and Wyffels, 2019). In this study, we present a new contribution to the field, with the intention of establishing the basis for the development of affordable methods of preserving elasmobranch sperm without the need for expensive or complex equipment.

5.1 Short-term storage

Sperm motility is one of the most used parameters to determine semen quality. Environmental temperature, pH, ionic composition and osmolality affect spermatozoa motility (Shazada., *et al.* 2024). The study's findings demonstrate how temperature and pH affect the motility of sperm samples that have been preserved. These conditions differ in terms of pH (6.5 and 7.8) and temperature (4 and 20°C). While all groups showed a decrease in sperm motility, we observed that the rate of decline and the final motility levels are significantly influenced by both pH and temperature conditions. When samples were stored at 4°C, they continuously showed more motility than when they were stored at 20°C, where they quickly lost motility after just one day of incubation.

In the groups stored at 4°C, sperm motility remained comparable to fresh samples (day 0) for up to three days, regardless of pH. Motility values ranged from 50-60% during this period, suggesting that cold storage is effective in maintaining sperm motility for short periods of time. However, after the initial three days, differences between the two pH conditions began to emerge. Samples stored at pH 6.5 maintained higher motility values than those stored at pH 7.8. This indicates that a slightly acidic environment may offer superior protection for sperm motility during extended storage. This is consistent with

previous findings showing that acidic environments, more closely to mimic the natural conditions of the female tract, help to retain the sperm function much longer.

The decline in motility after two weeks of storage was observed across all samples, with a progressive reduction in motility as the storage time increased. The rapid loss of motility stored at 20°C (both pHs) shows the harmful effect of high temperatures. In both cases, motility fell to almost zero within the first five days of storage. This suggests that higher temperatures accelerate sperm degradation, likely due to increased metabolic activity which will decline the motility (Shazada., *et al.* 2024). Cold temperatures, on the other hand, reduce metabolic rates, slowing down the degradation process.

The decline in sperm motility became especially apparent after two weeks of storage across all conditions. Day 17, sperm motility in samples stored at 4°C and pH 7.8 had dropped to around 10%, indicating that this combination of storage conditions was less effective for long-term preservation. On the other hand, samples kept at 4°C and pH 6.5 showed great resilience with the motility remaining higher for a long period. This supports the notion that pH6.5, when combined with cold storage, provides a more optimal environment for preserving sperm motility over extended periods. In other studies, like the one from Shazada., *et al.* (2024) shown that for the common carp, the pH of seminal plasma is between 7.5 and 8.5. On the other hand, for the shark sperm, the pH7.8 environment, being more alkaline, may increase metabolic activity, contributing to quicker declines in motility despite the cold storage conditions. This suggests that for shark sperm a neutral and acidic environment provides an appropriate environment for spermatozoa to protect their motility potential.

The findings suggest that storing sperm at 4°C are the best conditions in preserving motility in the short term, with pH 6.5 providing an additional benefit for extended storage periods. Regarding pH, sperm motility starts to gradually drop after two weeks, suggesting that further clarification of long-term storage procedures is necessary. Developing improved techniques to maintain sperm viability beyond this point will be important for the application in the ARTs and cryopreservation. The cool temperatures during the period of sperm storage maintain spermatozoa metabolism at a minimal level. However, if the storage time is prolonged, microbial contamination may reduce sperm motility and viability (Shazada., *et al.* 2024).

5.2 Gamete cryopreservation

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. Cryoprotectants effectiveness depends on its toxicity to the spermatozoa, its concentration, the equilibration duration time, and the diluents used. Regarding sharks, the most extensive study ever conducted was carried out during the doctoral thesis of Garcia-Salinas et al. (2023), in which authors were able to report the development of different formulas to achieve the long-term storage of elasmobranchs. Therefore, all the work carried out in this master's thesis has been based on the mentioned studies, where researchers were able to develop protocols and cryopreserved gametes on 10 elasmobranch species.

► *The use of different cryoprotocols for shark 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.

The cryoprotectants used in this study include two concentrations of 10 and 20% of DMSO, MET, GLY and EG. These cryoprotectants are evaluated in three contexts: fresh samples, under toxicity and after cryopreservation. The overall approach indicates substantial variation in the efficacy of the cryoprotectants. DMSO combined with MET provided the highest post-cryopreservation motility (around 30%), indicating its effectiveness in minimizing damage during the freezing and thawing processes compared to other cryoprotectants. On the other hand, glycerol has shown the worst results in terms of toxicity and cryopreservation processes. The chemical property of the GLY has a negative impact on the shark sperm and shows a high toxicity to it.

The fresh samples show a motility at around 70%, which is the highest motility that can be reached before cryopreserving. The fresh control samples are important for comparison, it helps to quantify the motility loss across the different cryopreservation processes. None of the cryoprotectants used managed to keep a high motility level. The highest one has been reached by DMSO+MET at a percentage of 10%. The motility had

reached around 30%. This means that damage can occur during the process of cryopreservation and the use of different cryoprotectants need to be more selected. The acidity of some is too pronounced for the shark sperm.

At the beginning of the toxicity, so after 15 minutes, the loss of motility decreased a lot. Going from 70% to 50% for the combination DMSO+MET. Glycerol, particularly at higher concentrations (20%) showed an important drop in motility, dropping around 20%. This indicates that GLY has a high toxicity even before the cryopreservation process. EG showed a low toxicity compared to GLY but remain worst that DMSO+MET. After the cryopreservation process, the effects of freezing and thawing the samples are highlighted in all the cryoprotectants used. DMSO+MET showed a motility around 25% and 20% at a concentration of 10% and 20% respectively. This would suggest that with a lower concentration of DMSO and MET the cryopreservation process would be more effective in preserving shark sperm samples.

This study indicates that the choice of the right concentration and the right cryoprotectants is very important for a successful cryopreservation process. DMSO combined with MET is at this day the most effective cryoprotectant in maintaining sperm motility, both in terms of high toxicity and post-cryopreservation. The observed differences between the two percentages used suggest that a lower concentration of the cryoprotectant may be more favorable as they provide adequate cryoprotection without excessive toxicity of an osmotic and an oxidative stress (Kolyada., *et al.* 2023). Modern methodological techniques are still largely explored and are yet to be standardized across cryopreservation protocols because of the effectiveness of these additives that are specific-specific (Kolyada., *et al.* 2023). It depends on many parameters, for example, their dose, their type and the sperm quality and integrity (Kolyada., *et al.* 2023). GLY, due to its high toxicity and an important loss of motility post-cryopreservation should be avoided in other cryopreservation procedures for shark sperm conservation.

These findings' deeper implications underline the necessity of continual improvement of cryoprotectant formulations and concentrations. The balance between toxicity and cryoprotection means that even small variations in the concentration of the cryoprotectant can have a big impact on the viability of the sperm. To increase the long-term survival of cryopreserved sperm, more study should concentrate on improving

freezing and thawing techniques, exploring alternative agents, and adjusting cryoprotectant combinations. Furthermore, the creation of less dangerous or non-toxic cryoprotectants may open new possibilities to improve sperm preservation, especially for animals with distinct physiological needs like for sharks.

► ***The use of different vials to cryopreserve shark sperm***

To find a biodegradable alternative to plastic containers for fish sperm cryopreservation, a pioneering method was reported by França *et al* (2023). This innovative approach uses hard gelatin capsules and hard hydroxypropyl methylcellulose (HPMC) capsules as storage containers for freshwater fish sperm. These capsules are made from biodegradable and biocompatible by-products of animal and plant production, making them environmentally friendly and compatible with biological samples. In addition, their wide market availability and cost effectiveness make them a practical and accessible solution. The use of these capsules represents a significant step towards reducing environmental impact compared to plastic containers.

This study showed the comparison between fresh and cryopreserved samples, highlighting how storage methods and cryopreservation impact sperm motility. It is then important to optimize sperm cryopreservation techniques for marine species like sharks, where sperm preservation can have significant impact on conservation and breeding programs. Among the tested containers, cryotubes provided the most effective protection for sperm motility, likely due to their material properties and potential for increased cryoinjury. According to the sperm motility, cryotubes are an effective container for maintaining sperm in its initial fresh state before cryopreserving. This makes cryotubes a reliable tool in sperm collection and handling prior to cryopreservation.

Among the cryopreserved samples, cryotubes exhibit the highest post-cryopreservation motility values close to 30%. Compared to the fresh samples in cryotubes the motility showed a significant decrease but suggests that cryotubes still provide a relatively favorable environment for sperm survival after freezing. The BC showed a lower post-cryopreservation motility than cryotubes with a motility around 20%. This suggests that while BC may still offer some protection for sperm during freezing, they are not as effective as cryotubes in maintaining motility post-thawing. The capsules provide a

certain level of protection against cryoinjury but may be less effective with shark sperm inside. Cryopreservation in other studies using biodegradable capsules with fish species like seabream or seabass, showed that the motility after cryopreservation was more efficient than with shark sperm (França., *et al.* 2024).

The lowest motility is observed in vegetable capsules with a motility around 15%. Vegetable-based capsules may not provide adequate protection against sperm sharks and freezing processes. The material composition of this capsule may be more evaluated to have better resistance to extreme conditions of cryopreservation and acidic environments of the sperm. The low motility in vegetable capsules indicated that the permeability of the capsule is not ideal for maintaining a stable internal environment during the freezing and thawing processes. França., *et al.* in 2024 also observed that after the thawing procedure, the vegetal capsules did not dissolve as well as the gelatine capsules. This can have an impact on shark sperm with the composition of the media that can differ, causing a decrease in motility.

The significant differences in post-cryopreservation motility between the three containers showed the importance of the storage environment in sperm cryopreservation. The cryotubes are the most effective container, likely due to its design and material properties. Cryotubes are typically made of propylene that can withstand freezing temperatures and provide stable internal environment, leading to a reduce of risk of mechanical damage to the sperm. Biodegradable capsules have not been used in a lot of studies but in ones we can observe that those capsules are as efficient as plastic straws preserving sperm kinetic parameters, membrane integrity, and DNA integrity after European eels, gilthead seabream, and European sea bass sperm cryopreservation (França., *et al.* 2024). It is important to now do a better adaptation of this protocol for the shark sperm and improve the results that have been obtained for now.

This study results have an important implication for the cryopreservation of shark sperm. The ability to preserve sperm with minimal motility loss is crucial for conservation efforts, breeding programs and species conservation. Even though biodegradable capsules have lower motility than the ones in cryotubes, it is important to underline their biodegradability. This important function will lead to in vitro fertilization without using needles or plastic components.

5.3 Comet assay

The goal of sperm is to pass on the male's genetic information to offspring, so it is imperative to prioritize the preservation of sperm genomic information when designing a cryopreservation protocol. Over the years, several studies have reported DNA damage in fish sperm after thawing, as assessed by the alkaline comet. This study is the first to report results on DNA fragmentation in fresh and thawed sperm from *S. canicula* sharks using comet assay analysis. The comet assay showed an elongated, bright tail, indicating DNA damage. The length and intensity of the tail are indicating with the degree of DNA fragmentation, likely due to either high exposure to damaging agents or impaired DNA repair mechanisms. The comet shape seen in this figure 4.7 indicates a significant level of DNA damage. The comet tail's length and brightness correlate with the number of DNA strand breaks.

The comet assay image suggests that the cells being tested experienced considerable DNA damage, as evidenced by the strong comet tail formation. Depending on the context of the experiment, this level of damage could reflect the effectiveness or severity of the test conditions.

Several studies have shown that the process of cryopreservation could induce sperm DNA damage, also indicating that oxidative stress is the major effector in DNA damage during sample cryopreservation (Gajski., *et al.* 2021). Using the comet assay after the cryopreservation process is an important tool as we can redefine DNA damage and adapt the protocol for the cryopreservation process.

This study has broad implications for natural and artificial reproduction, for species that rely on sperm motility for fertilization. Sperm banking or in vitro fertilization (IVF), where sperm needs to be stored for extended periods, understanding the synergistic effects of temperature and pH can significantly improve sperm preservation strategies (Contreras., *et al.* 2020). Research should focus on identifying mechanisms that underline sperm motility loss, such as oxidative damage and membrane destabilization. This would refine storage protocols and consequently increase sperm longevity and motility. Chemical composition, osmolality, and pH extender are important factors for short term storage success due to their physiological roles in spermatozoa motility (Shazada., *et al.* 2024).

5.3 Future perspectives in elasmobranch's gametes

In developing and applying techniques to control elasmobranch reproduction, we are still in the preliminary stages. Progress will require a broader understanding of the biology, anatomy, and ecology of various species, from the cellular level to the entire organism. The more thorough our knowledge becomes, the better we can address the unique challenges of working with diverse groups. So, future work should focus on:

- Improving cryoprotocols: It will be essential to broaden the range of cryoprotectants, test novel combinations and assess toxicity tolerances across species to account for interspecies variability. Recent studies suggest promising results with combinations of trehalose and DMSO or glycerol, as these effectively combine external and internal cryoprotectants (Montano et al., 2023). In addition, exploration of other additives, such as antioxidants, may improve post-thaw survival (Len et al., 2019).
- Standardizing the use of CASA is critical to advancing elasmobranch reproductive research. Previously, the unique size and shape of elasmobranch sperm cells presented challenges that prevented effective use of CASA. However, recent technical modifications introduced by Montano et al. (2023) have overcome these limitations, allowing more accurate analysis of sperm motility. These improvements should be widely adopted in future studies to refine our understanding of sperm performance in these species.
- Furthermore, developing specialized diluents will allow for more reliable assessments. With CASA, it will now be possible to accurately measure key motility parameters such as linear and curvilinear velocity, as well as other metrics such as sperm trajectory and progression, all of which contribute to a more comprehensive assessment of sperm health and viability in elasmobranchs.

Developing cryopreservation techniques for sperm would be futile if they cannot ultimately be applied in artificial insemination. While artificial insemination with fresh sperm has been trialed in elasmobranchs, yielding mixed success rates (Masuda et al., 2003; Luer et al., 2007; Daochai et al., 2020; Wyffels et al., 2021; Adams et al., 2022),

the method has yet to incorporate cryopreserved sperm critical area for future research. The implementation of this technique in aquaria would allow the controlled monitoring of the female's gestation and the careful observation of the development of the newborns, ensuring healthy development at every stage.

In summary, we are at the forefront of developing techniques that may be the last hope for many endangered species. However, there is still a huge amount of knowledge to be gained. The immense diversity and variability within elasmobranchs make this a challenging endeavor. However, similar obstacles have been overcome in other animal groups, from birds to mollusks, where innovative strategies have turned the tide. While not all species will necessarily require these interventions, and some may face practical constraints, it is far better to have the tools ready when needed than to be unprepared. The road ahead is long, full of learning and evaluation, but swift and decisive action is crucial as time is running out for many of these species.

6. CONCLUSIONS

This master thesis goes deeper into the improvement of gamete handling, sperm assessment, and gamete preservation protocols for elasmobranchs. Several goals were achieved both trying to adapt and apply several reproduction techniques and tools previously applied in other fish species; performing also the current preservation protocols developed on elasmobranch species. The main conclusions of this work are:

- Regarding short-term storage at chilled temperatures, this study reveals that the best method to store spermatozoa for a short-term period was keeping the samples at 4°C at a pH of 6.5, being possible to reach 40% of motility up to 14 days of storage.
- Regarding short-term storage in ice (for sending samples), the best method was to keep samples at room temperature ($\pm 20^{\circ}\text{C}$) with ice without the addition of cryoprotectants. The samples kept in the fridge ($\pm 4^{\circ}\text{C}$) have also good results, both protocols reaching a motility of close to 50% by the end.
- About cryopreservation trials, the best results for *S. canicula* were obtained with the combination of 5% methanol and 5% DMSO, with motility and post-freezing membrane integrity values close to 20-25 %.
- Biodegradable capsules were studied as a potential replacement for traditional vials, and results showed that traditional vials (cryotubes) showed better results (30-35%) of sperm post-thawing motility) than biodegradable vials (15-25%).
- Finally, this study reported that the comet assay technique was applied for the first time in elasmobranch species, showing oxidative stress as a primary factor in DNA damage after cryopreservation process.

7. REFERENCES

- Adams, L., Lyons, K., Larkin, E., Leier, N., Monday, J., Plante, C., ... & Wyffels, J. (2022). Artificial insemination and parthenogenesis in the zebra shark *Stegostoma tigrinum*. *Frontiers in Marine Science*, 9, 886616. <https://doi.org/10.3389/fmars.2022.886616>
- Asturiano, J. F., Cabrita, E., & Horváth, Á. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: A mini review. *General and Comparative Endocrinology*, 245, 69–76. <https://doi.org/10.1016/j.ygcen.2016.06.019>
- Barnett, L. A. K., Earley, R. L., Ebert, D. A., & Cailliet, G. M. (2009). Maturity, fecundity, and reproductive cycle of the spotted ratfish, *Hydrolagus coliei*. *Marine Biology*, 156(3), 301–316. <https://doi.org/10.1007/s00227-008-1084-y>
- Bath, G. E., Price, C. A., Riley, K. L., & Morris Jr, J. A. (2023). A global review of protected species interactions with marine aquaculture. *Reviews in Aquaculture*, 15(4), 1686-1719. <https://doi.org/10.1111/raq.12811>
- Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., & Herráez, M. P. (2005). Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. *Cryobiology*, 50(2), 144–153. <https://doi.org/10.1016/j.cryobiol.2004.12.003>
- Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J. G., ... & Risopatrón, J. (2020). Effects of short-term storage on sperm function in fish semen: a review. *Reviews in Aquaculture*, 12(3), 1373-1389. [doi:10.1111/raq.12387](https://doi.org/10.1111/raq.12387)
- Daly, J., & Jones, R. (2017). The use of reproductive technologies in breeding programs for elasmobranchs in aquaria. *The elasmobranch husbandry manual II: Recent advances in the care of sharks, rays and their relatives*, 363-374. <https://www.researchgate.net/profile/Mark-Smith-68/publication/319762551>
- Daochai, C., Keschumras, N., Chansue, N., & 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. <https://www.researchgate.net/profile/Chayanis-Daochai-2/publication/344898529>
- Davidson, L. N. K., Jaiteh, V. F., Chin, A., & Jabado, R. W. (2022). Conservation science for sharks and rays. In *Biology of Sharks and Their Relatives* (pp. 657–688). CRC Press. <http://dx.doi.org/10.1201/9781003262190-21>
- 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. <https://doi.org/10.7554/elife.00590>
- Dulvy, N. K., Pacoureau, N., Rigby, C. L., Pollom, R. A., Jabado, R. W *et al.* (2021). Overfishing drives over one-third of all sharks and rays toward a global extinction crisis. *Current Biology*, 31(22), 5118–5119. <https://doi.org/10.1016/j.cub.2021.11.008>
- Ellis, J. R., & Shackley, S. E. (1997). The reproductive biology of *Scyliorhinus canicula* in the Bristol Channel, UK. *Journal of Fish Biology*, 51(2), 361-372. <https://doi.org/10.1111/j.1095-8649.1997.tb01672.x>
- França, T.S., Gomes, I. C., Sanches, E. A., Atehortúa, M. P., dos Santos Teixeira, N., Rodrigues, R. B., ... & Streit, D. P. (2023). Fish sperm cryopreservation using biodegradable containers: New low-cost and environment-friendly methodology. *Reproduction*, 166(2), 89-97. <https://doi.org/10.1530/REP-23-0046>

- França, T. S., González-López, W. A., Sanchez, M. P., Ferrão, L., Fernández-García, F., Borges, L. P., ... & Asturiano, J. F. (2024). Successful cryopreservation in biodegradable containers of sperm from aquaculture Mediterranean fishes. *Theriogenology*, *216*, 53-61. <https://doi.org/10.1016/j.theriogenology.2023.12.016>
- Froehlich, H. E., Gentry, R. R., & Halpern, B. S. (2017). Conservation aquaculture: Shifting the narrative and paradigm of aquaculture's role in resource management. *Biological conservation*, *215*, 162-168. <https://doi.org/10.1016/j.biocon.2017.09.012>
- Gajski, G., Ravlić, S., Godschalk, R., Collins, A., Dusinska, M., & Brunborg, G. (2021). Application of the comet assay for the evaluation of DNA damage in mature sperm. *Mutation Research/Reviews in Mutation Research*, *788*, 108398. <https://doi.org/10.1016/j.mrrev.2021.108398>
- García-Salinas, P. (2023). Ph Doctorate: *Development and application of techniques for the control of captive breeding in elasmobranchs*. Defended in 12/2023 in the UPV: [File](#).
- García-Salinas, P., Gallego, V., & Asturiano, J. F. (2021). Development of sperm cryopreservation protocols for sharks and rays: New tools for elasmobranch conservation. *Frontiers in Marine Science*, *8*. <https://doi.org/10.3389/fmars.2021.689089>
- Gillis, J. D., Penfold, L. M., & Mylniczenko, N. D. (2021). Initial Characterization of Male Southern Stingray (*Hypanus americanus*) Reproductive Parameters and Preliminary Investigation of Sperm Cryopreservation. *Animals*, *11*(9), 2716. <https://doi.org/10.3390/ani11092716>
- Hamlett, W. C. (2005). Oviducal glands in chondrichthyans. *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras*, 311–346. [Reproductive Biology and phylogeny of Chondrichthyes](#)
- Heithaus, M., Frid, A., Vaudo, J., Worm, B., & Wirsing, A. (2010). Unraveling the ecological importance of elasmobranchs. In *Marine Biology* (pp. 611–637). CRC Press. <http://dx.doi.org/10.1201/9781420080483-c16>
- Horváth, Á., & Urbányi, B. (2020). Chapter 15 sperm cryopreservation of aquatic species. In *Reproduction in Aquatic Animals* (pp. 321–334). Springer Singapore. http://dx.doi.org/10.1007/978-981-15-2290-1_16
- Jones, C. J. P., Walker, T. I., Bell, J. D., Reardon, M. B., Ambrosio, C. E., & Almeida, A. (2005). “Male genital ducts and copulatory appendages in chondrichthyans.” *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras*, 361–393. <https://www.researchgate.net/profile/Terence-Walker/publication/297420300>
- Kolyada, M. N., Osipova, V. P., & Berberova, N. T. (2023). Use of cryoprotectors and antioxidants in sturgeon semen cryopreservation. *Cryobiology*, *111*, 30-39. <https://doi.org/10.1016/j.cryobiol.2023.02.003>
- Len, J. S., Koh, W. S. D., & Tan, S.-X. (2019). The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience Reports*, *39*(8). <https://doi.org/10.1042/bsr20191601>
- Liu, X., Pan, Y., Liu, F., He, Y., Zhu, Q., Liu, Z., Zhan, X., & Tan, S. (2021). A review of the material characteristics, antifreeze mechanisms, and applications of cryoprotectants (cpas). *Journal of Nanomaterials*, *2021*, 1–14. <https://doi.org/10.1155/2021/9990709>
- Luer C. A., Walsh C. I., Bodine A. B., Wyffels J. (2007). Normal embryonic development in the clearnose skate, *Raja eglanteria*, with experimental observations on artificial insemination. *Environ. Biol. Fishes.* *80*, 239–255. [doi: 10.1007/s10641-007-9219-4](https://doi.org/10.1007/s10641-007-9219-4)

- Mancia, A., Chenet, T., Bono, G., Geraci, M. L., Vaccaro, C., Munari, C., ... & Pasti, L. (2020). Adverse effects of plastic ingestion on the Mediterranean small-spotted catshark (*Scyliorhinus canicula*). *Marine environmental research*, *155*, 104876. <https://doi.org/10.1016/j.marenvres.2020.104876>
- Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T. R., & Cabrita, E. (2017). Cryobanking of aquatic species. *Aquaculture*, *472*, 156–177. <https://doi.org/10.1016/j.aquaculture.2016.05.042>
- Masuda, M., Izawa, Y., Kametuta, S., Ikuta, H., & Isogai, T. (2003). Artificial insemination of the cloudy catshark.
- Mayer, I. (2019). The role of reproductive sciences in the preservation and breeding of commercial and threatened teleost fishes. In *Reproductive Sciences in Animal Conservation* (pp. 187–224). Springer International Publishing. http://dx.doi.org/10.1007/978-3-030-23633-5_7
- Mayer, I., & Pšenička, M. (2024). Conservation of teleost fishes: application of reproductive technologies. *Theriogenology Wild*, *100078*. <https://doi.org/10.1016/j.therwi.2024.100078>
- Mizuta, D. D., Froehlich, H. E., & Wilson, J. R. (2023). The changing role and definitions of aquaculture for environmental purposes. *Reviews in Aquaculture*, *15*(1), 130-141. <https://doi.org/10.1111/raq.12706>
- Montano, G. A., Steinman, K. J., McDonnell, A., & 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. <https://doi.org/10.1016/j.therwi.2023.100017>
- Penfold, L. M., & Wyffels, J. T. (2019). Reproductive science in sharks and rays. *Reproductive sciences in animal conservation*, 465-488. <https://doi.org/10.1007/978-3-030-23633-5>
- Ragonese, S., Vitale, S., Dimech, M., & Mazzola, S. (2013). Abundances of demersal sharks and chimaera from 1994-2009 scientific surveys in the central Mediterranean Sea. *PloS one*, *8*(9), e74865. <https://doi.org/10.1371/journal.pone.0074865>
- Ridlon, A. D., Wasson, K., Waters, T., Adams, J., Donatuto, J., Fleener, G., ... & Grosholz, E. D. (2021). Conservation aquaculture as a tool for imperiled marine species: Evaluation of opportunities and risks for Olympia oysters, *Ostrea lurida*. *PloS one*, *16*(6), e0252810. <https://doi.org/10.1371/journal.pone.0252810>
- Shazada, N. E., Alavi, S. M. H., Siddique, M. A. M., Cheng, Y., Zhang, S., Rodina, M., ... & Linhart, O. (2024). Short-term storage of sperm in common carp from laboratory research to commercial production—A review. *Reviews in Aquaculture*, *16*(1), 174-189. <https://doi.org/10.1111/raq.12827>
- Shiffman, D. S., Macdonald, C. C., Wallace, S. S., & Dulvy, N. K. (2021). The role and value of science in shark conservation advocacy. *Scientific Reports*, *11*(1), 16626. <https://doi.org/10.1038/s41598-021-96020-4>
- Walker, T. I. (2020). Chapter 10 Reproduction of Chondrichthyans. *Reproduction in Aquatic Animals: From Basic Biology to Aquaculture Technology*, 193-223. https://doi.org/10.1007/978-981-15-2290-1_11
- Wyffels, J. T., Adams, L. M., Bulman, F., Fustukjian, A., Hyatt, M. W., Feldheim, K. A., & Penfold, L. M. (2021). Artificial insemination and parthenogenesis in the whitespotted bamboo shark *Chiloscyllium plagiosum*. *Scientific reports*, *11*(1), 9966. <https://doi.org/10.1038/s41598-021-88568-y>

8. ANNEXES

Annex 1. Congress posters

- l) This poster was presented at the X Iberian Congress of Ichthyology, Vic (Spain), in June 2024: “Gamete cryopreservation in elasmobranchs using biodegradable capsules”

GAMETE CRYOPRESERVATION IN ELASMOBRANCHS USING BIODEGRADABLE CAPSULES

Authors: Magny, Ana¹; Franca, Thales¹; García-Salinas, Pablo^{1,2}; Pérez, Luz¹; Asturiano, Juan F.¹; Gallego, Víctor¹
 Institutions: 1-Grupo de Acuicultura y Biodiversidad, Universitat Politècnica de València, Spain. 2-Research Department, Fundació Oceanogràfic, Spain.

INTRODUCTION

Elasmobranchs have life strategies that make them highly vulnerable to rapid environmental change, and are one of the most threatened vertebrate groups on the planet. In this sense, establishing breeding programs in captivity, often with assisted reproduction techniques, is one of the ex-situ conservation projects' primary goals.

OBJECTIVES

Due to the use of assisted reproductive control techniques have been scarcely developed in elasmobranchs, the general goal of this study was to perform the current cryopreservation protocols developed on elasmobranch species by using new alternative biodegradable containers, which have never been used in these species.

MATERIAL AND METHODS



HOW DO WE CRYOPRESERVED THE SAMPLES?

- Taking the sperm and diluting in extender
- Cheking the initial quality by sperm motility
- Diluting the sperm and filling the biocapsules
- Freezing the biocapsules in liquid NO (-196°C)
- Thawing the biocapsules in a warm bath (70°C)
- Cheking the finial quality by assessing sperm motility

RESULTS & DISCUSSION



Graph showing the motility of shark sperm before and after the cryopreservation process according to its container.

TAKE HOME MESSAGE

our research can potentially help with the development of artificial insemination projects in elasmobranchs, in which these biodegradable capsules could be introduced directly into females. However, further studies using these biodegradable containers should address the topic of freezing rates, which could improve the final motility of gametes.



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SIBIC 2024
X Iberian Congress of Ichthyology



ELASMOREP

- II) This poster was presented at the IX International Workshop on the Biology of Fish Gametes, León (Spain), in July 2024: “Short term storage in elasmobranchs using different pH and temperatures”

SHORT TERM STORAGE IN ELASMOBRANCH SPERM USING DIFFERENT pH_s AND TEMPERATURES

Magny, Ana¹; Franca, Thales¹; García-Salinas, Pablo²; Pérez, Luz¹; Asturiano, Juan F.¹; Gallego, Víctor¹
 1-Grupo de Acuicultura y Biodiversidad, Universitat Politècnica de València, Spain. 2-Research Department, Fundació Oceanogràfic, Spain.

INTRODUCTION

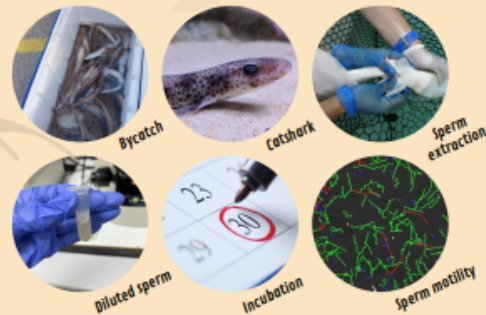
Elasmobranchs are among the most threatened groups of vertebrates on Earth, and the primary threats to their survival are overfishing and habitat destruction. To address this, both *in situ* conservation efforts and *ex situ* conservation programs are essential. Implementing assisted reproductive technologies, though not widely developed for elasmobranchs, is a crucial aspect of these *ex situ* measures.

OBJECTIVES

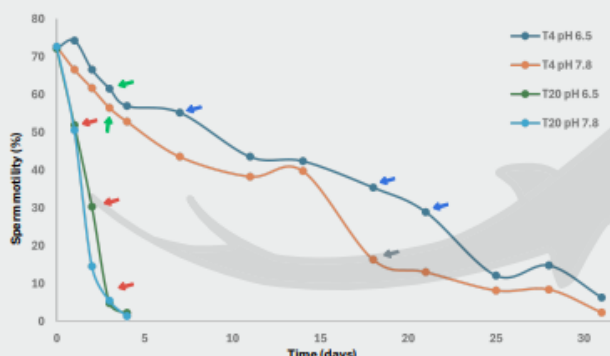
This study aims to develop tools for the establishment of *ex situ* conservation programs of elasmobranchs. Due to the use of assisted reproductive control techniques that have been scarcely developed in this group, the general goal of this study was to compare different methods of short-term storage in elasmobranch spermatozoa.

MATERIAL & METHODS

- ↓ Taking the sperm and diluting in extender
- ↓ Cheking the initial quality → sperm motility
- ↓ Adjusting the extenders with a pH at 6.5 and 7.8
- ↓ Adjusting the temperatures to 4 and 20 °C
- ↓ Check the motility twice a week for a month



RESULTS & DISCUSSION



Graph 1. Sperm motility values on the 4 experimental groups over 30 days

Sperm samples stored at 4 °C (regardless of pH incubated) showed higher motility values than samples incubated at 20 °C, which motility values decreasing rapidly from 1 day of incubation (red arrows).

On the other hand, sperm samples stored at 4 °C did not showed statistical differences respect to the fresh samples (day 0) until 3-day of storage time, showing values between 55-60% at both pHs (green arrows).

From that point, the motility values of sperm samples stored at 4 °C and pH 6.5 maintained at several points higher motilities than the samples incubated at 4 °C and pH 7.8 (day 7, 18 and 21; blue arrows).

From 2 weeks of storage, sperm motility samples showed a progressive reduction until day 30, and samples incubated at 4 °C and pH 7.8 reached very low values (around 10%) on the day 17 (grey arrow).

CONCLUSION

This study reveals that the best method to store elasmobranch sperm for a short-term period is keeping the samples at 4 °C and pH of 6.5, being possible to reach 40% of motility up to 14 days

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FUNDACIÓN OCEANOGRÀFIC



III) This poster was presented at the IX International Workshop on the Biology of Fish Gametes, León (Spain), in July 2024: “Trying to improve cryopreservation protocols in elasmobranch sperm using new cryoprotectants and biodegradable vials”

TRYING TO IMPROVE CRYOPRESERVATION PROTOCOLS IN ELASMOBRANCH SPERM USING NEW CRYOPROTECTANTS AND BIODEGRADABLE VIALS

Magny, Ana¹; Franca, Thales¹; Garcia-Salinas, Pablo²; Pérez, Luz¹; Asturiano, Juan F.¹; Gallego, Victor¹
 1-Grupo de Acuicultura y Biodiversidad, Universitat Politècnica de València, Spain. 2-Research Department, Fundació Oceanogràfic, Spain.

INTRODUCTION

Elasmobranchs, which include sharks, rays, and skates, are among the most threatened groups of vertebrates on Earth, and *in situ* conservation efforts and *ex situ* conservation programs are essential for their conservation. Implementing assisted reproductive technologies, though not widely developed for elasmobranchs, is a crucial aspect of these *ex situ* measures. This study aimed to improve the current cryopreservation protocols in elasmobranch sperm using new cryoprotectants and, in addition, assessing the use of biodegradable vials.

RESULTS & DISCUSSION

In general, data showed (Graph 1) that post-thawed samples had significantly lower motility values than the fresh samples (which had around 60-65%). The best results were obtained by the MD-10 combination, with 25-30% of motile spermatozoa post-thawing.

On the other hand, the other cryoprotectants (GLY and ETG) did not produce good results, probably due to the high toxicity of these cryoprotectants. This effect was more pronounced using the Glycerol, when sperm sample just before cryopreservation process (after incubation time), showed a hard decrease from 60 to 30% for a period of 15 min (data not shown).

MATERIAL & METHODS

The model species used on these trials was *Scyliorhinus canicula*. In the first trial, we used a series of different combinations of cryoprotectants (methanol, MET; dimethyl-sulfoxide, DMSO; glycerol, GLY; and ethylene-glycol, ETG) and two final concentrations (10 and 20%) according to 6 different protocols: MD-10: 5% MET + 5% DMSO; MD-20: 10% MET + 10% DMSO; GLY-10: 10% GLY; GLY-20: 20% GLY; ETG-10: 10% ETG; and ETG-20: 20% ETG. In the second trial and using the best cryoprotocol, we tested new biodegradable capsules.

HOW DID WE CRYOPRESERVE THE SAMPLES?

- Taking the sperm and diluting in extender
- Checking the initial quality → sperm motility
- Diluting the sperm and testing new cryoprotectants
- Thawing the cryotubes in a warm bath (75 °C)
- Checking the final quality → sperm motility
- Testing the best cryoprotocol using biocapsules

TAKE HOME MESSAGE

Our research can potentially help with the development of artificial insemination projects in elasmobranchs, in which these biodegradable capsules could be introduced directly into females. However, further studies using these biodegradable containers should address the topic of freezing rates, which could improve the final motility of gametes.

Acknowledgments:

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